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PHYSICAL CHEMISTRY OF CELLS AND TISSUES

PHYSICAL CHEMISTRY

OF

CELLS AND TISSUES

BY

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PREFACE

This book has grown to its present state of publication during more than three years of war time. Glanced at superficially, its outlines may appear to comprise a well-defined entity. However, those who have contributed to its growth are aware of many a wound inflicted upon its form by the war and of many a defect, the scars of which are conspicuous enough, though they have not abolished the integrity of the basic idea.

The subject of the book is physiology; not "physiology from above," but "physiology from below"; not physiology originated essentially to fill human needs and help suffering individuals, but physiology as a branch of physical chemical science dealing with life as a physical, though exceedingly complex system, that may be subjected to scientific analysis like any other natural object.

In this preface the idea of the book will be made clear to the reader by a brief abstract from the eight sections, which appear in the table of contents. It will be convincingly shown that it is not only possible, but of importance, to anchor physiology even deeper in physical chemistry than was done previously, i.e., even closer to the fundamentals, on which our concepts of inorganic nature are erected, and when one tries to segregate the elementary processes combined in the life of a cell and to analyze them with the new tools of modern physics, this attitude makes more discernible the great number of unsolved problems.

During the last four decades a tremendous revolution in our conception of the inorganic world has taken place, which during the last twenty years has progressively seized the aspect of the world of organisms. For qualities of matter spatially distributed quantities have been substituted; chemistry has more and more become a branch of physics.

In our book this trend is described in Sections 1 and 2. Beginning with a survey over the fundamentals of classical physical chemistry, a comprehensive part is devoted to outlining the numerous grades of structural complexity, beginning at the low level of atoms and molecules and defining the various electronic and nuclear forces which are involved in their linkage or disrupture. Then, ascending through levels of greater and greater aggregation, the rank of such giant molecules is attained as we meet in the synthetic polymerization products of organic chemistry on the one hand, and in the huge aggregates of amorphous colloidal micellæ, of anisodiametric thread-like and leaf-like units, from which by interaction and orientation emerge the fibrils and fibers, the films, membranes, and septa, so widespread in living cells.

With the subsequent sections, 3–8, the actual field of cell physiology is entered. Section 3 briefly refers to the structure of protoplasm. Here, reasons are offered for the existence of submicroscopic particles of an even

higher order of aggregation. As these particles are intermixed with macromolecules and molecules of smaller and smallest dimensions inside and around the protoplasm, it is made clear that they are subjected to spatial and temporal changes brought about especially by the metabolic reactions. Therefore, the next logical step is to assign functional significance to the various structures and to their changes.

Among these superstructures the most outstanding importance is claimed for the plasma membrane as an interphase between living and nonliving (Sections 4 and 5). The plasma membrane has often been thought a "mixed film," and it will be shown that, on the basis of studies upon red blood corpuscles, plant cells, and other objects, various fairly simple patterns could be proposed to portray its submicroscopic architecture, also changes of permeability due to disarrangements in this interfacial colloidal structure in response to the influences of ions, narcotics, the electric current, and others.

However, it also will be explained in these sections that these patterns are found entirely unsatisfactory in view of the fact that permeability is altered not merely following artificial alterations in the surrounding media or following external stimulation. Instead, this "mixed film" appears as an "active organ," where metabolic processes are somewhat associated with a transport of solutes or of solvent against the concentration gradients between the interior of the cell and outside. In other words, work derived from the expenditure of metabolic energy can be done by this superstructure, provided the free energy is disposed of in an adequate succession and characteristic involvement of the chemical reactions, as explained in Sections 6 to 8.

By this statement the reader will be confronted with one of the central problems of general cellular physiology, namely, the mechanisms, by which in such submicroscopical cellular structures the liberated energy can be utilized to perform work. Beside the plasma membrane there is another microstructure, the thread-like aggregates of myosin molecules in muscle, where the problem of interrelation between mechanism and metabolism seems to be more accessible. It will be shown (Section 7) that the myosin threads themselves seem to release the transformation of certain muscle metabolities which reciprocally cause the threads to shorten and to relax. Possibly, one likewise can arrive at an approximate interpretation of the mechanism of active transfer, this characteristic property of the numerous [illegible] and secreting membranes, by comparing them with the sieve membranes as analoga, which, endowed with pores of different widths, with pore walls carrying different electric charges and placed in solutions of different ion concentrations, may be assumed to establish anti-diffusion and anti-osmotic effects with water and ions (Section 8).

Certainly, such interpretations lie as yet rather far beyond the limits of exact and definite statements. However, perhaps the viewpoint of the physicist, which in this book has been pushed into the foreground, lends itself to such extrapolations, especially as physics is on the way to open new

and wide areas to problems in the field of molecular structure. On the other hand, in choosing a new start from a new base line, we have ignored or at least neglected in our text several important recent trends of physico-chemical investigation into physiology and also older observations and conclusions, which in the meantime have been turned over into the stock of common [illegible] In this respect the book cannot claim to offer a universal information in the field of physical chemistry of cells and tissues. But it may guide to new roads into the unknown.

Grateful acknowledgment is made to the Carnegie Corporation of New York for a grant-in-aid to complete and to edit this book.

Special thanks are due to The Blakiston Company for their liberal agreement to extend considerably, with respect of war time conditions, the time limit of the contract and for their unfailing co-operation and assistance.

RUDOLF HÖBER.

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Section 1

SELECTED PRINCIPLES OF PHYSICAL CHEMISTRY

By DAVID I. HITCHCOCK

INTRODUCTION

The recognition of physical chemistry as a separate branch of science is associated with the foundation, in 1887, of the *Zeitschrift für physikalische Chemie*. The early volumes of this journal contained the well known papers of Arrhenius, van't Hoff, Ostwald, and Nernst on electrolytic dissociation, osmotic pressure, dissociation constants, and electromotive force. These papers had been preceded, however, by a number of important contributions, including the work of Poiseuille (1843) on viscosity, Wilhelmy (1850) on reaction velocity, Fick (1855) on diffusion, Guldberg and Waage (1864–67) on mass action, and Gibbs (1875–78) on heterogeneous equilibrium. A number of the early workers in physical chemistry were men of biological training. Poiseuille was a physician, Fick was a physiologist, and the work of the botanists, Pfeffer (osmotic pressure, 1877) and de Vries (plasmolysis, 1882), as well as that of a physiologist, Hamburger (hemolysis, 1883), was used by van't Hoff in the development of his theory of solutions. In more recent years the application of physical chemistry to biological phenomena was particularly emphasized in the writings of Höber and of Bayliss.

Physical chemistry has been divided into the study of laws applicable to systems in equilibrium, and of laws governing the rates by which equilibrium is attained. Since living cells and tissues are not in equilibrium, although many of their properties show remarkably steady states, the discussion of rates must occupy a prominent place in the present work.

The treatment of physicochemical principles in the first part of this book is based on the assumption that its readers already have some knowledge of the subject. The study of this book might well be preceded by the reading of one of the elementary texts in the following list, or accompanied by the use of one of the intermediate or advanced texts.

Text Books of Physical Chemistry

Elementary

Findlay, A., Physical Chemistry for Students of Medicine: Longmans, London, 1931.

Chapin, W. H., and L. E. Steiner, Second Year College Chemistry: Wiley, New York, 1938.

Hitchcock, D. I., Physical Chemistry for Students of Biology and Medicine: Thomas, Springfield (Illinois), 1940.

West, E. S., Physical Chemistry for Students of Biochemistry and Medicine: Macmillan, New York, 1942.

Intermediate

Millard, E. B., Physical Chemistry for Colleges: McGraw-Hill, New York, 1941.

Gucker, F. T., Jr., and W. B. Meldrum, Physical Chemistry: American Book Co., New York, 1942.

Getman, F. H., and F. Daniels, Outlines of Physical Chemistry: Wiley, New York, 1943.
MacDougall, F. H., Physical Chemistry: Macmillan, New York, 1943.

Advanced

Taylor, H. S., ed., A Treatise on Physical Chemistry, 2nd ed., 2 vols.: Van Nostrand, New York, 1931 (also 3rd ed., vol. 1, 1942, vols. 2–5 to be published).
Glasstone, S., Text-Book of Physical Chemistry: Van Nostrand, New York, 1940.

Biological Applications

Höber, R., Physikalische Chemie der Zelle und der Gewebe, 1st ed., 1902; 6th ed.: Engelmann, Leipzig, 1926.
Bayliss, W. M., Principles of General Physiology, 1st ed., 1915; 4th ed.: Longmans, London, 1924.

Principal Notation Used in Chapters 1–5

a	area; activity; initial concentration; empirical constant.
b	initial concentration; empirical constant.
c	concentration (moles/cc.).
d	differential; density.
e	base of natural logarithms.
f	activity coefficient; frictional resistance.
g	osmotic coefficient.
k	velocity coefficient; any constant.
m	molality (moles/kg. H_2O).
n	number of moles or equivalents.
p	pressure; negative logarithm.
q	heat absorbed.
r	radius.
t	time; temperature (Centigrade).
t_c, t_a	transference numbers.
u, v	mobilities of ions.
v	volume.
w	work done.
x	distance; mole fraction; concentration of reaction product.
y	fraction transformed.
z	valence.
A	work content; empirical constant.
B	empirical constant.
C	concentration (moles/liter).
D	diffusion coefficient.
E	electromotive force.
E_a	energy of activation.
F	free energy.
$\mathbf{F}$	faraday.
H	heat content.
K	equilibrium constant.
M	molecular weight; molar (moles/liter).
N	Avogadro's number; normal (equivalents/liter).
P	osmotic pressure.

Q_{10} temperature coefficient.
R gas constant.
S entropy.
T temperature (absolute).
U internal energy.
V volume (of solvent).

α gas absorption coefficient (Bunsen).
β gas solubility coefficient (Ostwald).
η viscosity.
λ_c, λ_a ionic conductances.
μ chemical potential; ionic strength; micron.
π 3.1416.
Δ increment.
ΔT_f freezing point lowering.
Λ equivalent conductance.
Σ summation.

1

DIFFUSION IN LIQUIDS

1. Fick's Law of Diffusion.—Perhaps the simplest kind of physicochemical reaction is one which is hardly chemical at all; namely, the diffusion or spontaneous transfer of a dissolved substance from one part of a solution to another. It was shown by Thomas Graham as early as 1850 that the rate of diffusion varies with the nature of the dissolved substance, and the quantitative description of the diffusion process dates from 1855, the year of the publication of Fick's law.[1]

Fick made the reasonable assumption that the flow of matter in diffusion ought to follow the same law as the conduction of heat, which had been treated mathematically by Fourier, with the substitution of quantity of solute for quantity of heat, and of concentration of solute for temperature. That is, the rate of diffusion, or the amount of substance crossing a given area in an infinitesimally short interval of time, ought to be proportional to the cross-sectional area and to the concentration gradient. In symbols,

$$dn = -Da\left(\frac{dc}{dx}\right)_t dt. \qquad (1)$$

Here dn is the amount of substance which passes in the time dt across an area a where the concentration gradient is dc/dx. The constant of proportionality, or the diffusion coefficient of the substance, is represented by D, and the negative sign appears because diffusion takes place from a region of higher concentration to one of lower (Fig. 1). Fick realized that the value of D, for a given solute and solvent, tended to increase somewhat with temperature, and later work has shown that D also tends to increase toward a limiting value as the solution is diluted; the latter trend is especially characteristic of solutions of electrolytes.

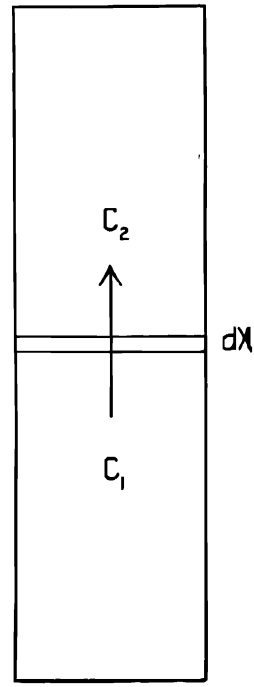

FIG. 1.—Diagrammatic side view of a diffusion cell. The arrow indicates the direction of diffusion if c_1 is greater than c_2. Since this direction is taken as positive, the concentration gradient, dc/dx, is negative.

[1] A. Fick, Ann. Physik und Chemie (Poggendorff), **94** (170): 59, 1855; Phil. Mag., [4] 10: 30, 1855.

Because of these minor variations, it is preferable to refer to D as the diffusion coefficient rather than as the diffusion constant. It will be seen from equation (1) that D has the dimensions of area divided by time. In the centimeter-gram-second system D is expressed in cm.^{2} sec.$^{-1}$, but it is sometimes expressed in square centimeters per day. If dn is expressed in moles and dx in cm., dc must be moles per cc. (not moles per liter).

If the minor variations in D are neglected and the area is fixed, equation (1) still contains four variables. This number may be reduced to three by a consideration of the relation between n and c. In an element of volume bounded by parallel planes of area a separated by the distance dx (Fig. 1), the concentration will be changing in the time dt. The number of moles of substance which enters the element of volume between these planes must differ from the number which leaves it because the concentration gradients at the two planes are not the same. If dn moles enter in the time dt, the number of moles which leave may be written $dn + \left(\frac{d(dn)}{dx}\right)_t dx$, and the amount of substance which accumulates is $-\left(\frac{d(dn)}{dx}\right)_t dx$. This amount divided by the volume adx gives the change in concentration in the time dt, or $-\frac{1}{a}\left(\frac{d(dn)}{dx}\right)_t$. The time rate of change in concentration is, therefore,

$$\left(\frac{dc}{dt}\right)_x = -\frac{1}{adt}\left(\frac{d(dn)}{dx}\right)_t.$$

The last factor may be evaluated by differentiation of equation (1), keeping the time constant:

$$\left(\frac{d(dn)}{dx}\right)_t = -Dadt\left(\frac{d^2c}{dx^2}\right)_t.$$

Multiplication of these two equations yields the second form of Fick's law, which is

$$\left(\frac{dc}{dt}\right)_x = D\left(\frac{d^2c}{dx^2}\right)_t. \tag{2}$$

Equation (2) is the most general equation for diffusion in one dimension. It represents a special case of a still more general equation for three-dimensional diffusion processes.[2]

Before equation (2) can be used, it must be integrated or solved. Such an equation has many different solutions, and the one which is to be applied in any case must be determined by the boundary conditions characteristic of the experiment. For the diffusion of a solute in a vessel of uniform cross-section from an infinitely long column of solution into a similar column of

[2] M. H. Jacobs, Ergebn. d. Biol., 12: 22, 1935; N. Rashevsky, Mathematical Biophysics: University of Chicago Press, Chicago, 1938.

solvent, Stefan[3] obtained a solution of equation (2) which is given by Svedberg[4] in the form

$$c = \frac{c_0}{2}\left(1 - \frac{2}{\sqrt{\pi}}\int_0^y e^{-v^2}dy\right), \tag{3}$$

where

$$y = \frac{x}{2\sqrt{Dt}}.$$

Here c_0 is the initial concentration of solute in the lower layer, and c is the concentration at time t and distance x from the initial boundary. The sign of x is positive if it is measured upward into the original solvent layer.

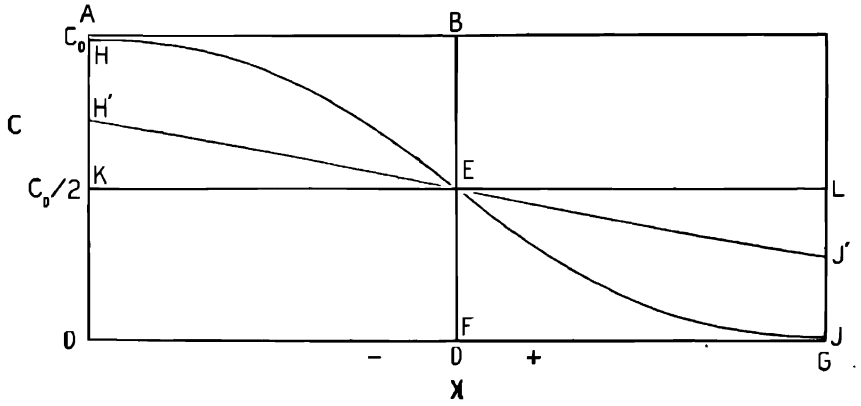

FIG. 2.—Diffusion of solute from a long column of solution into an equally long column of solvent. Abscissas represent distances from the initial boundary; ordinates represent concentrations. At zero time, values of c lie on the broken line ABFG. After a long time, c is constant throughout the system, as shown by the horizontal line KL. At any intermediate time, values of c at different distances fall on a curve of the type HEJ or H'EJ'.

Equation (3) applies to experiments with finite or even short columns if the time is short enough so that there is practically no change due to diffusion at the bottom and the top of the tube. The function

$$\frac{2}{\sqrt{\pi}}\int_0^y e^{-v^2}dy$$

is a probability integral whose values are to be found in mathematical tables.[5] The values of this integral lie between 0 and 1, passing from 0 to 0.9953 as y varies from 0 to 2.00. In using equation (3) for the deter-

[3] J. Stefan, Akad. Wiss. Wien, Sitzungsb. d. Math.-naturw. Cl., **79, II**: 161, 1879.

[4] T. Svedberg, Colloid Chemistry: Chemical Catalog Co., New York, 1928, p. 140.

[5] J. L. Coolidge, An Introduction to Mathematical Probability; Clarendon Press, Oxford, 1925, p. 209; J. B. Dale, Five-Figure Tables of Mathematical Functions: Arnold, London, 1903, p. 84; J. W. Mellor, Higher Mathematics for Students of Chemistry and Physics: Longmans, London, 1919, p. 621; Smithsonian Physical Tables: Smithsonian Institution, Washington, 1933, pp. 56, 60.

mination of D, a value of the integral is obtained by substituting numerical values of c and c_0, the table is used backward to get y, and finally D is obtained from the definition of y and the measured values of x and t. The nature of the variation of c with x, as given by equation (3), is shown in Fig. 2. At the level of the initial boundary ($x = 0$), the equation indicates that c will have the value $c_0/2$ at all times after diffusion has started; if the initial volumes of solution and solvent are equal, $c_0/2$ will also be the final concentration at any level.

In a somewhat different type of experiment, the concentration of the initial layer of solution is maintained at the value c_0 throughout the time of diffusion. For this case the solution of equation (2) is

$$c = c_0\left(1 - \frac{2}{\sqrt{\pi}}\int_0^y e^{-y^2}dy\right) \tag{3a}$$

with the same notation as in equation (3). Equation (3a) was found to apply to diffusion from a solution kept saturated by the presence of solid salt,[6] as well as to diffusion from a large volume of solution, not necessarily saturated, into a tube full of jelly containing the solvent.[7] In such cases the final concentration in the solvent layer will be c_0, and the relation of c to x is exactly like that shown in the lower half of Fig. 2 if the ordinate of the horizontal line KL is taken as c_0 instead of $c_0/2$.

Equations (3) and (3a) yield an important and simple relation which is characteristic of diffusion processes. If c is constant, y must also be constant, and the definition of y indicates that under these conditions x is proportional to $\sqrt{t}$. That is, the distance traveled by a layer of any given concentration should vary directly as the square root of the time of diffusion. This relationship has often been verified; the experimental method is particularly simple when the diffusion takes place in a jelly containing an indicator substance.[8]

2. Measurement of Diffusion Coefficients.—The classical method for the determination of diffusion coefficients involved extremely painstaking experimental work and the creation of special mathematical tables.[9,3] The solute was allowed to diffuse upward from a layer of solution, in the bottom of a uniform cylindrical vessel, into a layer of pure solvent, usually three times as thick as the initial layer of solution. At the end of the allotted time the mixture was separated, with as little disturbance as possible, into four equal layers, and the concentration in each layer was determined by appropriate chemical or physical means. Among those who used this method were Öholm,[10] whose results occupy a prominent place in tables of

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[6] R. Haskell, Physical Rev., **27**: 145, 1908.

[7] G. S. Adair, Biochem. J., **14**: 762, 1920.

[8] W. Stiles, Biochem. J., **14**: 58, 1920; Permeability: Wheldon & Wesley, London, 1924.

[9] W. Kawalki, Ann. Physik und Chemie (Wiedemann), **52** N.F.: 166, 1894.

[10] L. W. Öholm, Ztschr. f. physik. Chem., **50**, 309, 1904; Med. Nobelinst., **2**, 1912.

diffusion coefficients, and Cohen and Bruins, [11] who described refinements of technique which yielded values accurate to 0.3%. An elaborate refractometric method for the determination of the diffusion constants of proteins was developed in Svedberg's laboratory by Lamm and Polson.[12]

Diffusion coefficients are most simply measured by the porous diaphragm method of Northrop and Anson;[13] their improved apparatus is shown in Fig. 3. While this method was devised for approximate studies of biological materials of high molecular weight, it was found by McBain and Liu[14] that it could be made to yield values reproducible within a few tenths of one per cent for the diffusion coefficients of substances of smaller particle size such as sugar or simple electrolytes. The essential feature of the method is the establishment of a constant concentration gradient within the pores of a membrane, freely permeable to the solvent and to the substance under investigation. The membranes are filter disks of alundum or fritted Jena glass.[15] A compartment above the membrane is completely filled with solution (5 to 50 cc.), and diffusion takes place downward, through the horizontal membrane, into a second compartment containing the pure solvent. The material which diffuses through the membrane sinks by gravity,[16] so that the under side of the membrane remains in contact with practically pure solvent, while gravity likewise acts to keep the upper side of the membrane in contact with a solution of practically the original concentration. The attainment of a steady state is checked by removing and analyzing the lower liquid at intervals, replacing it with fresh solvent. When equal amounts of substance are found to pass the membrane in the same time interval, this constant rate of diffusion may be used for the calculation of the diffusion coefficient, provided that the membrane has been calibrated with a substance of known diffusion coefficient. (In their first

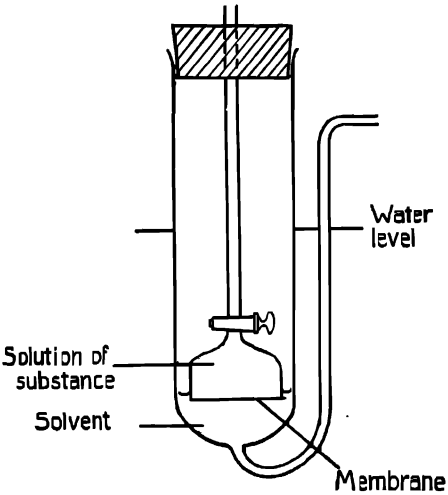

FIG. 3.—Membrane diffusion cell. (From H. W. Scherp, J. Gen. Physiol., 16: 795, 1933.)

[11] E. Cohen and H. R. Bruins, Ztschr. f. physik. Chem., 103: 349, 1923.

[12] O. Lamm and A. Polson, Biochem. J., 30: 528, 1936.

[13] J. H. Northrop and M. L. Anson, J. Gen. Physiol., 12: 543, 1929; 20: 575, 1937.

[14] J. W. McBain and T. H. Liu, J. Am. Chem. Soc., 53: 59, 1931.

[15] Cells with fritted disks of Pyrex glass are listed in the catalogue of the Corning Glass Works, Corning, N.Y.

[16] The specific gravity of the solution must be appreciably higher than that of the solvent; the difference due to one per cent of dissolved protein is sufficient. It is necessary to avoid all stirring, even of the water in the bath by which the temperature is controlled, because of the disturbing effect of vibrations (J. H. Northrop, 1941).

work, Northrop and Anson used 0.1 N HCl as standard, accepting a value of Öholm which they later concluded to be in error. More recently they recommended 2 N NaCl; this substance was preferred because its diffusion coefficient does not vary appreciably with concentration.)

The method is characterized not only by simplicity of technique, but also by the ease with which the diffusion coefficients are calculated. If the amount of substance which has diffused is so small that the initial concentration may be considered to be unchanged, while the lower liquid may be considered to be pure solvent, then the constant concentration gradient is simply $-c/x$, where x is the unknown length of the path of diffusion in the pores of the membrane. The analysis of the lower liquid gives the value of $\Delta n/\Delta t$, which replaces dn/dt in the first form of Fick's law if the rate is constant. The diffusion coefficient is then given by

$$D = \frac{x\Delta n}{ac\Delta t} \tag{4}$$

if a represents the effective but unknown cross-sectional area of the pores of the membrane. The ratio x/a, or the "cell constant" for the membrane, is obtained from the calibration data, and even if c is known only in terms of some arbitrary unit of biological activity per cc. the value of D will have the correct dimensions if Δn is expressed in comparable units. Northrop and Anson considered this simple calculation to be adequate if the amount of substance which diffused through the membrane during an experiment was not more than a few per cent of the total amount present; but they used for the calculation a mean value of c (e.g., $c = 0.98c_0$ if $\Delta n = 4\%$ of the total solute).

The membrane method is not limited to those diffusion experiments in which the initial concentration changes but little. After the diffusion gradient was established by a preliminary run of a few hours, McBain and Liu[14] replaced the lower solution by fresh solvent and started the experiment proper with the upper solution at an unknown initial concentration, c_0; the value of c_0 could be determined from the volumes of the two compartments and the analysis of both solutions at the end of the experiment. For such an experiment Fick's law may be written

$$dn = -\frac{D}{k}(c_2 - c_1)dt,$$

where c_1 and c_2 are the concentrations of the upper and lower solutions, respectively, at the time t, and k is the cell constant, x/a. If the two compartments have the same volume, v, and the diffusion is started with pure solvent in the lower compartment, this equation may be integrated and solved for D:

$$D = \frac{vk}{2t}\ln\frac{c_0}{c_0 - 2c_2}. \tag{5}$$

If the volumes of the upper and lower compartments are not equal, but are v_1 and v_2 respectively, the corresponding relation is

$$D = \frac{v_1 v_2 k}{(v_1 + v_2)t} \ln \frac{c_0}{c_0 - c_2(1 + v_2/v_1)}. \tag{6}$$

The membrane method has been applied in Northrop's laboratory to the study of proteolytic enzymes and of bacteriophage,[17] and in McBain's laboratory to the study of simpler substances, including electrolytes.

3. Diffusion Coefficient and Molecular Size.—Some values for the diffusion coefficients of substances in dilute aqueous solution, at about 20°C., are shown in Table I, together with approximate figures for the molecular weights of these solutes. Evidently there is some sort of inverse relation

TABLE I.—DIFFUSION COEFFICIENTS FOR DILUTE AQUEOUS SOLUTIONS

Substance	Molecular weight	Concentration mole/liter	Temperature °C.	$D \times 10^5$ (cm²/sec.) $\times 10^5$	Reference
H_2	2	0 0	21	5 2	[a]
N_2	28	0 0	22	2.02	[a]
O_2	32	0 0	18	1.98	[a]
HCl	36.5	0 1	19	2.5	[a]
CO_2	44	0 0	20	1.77	[a]
NH_4Cl	53.5	0 2	18	1.5	[a]
NaCl	58 5	0 05	20	1.39	[a]
KCl	74 5	0 1	20	1.676	[a]
Urea	60.	0 25	20	1.18	[a]
Glycerol	92.	0 125	20	0.83	[a]
Glucose	180.	0.0	20	0.60	[a,c] (interp.)
Lactose	342	0 1	20	0 43	[a]
Raffinose	504	0.1	20	0 36	[a]
Myoglobin	17,500	0.0	20	0.1125	[b]
Lactoglobulin	37,900	0 0	20	0 0727	[b]
Hemoglobin	68,000	0 0	20	0.063	[b]
Edestin	309,000	0 0	20	0 0393	[b]
Erythrocruorin (*Lumbricus*)	2,946,000	0.0	20	0.0181	[b]

[a] International Critical Tables, vol. 5, p. 63; McGraw-Hill, New York, 1929.
[b] T. Svedberg, Ind. Eng. Chem., Anal. Ed., 10: 113, 1938.
[c] L. Friedman and P. G. Carpenter, J. Am. Chem. Soc., 61: 1745, 1939.

between the values of D and M. The logarithms of these values are plotted in Fig. 4. The points for substances of high molecular weight show a certain tendency to approach a straight line. Such a linear relation may be obtained by the substitution of appropriate numerical values in the Sutherland-Einstein[18] diffusion equation. This equation may be derived as

[17] J. H. Northrop, Crystalline Enzymes: The Chemistry of Pepsin, Trypsin, and Bacteriophage: Columbia University Press, New York, 1939.
[18] A. Einstein, Ztschr. f. Elektrochemie, 14: 235, 1908.

follows: If a semipermeable, movable piston is imagined at the boundary between two unequally concentrated solutions of a single solute, it will be pushed into the more dilute solution as osmosis takes place. This motion could be prevented by the application to the piston of an external force, equal to the product of the osmotic pressure difference and the area of the piston, or adP. If there is no piston, the concentrations will tend to be equalized by diffusion instead of by osmosis. According to Einstein, the force of diffusion should be equal and opposite to the force required to prevent osmosis, or $-adP$. If the solutions are dilute the osmotic pressure

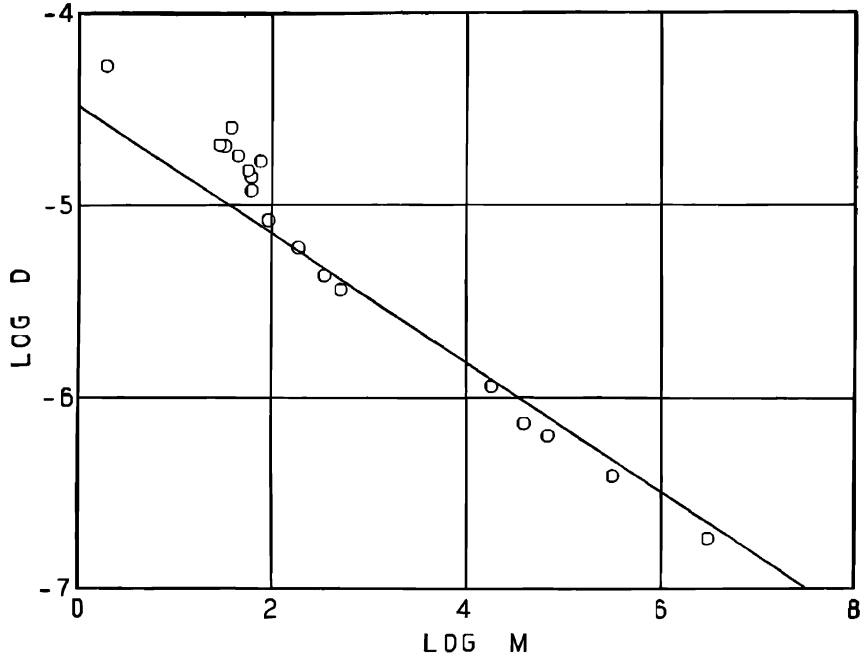

FIG. 4.—Relation between diffusion coefficients and molecular weights of substances in dilute aqueous solution. Logarithmic plot. (Data from Table I.)

will be given by the van't Hoff equation and the force of diffusion will be $-aRTdc$. This force acts on the molecules in a thin element of volume, having the cross-section a and the thickness dx. Its volume will be adx, and it will contain $cadx$ moles or $Ncadx$ molecules of solute if N is Avogadro's number (6.02×10^{23}). The force acting on each molecule will therefore be $-RTdc/Ncdx$. A force acting on a particle in a viscous medium like water produces a velocity proportional to the force; hence the velocity of diffusion may be written

$$\frac{dx}{dt} = -\frac{RT}{Ncf}\frac{dc}{dx},$$

if the constant of proportionality is $1/f$. The constant f may be called the frictional resistance per molecule. The amount of solute crossing the

boundary in the time dt must be equal to the product of the velocity, the concentration, the cross-sectional area, and the time, or

$$dn = -\frac{RT}{Nf} a \frac{dc}{dx} dt.$$

A comparison of this equation with the first form of Fick's law, equation (1), shows that

$$D = \frac{RT}{Nf}, \tag{7}$$

which is the Einstein diffusion equation.[19] If the units of the centimeter-gram-second system are used, D must be expressed in cm.^{2} sec.$^{-1}$ and RT in ergs mole^{-1}. Since N is molecules mole^{-1}, the frictional resistance, f, has the dimensions of force per molecule and per unit of velocity, or dynes sec. cm.$^{-1}$ molecule^{-1}.

The value of f is, in general, unknown, but it would be expected to decrease, as does the viscosity of a liquid, when the temperature is increased. For ordinary temperatures the value of T increases by only 3% for a 10° rise in temperature, while the viscosity of water decreases by about 21%. Actually the diffusion coefficients for many substances in aqueous solution increase by about 25% when the temperature is raised 10°, while equation (7) would suggest about 30%.

For one special case, as Einstein pointed out, the value of f in equation (7) is known. If the diffusing particles are spherical, and much larger than the molecules of the solvent, the frictional resistance is given by Stokes' law, which is

$$f = 6\pi\eta r. \tag{8}$$

Here π is 3.14 . . . , η is the viscosity of the solvent, and r is the radius of the particle. (The derivation of this equation may be found in advanced textbooks of physics.[20])

Equations (7) and (8) yield the Sutherland-Einstein equation,

$$D = \frac{RT}{6\pi\eta r N}, \tag{9}$$

which was verified experimentally by Svedberg[21] for the case of colloidal gold particles of radius 1.3 mμ.

The limitations of equation (9) may be shown as follows. If the dissolved substance consists of spherical particles of radius r and density d, its

[19] This derivation is also a derivation of Fick's law. Since the limiting law for osmotic pressure is not exact except for very dilute solutions, it is to be expected that D will vary with c for solutions of appreciable concentration.

[20] H. Lamb, Hydrodynamics: University Press, Cambridge, 1924, p. 567; L. Page, Introduction to Theoretical Physics: Van Nostrand, New York, 1928, p. 245.

[21] T. Svedberg, Colloid Chemistry: Chemical Catalog Co., New York, 1928, p. 96.

molecular weight should be

$$M = \tfrac{4}{3}\pi r^3 N d. \tag{9a}$$

If this equation is solved for r, and the resulting expression is substituted in equation (9), it follows that D should vary inversely as the cube root of M, other factors being constant. For aqueous solutions the viscosity in equation (9) is that of pure water, or 0.01 in c.g.s. units at 20°C. For substances of very high molecular weight—namely, the proteins—Svedberg found that the partial specific volume was close to 0.75; hence d may be taken as 1.33. The substitution of these figures, with the values of known constants, yields the simple equation:

$$D = \frac{3.21 \times 10^{-5}}{\sqrt[3]{M}}$$

or

$$\log D = -4.49 - 0.33 \log M$$

which should be applicable to dilute solutions of spherical protein molecules at 20°C. The last equation is represented by the straight line in Fig. 4. As might be expected, this linear graph fails to fit the facts for substances of low molecular weight. Surprisingly enough, the points for sugars fall fairly close to the line. The points for proteins appear to lie on a line of the calculated slope, $\frac{1}{3}$, but this observed line is lower than that calculated, or displaced to the left by about 0.3 in log M. This means that if M is calculated from D by combining equations (9) and (9a), the calculated values will be about twice as big as those obtained by the ultracentrifuge. Such results were recorded by Anson and Northrop,[13] who suggested that the discrepancies might be due to hydration of the protein molecules, to deviation from spherical shape, or to both factors.

The Einstein relation (equation (7)), without the assumption of spherical particles, is also used in the derivation of Svedberg's equation for the calculation of molecular weights from measurements of the velocity of sedimentation in the ultracentrifuge. Both this method, which requires an independent measurement of the diffusion coefficient, and the sedimentation equilibrium method, in which the rate of sedimentation is balanced by the rate of diffusion, yield molecular weights independent of molecular shape and hydration.[22] Since the two methods usually give concordant values for the molecular weight, its value as determined by sedimentation equilibrium may be combined with the sedimentation velocity to yield a value for the diffusion coefficient or the frictional resistance, f, of equation (7). The frictional resistance, f_0, for a spherical particle may be calculated by equation (8), using a value for r based on the molecular weight and the partial specific volume of the solute. If the particles are spherical, the ratio f/f_0 will be 1. Svedberg has stated that a deviation from the spherical

[22] T. Svedberg, Ind. Eng. Chem., Anal. Ed., 10: 113, 1938.

shape, or the existence of hydrated molecules, will make this value higher than unity.[23] Svedberg's values of f/f_0 for 44 proteins range from 1.0 to 1.9. The difficulties involved in translating these frictional ratios into ratios of molecular dimensions have been discussed by Neurath,[24] who has also calculated values of the molecular weight and frictional ratio for several proteins from measurements of viscosity and diffusion. As a result of revised sedimentation and diffusion data, Svedberg has assigned to egg albumin (formerly believed to have spherical molecules of weight 34,500) a molecular weight of 40,500 or 43,800 and a frictional ratio of 1.1. If the molecule is not hydrated, and is a prolate ellipsoid (i.e., more like a rod than a disk), Neurath has calculated that its long diameter is about 2.9 times its short diameter.

4. Diffusion of Electrolytes.—In the early days of the ionic theory, Nernst[25] pointed out that, although the two ions of an electrolyte may have different mobilities, they are prevented by electrostatic forces from becoming appreciably separated during free diffusion. In other words, the diffusion of an electrolyte may be described by a single diffusion coefficient. Nernst's relation between the diffusion coefficient and the mobilities of the ions may be derived as follows. At the junction between two unequally concentrated solutions of the same electrolyte there will be a tendency for the more mobile ions to get ahead, producing an electric potential difference dE. This potential difference will be established rapidly and will not increase as diffusion proceeds, since electrostatic forces prevent any appreciable separation of oppositely charged ions. Electrical neutrality must prevail in any tangible portion of the solution. The actual rate of diffusion of the electrolyte is due to the combined influence of electric and osmotic forces.

Nernst considered a diffusion cell containing two solutions of a completely dissociated, uni-univalent electrolyte such as HCl. If the cation were free to move under the influence of the potential gradient, its velocity would be $-udE/dx$, since the mobility u of the cation is defined as its velocity under unit potential gradient. The negative sign means that the motion is opposite to the direction of diffusion if the potential gradient is positive in that direction. The electric force acting on 1 mole of cations is $-FdE/dx$; if dE is expressed in volts, dx in cm., and F in coulombs per chemical equivalent, this force will be expressed in volt-coulombs per cm. and per mole of univalent cations. The ratio of ionic velocity to electric force per mole is, therefore, u/F. It is assumed that the product of this ratio and any kind of force which may be applied to a mole of cations will give the velocity due to this force, since the same sort of frictional resistance is opposing the motion of the ions.

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[23] T. Svedberg and K. O. Pedersen, The Ultracentrifuge: Clarendon Press, Oxford, 1940.

[24] H. Neurath, J. Am. Chem. Soc., **61**: 1841, 1939; H. Neurath, G. R. Cooper, and J. R. Erickson, J. Biol. Chem., **133**: 411, 1940.

[25] W. Nernst, Ztschr. f. physik. Chem., **2**: 613, 1888; Theoretische Chemie: Enke, Stuttgart, 1926.

If there were no electric forces, diffusion could be considered simply as the result of a gradient in osmotic pressure, as in Einstein's derivation of equation (7). In a thin element of volume, adx, the osmotic pressure gradient is $-dP/dx$ and the diffusion force is $-adP$. If the average concentration of the electrolyte in this slice of solution is c moles per cc., this force is exerted on $2cadx$ moles of ions, including both anions and cations. The osmotic force per mole of ions is therefore $-dP/2cdx$. If the solutions are dilute enough to follow van't Hoff's law, the total osmotic pressure, P, is $2RTc$, and dP/dx is $2RTdc/dx$. The osmotic force per mole of ions of either sign is therefore $-RTdc/cdx$. If this force were acting alone on one mole of cations, it would produce a velocity equal to $-uRTdc/\mathrm{F}cdx$.

The actual velocity of the cations must be the sum of the velocities due to electric and osmotic forces, or

$$-u\frac{dE}{dx} - \frac{uRT}{c\mathrm{F}}\frac{dc}{dx}.$$

By similar reasoning it follows that the actual velocity of the anions must be

$$+v\frac{dE}{dx} - \frac{vRT}{c\mathrm{F}}\frac{dc}{dx}$$

if v is the mobility of the anion.

The osmotic terms have the same sign for the two ions, and the sign is negative because diffusion occurs in the direction of a negative concentration gradient. The electrical terms have opposite signs because of the opposite charges of the two kinds of ions; if the electrical gradient is positive in the direction of diffusion, the cation will be retarded and the anion accelerated. The number of equivalents of ions which will pass a given plane as a result of this motion is given by the product of velocity, concentration, cross-section, and time. Because of the requirement of electric neutrality, this number must be the same for positive and negative ions. It follows that

$$dn = -uadt\left(c\frac{dE}{dx} + \frac{RT}{\mathrm{F}}\frac{dc}{dx}\right) = +vadt\left(c\frac{dE}{dx} - \frac{RT}{\mathrm{F}}\frac{dc}{dx}\right). \qquad (10)$$

If this equation is solved for $\frac{dE}{dx}$, we obtain

$$\frac{dE}{dx} = -\frac{u-v}{u+v}\frac{RT}{\mathrm{F}c}\frac{dc}{dx}. \qquad (11)$$

By substitution of this value for dE/dx in either member of equation (10), a simpler expression is obtained for the amount of substance which diffuses:

$$dn = -\frac{2RT}{\mathrm{F}}\frac{uv}{u+v}a\frac{dc}{dx}dt. \qquad (12)$$

A comparison of equation (12) with the first form of Fick's law, equation (1),

gives the Nernst diffusion equation, which is

$$D = \frac{2RT}{\mathbf{F}} : \frac{uv}{u+v}. \tag{13}$$

In this derivation mobility has been expressed in cm.^{2} sec.$^{-1}$ volt^{-1} and force in volt-coulombs cm.$^{-1}$ It follows that equation (13) will yield values of D in cm.^{2} sec.$^{-1}$ if RT is expressed in volt-coulombs mole^{-1} and $\mathbf{F}$ in coulombs equivalent^{-1}, if we recall that mole and equivalent are identical for univalent ions. The values of the constants in equation (13) are, therefore, 8.314 for R and 96,500 for $\mathbf{F}$.

Equation (13) was verified by Öholm's data for the diffusion coefficients of common 1:1 electrolytes in 0.01 N solutions; the observed and calculated values for D have been tabulated by Nernst and others.[25] For halides of the alkali metals the agreement is within about 0.5%, but for strong acids and bases divergences of 5 to 10% are shown.

Equation (13) was modified by Noyes (as reported by Haskell[27]) to cover the case of an electrolyte with ions of valence higher than 1; the factor 2 is replaced by the sum of the reciprocals of the valences of the ions, $\frac{1}{z_c} + \frac{1}{z_a}$. A further modification is convenient, in order to make it possible to use the limiting equivalent conductances of the ions, λ_c and λ_a, in place of their mobilities, u and v. Since u or v is $\lambda/\mathbf{F}$, the factor $1/\mathbf{F}$ must be replaced by $1/\mathbf{F}^2$. With these substitutions equation (13) assumes the form

$$D = \frac{RT}{\mathbf{F}^2} \frac{\lambda_c \lambda_a}{\lambda_c + \lambda_a} \left(\frac{1}{z_c} + \frac{1}{z_a} \right) \tag{14}$$

which is more generally applicable.

If it were possible to have a single electrolyte diffusing without the production of a potential difference, it follows from equation (10) that the amount of cations diffusing across the area a in the time dt would be

$$dn = -\frac{RTu}{\mathbf{F}} a \frac{dc}{dx} dt$$

and by comparison with equations (1) and (14) we may write an ideal diffusion coefficient for an ion of valence z as follows:

$$D_i = \frac{RT\lambda_i}{z\mathbf{F}^2}. \tag{15}$$

Such equations could have real significance for a single electrolyte only if λ_c and λ_a were equal. For the ions of KCl, λ_c and λ_a differ by less than 4%, but no salt is known for which they are exactly equal. To illustrate the difference between such ideal diffusion coefficients for single ions and the

[26] W. Nernst, Theoretische Chemie: Enke, Stuttgart, 1926, p. 435; H. S. Taylor, A Treatise on Physical Chemistry, 2: Van Nostrand, New York, 1931.

[27] R. Haskell, Physical Rev., 27: 145, 1908.

real diffusion coefficient of the electrolyte as a whole, we may apply equation (15) to the ions of HCl at 25°. Here λ_c is 349.8 and λ_a is 76.3; the calculated values of D_c and D_a are 9.31×10^{-5} and 2.03×10^{-5}, while equation (13) or (14) gives 3.33×10^{-5} as the actual diffusion coefficient of HCl in a very dilute solution. Evidently the electric forces produce a marked retardation in the motion of the faster ion and a smaller acceleration in that of the slower.

5. Diffusion in Mixed Electrolytes.—These hypothetical ionic diffusion coefficients have been regarded as limiting values of some real significance in the light of an interesting discovery made by Arrhenius[28] and used by him as additional evidence for the validity of the theory of electrolytic dissociation. Arrhenius found that the presence of a second solute generally retarded the diffusion of a dissolved substance, except in one special case. When both solutes were strong electrolytes, a marked acceleration of diffusion was observed. This occurred with HCl, for example, when it was allowed to diffuse into water containing NaCl, and also when the NaCl concentration was uniform throughout and only the lower layer contained HCl at the beginning of the experiment. By reasoning similar to that of Nernst, Arrhenius showed that this acceleration could be calculated from the mobilities of the ions and the concentrations of salt and acid in the diffusion layer. Even when the NaCl was initially present at the same concentration in all parts of the system, it was necessary to assume that its ions would diffuse; in the case of Na^+, this would be a back diffusion as a result of the electrical gradient produced by the unequal mobilities of H^+ and Cl^-. If there were a small backward movement of a large number of Na^+ ions, it would be possible for a small number of H^+ ions to go forward a considerable distance without disturbing electrical neutrality or bringing strong re[illegible] forces into play, even though the Cl^- ions diffused much less rapidly than the H^+ ions. The equations of Arrhenius showed that the acceleration depended on the ratio of salt to acid rather than on the absolute concentration of salt, and this was borne out by his experiments. His calculations also indicated that if this ratio were very large the rate of diffusion of the acid should approach the calculated limiting value for the H^+ ion, and that the liquid junction potential should approach zero. The highest diffusion constants reported by Arrhenius for HCl diffusing into salt solutions were about twice those found in the absence of salt, while the calculated limiting ratio is 2.8.

The effect of a salt with an ion in common on the diffusion of an acid was also formulated quantitatively by Abegg and Bose,[29] who reported qualitative experiments tending to verify the theory.

More recently, McBain has studied the acceleration or retardation of diffusion in mixed electrolytes. He found acceleration even beyond the

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[28] S. Arrhenius, Ztschr. f. physik. Chem., **10**: 51, 1892.

[29] R. Abegg, and H. Bose, Ztschr. f. physik. Chem., **30**: 545, 1899.

calculated limiting values in certain cases where one electrolyte diffused against another, or with another into pure water.[30] This was ascribed to the existence of electrical gradients produced by the ions of the second electrolyte. Such experiments are likely to prove useful in explaining the behavior of electrolytes in and around tissues.

6. Diffusion Across Membranes and Ionic Distribution.—Interesting experiments along these lines were carried out by Teorell, with the object of explaining the distribution of electrolytes between the stomach contents and surrounding tissues. He found[31] that, when a small volume of 0.16 *N* NaCl was dialyzed through parchment against a large volume of 0.16 *N* HCl, there was a temporary accumulation of chloride in the salt solution; its concentration became as high as 0.19 *N*, while that of the outer acid was still 0.16. This unequal distribution of chloride was in the same direction as that observed in a Donnan equilibrium when the membrane is impermeable to the positive ion of the inner solution. In a later paper, Teorell[32] worked out equations for the effect of a steady state produced by the continuous diffusion of an electrolyte between two solutions in which the concentration of this electrolyte was kept at two different but constant levels. He concluded that such a steady state should result in an unequal distribution of the ions of a second electrolyte, present in fixed amount, and that the ratios of ionic concentrations for the second electrolyte should follow Donnan's equation for membrane equilibrium, even though the membrane was permeable to all the ions present. His deductions were approximately confirmed by the results of a few experiments with simple electrolyte solutions separated by four sheets of permeable cellophane.[33] In an experiment in which 0.1 *N* perchloric acid diffused into 0.1 *N* ammonium chloride, the ion NH_4^+ was drawn back into the inside solution until its concentration was over twice that in the solution from which it came, while the reciprocal of this ratio represented the distribution of Cl^- ions after the steady state had been attained.

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J. W. Williams and L. C. Cady, "Molecular Diffusion in Solution," Chem. Rev., **14**: 171–217, 1934.

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[30] J. W. McBain and C. R. Dawson, J. Am. Chem. Soc., **56**: 52, 1934; J. R. Vinograd and J. W. McBain, J. Am. Chem. Soc., **63**: 2008, 1941.

[31] T. Teorell, J. Physiol., **78**: 11P, 1933.

[32] T. Teorell, Proc. Nat. Acad. Sc., U.S., **21**: 152, 1935.

[33] T. Teorell, J. Gen. Physiol., **21**: 107, 1937.

2

REACTION VELOCITY AND ENZYME ACTION

1. Chemical Kinetics.—This branch of physical chemistry includes the study of the rates of chemical reactions and the factors which influence such rates. The velocity of a chemical reaction may be defined as the rate of decrease in the concentration of one of the reacting substances; the dimensions of reaction velocity are those of concentration divided by time, or, in customary units, moles/(liters × sec.). Because of the speed of many inorganic reactions, particularly those between ions in solution, the laws of chemical kinetics were established by the study of slower, organic reactions, especially hydrolytic reactions. This class of reactions is of prime importance in biochemistry.

The fundamental principle of chemical kinetics is the law of mass action, which was formulated by Guldberg and Waage[34] in 1864 and 1867. Some of the consequences of this law were independently formulated and verified by other workers.[35] According to the law of mass action, the velocity of a chemical reaction is proportional to the product of the active masses of the reacting substances, each active mass being raised to a power equal to the number of molecules of that substance which appears in the chemical equation for the reaction. For a reaction which takes place in a uniform environment, the active mass is proportional to the concentration, and concentrations in moles per liter are generally used in kinetic equations in place of active masses. Guldberg and Waage recognized, however, that only a fraction of all the molecules of each kind would, in general, be in a condition capable of reacting on collision, and that the velocity would then depend on the product of several coefficients as well as on the product of the concentrations.

2. Reactions of the First Order.—If a reaction involves a decrease in the concentration of only one substance, the law of mass action may be written in the differential form

$$-\frac{dC}{dt} = kC, \tag{1}$$

[34] C. M. Guldberg and P. Waage, J. pract. Chem., **127** (N.F. **19**): 69, 1879. This and earlier Norwegian papers were reprinted in German in Ostwald's Klassiker der exakten Wissenschaften, No. 104: Engelmann, Leipzig, 1899.

[35] L. Wilhelmy, Ann. Physik. (Poggendorff) **81**: 413, 499, 1850; Ostwald's Klassiker der exakten Wissenschaften, No. 29, 1891; A. V. Harcourt and W. Esson, Phil. Tr. Roy. Soc. (London), **156**: 193, 1866; **157**: 117, 1867.

where C represents the concentration of the reacting substance and t represents time after the beginning of the reaction. The constant of proportionality, k, is termed the velocity constant, velocity coefficient, or specific reaction rate. Evidently k would be equal to the actual velocity if the concentration were unity. Since dC/C is $d \ln C$, equation (1) may be integrated to give the equation

$$kt = \ln \frac{C_0}{C} = 2.303 \log \frac{C_0}{C}, \qquad (2)$$

in which C_0 is the initial concentration, or the value of C when t is zero. Reactions whose rates are expressed by equation (1), which contains only the first power of one concentration factor, are called reactions of the first order. Equation (2) is also called the equation for a first-order reaction, or the unimolecular equation. Although reactions which are truly unimolecular in mechanism are relatively rare, the first-order equation applies to many reactions which might appear to be of higher order. Such a pseudo-unimolecular reaction must result if the concentrations of all reacting substances except one are so high that the amounts by which they change during the reaction are mathematically insignificant.

It was the study of such a reaction, the hydrolysis of sucrose in aqueous nitric acid, that provided the first verification of equation (2) in the classical experiments of Wilhelmy,[35] who followed the course of the reaction by measuring the optical rotation of the solution. Some of his results, recalculated to give the fraction of sucrose remaining at different times, are plotted in Fig. 5, in which the upper curve is a direct plot of this fraction against the elapsed time. The velocity of the reaction is proportional to the negative slope of this curve, and its regular decrease is consistent with equation (1) but is not a specific test of the applicability of the first-order equation. The lower graph is a plot of the logarithm of the reciprocal of the fraction unchanged. According to equation (2) this logarithm should be proportional to the time, and the graph should be a straight line passing through the origin and having a positive slope equal to $k/2.303$ (if the times are plotted in suitable units). The fit of the points on the straight line is sufficient to illustrate the verification of equation (2), and modern work of greater precision has served to confirm Wilhelmy's conclusion. Wilhelmy noted that the temperature of his laboratory rose from 15.5 to 18° during the first part of this experiment, and fell to 14.5° at the time of the last observation which is shown; this rise may account for the apparent acceleration shown by two points.

In testing a set of data to find out whether they follow the first-order equation, it is not necessary to calculate the fraction unchanged, or even the actual values of the concentration, provided that some property is measured which varies in a linear way with concentration. Such a property might be the reading of a polarimeter in angular degrees, or the refractive index of the solution, or the total volume of the solution, as measured by

the height of the meniscus of the solution in the capillary tube of a dilatometer. If such a property decreases linearly with the concentration, and if R is the reading at time t and R_e the reading after the reaction has stopped, equation (2) will be verified if a plot of log $(R - R_e)$ against t is a straight line. The intercept of such a line (at $t = 0$) is a measure of the total change, log $(R_0 - R_e)$, and it is sometimes convenient to determine

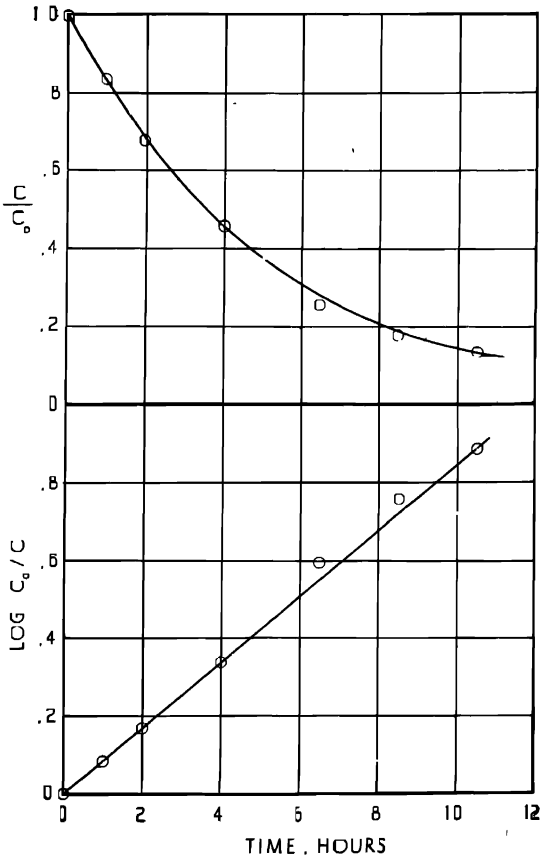

FIG. 5.—A first-order reaction. Hydrolysis of sucrose in dilute nitric acid. (Data of L. Wilhelmy, Ann. Physik, 81: 413, 1850.)

the value of the initial reading, R_0, by such a linear extrapolation. If the reading decreases as the reaction proceeds, and if the equation fits the data, the downward slope of the semilogarithmic plot is equal to $k/2.303$.

Equations (1) and (2) may look superficially different if other notation is used. If x represents the loss in concentration during the time t, and a the initial concentration, the equations become

$$+\frac{dx}{dt} = k(a - x) \qquad (1a)$$

and

$$kt = 2.303 \log \frac{a}{a - x}. \tag{2a}$$

If y represents the fraction of the substance which has been transformed in the time t, equation (2) may be written

$$kt = 2.303 \log \frac{1}{1 - y}. \tag{2b}$$

Evidently the graphical relations are quite similar; a plot of x or y against t, if inverted, will look like the upper curve in Fig. 5. Equation (2) may also be written in exponential form as

$$C = C_0 e^{-kt} \tag{2c}$$

and the corresponding forms of equations (2a) and (2b) may readily be derived.

It may be seen by inspection of any of the preceding equations for first-order reactions that the velocity coefficient is independent of the units in which concentration is expressed. In fact, the unimolecular k has the dimensions of the reciprocal of time, or 1/sec. Equation (2) provides a physical significance for the value of k; it is the reciprocal of that time required for the transformation of a fraction of the original material such that $\ln C_0/C$ is 1, and the fraction transformed in this time is $1 - \frac{1}{e}$, or 0.632. It is sometimes convenient to describe the velocity of a reaction in terms of the time required for the transformation of some other definite fraction of the reacting substance. Equation (2) shows that the half-transformation time or half-period is related to k by the equation

$$kt_{0.5} = 2.303 \log 2 = 0.693.$$

The half-period of a unimolecular reaction is therefore independent of the initial concentration.

3. Reactions of the Second Order.—If a reaction involves a decrease in the concentrations of two kinds of reacting molecules, the reaction is said to be bimolecular. Its velocity is proportional to the product of the two concentrations, and if each of these factors is raised only to the first power the reaction is described by an equation of the second order. The second-order equation assumes a particularly simple form if the initial concentrations of the two reacting species are identical. In this case the differential equation may be written

$$-\frac{dC}{dt} = kC^2 \tag{3}$$

and the integral form is

$$kt = \frac{1}{C} - \frac{1}{C_0}. \tag{4}$$

Equation (4) applies to the hydrolysis or saponification of an ester by an equivalent amount of a strong alkali. The course of such a reaction is illustrated by Fig. 6, which was plotted from data obtained long ago by Warder,[35] who prepared a solution containing 0.02 *M* ethyl acetate and 0.02 *M* sodium hydroxide and followed the course of the reaction at 20.5–20.6°C. by titrating measured samples at intervals. The upper part of

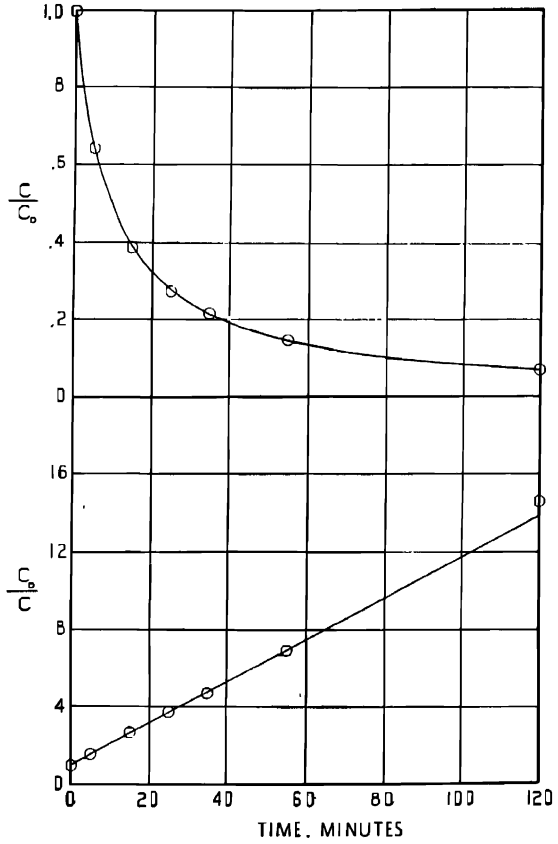

Fig. 6.—A second-order reaction. Saponification of 0.02 *M* ethyl acetate in 0.02 *M* sodium hydroxide. (Data of R. B. Warder, Am. Chem. J., 3: 340, 1881–82.)

the figure is a plot of the fraction remaining unchanged as a function of time, and it shows a smooth decrease in the velocity of the reaction as well as in the concentration of either substance. If this curve is compared with one for a unimolecular reaction by changing the time scale to bring the curves together at one point, say that at which half of the reaction has taken place, it may be seen that the bimolecular reaction is faster than the unimolecular in the early stages, but slower in the later stages of the reaction.

[35] R. B. Warder, Am. Chem. J., 3: 340, 1881–85

The applicability of equation (4) to a set of data may be tested by plotting the reciprocal of the concentration against the time; the result should be a straight line with an intercept equal to $1/C_0$ and a slope equal to k. A similar test is shown in the lower part of Fig. 6, in which the ordinates are values of C_0/C. Here the intercept must be unity and the slope should be C_0k. Inspection of Fig. 6 shows that Warder's data are well represented by equation (4) throughout at least 85 per cent of the total change.

If a bimolecular reaction is started with unequal initial concentrations, a and b, of the reacting substances, and the change in either concentration in the time t is represented by x, the differential equation for the velocity may be written

$$\frac{dx}{dt} = k(a - x)(b - x). \tag{5}$$

Before this equation can be integrated, one must use the method of partial fractions to show that

$$\frac{1}{(a - x)(b - x)} = \frac{1}{b - a}\left(\frac{1}{a - x} - \frac{1}{b - x}\right).$$

Then the integral form becomes

$$(b - a)kt = \ln \frac{a(b - x)}{(a - x)b}. \tag{6}$$

This equation may be tested as a straight line by plotting $\log \frac{b - x}{a - x}$ against t. A direct plot of $a - x$ or $b - x$ against t, for a reaction which follows equation (6), will be intermediate in shape between the upper curves of Figs. 5 and 6. If the value of b in equation (6) is very much larger than that of a, it will also be very much larger than any possible value of x. In that case the ratio $(b - x)/b$ may be considered equal to one and cancelled in the logarithm. Equation (6) then becomes identical with equation (2a), the unimolecular formula, except that the apparent unimolecular velocity coefficient is equal to the product of the bimolecular coefficient and the difference in initial concentrations, $b - a$.

It may be seen directly from equation (3) or (5) that any second-order velocity coefficient must have the dimensions of the reciprocal of a product of time and concentration, such as liters/(moles $\times$ sec.). In comparing the velocity coefficients of reactions which follow equations of the second order it is, therefore, important to note the units used for concentration as well as for time.

If the rate of a second-order reaction is expressed as the time for half-transformation of one of the reacting substances, the relation between $t_{0.5}$ and k depends on the ratio of the initial concentrations. If this ratio

is unity, equation (4) yields the relation

$$kt_{0.5} = \frac{1}{C_0} = \frac{1}{a} = \frac{1}{b}.$$

If b is greater than a, the half-period is the time when x is equal to $a/2$. For that case equation (6) shows that

$$kt_{0.5} = \frac{2.303}{b - a} \log \left(2 - \frac{a}{b}\right).$$

Equations for the rates of reactions of orders higher than the second may also be derived from the law of mass action, but such reactions are not often found. Complicated reactions are apt to proceed by a mechanism involving several steps, and the rate is controlled by the slowest step in the series, usually a reaction of the first or second order. Examples may be found in well known textbooks.[37]

4. The End of a Reaction.—In the preceding discussion it has been assumed that each reaction goes to completion; that is, that the concentration of at least one of the reacting substances becomes zero during the period of observation. The integrated equations, however, indicate that this would theoretically require an infinite time, and this might seem to make it impossible to calculate k by a method involving a final measurement of some property. For a first-order reaction, it may be calculated from equation (2) that an error of 1 part in 1000 in the measurement of the total change would produce an error of about 1.5 parts in 1000 in a value of k calculated from a determination near the time of half-transformation, and such an error is not excessive. Hence it is legitimate to take a reading at 99.9 per cent completion as final, and equation (2) shows that the time required would be about ten times the half-period.

In the case of a second-order reaction which follows equation (4), an error of 1 part in 1000 in the final reading would make an error of about 3 parts in 1000 in a value of k determined near the half-period, and this error is not unreasonable. The time for 99.9 per cent completion, however, would be 999 times that for 50 per cent completion! Evidently such reactions are best followed by a direct measurement of C. For a reaction which follows equation (6) the corresponding calculated values would lie between those obtained from equations (2) and (4).

5. Rates of Reversible Reactions.—If a reaction is reversible, it may appear to stop in a state of equilibrium, with finite amounts of the reactants and the products all present. The rate of such a reaction may sometimes be followed in either direction. If both the direct and the reverse reactions are of the first order, the differential equation for the observed rate is

[37] J. H. van't Hoff, Studies in Chemical Dynamics, trans. by T. Ewan: Williams and Norgate, London, 1896, p. 25; J. W. Mellor, Chemical Statics and Dynamics: Longmans, Green and Co., London, 1904, Chapter V; F. O. Rice and H. C. Urey, in H. S. Taylor's Treatise on Physical Chemistry: Van Nostrand, New York, 2nd ed., 1931, p. 964.

$$-\frac{dC}{dt} = k_1 C - k_2(C_0 - C). \tag{7}$$

This equation implies that the reaction is started with one reacting substance at the concentration C_0 but with none of the reaction product present. At the time t the concentration of the reactant is C, while that of the product is $C_0 - C$. Equation (7) is based on the principle that the two opposing reactions proceed simultaneously, and that the rate of each follows the law of mass action. The observed or net rate, $-dC/dt$, is equal to the difference between the rates of the opposing reactions. At equilibrium the net rate is zero or the opposing rates are equal, and this condition may be expressed by the equation

$$\frac{C_0 - C_e}{C_e} = \frac{k_1}{k_2}, \tag{8}$$

which defines the equilibrium constant of the reversible reaction in terms of the initial concentration, C_0, and the equilibrium concentration, C_e, of a single substance. If C_0 is eliminated from equations (7) and (8) the differential equation becomes

$$-\frac{dC}{dt} = (k_1 + k_2)(C - C_e) \tag{9}$$

and the integral form is

$$(k_1 + k_2)t = 2.303 \log \frac{C_0 - C_e}{C - C_e}. \tag{10}$$

Equations (9) and (10) differ from the equations for a nonreversible reaction of the first order only in the significance of the constant factor and in the use of a value of C_e other than zero. Kinetic experiments do not distinguish between reversible and nonreversible reactions of the first order if the constant is determined from initial and final readings of some property dependent on the concentration, together with readings at various times during the reaction. Such reactions can be distinguished if the actual concentration at equilibrium is determined. In the case of a reversible reaction, equation (8) gives the ratio, k_1/k_2, while equation (10) yields the sum, $k_1 + k_2$. Hence a study of the course of the reaction in one direction, together with a knowledge of the equilibrium condition, makes it possible to obtain values for the separate velocity coefficients of the opposing reactions.

It may be shown that equations (9) and (10) are still valid when some of the product as well as the reactant is present at the start of the reaction. A similar treatment of opposing bimolecular reactions results in more complicated equations, but again it is possible to obtain the velocity coefficients of the opposing reactions if the course of the reaction and the equilibrium condition are both investigated.[38]

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[38] J. W. Mellor, Chemical Statics and Dynamics: Longmans, London, 1904, Chapter IV; W. C. McC. Lewis, A System of Physical Chemistry: Longmans, London, 1920, Vol. I, p. 406.

6. Reaction Velocity and Temperature.—It is well known that most chemical reactions proceed more rapidly at higher temperatures. For any given reaction a plot of rate against temperature is far from linear, but has a decided upward curvature. The factor by which the rate is multiplied as a result of a rise in temperature of 10°C. is sometimes called the temperature coefficient of the reaction; it may be defined by the equation

$$Q_{10} = \frac{k_{t+10}}{k_t},$$

where the subscripts of the velocity coefficients refer to Centigrade temperatures. If the factor Q_{10} were constant over an extensive temperature range, the relation between k and t would be given by a logarithmic or exponential equation, and a plot of log k against t would be a straight line, represented by an equation of the form

$$\log k = a + bt.$$

By substituting two values of k, corresponding to an interval of 10° in t, it may readily be shown that the slope, b, is equal to 0.1 log Q_{10}. For any temperature interval, $t_2 - t_1$, the linear relation may therefore be written in the form

$$\log Q_{10} = \frac{10}{t_2 - t_1} \log \frac{k_2}{k_1}.$$

This equation can be used to calculate Q_{10} if the temperature interval of the observations is not 10°. In general, however, a plot of log k against t is not a straight line over a wide range of temperature, and a given reaction will have different values of Q_{10} for different 10° intervals. The left side of Fig. 7 shows a plot of log k against t for the hydrolysis of sucrose in hydrochloric acid solutions. The curve indicates that Q_{10} decreases as t is increased; in this case its actual values are 5.55 for the first two points and 4.27 for the last two. This reaction happens to have a rather high temperature coefficient. Many chemical reactions have Q_{10} values between 2 and 4, while a physical process such as diffusion or viscous flow is apt to have a temperature coefficient in the neighborhood of 1.2 to 1.3. At present the coefficient Q_{10} is not often used in describing the effect of temperature on reaction rates.

A better method of representing temperature effects was discovered by Arrhenius,[39] who noticed that an excellent straight line was obtained by plotting the logarithm of a velocity coefficient against the reciprocal of the absolute temperature (Fig. 7, right side). This finding may be described by the equation

$$\log k = B - \frac{A}{T}, \tag{11}$$

[39] S. Arrhenius, Ztschr. f. physik. Chem., **4**: 226, 1889.

where A and B are empirical constants. This equation may be put into the equivalent form

$$\ln k = 2.303B - \frac{E_a}{RT} \tag{11a}$$

or

$$\frac{d \ln k}{dT} = \frac{E_a}{RT^2} \tag{11b}$$

if the numerical value, A, of the slope of the linear plot is replaced by $E_a/2.303R$. Here R is the constant of the perfect gas law, 1.987 calories per mole and per degree; E_a is then $4.575A$, and it has the dimensions of

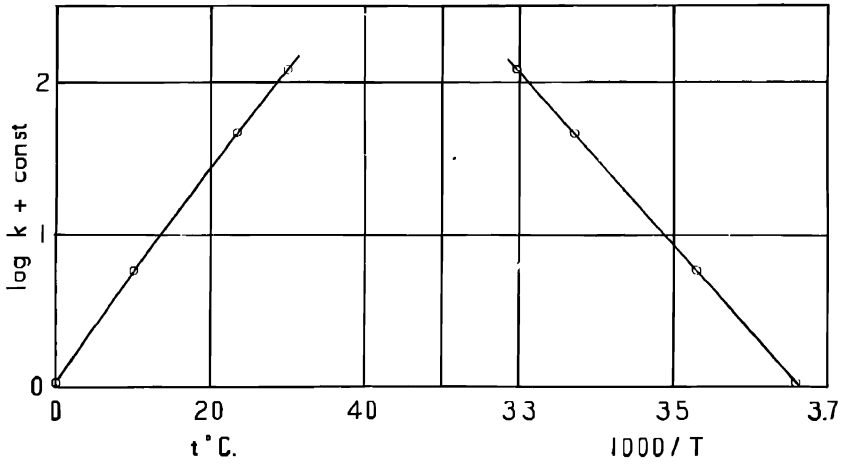

FIG. 7.—Influence of temperature on reaction velocity. Hydrolysis of sucrose (2 g. per 100 cc.) in 0.577 M hydrochloric acid. (Data of L. J. Heidt and C. B. Purves, J. Am. Chem. Soc., **62**: 1006, 1940.)

calories per mole. If E_a is to be evaluated from only two experimental points, equation (11a) may be rearranged to give

$$\ln \frac{k_2}{k_1} = \frac{E_a}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \tag{11c}$$

or

$$E_a = \frac{4.575 T_1 T_2}{T_2 - T_1} \log \frac{k_2}{k_1}. \tag{11d}$$

Any of these equivalent expressions may be called the Arrhenius equation for the effect of temperature on reaction velocity. Such effects are commonly described in terms of the value of E_a, which is called the energy of activation or the critical increment (of energy).

Arrhenius based his interpretation of E_a as an energy of activation upon the following considerations. He knew that the effect of temperature on

the equilibrium constant of a reaction was described by the equation

$$\frac{d \ln K}{dT} = \frac{q}{RT^2}, \tag{12}$$

in which q is the heat absorbed when the reaction proceeds as written in the chemical equation to which the equilibrium constant, K, applies. This equation had been derived thermodynamically by van't Hoff for an equilibrium involving perfect gases or perfect solutes. In order to deduce a similar relation for a velocity coefficient, Arrhenius assumed that the ordinary molecules of the reacting substance were always in equilibrium with a small proportion of active molecules, and that the equilibrium constant of this reaction was given by the law of mass action:

$$\mathrm{A} \rightleftharpoons \mathrm{A}'; \qquad K = \frac{C_{\mathrm{A}'}}{C_{\mathrm{A}}}.$$

He assumed further that the observed rate was determined by the rate of decomposition of the active molecules to yield the products of the reaction, and that this rate followed the law of mass action:

$$-\frac{dC_{\mathrm{A}}}{dt} = -\frac{dC_{\mathrm{A}'}}{dt} = k'C_{\mathrm{A}'} = k'KC_{\mathrm{A}} = kC_{\mathrm{A}}.$$

The observed velocity coefficient, k, would then be equal to the product of the hypothetical velocity coefficient, k', for the decomposition of the active molecules, and the hypothetical equilibrium constant, K. If k' were independent of temperature and K were influenced according to equation (12), then the influence of temperature on k would be given by equation (11b) and E_a would represent the energy absorbed in the transformation of one mole of ordinary molecules into the active state.

The Arrhenius hypothesis has been modified by the belief that the active molecules differ from ordinary molecules only in their energy content, but the idea that the slope of the empirical line gives a measure of the energy of activation constitutes the foundation of modern work in chemical kinetics. Numerous examples of the application of the Arrhenius equation to a wide variety of chemical reactions are to be found in special treatises.[40] The Arrhenius equation has also been shown to describe the effects of temperature on the rates of many biological processes.[41]

7. Homogeneous Catalysis in Solutions.—Many reactions which proceed with measurable velocity in aqueous solution are accelerated by the presence of relatively small amounts of certain substances which are not,

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[40] E. A. Moelwyn-Hughes, The Kinetics of Reactions in Solution: Clarendon Press, Oxford, 1933; F. Daniels, Chemical Kinetics: Cornell University Press, Ithaca, 1938; S. Glasstone, K. J. Laidler and H. Eyring, The Theory of Rate Processes: McGraw-Hill, New York, 1941.

[41] W. J. Crozier, J. Gen. Physiol., 7: 123, 1924, and numerous later papers in the same journal; T. C. Barnes, Textbook of General Physiology: Blakiston, Philadelphia, 1937, p. 515.

in general, used up by the reaction. Predominant among such catalysts are the hydrogen[42] and hydroxyl ions, which are responsible for the marked effects of *p*H on reaction rates. For example, the mutarotation of glucose proceeds very rapidly in acid or alkaline solutions, but has a zone of minimal velocity extending over a range of 2 or 3 *p*H units; in this case the center of the region of minimal velocity is on the acid side of the neutral point. The rates of this and similar reactions may be expressed by an equation of the type

$$k = k_0 + k_{\mathrm{H}} C_{\mathrm{H}} + k_{\mathrm{OH}} C_{\mathrm{OH}}, \tag{13}$$

where k_0 represents the minimal value of the velocity coefficient, and the last two terms represent the catalytic effects of H^+ and OH^-. The direct proportionality implied in equation (13) is consistent with the law of mass action if a catalyzed unimolecular reaction is treated as a bimolecular reaction in which the concentration of one reactant, the catalyst, does not change. If a reaction

$$\mathrm{A} \rightarrow \mathrm{B}$$

proceeded only in the presence of hydrogen ions, we might write the observed reaction as

$$\mathrm{A} + \mathrm{H}^+ \rightarrow \mathrm{B} + \mathrm{H}^+,$$

and the kinetic equation would be

$$-\frac{dC_{\mathrm{A}}}{dt} = k_{\mathrm{H}} C_{\mathrm{A}} C_{\mathrm{H}}. \tag{14}$$

The value of k_{H} could then be obtained by dividing the observed velocity coefficient, as obtained from the simple unimolecular formula, by C_{H}. If the rate were not determined solely by hydrogen ion catalysis, it would be equal to the product of C_{A} and the sum of several terms, as in equation (13).

The experimental verification of equation (13) is very good for the effect of hydrogen ions, but rather less quantitative for the effect of hydroxyl ions. This uncertainty results from the fact that the catalytic effect of hydroxyl ions is often very great. If it is studied in sufficiently dilute solutions of a strong base, the hydroxyl ion concentration is uncertain because of contamination from the air or from the containing vessel. If the solution is buffered, there may be additional catalytic effects due to acids or bases (in the sense of Brönsted) other than H^+ or OH^-; these effects are considered in Brönsted's theory of general acid-base catalysis.[43] An illustration of the catalytic effects of hydrogen and hydroxyl ions is

[42] In aqueous solutions the hydrogen ion is hydrated to form H_3O^+, the hydronium, oxonium, or hydroxonium ion. In the present discussion, the older name and symbol are used for this ion. The recognition of the state of hydration or solvation of the hydrogen ion is particularly important in comparative studies of reactions in various solvents.

[43] J. N. Brönsted, Chem. Rev., **5**: 231, 1928; R. P. Bell, Acid-Base Catalysis: Clarendon Press, Oxford, 1941.

given in Fig. 8, which shows the velocity coefficients for the mutarotation of glucose in dilute hydrochloric acid and in ammoniacal buffer solutions. Fig. 8 shows that the excess velocity above the minimum is directly proportional to the concentration of either catalyst, and the straight lines constitute a graph of equation (13). Because of the reciprocal relation between C_H and C_{OH}, it appears that hydroxyl ions have a negligibly small catalytic effect in acid solutions, while the same is true for hydrogen ions in alkaline solutions. This method of plotting compresses the region of minimal velocity, where both ions might be effective catalysts, into a single

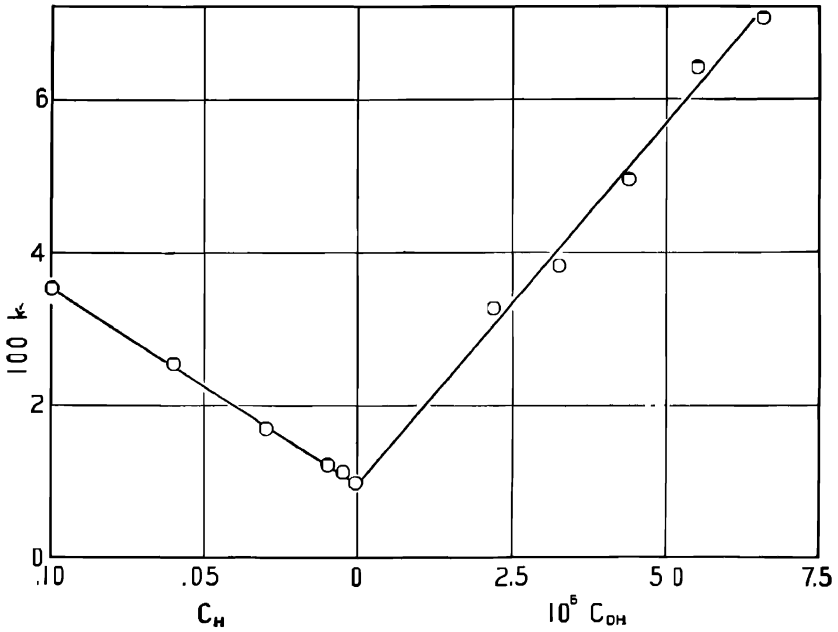

Fig. 8.—Catalysis by hydrogen and hydroxyl ions. Mutarotation of glucose at 25°. (Data of C. S. Hudson, J. Am. Chem. Soc., **29**: 1571, 1917, for hydrochloric acid solutions, and of Y. Osaka, Ztschr. f. physik. Chem., **35**: 661, 1900, for buffer solutions of ammonia and ammonium chloride (0.0225 *N*).)

point corresponding to the k_0 of equation (13). The true value of k_{OH} is probably less than the slope of the line in the right side of Fig. 8, for Brönsted and Guggenheim[44] have pointed out that some of the catalysis observed in these buffers was probably due to ammonia molecules, while Smith[45] has found evidence that a large fraction of the catalysis in alkaline solutions may be due to the catalytic effect of negative ions formed by the acidic dissociation of glucose itself.

In order to explain homogeneous catalysis, it is assumed that the reaction proceeds through a transition state in which there exists an intermediate

[44] J. N. Brönsted and E. A. Guggenheim, J. Am. Chem. Soc., **49**: 2554, 1927.

[45] G. F. Smith, J. Chem. Soc., **1936**: 1824, 1936.

compound or critical complex, of higher potential energy than that of the reactants or that of the products of the reaction. It was assumed by Bjerrum,[46] as in Arrhenius's original theory of active molecules,[39] that the critical complex is always in equilibrium with the reacting substances, including the catalyst. If such an equilibrium is governed by the classical mass law, in terms of concentrations, the kinetic equation resulting from this hypothesis is identical with equation (14), provided that the rate depends on the concentration of the critical complex existing at any time. If the hypothetical equilibrium is governed by the thermodynamic law of mass action, in terms of activities, an equation of the same form results, provided that the activity coefficients are not changed by the progress of the reaction. On the basis of this theory, a reaction

$$A \rightarrow B$$

catalyzed by hydrogen ions would be written in the form

$$A + H^+ \rightleftharpoons AH^+ \rightarrow B + H^+$$

with an equilibrium constant defined by the equation

$$K = \frac{C_{AH} f_{AH}}{C_A C_H f_A f_H}$$

in which f represents an activity coefficient. The kinetic equation would then become

$$-\frac{dC_A}{dt} = kC_{AH} = kKC_A C_H \frac{f_A f_H}{f_{AH}}$$
$$= k_H C_A C_H \frac{f_A f_H}{f_{AH}}. \qquad (15)$$

Evidently equations (14) and (15) are of the same form if the ratio of activity coefficients is constant. In comparative experiments with widely different concentrations, especially of electrolytes, this will not be the case, and equation (15) was originally proposed by Brönsted[47] to explain the effect of added salts on reaction velocity. If the substance A is a neutral molecule, the intermediate complex and the hydrogen ion will be ions of the same charge, and the activity coefficient ratio should be little influenced by an added salt. If A is a negative ion, the activity coefficient ratio will be decreased by added salt, and the rate of the reaction will show a corresponding decrease. If A is a cation, the complex will be a cation of higher valence, and the activity coefficient ratio will be increased by added salt, which will therefore increase the reaction rate. Salt effects of this kind, either positive or negative, are called primary salt effects, and their magnitude and direction have been shown by Brönsted and others[48] to agree with the Debye-Hückel theory of interionic attraction in salt solutions. There

[46] N. Bjerrum, Ztschr. f. physik. Chem., 108: 82, 1924; cf. R. P. Bell, ref. 43.

[47] J. N. Brönsted, Ztschr. f. physik. Chem., 102: 169, 1922; also ref. 43.

[48] V. K. La Mer, Chem. Rev., 10: 192, 1932; R. P. Bell, ref. 43.

are also secondary salt effects; here an added salt influences the activity coefficients in a second equilibrium, such as the ionization of a buffer, and thus indirectly produces a change in the concentration of a substance, such as hydrogen or hydroxyl ion, which is directly concerned in the reaction whose rate is measured. The Brönsted theory of salt effects on reaction velocity has been well verified for solutions of ionic strengths up to about 0.1. For a reaction involving a neutral molecule and a univalent ion—that is, for most reactions catalyzed by H^+ or OH^-—the primary salt effect at 0.1 ionic strength is not apt to be more than 5 to 12 per cent of the rate. A secondary salt effect may be of the order of 50 per cent or more under comparable conditions.

Electrolyte effects of an altogether different order of magnitude have been found in concentrated solutions of strong acids. In the acid hydrolysis of sucrose, for example, the classical proportionality between reaction velocity and hydrogen ion concentration is strictly exact only for concentrations well below 0.1 *M*. The upper part of Fig. 9 illustrates the magnitude of the deviations from linearity observed with concentrated solutions. The velocity coefficient obtained with 4 molar acid is about 600 per cent greater than that which would be expected from the results at low concentrations, as shown by the distance between the observed curve and the extrapolated broken line. The lower part of Fig. 9 shows that such a curve may be rectified by plotting the logarithm of the catalytic coefficient of the hydrogen ion, $\log (k/C_{HCl})$, against the acid concentration, as has been pointed out by recent workers.[49] This linear relation corresponds to an equation of the form

$$\log \left(\frac{k}{C_{HCl}} \right) = a + bC_{HCl},$$

and it is consistent with the existence of a primary electrolyte effect due to the acid itself, although the effect is far greater than most primary salt effects. This interpretation follows from equation (15) if k/C_{HCl} is identified with $k_H f_H f_A / f_{AH}$ and if the logarithm of this ratio of activity coefficients is a linear function of the ionic strength, as might be expected from a consideration of the ionic types involved. However, Brönsted has not claimed that his equation includes all effects to be found in such concentrated solutions.

An interesting relation between reaction rates in very acid solutions and the behavior of basic indicators was discovered by Hammett and Paul.[50] They plotted the logarithms of the velocity coefficients observed for the hydrolysis of sucrose in a number of concentrated solutions of strong acids against a newly defined acidity function, and obtained a straight line of unit slope. This result amounts to a linear relation, with unit slope,

[49] M. Duboux, Helvet. chem. acta, **21**: 236, 1938; P. M. Leininger and M. Kilpatrick, J. Am. Chem. Soc., **60**: 2891, 1938.

[50] L. P. Hammett and M. A. Paul, J. Am. Chem. Soc., **56**: 830, 1934.

between $\log k$ and $\log (C_{BH}/C_B)$ where BH is the acid (cationic) form and B is the basic (uncharged) form of the indicator. In other words, the velocity coefficient itself was directly proportional to the concentration ratio of the two forms of the indicator, even though this ratio was not simply related to the hydrogen ion concentration nor to any other measure of acidity which had previously been suggested. Their result may also be taken to mean

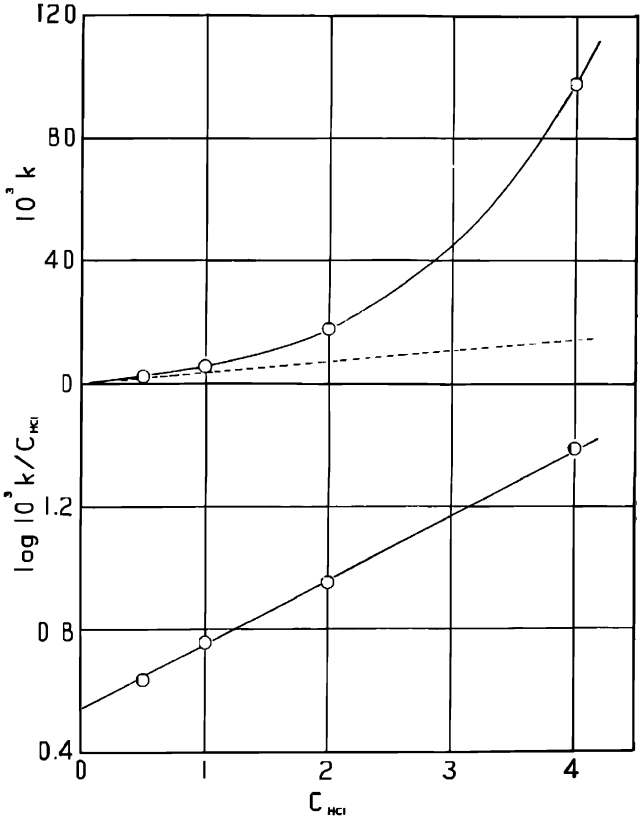

FIG. 9.—Catalysis by hydrogen ions in concentrated solutions. Hydrolysis of 10 per cent sucrose in hydrochloric acid solutions at 25°. (Data of A. Hantzsch and A. Weissberger, Ztschr. f. physik. Chem., **125**: 251, 1927.)

that k was directly proportional to $C_H f_H f_B / f_{BH}$, an expression which is of the same form as that given by the Brönsted theory in equation (15).

Experiments with reactions catalyzed by acids have brought to light another divergence, usually of lesser magnitude, from the requirements of classical theory as represented by equation (14). This is an effect of the concentration of the principal reactant or substrate on the value of the velocity coefficient. For example, the unimolecular constant for the hydrolysis of sucrose in 0.1 N sulfuric acid was found by Jones and Lewis[51] to be increased by about 34 per cent when the sucrose concentration was

[51] C. M. Jones and W. C. McC. Lewis, J. Chem. Soc., **117**: 1120, 1920.

increased from 10 to 50 g. per 100 cc. A similar effect at lower concentrations of sucrose is represented in Fig. 10. Here the rate of hydrolysis, at 25°, in 0.486 *M* hydrochloric acid, is contrasted with the rate produced by the enzyme invertase in dilute buffer solutions. The reciprocal of the half-period was used as a measure of the specific reaction rate because the enzyme reaction does not follow a first-order equation; for the acid hydrolysis, $1/t_{0.5}$ is proportional to the observed values of k. It may be seen that a fivefold increase in the sucrose concentration produced an increase of about 10 per cent in k for the acid hydrolysis; this effect may well be due to a change in the value of $f_H f_A / f_{HA}$ in equation (15). The far-greater opposite

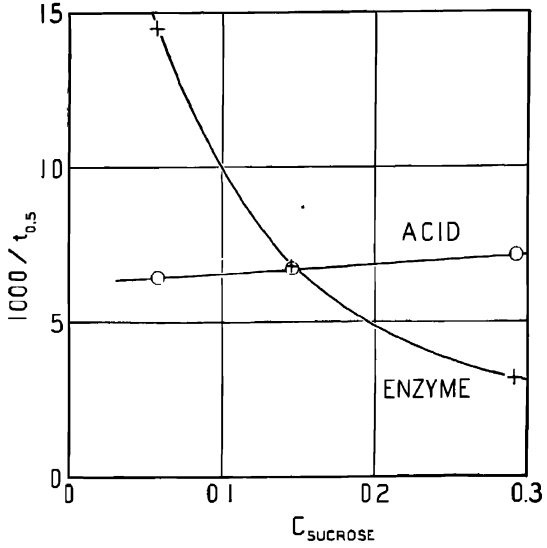

FIG. 10.—Effect of sucrose concentration on the specific velocity of hydrolysis catalyzed by hydrochloric acid (0.486 *N*) or by invertase (19.0 mg. per l.) at 25°. Abscissas, moles sucrose per liter. Ordinates, 1000 times reciprocal of half-period in minutes. (Data of D. I. Hitchcock and R. B. Dougan, J. Phys. Chem., **39**: 1177, 1935.)

effect which appears in the catalysis by the enzyme requires a different explanation.

8. Catalysis by Enzymes.—Enzymes are organic catalysts formed in living cells. Although no enzyme has yet been synthesized in the laboratory, several have been isolated in crystalline form, presumably as pure substances. The first enzyme to be crystallized was urease, isolated from jack beans by Sumner in 1926. Since that time, a number of proteolytic enzymes of animal origin have been crystallized by Northrop[52] and others. These crystalline enzymes appear to be proteins, but, since it is very difficult to apply the ordinary criteria of chemical purity to molecules as large as proteins, some workers still doubt that the crystalline enzymes are pure chemical individuals. When enzymes are dissolved or dispersed in

[52] J. H. Northrop, Crystalline Enzymes: Columbia University Press, New York, 1939.

aqueous media, they form colloidal solutions. This property has led some investigators to classify the action of enzymes as heterogeneous catalysis, with emphasis on the possible adsorption of the reacting substances on the surface of the enzyme particles. Other workers, however, have preferred to consider the enzyme solutions as molecular dispersions, laying emphasis on the analogies between enzyme action and homogeneous catalysis, and attempting to apply the laws of classical physical chemistry as in the case of true solutions.

The reactions catalyzed by enzymes may be classified roughly as those involving the hydrolytic splitting of chemical linkages, and those involving oxidation-reduction phenomena. Theoretically enzyme reactions, like other catalytic reactions, should be reversible, but such reversal has been definitely established in laboratory experiments in relatively few cases; enzymes which accelerate the hydrolysis of ester linkages have been shown to accelerate the reverse reaction. According to the usual definition of a catalyst, its presence ought not to shift the equilibrium point of the catalyzed reaction. Yet there are many reactions which do not take place at all in the absence of a catalyst, but go to completion if the proper enzyme is present. In such cases it may be argued that the original rate is not zero, but simply too low for convenient measurement. If so, the reactants without the catalyst are only in false or apparent equilibrium. In other cases the equilibrium concentrations seem to vary with the total enzyme concentration. This may be true if the enzyme is destroyed during the reaction, or if it combines with one of the reactants or products. In the latter case, there may be a second equilibrium which should be considered simultaneously with the first; if it is neglected, the first equilibrium will appear to be shifted.

There are a few principles governing the action of enzymes which seem to be quite general, and most of these were first established in the study of yeast invertase (also called saccharase or sucrase), an enzyme which catalyzes the hydrolysis of sucrose to form glucose and fructose. This enzyme has not yet been isolated as a pure or crystalline substance, and there is no good evidence that it can accelerate the reverse reaction. The frequency with which this enzyme has been studied is due to the ease with which it can be obtained in highly active and stable form, rather than to its intrinsic importance, but its behavior seems to be fairly typical of that found with other enzymes.

9. Effect of Enzyme Concentration.—In general, the velocity of a reaction catalyzed by an enzyme is directly proportional to the concentration of the enzyme. This simple relation was established for yeast invertase by O'Sullivan and Tompson in 1890, and is illustrated in Fig. 11. It was formerly believed (Schütz, 1885) that with pepsin the reaction rate increased only as the square root of the enzyme concentration, but Northrop[53] found that direct proportionality could be obtained with pepsin and

[53] J. H. Northrop, Harvey Lectures, 21: 36, 1925–26.

trypsin if inhibiting substances were first removed by dialysis of the enzyme preparations.

10. Effect of Hydrogen Ion Concentration.—If an enzyme reaction is studied in solutions of controlled and varied *p*H, it is usually found that there is an optimum region of acidity or alkalinity specific for the most efficient action of each enzyme on a particular substrate. Such *p*H effects were first described by Sørensen in 1909 for invertase, pepsin, and catalase. The *p*H effect is generally explained (Michaelis and Davidsohn, 1911) as due to two factors, an irreversible destruction of the enzyme by acid on one side of the optimum, and a reversible ionization of the enzyme itself on the less acid side. On this theory the active portion of an enzyme such as invertase consists of the unionized molecules or dipolar ions of the ampho-

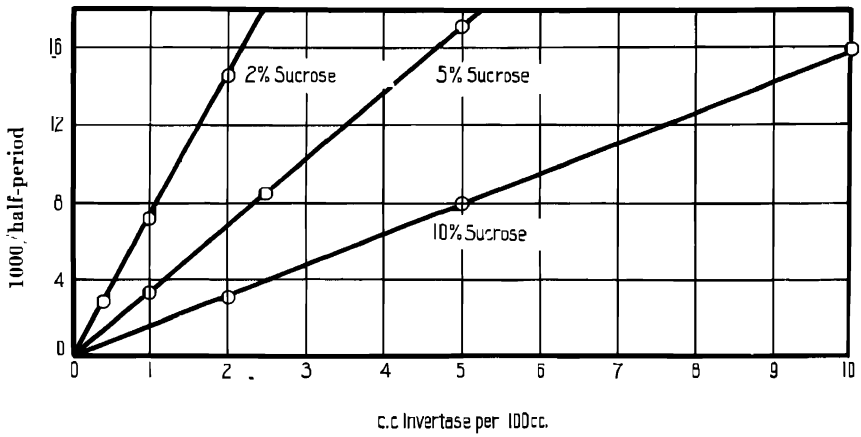

FIG. 11.—Direct proportionality of reaction velocity to enzyme concentration. (From D. I. Hitchcock and R. B. Dougan, J. Physical Chem., **39**: 1177, 1935.)

teric enzyme. The resemblance between the *p*H-activity curve for invertase, on the alkaline side, and the dissociation residue curve of a weak acid was confirmed for three different temperatures by Nelson and Bloomfield.[54] In the case of pepsin and trypsin, Northrop[53] pointed out that there was a certain parallelism between the *p*H-activity curves and the titration or ionization curves of the proteins used as substrates. In any case, it seems to be well established that the effect of *p*H on the velocity of most enzyme reactions is described by a curve shaped like a hump. Examples are to be found in books on enzymes.[55]

11. Effect of Substrate Concentration.—The marked effect of the sucrose concentration in decreasing the specific velocity of invertase action is shown in Fig. 10. This effect has usually been discussed in terms of the

[54] J. M. Nelson and G. Bloomfield, J. Am. Chem. Soc., **46**: 1025, 1924.

[55] J. B. S. Haldane, Enzymes: Longmans, London, 1930; E. Waldschmidt-Leitz, Enzyme Actions and Properties, trans. by R. P. Walton: Wiley, New York, 1929; H. Tauber, Enzyme Chemistry: Wiley, New York, 1937.

actual velocity rather than the specific velocity. A quantity proportional to the actual velocity may be obtained by multiplying the ordinates of Fig. 10 by the initial substrate concentration, or by noting the initial rate of change of the property which is measured in following the reaction. If the relative velocity is obtained in either of these ways, the curve assumes quite a different shape. While the specific velocity decreases with increasing substrate concentration, the actual velocity increases to a rather flat maximum, as shown in Fig. 12. Such curves have been interpreted, on the adsorption theory, by assuming that the surface of the enzyme becomes gradually covered or saturated with substrate as the initial concentration of the latter is increased. A purely chemical explanation was proposed by

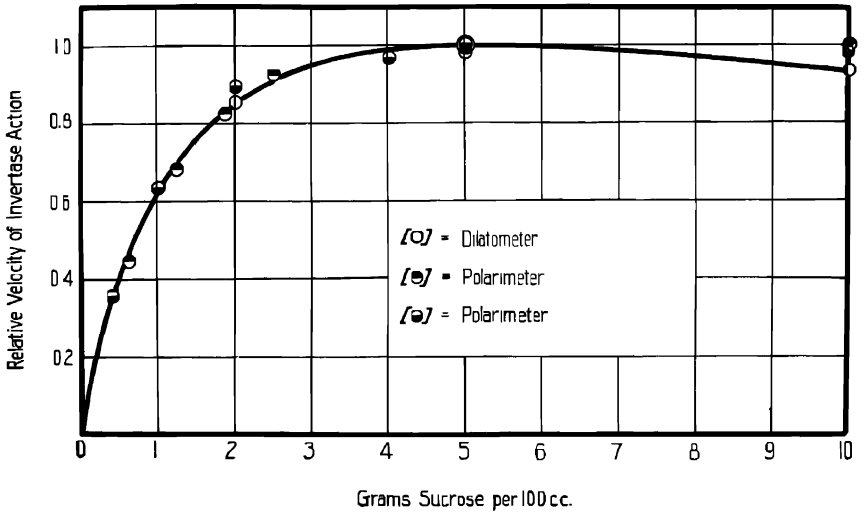

FIG. 12.—Effect of substrate concentration on the velocity of enzyme action. (From D. I. Hitchcock and R. B. Dougan, J. Physical Chem., **39**: 1177, 1935.)

Michaelis and Menten.[55,55] These workers plotted the relative velocity against the logarithm of the initial substrate concentration, and noticed that the curve then assumed the familiar sigmoid form characteristic of a mass law equilibrium, such as the dissociation of a weak electrolyte. They were able to derive an equation having such a curve by assuming that there was reversible combination between the enzyme and its substrate, and that the intermediate complex so formed was always in equilibrium, according to the law of mass action, with its constituents. They also assumed that the rate of the reaction was governed, at any time, by the concentration of the intermediate complex, but that the amount of substrate combined at any instant was a negligibly small fraction of the total. So far their assumptions are identical with those used by Arrhenius and Brönsted in explaining homogeneous catalysis. A difference is to be found in their final assumption,

[55] L. Michaelis and M. L. Menten, Biochem. Ztschr., **49**: 333, 1913.

which is that the amounts of free and combined enzyme are of the same order of magnitude. If the hypothetical equilibrium is

$$E + S \rightleftharpoons X,$$

its equilibrium constant (the "Michaelis constant") will be

$$K_m = \frac{C_E C_S}{C_X},$$

where C_E is the concentration of free enzyme, C_S, that of the total substrate and C_X, that of the complex. However, if C_T represents the total enzyme concentration, C_E may be obtained from the relation

$$C_T = C_E + C_X$$

with the result that

$$K_m = (C_T - C_X)\frac{C_S}{C_X}$$

or

$$C_X = C_T \frac{C_S}{K_m + C_S}.$$

If the rate is proportional to C_X, this equation makes it proportional to the total enzyme concentration, as it is known to be, while the last fraction accounts for the form of the saturation curve of Fig. 12. The equation shows that when C_S is much smaller than K_m, C_X is proportional to C_S; while when C_S is much larger than K_m, C_X is constant and equal to C_T. Evidently when C_S is equal to K_m, C_X will be equal to $C_T/2$. Accordingly the Michaelis constant is equal to that concentration of substrate which produces half of the maximal velocity. The reciprocal of K_m is considered to be a measure of the affinity of an enzyme for its substrate. Michaelis and Menten carried their theory still further by assuming a similar combination of the enzyme with each of the products of the reaction, and worked out an equation for the kinetics of invertase action on this basis. Although this theory has been widely adopted, there are several experimental results which it seems not to fit.[57]

Possible variations of the Michaelis theory which may be applicable to other enzyme reactions, as well as simple graphical methods of obtaining the hypothetical dissociation constants, were discussed by Lineweaver and Burk.[58]

A kinetic equation formally identical with that of Michaelis and Menten was derived from different assumptions by Van Slyke and Cullen (1914). They assumed that the time required for the formation and decomposition of the intermediate complex were of the same order of magnitude. The bearing of this hypothesis on the measurement of enzyme activity has been pointed out by Van Slyke.[59]

[57] J. M. Nelson, Chem. Rev., **12**: 1, 1933.

[58] H. Lineweaver and D. Burk, J. Am. Chem. Soc., **56**: 658, 1934.

[59] D. D. Van Slyke, Advances in Enzymology, **2**: 33, 1942, in which he has corrected the integrated equations on pp. 34, 35, and 39 by substituting x for S.

12. Very Rapid Enzyme Reactions.—The majority of enzyme reactions which have been studied *in vitro* have been relatively slow, with half-periods measured in minutes or hours. The range of velocities accessible to experimental study was greatly increased by the development of an ingenious apparatus (Hartridge and Roughton, 1923) by which extremely rapid reactions, with half-periods measured in fractions of a second, could be studied. The use of this technique led to the discovery of carbonic anhydrase (Meldrum and Roughton, 1933), an enzyme which accelerates the hydration of carbon dioxide to form carbonic acid, as well as the reverse reaction. The physiological significance of this enzyme was reviewed by Roughton.[60]

Another form of apparatus for recording the progress of fast reactions was described by Stern and Du Bois,[61] who were particularly interested in the enzyme catalase, which is obtained from liver and decomposes hydrogen peroxide. Stern reported the observation of spectroscopic changes which he interpreted as confirming the existence of a fugitive intermediate complex formed by this enzyme with another substrate, ethyl hydrogen peroxide, in agreement with the theory of Michaelis and Menten.

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S. Glasstone, Text-Book of Physical Chemistry: Van Nostrand, New York, 1940.
J. B. S. Haldane, Enzymes: Longmans, London, 1930.

[60] F. J. W. Roughton, Physiol. Rev., **15**: 241, 1935.
[61] K. G. Stern and D. Du Bois, J. Biol. Chem., **115**: 575, 1936.

3

ELEMENTS OF THERMODYNAMICS*

1. The Nature and Terminology of Thermodynamics.—Thermodynamics is that branch of physical science which deals with transformations of energy, particularly of heat and work. Classical thermodynamics consists of deductions made from two fundamental laws whose general validity is accepted as the result of experience. During the last century, these laws have been usefully applied in physics, engineering, and chemistry, and there is no reason to doubt their applicability to processes which take place in living organisms. Many of the conclusions drawn from thermodynamics are negative rather than positive. It may be shown that a certain process is thermodynamically impossible; if so, it will not occur spontaneously. On the other hand, if a process is shown to be thermodynamically possible, it does not follow that it will occur at a measurable rate. Thermodynamics does not deal with rates, but rather with states of equilibrium.

A thermodynamic system usually means a limited portion of space, with the matter which occupies that space. If the system is a closed system, matter is not transferred across its boundaries. If the system is isolated, there is no exchange of either matter or energy between the system and its surroundings. The exchanges of energy between a system and its surroundings, however, are the principal subject of thermodynamic discussions.

The state of a system is defined in terms of properties which are measurable on a macroscopic scale. These properties may be the statistical result of the behavior of individual molecules, but thermodynamics does not deal with single molecules. A knowledge of the state of a system implies a knowledge of the kind and quantity of matter, of its distribution between the solid, liquid, and gaseous states, and of the volume, pressure, and temperature. For any homogeneous portion of matter, the last three of these properties are related by an equation of state, which may or may not be explicitly known. In the application of thermodynamics to simple cases, it is assumed that influences of gravitational, electric, and magnetic fields are excluded or may be neglected.

A thermodynamic process takes place whenever there is a change, either physical or chemical, in the state of a system. A thermodynamic cycle includes processes by which a system undergoes a change of state along one

* The writer is indebted to Dr. Scott E. Wood, of the Department of Chemistry, Yale University, for reading and criticizing the manuscript of this chapter.

path, but is returned to its initial state by way of another path. An equilibrium state is one which does not change spontaneously with the lapse of time. Cases are known of apparent but false equilibria, as in a mixture of hydrogen and oxygen gases at ordinary temperatures. Here it may be assumed that the gases are actually reacting to form water, but that the rate is too slow to be measurable; it is well known that the presence of a trace of a suitable catalyst will cause a rapid and violent approach to a condition of stable thermodynamic equilibrium.

2. The First Law of Thermodynamics.—This law is nothing more than the principle of the conservation of energy, applied with the recognition that heat is a form of energy (Helmholtz, 1847). It is comprised in the statements that the energy of an isolated system is constant, and that any exchange of energy between a system and its surroundings must occur without the creation or destruction of energy. The first law may be expressed by the equation

$$\Delta U = q - w. \tag{1}$$

Here q represents the heat absorbed by a system from its surroundings, and w represents work done by the system on its surroundings. Energy can be transferred only as heat or work if there is no exchange of matter. The function represented by U is called the internal energy. Absolute values of U are not known; only changes in internal energy can be measured. The expression ΔU means a finite increment in U; it is the difference between the values of U at the end and the beginning of the process which is being considered. For a given change in state, the value of ΔU is independent of the path by which the change takes place. This is also expressed by saying that U is a function of the state of the system, or that dU is an exact differential. Similar statements would not be true of q or w; the amounts of heat and work which are transferred during a change in the state of a system do depend on the path.

Unless some special arrangement is made (as in a galvanic cell), the only sort of work done by a system on its surroundings is work of expansion. The term w in equation (1) may then be replaced by $p\,\Delta v$ if the pressure is constant, or by $\int_{v_1}^{v_2} p\,dv$ if the pressure varies during the change in state of the system. If a process occurs at constant volume, with no arrangement for the production of electrical or other work, the term w becomes zero. This is the case when a chemical reaction takes place in a constant volume calorimeter. The first law then becomes

$$-\Delta U = -q_v. \tag{2}$$

The heat evolved by a reaction which proceeds at constant volume is equal to the decrease in internal energy.

If a reaction takes place without any constraint other than that of constant pressure, the first law implies that the heat absorbed is equal to

$\Delta U + p\,\Delta v$. It is possible to state this fact in terms of another thermodynamic function called the heat content or enthalpy, which is defined by the equation

$$H = U + pv. \tag{3}$$

The heat content is also a function of the state of the system. If the pressure remains constant, it follows that ΔH is equal to $\Delta U + p\,\Delta v$, or

$$-\Delta H = -q_p. \tag{4}$$

The heat evolved by a reaction in a constant pressure calorimeter is equal to the decrease in the heat content function. The usefulness of this function is due to the fact that most processes are studied at constant pressure.

3. Spontaneous Processes and Reversible Processes.—Any system which is not in equilibrium undergoes a spontaneous change in the direction of equilibrium. Familiar examples include the falling of a weight toward the earth, the flow of heat from a hot object to a cooler one, and the diffusion of a gas or a dissolved substance into a region of lower concentration. It is possible to reverse the direction of some of these processes by the suitable application of energy. In thermodynamics, however, any actual or spontaneous process is said to be irreversible, because of a special meaning which is assigned to the word reversible. In order for a process to be thermodynamically reversible, it would have to be possible to restore the system to its original state by the expenditure of only as much energy as had been lost by the system in the original process. Conceivably this might be true for the expansion of a gas in a cylinder provided with a perfectly fitting but frictionless piston if the external pressure were infinitesimally lower than that of the gas. The reversible process is an ideal which is approached only by small and slow real changes when frictional and other forms of resistance are negligible. An infinitesimal change from a state of equilibrium is considered to be reversible. The concept of a reversible process is useful in the calculation of limiting values for the energy changes which accompany real processes.

4. The Second Law of Thermodynamics.—This law states a restriction which has been observed in the transformation of heat into work. This restriction has been stated by Planck[52] as follows: "It is impossible to construct an engine which will work in a complete cycle, and produce no effect except the raising of a weight and the cooling of a heat-reservoir." This law denies the possibility of perpetual motion of the second kind, by which work would be produced from heat without other changes. It does not deny that work can be produced from heat by means of a suitable heat engine, for in this case there is always a transfer of a considerable fraction of the heat absorbed from a hotter to a cooler portion of the surroundings. It was shown by Carnot (1824) that the theoretical maximum work done

[52] M. Planck, Treatise on Thermodynamics, trans. by A. Ogg: Longmans, London, 1927.

by a cyclical heat engine is

$$w = q_1 \frac{T_1 - T_2}{T_1} \tag{5}$$

if q_1 is the heat absorbed at the higher temperature, T_1. This maximum work could be obtained only if every step in the operation were thermodynamically reversible.

If such an ideal heat engine is considered as a thermodynamic system, the completion of a cycle restores the system to its original state. The value of ΔU is therefore zero for any number of cycles. It follows from the first law that

$$w = q_1 + q_2, \tag{6}$$

where $-q_2$ is the heat given up to the reservoir at the lower temperature, T_2. Equations (5) and (6) may be combined to give a relation,

$$\frac{q_1}{T_1} + \frac{q_2}{T_2} = 0, \tag{7}$$

which is valid for a cycle involving the reversible transfer of heat between two parts of the surroundings at different temperatures. This may be put into the form

$$\sum \frac{q_r}{T} = 0 \tag{8}$$

for any reversible cycle. The sum of such ratios, $\Sigma(q_r/T)$, for any reversible process was termed by Clausius (1854) the increase in entropy of the system. Changes in the entropy, S, accompanying a reversible process, are defined by the equation

$$\Delta S = \sum \frac{q_r}{T}. \tag{9}$$

The fact that ΔS is zero for a cyclical process is consistent with the idea that the entropy is determined by the state of the system, and it can be proved that the change in entropy, like the change in internal energy, is independent of the path by which a definite change in state occurs.

If the cycle of a heat engine includes an irreversible process, the work done is less than that given by equation (5), for some heat is lost by frictional or other resistance. Since $-q_2$ is greater than before, $\Sigma(q/T)$ is less than zero. Because the process is cyclical and S is a function of the state of the system, ΔS is still zero. It follows that

$$\Delta S > \sum \frac{q_{ir}}{T} \tag{10}$$

for a cycle which includes an irreversible process, and it can be shown that this inequality is valid for any irreversible process, whether it be cyclical

or not. The change in entropy accompanying an irreversible process is not given by the sum of the actual q/T terms, but must be obtained from the corresponding terms for a reversible process which produces the same change in state.

In terms of entropy, the second law of thermodynamics is comprised in the relations (9) and (10). If changes in the entropy of the surroundings as well as of the system are considered, this law may be stated in the form that any irreversible process is accompanied by an increase in the total entropy of all bodies concerned in the change, while a reversible process takes place with no change in entropy.

5. Work Content and Free Energy.—An alternative statement of the second law, in terms of work rather than heat or entropy, was once made by Lewis[63] as follows: "Every process that occurs spontaneously is capable of doing work; to reverse any such process requires the expenditure of work from outside." The significance of this statement was clarified by the use of two additional thermodynamic functions which had been employed by Gibbs[64] in 1875. The work content, A (maximum work function, Helmholtz free energy), and the free energy, F (thermodynamic potential, Gibbs free energy), are defined by the equations

$$A = U - TS \tag{11}$$

$$F = U - TS + pv = A + pv = H - TS. \tag{12}$$

These equations indicate that each of these quantities is a definite function of the state of a system.

For a process which takes place at constant temperature, the change in A is

$$(\Delta A)_T = \Delta U - T\,\Delta S. \tag{13}$$

If the process is reversible, equation (13) may be combined with equations (1) and (9) to give

$$-(\Delta A)_T = w_{max.} \tag{14}$$

since the work done by a reversible process is the maximum work which can be obtained from a given change in state. If the same change in state takes place along a path which is not completely reversible, the decrease in A will be the same, but the work done will be less. The work done approaches the decrease in A as the process becomes more nearly reversible.

For a process which takes place at constant temperature and at constant pressure, the change in F is

$$(\Delta F)_{T,p} = \Delta U - T\,\Delta S + p\,\Delta v. \tag{15}$$

If the process is also reversible, equation (15) may be combined with equations (1) and (9) to give

$$-(\Delta F)_{T,p} = w_{max.} - p\,\Delta v. \tag{16}$$

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[63] G. N. Lewis, J. Am. Chem. Soc., **35**: 1, 1913.

[64] J. W. Gibbs, Collected Works, Vol. I: Longmans, New York, 1928.

The decrease in free energy accompanying any process at constant temperature and pressure is equal to the maximum net work, exclusive of work of expansion. If the process is not completely reversible, the decrease in free energy is greater than the actual net work, which approaches $-\Delta F$ as the reversibility of the process is improved.

Since any spontaneous process is thermodynamically irreversible, such a process will yield an amount of work less than that obtainable from the same change of state by a reversible path. Instead of equations (14) and (16), the inequalities

$$-(\Delta A)_T > w \tag{17}$$

$$-(\Delta F)_{T,p} > w - p\,\Delta v \tag{18}$$

are valid for spontaneous processes. In the absence of any arrangement for the production of electrical work, or some other unusual form of work, the term w represents mechanical work of expansion and is equal to $p\,\Delta v$. If the volume of the system is kept constant, this work term becomes zero. Spontaneous processes may therefore be characterized by two additional inequalities,

$$-(\Delta A)_{T,v} > 0 \tag{19}$$

$$-(\Delta F)_{T,p} > 0. \tag{20}$$

A spontaneous process at constant temperature and volume must be accompanied by a decrease in the value of the work content, while one which occurs at constant temperature and pressure is possible only if the free energy decreases. Either process can be reversed only by the expenditure of energy from outside the system.

Spontaneous processes tend to go on until equilibrium has been attained. When this state has been reached under the given conditions, the value of A or F will no longer change. The equations

$$(dA)_{T,v} = 0 \tag{21}$$

and

$$(dF)_{T,p} = 0 \tag{22}$$

may therefore be used as criteria of equilibrium. These and other criteria, corresponding to other restricting conditions, were stated by Gibbs,[64] but it was Lewis[63,65] who pointed out the practical advantage of using relations containing the free energy function; this advantage arises from the fact that most processes are studied at constant pressure. As a result of Lewis's work, our knowledge of chemical reactions has been greatly increased by the systematic study of free energy changes.

6. Chemical Potentials.—For an infinitesimal, reversible change in which the only work is that of expansion, equations (1) and (9) may be combined in the form

$$dU = TdS - pdv. \tag{23}$$

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[65] G. N. Lewis and M. Randall, Thermodynamics and the Free Energy of Chemical Substances: McGraw-Hill, New York, 1923.

If such a change involves the introduction of additional quantities of matter into the system, there is an additional increment in internal energy for each added substance. Equation (23) must then be replaced by the expression

$$dU = TdS - pdv + \mu_1 dn_1 + \mu_2 dn_2 + \cdots \tag{24}$$

in which n_1 and n_2 represent the quantities of different substances in the system. Each of the coefficients μ is a partial derivative of U with respect to the amount of one substance only. If the change takes place at constant temperature and pressure, the increase in free energy, according to equation (12), is

$$(dF)_{T,p} = dU - TdS + pdv. \tag{25}$$

Addition of equations (24) and (25) gives the relation

$$(dF)_{T,p} = \mu_1 dn_1 + \mu_2 dn_2 + \cdots \tag{26}$$

from which it appears that μ is also a partial derivative of F with respect to the amount of a single substance. The coefficients μ were first used by Gibbs, who referred them to unit masses of substances. If the unit mass of each substance is taken as its molecular weight in some specified state, n represents a number of moles and μ is the chemical potential per mole, which is identical with the partial molal free energy of Lewis.

For a change which does not involve the introduction of new matter into the system, but only the transfer of matter from one phase to another, the corresponding relation is

$$(dF)_{T,p} = \mu' dn' + \mu'' dn'', \tag{27}$$

where the prime marks refer to two phases. If only one kind of matter is transferred, dn' is equal to $-dn''$, and if the system is in equilibrium $(dF)_{T,p}$ is zero. It follows that the simple relation

$$\mu' = \mu'' \tag{28}$$

is a criterion of equilibrium for the distribution of a substance between two phases. The chemical potential is a measure of the tendency of a substance to pass from one phase to another; that is, of its escaping tendency. Equation (28) applies to the equilibrium between a liquid and its vapor or solid phase, and to that between a pure substance and its saturated solution. It also provides a basis for the law of distribution of a solute between two solvents, as well as for the equations describing osmotic equilibrium and the Donnan membrane equilibrium.

The chemical potential was employed by Gibbs in the deduction of the phase rule, and it has also been used to deduce many other fundamental laws of physical chemistry, including the law of mass action for physical or chemical equilibrium. Numerical values of the chemical potential, however, are not often used; for a dissolved substance the chemical potential has the inconvenient property of approaching minus infinity as the concen-

tration becomes very low. It is preferable, for many chemical calculations, to replace the chemical potential by a related function, the activity.

7. Activity and Activity Coefficient.—In eary applications of thermodynamics to physical chemistry (van't Hoff, Nernst, and others), important equations were derived by combining the first and second laws with the equations for perfect gases or ideal, dilute solutions. These equations were exact only when applied to highly dilute systems. Equations of similar form were made applicable to all regions of concentration by the introduction of a function called the activity (Lewis, 1907). The activity of a substance may be defined by the equation

$$\mu - \mu^\circ = RT \ln a \tag{29}$$

in which a represents activity and R is the constant of the perfect gas law. The quantity μ° is the chemical potential of the substance in an arbitrary standard state in which its activity is taken as unity. The standard state is chosen in such a way that the activity becomes equal to the pressure for a perfect gas, to the mole fraction for the solvent in an ideal solution, and to the molality (moles solute per 1000 g. water) for a solute in an ideal dilute aqueous solution. The activity of a pure solid or liquid is taken as 1. The activity may always be replaced by the product of an actual pressure, mole fraction or molality, and a number called the activity coefficient. The activity coefficient is an expression of the difference between actual and ideal behavior; if the laws of ideal gases or solutions were accurately followed, the activity coefficient would always be 1. Actually its value is within a few per cent of 1 for most gases at moderate pressures (up to 1 or 2 atmospheres) and for nonelectrolytes in aqueous solutions of moderate concentration (up to about 1 molal). For strong electrolytes in aqueous solution, however, marked deviations may appear at much lower concentrations. Approximate values for some common salts in water at 25° are given in Table II, which was prepared by rounding off a few of the more exact figures given by Robinson and Harned.[55] The effect of temperature on the activity coefficients of strong electrolytes is generally rather small; that of 0.1 *M* NaCl decreases about 1% between 0° and 40°C., while that of 1.0 *M* NaCl increases 3% over the same range. The activity coefficient of NaCl in sea water is about 0.67, while that of a weak acid (or of the ions of water) in sea water is approximately 0.73 (Robinson and Harned).[55]

TABLE II.—ACTIVITY COEFFICIENTS OF SALTS IN WATER AT 25°

Molality	NaCl	$CaCl_2$	Na_2SO_4	$MgSO_4$
0.1	0.78	0.53	0.45	(0.150)
0.2	0.73	0.48	0.37	0.108
0.5	0.68	0.45	0.27	0.068
1.0	0.66	0.51	0.20	0.049

[55] R. A. Robinson and H. S. Harned, Chem. Rev., **28**: 419, 1941.

Methods for obtaining the values of activity coefficients were discussed by Lewis and Randall,[55] Robinson and Harned,[56] and MacInnes.[57]

One of the simplest ways of determining the activity coefficient of a dissolved substance is based on solubility measurements. Since the chemical potential of the solute in a saturated solution is equal to that of the solid saturating body, the activities of the same substance in the two phases are proportional, or

$$\frac{a \text{ (dissolved)}}{a \text{ (solid)}} = K. \tag{30}$$

It will be seen that equation (30) is nothing more than the law of mass action, as applied to a simple case of physical equilibrium. The value of K would be 1 if the same standard state were used for both phases, but this is not the case. The activity of the solid is taken as 1. That of the dissolved substance may be replaced by the product of its molality, m, and its activity coefficient, γ, or by the product of its molar concentration, C, and a slightly different activity coefficient, f. Accordingly we may write

$$Cf = K. \tag{31}$$

The solubility may be varied by equilibrating the solid with solvent containing varied amounts of a second solute, of concentration C'. By a suitable plot (e.g., $\log C$ vs. $C + C'$ or $\sqrt{C + C'}$) it may be possible to make a linear extrapolation to infinite dilution. This procedure yields a fictitious value, C_0, for the solubility of the first substance at infinite dilution of both substances. C_0 is equal to K in equation (31), since the value of f for infinite dilution is 1. The activity coefficient of the first substance in a solution in which its solubility is C is then obtained by the simple relation,

$$f = \frac{C_0}{C}. \tag{32}$$

8. Osmotic Work and Free Energy of Dilution.—In the historical development of physical chemistry, considerable use was made of the concept of maximum osmotic work and of the analogy between the behavior of dilute solutions and perfect gases. If a gas is allowed to expand reversibly, the maximum work done on the surroundings is $\int_{v_1}^{v_2} p\,dv$. If the gas follows the perfect gas law, p may be replaced by nRT/v, and if the temperature is constant the work done is

$$w = nRT \ln \frac{v_2}{v_1} = nRT \ln \frac{C_1}{C_2}, \tag{33}$$

where n is the number of moles of expanding gas.

If a solution is separated from the pure solvent by a semipermeable piston, it may also expand reversibly, drawing in solvent by osmosis, if the

[57] D. A. MacInnes, Principles of Electrochemistry: Reinhold, New York, 1939.

external pressure on the piston is infinitesimally lower than the osmotic pressure of the solution at all times during the process. The maximum work done in raising the piston is given by a similar integral in which the osmotic pressure, P, replaces the gas pressure, p. If the osmotic pressure follows van't Hoff's law, P is equal to nRT/v or RTC. Accordingly, the maximum work obtained by such a reversible dilution process is also given by equation (33) if n is taken as the constant number of moles of solute in the system. If the external pressure on the piston is increased above the osmotic pressure of the solution, the latter becomes more concentrated by the removal of solvent through the semipermeable piston. In this case C_1 is less than C_2, w is negative, and work at least equal to $-w$ must be done on the system by some external agency. If the pure solvent and the piston are removed, the change of state may be described by saying that n moles of solute have passed from the condition described by C_1 and T to that described by C_2 and T, the initial and final total pressures being identical with that of the atmosphere. Now n moles of solute may also experience the same change of state by being transferred bodily from a large volume of solution of concentration C_1 to another large volume of solution of concentration C_2. If this transfer is accomplished in a reversible way, the process will yield an amount of energy equal to the w of equation (33), and this change in energy has often been called osmotic work. Since w is the maximum work obtainable from a process at constant temperature, it is also equal to $-(\Delta A)_T$ for the whole system, according to equation (14).

The change in energy accompanying the transfer of a solute from one concentration to another is better expressed in terms of free energy and activity, because it is then no longer necessary to limit the equations to the case of solutions which follow van't Hoff's law. For the reversible transfer of n moles of solute from solution 1 to solution 2, the decrease in free energy at constant temperature and pressure is given by an equation similar to equation (27),

$$-(\Delta F)_{T,p} = n(\mu_1 - \mu_2). \tag{34}$$

The chemical potentials may be expressed in terms of activities by equation (29), with the result that

$$-(\Delta F)_{T,p} = nRT \ln \frac{a_1}{a_2}. \tag{35}$$

If the activity coefficients of the solute in the two solutions are identical, the right members of equations (35) and (33) are the same. Actually the changes in free energy and work content must differ by $\Delta(pv)$, but in the application of equation (33) to the transfer of solute it was assumed that no change in volume or pressure occurred.

Equation (35) was used by Borsook and Winegarden[88] in an interesting application of thermodynamics to the calculation of the work done by the

[88] H. Borsook and H. M. Winegarden, Proc. Nat. Acad. Sc. U. S., 17: 3, 13, 1931.

human kidney in the formation of urine. These authors showed that their equation could be reduced, by simplifying assumptions, to the form of an equation previously obtained by Hill,[59] who made use of the concept of osmotic work rather than free energy.

9. Some Thermodynamic Equations of Physical Chemistry.—A few of the physicochemical relationships which have been deduced by means of thermodynamics are summarized below. Derivations of these equations are to be found in textbooks of physical chemistry and chemical thermodynamics.

Law of Mass Action for Equilibrium.—For a physicochemical equilibrium of the type

$$mA + nB \rightleftharpoons pC + qD$$

the law of mass action is

$$K = \frac{(C)^p(D)^q}{(A)^m(B)^n}. \tag{36}$$

This is an exact equation if the parentheses refer to the activities, at equilibrium, of the substances whose symbols are enclosed.

Free Energy of a Chemical Reaction.

$$-\Delta F = RT \ln K - RT \ln Q. \tag{37}$$

Here K is the equilibrium constant, as defined by the preceding equation. Q is a similar quotient of activities which do not refer to the equilibrium state. These activities are specified or implied in the chemical equation for the process, which is the complete conversion of the indicated numbers of moles.

Free Energy and Temperature.

$$\left(\frac{d\Delta F}{dT}\right)_p = \frac{\Delta F - \Delta H}{T}; \qquad \frac{d(\Delta F/T)}{d(1/T)} = \Delta H. \tag{38}$$

Here ΔF and ΔH refer to the same process at constant temperature and pressure.

Equilibrium Constant and Temperature.

$$\frac{d \ln K}{dT} = \frac{\Delta H}{RT^2}; \qquad \frac{d \log K}{d(1/T)} = \frac{-\Delta H}{2.303R}. \tag{39}$$

In this equation ΔH refers to the process indicated by the chemical equation for which K is calculated.

Vapor Pressure and Temperature.

$$\frac{dp}{dT} = \frac{\Delta H}{T\Delta v}; \qquad \frac{d \log p}{d(1/T)} = \frac{-\Delta H}{2.303R}. \tag{40}$$

[59] A. V. Hill, in J. Barcroft, The Respiratory Function of the Blood, 1st ed., pp. 92–94. University Press, Cambridge, 1914.

Here ΔH refers to the formation of 1 mole of vapor from the liquid or solid, and Δv is the corresponding increase in volume. The second form is exact if the volume of the liquid or solid is negligible with respect to that of the vapor, and if the vapor behaves as a perfect gas.

Osmotic Pressure and Vapor Pressure.

$$P = \frac{RT}{V_0} \ln \frac{p_0}{p}. \tag{41}$$

In this equation P is the osmotic pressure of a solution over which the vapor pressure of the solvent is p; p_0 is the vapor pressure of the pure solvent and V_0 is the molar volume of the liquid solvent in the solution. The equation is exact if V_0 is independent of pressure and if the vapor of the solvent follows the perfect gas law.

Osmotic Pressure and Concentration.

$$P = \frac{RT}{V_0} \ln \frac{1}{1 - x}. \tag{42}$$

Here x is the mole fraction of solute in the solution. The equation is exact if the conditions for the preceding equation are satisfied and if the vapor pressure follows Raoult's law. The simpler equation of Morse,

$$P = RTm, \tag{43}$$

gives values of P which are somewhat higher, but the difference is less than 1 per cent for concentrations below 1 molal. For concentrations of the order of 0.1 M, either equation becomes practically identical with the original equation of van't Hoff,

$$P = RTC. \tag{44}$$

None of these equations is valid for electrolytes unless the concentration of the ions is taken into account, and even then it may be necessary to introduce an empirical osmotic coefficient to make the equations fit experimental data.

Freezing Point Depression and Concentration.

$$\Delta T_f = k_f m. \tag{45}$$

This is an approximate equation for dilute solutions. For aqueous solutions, k_f is 1.858°C.

Osmotic Pressure and Freezing Point Depression.

$$P = RT \frac{\Delta T_f}{k_f}. \tag{46}$$

This equation is valid over a much wider range than the preceding formula.

For aqueous solutions it may be written

$$P \text{ (atm.)} = 0.04416T\,\Delta T_f \tag{47}$$

or

$$P \text{ (atm., 0°C.)} = 12.06\,\Delta T_f. \tag{48}$$

Electromotive Force and Temperature.

$$\left(\frac{dE}{dT}\right)_p = \frac{E + \Delta H/n\mathbf{F}}{T}. \tag{49}$$

In this equation E is the electromotive force, in volts, of a reversible galvanic cell; ΔH is the increase in heat content, in joules, of the cell reaction which involves n electrochemical equivalents; and **F** is the faraday, 96,500 coulombs per equivalent.

Other thermodynamic relations involving electromotive force are discussed in the following chapter.

General References

A. A. Noyes and M. S. Sherrill, Chemical Principles: Macmillan, New York, 1922, 1938.

F. H. MacDougall, Physical Chemistry: Macmillan, New York, 1943.

G. N. Lewis and M. Randall, Thermodynamics and the Free Energy of Chemical Substances: McGraw-Hill, New York, 1923.

L. E. Steiner, Introduction to Chemical Thermodynamics: McGraw-Hill, New York, 1941.

4

ELECTROMOTIVE FORCE

Electrochemistry and electrophysiology may be said to have had a common origin, shortly before 1800, in the well known experiments of Galvani with frogs' legs in contact with two metals, and in the controversy between Galvani and Volta concerning the interpretation of these experiments. This dispute led Volta to the discovery of the first electrochemical cell, the voltaic pile. Electrochemistry has developed along quantitative lines since the statement of Faraday's law (1833), while the modern development of electrophysiology dates from the discovery by du Bois-Reymond (1843) of the current of injury from a muscle or nerve. In recent times, much of the experimental work in both of these branches of science has involved the exact measurement of electromotive force.

1. Galvanic Cells.—If a single piece of metal is connected, at two points, to the terminals of an instrument for measuring electromotive force (a potentiometer), there is, in general, no difference in electrical potential; the electromotive force is zero. Yet the only sort of potential difference which can be measured is one existing between two pieces of metal of the same kind. If there are connected between these two pieces of similar metal one or more pieces of dissimilar metal, there may be a measurable electromotive force if two of the junctions are kept at different temperatures; this arrangement is a thermocouple. A measurable electromotive force may also be obtained from a cell containing two electrodes, of different kinds of metal, both in contact with a single solution of suitable chemical nature. The existence of this electromotive force does not require a difference in temperature. Such an arrangement is a galvanic cell. Again, the measured electromotive force is that between two pieces of metal of the same kind; namely, the wires leading to the measuring instrument. The electromotive force is generally considered by chemists to be due to unequal potential differences at the phase boundaries in the cell; that is, the interfaces between each of the electrodes and the solution. It should be emphasized at the outset that no way is known to measure the single potential difference at any such phase boundary.

Of the galvanic cells which are employed in physicochemical investigations, the majority have electromotive forces of the order of 1 volt or less. The E.M.F. of the lead storage cell or accumulator is about 2 volts, that of a fresh dry cell is about 1.5, and that of the Weston standard cell is close to 1.018. Potentiometers used in the exact measurement of E.M.F.

have a range extending to 1.6 volts, and this range has been found ample in most scientific investigations of individual galvanic cells.

2. Reversible Galvanic Cells.—In certain cases the free energy of a chemical reaction may be obtained from the measurement of the electromotive force of a reversible galvanic cell. This is possible if the cell is what Gibbs called a perfect electrochemical apparatus; that is, if no change takes place in the cell except during the passage of current, and all changes which accompany the current can be reversed by reversing the current. The electromotive force of such a cell, if measured at constant temperature without drawing current from the cell, is proportional to the free energy of the cell reaction. If a very large cell of this sort were allowed to deliver n faradays of electricity, the cell reaction would proceed reversibly in the direction of spontaneous approach to equilibrium; the number n is the number of electrochemical equivalents corresponding to the numbers of moles indicated by the chemical equation for the cell reaction. Since electrical work in joules is given by the product of electromotive force in volts and the number of coulombs of electricity which flow, the maximum electrical work of the process is $nE\mathbf{F}$ where $\mathbf{F}$ is the number (96,500) of coulombs in one faraday of electricity. The maximum work of the process is $-\Delta A$; this includes any work of expansion as well as the electrical work. It follows from equations (14 and 16, chap. 3), since only these two kinds of work are involved, that the maximum electrical work is the free energy decrease accompanying the cell reaction, or

$$-\Delta F = nE\mathbf{F}. \tag{1}$$

It is therefore possible to relate the electromotive force to the equilibrium constant of the cell reaction, and the activities of the substances involved, by combining equation (1) with (37, chap. 3).

For the cell

$$(\mathrm{Pt})\mathrm{H_2}\ (1\ \mathrm{atm.}),\ \mathrm{HCl}(C),\ \mathrm{AgCl},\ \mathrm{Ag} \tag{I}$$

the cell reaction is

$$\tfrac{1}{2}\mathrm{H_2}\ (\mathrm{gas,\ 1\ atm.}) + \mathrm{AgCl}\ (\mathrm{solid}) \rightleftharpoons \mathrm{H^+}\ (\mathrm{aq.\ soln.},\ C) + \mathrm{Cl^-}\ (\mathrm{aq.\ soln.},\ C) + \mathrm{Ag}\ (\mathrm{solid})$$

for one faraday of electricity. The activities of the two solids and the hydrogen gas are unity by definition. Equation (37, chap. 3) may be applied in the form

$$-\Delta F = RT \ln K - RT \ln C_{\mathrm{H}} C_{\mathrm{Cl}} f^2, \tag{2}$$

in which K is the equilibrium constant and f^2 is the product of the activity coefficients of the ions $\mathrm{H^+}$ and $\mathrm{Cl^-}$. Equations (1) and (2) may be combined to give a formula for the electromotive force,

$$E = E_0 - \frac{RT}{n\mathbf{F}} \ln C_{\mathrm{H}} C_{\mathrm{Cl}} f^2. \tag{3}$$

The quantity E_0 is a constant characteristic of the cell reaction at the given temperature; its value may be obtained by an extrapolation to zero ionic strength of a suitable function of the E.M.F. and the ionic concentrations.[70] In using equation (3), E is generally expressed in volts; if so, R must have the value 8.314 joules per mole and per degree. If logarithms to the base 10 are used, the coefficient of the logarithmic term is $1.984 \times 10^{-4}T/n$. The numerator of this expression is 0.05915 for 25° or 0.06173 for 38°. In subsequent equations this coefficient will be abbreviated as 0.06, with the understanding that its exact value should be used in calculations. After E_0 has been obtained, equation (3) may be solved for f and equation (2) for $-\Delta F$.

Cells of type I (reversible galvanic cells without liquid junction) have been widely used by physical chemists in the measurement of the activity coefficients of strong electrolytes and the dissociation constants of weak electrolytes.[71]

3. Concentration Cells Without Transference.—If two cells of type I are connected in series, with like poles together, the resulting double cell is called a concentration cell without liquid junction, or without transference. If the concentration of the electrolyte is the same in both parts of the cell, the electromotive force will obviously be zero. If two different concentrations are used, a finite electromotive force is obtained. If current is drawn from the cell, the same reaction proceeds in opposite directions in the two parts of the cell, and the net result is the removal of ions of the electrolyte, in equivalent amounts, from one solution, and the production of similar ions, in the same amounts, in the other. This transfer is not called transference in the electrochemical sense, because the ions do not migrate directly from one solution to the other. In this case hydrogen, silver, and silver chloride will also appear or disappear on one side or the other; since each of these substances is in the same state in the two parts of the cell, the changes in their amounts do not involve a change in the free energy of the whole system. The free energy change of the net cell reaction is therefore that of the transfer of the electrolyte from the more concentrated to the more dilute solution; it is given by the difference between two equations of the form (2), or by the sum of two equations like (35, chap. 3), one for each type of ion. The electromotive force of the cell is therefore related to the concentration of the acid (since C_H and C_{Cl} are equal in this case) by the equation

$$E = 0.06 \log \frac{C_1^2 f_1^2}{C_2^2 f_2^2} \tag{4}$$

or

$$E = 2 \times 0.06 \log \frac{C_1 f_1}{C_2 f_2}, \tag{5}$$

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[70] D. A. MacInnes, Principles of Electrochemistry: Reinhold, New York, 1939.

[71] H. S. Harned, in H. S. Taylor's Treatise on Physical Chemistry, 1: Van Nostrand, New York, 1931; H. S. Harned and B. B. Owen, Chem. Rev., 25: 31, 1939.

where the subscripts refer to the two solutions and f is the geometric mean activity coefficient of the two ions.

4. Concentration Cells with Transference. A cell of this type has two identical electrodes reversible with respect to the same kind of ions, and two solutions containing the same electrolyte at two different concentrations; the cell is completed by a direct liquid junction between the two solutions. Such a cell is

$$(\mathrm{Pt})\mathrm{H_2},\ \mathrm{HCl}(C_2),\ \mathrm{HCl}(C_1),\ \mathrm{H_2}(\mathrm{Pt}) \qquad (\mathrm{II})$$

and its cell reaction involves not only the changes at the electrodes but the migration of the ions at the liquid junction. The electromotive force therefore depends on the transference number or the fraction of current carried by one kind of ion in this direct migration. For small differences in concentration the electromotive force is given by an equation

$$E = 2 \times 0.06\, t_a \log \frac{C_1 f_1}{C_2 f_2} \qquad (6)$$

obtained from equation (35, chap. 3) by replacing n by $2t_a$. Here t_a is the transference number of the ion to which the electrodes are not reversible (in this case, the anion). Since the transference number, t_a, is related to the mobilities, u and v, of the cation and anion of an electrolyte by the equation

$$t_a = \frac{v}{u + v} \qquad (7)$$

equation (6) may be written

$$E = \frac{2 \times 0.06\, v}{u + v} \log \frac{C_1 f_1}{C_2 f_2} \qquad (8)$$

or

$$E = 0.06 \log \frac{C_1 f_1}{C_2 f_2} - 0.06 \frac{u - v}{u + v} \log \frac{C_1 f_1}{C_2 f_2}. \qquad (9)$$

These equations, without activity coefficients, were obtained by Nernst in 1889. If u and v were exactly equal, equation (7) shows that t_a would be 0.5, and equation (6) or (9) would then reduce to a form resembling the simple formula which is more commonly associated with Nernst's name.

5. Liquid Junction Potentials.—In the case of hydrochloric acid, the transference number of the anion is only 0.17; that is, the hydrogen ion is about five times as mobile as the chloride ion. At the junction between two solutions of hydrochloric acid, both ions diffuse from the more concentrated solution to the more dilute. The higher mobility of the hydrogen ion has the effect of increasing the positive electric potential of the dilute solution, even though electrostatic forces prevent any analytically detectable separation of the oppositely charged ions. The existence of this diffusion potential may be inferred from the fact that the electromotive force of Cell II, with 0.1 and 0.01 N solutions of the acid, is only 19.3 milli-

volts (at 25°) while that of the similar cell with silver-silver chloride electrodes is 92.5 mv. In the first case the liquid junction potential opposes the difference in electrode potentials, while in the second case it is added to it. In this argument it is implied that the difference between the electrode potentials ought to be the same for the two cells. This would be the case if the hydrogen and chloride ions, in a given solution of hydrochloric acid, had identical activity coefficients. If this assumption could be justified, we could say, in agreement with Nernst, that the first term on the right in equation (9) represented the difference between the electrode potentials, while the last term represented the liquid junction potential or diffusion potential. The former is just half the electromotive force of a concentration cell without transference. Since this cell has an E.M.F. of 111.8 mv. with these concentrations of acid, the diffusion potential would be 55.9 − 19.3, or 92.5 − 55.9, which is 36.6 mv. Unfortunately these calculations rest on an assumption which has not been verified. No way is known to obtain the activity coefficient of a single kind of ion, nor to obtain the value of a single junction potential. If one could be measured, the other would be known. Any value for either quantity rests on an arbitrary assumption; various plausible assumptions lead to calculated values for the same liquid junction potential differing by 1 or 2 millivolts, even in simple cases.[70]

For a cell containing solutions so nearly alike as to have the same activity coefficient, Nernst's original interpretation would be correct, and the diffusion potential between two solutions of the same 1:1 electrolyte would be

$$E_D = 0.06 \frac{u - v}{u + v} \log \frac{C_1}{C_2}. \tag{10}$$

(This is the integral form of equation (11, chap. 1), which occurred in Nernst's derivation of the relation between diffusion coefficient and ionic mobility.)

It was later pointed out by Nernst (1898) that the diffusion potential in a cell of type II could be practically eliminated by using as the solvent not pure water, but a solution of a suitable indifferent electrolyte. According to more recent ideas, a second electrolyte, if present in sufficient concentration, will have a "swamping" effect on the forces between the ions of the first electrolyte, giving it a constant activity coefficient independent of its own concentration. In that case equation (9) becomes

$$E = \frac{0.06}{n} \log \frac{C_1}{C_2}, \tag{11}$$

which is the original Nernst formula. The swamping effect of an added salt was also shown by Brönsted (1919) to make the classical law of mass action, in terms of concentrations, valid for reactions involving ions. It is this influence of a relatively constant electrolyte content which has made

possible the application of simple equations in studies of the acid-base balance in blood.

In spite of the uncertainty as to the exact measurement of liquid junction potentials, fair estimates of their probable magnitude may be obtained from these equations based on the laws of ideal dilute solutions. A comparison of equations (10) and (11) shows that the diffusion potential in a concentration cell with transference is less than the calculated difference in electrode potentials. The latter may approach 60 mv. for a 10:1 ratio of concentrations, while the diffusion potential is calculated to be about −36 mv. (dilute solution positive) for hydrochloric acid, +12 mv. for sodium chloride, and only about 1 mv. for potassium or ammonium chloride solutions. For the case of two equally concentrated solutions containing different uni-univalent electrolytes with a common ion, the diffusion potential may be calculated approximately by an equation of Planck (1890), modified by Lewis and Sargent (1909),

$$E_D = 0.06 \log \frac{\Lambda_1}{\Lambda_2}, \tag{12}$$

in which Λ_1 and Λ_2 are the equivalent conductances of the two electrolytes at the given concentration. The sign of E_D may be obtained by considering the relative mobilities of the ions not shared in common. At the junction between 0.1 N solutions of hydrochloric acid and sodium chloride, the salt solution will become more positive by about 33 mv.; at the junction of 0.1 N sodium chloride with 0.1 N potassium or ammonium chloride, the former solution will be positive by about 5 mv. These calculated values indicate that the potential difference at a free junction between two solutions is hardly of sufficient magnitude to account for the injury potential in muscle or nerve; such bioelectric potentials are of the order of 50 mv.; and they occur in the absence of any high concentration of H^+ or OH^-, the ions of highest mobility.

6. Salt Bridges.—In the case of potassium chloride it happens that the two ions have nearly equal mobilities; the transference number of the cation is close to 0.49 at all concentrations. If a concentrated solution of this salt is interposed between two solutions containing other electrolytes at much lower concentrations, most of the diffusion at the boundaries will consist in the movement of K^+ and Cl^- ions at nearly the same rate. Hence it might be expected that a salt bridge of saturated potassium chloride solution would tend to obliterate diffusion potentials. If the hydrochloric acid solutions of cell II (0.1 and 0.01 N) are not connected directly, but only by means of such a bridge, the electromotive force at 25° becomes 57.4 mv. instead of 19.3 mv. If this procedure cancelled diffusion potentials, the electromotive force of the cell

$$(Pt)H_2,\ HCl(0.01\ N),\ KCl\ (satd.),\ HCl(0.1\ N),\ H_2(Pt) \qquad (III)$$

would be given by the first term of equation (9), provided that the ions H^+

and Cl^- had the same activity coefficient. The electromotive force would then be

$$E = 0.05915 \log \frac{0.1 \times 0.799}{0.01 \times 0.906} = 0.0559 \text{ volt.}$$

This set of assumptions thus leads to a result which is only 1.5 mv. below the observed value. This is an extreme case, because of the unusually large difference between the mobilities of H^+ and Cl^-. The limiting conductances of ions are proportional to their mobilities; some approximate values for limiting ionic conductances at 25° are as follows: H^+, 350; K^+, 74; Na^+, 50; $\frac{1}{2}Ca^{++}$, 60; OH^-, 198; Cl^-, 76; HCO_3^-, 44; CH_3COO^-, 41. In most solutions not containing a high concentration of H^+ or OH^-, differences in ionic mobility will be relatively small. It is therefore inferred that if solutions of moderate acidity or alkalinity are connected by a salt bridge of saturated potassium chloride solution the elimination of the diffusion potential will be nearly complete. This assumption forms the basis for the common use of the salt bridge in measurements of *p*H and of oxidation-reduction potentials.

7. The Determination of *p*H Values.—If the solutions of cell III are replaced by a solution of known *p*H and one whose *p*H is to be determined, the cell becomes

$$(Pt)H_2, \text{ unknown } (pH_2), KCl \text{ (satd.)}, \text{ standard } (pH_1), H_2(Pt). \quad \text{(IV)}$$

Under suitable conditions the hydrogen electrodes may be replaced by quinhydrone electrodes or glass electrodes. If liquid junction potentials are eliminated by the salt bridge, the electromotive force of cell IV will be given by a modified Nernst formula,

$$E = 0.06 \log \frac{a_1}{a_2}, \quad (13)$$

in which the activities are those of the hydrogen ion. If *p*H is interpreted as $-\log a_H$, this equation may be written

$$pH_2 = \frac{E}{0.06} + pH_1. \quad (14)$$

Equation (14) may be used as an experimental definition of *p*H if the value for a standard solution is agreed upon. This is essentially the common practice. One suitable standard solution is 0.1 *N* acetic acid in 0.1 *N* sodium acetate; its *p*H has been located, as the result of two investigations,[70,72] within 0.01 of 4.645 at either 25° or 38°C. Actually the use of cell IV is less convenient than that of a cell composed of a single hydrogen (or glass or quinhydrone) electrode and a calomel half-cell,

$$(Pt)H_2, \text{ standard or unknown solution, } KCl \text{ satd.}, HgCl, Hg. \quad \text{(V)}$$

If such a cell is used, the *p*H may be calculated from a single measurement

[72] D. J. Hitchcock and A. C. Taylor, J. Am. Chem. Soc., 60: 2710, 1938.

with the unknown solution by the equation

$$p\mathrm{H}_2 = \frac{E_2 - E_0}{0.06}, \tag{15}$$

in which E_0 represents the electromotive force which would be obtained if the hydrogen electrode were bathed by a solution of pH zero. The behavior of the electrodes may be checked by measurements with one or more standard solutions. The difference between two such values of the electromotive force of cell V is equivalent to the E of cell IV, and equation (14) or (15) may be written in the form

$$p\mathrm{H}_2 = \frac{E_2 - E_1}{0.06} + p\mathrm{H}_1$$

for this case.

The practical value of pH determinations, irrespective of any theoretical significance, is now widely recognized by industrial as well as biological chemists. The standardization of the pH scale is of some importance in the comparison of pH values from different sources, and in the use of pH values in equations involving dissociation constants of electrolytes. The most common equation of this type is the application of the law of mass action to a buffer system (Henderson-Hasselbalch equation). This equation is generally written

$$p\mathrm{H} = pK' + \log \frac{[\text{salt}]}{[\text{acid}]}. \tag{16}$$

It must be used with the understanding that [salt] refers to the concentration of a completely dissociated salt of an acid so weak that the concentration of its undissociated molecules is practically equivalent to its total concentration, [acid]. In the case of carbonic acid, [acid] means the concentration of free, dissolved CO_2, whether hydrated to form H_2CO_3 or not, as obtained from a solubility coefficient and a measurement of CO_2 tension, while [salt] means the difference between the total CO_2, bound or free, and this value for the free CO_2. The quantity pK' is the negative logarithm, not of the classical constant (K_c) nor of the true thermodynamic constant (K), but of an apparent ionization constant (K') obtained from pH measurements under similar conditions of temperature, ionic strength, and concentration of other solutes. Evidently pK' is the value of pH when the ratio, [salt]/[acid], is unity, and its value is therefore dependent on the standardization of the pH scale.

At the present time most pH measurements are made by means of the glass electrode; the technique and principles underlying the successful use of this important tool have been fully discussed by Dole.[73] The most useful earlier work on pH measurements and buffer solutions is that of

[73] M. Dole, The Glass Electrode: Wiley, New York, 1941.

Clark.[74] The shorter book of Kolthoff and Laitinen[75] may also be recommended to students of these subjects.

8. Membrane Potentials in Systems at Equilibrium.—If a membrane permeable to some components separates two parts of a system, and the system is in equilibrium, the chemical potentials of a permeant substance in the two phases must be equal, as indicated by equation (28, chap. 3). If the permeating substance is hydrochloric acid, for example, this means that

$$\mu'_{HCl} = \mu''_{HCl} \tag{17}$$

where the prime marks refer to the two phases separated by the membrane. The chemical potential of a strong electrolyte is the sum of the chemical potentials of its ions, or

$$\mu_{HCl} = \mu_H + \mu_{Cl}. \tag{18}$$

By combining equations (17), (18), and (29, chap. 3) we may obtain a relation between ionic activity products,

$$RT \ln a'_H a'_{Cl} = RT \ln a''_H a''_{Cl}$$

or

$$C'_H C'_{Cl} (f')^2 = C''_H C''_{Cl} (f'')^2. \tag{19}$$

For sufficiently dilute solutions the activity coefficients for the two phases may be considered identical, and equation (19) takes the form

$$C'_H C'_{Cl} = C''_H C''_{Cl}, \tag{20}$$

which is typical of the law deduced for membrane equilibria by Donnan (1911). If the membrane is permeable to all substances present, there will be no difference between the two phases at equilibrium, and there will be equality of the individual ionic concentrations as well as their products. However, if one phase contains an additional electrolyte RCl, whose cation R^+ cannot penetrate the membrane, the requirement of electric neutrality in this solution demands an inequality between C'_H and C'_{Cl}, for

$$C'_{Cl} = C'_H + C'_R. \tag{21}$$

It follows from equation (20) that

$$C'_H(C'_H + C'_R) = (C''_H)^2 = (C''_{Cl})^2 \tag{22}$$

and

$$C''_H > C'_H; \qquad C''_{Cl} < C'_{Cl}. \tag{23}$$

This sort of unequal distribution of diffusible ions is characteristic of the condition known as the Donnan equilibrium. For systems composed of a protein hydrochloride in aqueous hydrochloric acid, separated by a collodion membrane from aqueous hydrochloric acid containing no additional substance, relations (20) and (23) were amply confirmed by the extensive

[74] W. M. Clark, The Determination of Hydrogen Ions: Williams & Wilkins, Baltimore, 1928.

[75] I. M. Kolthoff and H. A. Laitinen, *p*H and Electro Titrations: Wiley, New York, 1941.

experiments of Loeb.[76] Approximate figures for C_H were obtained from *p*H measurements, while those for C_{Cl} were obtained by volumetric analysis for total chloride as well by electrometric methods.

If such a system is provided with electrodes reversible to one type of diffusible ion in both phases, thermodynamic equilibrium implies that the electromotive force must be zero. Loeb's experiments fulfilled this requirement, as shown by measurements with silver-silver chloride electrodes. The same would be true if hydrogen electrodes were used, as in the cell

$$(\mathrm{Pt})\mathrm{H_2},\ \mathrm{RCl} + \mathrm{HCl},\ \text{membrane},\ \mathrm{HCl},\ \mathrm{H_2(Pt)};\qquad E = 0. \qquad \text{(VI)}$$

When the solutions were removed from contact with the membrane and put separately into a *p*H cell, a difference in *p*H was found, amounting in some cases to 0.6 *p*H units. This difference means that in the cell

$$(\mathrm{Pt})\mathrm{H_2},\ \mathrm{RCl} + \mathrm{HCl},\ \mathrm{KCl\ (satd.)},\ \mathrm{HCl},\ \mathrm{H_2(Pt)};\qquad E = 0.06(p\mathrm{H}' - p\mathrm{H}'') \qquad \text{(VII)}$$

the hydrogen electrode in the external acid was positive, by nearly 36 millivolts in some cases. Loeb also made measurements of the cell

$$\mathrm{Hg},\ \mathrm{HgCl},\ \mathrm{KCl\ (satd.)},\ \mathrm{RCl} + \mathrm{HCl},\ \text{membrane},\ \mathrm{HCl},\ \mathrm{KCl\ (satd.)},\ \mathrm{HgCl},\ \mathrm{Hg} \qquad \text{(VIII)}$$

which is commonly regarded as yielding a measurement of the membrane potential. This cell is illustrated in Fig. 13. A comparison of cells VI, VII, and VIII indicates that the E.M.F. of cell VIII must be equal and opposite to that of cell VII if equilibrium prevails, and this is what Loeb found in numerous experiments. For the Donnan membrane potential, the E.M.F. of cell VIII therefore followed the equation

$$E_M = 0.06 \log \frac{a'_{\mathrm{H}}}{a''_{\mathrm{H}}} = 0.06 \log \frac{a''_{\mathrm{Cl}}}{a'_{\mathrm{Cl}}} \qquad (24)$$

and the electrode connected to the external acid solution was negative. In experiments in which the hydrochloric acid was replaced by sodium hydroxide, the polarity of both cells VII and VIII was reversed. This is a result of the amphoteric nature of proteins, which form negative, nondiffusible ions in alkaline solutions.

Further experiments have shown that the Donnan membrane potential, as given by cell VIII, persists after a hole has been made in the membrane. This indicates that the Donnan membrane potential partakes of the nature of a diffusion potential, enhanced by the very low mobility of the colloidal protein ions. Similar ideas were expressed by Murray.[77]

The concept of a membrane potential, like that of a diffusion potential, is unsatisfactory to theoretical physical chemists, because a single potential

[76] J. Loeb, Proteins and the Theory of Colloidal Behavior: McGraw-Hill, New York, 1922, 1924; D. I. Hitchcock, Physiol. Rev., 4: 505, 1924.

[77] C. D. Murray, J. Gen. Physiol., 5: 759, 1924.

difference cannot be directly measured. It will be seen that cell VIII includes at least three phase boundaries which might be the seat of potential differences. Only if the membrane potential is defined as the observed electromotive force of a complete cell does the term acquire a definite physical significance. Such a definition is adequate for comparisons with bioelectric potentials, because the latter can likewise only be obtained in terms of a measured electromotive force.

Loeb's highest values for membrane potentials were obtained in the presence of only small amounts of acid or alkali, with no added salt. It is a

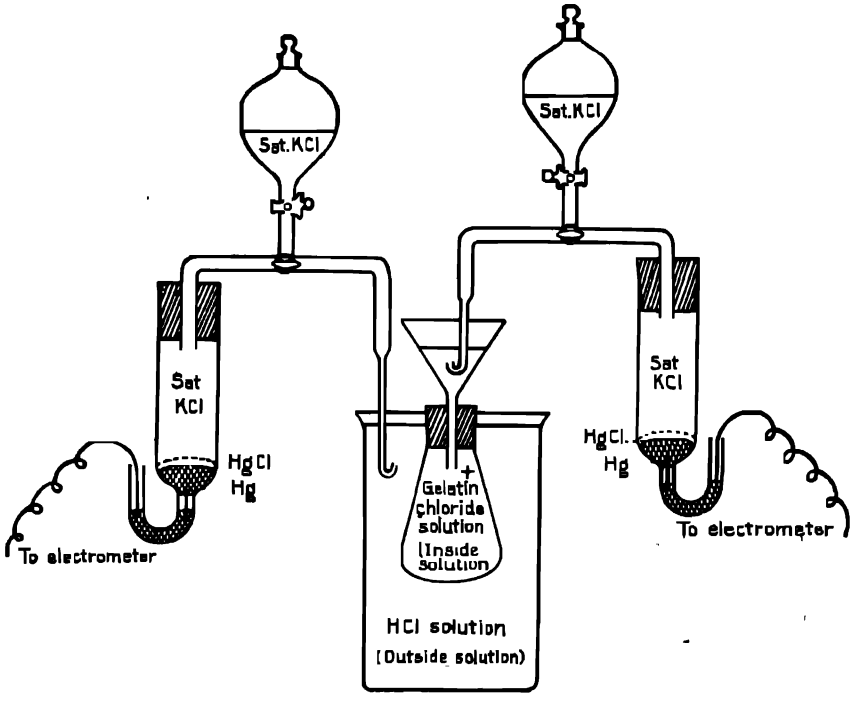

FIG. 13.—Cell for the measurement of membrane potentials. (From J. Loeb, Proteins and the Theory of Colloidal Behavior: McGraw-Hill, New York, 1922.)

property of the Donnan equilibrium that the unequal distribution of ions is repressed by the presence of any diffusible electrolyte at high concentration. Only if the concentration of the nondiffusible ions were very high could there be a Donnan effect of any great magnitude in saline solutions of physiological concentration. Loeb's hope that the Donnan membrane potential might serve to explain the origin of electric charges in living tissues has not yet been fulfilled.

9. Membrane Potentials in Systems Not at Equilibrium.—Other models for bioelectric potentials have been constructed by Beutner,[78]

[78] R. Beutner, Die Enstehung elektrischer Ströme in lebenden Geweben: Enke, Stuttgart, 1920; Physical Chemistry of Living Tissues and Life Processes: Williams & Wilkins, Baltimore, 1933.

who has been especially concerned with cells which include a nonaqueous layer between two aqueous solutions. In such systems there appear to be unequal concentrations of dissolved electrolytes in different parts of the nonaqueous layer, and the cell as a whole is not in equilibrium. These cells are remarkable because of the magnitude of the electromotive force, which amounted to 0.1 volt in some cases, but their value in explaining bioelectric potentials is somewhat doubtful.

Another type of cell in which equilibrium appears to be absent includes two salt solutions separated by a dried collodion (nitrocellulose) membrane. Such a membrane is very different from the common dialyzing membranes; it is practically impermeable, at least in experiments not prolonged for days or weeks, to water and to dissolved electrolytes. It was shown by Michaelis,[79] however, that there might be a slight exchange of cations but not of anions between two solutions separated by such a membrane over a long period. Michaelis found for the cell

Hg, HgCl, KCl (satd.), KCl(0.1 *M*), membrane, KCl(0.01 *M*), KCl (satd.), HgCl, Hg (IX)

an electromotive force of the order of 50 mv. He explained this as a diffusion potential, of the sort given approximately by equation (10), modified by the fact that the mobility of the anion in the membrane was practically zero. If this were true, equation (10) would take the form of equation (11), and the E.M.F. would approach 53 to 57 mv. at 15°C. The dilute solution would be more positive because of the tendency of the cation to diffuse into it as far as electrostatic forces would permit, while the anion would be prevented from diffusing by the impermeability of the membrane. If Michaelis's calomel electrodes and salt bridges are replaced by silver chloride electrodes dipping directly into the 0.1 and 0.01 *N* solutions, the electromotive force is about 90 to 100 mv. instead of the 54 mv. observed when the membrane is pierced or entirely absent. These cells are not in thermodynamic equilibrium, since the electromotive force is not zero when identical reversible electrodes are in contact with the two solutions. The membrane potential, defined as the E.M.F. of cell IX, was found by Michaelis to be even higher when the solutions separated by the membrane were of different chemical nature though of the same concentration. With 0.1 *M* hydrochloric acid and 0.1 *M* sodium chloride he obtained an E.M.F. of 140 mv. If this E.M.F. were a diffusion potential, it would be given by equation (12), which may be written in the form

$$E_D = 0.06 \log \frac{u_1 + v}{u_2 + v}.$$

If the mobility v of the anion is taken as zero in the membrane, while the cations are assumed to retain their ordinary mobilities, the equation gives

[79] L. Michaelis, J. Gen. Physiol., **8**: 33, 1925; Colloid Symposium Monographs, **5**: 135, 1928.

an E.M.F. of only 0.06 log ($^{350}/_{50}$), or about 50 mv. instead of the observed 140. It was therefore concluded that the dry collodion membrane decreased the mobilities of some cations more than others, even though it did not reduce them to zero as in the case of anions.

Dried collodion membranes were also employed by Labes and Zain[80] and Ebbecke[81] in the construction of interesting models which could be polarized by the passage of a direct current. These workers were able to imitate with their models some of the electrical behavior associated with electrotonus in muscle or nerve.

10. Extension of the Theory of Membrane Potentials.—When Michaelis[79] measured the E.M.F. of cell IX with higher concentrations of salt, the results were considerably less than the theoretical maximum given by equation (11) for a 10:1 ratio. Since this equation takes no account of the absolute values of ionic concentrations or activities, but only of their ratios, further explanation was needed. An extension of the classical theories of Nernst, Planck, and Donnan was developed independently by Teorell[82] and by Meyer and Sievers.[83] These workers consider a charged membrane as having a fixed concentration or activity of immobile ions whose charges are balanced by those of mobile ions; if the latter are not originally identical with ions in the solutions bathing the membrane, a rapid exchange of ions of like charge occurs. They assume that a local Donnan equilibrium prevails at each surface of the membrane, with the existence of two opposite but unequal Donnan membrane potentials (or Nernst phase boundary potentials). Inside the membrane there is assumed to be a linear concentration gradient of both ions of the electrolyte furnished by the external solutions. The interior of the membrane is therefore the seat of a third potential difference, a diffusion potential whose value should be approximately given by a formula derived by P. Henderson (1907). The whole membrane potential is set equal to the sum obtained by adding the Henderson diffusion potential to the difference between the two Donnan boundary potentials. The result is an equation which cannot be solved directly for either of the two unknowns; namely, the hypothetical concentration of immobile ions ("constant of selectivity") and the ratio of ionic mobilities in the membrane. By assuming values for these quantities Teorell showed that the formula would predict variations in the membrane potential with concentration, in the right direction to fit observed results. Meyer and Sievers worked out a graphical method of getting the unknown quantities from several measurements of the E.M.F. of cells containing a single salt,

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[80] R. Labes and H. Zain, Arch. f. exper. Path. u. Pharmakol., **125**: 352, 1927.

[81] U. Ebbecke, Ztschr. f. Biol., **91**: 247, 1931.

[82] T. Teorell, Proc. Soc. Exper. Biol. & Med., **33**: 282, 1935; Tr. Faraday Soc., **33**: 1052, 1986, 1937.

[83] K. H. Meyer and J.-F. Sievers, Helvet. chem. acta, **19**: 649, 1936; Meyer, Tr. Faraday Soc., **33**: 1973, 1937; Meyer *et al.*, Helvet. chem. acta, **19**: 665, 948, 987, 1936; **20**: 634, 1937; **23**: 795, 1940.

with fixed ratio of concentrations but varied actual concentrations. Meyer and his coworkers used the selectivity constant as a means of characterizing membranes, and prepared artificial membranes of various types. Some membranes gave E.M.F. values indicating that they were permeable only to cations; others, to anions. Meyer[84] has suggested that bioelectric phenomena may be due to the existence and morphological arrangement of membranes of both types in tissues, as well as to chemical reactions which produce or remove dissolved ions.

Although this extended theory of membrane potentials provides an explanation for some experimental facts, its general validity is still "not proven." On recalculating the data observed by Meyer[83] in experiments with artificial membranes, Fetcher[85] obtained a good many figures which he could not reconcile with the theory.

11. Oxidation-Reduction Potentials.—The chemical reaction which takes place when current is drawn from any galvanic cell involves an oxidation and a reduction. In the reaction for cell 1,

$$\tfrac{1}{2}H_2 + AgCl \rightleftharpoons H^+ + Cl^- + Ag,$$

hydrogen is oxidized to hydrogen ion at the hydrogen electrode, while ionic silver from silver chloride is reduced to metallic silver at the other electrode. When a cell operates spontaneously, the oxidation occurs at that electrode which becomes the negative pole in the external circuit, while reduction occurs at the electrode which becomes the positive pole. If a current is driven through the cell in the opposite direction from an external source, the direction of the cell reaction is reversed and the opposite changes take place at the electrodes. By the use of arbitrary conventions it is possible to express the standard potential, or E_0, for a whole cell as the sum of two standard potentials assigned to the individual electrode reactions. Lewis and Randall[86] and other physical chemists have emphasized the value of the accurate determination of standard electrode potentials by means of cells without liquid junction. Tables of standard electrode potentials are familiar to all students of chemistry, and a very extensive tabulation of such data for inorganic systems has been made by Latimer.[87]

The term oxidation-reduction potential is generally used in a more restricted sense to denote the potential difference between a solution, containing both the oxidized and reduced forms of the substance in question, and an inert electrode, usually of platinum or gold. Here the substance of the electrode takes no chemical part in the cell reaction; it furnishes no ions to the solution, but merely takes up or gives off electrons. In the absence of a metal electrode bathed by a solution containing the oxidant

[84] K. H. Meyer, Tr. Faraday Soc., **33**: 1049, 1937.

[85] E. H. Fetcher, Jr., J. Physical Chem., **46**: 570, 1942.

[86] G. N. Lewis and M. Randall, Thermodynamics: McGraw-Hill, New York, 1923.

[87] W. M. Latimer, The Oxidation States of the Elements and Their Potentials in Aqueous Solutions: Prentice-Hall, New York, 1938.

and reductant in question, there is no mechanism for the existence of an oxidation-reduction potential; accordingly bioelectric potentials cannot be oxidation-reduction potentials.

It is possible, as was shown by Conant and Fieser,[88] to obtain the value of the oxidation-reduction potential of an organic system from the electromotive force of a cell without liquid junction. A derivation of the equation for the E.M.F. of such a cell has been presented elsewhere.[89] This procedure has not come into general use, but most workers have employed cells of the type

$$\text{Hg, HgCl, KCl (satd.), buffer + oxidant + reductant, Pt.} \qquad \text{(X)}$$

If the E.M.F. of this cell is added to the E.M.F. observed for cell V, containing the same buffer but no oxidant nor reductant, the sum is the E.M.F. of a cell with negligible liquid junction potential. It is customary to express these potentials on the normal hydrogen electrode scale. This is accomplished by adding only the E.M.F. which cell V would have if its solution had unit activity of hydrogen ions or zero pH; that is, the E_0 of cell V. The sum of this quantity and the observed E.M.F. of cell X was designated by Clark[74] as E_h. It is related to the concentrations of oxidant and reductant by an equation first used by R. Peters (1898). Peters' equation is

$$E_h = E_0 - \frac{0.06}{n} \log \frac{[\text{reductant}]}{[\text{oxidant}]} \qquad (25)$$

for a system in which the chemical formulas of the oxidant and reductant differ only in their electric charge; n is here the difference between these charges, in faradays per mole or electrons per molecule. Strictly, the concentrations should be replaced by activities. When the oxidant and reductant are Fe^{+++} and Fe^{++}, n is 1. For a large number of organic systems, such as that composed of quinone ($C_6H_4O_2$) and hydroquinone ($C_6H_4(OH)_2$), the value of n is 2. The electrochemical reductant in this system is not hydroquinone itself, but its bivalent anion, $C_6H_4O_2^{=}$. Hydroquinone is a very weak, dibasic acid; accordingly, the law of mass action may be applied to both stages of its dissociation. The concentration of the anion may therefore be expressed in terms of the two dissociation constants, the concentration of hydrogen ions, and the concentration of undissociated molecules of hydroquinone. If the hydrogen ion concentration is much larger than either of the dissociation constants, the total concentration of the reductant is practically identical with the concentration of undissociated molecules, and Peters' equation becomes

$$E_h = E_0' - 0.03 \log \frac{[\text{total reductant}]}{[\text{oxidant}]} - 0.06\ p\text{H} \qquad (26)$$

[88] J. B. Conant and L. F. Fieser, J. Am. Chem. Soc., **44**: 2480, 1922.

[89] D. I. Hitchcock, Physical Chemistry for Students of Biology and Medicine: Thomas, Springfield, Illinois, 1940.

for systems of this type in not too alkaline solutions. The quantity E_0' in equation (26) differs from the E_0 of equation (25) because it includes the ionization constants of the reductant. The behavior of organic oxidation-reduction systems is commonly described in terms of values of E_0', or of E_h at a specified pH and a 1:1 ratio of reductant to oxidant. If such values for different systems are known, it can be predicted that a system having a higher value, under given circumstances, will tend to oxidize a system of lower E_0'. The effect of the degree of oxidation on the E_h for a single system is represented by a sigmoid curve not unlike the dissociation curve of a weak electrolyte. For systems having the same value of n, the curves can be superimposed by shifting them along the E_h axis. If n has different values for two systems, the curves will have different slopes. Examples may be found in the work of Clark[74,90] and of Michaelis.[91] Oxidation-reduction potentials of systems of biological importance have been considered in recent symposia.[92]

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5

SOME PROPERTIES OF AQUEOUS SOLUTIONS

1. Osmotic Pressure.—The direct measurement of osmotic pressure requires a membrane which is perfectly semipermeable; that is, one which allows the solvent to pass freely, but holds back all dissolved substances. The membrane must also be sufficiently rigid to withstand the pressure, which may be very high. In the case of ordinary aqueous solutions of substances of low molecular weight (crystalloids), the existing data consist principally of those obtained with sugars by Morse, Frazer, and collaborators (1901–1923) and by Berkeley and Hartley (1906–1916). Because of the difficulty attending such measurements, it is fortunate that the osmotic pressure may be accurately calculated from the value of any of the other colligative properties such as the freezing point depression, as indicated in equations (46–48) of Chapter 3. The adequacy of such calculations, even for very concentrated solutions, is illustrated by the constancy of the ratios of the values of these two properties for sucrose solutions. Table III gives experimental data for both properties at or near 0°C., and the ratios in the last column are not far from the theoretical value, 12.06.

TABLE III.—OSMOTIC PRESSURES AND FREEZING POINT DEPRESSIONS OF AQUEOUS SOLUTIONS OF SUCROSE
(Data from International Critical Tables)

Molality	P, atm., at 0°C.	ΔT_f, °C.	$P/\Delta T_f$
1	24 76	2 06	12 0
2	54 9	4 6	11 9
3	90 0	7 5	12 0
4	129 7	10 8	12 0

The relation between osmotic pressure and concentration is usually found to show deviations from the logarithmic equation for ideal solutions, as well as from the linear equations of Morse and van't Hoff (chap. 3, equations 42–44). These deviations may be expressed in terms of an empirical osmotic coefficient, g, defined as the ratio of the observed to the ideal osmotic pressure. The exact value of g depends on the equation which is taken to represent ideal behavior. For solutions of physiological interest, the osmotic coefficient may be defined with sufficient accuracy by the relation

$$g = \frac{P}{RT\Sigma m}, \tag{1}$$

in which Morse's equation is taken as the ideal law. The osmotic coefficient may also be obtained from the freezing point depression of an aqueous solution by the relation

$$g = \frac{\Delta T_f}{1.858\Sigma m} \tag{2}$$

in which equation (45, chap. 3) is used as a law for ideal solutions. The results in Table III indicate that equations (1) and (2) will yield the same

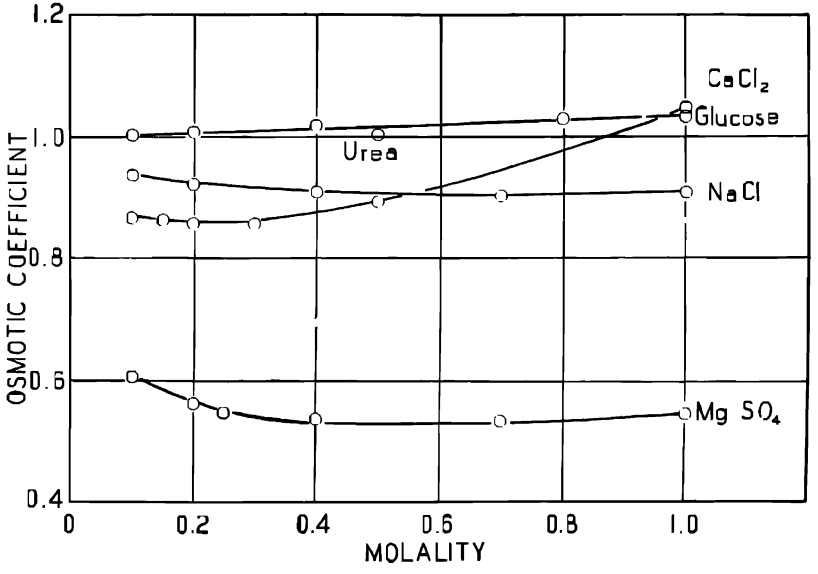

FIG. 14.—Osmotic coefficients of aqueous solutions, as calculated from freezing point depressions. (Data from International Critical Tables: McGraw-Hill, New York, 1928.)

value of g for an aqueous solution of sucrose, of any given concentration up to 4 M. In these equations Σm is the total molal concentration of all particles which are osmotically effective; that is, all molecules or ions. Osmotic coefficients for strong electrolytes are calculated on the assumption of 100 per cent ionization.

The values of the osmotic coefficient for a few typical salts and nonelectrolytes, as calculated from freezing point data by equation (2), are plotted in Fig. 14. In each case the value of g is 1 at infinite dilution. If g were taken as 1 for a solution of glucose or urea having the same freezing point as mammalian blood serum (−0.56°C.), the calculated concentration of such an isotonic solution would be in error by only 1 or 2 per cent. If a similar assumption were made for salt solutions, the curves in Fig. 14 indicate that the calculated isotonic concentrations would be too low, by 7 per cent in the case of NaCl, 13 per cent in the case of $CaCl_2$, and 45 per

cent in the case of $MgSO_4$. In general it is true that deviations from the laws of dilute solutions increase markedly with the valence of ions.

When only one solute is present, the osmotic coefficient is related to the activity coefficient and concentration of the solute by the differential equation

$$d \ln f = dg - (1 - g) d \ln C. \tag{3}$$

If a set of values of g is known for corresponding values of C, f may be calculated by the use of graphical integration. However, if either $1 - g$ or $-\ln f$ is directly proportional to $\sqrt{C}$, as in the Debye-Hückel limiting law (see below), the relation between f and g reduces to the simple form,

$$-\ln f = 3(1 - g). \tag{4}$$

Equations (3) and (4) were first obtained by Bjerrum (1916, 1923); their derivation is given in advanced textbooks.[93] If the solute is a nonelectrolyte, $1 - g$ or $-\ln f$ is generally proportional to C, and in that case the 3 in equation (4) is replaced by the factor 2.

While the osmotic pressure of blood plasma or of a physiological saline solution is about 7.6 atmospheres at 38°, it should be emphasized that an actual pressure of this magnitude is developed only when the solution is separated from the pure solvent by a perfectly semipermeable and rigid membrane. This condition is never fulfilled in living organisms. Nevertheless a knowledge of the magnitudes of osmotic pressures has served to explain many observations involving osmosis in biological systems, as in the classical experiments of de Vries (1882) on plasmolysis and of Hamburger (1883) on hemolysis. Even if a membrane shows some permeability to dissolved substances, it is generally true that water passes through it more rapidly than most common solutes. Osmosis therefore occurs in that direction which tends to equalize the concentrations of water, even though dissolved substances may later penetrate the membrane.

2. Negative and Anomalous Osmosis.—It was observed many years ago (Dutrochet, 1835; Graham, 1854) that in certain cases there may be a temporary flow of water from a solution through a membrane into the pure solvent. Such negative osmosis was found when an aqueous solution of tartaric or oxalic acid was separated from pure water by a pig's bladder. It may also be demonstrated by an experiment described by Loeb.[94] If a permeable membrane of collodion (nitrocellulose) is soaked in a gelatin solution and washed with several changes of warm water, the membrane retains a coating or film of protein. If the membrane (in the form of a sack or test tube) is filled with water, fitted with a stopper bearing a vertical

[93] H. S. Harned, in H. S. Taylor (ed.), A Treatise on Physical Chemistry: Van Nostrand, New York, 1931, 2nd ed., 1: pp. 778–780; E. A. Guggenheim, Modern Thermodynamics by the Methods of Willard Gibbs: Methuen, London, 1933, p. 131; G. N. Lewis and M. Randall, Thermodynamics and the Free Energy of Chemical Substances: McGraw-Hill, New York, 1923, pp. 286, 342. The j of Lewis and Randall is identical with $1 - g$, as here defined.

[94] J. Loeb, J. Gen. Physiol., 2: 173, 1919.

glass tube, and submerged in a solution of phosphoric acid, water is found to rise in the tube for the first 20 or 30 minutes. In most cases of negative osmosis, the membrane has contained protein material and the solution has contained a fairly strong acid. Such experiments can be qualitatively explained as due to electro-osmosis. It is known that an electromotive force, applied across a protein membrane in contact with acid, will cause water to move toward the positive pole. Although no external E.M.F. is applied in the demonstration of negative osmosis, it may be assumed that a diffusion potential, arising from the high mobility of the hydrogen ion, has a similar effect. If the experiment is repeated with a collodion membrane not treated with protein, negative osmosis is not observed; in this case the membrane does not acquire a positive charge in the presence of acid. Loeb also found negative osmosis when a gelatin-treated membrane was placed between a barium hydroxide solution and pure water. In this case the alkali makes the protein negative with respect to the liquid, and the diffusion potential, arising from the high mobility of the hydroxyl ion, makes the water negative with respect to the barium hydroxide solution. Negative osmosis has long been of interest to physiologists as a possible explanation of secretion, which involves the movement of water and ions against a gradient of pressure or concentration.

If a permeable membrane such as collodion is placed between water and a solution of a nonelectrolyte (sucrose, glucose, glycerol, etc.) the direction of osmosis is normal. In such experiments Loeb[94] found that the increase in volume of the solution, during the first 20 minutes, was directly proportional to the initial concentration of the nonelectrolyte, even up to concentrations of 1.0 *M*. This simple relation was found not to hold for many salts, particularly at low concentrations. While the osmosis was always positive, the increase in volume in a given time was greater at very low and high concentrations than in an intermediate region, where the curve of volume against initial concentration passed through a minimum. Similar results were obtained by Bartell[95] and others with membranes of various sorts. Apparently the normal course of osmosis is opposed by electrical effects in a certain region of concentration. Since the results depend on the nature of the membrane and its possible electric charge, as well as on the nature and concentration of the salt, the complete explanation is rather involved. Reference may be made to the discussion by Greenberg.[95]

3. Colloid Osmotic Pressure.—Although the total osmotic pressure of a physiological fluid is not readily obtained by direct measurement, the partial osmotic pressure due to colloids, or very large molecules such as proteins, can be measured without difficulty with the aid of a collodion or cellophane membrane. The colloid osmotic pressure of blood serum was first measured by Starling (1896), and such measurements have been of

[95] F. E. Bartell, Colloid Symp. Monographs, **1**: 120, 1923.

[95] D. M. Greenberg, in C. L. A. Schmidt (ed.), The Chemistry of the Amino Acids and Proteins: Thomas, Springfield, 1938, p. 814. See also R. Höber, chap. 38 of this book.

great value in explaining the movement of fluid across the walls of capillary blood vessels. Apparatus and technique have been described by numerous workers, including Adair,[97] Krogh and Nakazawa,[98] and Simms.[99] The colloid osmotic pressure of serum was discussed by Krogh[100] and by Meyer.[101]

Measurements of the colloid osmotic pressure of solutions of purified proteins (Sørensen, 1917; Adair, 1924) yielded values for the molecular weights of egg albumin and hemoglobin which were later confirmed by the use of the ultracentrifuge (Svedberg, 1926). In such measurements the increase in osmotic pressure with the concentration of the protein is much more than linear. The molecular weight is obtained by applying the van't Hoff equation to a value, extrapolated to zero concentration, of the ratio P/C, where P is the observed osmotic pressure and C is the concentration in grams per 100 cc. of solution. According to Adair and Robinson,[102] a linear plot suitable for this extrapolation is obtained for some proteins by plotting P/C against C, while in other cases it is preferable to plot C/P against C. Similar methods of getting the molecular weights of proteins from osmotic pressure data were employed by Burk.[103]

4. Colloid Osmotic Pressure and Membrane Equilibrium.—The partial osmotic pressure of an electrically neutral colloid may be measured directly, without much error, in the presence of any other dissolved substance, either ionized or non-ionized, to which the membrane is freely permeable. However, if the colloidal material bears an electric charge, as is often the case, the observed osmotic pressure will be due in part to the unequal distribution of diffusible ions characteristic of the Donnan equilibrium. For example, we may suppose that a colloidal salt, Na_nR, is dissolved in water and placed inside a collodion sack. Let the sack be provided with a manometer and submerged in a sodium chloride solution free from colloidal ions. If osmotic equilibrium is attained, the colloidal solution must be under an excess of hydrostatic pressure, P. The value of P will depend not only on the concentration of the colloid, but on the difference between the concentrations of the diffusible ions, Na^+ and Cl^-. The concentration of each of these ions in the external solution at equilibrium may be represented by the same symbol, x. If y represents the concentration of Cl^- in the colloidal solution at equilibrium and z represents the equivalent

[97] G. S. Adair, Proc. Roy. Soc., **A108**: 627, 1925; Biochem. J., **29**: 2576, 1935.

[98] A. Krogh and F. Nakazawa, Biochem. Ztschr., **188**: 241, 1927; R. Dubach and R. M. Hill, J. Biol. Chem., **112**: 313, 1935; C. H. Wies and J. P. Peters, J. Clinical Investigation, **16**: 93, 1937.

[99] H. S. Simms, R. L. Zwemer, and B. E. Lowenstein, J. Lab. Clin. Med., **28**; 113, 1942.

[100] A. Krogh, The Anatomy and Physiology of Capillaries: Yale University Press, New Haven, 1929.

[101] P. Meyer, Ergebn. d. Physiol., **34**: 18, 1932.

[102] G. S. Adair and M. E. Robinson, Biochem. J., **24**: 1864, 1930.

[103] N. F. Burk and D. M. Greenberg, J. Biol. Chem., **87**: 197, 1930; N. F. Burk, *ibid.*, **98**: 353, 1932; *ibid.*, **121**: 373, 1937

concentration of the colloidal anion, R^{n-}, the condition of electroneutrality demands that the concentration of Na^+ in the same solution be equal to $y + z$. The distribution of ions at equilibrium will be as indicated in the accompanying diagram, in which the vertical line represents the membrane.

I		II	
z/n	R^{n-}		
$y + z$	Na^+	Na^+	x
y	Cl^-	Cl^-	x

According to Donnan's theory of membrane equilibrium, the concentrations of diffusible ions at equilibrium must conform to the ion-product equation (20, chap. 4), which may be written

$$x^2 = y(y + z). \tag{5}$$

The total osmotic pressure of each solution may be calculated approximately by equation (1), on the assumption that g is 1. The observed pressure at equilibrium must be the difference between the total osmotic pressures of the two solutions, or

$$P = RT\left(z + \frac{z}{n} + 2y - 2x\right). \tag{6}$$

The factor in parenthesis is the algebraic sum of the molal concentrations of the ions. If y is eliminated from equations (5) and (6), it follows that

$$P = RT\left(\frac{z}{n} + \sqrt{z^2 + 4x^2} - 2x\right). \tag{7}$$

The total osmotic pressure of both ions of the colloidal salt should be $RT(z/n + z)$, but equation (7) indicates that the measured pressure could attain this value only if x were zero (and if the ions of water played no part in the equilibrium). The osmotic pressure of the colloidal ion alone would be $RT\ z/n$; equation (7) shows that the measured pressure would approach this value if $4x^2$ were much larger than z^2. If the solution also contained nonionized colloidal material, its molal concentration would have to be added to z/n in calculating the true colloid osmotic pressure.

The part played by the Donnan effect in the determination of colloid osmotic pressure will be illustrated by calculations which may have some bearing on the interpretation of measurements made with blood serum. The concentration of the proteins in serum is about 75 grams per 1000 g. water, and the protein is believed to exist as anions whose charges are balanced by about 0.017 equivalents of cations, principally sodium ions.[104] The colloid osmotic pressure of serum is determined largely by the albumin fraction; its molecular weight is about 70,000, while that of the globulins is much greater (Adair and Robinson[102]). We may therefore consider

[104] J. P. Peters and D. D. Van Slyke, Quantitative Clinical Chemistry: Williams and Wilkins, Baltimore, 1931, 2: 661; J. P. Peters, Body Water: Thomas, Springfield, 1935, pp. 70, 99.

a hypothetical model consisting of an aqueous solution of a colloidal sodium salt having a molal concentration of 0.001 with respect to the colloidal ion, but 0.017 with respect to Na^+. The value of z is therefore 0.017, while that of z/n is 0.001. Samples of this solution are to be equilibrated across a membrane, permeable to salt and water, but impermeable to the colloidal ions, against a series of solutions of sodium chloride. If the system is kept at 38°, equation (7) indicates that osmotic equilibrium will be attained when the colloidal solution is under the excess pressures, P, indicated in Table IV, for assumed values, x, of the external salt concentration. The value calculated for equilibration against a physiological salt solution, 28.3 mm., is in reasonable agreement with observed results[98] for blood serum. The osmotic pressure due to the colloidal ion alone, $RT\ z/n$, is 19.4 mm. if z/n is 0.001. The last column of the table gives the differences between the values of P and this figure; that is, the last column represents the pressure due to the Donnan effect. Unless the salt concentration is very high this pressure is a considerable fraction of the total. When colloid osmotic pressure is applied in the explanation of physiological phenomena, it is the whole effective pressure, P, that is wanted. On the other hand, if the results of such measurements are to be used to calculate the molecular weight of a colloidal electrolyte, the Donnan pressure must be subtracted from P to obtain the pressure due to the colloidal material alone, which is $RT\ z/n$.

TABLE IV.—CALCULATED VALUES FOR THE EFFECTIVE OSMOTIC PRESSURES AT 38° OF A COLLOIDAL SODIUM SALT ($Na_{17}R$, 0.001 M) AGAINST SODIUM CHLORIDE SOLUTIONS

External NaCl (x) moles per kg. H_2O	Effective Pressure (P) mm. Hg	Donnan Pressure ($P - 19.4$) mm. Hg
0	349	330
0 001	312	293
0 01	141	122
0 1	33 4	14.0
0 16	28 3	8 9
0 2	26 4	7 0
0 5	22 1	2 7
1 0	20 8	1 4

The theory of membrane equilibrium and its application to systems containing colloidal electrolytes have been discussed by Donnan,[105] Loeb,[106] and others.[107,108,109]

[105] F. G. Donnan, Chem. Rev., **1**: 73, 1924; Kolloid-Ztschr., **61**: 160, 1932; Ztschr. f. physik. Chem., **A168**: 369, 1934; J. Chem., Soc., **1939**: 707, 1939.

[106] J. Loeb, Proteins and the Theory of Colloidal Behavior: McGraw-Hill, New York, 1924.

[107] D. I. Hitchcock, Physiol. Rev., **4**: 505, 1924; Ergeb. d. Physiol., **23**: 274, 1924; J. Gen. Physiol., **9**: 97, 1925

[108] T. R. Bolam, The Donnan Equilibria: Bell, London, 1932.

[109] D. M. Greenberg, in C. L. A. Schmidt (ed.), The Chemistry of the Amino Acids and Proteins: Thomas, Springfield, 1938, Chapter XIV.

5. Amphoteric Behavior of Proteins. —As a result of electrochemical and physicochemical measurements made by Hardy, Osborne, Robertson, Michaelis, Loeb, and others, the amphoteric nature of proteins was established. These substances bind hydrogen ions, acquiring positive charges, when they are placed in acid solutions, and they appear to bind hydroxyl ions, acquiring negative charges, when placed in alkaline solutions. Since protein particles in solution are of colloidal dimensions, some have regarded this binding of ions as due to adsorption on the surfaces of the particles. Most workers now consider that the charges of colloidal protein ions are best ascribed to chemical combination of hydrogen ions with basic groups in the protein molecule, or removal of hydrogen ions, by an ionization process, from acidic groups. Evidence for the belief that proteins are amphoteric electrolytes was discussed elsewhere,[110] and the amphoteric properties of certain proteins were considered in detail from this viewpoint at a symposium.[111]

The ionization of a protein or an amino acid in solutions of varied acidity or alkalinity is best described in terms of the ideas of Brönsted[112] and Bjerrum[113] concerning acids, bases, and ampholytes. If an amphoteric substance is placed in a solution containing also a strong acid in sufficiently high concentration, the ampholyte binds hydrogen ions to form a cation of maximal positive charge. If the solution is titrated with successive increments of a strong alkali, this cation behaves as a polybasic acid, losing hydrogen ions in successive stages. If the ampholyte is an amino acid, the successive stages of the titration and ionization may be revealed by breaks in the titration curve, and each step in the curve often resembles the typical titration curve of a single weak acid. In that case the negative logarithm of an acidic dissociation constant, or pK_a, is practically equal to the pH value of the solution at the mid-point of a section of the curve. In the case of a protein, the number of steps in the curve is much less than the number of ionizable groups known to exist in the molecule, but this is readily explained, by analogy with the titration curves of peptides, on the assumption that the steps in the curve are masked, or overlap, when several groups have closely similar dissociation constants. The first part of the titration, from about pH 1 to 4, is ascribed to the removal of hydrogen ions from carboxyl groups. A buffering effect between pH 5 and 7 may be due to the removal of hydrogen ions which were attached to the basic imino group of histidine. The latter part of the titration, from pH 8 or 9 to 12 or above, is thought to consist in the removal of hydrogen ions which were combined with amino or guanidine groups. In this way it has proved possible to

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[110] D. I. Hitchcock, in C. L. A. Schmidt (ed.), The Chemistry of the Amino Acids and Proteins: Thomas, Springfield, 1938, Chapter XI; Cold Spring Harbor Symp., **6**: 24, 1938.

[111] R. K. Cannan and others, Ann. New York Acad. Sc., **41**: 243, 267, 287, 321, 1941.

[112] J. N. Brönsted, Rec. trav. chim., **42**: 718, 1923.

[113] N. Bjerrum, Ztschr. f. physik. Chem., **104**: 147, 1923.

reproduce the whole titration curve of an amino acid, peptide, or protein by a curve calculated from mass law equations involving a small number of acidic ionization constants.[114]

The belief that these definite kinds of groups are titrated, in the regions of *p*H which have been indicated, was founded, in the first instance, on the probable magnitude of their pK_a values, as inferred from comparisons with those of similar groups in nonamphoteric substances. Additional support was found in comparisons of the temperature coefficients of pK_a values, or of the heats of ionization derived from these coefficients. It followed from this assignment of groups to *p*H regions that an amino acid or protein at its isoelectric point should exist, not as an uncharged molecule, but as a dipolar or multipolar ion (*Zwitterion*) carrying equal numbers of positive and negative charges. The validity of this inference has been supported by studies of solubility, dielectric constant, and the Raman effect.[115] It should be pointed out, however, that while the natural amino acids and proteins are believed to exist largely in the *Zwitterion* form at their isoelectric points, this is not true of all amphoteric substances, since ortho- and para-aminobenzoic acids appear to exist chiefly as undissociated molecules.

Many proteins and some amino acids exhibit minimal solubility at *p*H values at or near their isoelectric points, and this phenomenon has sometimes been used as a means of locating the position of the isoelectric point. Since the term isoelectric implies the absence of any excess of positive or negative charge, an isoelectric point is preferably determined by electrokinetic measurements. Other properties of proteins which show minima at or near the isoelectric point include osmotic pressure, viscosity, and swelling, while the membrane potential changes its sign at the isoelectric point. The variations in these properties, over a wide range of *p*H values and electrolyte concentrations, were explained by Loeb's theory of colloidal behavior,[106,107] which was based on the Donnan theory of membrane equilibrium[105] and on the treatment of proteins as amphoteric electrolytes. An inspection of tables of isoelectric points[110,116] indicates that most proteins are negatively charged at the *p*H values of physiological fluids other than gastric juice.

6. Electrokinetic Phenomena.—This term includes electrophoresis or cataphoresis, electro-osmosis, streaming potentials, and sedimentation potentials. The last of these phenomena may be regarded as the converse of the first, while the third is the converse of the second. In each case there are two quantities which may be measured directly; namely, the

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[114] E. J. Cohn, Physiol. Rev., **5**: 349, 1925; Cold Spring Harbor Symp., **5**: 8, 1938; A. A. Green, J. Am. Chem. Soc., **60**: 1108, 1938.

[115] J. T. Edsall, in C. L. A. Schmidt (ed.), The Chemistry of the Amino Acids and Proteins: Thomas, Springfield, 1938.

[116] D. J. Lloyd and A. Shore, Chemistry of the Proteins: Blakiston, Philadelphia, 1938, p. 316.

motion of one phase of matter with reference to another, and a potential difference which is applied to or produced by the system in question. The electrokinetic phenomena are explained by assuming that an additional potential difference, the electrokinetic potential or zeta potential, exists between the moving and stationary phases. The theory of electrokinetic phenomena was first worked out by Helmholtz (1879); it was further developed by later workers. Discussions of the theory are to be found in textbooks[117] and in special treatises.[118]

Electrophoresis usually refers to the motion of particles through a liquid under the influence of an electric field. The electric field is provided by an external source of potential connected with two electrodes, usually of a reversible type, dipping either into the experimental liquid or into other solutions in contact with it. The particles may be ions in true solution, colloidal ions like those of a protein, suspensoid particles such as colloidal gold or platinum, or relatively large particles such as gas bubbles, fat droplets, microscopic crystals, or other particles of suspended solids. Electrophoresis has been observed with bacteria, erythrocytes, and other biological cells.

The velocities of ions in true solution were first obtained by the transference method (Hittorf, 1853), which has also been applied to the study of proteins (Greenberg and Schmidt, 1924). Ionic velocities were obtained more directly by the moving boundary method (Lodge, 1886); this method, as improved by later workers, especially MacInnes[117] and his collaborators, has yielded extremely accurate values. The moving boundary method was also applied to colloids (Burton, 1906) and was later developed (Tiselius, 1937) into a precise method for the investigation of proteins. Since the Tiselius apparatus may be used to test the homogeneity of a preparation or to separate it into definite fractions, as well as to measure mobilities, it has rapidly become a valuable tool in biochemical investigations.[119]

The electrophoresis of larger particles may be observed directly under the microscope. The microscopic method (Ellis, 1912), in improved form, has been used especially by Abramson.[118] This worker was able to show that the mobilities of foreign particles which had been bathed in a protein

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[117] H. Freundlich, Colloid and Capillary Chemistry, trans. by H. S. Hatfield: Dutton, New York, 1926; M. Dole, Principles of Experimental and Theoretical Electrochemistry: McGraw-Hill, New York, 1935; D. A. MacInnes, Principles of Electrochemistry: Reinhold, New York, 1939; S. Glasstone, Text-Book of Physical Chemistry: Van Nostrand, New York, 1940.

[118] H. A. Abramson, Electrokinetic Phenomena and Their Application in Biology and Medicine: Chemical Catalog Co., New York, 1934; H. A. Abramson, L. S. Moyer and M. H. Gorin, Electrophoresis of Proteins and the Chemistry of Cell Surfaces: Reinhold, New York, 1942; E. J. Cohn and J. T. Edsall, Proteins, Amino Acids and Peptides as Ions and Dipolar Ions: Reinhold, New York, 1943, Chapter 25, by H. Mueller.

[119] L. G. Longsworth and D. A. MacInnes, Chem. Rev., **24**: 271, 1939; K. G. Stern, Ann. New York Acad. Sc., **39**: 147, 1939; L. G. Longsworth, Ann. New York Acad. Sc., **41**: 267, 1941; A. Rothen, J. Gen. Physiol., **25**: 487, 1942. See also Sec. 5, Chap. 18.

solution were independent of the size, shape, or chemical nature of the particles themselves. He also found that the curve of mobility as a function of pH could usually be superimposed upon the titration curve of the protein. The microscopic method is more rapid and convenient than the moving boundary method, but a good many measurements are usually averaged before a reliable result is obtained. When the microscopic method is applied to protein-coated particles, it must be recognized that the observed mobility is a property of the protein as it exists in an adsorbed film, and that the result may not always be identical with the mobility of the protein ions in a colloidal solution free from foreign particles.

Electro-osmosis is the passage of a liquid through a diaphragm (membrane, capillary tube, etc.) due to an electric field. An external potential difference is impressed upon two electrodes in contact with the liquid on opposite sides of the diaphragm, and the motion of the liquid as a whole through the diaphragm may be followed by observing a meniscus in a horizontal capillary tube. Just as the isoelectric point of amphoteric particles is determined in terms of a solution in which the velocity of electrophoresis is zero, so the isoelectric point of a membrane may be obtained by finding a liquid of such composition that there is no electro-osmosis. Both electrophoresis and electro-osmosis seem to be involved in the introduction of drugs through the human skin by means of an electric current (Abramson, Moyer and Gorin[118]); this process has sometimes been called iontophoresis.

Measurements of electrophoresis or electro-osmosis are usually expressed in terms of mobility; that is, velocity per unit potential gradient (cm./sec. per volt/cm.). According to classical theory (Helmholtz, Smoluchowski, Perrin) the mobility should, in either case, be given by the equation

$$u = \frac{\zeta D}{4\pi\eta}.$$

Here ζ is the electrokinetic potential, D is the dielectric constant of the liquid medium and η is its viscosity coefficient. The factor 4π was obtained by considering the zeta potential as that of a condenser with parallel plates. Since the zeta potential cannot be measured directly, it was formerly customary to calculate its value from the measured mobility by means of this equation. More recently it was pointed out (Debye and Hückel, 1924) that for small spherical particles in an insulating medium the factor 4π should be replaced by 6π. If the thickness of the double layer is much less than the radius of curvature of the surface, the classical equation is still believed to be valid for particles of any shape.

Helmholtz treated the zeta potential as if it existed only between opposite charges in a thin double layer. Modern workers prefer the idea of Gouy (1910), who conceived of the charges in the liquid as dispersed through a diffuse layer or ion atmosphere. The potential difference responsible for electrokinetic phenomena is not necessarily identical with a potential

difference of the sort postulated by Nernst (1889) as existing between an electrode and the solution bathing it. (Some writers refer to these Nernst potentials as thermodynamic or epsilon potentials.) Freundlich[117] and Rona (1920) compared the potential difference across a thin glass membrane with that deduced from measurements of the streaming potential produced in tubes of the same kind of glass. The former depended almost solely on hydrogen ion concentration, while the latter was markedly influenced by minute concentrations of aluminum chloride or crystal violet. To explain such results it is assumed that electrokinetic behavior depends only on a portion of the total potential difference; namely, on that existing between the fixed and movable portions of the liquid. A full understanding of the present state of electrokinetic theory is possible only for those trained in mathematical physics.

Recent important progress has resulted from the application of improved electrokinetic technique (Tiselius, 1937) to materials of biological interest.[119] This progress has depended on the accurate measurement of mobilities, and has not involved the calculation of zeta potentials. Electrophoresis has been employed more often than electro-osmosis, while streaming potentials and sedimentation potentials have received little attention.

7. Dielectric Constant and Dipole Moment.—According to Coulomb's law of electrostatics, the force exerted between two electric charges is proportional to the product of the charges (amounts of electricity) and inversely proportional to the distance between the charges. Faraday showed that the force depends on the nature of the medium which separates the charges, and accordingly the constant of proportionality in Coulomb's law is different for different media. The dielectric constant is the reciprocal of this proportionality constant, so that the force between two charges tends to be low if they are separated by a medium of high dielectric constant. This is the basis of the Nernst-Thomson rule (1893), according to which the ionizing power of different solvents varies with their dielectric constants. Since ionization in solution often depends on the chemical properties of the solvent (for example, the affinity of water for protons), the rule is often inexact. The dielectric constant is always relative to that of a vacuum, which is taken as 1, and practically that of air is also 1. The dielectric constant of water is about 80, a value which is much higher than those for other common liquids (alcohol, 25; ether, 4; benzene, 2). The dielectric constant is equal to the quotient obtained by dividing the electrical capacity of a condenser, with its plates separated by the medium in question, by the capacity of the same condenser with only air or a vacuum between the plates. Methods of measurement, as well as the interpretation of the results, have been discussed by MacInnes.[120]

The dipole moment of a molecule, in which two equal and opposite electric charges are separated by a definite distance, is equal to the product of

[120] D. A. MacInnes, Principles of Electrochemistry: Reinhold, New York, 1939.

the distance and the magnitude of one of the charges. A molecule may have a permanent electric moment, or it may have a moment induced by an applied electric field. Dipole moments are obtained by calculation from measurements of dielectric constant, but the relation between the two quantities is not always a simple one. Theoretical and experimental work in this field has been summarized in monographs,[121] and there is also much important work in current journals.

Dielectric measurements indicate that water is a dipole, while carbon dioxide is not; it is inferred that the atoms in CO_2 are arranged symmetrically along a line, while the hydrogen atoms in H_2O make an angle of less than 180° with the oxygen atom. Symmetrical molecules such as methane and carbon tetrachloride are not dipoles, and benzene likewise has no dipole moment. A *Zwitterion* is obviously a dipole, and the assignment of this structure to isoelectric amino acids, peptides, and proteins rests on the fact that their aqueous solutions have dielectric constants higher than that of water. The dipolar structure of these substances has been studied especially by Cohn,[122] who mentions that urea and the phospholipoids also increase the dielectric constant of water, while fats have low dielectric constants. "The dielectric constants of different tissues may thus be expected to vary widely, depending upon the electrical properties of the molecular components."

8. Interionic Attraction Theory.—Although it has not been possible to measure the dielectric constant of any solution containing electrolytes in appreciable concentration, the dielectric constant of the solvent is a factor in the interionic attraction theory of Debye and Hückel (1923). One result of this theory is a simple equation relating the activity coefficient of an electrolyte to the ionic concentration of the solution,

$$-\log f = z_1 z_2 A \sqrt{\mu}. \tag{8}$$

Here f is the activity coefficient of an electrolyte whose ions have the valences z_1 and z_2, A is a theoretical constant, and μ is the ionic strength, defined in terms of the concentrations of all ions in the solution. The value of A depends on the dielectric constant of the solvent and the temperature; for aqueous solutions it is 0.503 at 18°, 0.509 at 25°, and 0.522 at 38°. The ionic strength, μ, may be defined as $0.5\Sigma C_i z_i^2$ or half of the sum of all the terms obtained by multiplying the molar concentration of each ion by the square of its own valence. (Lewis and Randall originally defined μ in terms of the molalities of the ions, but the Debye-Hückel theory uses concentrations. The ionic strength is quite different from the chemical potential of Gibbs, although the same symbol is commonly employed for either quantity.) Equation (8) is a limiting law which should be valid only

[121] P. Debye, Polar Molecules: Chemical Catalog Co., New York, 1929; C. P. Smyth, Dielectric Constant and Molecular Structure: Chemical Catalog Co., New York, 1931.

[122] E. J. Cohn, Chem. Rev., **19**: 241, 1936; *ibid.*, **24**: 203, 1939; Harvey Lectures, **34**: 124, 1938–39.

if certain terms may be neglected because the ionic strength is low. The activity coefficients of strong electrolytes are correctly given by this equation if μ is of the order of 0.01 or less. For higher ionic strengths, of the order of 0.1, the equation

$$-\log f = z_1 z_2 A \sqrt{\mu} - B\mu \tag{9}$$

is sometimes useful. The value of B depends on the specific nature of the electrolyte and must be determined from the data themselves. The theory has yielded more elaborate equations for wider ranges of concentration, but these also contain empirical constants. The importance of the Debye-Hückel theory lies in the fact that it provided the first satisfactory theoretical interpretation of the variations from unity of the activity coefficients and conductance ratios of strong electrolytes, which are believed to be completely ionized. A full discussion of the theory, and its application to weak as well as strong electrolytes, is to be found in the book by MacInnes.[120]

9. Solubility Relations and Distribution Coefficients.—According to the phase rule (Gibbs, 1875–1876), "a system of r coexistent phases, each of which has the same n independently variable components, is capable of $n + 2 - r$ variations of phase." If this rule is applied to the equilibrium between a gas and a pure liquid in which the gas dissolves (e.g., oxygen and water), the number of components is two. At ordinary temperatures there will be two phases, a liquid and a gas. Accordingly the phase rule allows two variations or degrees of freedom; that is, if the temperature and pressure are both fixed, the composition of the system at equilibrium is also fixed. It follows that the solubility of a gas in a liquid must depend on the temperature and on the pressure. The nature of this dependence becomes evident if we treat the dissolving process as a reversible, physicochemical reaction. If it is found that heat is evolved when the gas dissolves, ΔH for the reaction is negative, and equation (39, chap. 3) indicates that the equilibrium constant must decrease as the temperature is increased. In other words, such a gas will be less soluble at higher temperatures; this is generally true for most gases. In the few cases in which the opposite effect is observed, it is also found that heat is absorbed during the process of solution.

If the equilibrium constant for the solution process is expressed in terms of concentrations, it becomes identical with the Ostwald solubility coefficient, β. This coefficient may be defined by the equation

$$\beta = \frac{C_2}{C_1} \tag{10}$$

in which C_2 is the concentration of the dissolved gas and C_1 is the concentration of gas in the gaseous phase in equilibrium with the liquid. Evidently β will be constant if the mass law is valid in terms of concentration; this will be true if neither C_1 nor C_2 is very high. If the gas follows the perfect gas law, C_1 will be proportional to the partial pressure of the gas. In that case, if β is constant, equation (10) is equivalent to Henry's law,

which may be written in the form

$$C_2 = kp. \tag{11}$$

Although Henry's law, as is indicated by this derivation, is a limiting law for ideal, dilute systems, it is found to fit the facts in many cases of practical importance, at ordinary temperatures and pressures.

The solubilities of gases are often expressed in terms of the Bunsen absorption coefficient, α, which may be defined by the equation

$$\alpha = \frac{p_0 v_0}{pV}. \tag{12}$$

Here V is the volume of solvent which contains, when in equilibrium with a gas of partial pressure p, an amount of dissolved gas which would occupy the volume v_0 under standard conditions, 0°C. and 1 atmosphere partial pressure; p_0 is this standard pressure. If equation (10) is combined with the definition of concentration ($C = n/v$) and the perfect gas law ($pv = nRT$), it follows that

$$\beta = \frac{p_0 v_0 T}{pVT_0} = \frac{\alpha T}{T_0} \tag{13}$$

That is, the Ostwald solubility coefficient may be obtained by multiplying the Bunsen absorption coefficient by the absolute temperature of the system and dividing by 273. In physiological work the solubility of a gas is often expressed in volumes per cent; this refers to the volume which would be occupied, under standard conditions, by the amount of gas dissolved in 100 volumes of solvent under the experimental conditions. The solubility in volumes per cent is given by the following relations:

$$\text{Volumes per cent} = \frac{100 v_0}{V} = \frac{100 \alpha p}{p_0} = \frac{100 \beta p T_0}{p_0 T}. \tag{14}$$

Data on the solubilities of gases may be found in well known reference books.[123]

The distribution of a dissolved substance between two immiscible liquids may also be treated according to the law of mass action. For the reversible, physicochemical reaction

$$\text{A (dissolved in liquid 1)} \rightleftharpoons \text{A (dissolved in liquid 2)}$$

[123] International Critical Tables: McGraw-Hill, New York, 1928, vol. 3, p. 254; A. Seidell, Solubilities, 3rd ed., vol. 1: Van Nostrand, New York, 1940; Handbook of Chemistry and Physics: Chemical Rubber Publishing Co., Cleveland; for O_2 and CO_2 in blood, see J. P. Peters and D. D. Van Slyke, Quantitative Clinical Chemistry: Williams and Wilkins, Baltimore, 1931, vol. 1, pp. 525, 878–882.

the equilibrium constant is

$$K = \frac{C_2}{C_1} \tag{15}$$

if the active masses are proportional to the concentrations. The distribution coefficient (Berthelot, 1872; Nernst, 1891) is a ratio of concentrations of the dissolved substance in the two liquid phases at equilibrium. The value of K is independent of the concentration in either phase only if the activity coefficients of the dissolved substance in the two phases remain in a constant ratio. If this were true up to the limit of saturation, the value of K would be simply the ratio of the solubilities of the solute in the two pure liquids.

Variations in K are to be expected if the presence of varying amounts of solute alters the mutual solubility of the two liquids in one another, and if the solute undergoes partial dissociation or association in one or both of the liquids. In the latter case the distribution coefficient for a single molecular species may be constant, but the actual stoichiometric distribution ratio will depend on the variable degrees of dissociation or association; these may be calculated if the proper equilibrium constants are known. Even in such cases an approximate simple relation is often valid over a limited range. For example, benzoic acid in water is electrolytically dissociated, but the degree of dissociation is small, so that most of the acid in the aqueous phase exists as single, undissociated molecules. Benzoic acid in benzene is not ionized, but forms associated molecules of twice the ordinary molecular weight. If it is assumed that this association in benzene is practically complete, while the dissociation in water is negligibly small, the mass law gives the expression

$$K' = \frac{\sqrt{C_2}}{C_1} \tag{16}$$

which has been approximately verified by experiment. In general, if a substance exists largely as single molecules (A) in phase 1 and as molecules of a definite polymer (A_n) in phase 2, the distribution law assumes the form

$$K' = \frac{\sqrt[n]{C_2}}{C_1}. \tag{17}$$

This equation may be put into a form which bears a superficial resemblance to the empirical adsorption isotherm of Freundlich. Attempts to deduce the latter equation from the distribution law have not been satisfactory, for one would have to make improbable assumptions: either that single molecules exist in the solution and fractional molecules in the adsorbed phase, or that only multiple molecules exist in the solution and single molecules in the adsorbed phase. Since Freundlich's equation does not apply in all cases of adsorption, it is best regarded as an empirical interpolation formula, useful within certain limits.

More complicated forms of the distribution law are given in advanced textbooks,[124] and the values of many distribution coefficients are given in tables.[125]

The influence of one solute on the solubility of another is well known. For example, sodium chloride may be recrystallized by passing hydrogen chloride gas into its saturated aqueous solution, while alcohol or acetone may be salted out of an aqueous solution by the addition of a salt which is very soluble in water. Carbon dioxide is much less soluble in a strong calcium chloride solution than in water, and even a nonelectrolyte may decrease the solubility of a gas in water. There are also "salting-in" effects, as in the solution of a globulin in a dilute saline solution, and the increased solubility of silver chloride in concentrated potassium chloride solutions. Some of these solubility effects have been satisfactorily explained; for example, the Debye-Hückel theory was verified by studies of the solubility of difficultly soluble electrolytes in salt solutions (Brönsted and La Mer, 1924). A general theoretical treatment of solubility, especially in systems composed of nonelectrolytes, is to be found in a monograph by Hildebrand.[126]

General References

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[124] A. E. Hill, in H. S. Taylor (ed.), A Treatise on Physical Chemistry, 2nd ed., vol. 1: Van Nostrand, New York, 1931; S. Glasstone, Text-Book of Physical Chemistry: Van Nostrand, New York, 1940.

[125] International Critical Tables: McGraw-Hill, New York, 1928, vol. 3, p. 418. See also Sec. 5, Chap. 23.

[126] J. H. Hildebrand, Solubility of Non-Electrolytes: Reinhold, New York, 1936.

Section 2

LARGE MOLECULES: THEIR PHYSICO-CHEMICAL PROPERTIES AND THEIR ARCHITECTURAL AND FUNCTIONAL SIGNIFICANCE IN LIVING MATTER

By J. B. BATEMAN

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INTRODUCTION

The four succeeding chapters deal with some properties of large molecules. We have refrained from classifying large molecules, or even defining them in any precise manner, preferring to illustrate their manifold behavior by describing their occurrence in the principal structural forms met with in nature: in fibers, in interfaces and membranes, in condensed masses, and in the dispersed state. This discussion we have prefaced with a short account of the forces operating in material systems, an appreciation of which is essential to a clear comprehension of the properties of colloids and the inherent vagueness of the classical definition of molecules when applied willy-nilly to systems in which the discontinuities are so ill-defined that a molecular boundary cannot be fixed, or, if arbitrarily fixed for one set of conditions and with one set of criteria, is so displaced in an altered environment that the molecule may lose its original identity, without, however, having undergone any change that would in simpler circumstances be called chemical. While recognizing this indefiniteness, it is nevertheless expedient to preserve the term, without being committed to any definition that would demand either the strict preservation of molecular identity through a series of physical manipulations, or the identical similarity of all molecular units formed in a given process under set conditions. The size of a macromolecule so limits the inevitability of its interactions that even in gross measurements we sometimes have to deal with statistical assemblies of nonidentical particles, while the changes occurring in the microscopic volumes of the living cell must often be so dependent upon fluctuations as to appear quite irregular and nonreproducible. To be sure, these deviations from statistical behavior will not enter greatly into the discussion which follows, for the study of macromolecules in biology is still at the preliminary stage, in which we try to define the repeating units in the fine structure of tissues, but it is well to recognize their critical significance to the future development of analytical biology.

6

INTERATOMIC AND INTERMOLECULAR FORCES[1]

1. Introduction.—The disintegration of a mass of tissue or, indeed, of any colloidal system into its component atoms demands an increasing expenditure of energy as the subdivision proceeds. It is, of course, a simple matter to degrade the elaborate structure by mechanical deformation, to remove a certain small number of atomic ions and small molecules by diffusion, and to segregate particles of different size and density by applying a gravitational field. Even these preliminary measures can have such spectacular effects upon the properties of the system, as striking in their way as the resolution of common salt into an inflammable metal and a corrosive gas, that it is tempting to regard any biological system as a single giant molecule, or, rather, a time series of giant molecules, continually changing in their detailed organization, but preserving a subtle integrity that can be ruptured by forces customarily believed incapable of producing chemical change. It can, indeed, be argued that an uncertainty principle applies in biology, operating in such a manner as to insure that the process of observation invariably changes the nature of the property observed. Although we do not wish to subscribe to this view or to enter into any of the sterile arguments that it has provoked, it is useful to insist on the essential unity and indivisibility of the forces responsible for the unstable organization of living systems and those involved in the formation of stable chemical compounds. The succeeding discussion will perhaps serve to indicate roughly some reasons for the extreme quantitative variation in the fundamentally similar forces causing cohesion and repulsion of atoms.

At a certain early stage of disintegration, protoplasm yields particles of colloidal dimensions which satisfy many of the conventional chemical criteria of molecular individuality. To some degree these, or their unstable derivatives, were present as aggregates in the undisturbed tissue, and could be shown to be present by their effect on various physical disturbances too weak to alter materially the unique properties of the tissue. To some degree also they were present in molecularly dispersed form interacting kinetically with molecules of the continuous phase and other solute molecules, but to a first approximation, at least, showing physical identity

[1] L. Pauling, The Nature of the Chemical Bond: Cornell University Press, Ithaca, N.Y., 1939.

with their derivatives extracted from the tissue and redispersed. In addition to a certain tendency to show uniformity of size and elementary composition—a tendency that can be exaggerated by various fractionation procedures—the protein molecules to which we especially refer have sufficiently well-defined chemical and physicochemical properties for the gross features of their structure to be elucidated by the methods of organic chemistry, even though chemical synthesis has not yet been achieved. This means that however extreme the lability of the association occurring in lipoproteins, nucleoproteins, and so forth, in the living cell, and however weak those linkages which are broken in denaturation, the protein molecule does represent a fairly sharp dividing line between the irreversible changes which can be brought about in protoplasm by weak mechanical forces and those requiring the more energetic operation of chemical agencies and high temperatures.

We can thus contemplate in protoplasm and the various dead structures created by its organizing activity or its degeneration the operation of the whole range of forces answerable for the orientation and cohesion of molecules and their component atoms. The extreme mechanical manifestations of this cohesion are seen in the fluid or plastic nature of single cells, the softness and high compressibility of protein crystals, the hardness of bone, the lateral weakness and high longitudinal tensile strength of animal and vegetable fibers, the extreme brittleness and low compressibility of sodium chloride crystals, and the volatility, combined with great chemical stability, of the molecules of oxygen or carbon dioxide. The same contrast extends in large degree to other properties, such as optical anisotropy, X-ray diffraction, light absorption, electric conductivity, dipole moment, and so forth. For a first description of interatomic and intermolecular forces, however, we shall attempt to find simpler illustrations, in order to emphasize the qualitative differences in behavior that can result from quantitative differences in atomic constitution.

2. The Interaction of Atoms.—The early theory of the directed valence bond resulted in a distinction between primary, or chemical, and secondary, or residual, bonds which is still in use, although the original idea of fixed valence has to some extent required modification. The distinction arose not only from the difference of stability between primary and secondary binding, but also from the complete mystery surrounding the nature of the forces involved in the formation of the valence bond. Thus the secondary forces were regarded as forces of cohesion, physical rather than chemical in character. The electronic theories of valence postulated the physical nature of chemical forces and led to theories interpreting both primary and secondary bonds in terms of the mutual potential energies of atomic nuclei and their associated electrons.

The essential features of the nuclear atom, and the theoretical foundations thus provided for a general theory of valence, have become widely known. The lack of clarity which exists, by contrast, among physicists

concerning the more general aspects of intermolecular action may justify the inclusion here of a short discussion of the subject.[2]

The positions of the electrons within an atom or a molecule are continually changing. Average positions can, however, be assigned, so that the potential at a remote point X due to an atom can be calculated as that due to an assembly of point charges and expressed as a series of terms in $1/r$, $1/r^2$, $1/r^3$, and so on. The term in $\Sigma e/r$ (e is charge, r is distance), representing a field strength varying according to Coulomb's Law as e/r^2, is zero for a neutral atom; for an atom with a spherically symmetrical time average of charge distribution, the higher terms are also zero, so that there is no external field. An atomic ion, such as Na^+ or Cl^-, will have a Coulomb term, but no higher terms, since it has the symmetrical electron configuration of the inert gases. More complex neutral molecules or ions in general give rise to potentials varying as $1/r^2$, $1/r^3$, etc. The term in $1/r^2$ contains the sum of the products: elementary charge $\times$ distance from center, and can thus be regarded as representing the field of a dipole. Similarly the term in $1/r^3$ refers to a quadrupole moment which may be important when the effective dipole is small.

By an extension of this treatment it is possible to calculate the force on a second particle placed in the potential field of the first at the remote point X. It is found that an ion in any of the types of field enumerated above experiences a force of translation, while a neutral molecule with an electric moment does so only if the field is nonuniform. The characteristics of the various forces of interaction are summarized in Table V (rows 1, 2, 3). In addition, dipoles and higher poles experience a torque. This makes certain orientations more probable than others, so that in the field of an ion the electric axis tends to point toward the ion, while in the field of another dipole the electric axes tend to become parallel. Such effects will in general be opposed by kinetic movements, so that reasonably complete orientation of a group of dipole molecules will occur only in very powerful fields.

TABLE V.—CHARACTERISTICS OF MOLECULAR INTERACTION

Interacting Particles	Attractive Force Proportional to
1. Ion—Ion	$e_i e_i' \cdot r^{-2}$
2. Ion—Permanent dipole	$\pm \mu' e_i \cdot r^{-3}$
3a. Permanent dipoles, coaxial	$\mu\mu' \cdot r^{-4}$
3b. Permanent dipoles at right angles	0
4. Ion—Induced dipole	$\alpha e_i^2 \cdot r^{-5}$
5. Permanent dipole—Induced dipole	$\alpha\mu^2 \cdot r^{-7}$
6. Transient dipole—Transient induced dipole	$\alpha^2 \cdot r^{-7}$

e_i, e_i' denote ionic charge.
μ, μ' denote dipole moment.
α denotes polarizability.
r denotes distance between centers.

[2] The treatment resembles that of J. C. Slater, Introduction to Chemical Physics: McGraw-Hill, New York, 1939. See also P. Debye, Polar Molecules: Chemical Catalog Co., New York, 1929.

When the two interacting systems of charges interpenetrate, it can be seen qualitatively that at first, as the electrons of the first atom come into the field of the nucleus of the second, there will be a considerable attractive force; with further penetration, powerful repulsive forces must come into play, since the effective fields will be those between the two nuclei and between pairs of electrons. The calculated cohesion at the equilibrium point is, however, far too weak to account for the strength of primary chemical binding. Even when the mutual displacement or polarization of the electrons is taken into account, classical electrostatics fails to explain the primary bond, but the polarization effect does explain some types of residual force and therefore merits further mention.

Even spherically symmetrical atoms or molecules are polarized in an external field, and the forces between the inducing particle and the induced dipole are summarized in rows 4 and 5 of Table V. To complete the enumeration of residual forces, it must be added that even spherically symmetrical atoms with a zero average field show instantaneous asymmetry, and therefore give rise to a fluctuating field. The transient fields of interacting particles induce transient electric moments, and give rise to the permanent "dispersion forces" of attraction specified in the last row of Table V.

The close approach and "chemical" interaction of atoms require quantum mechanical treatment, and it is sufficient to say here that under certain clearly defined conditions during the interpenetration of atoms, there occurs a concentration of outer shell electrons in the internuclear region, amounting, in effect, to the electron sharing postulated in the early electronic theories of valence. Further interpenetration is limited by repulsive forces arising from the interaction of the inner closed shells, with operation of the Pauli principle, and by electrostatic repulsion between nuclei.

All forces of interaction, from the dispersion to the covalent, may be generalized in the form:

$$F = \frac{A}{r^m} - \frac{B}{r^n} \tag{1}$$

or

$$F = \frac{A}{r^m} - Be^{\rho}, \tag{2}$$

where F is force, r is distance, and A, B, m, n, ρ are constants. Whatever the type of interaction, there will always be a certain equilibrium distance of separation, r_0, at which the force is zero and the potential energy minimal; with increasing distance, the force of attraction will rise and then decrease. There is therefore a tendency for all particles to associate, vibrating by virtue of thermal energy about the point of minimal potential energy.

Often the rupture energy is far less than the kinetic energy, so that a tendency to associate shows itself only in a deviation from the perfect gas law, the terms of equation (1) finding expression in a and b of Van der Waals' equation. Attractive forces of types 3, 5 and 6 (Table V) are in

varying degrees responsible for this effect and are sometimes known as Van der Waals forces; their respective contributions are shown in Table VI, which also shows how a depends upon dipole moment and b upon molecular volume. When the moment is sufficiently high, as in water, the rupture energy is of the order of magnitude of the thermal energy, resulting in condensation to the liquid state, in which bonds between pairs of molecules have a longer life, but are not strong enough to give rise to a structure with any mechanical stability.

TABLE VI.—VAN DER WAALS CONSTANTS AND DIPOLE MOMENTS

Gas	(1) Exp. $10^{-12}a$	(2) Calculated $10^{-12}a$ London	(3) Calculated $10^{-12}a$ Slater	(4) b	(5) V	(6) Contribution to lattice energy in ergs $\times 10^{-60}$ Orientation	Induction	Dispersion	(7) $10^{18}\mu$
CO	1 50	1 24	1 86	39 7	32 7	— 0 0034	— 0 057	— 67 5	0 11
HI	....	...	.	.	....	— 0 35	— 1 68	—382 0	0 38
HBr	4 51	3 83	3 93	44 1	37 5	— 6 2	— 4.05	—176 0	0 78
HCl	3 72	2 12	2 56	40 7	30 8	— 18 6	— 5 4	—105 0	1 03
NH_3	4 22	....	. ..	36 9	24 5	— 84 0	—10 0	— 93 0	1 49
H_2O	5 53	....	. .	30 4	18 0	—190 0	—10 0	— 47.0	1.85
CH_4	2 28	1 92	2 23	42 6	49 5				
C_2H_2	4 43	2 22	3 02	51 3	50 2				
C_2H_4	4 46	3 10	3 84	56 1	54 9				
C_2H_6	5 46	3 42	4 53	63 5	49 3				

a in column (1) is the experimental value of the Van der Waals constant.

a in columns (2) and (3) denotes values calculated by London and by Slater and Kirkwood respectively.

b is the second Van der Waals constant.

V is the molecular volume of the substance in the liquid state.

Values in column (6) are contributions to lattice energy of the three forms of interaction, calculated by London, for unit intermolecular distance.

μ is dipole moment in electrostatic units.

The table has been compiled from data given by Slater, Introduction to Chemical Physics, p. 408; Mark, Physical Chemistry of High Polymeric Systems, p. 95; Evans, Introduction to Crystal Chemistry, p. 52; Debye, Polar Molecules, p. 40; and Smyth, Dielectric Constant and Molecular Structure, p 192.

3. The Homopolar Bond.—Homopolar or covalent linkages, with an energy of the order of magnitude 100 kcal./g. equiv., are well known to be governed by the rule that the number of such linkages between one atom and others is limited by the number of additional electrons needed to complete the inert gas configuration. A necessary consequence is that monovalent atoms can form only binary homopolar associations, which must then depend upon secondary forces for higher order aggregation. The mechanical properties of such compounds can depend only upon these secondary forces, and the rupture energy of the homopolar bond can only be measured

indirectly. Substances in which two or more types of binding are present are said to be heterodesmic. Homopolar associations of bivalent atoms are also heterodesmic, since they may form homopolar chains or rings, but cannot have homopolar binding in three dimensions; homopolar homodesmic compounds are confined to tetravalent atoms, such as carbon, which in diamond forms a homopolar structure of indefinite extension in three dimensions, with consequent loss of molecular individuality. The majority of carbon compounds are, however, heterodesmic, since the three-dimensional linkage is usually interrupted sooner or later by atoms of lower valence.

Two further consequences of exchange interaction or electron sharing are important, namely, the existence in homopolar compounds of characteristic bond angles and interatomic distances, r_0. The semiempirical theories of Pauling and Slater stress the importance of the state of quantization of the electrons participating in homopolar binding in determining the bond angle. Electrons in p states tend to form linkages perpendicular to one another, and this pronounced orientation, with a well-marked resistance to deformation, imposes a limitation upon the possible modes of packing of homopolar molecules. This is responsible for many of the facts of stereochemistry and morphology. Repulsive forces between substituent atoms usually cause deformation of the 90° valence angle, for this needs considerably less energy than does linear extension in the direction of strongest binding: about 1 kcal./g. mol. for 10 degrees, compared with 10–100 kcal. for an extension of 0.1–0.3 Å. This is seen, for example, in the water molecule, with an angle of 104°31′,[3] in the pyramidal structure of ammonia, where the bond angles are all 106°, in the well-known but approximate tetrahedral distribution of valence bonds around the saturated carbon atom, and again in the series CCl_4, $CHCl_3$, CH_2Cl_2, which show progressive increase in the Cl—Cl distance, corresponding to increasing distortion of the tetrahedral configuration of CCl_4. Double and triple bindings between carbon atoms, which may be regarded as extreme instances of distortion of bond angle, involve also some decrease in interatomic distance, which in typical cases may be given as 1.55, 1.34, and 1.19 Å for single, double, and triple bonds respectively. In different compounds it is, however, possible to find a series of intermediate values for the carbon-carbon distance, and for the corresponding dissociation energies.[1,4] The position of double bonds in a conjugated system has ambiguity, arising from the fact that each atom contains a single electron with unneutralized spin; from the point of view of wave mechanics, there is difficulty in assigning these to a series of localized double bonds with spins uniquely paired. Rather is it necessary to assume each electron "to move in the field of nuclear framework . . . (being) allotted to a polycentric orbital embracing all the carbon atoms

[3] R. Mecke and W. Baumann, Physik. Ztschr., **33**: 833, 1932; B. T. Darling and D. M. Dennison, Phys. Rev., **57**: 128, 1940.

[4] J. E. Lennard-Jones and C. A. Coulson, Tr. Faraday Soc., **35**: 811, (1939).

which are conjugated together, in conformity with the Pauli Principle."[4,5] Such a "resonance" system has lower energy than would be anticipated from a conventional formula with localized double bonds,[6] while all the C—C bond lengths are equal and slightly longer than a pure double bond.[7] An alternative to the theory of mobile electrons is to attribute the resonance effect to tautomerization among various possible canonical[8] structures of conventional type; both theories have been applied to the benzene and naphthalene molecules,[9] and both have been used semiempirically with some success in the calculation of energies and bond lengths in resonating structures.

Apart from this peculiarity of conjugated systems, the repulsion between electrons concentrated in the internuclear region in a pure double bond leads to an electron distribution with twofold axial symmetry, so that there are two bond orientations of minimum energy, at 180° to each other corresponding to the stable cis- and trans-isomers of organic chemistry.[1], A second consequence of this restriction conferred by the double bond is the anisotropy of polarizability of organic molecules containing this linkage—a property which, indeed, extends to the single linkage in molecules with unsymmetrically occupied carbon bonds. The polarizability is usually considered localized between atoms, in the region of high electron cloud density, and is described in terms of the three axes of a polarization ellipsoid.[11] In conjugated systems the polarization ellipsoid, instead of coinciding with each bond, is of considerable length. Since the electrons concerned in polarization phenomena are those which form the virtual oscillators responsible for dispersion interaction, this increase in length and decreased frequency is of importance in the calculation of Van der Waals forces and will be referred to later.

4. Linkage between Ions: the Heteropolar Bond.—Electron sharing represents only one method by which pairs of atoms can attain the inert gas configuration. A donor atom, with a small number of outer electrons, can do so by ionization, while an acceptor atom with a nearly completed outer shell can take up these electrons and become a negative ion. Such a reaction will result in an assembly of ions in which every ion of one sign is surrounded by an atmosphere of the opposite sign, the precise geometrical arrangement being determined by the repulsive forces, which also define

[5] R. Kuhn, Angew. Chem., **50**: 703, 1937.

[6] L. Pauling and J. Sherman, J. Chem. Phys., **1**: 606, 1933.

[7] E.g., C. A. Coulson, Proc. Roy. Soc., **164A**: 383, 1938.

[8] L. Pauling, J. Chem. Phys., **1**: 280, (1933).

[9] a. L. Pauling and G. W. Wheland, J. Chem. Phys., **1**: 362, 1933; b. C. A. Coulson, cited by J. E. Lennard-Jones and C. A. Coulson, Tr. Faraday Soc., **35**: 811, 1939.

[10] a. E. Hueckel, Ztschr. Phys., **60**: 43, 1930; b. J. C. Slater, Phys. Rev., **38**: 1109, 1931; c. L. Pauling, *ibid.*, **40**: 891, 1932.

[11] a. Lord Rayleigh, Scientific Papers, **6**: 540; b. R. Gans, Ann. Physik, **37**: 881, 1912; *ibid.*, **62**: 331, 1920; *ibid.*, **65**: 97, 1921; c. M. Born, *ibid.*, **55**: 177, 1918; d. For experimental methods see H. A. Stuart, Ergebn. d. exakt. Naturwiss., **10**: 159, 1931.

the ionic radii. Such a structure is homodesmic and devoid of molecularly distinct units; other ionic structures are, however, possible, in which ions of several types participate, with different interionic bond strengths.

The very sharp localization of ions in the typical ionic lattice has been demonstrated by X-ray analysis.[12] The outstanding physical properties are to be expected from this predominantly Coulomb binding: low compressibility, hardness, high rupture energy, low thermal expansion, and characteristic lattice vibration frequency. Low electrical conductivity in the solid state gives way to ionic conduction on fusion. These properties have been accounted for theoretically, with varying degrees of refinement, by addition of the potential energies of interaction of pairs of point charges over the entire lattice.

5. Partial Ionic and Homopolar Bonds:[13] Origin of Dipole Moments.—It can be imagined, however, that in some cases the electrostatic interaction of ions will cause deformation, so that the charge-free interionic region will tend to become occupied by electrons attracted away from the negative ion. Since the atoms still tend to preserve their inert gas configurations we may expect this process to be accomplished in suitable cases by electron sharing and not by the formation of an assembly of unbound neutral atoms. This will result in a decrease of dipole moment of each ion pair and in a closer approach of the nuclei, since the shared electrons take up less room than the corresponding unshared pair. On the other hand, it may be imagined that the shared electrons in an homopolar bond can confer polarity upon the molecule by being more closely attached to one atom than to the other. We shall not defend this description of the mixed covalent and ionic character of bonds; it is sufficient to mention that it has proved fruitful in the systematizing of a considerable body of chemical information. Pauling's semiempirical method is to enumerate all the reasonable ionic and covalent structures of a compound that are possible without actual atomic rearrangement, and by semiquantitative arguments based upon a comparison between the observed bond distances, bond energies, dipole moments, and any other relevant quantities, and those calculated for the several separate structures, to arrive at an estimate of their respective contributions to the measured values.

The energies of various purely homopolar bonds should be interrelated by simple rules; the energy of the C—H bond should be, for example, the arithmetic mean of the energies C—C and H—H. The energies for various single bonds between unlike atoms, calculated from thermochemical data, do not, in fact, obey this rule; if we assume that bonds between like atoms are completely homopolar, it is reasonable to attribute the discrepancy in the case of other bonds to their partial ionic character. If we denote by Δ this extra ionic resonance energy, it is found empirically that values of $\sqrt{\Delta}$

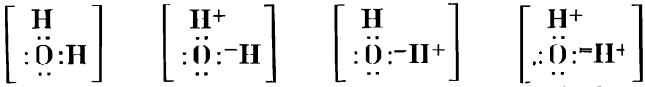
[12] H. G. Grimm, R. Brill, C. Hermann and C. Peters, Naturwissenschaften, **26**: 29, 479, 1938, Ann. Physik, (5), **34**: 393, 1939.

[13] See chap. 2 of ref. (1).

are additive, in the sense that a series of values, x, can be assigned to the elements (with x set arbitrarily at 2.5 for carbon) such that the difference $(x_a - x_b)$, or δx, for any two atoms a and b, is proportional to the value of $\sqrt{\Delta}$ for a single bond between those atoms. Pauling calls x the "electronegativity" of an element, the electronegativity scale defined in the above manner being closely similar to that of classical electrochemistry. The values of δx for single bonds correspond, very roughly, with a proportionality factor 10^{18}, to their dipole moments, the latter being regarded as components of a vector defining the observed dipole moment of a molecule, and derived by analysis of this quantity with the aid of observed bond angles and interatomic distances. An alternative relationship given by Pauling may be written in the form

$$0.25(\delta x)^2 = -\ln(1 - p) \tag{3}$$

where p is the fractional ionic character of the bond, denoting the ratio of the observed dipole moment of the bond to the moment calculated from the observed interatomic distance and the valence of the ions. This relationship was based on a calibration with the hydrogen halides, and it is of some value in assessing the relative importance of various resonating structures. Such considerations give, for instance, the following proportions of the four structures of water:



The bond C=O is of some interest in biology. This, in various aldehydes and ketones, has a moment around $2.8.10^{-18}$ e.s.u., instead of $5.95.10^{-18}$ for the pure ionic structure $R_2C^+:\ddot{O}:^-$, corresponding to an ionic character of 47%, or about twice that of the single bond C—O. Triple bonds, however, as in the alkyl cyanides, have much higher ionic character than would be expected from this "rule," a fact that must be attributed to mobile electrons in the triple bond.

6. The Hydrogen Bond.[14]—The partial ionic character of covalent bonds, increasing in importance with the difference in electronegativity of the bonded atoms, provides for the occurrence of a series of secondary interactions of electrostatic character. These would be expected, *a priori*, to resemble the interaction of dipoles in some cases; in others, through the operation of steric factors hindering orientation, the interaction of ions would

[14] The subject has been reviewed up to 1939 in ref. (1), to which reference should be made for points of historical interest and for qualification of some of the statements made in this section. A further question of nomenclature, with reference to the distinction between "hydrogen" and "hydroxyl," or "long" and "short," hydrogen bonds, is discussed by J. D. Bernal and H. D. Megaw, Proc. Roy. Soc., **151A**: 384, 1935; J. D. Bernal, Tr. Faraday Soc., **35**: 922, 1940; A. R. Ubbelohde, Proc. Roy. Soc., **173A**: 417, 1939; Tr. Faraday Soc., **35**: 886, 1940.

better describe the situation. In the latter case, a bond energy much smaller than that of a purely ionic system would be expected, because of the partial covalent character of the primary bonds. At the same time, the system might be stabilized by resonance between two covalent structures:

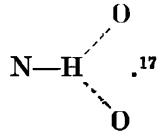
$$X—AY \longleftrightarrow XA—Y$$

and

$$X^-A^+Y^-:$$

Such a covalent contribution, involving directed bonds, will impose a geometrical restriction upon the electrostatic arrangement. The energy of the latter, considered alone, should depend upon the electronegativities of the atoms or groups X and Y, and upon their ionic radii and that of the ion A^+. The special importance of the hydrogen bond, in which A^+ is a hydrogen ion,[15,16] is due largely to the minute size of the proton; and the strength of hydrogen bonds depends, in a very rough way, upon the electronegativity of X and Y, upon their size, and upon the number of hydrogen bonds in which they participate.

The small size of a proton is likewise responsible for the further peculiarity that hydrogen can rarely associate with more than two anionic groups. These usually form a linear sequence X—H—X so that the close approach of further X groups is prevented by the electrostatic repulsion of those already bound to hydrogen. Exceptions to this rule are found in the case of glycine and alanine crystals, which may contain bifurcated hydrogen bonds



.[17]

Given these facts: the twofold coordination of protons, the symmetrical electrostatic field of the anionic groups, and the directional character of the covalent bond, the possible modes of molecular association through hydrogen bonds can be seen to have great variety. Consider, for example, the compounds CH_4, NH_3, OH_2, FH. The conditions here are reminiscent of

[15] For discussions of bi-covalent hydrogen see N. V. Sidgwick, The Electronic Theory of Valency; Oxford, 1929; Ann. Rep. Chem. Soc., **31**: 34, 1935; L. Pauling, Proc. Nat. Acad. Sc., Wash., **14**: 359, 1928.

[16] The relative importance of resonant covalent contributions and electrostatic effects has been much discussed. R. H. Gilette and A. Sherman (J. Am. Chem. Soc., **58**: 1135, 1936) give quantum mechanical calculations for carboxylic acids which do not agree closely with experimental results; on the other hand, purely electrostatic models have been used with some success by Moelwyn-Hughes, J. Chem. Soc., p. 1243, 1938; M. M. Davies, Tr. Faraday Soc., **36**: 333, 1940; E. Bauer and M. Magat, J. Phys. Rad., **9**: 319, 1938; *cf.* J. D. Bernal, Tr. Faraday Soc., **35**: 912, 1940.

[17] G. A. Albrecht and R. B. Corey, J. Am. Chem. Soc., **61**: 1087, 1939; H. A. Levy and R. B. Corey, *ibid.*, **63**: 2095, 1941.

those governing formation of homodesmic or heterodesmic covalent or ionic structures. The covalent binding of the first three molecules predisposes to a tetrahedral configuration; this is complete in CH_4. In NH_3 it can be completed by sharing one additional proton from a neighboring molecule, so that the substance might be expected to associate to form chains and rings, but never to give a three-dimensional structure. The same is true of FH, in which the tetrahedral configuration can obviously be at most rudimentary, so that new protons will tend to take a position diametrically opposite the first.[18] In water, the tetrahedral arrangement is already implied in the V-shaped H—O—H chain,[19] and the presence of only two hydrogen atoms per molecule makes it possible to complete the configuration by attachment of two further water molecules through their H atoms. In this way, a self-perpetuating three-dimensional structure can be formed, and this is largely responsible for the striking physical differences of water from other neonlike substances.[20] Many similar examples are to be found among organic compounds, where the presence of hydrogen bonds is often detected by the indirect methods of X-ray and electron diffraction. These methods are not, however, readily applicable to complex molecules in which the detailed atomic configuration is unknown. Argument by analogy, doubtless, has some value: the known structures of glycine and diketopiperazine crystals certainly suggest that cohesion must occur in dry proteins and polypeptides by formation of intermolecular hydrogen bridges involving the ketoimino linkage and amino and carboxyl side-chains.[21] At present, other methods must be applied, also largely by analogy or by questionable simplification; nevertheless, we shall mention briefly two methods which have given information in simple systems, and the extent to which they have found application to macromolecules.

Hydrogen bond formation between different molecular species has been extensively studied by measurement of solubilities and of heats of mixing, especially in connection with bonds involving CH groups.[22] In general, abnormal solubility or high heat of mixing is attributed to intermolecular association of some kind, and attempts are made to identify the atoms

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[18] There is probably a tendency of the HFH bond angle to be 140° instead of 180° as suggested here. See S. H. Bauer, J. Y. Beach, and J. H. Simons, J. Am. Chem. Soc., **61**: 19, 1939.

[19] E.g., R. Mecke and W. Baumann, Physik. Ztschr., **33**: 833, 1932.

[20] J. D. Bernal and R. H. Fowler, J. Chem. Phys., **1**: 515, 1933.

[21] The importance of hydrogen bridging in proteins is discussed by W. T. Astbury, Tr. Faraday Soc., **36**: 871, 1940.

[22] G. F. Zellhoefer, M. J. Copley, and C. S. Marvel, J. Am. Chem. Soc., **60**: 1337, 1938; M. J. Copley, G. F. Zellhoefer, and C. S. Marvel, *ibid.*, **61**: 3550, 1939; M. J. Copley, C. S. Marvel, and E. Ginsberg, *ibid.*, **61**: 3161, 1939; M. J. Copley, G. F. Zellhoefer, and C. S. Marvel, *ibid.*, **62**: 227, 1940; M. J. Copley, E. Ginsberg, G. F. Zellhoefer, and C. S. Marvel, *ibid.*, **63**: 254, 1941; C. S. Marvel, M. J. Copley, and E. Ginsberg, *ibid.*, **63**: 3109, 3263, 1940; C. S. Marvel, F. C. Dietz, and M. J. Copley, *ibid.*, **62**: 2273, 1940; C. S. Marvel, J. Harkema, and M. J. Copley, *ibid.*, **63**: 1609, 1941; C. S. Marvel and J. Harkema, *ibid.*, **63**: 2221, 1941.

involved by the method of elimination familiar in organic chemistry; the results are often complicated by homomolecular association in one or other of the two components, so that the observed solubility or heat of reaction is actually a resultant of several processes which may be difficult to disentangle. It is obvious that in labile systems of unknown constitution the methods are not likely to be very useful. From a brief study of the solubilities of polyesters, polyketones, and polymeric halogenated hydrocarbons it has been concluded that hydrogen bonding is only one of several unknown factors determining the observed relationships. Polyvinyl chloride $(—CH_2 \cdot CHCl—)_n$, which should form hydrogen bonds, has about the same low solubility in chloroform, tetrachlorethane, carbon tetrachloride, and tetrachlorethylene; the polyesters and polyketones, on the other hand, show the expected behavior, being far more soluble in $CHCl_3$ and $C_2H_2Cl_4$ than in CCl_4 or C_2Cl_4.

The second method to which we refer is that of infra-red spectroscopy in the wave length region around 3μ.[23] The absorption band corresponding to vibration of a singly bound hydrogen atom, for example, in —OH, ≡CH, etc., is usually spread out and displaced to a lower frequency when the hydrogen is subjected to some additional force. Perturbation of the XH bond, which can thus be measured spectroscopically, is usually attributed to molecular association through hydrogen bonds. This conclusion has been well substantiated in certain cases, but the interpretation of the observed bands is less definite in the case of large molecules. Features in the complicated spectra of cellulose[24] and gelatin[25] have been explained by analogy with those of sucrose and of various associated amides respectively. Crystalline sucrose[26] shows several first overtone bands at $1.5–2.0\mu$, attributed to valence vibration (stretching) and valence vibration combined with distortion (bending) of perturbed and unperturbed OH groups. These can be identified in modified form in the spectrum of dried ramie fiber, the unperturbed components being practically absent. In studies on dry proteins, certain bands have been ascribed to double hydrogen bonds, producing eight-membered rings, between parallel ketoimino linkages,[27] and intensity changes have been observed on absorption of moisture.[28] The detailed interpretation of these results, however, requires much further work; this may, indeed, be said of all methods of investigating hydrogen bonds in complicated systems. The lability of the bond contributes at once to the difficulties attending its investigation and to its extreme impor-

[23] Recent reviews: ref. (1); also G. B. B. M. Sutherland, Tr. Faraday Soc., **35**: 389, 1940; J. J. Fox and A. E. Martin, *ibid.*: 897, 1940.

[24] J. W. Ellis and J. Bath, J. Am. Chem. Soc., **62**: 2859, 1940.

[25] A. M. Buswell, W. H. Rodebush, and M. F. Roy, *ibid.*, **60**: 2444, 1938; A. M. Buswell, J. R. Downing, and W. H. Rodebush, *ibid.*, **61**: 3252, 1939.

[26] J. W. Ellis and J. Bath, J. Chem. Phys., **6**: 221, 1938.

[27] A. M. Buswell, J. R. Downing, and W. H. Rodebush, J. Am. Chem. Soc., **61**: 3252, 1939. A. M. Buswell, K. F. Krebs, and W. H. Rodebush, J. Phys. Chem., **44**: 1126, 1940.

[28] J. W. Ellis and J. Bath, J. Chem. Phys. **6**: 723, 1938.

tance, so that a linkage which can be said to be more significant for physiology "than any other single structural feature"[29] can also be described as "no more than an attractive possibility"[30] in protein structure.

7. Van der Waals and Other Secondary Forces.—Molecular association is often observed in cases where formation of hydrogen bonds is unlikely; calculations based on the heats of sublimation of organic compounds give values of the molar cohesion of various groups,[31] ranging from 1 kcal./mol. for the methylene group to 7.25 for hydroxyl and 16.2 for ketoimino. The higher values refer to polar groups, including those which can take part in hydrogen bridges. Well-marked association in absence of the latter usually involves dipole-dipole interaction, or the interaction of permanent and induced dipoles, with stabilizing contributions from dispersion forces; it is clear from the formulæ in Table V that, under favorable conditions, the former effects, depending on the square of the permanent moment, will greatly exceed the latter, and will, moreover, cause mutual orientation of a very specific kind, depending on the shape of the inducing dipole and on the local distribution and anisotropy of polarizability of the induced dipole. These relationships have been interestingly worked out in the case of associations between the lamellar molecules of naphthalene, anthracene, and phenanthrene with various nitrobenzenes.[32] In absence of dipole moment, as with cohesion between CH_2 or CH_3 groups, only dispersion effects can be involved. The magnitude of these is difficult to estimate, but there is little doubt of their importance. It is perhaps a good thing at this point to review the subject of Van der Waals forces more or less historically; this will lead to a clear statement of the conditions to which the well-tested equations are limited, and even without detailed information will enable us to view critically some of the more reckless references that have been made to Van der Waals forces in condensed systems.

The fundamental observation is that of the "imperfect" nature of all real gases, including even the inert gases at very low temperatures. Theoretical and empirical equations have been devised which account for this fact with varying degrees of success, those of Van der Waals and Dieterici and the semiempirical power series of Kamerlingh-Onnes being the most familiar. Experimental data are still most frequently expressed in terms of the Van der Waals constants a and b, and it is generally agreed that these correspond respectively to intermolecular forces of attraction and repulsion. The "a" term of Van der Waals' equation was originally deduced by assuming forces of attraction extending over many molecular diameters, but it was later shown, by statistical analysis of collision frequencies, that the same result can be obtained with short-range forces.[33] The mechanism of the

[29] Ref. (1), p. 265.

[30] D. Jordan-Lloyd, Tr. Faraday Soc., **36**: 886, 1940.

[31] M. Dunkel, Ztschr. f. phys. Chem., **138A**: 42, 1928.

[32] E.g., J. E. Lennard-Jones, Proc. Roy. Soc., **45**: 475, 1931; Tr. Faraday Soc., **32**: 1, 1936.

[33] E.g. P. Debye, Physik. Ztschr., **21**: 178, 1920, where earlier work is also referred to.

Van der Waals attraction probably varies. Keesom[34] showed that the temperature variation of a could be well represented in some cases by equations expressing the interaction of rigid quadrupoles, giving an intermolecular force inversely proportional to r^7T. Debye[35] introduced a temperature-independent term in r^{-7} to account for the fact that Van der Waals forces do not vanish at high temperatures, by assuming an induction effect. Neither the Keesom nor the Debye effects are, however, applicable to spherically symmetrical molecules, and it is in these cases that dispersion forces[36] become really important.

The detailed calculations have been made only for hydrogen and helium, but approximate generalized equations for nonpolar gases have been given; it is agreed that the force between the molecules of such substances at large distances can be represented by a function that varies as the inverse seventh power of the distance. Some idea of the kind of agreement that can be obtained is given by the data in Table VI, columns 1, 2 and 3, while the relative importance of the three components of residual force in several cases can be seen from column 6.[37] For the larger molecules the agreement between experiment and theory is poor; and even for the inert gases "it is not yet certain that there is not an error by a factor of two."[38] Nevertheless, the formulæ have been applied to larger molecules and even to bodies of colloidal dimensions by integrating or summing the reciprocal energies of every pair of atoms in the interacting bodies. The principle invoked here is that the attraction between two atoms is independent of the presence of a third; thus, given the dispersion formula, the question is merely a mathematical one. Such manipulations have given forces varying as r^{-2}, where r is the shortest distance of separation, for two spheres in "contact"[39,40] or a sphere and an infinite plane surface,[40] as r^{-4} for an atom near a plane surface[41] and as r^{-3} for two plane surfaces[40,42] and it has been claimed[39,43] that formulæ obtained in this way justify the assumption[44] of interparticle attractive forces of sufficient range and intensity to account for the formation of coacervates and thixotropic gels. Hamaker[40] also attempts to show that the forces of attraction between two similar particles are unaffected by the presence of an intervening fluid medium, provided

[34] W. H. Keesom, Physik. Ztschr., **22**: 129, 1921; *ibid.*, **23**: 225, 1922.

[35] P. Debye, *ibid.*, **21**: 178, 1920; H. Falkenhagen, *ibid.*, **23**: 87, 1922.

[36] F. London, Ztschr. f. physik. Chem., **11B**: 222, 1930.

[37] Cf. A. J. Staverman, Physica, **4**: 1141, 1937.

[38] J. E. Lennard-Jones, Tr. Faraday Soc. **32**: 37, 1936.

[39] R. S. Bradley, Phil. Mag., **13**: 853, 1932.

[40] H. C. Hamaker, Physica, **4**: 1058, 1937.

[41] M. Polanyi and F. London, Naturwissenschaften, **18**: 1099, 1930.

[42] J. H. de Boer, Tr. Faraday Soc., **32**: 10, 1936.

[43] H. C. Hamaker, Rec. trav., **55**: 1015, 1936; *ibid.*, **56**: 3, 727, 1937; *ibid.*, **57**: 51, 1938; Tr. Faraday Soc., **36**: 186, 1940.

[44] H. Kallmann and M. Willstaetter, Naturwissenschaften, **20**: 952, 1932; H. Freundlich, Thixotropy: Paris, 1935.

the molecules of this are not oriented, but his arguments are formal ones and no attempt is made to justify them on physical grounds.[45]

Although Van der Waals forces are probably not, as a rule, important in the structure of aqueous disperse systems, they do to a large extent determine the molecular volume of liquids (Table VI), and in absence of dipole or ionic forces they are responsible for the cohesion of molecular crystals, the form being determined largely by the shape of the homopolar units, and other properties by the strength of intermolecular binding. In particular, the intermolecular distances in molecular crystals and nonpolar liquids usually have the large value 3–5 Å, and the existence of such spacings is taken to indicate the residual nature of the binding. The crystals are soft, compressible, have high coefficients of thermal expansion and low heats of sublimation. The simple dispersion formulæ have been used to calculate the tensile strength of a number of synthetic resins,[46,47] with results at least one order of magnitude larger than those observed;[48] this has been attributed to the presence of structural faults or "Lockerstellen" in the materials.[47,48] An estimate[46] of the tensile strength of cellulose fibers, on the other hand, made by assuming this to be composed of the force necessary to tear loose certain Van der Waals linkages and that needed to cause the stretched molecules to slide over one another, has given results of the same order of magnitude as those observed.[49] This probably gives cause for astonishment; in another case studied by de Boer[46] the approximate formula gives unexpected agreement with experimental data—namely, in the calculation of the sublimation energy of benzene. One imagines the agreement to be fortuitous, since molecules with nonlocalized electrons probably require special theoretical treatment,[50] while the practice of postulating distributed centers of Van der Waals attraction in order to facilitate calculation has been criticized by London.[51]

London[51] has at the same time drawn attention to the possibility of long-range Van der Waals forces of considerable biological importance between molecules containing regions of conductivity such as those produced by conjugated double bonds. He makes a start by considering the dispersion forces between elongated molecules of this kind. In these the virtual oscillators are of low frequency and are comparable in length to the length of the chain. Their extent is taken into account by separating each into two suitably situated regions of positive and negative density, and the results of the calculation are expressed in terms of the positions of

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[45] Cf. I. Langmuir, J. Chem. Phys., **6**: 873, 1938.

[46] J. H. de Boer, Tr. Faraday Soc., **32**: 10, 1936.

[47] R. Houwink, Elasticity, Plasticity and Structure of Matter: Cambridge, 1937.

[48] R. Houwink, Tr. Faraday Soc., **32**: 122, 1936.

[49] K. H. Meyer and H. Mark, Der Aufbau der hochpolymeren organischen Naturstoffe: Leipzig, 1930, p. 152.

[50] Cf. J. E. Lennard-Jones, Tr. Faraday Soc., **32**: 37, 1936.

[51] F. London, J. Phys. Chem., **46**: 305, 1942.

these "monopoles" in the interacting molecules and the energy of the quantum jump believed, from spectroscopic data, to be associated with interatomic charge transfer. At intermolecular distances very small compared with the oscillator length, for molecules with similar monopoles in juxtaposition, the attractive force varies as $1/r^3$; the force on a small spherical molecule near a long oscillator varies as $1/r^5$. It is suggested that the existence of such long-range forces, operating at widely separated points of attraction along a conjugated chain, may account for the elasticity of rubber, in contrast to the brittle long-chain paraffins, in which short-range ($1/r^7$) forces act between separate atoms.

We opened this section with a reference to molecular association resulting from orientation and induction effects. It should be added that the dispersion effect, to which we have at least by implication attributed part of the lateral cohesion of chain molecules in fibrous structures, and the molecular binding of homopolar molecules in crystals, can also under other circumstances play an important part in restricting the random motion of molecules, and, therefore, in promoting their morphological organization. The calculations of de Boer,[52] although open to criticism, suggest that in liquid benzene there may be a tendency to formation of unstable dimers consisting of benzene rings lying in parallel planes about 3.5 Å apart; this tendency is opposed by thermal motion and by a polarization anisotropy which would favor another configuration, but there is evidence[53] that the parallel orientation does occur. This is not, however, the configuration adopted in benzene crystals.[54] Intramolecular dispersion forces can similarly give certain configurations a higher probability than others, leading in the case of chain molecules to an average chain length different from that which would be anticipated on the basis of unrestricted rotation about single bonds. Such preferred orientations have been discussed in the case of polystyrene, $(—CH(C_6H_5)—CH_2—)_n$,[55] where the orienting effect of benzene rings would be expected to play a part. These effects are better understood in small molecules, and, even in ethane, studies of heat capacity and vibrational spectra indicate that a potential barrier of 3000 cal./mole restricts internal rotation in favor of a configuration in which the six C—H bonds, projected on a plane perpendicular to the C—C axis, are staggered at angles of 60° to one another.[56] In ethylene halides, where dipole forces come into play, electron diffraction studies show that rotation is considerably impeded in favor of the transisomer.[57] In substituted ben-

[52] J. H. de Boer, Tr. Faraday Soc., **32**: 10, 1936; J. H. de Boer and G. Heller, Physica, **4**: 1045, 1937.

[53] G. Briegleb, Ztschr. f. physik Chem., **14B**: 97, 1931; *ibid.*, **16B**: 249, 1932; J. Selman, cited by J. H. de Boer, Tr. Faraday Soc., **32**: 10, 1936.

[54] E. G. Cox, Proc. Roy. Soc., **135A**: 491, 1932.

[55] J. H. de Boer, Tr. Faraday Soc., **32**: 10, 1936.

[56] G. B. Kistiakowsky, J. R. Lacher, and F. Stitt, J. Chem. Phys., **7**: 289, 1939; F. Stitt, *ibid.*, **7**: 297, 1939; cp. H. Eyring, J. Am. Chem. Soc., **54**: 3191, 1932.

[57] J. Y. Beach and K. J. Palmer, J. Chem. Phys., **5**: 639, 1938; J. Y. Beach and A. Turke-

zenes it appears that polar groups impede one another's rotation about single bonds whenever they approach to within about 3 Å.[58]

8. Long-range Forces in Macromolecular Systems.—Quantitative arguments have obviated any need for the assumption of long-range Van der Waals forces in explaining the stability of hydrophobic systems. Other biological phenomena have been mentioned, however, which seem at first sight to require some mechanism for the transmission of "stimuli" to remote points. The best-known case is that of photosynthesis, in whch it seems to be necessary, in order to explain the observed fractional quantum efficiency,[59] for a large assembly of chlorophyll molecules in the interior of the chloroplast to absorb four light quanta simultaneously[60] and then to transmit the electronic excitation energy from one molecule to the next by some sort of resonance effect, until it reaches a point on the surface occupied by an adsorbed CO_2 molecule.[61] Other cases have been mentioned in a recent article by Szent-Györgyi,[62] who seems to suggest that common energy levels associated with the presence of nonlocalized electrons may be responsible for the transmission of "chemical" stimuli in genes, muscle fibers, and even through an entire organism. London, whose treatment of long-range "monopole" forces has already been referred to, has expressed an opinion in this connection that is worth quoting: "There can be, however, some doubt as to whether at present we are able to survey all consequences of quantum mechanics to such an extent that we could, with certainty, preclude the existence of specifically macromolecular forces which could not be built up by the well-known elementary atomic or molecular forces, but would rather depend on properties of the molecule *as a whole*. . . . It might be of interest to establish in full generality the conditions under which, in the interior of molecules, such regions of 'electric conductivity' may occur as we have recognized to be responsible for the presence of these characteristic long-range forces. It might well be that still other phenomena and mechanisms are connected with this peculiar kind of intramolecular charge transfer."[63] It is a long step from this to the conducting assemblies of macromolecules suggested by Szent-Györgyi and by Weiss, and still further to the conception of the "cell as a whole," or as a single giant molecule, as we facetiously spoke of it at the opening of this chapter.

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vich, J. Am. Chem. Soc., **61**: 303, 1939; cp. also A. Turkevich and J. Y. Beach, *ibid.*, **61**: 3127, 1939; C. T. Zahn, Phys. Rev., **37**: 1516, 1931; E. W. Greene and J. W. Williams, *ibid.*, **42**: 119, 1932.

[58] R. Tiganik, Ztschr. f. physik. Chem., **14B**: 135, 1931.

[59] O. Warburg and E. Negelein, Ztschr. f. physiol. Chem., **106**: 191, 1923; F. F. Rieke, J. Chem. Phys., **7**: 238, 1939.

[60] R. Emerson and W. Arnold, J. Gen. Physiol., **16**: 191, 1930; H. Gaffron and K. Wohl, Naturwissenschaften, **24**: 81, 1936; H. I. Kohn, Nature, **137**: 706, 1936.

[61] J. Weiss, Nature, **137**: 997, 1936.

[62] A. Szent-Györgyi, Science, **93**: 609, 1941.

[63] F. London, J. Phys. Chem., **46**: 305, 1942.

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7

SOME PROPERTIES OF LARGE MOLECULES IN SOLUTION[64,65]

1. Introduction.—The labile organization of protoplasm is closely bound up with its colloidal nature. Many experimenters in the past, recognizing the complexity of colloidal systems, and finding ample experimental evidence for the belief that reproducible properties can hardly be expected of random aggregates of dissimilar molecules, described colloids and the peculiar forms and reactions of protoplasm very much as a naturalist describes variations among species—as something amenable to meticulous description, but beyond the reach of quantitative experimental study. If invited to account for the extraordinary uniformity and reproducibility of, let us say, sea-urchin eggs, in terms of the extremely variable aggregates of which they are supposed to be composed, they would appeal to some external force, a vital principle, or an entelechy, which would impose form and vital function upon lifeless matter, much as a potter moulds his clay. Instead of thus confusing increasing complexity with increasing lack of organization, it is probably better to discern in the specificity of colloidal systems the emergence of new qualities which depend for their very existence upon an intrinsic tendency of matter to become oriented both in the geometrical or morphological sense and in the sense of a correlated localization of energy transfers. Except, perhaps, in the experimental study of biological specificity, this view has received no stronger support than in the discovery that the colloidal components of living matter can by the mildest methods be resolved into colloidal substances which yet have all the earmarks of chemical individuals. In a majority of cases they can be molecularly dispersed, and there is an increasing body of chemical and physical evidence that serves to identify these well-defined particles with some of the most highly specific components of protoplasm. It is the purpose of this chapter to review briefly the properties of such homogeneous colloidal substances, without particular reference to their origin; the common characteristic of immediate interest is their colloidal nature, which implies large molecular size and, therefore, the use of special methods of study. Important substances of this class are obtained synthetically, and since

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[64,65] *References:* In Chapter 7, frequent reference is made to [64] T. Svedberg and K. O. Pedersen and others, The Ultracentrifuge: Oxford, 1940; and [65] Advances in Colloid Science, Vol. I, edited by E. O. Kraemer: Interscience Publishers, Inc., New York, 1942.

some of these have properties that stand in interesting opposition to many of biological origin, they will be referred to. Particle size and shape are the most elementary properties to be discussed; the progress made possible by the use of physical methods in defining the net charge and the distribution of nonpolar, polar, and charged groups will be mentioned, and convergence of physical and chemical evidence to provide a more or less consistent general conception of macromolecules will complete our discussion of these substances as they probably exist in very dilute solutions. It will then be possible, again with the utmost brevity and incompleteness, to indicate, with especial emphasis upon the behavior of biological fluids and their derivatives, some of the interactions that occur in polydisperse solutions.

2. The Determination of Size and Shape of Large Molecules in Dilute Solution.—The determination of molecular size and shape can seldom be accomplished by use of a single experimental method. Moreover, application of a single method usually involves additional measurements often regarded as accessory in nature but actually as important and frequently as difficult as the principal determination. The results obtained by the various methods can, as a rule, only be expressed in comparable terms by the application of theoretical equations, and these, which are invariably approximate, impose a new uncertainty upon the errors of measurement.

The methods available are of two kinds; by their means one measures either a thermodynamic equilibrium or a velocity. The former have the important advantage that, involving as they do the establishment of an equilibrium between opposing tendencies regulated by the same dynamic quantities, they finally give a measure only of the number of particles present, irrespective of their size and shape. The classical thermodynamic method is, of course, that of osmotic pressure (cryoscopy being too untrustworthy and vapor pressure determinations usually too inaccurate), in which a mechanical barrier opposes diffusion of the colloidal solute. Scrupulously applied, it is still probably the most accurate, although by no means the most fashionable, method for determining the number of particles. It must be recognized that for a colloidal electrolyte the observed osmotic pressure arises only in part from the colloidal ions, and in part from the unequal distribution of diffusible ions: the relation to the molecular weight is given by a combination of Van't Hoff's law and Dalton's law of partial pressures.[56]

$$p = p_i + RT\varphi \frac{c}{M_1}, \tag{4}$$

where p = the observed osmotic pressure
p_i = the ion pressure difference

[56] a. G. S. Adair, Proc. Roy. Soc., **120A**: 573, 1928; b. G. S. Adair and M. E. Robinson, Biochem. J., **24**: 1864, 1930.

φ = the osmotic coefficient, a function of pH, salt concentration, and degree of interaction of colloidal ions and their shape.[57]

c/M_1 = the ratio of concentration to molecular weight; thus, if c refers to dry substance, M_1 is the corresponding molecular weight; the actual molecular weight in solution may be entirely different, as a result of solvation.

The measurement of osmotic pressure gives the correct value of c/M while the correct determination of the molecular weight depends upon a knowledge of solvation. This is, of course, still true when φ and p, have been eliminated, as is customarily done, by extrapolation to zero colloid concentration:

$$\frac{1}{M_1} = \left(\frac{p}{c}\right)_{c \to 0} \frac{1}{RT}. \tag{5}$$

The same limitations naturally apply to the sedimentation equilibrium method, in which the osmotic membrane is replaced by a gravitational force; there is the added complication that, since the gravitational field is not uniform, it is necessary to measure a concentration gradient. The molecular weight is then given by the expression:[58]

$$M_1 = \frac{2RT \ln (c_2/c_1)}{(1 - V_1\rho)\omega^2(x_2^2 - x_1^2)}, \tag{6}$$

where c_2, c_1 are relative concentrations at distances x_2, x_1 from the center of rotation, ω is angular velocity, V_1 is the partial specific volume of the colloidal solute, defined as $\partial v/\partial g$, where v is volume and g is mass of solute, while ρ is the density of the solution. If V_1 refers to the unhydrated substance, M_1 determined by equation (6) at high dilutions should be comparable to the value found by osmotic pressure determinations.

The dynamic methods have in common their dependence upon an imposed translational or rotational movement of the particles, or both, in opposition to their Brownian movement; all the equations employed must therefore contain terms expressing frictional resistance to motion, and these must necessarily be functions of the shape and flexibility, and often also of the size, of the particles. In other words, if the reference substance is the anhydrous solute, it is always necessary to take into account the degree of solvation and the shape of the resulting solvated particle. In general it is not possible to calculate both these quantities, and until recently it has been customary to express results in terms of an apparent shape factor calculated for a hypothetical unsolvated particle. As far as possible we shall avoid placing too much weight upon results obtained in this way.

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[57] Cf. R. E. Powell and H. Eyring, Frictional and Thermodynamic Properties of Large Molecules, in ref. (65), p. 183, 1942.

[58] a. T. Svedberg, Kolloid-Ztschr., Erg. Bd., zu 36, 53, 1925; b. Colloid Chemistry, 2nd ed.: Chemical Catalog Co., New York, 1928, p. 150. c. ref. (64), pp. 6, 48.

The dynamic methods further divide themselves according to the nature of the property measured. In measurement of sedimentation rate, diffusion constant, and viscosity, we deal with properties directly related to the mechanical interaction between solute and solvent, so that all interpretations of these quantities in terms of the properties of the solute must contain some function of a friction coefficient, f, which gives the frictional force opposing translational motion of a solute molecule as (fv), where v is the rate of movement. For the motion of a spherical molecule, Stokes' law gives the frictional coefficient f_0 as

$$f_0 = 6\pi\eta r = 6\pi\eta N \left(\frac{3MV}{4\pi N}\right)^{1/3}, \tag{7}$$

where η is viscosity, r radius of the molecule, N Avogadro's number $(6.06 \cdot 10^{23})$, M molecular weight, V partial specific volume. If the particle is not spherical, f is some other function of shape. The functions for both elongated and flat ellipsoids of revolution (rods and discs) in random configuration have been derived theoretically in the form

$$\frac{f}{f_0} = \Phi\left(\frac{a}{b}\right) \tag{8}$$

where f_0 is the frictional coefficient of a sphere of equal volume and a, b are the axes of the ellipsoid.[59] These equations are applicable to measurements in which orientation of the solute molecule does not occur.

In the second group of dynamic methods, namely, the measurement of dielectric dispersion and streaming double refraction, the effects observed depend similarly upon a frictional coefficient, ζ, in this case representing the force opposing rotation, and defined by $\zeta\omega$, where ω is angular velocity. For rotation of a spherical molecule

$$\zeta = 8\pi\eta r^3, \tag{9}$$

while for an ellipsoid of revolution any mode of rotation can be expressed as the resultant of components of rotation of each axis about the other, each with its own frictional coefficient, ζ_a and ζ_b. It is convenient in practice to replace the friction coefficient by a closely related quantity, the time required for a system of oriented molecules to regain a certain degree of random distribution when the orienting force is relaxed; this is known as the relaxation time, τ,[70] and for a spherical molecule is given by

$$\tau_0 = \frac{\zeta}{2kT} = \frac{4\pi\eta r^3}{kT} = \frac{\pi\eta MV}{3RT}, \tag{10}$$

where k is Boltzmann's constant $(1.372 \cdot 10^{-16}$ erg. deg.$^{-1})$. For ellipsoids

[59] a. R. O. Herzog, R. Illig, and H. Kudar, Ztschr. f. physik. Chem., 137A: 329, 1934; b. F. Perrin, J. Phys. Rad., (7), 7: 1, 1936.

[70] For precise definition see P. Debye, Polar Molecules: Chemical Catalog Co., New York, 1929, pp. 83ff.

of revolution the values of τ_a and τ_b have been expressed in terms of the axial ratios by equations analogous to (8):[71]

$$\frac{\tau_a}{\tau_0} = \Phi'\left(\frac{a}{b}\right) \frac{\tau_b}{\tau_0} = \Phi''\left(\frac{a}{b}\right). \tag{11}$$

where, again, τ_0 refers to the equivalent sphere and can be calculated from equation (10).

We can now consider more explicitly the various experimental methods. The instrument that has probably contributed most to the physical knowledge of large molecules is the ultracentrifuge,[72] the use of which in determining sedimentation equilibria has already been referred to. Before such equilibrium is attained, the rate of sedimentation during centrifuging can be determined and expressed as a sedimentation constant, s, defined as the rate of movement of the substance, dx/dt, in a field of unit centrifugal force. Equating centrifugal and frictional forces gives the expressions

$$\left(\frac{dx}{dt}\right) \cdot \frac{1}{\omega^2 x} = s = \frac{M_{12}(1 - V_{12}\rho)}{f_{12}} = \frac{M_1(1 - V_1\rho)}{f_1}, \tag{12}$$

where the symbols have the meanings previously assigned to them, the suffix 12 referring to the actual solvent-solute complex comprising the colloidal kinetic unit in solution, and 1 to the unsolvated molecules. It is easy to show that

$$f_1 = f_{12} \tag{13}$$

approximately. Hence the use of V_1 and M_1 (obtained by some other method) in conjunction with the experimental value of s will give the correct value f_{12} for the frictional coefficient of the solvated molecule. The calculation of a shape factor by use of equation (8) is, however, not possible unless we know $(f_0)_{12}$, the frictional coefficient for a spherical molecule of molecular weight M_{12}. If w is the number of grams of solvent bound to one gram anhydrous substance, it may easily be shown that for a very dilute aqueous solution ($\rho = 1.00$),

$$\frac{f_{12}}{(f_0)_{12}} = \frac{f_{12}}{(f_0)_1} \cdot \frac{(f_0)_1}{(f_0)_{12}} = \left(\frac{V_1}{w + V_1}\right)^{2/3} \cdot \frac{f_{12}}{(f_0)_1} = g\,\frac{f_{12}}{(f_0)_1}, \tag{14}$$

where the fraction $f_{12}/(f_0)_1$ is the value usually, and erroneously, given as the "frictional ratio," f/f_0. Clearly, we must have some independent way of finding w, the hydration, if the correction factor g is to be applied.

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[71] a. R. Gans, Ann. Physik, (4), **86**: 628, (1928); b. F. Perrin, J. Phys. Rad., (7), **5**: 497, 1934.

[72] a. Exhaustively discussed in ref. (64). b. Reference should also be made to a group of papers in Ann. New York Acad. Sc., **43**: Art. 5, 173, 1942. Errors due to failure to correct for variation of viscosity with centrifugal force are pointed out by R. E. Powell and H. Eyring in ref. (65), p. 212.

The molecular weight M_1 is often obtained from the sedimentation constant by inserting in equation (12) a value of f_{12} calculated from the diffusion constant, D:

$$f_{12} = \frac{RT}{D} \tag{15}$$

The information gained from viscosity measurements, even at great dilution, is far more uncertain; it has been shown repeatedly that elongated particles form more viscous solutions than spherical ones, but the precise relationship between viscosity and shape is still uncertain.[73] Many of the equations that have been suggested are of the form

$$\frac{(\eta - \eta_0)}{\eta_0} = kVc, \tag{16}$$

where η and η_0 are viscosities of solution and solvent respectively, V is partial specific volume, c is concentration in g./100 cm.^{3}, and k is a function of particle shape. The practical determination of k is best done by extrapolation, since in many cases deviations from the simple proportionality of equation (16) occur at quite low concentrations; accordingly, we may define k as the volumetric intrinsic viscosity, $[\eta]_V$:[74]

$$[\eta]_V = \frac{1}{V}\left(\frac{\eta - \eta_0}{\eta_0 c}\right)_{c\to 0} = k. \tag{17}$$

For spherical particles, k is equal to 0.025.[75] For ellipsoids of revolution, a theoretical expression for k obtained by Simha[76] has met with some success in application to proteins.[77] The correct application of any equation, however, requires the use of an appropriate value for the partial specific volume, and hence, as in all other methods, a knowledge of solvation. The correct value of $[\eta]_V$ in equation (17) is

$$\frac{1 + w}{V_1 + w}\left[\frac{\eta - \eta_0}{\eta_0}\frac{1}{c(1 + w)}\right]_{c_1\to 0}$$

An empirical equation relating intrinsic viscosity of a number of proteins to axial ratios derived from sedimentation and diffusion data (equations 12, 15 and 8) has been given by Polson.[78] Working backward, Polson then

[73] See especially the review by Lauffer, Chem. Rev., Dec., 1942, which appeared after the completion of this chapter.

[74] a. E. O. Kraemer and W. D. Lansing, Nature, **133**: 870, 1934; b. E. O. Kraemer and J. B. Nichols in ref. (64), p. 417.

[75] a. A. Einstein, Ann. Physik, **19**: 371, 1906; *ibid.*, **34**: 591, 1911; b. see also the qualitative statement in H. Mark, Physical Chemistry of High Polymeric Systems: Interscience Pub., Inc., New York, 1941, pp. 277ff.

[76] R. Simha, J. Phys. Chem., **44**: 25, 1940.

[77] For a discussion of other viscosity equations see, e.g., H. Mark, Physical Chemistry of High Polymeric Systems; Interscience Pub., Inc., New York, 1941, pp. 255ff.

[78] a. A. Polson, Kolloid-Ztschr., **87**: 149, 1939; b. *ibid.*, **88**: 51, 1939.

derives the molecular weights of a number of proteins from their intrinsic viscosities by means of the same three equations. The values of a/b obtained during this procedure are not to be taken seriously, since hydration is ignored, but the method may have certain value in determining M approximately when sedimentation data are not available.

The determination of molecular weights by interpolation from viscosity measurements is possible only in cases where a number of members of a polymeric homologous series have been examined. The equation of Staudinger,[79] which may be expressed in the form:

$$[\eta]_V = kZ, \tag{18}$$

where Z is the degree of polymerization, k is a constant characteristic of a given homologous series, was derived for rigid rods with effective volumes equal to the volume of the disc described by rotation of the long axis of the rod; other evidence, theoretical and experimental, suggests strongly that linear polymers are flexible or worm-like structures.[80,81,82,83] This greatly limits the theoretical value of Staudinger's equation.[84]

The experimental study of molecular rotation and the interpretation of the data (cp. equations 10 and 11) are difficult. This applies especially to the results of dielectric dispersion measurement because of the extreme generality of the Debye equation for the relaxation time, which will apply to "any property which measures how much a system changes from one state to another when a periodic force is applied"[85] and which, in almost any system, could result from one of, or a combination of, several different mechanisms. In the simplest case the quantity derived from dispersion curves for dielectric constant or conductivity is a single critical frequency, ν_c, the frequency at which

$$\epsilon' = \tfrac{1}{2}(\epsilon_0 + \epsilon_\infty) \tag{19}$$

and $2\lambda/\nu$, or ϵ'', has its maximum value,

where ϵ' = dielectric constant at any frequency,
ϵ_0 = static dielectric constant,
ϵ_∞ = high frequency dielectric constant,
λ = conductivity.

[79] E.g., H. Staudinger, Die hochmolekularen organischen Verbindungen: Kautschuk und Cellulose: H. Springer, Berlin, 1932, p. 96.; more recent work from Staudinger's laboratory is summarized by E. O. Kraemer, J. Franklin Inst., **230**: 514, 1940.

[80] a. W. Kuhn, Kolloid-Ztschr., **68**: 2, 1934; b. M. L. Huggins, J. Phys. Chem., **42**: 911, 1938; c. *ibid.*, **43**: 439, 1939.

[81] E.g., R. Fordyce and H. Hibbert, J. Am. Chem. Soc., **61**: 1910, 1912, 1939.

[82] a. P. J. Flory, J. Am. Chem. Soc., **62**: 1057, 1940; b. J. Phys. Chem. **46**: 870, 1942.

[83] R. E. Powell and H. Eyring in ref. (65), p. 183, 1942.

[84] a. Compare E. O. Kraemer, J. Franklin Inst., **230**: 514, 1940; b. see also the discussion of the viscosity of polyvinyl chloride solutions by D. J. Mead and R. M. Fuoss, J. Am. Chem. Soc., **64**: 277, 1942, and a review of data based on Staudinger's equation by c. K. H. Meyer, Kolloid-Ztschr., **95**: 70, 1941.

[85] R. E. Powell and H. Eyring, in ref. (65), p. 215, 1942.

From this we can calculate a single relaxation time τ provided the relation between polarizability and dielectric constant is known; in many cases for solutions in polar solvents the relation is linear[86] and then

$$\nu_c = \frac{1}{2\pi\tau}. \tag{20}$$

If the solute molecules are known or suspected to have dipole structure it is quite probable that the measured relaxation time is connected with the rotation of dipoles in a viscous medium under the influence of the applied field, and this interpretation can in many cases be supported by other evidence; particularly, when the structure of the molecule is known in some detail, it can be determined whether or not the observed relaxation time is compatible with rotation of a spherical unsolvated molecule, or with oscillation of a single group, or whether a solvation shell or a non-spherical shape must be assumed to account for the observed values. The latter case can occur if the second relaxation time is associated with a very minute dipole moment.[87] In the case of large multipolar molecules, it is quite usual to find the dispersion effect spread over a wider frequency range than that required by the Debye equation, and it may be possible to attribute this to the modes of rotation of an ellipsoid of revolution and to calculate the axial ratio, the molecular volume (MV), the direction of the dipole moment vector, and the hydration.[87] If two relaxation times are insufficient, other interpretations are possible; in the case of thread-like molecules, there may be many relaxation times, because "in the various configurations which a chain molecule can assume we can find now one, now another, segment of the chain acting effectively as a co-operating electrical unit."[88,89]

Several other relaxation mechanisms have been considered.[90] The only one which appears to require careful consideration in connection with protein solutions is that discussed by Fricke and Jacobson,[91] whose measurements on the dispersion of gelatin solutions and the effect of electrolytes and pH changes led to the conclusion that neither the orientation of dipoles,[92] nor the Debye-Falkenhagen effect[93] could wholly account for the results observed. The important mechanism, according to Fricke and Jacobson, is similar to that operating in coarse suspensions,[94] and is connected with

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[86] E.g., J. Wyman, Chem. Rev., **19**: 213, 1936.

[87] E.g., J. L. Oncley, Ann. New York Acad. Sc., **41**: 121, 1941.

[88] R. M. Fuoss and J. G. Kirkwood, J. Am. Chem. Soc., **63**: 385, 1941.

[89] a. J. G. Kirkwood and R. M. Fuoss, J. Chem. Phys., **9**: 329, 1941; b. compare W. A. Yager, Physics, **7**: 434, 1936.

[90] Some are enumerated by Powell and Eyring in ref. (65).

[91] H. Fricke and L. E. Jacobson, J. Phys. Chem., **43**: 781, 1939.

[92] P. J. Denekamp and H. R. Kruyt, Kolloid-Ztschr., **81**: 62, 1937.

[93] Relaxation of Debye-Hueckel ionic atmosphere: cf. H. Falkenhagen, Electrolytes, 1934, p. 107, etc.

[94] Studied in a considerable series of papers by Fricke, referred to in ref. (91).

polarization of an "interphase" consisting, probably, of oriented water molecules attached to the particle surface.[95,96,97]

Whereas in measurements of dielectric dispersion only a very small proportion of solute molecules is actually affected by the field, the measurement of streaming double refraction depends upon the mass orientation of particles. The orienting torque is applied hydrodynamically by a velocity gradient at right angles to the direction of flow, and the degree of orientation, subject of course to a distribution law, is a function of the velocity gradient and the Brownian movement or relaxation time of the particles. The intrinsic optical anisotropy of the particles is merely used as the index to the degree of orientation, and the optical quantities measured as a function of the velocity gradient must be interpreted theoretically in terms of a relaxation time, from which, in turn, shape factors may be obtained by the application of equation (11).[98] The method has given valuable results for elongated molecules with large relaxation times; it has only recently been extended to molecules which are also amenable to study by dielectric dispersion determinations. Consequently, it is too early to attempt a final estimate of its accuracy.

3. The Measurement of Hydration.—Without a knowledge of hydration, the shape and size of molecules in solution are not susceptible to precise definition, unless the molecules are so elongated that the effects of shape greatly outweigh the effect of any possible degree of hydration. Exact size and shape may indeed always defy definition, for the concept of hydration is itself manifold. Little effort has been made to reconcile the conflicting and certainly too circumscribed points of view, which have bred unnecessary controversy. From the unique character of water, it must be evident that there may be many degrees of hydration, ranging from mere mechanical entanglement[99] to the close-packed structure of dielectrically "saturated" water (q.v.) in an intense electrostatic field.[100] The subject has never been adequately reviewed,[101] nor has any sufficiently

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[95] Cf. Bernal and Fowler's discussion of the dielectric constant of ice: J. Chem. Phys., **1**: 515, 1933.

[96] E.g., M. von Ardenne, O. Groos, and G. Otterbein, Physik. Ztschr., **37**: 533, 1936; G. Baez, *ibid.*, **40**: 394, 1939.

[97] A number of early papers bearing on this question are discussed by E. Heymann, Kolloid-Ztschr., **56**: 229, 1934, including those of Piekara and of Errera, which cannot be dealt with here.

[98] a. See the excellent review by J. T. Edsall in ref. (65), pp. 269-316, 1942; b. a very concise series of relevant optical definitions is given by F. O. Schmitt and R. S. Bear, Biol. Rev., **14**: 27, 1939 (appendix).

[99] The mode of packing of water molecules into cavities has been interestingly discussed by D. M. Wrinch, Phil. Mag., (7) **25**: 705, 1938.

[100] a. Cf. Wo. Ostwald, cited by F. Thoenes, Biochem. Ztschr., **157**: 124, 1925; b. W. Kuhn, Kolloid-Ztschr., **36**: 275, 1924; c. H. Mark, Physical Chemistry of High Polymeric Systems: Interscience Pub., Inc., New York: 1940, pp. 254ff.

[101] But see a. O. Bluch, Protoplasma, **3**: 81, 1927; b. T. C. Barnes and T. L. Jahn, Quart. Rev. Biol. **9**: 292, 1934, c. H. Mark, (ref. 100c); d. R. A. Gortner, Outlines of Biochemistry:

comprehensive experimental study been made during the years in which other physical data concerning large molecules in solution have been accumulating. The resulting uncertainty of definition of various molecular magnitudes stands in contrast to the remarkable engineering skill and patient measurement that have given accuracy to many of the primary empirical quantities.

Thermodynamic methods have obvious disadvantages in non-ideal systems. The experimental data are in many cases reliable, but their interpretation may be dubious. In a number of investigations, the activity of water has been varied by mechanical pressure across a semipermeable membrane,[102] or by changing the water vapor pressure at constant temperature;[103] in others, by lowering the temperature.[102] Exposed to an atmosphere of constant water vapor pressure, a specimen will eventually attain a constant weight, which can be measured.[103] Alternatively, exposed to a constant temperature below its freezing point, a substance containing free water will in general separate into pure ice and an aqueous phase or phases of definite composition, and the amount of ice formed can be determined by measuring the heat of fusion,[104,105,106,107] or the volume change accompanying it,[108,109] or sometimes by weighing the ice extruded on the surface of a thin slice of the material;[110] the average composition of the residue can then be determined if its original composition be known. For a simple solution of sodium chloride at room temperature a continuous series of equilibria between water vapor and salt solution is established as the water vapor pressure is reduced, until it reaches a value slightly below 4 mm. Hg; at this point equilibrium is attained only when all the water has evaporated. At all lower vapor pressures the system is invariant. Similarly, on cooling, a series of equilibria between ice and solution is established until the temperature is below $-22°$C., when a mixture of salt and ice separates out and the system becomes entirely solid. Most colloids show a different behavior when the activity of water is lowered; an electrolyte-free gelatin gel, for

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Wiley, New York, 1929; e. R. A. Gortner, Tr. Faraday Soc., **25**: 678, 1930; f. K. C. Blanchard, Cold Spring Harbor Symp., **8**: 1, 1940; g. D. M. Greenberg in The Chemistry of the Amino Acids and Proteins, edited by C. L. A. Schmidt: C. C. Thomas, Springfield, Ill., 1938, p. 478.

[102] E.g., a. D. Jordan-Lloyd and T. Moran, Proc. Roy. Soc., **147A**: 382, 1934; b. T. Moran, *ibid.*, **118B**: 548, 1935.

[103] a. J. D. Babbitt, Canad. J. Research, **20**: 143, 1942, etc.; b. J. Brooks, Proc. Roy. Soc., **114B**: 258, 1933; c. J. Gen. Physiol., **17**: 783, 1934; d. For an early discussion of this factor see J. M. Van Bemmelen, Rec. trav. Chim., **7**: 87, 1888, reprinted in The Foundations of Colloid Chemistry, edited by E. Hatschek: Benn, London, 1925.

[104] M. Rubner, Abh. Preuss. Akad. Wiss., Nr. 1, 1922.

[105] F. Thoenes, Biochem. Ztschr., **157**: 124, 1925.

[106] W. Robinson, J. Biol. Chem., **92**: 699, 1931.

[107] R. A. Gortner, Outlines of Biochemistry: Wiley, New York, 1929.

[108] T. Moran, Proc. Roy. Soc., **107B**: 182, 1930.

[109] I. D. Jones and R. A. Gortner, Colloid Symp. Monographs, **9**: 387, 1931.

[110] a. T. Moran, Proc. Roy. Soc., **112A**: 30, 1926; b. *ibid.*, **118B**: 548, 1935.

example, forms a solid solution[111] containing about 0.5 g. water/g. gelatin on cooling to about −10°C., and the composition of this solid solution does not change significantly on further cooling to −45°C.[110,112] Analogous observations can be made when the water activity is reduced by other methods, although in some cases these give a somewhat less definite plateau. Moran[110b] considers that the water content of the solid solution of nearly constant composition represents a strongly bound fraction of the water, while at higher activities of water a further considerable amount is more loosely bound. The state of the latter portion has not been at all clearly defined, but in some sense the interpretation of the plateau may be a correct one, provided the curves represent true equilibrium states. This has been very plausibly doubted,[111,113] although the objections may be equivalent to the ostensibly opposed point of view adumbrated by Moran. Single points on the vapor pressure isotherms of some solid proteins have been obtained by Adair and Robinson.[114]

The amount of water bound by a protein is probably reduced by the presence of neutral salts or other crystalloids.[108] Some such effect would be expected if the bound water were also nonsolvent water. The latter property has been used, perhaps rather naïvely, as a second criterion of water binding,[115] the amount of bound water being calculated by comparing the apparent content of solvent water with the total water determined by drying. The former can be found by measuring the effect of added soluble substances on the freezing point or vapor pressure of the system, if it be assumed that the colloidal or other components do not adsorb or otherwise modify the activity coefficients of the various substances present.[116] Such an assumption is frequently unjustified, and it is only with very simple solutions and carefully chosen test substances that, if ever, the method can be trusted;[117] it may be doubted whether any accurate data have been published. A variant of the method has been applied to solid proteins by Sørensen[118] and others. Analyses of mother liquor and of protein crystals with adhering mother liquor often show the presence of a higher proportion

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[111] For microscopic description of this solid solution and discussion of its nature see W. B. Hardy, Proc. Roy. Soc., **112A**: 30, 1926.

[112] J. H. Mennie, Canad. J. Research, **7**: 178, 1932.

[113] S. S. Kistler, J. Am. Chem. Soc., **58**: 901, 1936.

[114] a. G. S. Adair and M. E. Robinson, Biochem. J., **24**: 993, 1930; b. J. Physiol., **72**: 2P, 1931.

[115] a. R. Newton and R. A. Gortner, Bot. Gaz., **74**: 442, 1922; b. R. A. Kruyt and K. C. Winkler, Ztschr. f. anorg. allgem. Chem., **188**: 200, 1930; c. A. V. Hill, Proc. Roy. Soc., **106B**: 477, 1930; d. E. Hatschek, Tr. Faraday Soc., **32**: 787, 1936, uses inability to hydrate anhydrous cobaltous chloride as an index for bound water in gelatin gels. The results (hydration around 0.5) are in agreement with those of Moran, but are perhaps open to other interpretations.

[116] Cf. G. S. Adair, Tr. Faraday Soc., **26**: 698, 1930.

[117] A. Grollman, J. Gen. Physiol., **14**: 661, 1931; but cf. F. W. Sunderman, J. Biol. Chem., **96**: 271, 1932.

[118] S. P. L. Sørensen, Compt. rend. d. trav. du lab. Carlsberg., **12**: 164, 1917.

of water to salt in the crystal than in the mother liquor. Adair and Adair[119] have used Sørensen's distribution data to calculate the hydration of several proteins, with results again falling in the range 0.2 -0.3. In a further slight variant, it has been found that a gram of dry silk fibroin immersed in a solution of the disaccharide trehalose adsorbs about 0.26 g. pure water, the surrounding solution becoming more concentrated.[120] From the equilibrium distribution of glucose between protein residue and ultrafiltrate, on the other hand, much lower values have been found for casein (0.01) and gelatin (0.05).[121] This might, however, result from combination of glucose with protein. It is often found that there is excess salt instead of excess water in protein crystals,[119,122] although there is evidence that the crystals, nevertheless, contain water of hydration.[119]

The hydration of protein crystals is amenable to more exact study if there is reason to believe that all the water in a well-defined crystal takes part in the structure of the crystal. This is not necessarily so. It is possible for water to exist merely in cavities from which it can be removed and replaced by air without disrupting the crystal. In recent studies of β-lactoglobulin it has been shown, however, that the unit cell shrinks when the crystal is dried[123,124] and that a proportional change in crystal dimensions takes place.[125,126] The change in volume is very nearly the same as that of the water lost on drying (0.84), and other evidence has been given[125] that the dry crystals are compact and free from air. Comparison of the densities of the wet and dry crystals, measured by a flotation method with organic media, leads to a similar value for the hydration of β-lactoglobulin.[125] Application of Sørensen's method, however, gives 0.294, suggesting that the bound water is present in two forms, only one of which is available as a medium for the free diffusion of dissolved substances.

Calculations of hydration from crystal densities were given earlier for a number of proteins by Adair and Adair,[119] using aqueous flotation media. Exchange of dissolved substances between medium and crystal water undoubtedly leads to changes of density,[120] and it seems probable that the lower hydration values obtained in these experiments are in part due to this source of error.[119] Adair and Adair stress this point in discussing the relation between the degree of hydration of proteins in the crystalline and dissolved forms. They point out that values found when the activity of

[119] G. S. Adair and M. E. Adair, Proc. Roy. Soc., **120B**: 422, 1936.

[120] L. F. Gleysteen and M. Harris, Nat. Bur. Standards J. Res., **26**: 71, 1941.

[121] D. M. Greenberg and W. F. Cohn, J. Gen. Physiol., **18**: 93, 1933.

[122] M. Sørensen and A. H. Palmer, Compt. rend. d. trav. du lab. Carlsberg, **21**: 283, 1938.

[123] a. D. Crowfoot, Chem. Rev., **28**: 215, 1941; b. D. Crowfoot and D. Riley, Nature, **141**: 521, 1938.

[124] I. Fankuchen, J. Am. Chem. Soc., **64**: 2504, 1942.

[125] T. L. McMeekin and R. C. Warner, J. Am. Chem. Soc., **64**: 2393, 1942.

[126] Cf., however, the view of F. Haurowitz (Ztschr. f. physiol. Chem., **135**: 147, 1924) that the water associated with hemoglobin crystals is to be regarded as water of imbibition, and not as water of crystallization.

water is low are likely to increase at the higher activities existing in solutions; moreover, since inorganic ions can diffuse into the water of hydration of the crystal, the environment of the protein molecule in this condition corresponds closely to that in solution. The former thermodynamic argument should not be unduly stressed, since the state of the protein molecules in the crystal can certainly not be defined in terms of the thermodynamic constants that are applicable in dilute solution. Crowfoot[123] considers that only part of the crystal water is likely to remain in association with protein molecules dispersed in aqueous solution.

We have seen (subsec. 2) how hydration can be calculated from dielectric relaxation data if the molecular weight of the anhydrous particle be known. A few values calculated in this way are given in Table VII, although they should be regarded as tentative. Some, if not all, of the water which is associated with the rotating particle in this way exists presumably in an oriented close-packed state in regions of high potential gradient. Water thus oriented loses its high permanent moment[127] and can be distinguished, in theory at least, from dielectrically free water by reason of its smaller contribution to the polarization. The total polarization of a solution thus consists of at least three additive components, resulting from the free water (dielectric constant around 80), the oriented water (d.c. around 2), and the anhydrous solute. In principle it should be possible to calculate the hydration from the dielectric constant of the solution and of the dry solute at frequencies high enough to eliminate any contribution from the permanent moment of the solute molecules, provided a satisfactory relationship between polarization and dielectric constant is available. The classical Clausius-Mosotti equation has been employed by Marinesco and others in such calculations,[128] but there is reason to believe that for many polar solutions this should be replaced by an empirical expression.[129] Marinesco's results are thus doubly in error, for the difficult experimental methods are not beyond criticism. Nevertheless some of the results are included in Table VII for comparison with those of other methods; the whole matter should be reinvestigated very completely. Unfortunately in most recent measurements the emphasis has been upon relaxation phenomena and the methods used[130] have not been well suited to measurement at high frequencies.

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[127] a. Ref. (70), pp. 109ff. Attempts to demonstrate the low dielectric constant of oriented water have not been very successful. In electrolyte solutions any such effect of hydration is masked by the Debye-Falkenhagen effect; b. H. Kallmann and K. E. Dorsch, Ztschr. f. physik. Chem., **125**: 305, 1927, as a result of direct measurement consider the dielectric constant of a water layer 5 Å thick at a platinum surface to be greater than 40. Compare, however, c. W. G. Palmer, Proc. Roy. Soc., **106**: 55, 1924; d. B. Derjaguin, Tr. Faraday Soc., **36**: 203, 1940.

[128] N. Marinesco, Kolloid-Ztschr., **58**: 285, 1932.

[129] a. J. Wyman, J. Am. Chem. Soc., **56**: 536, 1934; b. *ibid.*, **58**: 1482, 1936; c. Chem. Rev., **19**: 213, 1936.

[130] J. L. Oncley, J. Am. Chem. Soc., **60**: 1115, 1938; J. Phys. Chem., **44**: 1103, 1940.

Information obtained by other methods tends to be scanty and uncertain. Numerous qualitative observations have been made on gelating systems which have suggested that the phenomenon is closely associated

TABLE VII.—SOME RESULTS OF MEASUREMENT OF HYDRATION BY SEVERAL METHODS

	Egg albumin	Hemoglobin (horse)	β-lactoglobulin	Gelatin	Methemoglobin	Cholesterol	Lecithin	Cotswold wool
Freezing curves								
Weight of ice	0.26[a]			0.5[a,h]				
Heat absorbed				2.0[j]				
Volume change				1–5[k]				
Vapor pressure isotherm				0.5[l]*				0.32[r]
Nonsolvent water								
Analysis of crystals and liquor	0.243[b]	0.306[b]	0.247[f]	0.05[m]				
Vapor pressure depression				2.3[n]				
Freezing-point depression				1.2[o]				
Crystal density	0.21–0.32[b]	0.16–0.34[b]						
X-rays: shrinkage of unit cell		0.72†[e]	0.7†[g]		0.73†[g]			
			0.6†[e]		0.86†[g]			
Dielectric dispersion	0.2[r]		0.35[r]					
High frequency dielectric constant	1.5[d]		0.01[d]	2.7[d]		0.23[d]	1.9[d]	
Electrostriction of solvent				0.08[p]				
Water content of crystals			0.83[f]					

The methods are identified more exactly in text.

* Air-dried specimens. Values probably too low.

† Water activity too low for combination with anhydrous cobaltous chloride.

[a] T. Moran, (102b)
[b] Adair and Adair, (119)
[c] Oncley, (130b)
[d] Marinesco, (128)
[e] Crowfoot, (123b)
[f] McMeekin and Warner, (125)
[g] Crowfoot, (123a)
[h] Mennie, (112)
[i] Thoenes, (105)
[k] Jones and Gortner, (109)
[l] Hatschek, (115d)
[m] Greenberg and Cohn, (121)
[n] Grollman, (117a)
[o] Newton and Gortner, (115a)
[p] T. Svedberg, J. Am. Chem. Soc., **46**: 2673, 1924.
[q] J. D. Bernal, I. Fankuchen, and M. Perutz, Nature, **141**: 523, 1938.
[r] J. B. Speakman, Tr. Faraday Soc., **25**: 92, 1929.

with water binding, but it has been shown that neither dielectric constant,[131,132] nor vapor pressure[117] changes measurably during the process, while anomalies in viscosity and diffusion constant are probably due to

[131] P. Girard and N. Marinesco, C. r. Soc. Biol., **102**: 302, 1929.

[132] a. S. S. Kistler, J. Phys. Chem., **35**: 815, 1931; b. cf. H. Kallmann and W. Kreidl, Ztschr. f. physik. Chem., **159A**: 322, 1932.

molecular asymmetry and the tendency to net and chain formation rather than to hydration. Neglect of these factors sometimes leads to remarkable conclusions; Hammarsten, for example, has stated that *all* the water in a 0.5% solution of sodium thymonucleate is bound.[133] Similarly are such phenomena as the absence of Brownian movement in certain Bentonite suspensions and the high sedimentation volume of talcum powder[134] probably due to electrical repulsion between particles[135] and not to solvation. X-ray analysis has to some extent revealed the position of water in fibers and protein gels,[136,137,138] and water binding by formation of hydrogen bonds has been suggested from a study of infra-red spectra.[139] These methods do not at present give quantitative data, but in the case of gelatin it appears that they are at least consistent with a hydration of about 0.35.[139b]

It will be seen from a perusal of the data in Table VII, which is very far from exhaustive, that despite the diversity of criteria for bound water, a fair degree of consistency among the values found warrants the conclusion that hydration is by no means a negligible phenomenon. For several proteins, a probable value is 0.2–0.5, although there are certainly plenty of discrepancies calling for resolution. If, as has often been suggested, the water of hydration exists in two fractions, each with its own properties, it would be surprising to find complete agreement among the data. This is a dangerous, if plausible, statement, and the extreme skepticism of some reviewers is perhaps due to a feeling that such a flexible idea can be too readily used as a mask for experimental error.

4. Experimental Results Relating to Size and Shape of Large Molecules in Solution.—We can differentiate two kinds of dissolved macromolecules: linear polymers and globular molecules. The former are flexible thread-like particles, ideally unbranched, but often complicated by some degree of branching. The globular molecules are essentially rigid; they may be tightly coiled threads (the tight or the spongy spherical clews of Signer), very highly branched linear polymers with interacting segments which produce rigidity, or strictly three-dimensional covalent lattice structures of varying degrees of regularity. These last tend, however, to be self-perpetuating and to give rise to insoluble masses, homodesmic in the sense of chapter 6, such as the resins. They need not be considered here.

The two classes naturally overlap. Some elongated molecules are not simple threads, with a single polymeric backbone, but have a more elaborate

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[133] a. E. Hammarsten, Biochem. Ztschr., **144**: 383, 1924; b. cf. E. Hatschek, The Viscosity of Liquids: Van Nostrand, New York, 1928.

[134] E. Hatschek, Tr. Faraday Soc., **25**: 592, 1930.

[135] I. Langmuir, J. Chem. Phys., **6**: 873, 1938.

[136] W. T. Astbury and H. J. Woods, Phil. Tr. Roy. Soc., **232A**: 333, 1933.

[137] K. Hermann, O. Gerngross, and W. Abitz, Ztschr. f. physik. Chem., **10**: 371, 1930.

[138] J. R. Katz, and J. C. Derksen, Rec. trav. **51**: 513, 1932; cf. K. Hess and J. Gundermann, Ztschr. f. physik. Chem., **34B**: 151, 1936.

[139] a. J. W. Ellis and J. D. Bath, J. Chem. Phys., **6**: 723, 1938; b. O. L. Sponsler, J. D. Bath, and J. W. Ellis, J. Phys. Chem., **44**: 996, 1940.

internal structure which lends rigidity and causes the particle to behave more like a stiff rod than a thread. This provides an important practical distinction, for rigid molecules are much more susceptible to exact description than flexible ones. We must mention also the important accidental circumstance that while, for reasons that are not in the least understood, many naturally occurring molecules can be obtained in the virtually monodisperse condition, some degree of polydispersity of size and shape is almost universal in solutions of synthetic or natural "linear" polymers. On the other hand, the latter have the advantage that they are formed from relatively simple repeating units, so that average molecular weights and average degrees of branching can frequently be determined by chemical estimation of free terminal groups. In the case of the globular proteins, which may be regarded as heteropolymers, although some evidence as to minimum molecular weights can be obtained from chemical analyses, the physical data are at first sight completely at variance with the chemical, and have to be considered on their own merits.

Because of the comparative simplicity of the physical data, let us consider first the globular proteins. To a first approximation, all physical methods, properly applied, support the view that most soluble proteins, with the notable exceptions of myosin, fibrinogen, tobacco mosaic virus, and perhaps gelatin, behave in solution as more or less hydrated rods or spheres of definite molecular weight. Numerous molecular weight measurements have served to establish the range of molecular sizes encountered, and to demonstrate a somewhat remarkable uniformity in the values found for particular proteins from a given source. Beyond this, and a further tendency for the molecular weights of chemically similar proteins to fall sometimes within the same range of values, there is really very little regularity to be discerned, especially in relation to the extraordinarily wide range of biological function covered by these substances. Proteins with widely different functions may fall within the same molecular weight range; others with apparently identical function and apparently close chemical similarity, such as the antibody globulins or the respiratory pigments from different species, cover a considerable range of molecular weights. Association or attachment to indifferent carriers might be suggested to account for this, and indeed there is the remarkable reversible splitting of proteins in presence of urea, clupein, and certain amino-acids[140] to favor the hypothesis, as well as the alleged grouping of molecular weights in multiples of a basic unit of 17,000,[140,141] which has, however, been disputed.[142] But there is no direct evidence.

The shape of protein molecules has been discussed on the basis of all the methods outlined in subsection 2, but without proper allowance, until

[140] a. Ref. (64), pp. 406 ff., b. N. F. Burk, J. Biol. Chem., **120**: 63, 1937; c. J. Steinhardt, *ibid.*, **123**: 543, 1938.

[141] T. Svedberg, Nature, **123**: 871, 1929.

[142] E.g., H. Neurath, Cold Spring Harbor Symp., **8**: 80, 1940.

recently, for the hydration factor. Thus somewhat extreme points of view have been taken. Workers of the Svedberg school and others, as a rule, gave the conventional frictional ratios, f/f_0 or the intrinsic volumetric viscosities based on the partial specific volume of the anhydrous solute, from which they deduced axial ratios. Adair and Adair contended that in several cases, at least, the apparent asymmetry vanished if hydration was taken into account.[143] A review by Oncley of the most trustworthy available data suggests a less extreme point of view.[87,144] For each protein and each experimental method Oncley plots hydration against axial ratio, in such a way that any pair of values of these variables falling on the curve represents a possible interpretation of the data for the method in question. For a given protein, the curves obtained for all methods often tend to intersect in a certain region; the values of hydration and axial ratio in this region are those that agree with most, but not necessarily all, of the experimental data, and may therefore be adopted tentatively. Some of these, taken from Oncley's diagrams, are given in Table VIII together with the experimental values upon which they are based, and, for comparison with selected values of hydration determined independently (cf. Table VII). The results have been weighted in favor of the X-ray data, since any value of the axial ratio must conform to the dimensions of the unit cell.[123] For this reason insulin, alone among the proteins considered, is best regarded as a disc.

As a convenient bridge to a discussion of linear molecules, we may refer to certain classes of polysaccharides, the structure of which can be inferred independently from chemical data:[145] these form a series starting from glycogen, with an elaborately branched structure and short average chain-length, passing through the progressively less branched amylopectin and amyloamylose, the two principal constituents of starch, to cellulose and its derivatives, which are essentially linear. The polymeric unit is in each case the pyranose ring. The transition is marked by a progressive increase in intrinsic viscosity,[116] by an increase in frictional ratio, and by the appearance, in cellulose, of streaming birefringence. Other effects become evident, notably a pronounced tendency of the sedimentation constant to fall off with increasing concentration, and for the osmotic coefficient to rise. These effects, which may occur at such low concentrations as to interfere seriously with the extrapolation to zero concentration, upon which reliable molecular weight values must be based, are highly characteristic of thread-like molecules. Even when the extrapolation is performed, s may be practically independent of molecular weight[147] on account of the increase of frictional constant with chain length; thus molecular weight determinations become

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[143] G. S. Adair, Proc. Roy. Soc., **170A**: 57, 1939.

[144] See also a. J. W. Mehl, J. L. Oncley, and R. Simha, Science, **92**: 132, 1940; b. H. Neurath, G. R. Cooper, and J. O. Erickson, J. Biol. Chem., **138**: 411, 1941.

[145] Cf. K. Freudenberg, Ann. Rev. Biochem., **8**: 81, 1939.

[146] H. Staudinger and E. Husemann, Ann. Chem., **530**: 1, 1937.

[147] R. Signer, in ref. (64), pp. 431–442.

difficult, and are further complicated by the polydispersity of nearly all linear polymers, which causes the sedimentation boundary to become very diffuse. Methods have been developed for calculating weight distribution curves from the boundary diagrams.[148]

TABLE VIII.—SOME WELL-ESTABLISHED PHYSICAL DATA ON CERTAIN PROTEINS

	Insulin	Lacto-globulin	Pepsin	Egg albumin	Serum albumin	Hemo-globin	Edestin
Molecular weight, $10^{-4}M$							
Sedimentation equilibrium	3 50	3.80	3 90	4 05	6 80	6 80	.....
Osmotic pressure	.....	.....	3 50	3 40–3 60	7 30	6 70	..
Sedimentation rate and diffusion	4 10	4.15	3 55	4 40	7 00	6 30	31 00
Sedimentation constant, $10^{13}s$	3 5	3.12	3 3	3 55	4 46	4 41	12.8
Diffusion constant, $10^{7}D$ (cm./sec.)	8 2	7 3	9 0	7 8	6 1	6 9	3 93
Partial specific volume, V'	0 749	0 751	(0 750)	0 749	0 748	0 749	0 744
Relaxation times, $10^{8}\tau$.	1 7	16	.	18	36	8 4	250
		5 6	....	4 7	7 5	....	28
Volumetric intrinsic viscosity	...	6 0	5 2	5.5	6 8	5 1	.....
Apparent frictional ratio, f/f_0	1 13	1.26	1 08	1 16	1 27	1.16	1 21
Most probable hydration, w (calc.)	0 2	0 6	?	0 2	(0 2)	0 3	0 1
Most probable axial ratio, a/b (calc.)	0 6	1 0	?	4 0	(5 0)	1 6	4–8
Hydration, experimental.	0 36	0 8	.	0 25	0 3	0 3	0 1
Axial ratio from X-ray data	0 6	1–2	... ..	.....	....	1 63	
Axial ratio from electrophoresis*	....	.. .	...	4 4	6 3	.....	.

* H. A. Abramson, M. H. Gorin, and L. S. Moyer, Chem. Rev., **24**: 345, 1939.

A similar situation exists concerning shape factors, for linear molecules can exist in an enormous number of configurations, and attempts to represent them in terms of rigid ellipsoids are highly artificial; the results can only represent, in very crude terms, an average configuration. This is clearly shown in the data of Kraemer,[149] who compares axial ratios obtained in the conventional way from viscosity and sedimentation data with those corresponding to the fully extended chain; some representative figures are given in Table IX. Probably the proteins included in the table are less

[148] E. O. Kraemer, in ref. (64), pp. 325–353.

[149] E. O. Kraemer and J. B. Nichols, in ref. (64), p. 416.

TABLE IX.—PHYSICAL PROPERTIES OF CERTAIN ELONGATED MOLECULES

	$10^{-4}M$	$[\eta]_V$	$10^4[\eta]_V/M$	$(f/f_0)_s$	$(f/f_0)_\eta$	$(a/b)_s$	$(a/b)_\eta$	(a/b)	Electron microscope	Flow birefringence
Cellulose	5 5	1.32	0.24	4.7	4.2	107	85	102		
	22	5.38	0.24	7.7	6 1	325	190	410		
Poly-ω-hydroxydecanoic acid	2.7	1 77	0.66	1.6	4.5	6 5	100	345		
Polychloroprene	22 5	1.70	0.076	4 5	4 5	98	98	1450		
Rubber	6.4	0.46	0 072	4 0	3 3	76	49	510		
Gelatin	5 0	0 9	0.18	1.34	3 8	3 8	68			
Thymonucleic acid	20[a]									
	50–100[e]	....	.....	2.5[a]	..	. ..	300[e]	.	.....	4,500
Nucleohistone from calf thymus	215[b]	9 0[d]	0 042	2.5[b]	..	35				
Fibrinogen	.....	....	. ...	.....	..	. .	...	. .	.....	1,800
Myosin	100[g]	2 40[h]	0 024	...	...	....	.	...	. ... . .	11,000–28,000
Tobacco mosaic virus	2,000–4,000	2 2[f]	0 0007	2 0	..	....	.	...	1,400–3,000[c]	7,200–24,000

Axial ratios calculated from sedimentation and viscosity data, $(a/b)_s$ and $(a/b)_\eta$ respectively, are compared with maximum values calculated from the molecular weight and the supposed linear structure of the polymers. Similar evidence for flexibility may be seen in the values $10^4[\eta]_V/M$; for elongated molecules growing by increase in length only we may write $[\eta]_V = kk'M$, where kk' is a constant for a particular type of molecule. If, on the other hand, the effective shape factors are not very greatly different over a wide range of molecular weights and types of substance, we may expect to find a wide variation in $[\eta]_V/M$. It will be seen in the table that the wide variation in this quantity is in sharp contrast to the comparative steadiness of $[\eta]_V$. Cellulose is an exception, conforming instead to the behavior anticipated from Staudinger's postulate and the equation connected with it.

Most of the data are taken or derived from figures given by Kraemer and Nichols (149). Other sources denoted by italic letters are as follows: (a) Pedersen in ref. (64), p. 443; (b) R. O. Carter, J. Am. Chem. Soc., **63**: 1960, 1941; (c) G. A. Kausche, E. Pfankuch, and H. Ruska, Naturwissenschaften, **27**: 292, 1939; W. M. Stanley and T. F. Anderson, J. Biol. Chem., **139**: 325, 1941; (d) R. O. Carter and J. L. Hall, J. Am. Chem. Soc., **62**: 1194, 1940; (e) R. Signer, T. Caspersson, and E. Hammarsten, Nature, **141**: 122, 1938; (f) J. R. Robinson, Nature, **143**: 923, 1939; (g) H. H. Weber, cited in: (h) J. T. Edsall and J. W. Mehl, J. Biol. Chem: **133**: 409, 1940.

flexible than true linear polymers and therefore more accurately described as ellipsoids.

To some extent these thread molecules seem to function in independent segments, each with its own Brownian movement, behaving in some respects as separate molecules of low molecular weight, in others as a macromolecule; the anomalies of osmotic and vapor pressure arise in part from the former effect,[150] while the viscosities of polyesters in the molten condition[151] and in fairly concentrated solution[152] require for their interpretation[153] the combination of a term involving flow in segments with one expressing the condition that the random motion of segments shall be co-ordinated in order to result in movement of the whole molecule in the direction of flow. The latter condition would not apply in absence of translational motion. Dielectric dispersion data should give information as to the modes of independent oscillation or stretching of polar chain segments but would not be expected to indicate, in absence of special theoretical treatment, the size or shape of the macromolecule. We have referred elsewhere to such a theoretical treatment of this question; it is worth while to mention some results obtained in the experimental study of simple linear molecules in solution which might be expected to show clearly the effect of oscillation of segments, uncomplicated by polydispersity. A lot depends upon the position of the polar groups; aliphatic alcohols, with a single polar group, behave as if only a small segment, perhaps an OH group, were free to oscillate, while linear molecules with terminal zwitterions, such as the simple peptides and betaines, seem to behave far more like rigid molecules for which reasonable shape constants can be calculated. Thus the axial ratios for the glycine peptides appear to be proportional to the square root of the number of residues.[154] This behavior appears to hold for peptides up to alanyl leucyl glycine[154] and for heptadecyl betaine, which is reported[155] to act like a sphere of diameter equal to the length of the fully extended chain. The reason for this is not at all clear. On the other hand, when there are distributed zwitterions or polar groups at intervals along the chain, we find independent oscillation of segments, as the data on poly-ω-hydroxydecanoic acids[156] show. Actual distribution curves have not been obtained for any of these substances. For larger molecules such as the vinyl polymers an "average" relaxation time, given by the frequency for maximum loss factor (ϵ''), has been found proportional to molecular weight,[157] in con-

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[150] a. R. E. Powell and H. Eyring, ref. (65), p. 183; b. K. H. Meyer and R. Luehdemann, Helvet. chem. acta, **18**: 307, 1935.

[151] P. J. Flory, J. Am. Chem. Soc., **62**: 3036, 1940.

[152] P. J. Flory, J. Phys. Chem., **46**: 870, 1942.

[153] W. Kauzmann and H. Eyring, J. Am. Chem. Soc., **62**: 3113, 1940.

[154] W. P. Conner and C. P. Smyth, J. Am. Chem. Soc., **64**: 1870, 1942.

[155] I. Hausser, Sitzungsber. Heidelberger Akad. Wiss., Math.-naturwiss. Klasse, Nr. 6 1935.

[156] a. J. Wyman, J. Am. Chem. Soc., **60**: 328, 1938; b. W. B. Bridgman, *ibid.*; 530.

[157] a. R. M. Fuoss, J. Am. Chem. Soc., **63**: 2401, 1941; b. D. J. Mead and R. M. Fuoss, *ibid.*: 2832.

formity with the theory of Kirkwood and Fuoss.[158] In none of these cases are exact calculations of segment lengths possible without a more precise knowledge of the relationship between macroscopic and "molecular" viscosity; the irrelevance of the former in calculations involving rotational motion has been shown quite clearly,[159] and is particularly obvious when the molecules of solid substances are able to rotate freely.[160] Comparisons of the free energies of activation for viscous flow and for rotation[150] likewise suggest that rotation is often much easier than translation.

5. Number and Distribution of Charged Groups in Proteins and Their Relation to Chemical Constitution. —"Neither the diversity nor the general properties of the proteins can be adequately explained by differences in the size or shape of their molecules."[161] Knowledge of size and shape can only provide the framework for a chemical topography of which the elements are already in existence, and as this grows in detail we may hope to observe the gradual convergence of our knowledge of chemical and biological specificities.

Underlying the rather definite size and shape of fractionated protein molecules, there must be an ordered arrangement of polymeric units; the nature of the units and the elementary plan of polymerization are, in fact, fairly well known, but the resulting model of the protein molecule is so much at variance with its properties that there must be a secondary structural principle, the nature of which is still not understood. There is wide agreement that the important polymeric unit is the peptide linkage,

$$-\mathrm{CH}(\mathrm{R})-\mathrm{CO}-\mathrm{NH}-,$$

and that the immensely varied properties of the proteins depend largely upon the nature of the side-chains, R;[162] the chemical evidence shows that if this structure is substantially correct, the side-chains cannot be identical throughout the peptide chain, since α-amino-acids in considerable variety are obtained by the hydrolysis of proteins. In no single case has the entire protein molecule been accounted for in this way, but this is not surprising, since according to Vickery[163] adequate quantitative methods exist only for nine amino-acids, and somewhat less satisfactory ones for six others out of the twenty-five "concerning which there is no doubt whatever," while there are yet others which may be present. Table X summarizes data for several proteins selected "from

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[158] a. R. M. Fuoss and J. G. Kirkwood, J. Am. Chem. Soc., **63**: 385, 1941; b. J. G. Kirkwood and R. M. Fuoss, J. Chem. Phys., **9**: 329, 1941.

[159] J. L. Oncley and J. W. Williams, Phys. Rev., **43**: 431, 1933.

[160] a. C. P. Smyth, Chem. Rev., **19**: 329, 1936; b. S. O. Morgan and W. A. Yager, Ind. Eng. Chem., **32**: 1519, 1940; c. A. Mueller, Proc. Roy. Soc., **158A**: 403, 1937.

[161] E. J. Cohn, Chem. Rev., **24**: 293, 1939, (p. 223).

[162] E.g., a. H. B. Vickery and T. B. Osborne, Physiol. Rev., **8**: 393, 1928; b. M. Bergmann, Chem. Rev., **22**: 423, 1938; c. M. Bergmann and J. S. Fruton, Compt. rend. d. trav. du lab. Carlsberg, **22**: 62, 1938; d. The Chemistry of the Amino Acids and Proteins, edited by C. L. A. Schmidt: C. C. Thomas, Springfield, Illinois, 1938.

[163] H. B. Vickery, Ann. New York. Acad. Sc., **41**: 87, 1941.

TABLE X.—COMPOSITION OF PROTEINS

	Hemoglobin 66,700				Egg albumin 40,000				Insulin 35,100				Serum albumin 73,000				Edestin (Hempseed) minimum 56,000 centrifuge 304,000			
	10^2c	$10^5c/m$	N	M	10^2c	$10^5c/m$	N	M	10^2c	$10^5c/m$	N	M	10^2c	$10^5c/m$	N	M	10^2c	$10^5c/m$	N	M
Fe	0.335	5.99	4	66,640																
S	0.390	12.17	8	65,760	1.616	50.43	20	39,660	3.34	104 2	36	34,560					0.880	27.46	15	54,630
	0.57	17.8	12	67,480																
Sulfide S	0.191	5.96	4	67,120	0.491	15.32	6	39,170	....	....		.....		..		. ..	0.346	10.79	6	55,580
P	....	.....	...	.....	0 097	3 13	1	32,010												
Amide N	1 01	72.1	48	66,580	0 97	69.3	28	40,390	1 55	110 8	39	35,220		. . .		...	1.88	134 3	75	55,870
Zn	.....	.....	..	.....	.....	.....	.	.. .	0.52	7 95	3	37,710								
									0.36	5.51	2	36,320								
Cd	.....	.....	...	.....	.....	.....	.	.....	0 77	6.85	2	29,200								
Co	....	.....	...	.....	.....	....		.....	0 44	7.47	3	40,200								
Arginine	3 59	20 62	14	67,890	5.66	32.52	13	39,970	....	.....		.....	....	.....	..	.....	16 76	96.29	54	56,100
Histidine	7 64	49 26	33	66,990	1 48	9.766	4	40,960	....	.....	.	.....	..	.....	..	.....	2 08	13.42	8	59,650
Lysine	8 10	55 44	37	66,750	4 97	34.02	14	41,160	... .	.....	..	.....	....	.....	..	..	2 19	14.98	8	53,380
Tyrosin	3 15	17 4	12	68,980	3 97	21 93	9	41,050	12 2	67.4	24	35,640	4.79	26 4	19	71,840	4.54	25.07	14	55,830
Tryptophane	1.28	6 27	4	63,760	1 18	5 78	2	34,600		.....	..	... .	0 53	2 597	2	77,000	1.46	7.16	4	55,880
Cystin	0 41	1.71	1	58,590	1 78	7.41	3	40,500	12 5	52.0	18	34,590	6.04	25.1	18	71,590	1 36	5 66	3	52,980
Cysteine	....	....	..	.....	1 78	14.7	6	40,820	12 6	104.1	36	34,590	....	.....	..	.....	1.36	11.23	6	53,420
Methionine	.....	.....		.....	5 23	35 05	14	39,940	.	.....	..	.....	...	.....	..	.....	2 39	16 02	9	56,190
Glutamic acid	6.3	42 8	29	67,710	13 96	95 13	38	39,980	.	.....	..	.....	....	.....	..	.....	19.2	130 5	73	55,920
Aspartic acid	8.9	66 9	45	67,320	6 07	45 60	15	41,420	..	....		.....	..	.....	.	.....	10.2	76.6	43	56,120
Total amino-acids	....	.....	175	.....	.....	.....	118	.....	.	...	78	.....	....	.....	45	.....	.....	.....	222	.....

Data selected by H. B. Vickery, Ann. New York Acad. Sc., **41**: 87, 1941, and compiled from his tables 3 to 7.

c = content of constituent in grams per gram of protein.

m = molecular weight of constituent.

c/m = molecular concentration of constituent.

N = number of molecules per molecule of protein which must be assumed in order to give a value of the protein molecular weight nearest to that at head of table.

M = molecular weight of protein on basis of the above values of c/m and N.

In the case of edestin, the minimum molecular weight has been taken on the assumption that the tryptophane analyses are correct. Physical chemical estimates of M are not a simple multiple of this value.

studies that show evidence of unusual care and attention to controls."[163] It will be seen that at best the figures account for only about 60% of the protein molecule; in the case of insulin, only for 12%. More complete analyses exist for these and numerous other proteins, but are not considered reliable enough for use in the calculation of molecular weights. The minimal molecular weight given in the table is that implied by expressing all the molar concentrations as integers, taking the smallest values consistent with the experimental figures and their probable error. The N values in the table have been obtained more or less in this way. The agreement with physical values of M is as satisfactory as could be expected.

Mere increase of molecular weight does not, it is clear, give rise to any such continuous change of properties as is encountered with "simple" polymers; the multiplicity of side-chains attached to the backbone, however, suggests an explanation, for it is obvious that the relative numbers and the sequence of the individual amino-acid residues can be almost infinitely varied. Attempts have been made to show that actually neither number nor sequence is entirely arbitrary, and it is scarcely conceivable that this should be so. In the relatively simple case of the protamine, clupein, formulæ have been given[154] involving an imperfectly repeating unit, and more recently Bergmann has claimed that "the molecules of various proteins fall into classes containing a definite number of amino-acid units . . . " which " . . . may be arranged in such a way that each individual residue repeats itself throughout the protein molecule . . . with a regularly recurring frequency."[162b] It would appear from the published criticisms of this hypothesis and of the experimental data underlying it[165] that such a rigid periodicity has not been demonstrated; certainly, the residue numbers given in Table X for egg albumin do not agree very closely with the values $(2^m \times 3^n)$ claimed by Bergmann. The structural principles involved are probably more complex and are unlikely to be amenable to such simple arithmetical expression.

Up to a point, the properties of the proteins are those of the structure described above. This is particularly true of their amphoteric nature. The side-chains behave chemically as if they were free; naturally, in absence of complete analyses, this cannot be considered rigorously established, but as a rule the acid and base combining power suggests the presence of more rather than of fewer reactive groups than those found analytically. A complete quantitative correlation is hampered also by our imperfect knowledge

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[164] a. K. Linderstrøm-Lang, Tr. Faraday Soc., **31**: 324, 1935; b. E. Waldschmidt-Leitz and E. Kofranyi, Ztschr. f. physiol. Chem., **236**: 18, 1935.

[165] E.g., a. A. Neuberger, Proc. Roy. Soc., **127B**: 25, 1939; b. H. B. Bull in Advances in Enzymology, edited by F. F. Nord and C. H. Werkman: Interscience Pub., Inc., New York, Vol. 1, 1941, pp. 1–42; c. R. D. Hotchkiss, J. Biol. Chem., **131**: 387, 1939; d. Chibnall's Bakerian Lecture (A. C. Chibnall, Proc. Roy. Soc., **131B**: 136, 1942): "I think that those interested in proteins would be wise to regard the Bergmann-Niemann hypothesis as still tentative . . . for much of the evidence hitherto brought forward to support it has been based on inadequate experimental data and has demonstrated nothing more than the hypnotic power of numerology."

of the change in electrochemical properties of amino-acids resulting from their incorporation in the peptide chain,[166] and by the combination of other ions than those of water during titration;[167] nevertheless, some progress, reviewed elsewhere in this volume, has been made in the analysis of titration curves in terms of the amino-acid residues responsible for them. The instructive work of Simms[168] was of preliminary value; the data on egg albumin more recently analyzed by Cannan[169] illustrate the present situation, with its rather more refined electrostatic theory and its leaven of empiricism. In this instance the analytical figures give too few acid groups to account for the titration curves, but in other respects the agreement is good, perhaps surprisingly so, when it is remembered that only 46% of the molecule is accounted for analytically.

It would follow qualitatively that if the net charge varies with *p*H as the result of ionization, the mobility in an electric field must vary also; this is, indeed, the case, and fairly close proportionality between titration and mobility data has been found.[170] Calculation of net charge from mobilities is, however, difficult; several factors have to be taken into account, such as the electrostatic screening effect of the Debye-Hueckel ion atmosphere, its effect on the viscous flow of solvent, the influence of particle asymmetry and nonuniform distribution of charge, and the possible binding of specific ions. Preliminary work has been done, taking the above-mentioned proportionality for granted, in the calculation of shape factors,[171] with results that agree fairly well with those of other methods (Table VIII). The present importance of electrophoresis, however, lies more in its use as a very sensitive indicator of small differences in the charged condition of proteins and in the analysis of polydisperse systems, such as plasma,[172] than as a source of absolute values of the net charge.

The Gibbs-Donnan distribution law also provides methods for measurement of valence, either by the analysis of osmotic pressures or by measurement of membrane potentials; the latter, in particular, has been advocated with considerable success by Adair and Adair.[173]

Although the effective charge on a protein molecule varies roughly in the manner shown by the titration curve, becoming zero at the isoelectric point, the

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[166] E.g., a. A. Neuberger, Biochem. J. **30**: 2085, 1936; b. J. P. Greenstein, J. Biol. Chem., **93**: 479, 1931; c. *ibid.*, **95**: 465, 1932; d. *ibid.*, **95**: 499, 1932; e. J. P. Greenstein, and F. W. Klemperer, *ibid.*, **128**: 245, 1939; f. J. P. Greenstein, F. W. Klemperer, and J. Wyman, *ibid.*, **129**: 681, 1939; g. M. Zief and J. T. Edsall, J. Am. Chem. Soc., **59**: 2245, 1937; h. E. J. Cohn, Cold Spring Harbor Symp., **6**: 8, 1938.

[167] E. g., a. J. Steinhardt, C. H. Fugitt, and M. Harris, Nat. Bur. Standards, J. Res., **26**: 293, 1941; b. J. Steinhardt, Ann. New York Acad. Sc. **41**: 287, 1941.

[168] a. H. S. Simms, J. Am. Chem. Soc., **48**: 1239, 1926; b. J. Gen. Physiol., **11**: 629, 1928.

[169] R. K. Cannan, A. Kibrick, and A. H. Palmer, Ann. New York Acad. Sc., **41**: 243, 1941.

[170] a. H. A. Abramson, J. Gen. Physiol., **15**: 575, 1932; b. H. A. Abramson, M. H. Gorin, and L. S. Moyer, Chem. Rev., **24**: 345, 1939; c. L. G. Longsworth, Ann. New York Acad Sc. **41**: 267, 1941.

[171] H. A. Abramson, M. H. Gorin, and L. S. Moyer, Chem. Rev., **24**: 345, 1939.

[172] E.g., E. J. Cohn, Chem. Rev., **28**: 395, 1941.

[173] a. G. S. Adair and M. E. Adair, Tr. Faraday Soc., **31**: 130, 1935; b. *ibid.*, **36**: 23, 1940.

dissociation constants of the various groups present overlap in such a way that the total number of charged groups is not zero at this point; the number of electrically balanced charged groups is maximal, while the total number of charged groups, balanced and not balanced, is maximal at some *p*H not necessarily identical with the isoelectric point. The maximum in the number of dipoles present at the isoelectric point does not mean, however, that the dipole moment is likewise maximal, since this quantity depends upon the distribution of charge; a molecule with very symmetrical charge distribution might have a very small permanent moment at any given *p*H, but in general one would expect the symmetry to vary with *p*H, so that a point of zero moment might be either unique or nonexistent for a particular protein. There are very few studies of the variation of dipole moment with *p*H, though such information might be useful in plotting the distribution of polar groups on the protein surface;[174] the moments of a number of isoelectric proteins are well known, however.[175] In the case of nonspherical molecules, assuming the zwitterion origin of the moment to be established, relaxation studies should provide a further characterization of the moment at various *p*H values in terms of the angle between the electric and the geometric axes. The moments recorded vary from 210.10^{-18} e.s.u. for egg albumin to 1200.10^{-18} for horse serum pseudoglobulin, the angle between the major axis of the ellipsoid and the dipole moment vector being estimated in the latter case as 45°. The moment of egg albumin is that expected from a singly charged dipolar ion with a dipole separation equal to the diameter of the molecule;[166b] since the molecule contains around 24 pairs of charges, this value represents a highly symmetrical distribution.

These facts are in accord with the simple polypeptide structure of proteins, according to which the amino-acids are arranged in a line, with charged groups attached to side-chains at various points along the line and distributed sufficiently at random to give a very small resultant electric moment. This picture is, however, at variance with the dielectric dispersion, sedimentation, and viscosity data, which show that the molecules behave as more or less rigid spheres or rods; moreover, the preponderance of nonpolar groups in the polypeptide model is such that proteins would be practically insoluble in water if they had such a structure. The conclusion forces itself that the chain is folded, and folded in such a manner that the acid and base combining groups are either free or very weakly bound, while the nonpolar groups are to some extent enclosed. The reversibility of titration curves—within certain limits—proves that even if there is a certain amount of side-chain interaction it must be supplemented by some other type of binding, since there is no evidence that the molecules assume an extended configuration at acid or alkaline reactions.

Degradation of the extremely sensitive protein structure without change of molecular weight can be caused by many means. There is good reason

[174] Cf. a. W. J. Shutt, Tr. Faraday Soc., **30**: 893, 1934; b. P. Girard and N. Marinesco, Compt. rend. Soc. de Biol., **102**: 726, 1929; c. H. Fricke and L. E. Jacobson, J. Phys. Chem., **43**: 781, 1939; d. J. B. Bateman and C. Pecher, unpublished measurements by Oncley's method in E. J. Cohn's laboratory.

[175] Recent literature in J. L. Oncley, J. D. Ferry, and J. Shack, Ann. New York Acad. Sc., **40**: 371, 1940.

for thinking of the processes involved as the introduction of disorder into an originally very regular structure, to use Langmuir's phrase, and since there is an almost infinite number of possible configurations of a polypeptide chain folded or contorted at random, it is not surprising to find comparatively little agreement as to the precise nature of the product obtained by the action of the various denaturing agencies. It is certain that the product has usually lost the most highly specific of its original properties, sometimes irreversibly, as in the case of antibodies,[176] sometimes reversibly, as with several proteins, including certain enzymes and antigens.[177] It is certain also that the product is often more viscous[178] and therefore less symmetrical, and less soluble[179] than the native material; that it contains free side-chain groups—tyrosine hydroxyl, sulfhydryl, disulfide—that were not originally detectable.[180] On the other hand, it is a matter of dispute whether the titration curves [181] and the electrostriction of water[182] are different for native and "denatured" proteins; it seems probable that such changes in the former as occur are due to changes in the titration constants and not in the number of available groups.[183]

The facts have not proved sufficiently definite or unequivocal to favor any clear picture of the structure of the native protein. The one coherent hypothesis[184] postulates covalent polymerization in two dimensions, made

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[176] a. J. R. Marrack, The Chemistry of Antigens and Antibodies: Medical Research Council, London, 1938; b. J. F. Danielli, M. Danielli, and J. R. Marrack, Brit. J. Exper. Path., **19**: 393, 1938.

[177] a. M. L. Anson and A. E. Mirsky, J. Gen. Physiol., **9**: 169, 1925; b. J. Phys. Chem., **35**: 185, 1931; c. B. F. Miller, J. Exp. Med., **58**: 625, 1933; d. R. M. Herriot, J. Gen. Physiol., **21**: 501, 1938; e. cf. I. Langmuir, Cold Spring Harbor Symp., **6**: 159, 1938.

[178] a. M. L. Anson and A. E. Mirsky, J. Gen. Physiol., **15**: 341, 1932; b. H. B. Bull, J. Biol. Chem., **133**: 39, 1940.

[179] a. H. Chick and C. J. Martin, J. Physiol. **40**: 404, 1910; b. *ibid.*, **43**: 1, 1911–1912; c. *ibid.*, **45**: 61, 1912–13; d. *ibid.*, **45**: 261, 1912–13; e. S. P. L. Sørensen, Compt. rend. d. trav. du lab. Carlsberg, **15**: 1, 1925; f. H. Wu, Chinese J. Physiol., **5**: 321, 1931.

[180] a. A. E. Mirsky and M. L. Anson, J. Gen. Physiol., **18**: 307, 1934–5; b. *ibid.*, **19**: 427, 1936; c. *ibid.*, **19**: 439, 1936; d. J. P. Greenstein, J. Biol. Chem., **125**: 501, 1938; e. *ibid.*, **128**: 233, 1939; f. *ibid.*, **130**: 519, 1939; g. R. M. Herriot, ref. (172); h. A. E. Mirsky, Cold Spring Harbor Symp., **6**: 157, 1938; i. J. T. Edsall, J. P. Greenstein, and J. W. Mehl, J. Am. Chem. Soc., **61**: 1613, 1939; j. J. P. Greenstein and J. T. Edsall, J. Biol. Chem. **133**: 397, 1941; k. J. T. Edsall and J. W. Mehl, *ibid.*: 409, 1940.

[181] a. W. J. Loughlin, Biochem. J., **27**: 97, 1933; b. C. Y. Chow and H. Wu, Chinese J. Physiol., **8**: 145, 1934; c. H. Nagaoka, J. Biochem., **23**: 101, 1935.

[182] a. Wo. Pauli and R. Weiss, Biochem. Ztschr., **233**: 381, 1931; b. F. Haurowitz, Kolloid-Ztschr., **71**: 198, 1935; c. cf. T. Moran, Proc. Roy. Soc., **118B**: 548, 1935; H. Neurath and H. B. Bull, J. Biol. Chem., **115**: 519, 1936; e. E. Heymann, Biochem. J., **30**: 126, 1936.

[183] E.g., a. H. B. Bull, Cold Spring Harbor Symp., **6**: 140, 1938; b. cf. F. Haurowitz, Kolloid-Ztschr., **74**: 208, 1936.

[184] a. D. M. Wrinch, Nature, **137**: 411, 1936; b. **138**: 241, 1936; c. **139**: 651, 972, 1937. d. Proc. Roy. Soc., **160A**: 59, 1937; e. **161A**: 505, 1937; f. Tr. Faraday Soc., **33**: 1368, 1937; g. Phil. Mag., **26**: 913, 1938; h. Nature, **143**: 482, 1939; i. Science, **88**: 148, 1938; j. J. Am. Chem. Soc., **60**: 2005, 1938; k. D. M. Wrinch and I. Langmuir, *ibid.*, **60**: 2247, 1938; I. Langmuir and D. M. Wrinch, Nature, **142**: 581, 1938.

possible by enolization of the peptide carbonyl group; the resulting "cyclol" network can be folded into polyhedral cages. The hypothesis appears to be too much at variance with experimental facts to find wide acceptance;[185] it is admirable as a piece of ingenious crystallography, but as a vehicle for the diversity of the proteins the cyclol cage seems remarkably inflexible. Whatever the stereochemical situation may be, it seems certain, from entropy and energy considerations, that many weak bonds are involved in the maintenance of a protein molecule in the native condition; a few of these may be covalent, such as disulfide[185] or secondary peptide[185] linkages between side-chains, but most are of residual character and may be salt linkages or hydrogen bonds.[186] The latter appear to be present in solid proteins;[187] in presence of water they would tend to become disrupted.[188] A convoluted peptide chain, held in a specific configuration by a number of such linkages, might still have a certain number of degrees of freedom without loss of specificity, but, if a sufficiently large number of bonds were broken, practically all specificity would be lost. If the unfolded chain be imagined restored to its original environment, one may conceive that in the course of time, as various random configurations are assumed during Brownian movement, certain of these will bring reactive groups into apposition, and the portion of the chain involved in this fluctuation will become immobilized, the structure being subsequently stabilized by the operation of other residual forces. Continuation of this process will result in the formation of a globular molecule which may or may not have all the specific properties of the original native protein. If the reactive groups which were bonded in the original structure happen to be so disposed along the polypeptide chain that their positions correspond to probable configurations of the chain in a particular environment, they will again react, and the denaturation will be completely reversible, or nearly so; if the reactive groups were originally linked in an unstable configuration under the influence of a special environment, such as the field of an antigen during the synthesis of an antibody molecule, then the original linkage will correspond to such an unlikely fluctuation that denaturation will result in permanent loss of specificity. It is, perhaps, in terms of some such model as this that we must in future discuss the protein molecule, but the picture will become sharp only when more is known about the sequence of amino-acid residues in the primitive polymer, and its relation to the distribution of polar groups in the globular molecule.

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[185] E.g., a. L. Pauling and C. Niemann, J. Am. Chem. Soc., **61**: 1860, 1939; b. M. L. Huggins, J. Am. Chem. Soc., **61**: 755, 1939.

[186] a. A. E. Mirsky and L. Pauling, Proc. Nat. Acad. Sc., **22**: 439, 1936; b. D. Jordan-Lloyd, Tr. Faraday Soc., **36**: 886, 1940.

[187] a. A. M. Buswell, J. R. Downing, and W. H. Rodebush, J. Am. Chem. Soc., **61**: 3252, 1939; b. A. M. Buswell, K. F. Krebs, and W. H. Rodebush, J. Phys. Chem., **44**: 1126, 1940; c. J. W. Ellis and J. Bath, J. Chem. Phys., **6**: 723, 1938.

[188] Cf. J. D. Bernal, Tr. Faraday Soc., **36**: 886, 1940.

6. Molecular Interaction and Molecular Individuality.—The study of molecular associates in systems of several components encompasses a great many phenomena which are not encountered, or are arbitrarily ignored, when interest is confined to solutions which have been artificially fractionated until they obey one or another of the physical criteria of homogeneity. The associations which occur must involve all the types of interaction enumerated in chapter 6, and the products will vary from the one extreme of instability, represented by a slightly increased duration of kinetic encounters between the reactants, to the other extreme at which definite complexes of considerable persistence are formed. It may be anticipated that in the future many of these interactions will be defined physicochemically. Although an important start has been made in thermodynamic and other studies of simple mixed systems,[189] such efforts at semiempirical definition of behavior are not yet broad enough in scope to be usefully applied to biological systems, except for purely descriptive purposes. In the meantime, the empirical study of various types of macromolecular aggregates continues, but, in the absence of any unifying concept, we do not propose to review these studies, significant as they are, of the nature of specific precipitates,[190] of the various naturally occurring complexes of proteins and nucleic acids,[191] or of the organ-specific ultramicroscopic particles which can be obtained from broken-down cells.[192] Instead, we shall refer to a more general question, basing our remarks upon an excellent review by Pirie.[193]

Pirie, examining criteria of purity of large molecules, extends classical conceptions to fit the peculiar circumstances attending the investigation of biochemical systems, including the limitations of present technical methods. His treatment almost compels a more radical approach, in which the very idea of purity, considered apart from a particular functional criterion, could be made to appear illusory. The size of a giant molecule, in itself, suggests a localization of chemical reactivities, and, by extension, of biological specificities, analogous to the independent kinetic behavior of chain segments or branches in a polymer molecule. Thus it can readily be imagined that activity, measured according to a given chemical, immunological, or

[189] See, for example, numerous papers by E. J. Cohn and collaborators; reviews: E. J. Cohn, Ann. Rev. Biochem., **4**: 93, 1935; Chem. Rev., **19**: 241, 1936; The Harvey Lectures, 1938–39, p. 124; see also E. J. Cohn, T. L. McMeekin, J. D. Ferry, and M. H. Blanchard, J. Phys. Chem., **43**: 159, 1939.

[190] E.g., J. R. Marrack, The Chemistry of Antigens and Antibodies: H. M. Stationery Office, London, 1939.

[191] E.g., M. G. Sevag, D. B. Lackman, and J. Smolens, J. Biol. Chem., **124**: 425, 1938; A. E. Mirsky and A. W. Pollister, Proc. Nat. Acad. Sc., **28**: 344, 1942; cf. E. Hammarsten, Biochem. Ztschr., **144**: 383, 1924; E. Hammarsten and G. Hammarsten, Acta med. Scandinav., **68**: 199, 1928.

[192] A. Claude, Proc. Soc. Exper. Biol. & Med., **39**: 398, 1938; W. Henle and L. A. Chambers, Science, **92**: 318, 1940; E. A. Kabat and J. Furth, J. Exper. Med., **71**: 55, 1940; B. Sigurdsson, *ibid.*, **77**: 315, 1943.

[193] N. W. Pirie, Biol. Rev., **15**: 377, 1940.

enzymatic process, may be equally associated with particles of different size[194] or chemical constitution[195] and with particles which, seen in terms of some other mode of activity, may have little or nothing in common. Conversely, particles which prove to be indistinguishable by a given physical measurement may well be entirely different in biological specificity.[196] From this point of view, the physical and chemical approach to the problems of specificity can prove misleading; such methods of fractionation may be of doubtful value if they do not possess some degree of specificity closely related to that involved in the biological process which serves as the final index of uniformity. Fractionation based on the known properties of a prosthetic group, for example, might be effective in concentrating an enzyme, while separation of a polydisperse bacterial antigen into fractions homogeneous in respect to particle size might be useless except as a means of establishing the point now under discussion. Examples pointing in the same direction could be multiplied to show that the most extreme specificity may reside variously in the molecule as a whole, or in a limited region only, the nonspecific residue in the latter case being amenable to more or less radical alteration without interfering with the specificity. In the limiting case, the specific group may be detachable and able to function in the absence of a carrier; the literature on viruses and enzymes abounds in examples of adsorption. On the other hand, the specific group, although detachable, may retain its reactivity only in conjunction with a carrier or some other molecule suitably situated; the functional unit might comprise, for example, a large kinetic unit, characterized by its sedimentation constant, acting in combination by residual forces with other molecules and with an atmosphere of solvent molecules. It could be argued that such a complex, to which a definite mean life could be assigned as an index of stability, has as much right to be given molecular status as does any covalent compound which has only a transient existence under given experimental conditions. It is clear, however, that such arguments substitute for the simplicity of the classical definition a highly ambiguous conception of the molecule, and it may be desirable to introduce a new term to distinguish the variable functional unit from the molecules of chemistry and physics. Our present intention, however, is solely to indicate some of the difficulties which are encountered as soon as any attempt is made to correlate the properties of systems at different levels of organization. It is hardly to be expected that at this stage any useful generalization will become evident: there is too little detailed information at either level.

[194] E.g., A. A. Miles and N. W. Pirie, Brit. J. Exper. Path., **20**: 83, 1939; J. Bourdillon, J. Gen. Physiol., **25**: 263, 1941; L. A. Chambers, W. Henle, M. A. Lauffer, and T. F. Anderson, J. Exper. Med., **77**: 265, 1943.

[195] E.g., G. Ågren and E. Hammarsten, J. Physiol., **90**: 330, 1937; E. Jorpes and S. Bergstrom, Biochem. J., **33**: 47, 1939; V. Desreux and R. M. Herriot, Nature, **144**: 287, 1939; A. H. Eggerth, J. Immunol., **42**: 199, 1941; *ibid.*, **45**: 303, 1942.

[196] E.g., K. Landsteiner, L. G. Longsworth, and J. Van Der Scheer, Science, **88**: 83, 1938, and numerous other examples.

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Pirie, N. W., The criteria of purity used in the study of large molecules of biological origin: Biol. Rev., **15**: 377, 1940.

Powell, R. E. and H. Eyring, Frictional and Thermodynamic Properties of Large Molecules. Advances in Colloid Science, ed. E. O. Kraemer: Interscience, New York, 1942, p. 183.

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8

CONDENSED SYSTEMS OF LARGE MOLECULES, WITH SPECIAL REFERENCE TO THE STRUCTURE OF FIBERS

1. Introduction on Crystal Form and Habit Considered in Relation to the Aggregation of Macromolecules.—Some of the macromolecules identified by their behavior in the disperse state, and particularly the highly anisodiametric particles represented by the synthetic linear polymers and by cellulose, are obtained in this condition by the breakdown of solids distinguished in the former case by their amorphous character, and in the latter by the fibrous habit in which they occur in plants. It is not surprising that thread-like molecules should tend toward random distribution, nor that under proper constraint they should readily take up a parallel orientation. The latter tendency is so marked, however, as to be of inestimable significance in biology. Indeed it may be said that the fibril is by far the most widespread of all the structural forms of living matter, and the most adaptable, providing on the one hand the basis for nearly all supporting structures, and a labile morphological foundation for nuclear processes on the other. It is this versatility of the fibril that lends such considerable interest to the study of its molecular organization.

This question of molecular organization is fundamental. There are others, perhaps no less important, which have been scarcely touched upon. Given the structure of the primitive molecular crystallite or its analogue in the more random world of high polymers, we are still confronted with the gap between the crystal unit and the complex histological pattern formed by the repetition of the unit; a gap that can be closed only with an intimate knowledge of the chemical vectors defining the dynamics of growth. The problem thus overflows its boundaries, to cover at length the entire field of morphogenesis.

Molecular organization in fibrils has its parallel in crystallography, in the relation between crystalline form and the stereometry of the molecular unit. The more general question of morphogenesis can, perhaps, be similarly regarded as a biological counterpart to the mineralogical problem of crystal habit, for the two have in common their need for a description of the environmental factors operating to give rise to aggregates in which the gross morphology is not in any obvious way related to the fine structure. The essential unity of all intermolecular and interatomic forces, our thesis

in chapter 6, would appear to leave no room in biological organization, on the purely physical level, for forces qualitatively different from those involved in inert matter. It is perhaps worth while, therefore, to consider briefly the application of some [illegible] facts to aggregates of large molecules.

We have in chapter 6 considered in rather general terms the condensation of an assembly of atoms to a heterodesmic solid, and have, at least by implication, referred to the crystalline nature of the solid, meaning, by the use of this term, the systematic repetition of units throughout the mass. However numerous the types of linkage in the system, we may imagine that, left to itself, an ordered arrangement will be attained in which all linkages standing in a given space relationship to the repeating unit will recur in parallel equidistant planes; generally speaking, planes containing only weak bonds, with correspondingly great bond lengths, will be planes of mechanical weakness in the crystal and will also tend, under uniform conditions of crystal growth, to be parallel to an important and frequently occurring crystal face. This rough-and-ready generalization embodies the law of Bravais and its recent extension by Donnay,[197] themselves rather strictly applicable to a number of ionic crystals.[198] The minerals mica and asbestos provide admirable illustrations.[199] In mica, tetrahedral groups, $Si^{4+}O_4^{2-}$, are united in sheets, so that three of every four oxygen atoms participate in two SiO_4 groups; the linkages are probably partly covalent in character. The residual polarity of the fourth oxygen atom is satisfied by formation of weak cross-linkages with metal ions; these bind the sheets together and are responsible for the lamellar habit and the very strong basal cleavage of the mineral. In asbestos the SiO_4 groups form chains, again partly covalent, which are feebly connected laterally through metal ion links; in a single well-formed crystal the chains may be several inches in length and can be easily teased out in the form of long fibers.

In molecular crystals with covalent molecules bound by ionic or residual forces, the mode of packing, and therefore the crystal form, may be so influenced by the position of polar groups that any obvious relationship between molecular asymmetry and crystal form may be lost. This is well and appropriately illustrated by the behavior of elongated molecules. Such molecules tend to arrange themselves with their long axes parallel; either they may form laminæ, in which all the ends are coplanar, as in a bundle of toothpicks, or else alternate molecules may overlap. The first type is exemplified by the monobasic fatty acids, which form laminæ of double molecules, joined by their carboxyl groups, with residual CH_3 to CH_3 linkages between the laminæ. The crystals likewise are laminar, with marked basal cleavage. In the overlapping type of arrangement the strong

[197] J. D. H. Donnay and D. Harker, Amer. Min., **22**: 446, 1937.

[198] Cf. M. A. Peacock and D. A. Moddle, Min. Mag., **26**: 105, 1941, and a forthcoming paper on muscovite mica, *ibid.*

[199] Cf. R. C. Evans, An Introduction to Crystal Chemistry: Cambridge, 1939.

cleavage is absent and the crystals are elongated in the direction of the long molecular axes. The same thing happens when two polar groups are present, the molecules then forming chains and crystallizing in needles, as does anthraquinone. When there are several polar groups distributed along the chain the behavior is less predictable, and we have for the first time the possibility that several modes of parallel association may be almost equally probable; the resulting crystal will have a certain degree of randomness in the transverse direction, combined with strict parallel alignment of chain molecules. At this point we encounter the first departure from the conventional lattice structure. When a molecule with distributed polar groups becomes sufficiently difform to be regarded as a colloidal particle in the direction of the chain and a crystalloid at right angles to it, we have still greater possibility of random behavior, for several segments of neighboring polymeric chains may take up a parallel orientation, while the remaining segments are free, by reason of the flexibility of the molecule, to unite with other chains or even to form closed rings. The number of possibilities is enormous and may range, according to the constitution of the chain, from the globular self-linked monomeric molecules of the soluble proteins to fibers with a very perfect alignment. In the latter case we can visualize "crystallization" as a continuation of the process of random parallel packing. This may result in a fiber, longer than the lengths of the polymeric chains which compose it, consisting of chains in parallel orientation, overlapping at random in the longitudinal direction, and joined laterally, also more or less at random, but with "cemented" regions containing on the average a certain number of segments and a certain number of interlinked chains.[209] In this partly random structure we have, for the first time, and to a degree never encountered when the molecular packing was more rigorously governed by the laws of spatial symmetry, a striking correlation between the form and properties of the crystal and those of the molecular units. The fiber owes its great longitudinal strength, however, not to the strength of the covalent bonds in the polymeric chain, since all the constituent chains are joined end to end by residual forces only, but to the numerous points of lateral cohesion; these bonds must first be broken if the chains are to slide over one another, as they must do when the fiber is ruptured.

This picture of the building of a fiber, in which we have anticipated some of the results to be given later, carries us back to the second question referred to above, namely, the regulation of crystal habit; for we can readily imagine the building of a fiber from elementary polymeric threads without understanding how its direction and relation to other structures can be regulated. In the growth of crystals, the ideal crystal displaying the relationships required by Donnay's rule or some more comprehensive state-

[209] It is not seriously suggested that the actual process of fiber formation is as simple as this. For example, association of chains and their continued growth by synthetic reactions may occur simultaneously. Cf. L. E. R. Picken, Biol. Rev., Cambridge, 15: 138, 1940.

ment can only be produced under very constant conditions. Small changes in the composition of the solution or of the melt may cause the appearance of new faces or the suppression of others, as in the unusual crystals of rock salt obtained in presence of glycine or formamide,[201] while traces of adsorbable substances can sometimes inhibit altogether the continued growth of a crystal.[201] The factors that can combine to produce deviations from the normal crystal habit must be very numerous and often very specific; crystals and crystal aggregates may be needle-shaped, thread-like, arborescent, botryoidal, or even spherical, without having any of the properties implied by these forms. The influence is not invariably in the direction of decreased organization; an appropriately periodic molecular field may induce oriented crystallization, as in the well-known case of potassium iodide deposited upon a basal cleavage surface of mica.[202] This phenomenon appears to be the result of a fortuitous agreement between the lattice period in the two compounds concerned, and it must have its counterpart in some of the specific interactions of biological components. W. J. Schmidt[203] has pointed to the influence of collagen fibers in regulating the oriented deposition of the mineral components of bone. Thus it is not difficult to understand, in principle, how the growth of structural elements in a cell can be directed by the peculiarities of the prevailing local environment; we can imagine the primitive spherical interface, deformed by adsorption of some surface-active component, acting locally as nucleus for aggregation of fibrils. The new structures, in their turn becoming seats of metabolic activity, may exert reciprocal influences on the behavior of other parts of the surface by the production of diffusible substances, so that a further deformation of the interface, perhaps, will stretch the existing fibers and thus establish a pattern for the continued deposition of oriented material. This crude statement gives nothing more than a hint of the extent to which complex influences analogous to those responsible for the habit of minerals, but inestimably more subtle, may give rise to some of the structural features of living systems. There is in fact more than a hint of the operation of such influences in some of the processes of development, such as the formation of filopodia and flagellæ.[204] "It seems clear that the form of cell components may be decided by factors similar to those which fix the form of true crystals. . . . In the last analysis the orientation of a fibril is equivalent to the orientation of a molecular structure, but it is clear that the orientation depends (also) on the morphological properties of the embryo considered as a whole."[204a]

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[201] Cf. F. Rinne, Crystals and Fine-structure of Matter, Trans. by W. S. Stiles: Methuen, London, 1924, p. 127.

[202] Cf. O. Lehmann, Fluessige Krystalle, Engelmann, Leipzig, 1904.

[203] W. J. Schmidt, Die Bausteine des Tierkoerpers in polarisiertem Lichte: Cohen, Bonn, 1924, pp. 296 ff.

[204] a. L. E. R. Picken, Biol. Rev., Cambridge, 15: 133, 1940; b. C. H. Waddington, Organisers and Genes: Cambridge, 1940; c. J. Needham, Perspectives in Biochemistry: Cambridge, 1938, p. 66.

With this introduction, which has perhaps exceeded the legitimate bounds of a treatise on large molecules, we may return to a more circumstantial account of the structure of solids, retaining the emphasis already placed upon those of biological interest.

2. The Study of the Arrangement of Particles in Bulk Systems.—In the following notes will be found a brief account of the physical principles underlying the use of X-rays in elucidating crystal structure.

The crystallographic basis is the space-lattice (cf. Fig. 15, top). If placed in a beam of X-rays, each lattice point in a single row of lattice points will act as source of a new spherical wave front, and in certain directions the wave from every lattice point will be in phase. There will be several diffracted plane wave fronts (Fig. 15, middle). The locus of each diffracted ray is obviously a right conical surface with apex at the "point" of incidence of the beam (right side of Fig. 15, middle), and with the row of lattice points as axis. A second row of lattice points intersecting the first will also give conical diffracted rays; the diffracted rays from a two-dimensional lattice, each point of which belongs to both rows, must therefore fall along the lines of intersection of the cones, which will generally be two in number. In three dimensions, three cones must have a common intersection if there is to be a diffracted beam. Thus a crystal will not in general produce a diffracted ray except in a certain series of orientations with respect to the incident beam. This concept of intersecting cones can often be replaced without loss of precision by the simpler idea of optical reflection from parallel lattice planes (cf. Fig. 15, bottom): the plane containing the inci-

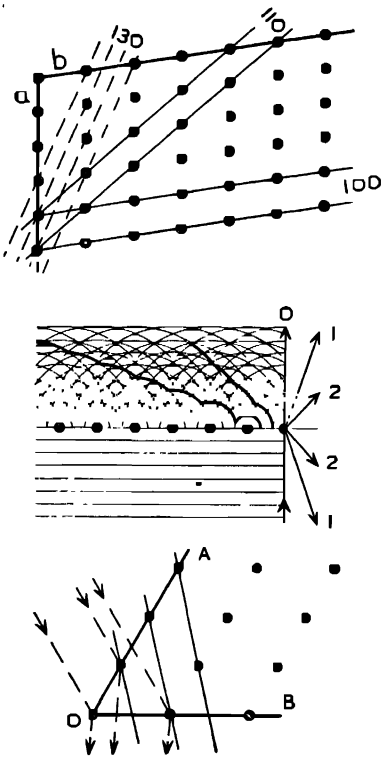


FIG. 15.—Lattice planes and the diffraction of X-rays.

Top: Illustrating in two dimensions the formation of lattice planes. The sets of planes shown are indexed in the Miller notation described in the text.

Middle: Scattering of X-rays by a linear array of particles. The circular arcs represent the secondary rays. The heavy lines represent the resultant first and second order wave fronts. On the right are shown (1 and 2) the corresponding diffracted rays.

Bottom: Illustrating, but not proving, that when the lattice line OA diffracts in the m th order and OB in the n th order, the direction of the diffracted ray corresponds to optical reflection of the incident ray from the lattice line (mn). The case illustrated is when m = 1 and n = 2. The heavy lines define the lattice lines; the dotted lines show the incident and diffracted rays, and the thin continuous lines define the "reflecting" lattice line (12)

dent and diffracted rays, and the angle between those rays, serve to identify the lattice plane responsible for the reflection, while the corresponding lattice spacing, d, is given by Bragg's equation

$$2d = \frac{\lambda}{\sin \theta} = \frac{\lambda}{\sin (90^\circ - i)}$$

where λ is wave-length, θ is the glancing angle and i is the angle of incidence on the reflecting plane. This represents the restriction imposed by interference between rays reflected from successive lattice planes.

In the Bragg spectrometer, each recorded point defines a particular value of d and i. Results are obtained more rapidly from rotation photographs for which the crystal is mounted with a crystallographic axis—say the c axis—coincident with an axis of rotation and normal to the incident X-ray beam. During rotation each set of planes capable of reflection comes in turn into a suitable position, so that a point pattern is produced on a cylindrical film perpendicular to the incident ray. The points, besides being placed symmetrically with respect to the rotation axis and a line perpendicular to it in the plane of the plate, also fall upon horizontally disposed lines, the so-called *layer-lines*. Successive layer-lines correspond to successive values, 1, 2, 3 . . . of the Miller index l, and points on the different layer lines correspond to reflections from lattice planes with the general indices $(hk0)$, $(hk1)$, etc. The reason for this is obvious from Fig. 15 (middle), for since the c axis remains perpendicular to the incident ray the angles of the diffraction cones do not change during rotation, and all diffracted rays must therefore lie on these cones, which intersect the cylindrical film in straight lines. For this reason, the positions of the layer-lines are of great value in determining the dimensions of the unit cell, the corresponding values of a and b being of course obtained by rotating the crystal about the a and b axes. From the dimensions of the unit cell, the number of molecules it contains can often be calculated if we know the density and molecular weight of the substance. The separate points on the rotation diagram are ambiguous, since we know their position only with respect to the incident X-ray beam and not with respect to the crystal. Nevertheless, their significance can often be deduced with considerable certainty. Many difficulties of interpretation are removed by using Weissenberg's device of a recording film moving in such a manner that its position corresponds always to that of the rotating crystal. This introduces the additional variable required to establish a one-one relationship between the position of a point and the lattice plane producing it. From the Weissenberg diagrams, projections can readily be prepared on which the points can be unequivocally indexed.

When single crystals are not available for examination, it is possible to make use of powder photographs: here each point of the three rotation photographs is replaced by a circle, since crystals are present in all possible configurations. Consequently the number of circles is often too large to be of any more than qualitative value, but when the three axes of

the unit cell are equal and at right angles the photographs can be used in the analysis of structure. If the particles were not entirely in random positions, but had one axial direction in common, the "powder" photograph would be expected to resemble the rotation diagram of a single crystal; diagrams of this type are characteristic of fibers and will be referred to later. On the other hand, if a body were amorphous, no diagram would be expected, although substances like rubber commonly show a single diffuse circular pattern which indicates the existence of some rather indefinite undirected periodicity. Liquids similarly show diffuse rings, the interpretation of which in terms of a distribution function for the distance of separation of molecules or groups of molecules has given rise to considerable discussion. The Bragg equation is not applicable in such cases.[205]

The space-group can be determined by a detailed examination of diffraction patterns. What has been considered above as a series of diffracting points coinciding with the lattice points is actually a series of groups of points within the unit cell, so that each lattice plane should be replaced by a number of planes regularly or irregularly spaced according to the degrees of symmetry of the particular space-groups concerned. This subdivision of the primitive translations is responsible for differences in intensity of the various spots on the X-ray diagram, since each plane participates in the reflection phenomenon and the various components of the final reflected ray differ in phase. When certain definite types of symmetry operation occur in the space-group, the phase-difference may be exactly a half-period, with the result that certain reflections may be absent from the diagram. The identification of these systematic extinctions, each of which is characteristic of the symmetry operation which produced it, thus serves to determine the space-group uniquely. This knowledge usually suggests possible arrangements of molecules in the unit cell.

Knowledge of the structure of a crystal in further detail depends upon accurate measurement of the absolute intensities of a large number of diffracted rays. The intensity of a ray diffracted in a particular direction represents the result of superposition of rays scattered in that direction by each electron. The scattered intensity, f, of a single atom can be calculated as a function of the angle between incident and scattered rays; when this angle is zero, f is approximately N times the amplitude scattered by a single electron, N being the number of electrons in the atom, since the path through the atom is then the same for all rays. It falls off with increasing deviation of the ray. The amplitude F_{hkl} (including numerical amplitude and phase) of the scattered ray from the entire unit of structure can be calculated by superimposing all the rays f_A, f_B, f_C, . . . scattered by the various kinds of atom, A, B, C, . . . present in the unit. Clearly a particular value of F for a particular angle does not define an unique structure;

[205] For a full discussion of the theories of X-ray diffraction by liquids, see J. T. Randall, The Diffraction of X-rays and Electrons by Amorphous Solids, Liquids and Gases: Chapman and Hall, London, 1934.

a curve relating F to the angle of scattering would be open to fewer conflicting interpretations, and the customary use of a complete diffraction diagram amounts almost to this, with the important qualification that the measured intensities are proportional to F^2, so that we actually know F only in magnitude and not in sign. Despite this limitation it has been possible in a number of cases, by calculating the F curve for several plausible structures, to decide which of these corresponds most closely to the experimental data.

The intensity curves can be interpreted in other useful ways. The electron density at any point is repeated periodically in any arbitrary direction, the period being related in a simple manner to the Miller indices of the plane normal to the direction in question. This can be expressed formally by a three-dimensional Fourier series giving the electron density as the sum of these different periodic functions, the amplitudes of which are given in the formal expression as unknown coefficients A_{hkl}. Now F_{hkl} is an integral of the electron density at every point in the unit cell, and it is possible by substituting the Fourier series in the expression for F to calculate the coefficients A in terms of F. The result is of surprising simplicity, the coefficients of the harmonic terms corresponding to the various possible crystal planes being proportional to F. Thus the observed values of F can be used to calculate the electron distribution in the unit cell, provided the signs are known.[206]

The application of such series is, however, limited to cases where the phase can be calculated or can be deduced from symmetry considerations. This difficulty of sign is a serious obstacle to the construction of density diagrams for complex unit cells. It is possible, however, to compound the various intensities by means of Fourier series containing these values, proportional to F^2, instead of the amplitudes, as coefficients. The theory of such series was considered by Patterson,[207] who was able to provide a physical interpretation and to show that diagrams obtained from F^2 series are capable of being used in the determination of structure. "The density in a Patterson projection at the end of a vector drawn from the origin represents the integration over the unit cell of the product of densities at all pairs of points in the crystal structure which have that vector relationship"[208]—in other words, in sufficiently simple cases it gives the position of every atom with respect to every other atom. For a complex structure the interpretation of a Patterson diagram (two-dimensional projection) may be extremely involved, and that of a section impossibly so; it is quite difficult to trace the relationship between the density diagram and the Patterson diagram even in cases where the structure is known. "Not only the relative positions within the molecule, but also those of atoms in [illegible] molecules related by the symmetry elements, are represented by super-

[206] See the excellent review by J. M. Robertson, Reports on Progress in Physics, Physical Society, London, **4**: 332, 1938.

[207] A. L. Patterson, Ztschr. f. Krist., **90**: 517, 543, 1935.

[208] W. L. Bragg, Nature, **143**: 73, 1939.

imposed diffuse peaks . . . and these combine in such a way that it is extremely hard to unravel the tangle."[208]

3. Notes on the Structure of Certain Crystals.—The study of the arrangement of large molecules in undispersed substances, at least insofar as it has reference to biological materials, is largely the study of difform systems: thin films and fibers. These are not strictly crystalline. Some large molecules, such as the globular proteins, do, however, form true crystals; others, such as starch and glycogen, occur in granular habit. These facts alone demand the inclusion here of some reference to the structure of true crystals. Other reasons also have determined the selection of the several groups of substances referred to in the following notes. The chief of these is the special structural relationship between the small molecules and the large—between the simple sugars and the cellulose fiber, or between the peptide linkages in diketopiperazine and those in keratin, for instance—a relationship that makes a knowledge of the molecular dimensions and packing of the small molecules indispensable to an understanding of the large. Reference to long chain aliphatic compounds and to the lipids and sterols is amply justified by the peculiar importance of these substances as components of the cell membrane, in which they are undoubtedly present in the form of macromolecular complexes with proteins, while preserving some of the structural features characteristic of their configuration in the crystalline state.

a). *Calcite, aragonite, and apatite.*—Skeletal structures, external and internal, are largely inorganic in nature; although deposited under the guidance of organic formative influences, and often giving little morphological evidence of their inorganic nature, they have the fine structure of substances commonly found as minerals. The immediate circumstances may be very deceptive. It may happen that a calcareous spicule of obviously organic origin is actually a single crystal, highly eccentric in habit; on the other hand, morphologically identical crystallites in comparable biochemical situations may be composed of chemically different substances.

Apart from the siliceous exoskeletons of some organisms, the most common mineral components of invertebrates are identical with one or other of the two forms of calcium carbonate, calcite and aragonite, while the mineral constituents of bone, including the enamel and dentine of teeth, are very closely related to apatite, $Ca_5F(PO_4)_3$, probably by substitution of hydroxyl for the fluorine atom. These are all ionic crystals.

The crystal structures of calcite and aragonite are rather simple, and can be readily understood as distorted examples of cubic and hexagonal close-packing. Fig. 16 illustrates these two alternative ways of packing equal spheres into the smallest possible volume. To avoid confusion, the circles representing the spheres have been shrunk; they are to be imagined large enough to be in contact with one another. There is only one way of packing spheres into a single layer; this is represented by the rows of black circles, *AA*. Between each pair of lines of spheres thus packed there are two rows of "triangular" spaces, *BB* and *CC*, and there are only two ways in which new layers of spheres can be packed above

and below the layer *AA*: either the upper layer can occupy the row of depressions *BB* and the lower layer the row *CC* (or vice versa), or else both upper and lower layers must occupy the rows *BB* (or *CC*). In the former case the structure, if repeated, would give a vertical pattern *CABCABCAB* . . . , in the latter, the pattern *BABABA*. . . . Apart from the distortion already men-

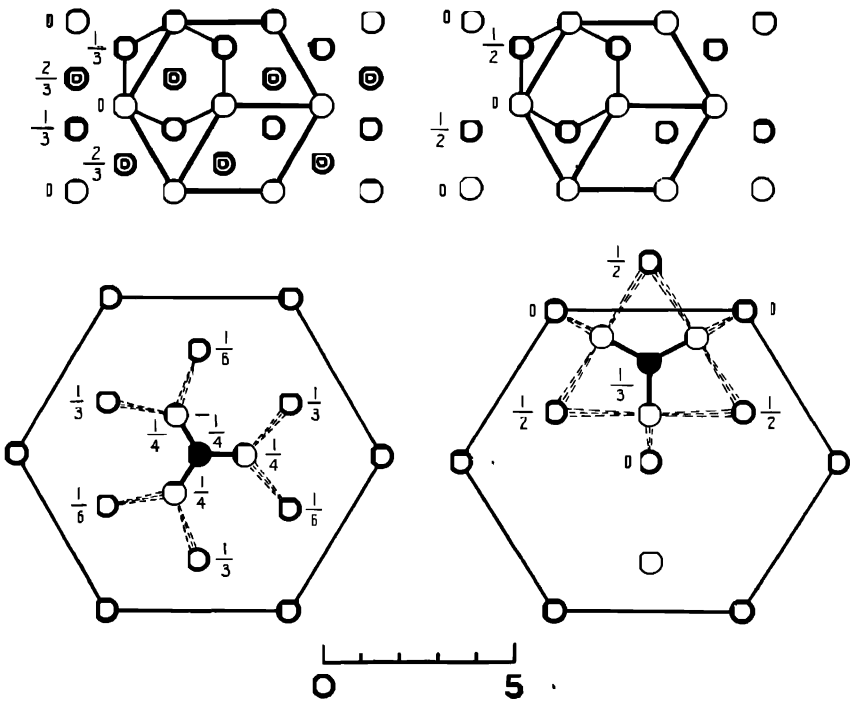


FIG. 16.—Above: Illustrating in plan the two modes of closest packing of spheres. Similar circles are coplanar, at distances above the basal plane represented by the numerals, which are given as fractions of the identity period. Connecting lines are drawn to aid comparison of the two methods of packing. The rows of thin circles indexed 0 define the basal rows referred to in the text as *AA*; the heavy circles are *BB* and the double circles *CC*.

Below: Comparing the environments of the carbonate ion in calcite (left) and aragonite (right). The diagrams are basal projections in which the atomic positions perpendicular to the plane of the paper are represented by fractions. Heavy open circles are calcium, light open circles are oxygen, black circles are carbon. It will be noted that the packing of the calcium ions in calcite and aragonite resembles that shown above on the left and right respectively. The carbonate ions are so placed as to give each oxygen two calcium ions as closest neighbors in calcite and three in aragonite.

tioned, these structures represent the packing of calcium ions in calcite and aragonite respectively (Fig. 16).

Apatite forms a denser and much harder structure,[209] in which the fluorine ions, or their analogue, occupy a key position on the edges of the hexagonal unit cell, from which they are linked, each through three $Ca^{\cdot\cdot}$ ions, to the phosphate

[209] Cf. W. L. Bragg, Atomic Structure of Minerals: Cornell University Press, Ithaca, 1937, p. 132; references, see R. W. G. Wyckoff, The Structure of Crystals, 2nd. ed.: Chemical Catalog Co., New York, 1931, p. 292; *ibid.*, 2nd ed. suppl., 1930–1931, p. 68.

structure. It is suggested that the carbonate in bone replaces fluorine in the apatite structure, but this would require such a considerable distortion of the lattice that the carbonate is probably present only in regions where the apatite lattice is imperfectly developed.[210,211]

b). *Hydrocarbons, fatty acids, lipids, and sterols.*—These substances, covering a very wide range of molecular complexity, have in common some very striking features of intermolecular organization, and as these are pertinent in any discussion of the mode of association of the substances in

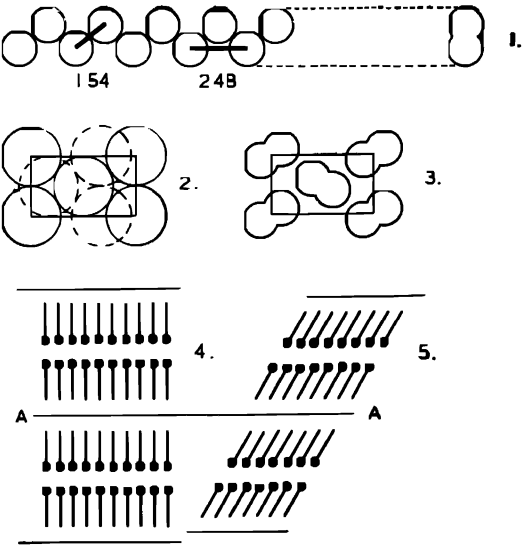


FIG. 17.—1. Projection of fully extended carbon chain in polymethylene compounds, showing distances between adjacent and alternate chain atoms. On the right, projection on a plane perpendicular to chain axis.

2. Showing the effective cross-section of freely rotating polymethylene chains and the resulting mode of packing.

3. Showing the decreased symmetry of polymethylene crystals when free rotation is prevented by cooling.

4. and 5. Illustrating the formation of bimolecular leaflets by aliphatic molecules with a single terminal polar group. AA represents the basal or cleavage plane. In 4 the chains are perpendicular to the basal plane; in 5 they are tilted.

living matter, we prefer to underline them, rather than to enter into a detailed description of specific complexities. We wish to stress in particular the predominant modes of packing of unsymmetrical molecules, the one assumed when the intermolecular forces are uniform, and the other when there is a specifically reactive terminal group.

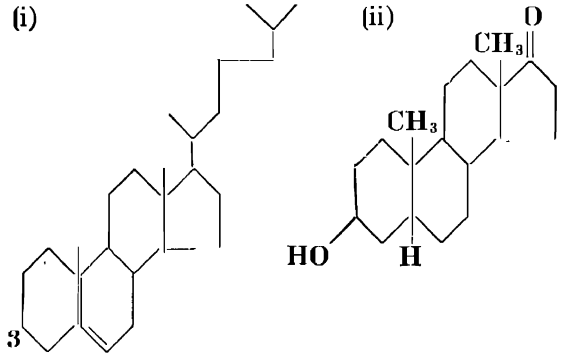
[210] W. F. Bale, Am. J. Roentgenol., **43**: 735, 1940.

[211] Further literature on X-ray structure of bone and tooth substance and related inorganic compounds, see W. F. Bale and H. C. Hodge, Naturwissenschaften, **24**: 141, 1936; W. F. Bale, M. L. Le Fèvre and H. C. Hodge, *ibid.*, **24**: 636, 1936; M. L. Le Fèvre, W. F. Bale and H. C. Hodge, J. Dent. Research, **16**: 85, 1937; H. C. Hodge, M. L. Le Fèvre and W. F. Bale Ind. Eng. Chem., **10**: 156, 1938.

An unusually symmetrical packing is shown by the long chain hydrocarbons at temperatures near the melting point. Here the chains become effectively cylindrical by virtue of their freedom to rotate about their long axis; the molecules in this case assume the hexagonal close-packed configuration (Fig. 17, diagram 2).[212] At lower temperatures Van der Waals attraction between the chains is sufficient to cause loss of rotational freedom, and the chains then pack with a less symmetrical mutual configuration, allowing the maximum area of contact. This is characteristic not only of the paraffin hydrocarbons but also of benzene, anthracene and other chemically inert ring structures.

When the hydrocarbon chain is terminated at one end by a polar or ionic group, as in the monobasic acids and alcohols, the units are double molecules joined end to end by their reactive groups; in general the substances are polymorphic, with chains oriented vertically or obliquely with respect to the basal plane *AA* (Fig. 17, diagrams 4 and 5) which is also a cleavage plane. At higher temperatures these substances, like the paraffins, may assume the hexagonal arrangement with rotational freedom.[213] The same bimolecular units occur when the terminating group is quite complex. They are found in the triglycerides[214] and apparently in the lipids. Bear, Palmer, and Schmitt[215] have noted in the X-ray diagrams of several dry lipids—lecithin, cephalin, sphingomyelin, phrenosin, kerasin—a common short-spacing reflection at about 4.2 Å, corresponding probably to the close-packing of parallel hydrocarbon chains, and long spacings which, allowing for some uncertainty in the length of the chains, agree quite well with the values calculated for fully extended double molecules. Similar spacings are found in the total dried lipid from brain or nerve.

Among the sterols (i) and the related sex hormones, which show an astonishing diversity of crystal structures, it is possible to distinguish single- and double-layer types of unit cell.[216] The most frequently occurring cells



[212] A. Mueller, Proc. Roy. Soc., **127A**: 417, 1930; *ibid.*, **138A**: 514, 1932.

[213] E.g., J. D. Bernal, Nature, **129**: 870, 1932.

[214] C. E. Clarkson and T. Malkin, J. Chem. Soc., p. 666, 1934.

[215] R. S. Bear, K. J. Palmer, and F. O. Schmitt, J. Cell. Comp. Physiol., **17**: 355, 1941.

[216] J. D. Bernal, D. Crowfoot, and I. Fankuchen, Phil. Tr. Roy. Soc., **239A**: 135, 1940.

are of the single-layer type, with dimensions that suggest a molecule about 4.5 Å thick, 7 Å wide, and 20 Å long; Patterson projections of cholesteryl chloride and bromide, showing two ridges running the length of the c-axis, confirm this interpretation. These dimensions are in conformity with a puckered ring system and a chain continuing along the line of the rings, and the packing of these long flat molecules is closely similar to that of the linear or cyclic hydrocarbons. It is significant that often the single-layer crystals have no polar groups; if such groups are present, either they are in an unfavorable position for association, or else there are two of them at opposite extremities of the molecule, as in androsterone (ii), which can interact without any considerable distortion of the one-layer structure. On the other hand, out of 48 compounds giving double-layer cells, 33 are known to have an hydroxyl group in the 3-position; in 7 the position of the groups is uncertain; and in the remaining 8, the reason for the double-layer structure is unknown. Pyrocalciferol, which certainly has an OH group in the 3-position, forms single-layer cells, while anhydrous cholesterol and anhydrous ergosterol form very complex crystals, with a unit cell containing as many as 32 molecules in the latter case and 8 in the former. Despite these and other anomalies, there is not much doubt that the double-layer structure is usually associated with interaction between two OH groups in the 3-position.

c). *Sugars.*—The accepted view that cellulose is made up largely of *β-d*-glucose residues, and starch of *α-d*-glucose, makes the structure of these relatively simple compounds a matter of peculiar importance in the study of macromolecules. Although recent chemical studies have established beyond peradventure the constitution of many of the stereoisomeric sugars, the precise spatial arrangement is still uncertain. This is mainly due to doubt as to the form of the pyranose ring, which may be built in various puckered forms with bond angles approaching the normal values, or in other forms with distorted bond angles, giving in an extreme case a flat ring in which the pyranose oxygen lies above or below a plane containing the four carbon atoms. The puckered rings add considerably to the number of possible stereoisomers, and the fact that these have not been shown to exist is perhaps a point in favor of a plane ring; the energies of activation for tautomeric interchange between the various theoretical forms appear not to have been worked out. In any event it is important to know an average configuration, since a small change may alter considerably the extra-cyclic valency directions, and may be the principal factor in determining the conformation and mode of packing of rings in a pyranose polymer.

The study of the sugars with X-rays is singularly difficult. The variety of different structures indicated by the extensive data available concerning crystal class, dimensions of the unit cell, and space-group is perhaps as considerable as among the sterols. Apart from possible variations in the ring, this is largely the result of interaction between hydroxyl groups, to which the crystals also owe their hardness, density, and high melting-point.

Replacement of a hydroxyl group or a change in its configuration may necessitate a complete readjustment of the relative positions of the molecules. Such rearrangement may make it difficult to arrive at any reasonable estimate of the effect of the change upon molecular dimensions, although this may sometimes be possible.

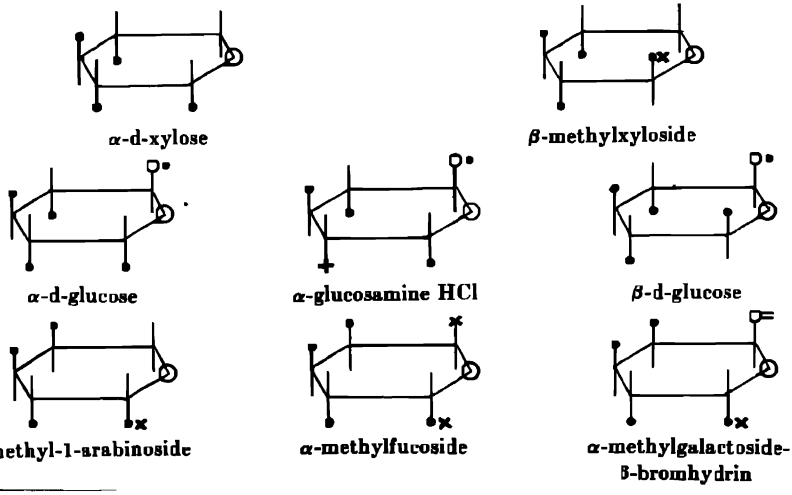
Consideration of the relationship between the unit cells of α-*d*-xylose,[217] β-methyl xyloside,[218] and α-*d*-glucose,[219] for instance, leads to the following values for the molecular dimensions:

α-*d*-xylose	5.62 × 6.32 × 4.60 Å
β-methyl xyloside	6.89 × 6.50 × 4.30
α-*d*-glucose	5.20 × 7.44 × 4.99

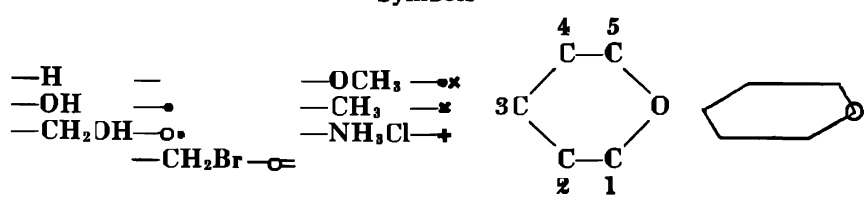
from which it has been concluded that the change from the α to the β configuration does not alter the thickness of the molecule, although it should do so if the ring were puckered; furthermore, some indication of the positions of the extracyclic CH_2OH group in glucose and of the methyl group in methyl xyloside is given by the changes in length and width. Only occasionally is it possible by increasing the volume of a single substituent to expand the lattice without causing any other change in the unit cell; the following example[220] gives the molecular dimensions in such a case:

β-methyl-*l*-arabinoside	7.74 × 8.10 × 5.89 Å
α-methyl-*l*-fucoside	7.87 × 9.96 × 5.72
α-methyl galactoside-6-bromhydrin	7.81 × 10.58 × 5.62

Key to Stereochemistry of Sugars Referred to in Text



[217] E. G. Cox, J. Chem. Soc., 2313, 1931.
[218] E. G. Cox, *ibid.*, 138, 1932.
[219] J. Hengstenberg and H. Mark, Ztschr. f. Krist. **72**: 301, 1929.
[220] E. G. Cox, T. H. Goodwin, and A. I. Wagstaff, J. Chem. Soc., 978, 1935.



Constancy of thickness of α- and β-derivatives, which would provide important evidence in favor of the flat pyranose ring, is difficult to establish in the majority of sugars, because of the uncertainty attaching to the mode of packing. Apart from the cases mentioned already, *d*-cellobiose (4-β-*d*-glucosido-*d*-glucose)[221] alone has a unit cell dimension of 5.0 Å or less. Conditions are far simpler when the effects of hydroxyl interaction are eliminated by methylation.[222] The methyl derivatives characteristically form acicular crystals with a very short cell dimension, parallel to the long axis, of about 4.5 Å; the cases listed in illustration include derivatives of glucose, cellobiose, and cellotriose,[223] from which it may perhaps be concluded that the flat ring is the form assumed in these substances. A certain amount of other evidence has been adduced in favor of a similar ring structure in the free sugars. It has been noted[224] that β-*l*-arabinose gives a particularly strong 18th order reflection parallel to (010), indicating a concentration of scattering mass along the *b*-axis at intervals of $b/18$, or 1.08 Å; this period corresponds roughly to the positions of hydroxyl oxygens above and below a plane pyranose ring, but is difficult to reconcile with a strainless ring. More recent evidence concerning the unmethylated sugars, however, appears to favor a slightly puckered ring. A complete X-ray synthesis of the structure of α-glucosamine hydrochloride and hydrobromide has been achieved[225] without recourse to any stereochemical assumptions. Both 3-dimensional Patterson sections and Bragg sections have been made, the signs of the structure factors required in the latter having been determined by a comparison of corresponding intensities for the chloride and the bromide. The results demonstrate for the first time the real existence of the pyranose ring in a crystalline sugar, whereas the chemical evidence has hitherto been strictly applicable only to glucosides. The data apparently exclude the boat-shaped strainless ring, but the ring is nevertheless slightly puckered. It is interesting that the No. 1 oxygen atom is found to project almost vertically from the ring, so that this atom in the corresponding β-sugar must lie practically flat. The same difference, if it persists in maltose and cellobiose, must be of great significance in the stereochemistry of starch and cellulose.

[221] J. Hengstenberg and H. Mark, Ztschr. f. Krist., **72**: 301, 1929.
[222] E. G. Cox, T. H. Goodwin, and A. I. Wagstaff, J. Chem. Soc., 1495, 1935.
[223] C. Trogus and K. Hess, Ber. d. dentsch. Chem. Ges., **68**: 1605, 1935.
[224] E. G. Cox, J. Chem. Soc., 2313, 1931.
[225] E. G. Cox and G. A. Jeffrey, Nature, **143**: 894, 1939.

Starch, despite its chemical similarity to cellulose, both substances being polymers of *d*-glucose, occurs always in granular habit. The granules possess a concentric layer structure with radial symmetry resulting from the presence of small elongated crystallites, probably not more than 100 Å in length, arranged with random orientation about an axis which coincides roughly with the radial direction.[226] The unit cell corresponds to two maltose, or four glucose, residues. The crystal structure is very imperfect, as would be expected of a substance which contains at least two polymeric components, each probably polydisperse; these may be distinguished as amylose, the water-soluble component of low molecular weight, and the giant molecule of amylopectin. Amylose is probably an unbranched chain, and amylopectin an elaborately ramified structure of polymerized maltose (4-α-*d*-glucosido-*d*-glucose) residues, the degrees of branching and molecular weight of the latter showing pronounced differences in starch from different sources. As a result of these differences and probably also of differences in the degree and mode of aggregation of crystallites, the reactions of starch granules from different sources vary specifically in relation to the systematic botanical position of the source.[227] The X-ray diagrams, similarly, vary according to origin, but the differences can be abolished without destroying the typical features of the starch diagram.[228] According to K. H. Meyer,[229] nothing more than the fact of crystallinity and the existence of different modifications has been established. The optical properties of the grains and the complicated changes observed on swelling suggest that the radial arrangement of crystallites is a very loose one, with intermediate regions in which the crystallites are widely separated from one another, being connected only by molecular filaments; it is the size and the highly ramified structure of the amylopectin molecule "which makes it possible for part of the molecule to remain in lattice-like micelles and another part of the same molecule to be surrounded by solvent," while the unbranched amylose probably is present in dissolved form.[230]

d). *Diketopiperazine, glycine, alanine.*—Tentative models of the polypeptide chain have played a considerable part in the elucidation of fiber structure and have figured in speculations on the structure of globular proteins. These models have lacked precision because until recently there have been no complete analyses even of the simplest related substances.

[226] E.g., R. S. Bear and D. French, J. Am. Chem. Soc., **63**: 2298, 1941.

[227] E. T. Reichert, A. Biochemic Basis for the Study of Problems of Taxonomy, Heredity, Evolution etc., with Especial Reference to the Starches and Tissues of Parent-Stocks and Hybrid Stocks and the Starches and Hemoglobins of Varieties, Species and Genera: Carnegie Institute, Washington, D.C., Publication 270, 1919.

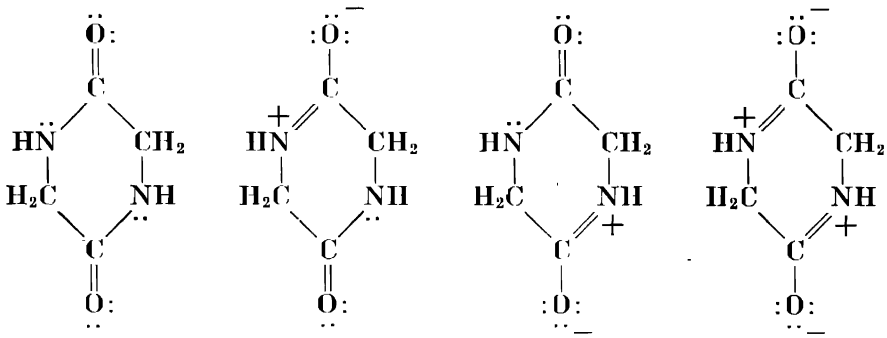
[228] J. R. Katz, Ztschr. f. physik. Chem., **150A**: 37, 1930; J. R. Katz and L. M. Rientsma, *ibid.*, **150A**: 60, 1930; J. R. Katz and T. B. Van Itallie, *ibid.*, **150A**: 90, 1930.

[229] K. H. Meyer, Advances in Colloid Science, edited by E. O. Kraemer: Interscience Pub., New York, 1941, p. 143.

[230] For recent reviews see particularly (229); also G. V. Cæsar and M. L. Cushing, J Phys. Chem., **45**: 776, 1941.

Such data are now available for diketopiperazine,[231] glycine[232] and alanine.[233] The results are of considerable interest, providing information which we cannot yet expect from the X-ray analyses of proteins themselves.

The diketopiperazine, or "glycine anhydride," molecule is a nearly plane hexagon (Fig. 18a). Since six-membered rings with only single bonds are invariably puckered, it has been inferred that the ring contains double bonds, resonating among the structures:



The molecules lie nearly flat in the (101) plane, in straight parallel rows, the molecules within a row being tilted so as to equalize the two NHO distances between neighboring molecules. The structure is thus determined largely by these two hydrogen bonds, the cohesion between neighboring strings of bonded molecules, both within the (101) plane and perpendicular to it, being considerably less and giving rise to marked cleavage along (101) and (010).

In glycine and alanine, the possibilities of hydrogen bonding are far more numerous than in diketopiperazine. The glycine molecule has the form shown in Fig. 18b. In the crystal, the molecules are packed in bimolecular sheets; between the sheets, only Van der Waals forces are involved, so that the plane of the sheets, (010), is a cleavage plane. Within each sheet the cohesion results from the system of hydrogen bonds indicated diagrammatically in Fig. 18c, d, e. The molecules are joined symmetrically in pairs, head to tail, by two bonds of length 3.05 Å; each is linked with others in its own plane by four bonds, one for each oxygen atom and two for the nitrogen, the NO_I bond being 2.88 Å and the NO_{II} 2.76 Å long. Additional NO_I bonds, 2.93 Å, link each molecule to one of its neighbors in the other molecular layer of the bimolecular sheet. Thus, in all, each nitrogen atom participates in four hydrogen bonds, involving only three hydrogen atoms. It has been suggested that the hydrogen atoms and the α-carbons are disposed tetrahedrally about the nitrogen atom, and that the 3.05 and 2.93 Å bonds are formed by a single hydrogen atom situated at 2.34 Å from O_I, 2.22 from O_{II}, and 0.99 from N. Besides necessitating a forked hydro-

[231] R. B. Corey, J. Am. Chem. Soc., **50**: 1598, 1938.

[232] G. Albrecht and R. B. Corey, *ibid.*, **61**: 1087, 1939.

[233] H. A. Levy and R. B. Corey, *ibid.*, **63**: 2095, 1941.

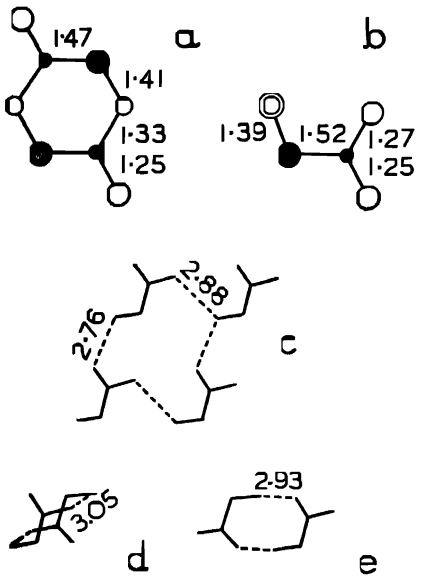


FIG. 18.—a. Plan of molecule of diketopiperazine. (Drawn from data given by R. B. Corey, J. Am. Chem. Soc., **60**: 1598, 1938.)

Large open circles: oxygen
Small open circles: NH
Large closed circles: CH_2
Small closed circles: carbon.

Bond lengths are in Ångstrom units.

b. The glycine molecule. (Drawn from data of G. Albrecht and R. B. Corey, J. Am. Chem. Soc., **61**: 1087, 1939.)

Double open circles: nitrogen.
Other symbols as in a.

c, d, e. Showing the different kinds of hydrogen bond in glycine. All these diagrams are projections on the same plane.

c. shows bonds between glycine molecules within a single sheet parallel to the plane of the paper.

d. shows bonds between corresponding glycine molecules within a bimolecular sheet. The bimolecular sheet may be visualized by imagining a second layer like that shown in c to be rotated through 180° and then imposed upon it.

e. shows bonds between a glycine molecule in one layer of the bimolecular sheet and its second nearest neighbor in the other layer.

It will be noted that according to these diagrams each nitrogen atom participates in four hydrogen bonds. The fact that only three hydrogen atoms are available for these bonds led to the suggestion that the 2.93 Å and 3.05 Å bonds of d and e are actually split hydrogen bonds involving only one hydrogen atom.

gen bond, the tetrahedral arrangement has been taken as further evidence for the dipolar ion structure of glycine.

The methyl group in alanine is responsible for a more complex structure than is found in glycine. The pairing of molecules does not occur, and the structure may be likened to a tubular framework of hydrogen bonds, with methyl groups packed inside the tubes, separated from one another by the unusually short distance of 3.64 Å. A more usual value is 4.0 Å. An interesting point in the final structure determination is that the contribution of the hydrogen atoms, assigned to plausible positions, had to be included in the calculations before the intensity data could be accounted for satisfactorily.

Besides showing the extreme importance of hydrogen bonds in determining the configuration of structures containing peptide or α-amino-acid groupings, these measurements give improved values for the probable bond lengths and bond angles of polypeptides. It is significant that the introduction of a methyl group into glycine causes no appreciable alteration in the N—C_α bond length, although the actual value, 1.39 Å, is for an unknown reason considerably less than the normal value, 1.47 Å. The bond lengths in diketopiperazine are also shorter than the usual values, probably as a result of the electric

charge. This is to be expected for the C—N bonds but is surprising for C—C, which has the value 1.47 Å instead of 1.54 Å. In glycine it is 1.52 Å. On account of these variations it is not reasonable to assume that no further revisions will have to be made when more complex structures have been examined, but the following values for the bonds in polypeptide chains, —NH—C'HR—C''O—, can perhaps be accepted provisionally on the basis of these and other recent X-ray analyses:[234]

N—C'	C'—C''	C''—O	C''—N	O—H
1.41	1.47-1.52	1.25	1.33	1.09 Å
	NC'C''	C'C''N	C''NC'	
	112°	118°	118°	

e). *Protein crystals.*—The discovery of crystalline proteins, around the middle of the last century, aroused immediate dispute. Those who considered that Nature would never do violence to scientific definition (which they were inclined to promote to the status of heavenly decree) denied the ability of a colloid to crystallize. In this they were supported by certain genuine peculiarities of protein crystals, and the sting of controversy, added to the great intrinsic interest of the subject, stimulated a number of meticulous researches to which we owe most of our present knowledge, and, indeed, a good deal more which awaits resuscitation. Only now, with the advent of X-ray analysis, is there a promise of further advance.

Much of the argument was unnecessary, for the occurrence of definite crystal forms, admittedly with a certain variability of interfacial angles, was an experimental fact, and, as Naegeli[235] pointed out, must indicate a periodic arrangement of particles; the dispute really centered around a secondary quality, namely, the ability of water and dissolved substances to penetrate the crystal fabric without disrupting or essentially altering the form of the crystal.[236] It was clear to Cohn, Naegeli, and many others[237] that this involved the separation of molecules, in contact with each other in the dry crystal, by thick layers of water, and the resemblance of the phenomenon to the swelling of cells and starch granules received a lot of attention. It was said that since protein crystals swell like starch, they cannot be true crystals, but only "crystalloids"; it was also deduced, from the same facts, that starch granules must be made up of minute crystallites, their essentially crystalline nature being masked, in the rounded form of the granule, by the tendency of the particles to assemble in crooked rows and bent surfaces. This view gains somewhat in credibility when one considers the genesis of protein crystals, which often start as "globuliths" of amorphous aspect, the crystalline form being assumed by subsequent rearrangement of

[234] Cf. M. L. Huggins, Ann. Rev. Biochem., **11**: 27, 1942.

[235] C. Naegeli, Sitzungsb. d. kgl. bayr. Akad. Wiss., **1862**: p. 120.

[236] F. Cohn, J. practische Chem., **80**, 129, 1860.

[237] For early literature see A. F. W. Schimper, Ztschr. f. Krist., **5**: 131, 1881, F. N. Schulz, Die Kristallisation von Eiweissstoffen und ihre Bedeutung fuer die Eiweisschemie: G. Fischer, Jena, 1901.

the particles constituting the aggregates. Gabriel,[238] believing that dissolved protein molecules are too large to form true crystals, attributed the rearrangement to depolymerization—an interesting hypothesis, upon an insecure foundation, which has recently reappeared in an attempt to explain certain features of the Patterson projections of protein crystals.[239]

The systematic description of these crystals has been the subject of a number of early papers[240] and has since been rather neglected, with the deflection of interest to the use of crystallization as a part of the preparative technique of protein chemistry, which received impetus from the isolation of urease[241] and of insulin[242] and has since extended to the preparation of other enzymes, enzyme precursors, and viruses in crystalline or paracrystalline form.[243] The variability of interfacial angles in protein crystals was noted in Naegeli's measurements and shown to be due, at least in part, to the anisotropy of swelling, with consequent change of axial ratios. A further cause is undoubtedly to be found in the plasticity of the crystals, a possible response to external forces being a structural rearrangement, with change of axial ratios and development of a higher grade of symmetry,[240c] so that it is not surprising that some uncertainty arose as to the crystal class to which various protein crystals should be assigned. The extensive work of Reichert and Brown[240e] on the crystalline hemoglobins of many animal species showed that while the crystals from each species are easily recognizable by their characteristic habit and appearance, and apparently belong to several different crystal classes, they are actually all members of an isopolymorphous series, the crystals belonging to the higher symmetry groups being derived by mimetic twinning from the orthorhombic and monoclinic types. The relative importance of environmental factors and of possible specific differences in the globin components is not yet clear. Wichmann[244] has described the isomorphism of various albumins and it is likely that many other instances could be found.

The study of protein crystals with X-rays is still in its early stages. Powder photographs[245] show sharp diffraction rings which could be either

[238] S. Gabriel, Ztschr. f. physiol. Chem., **15**: 456, 1891.

[239] J. D. Bernal, Proc. Roy. Soc., **170A**: 75, 1939; Nature, **143**: 663, 1939.

[240] E.g., a. C. Naegeli, Sitzungsb. d. kgl. bayr. Akad. Wiss., 1862, p. 120; b. A. Rollet and V. von Lang, Sitzungsb. Wiener Akad. Wiss., Math.-Naturwiss. Kl., **46**: 65, 1862; c. A. F. W. Schimper, Ztschr. f. Krist., **5**: 131, 1881; d. A. Wichmann, Ztschr. f. physiol. Chem., **27**: 575, 1899; e. E. T. Reichert and A. P. Brown, The Differentiation and Specificity of Corresponding Proteins and Other Vital Substances in Relation to Biological Classification and Organic Evolution: The Crystallography of Hemoglobins: Carnegie Institute, Washington, D.C., Publication No. 116, 1909.

[241] J. B. Sumner, J. Biol. Chem., **69**: 435, 1926.

[242] J. Abel, Proc. Nat. Acad. Sc., **12**: 132, 1926.

[243] *Inter alia*, J. H. Northrop, J. Gen. Physiol., **13**: 747, 1930; R. M. Herriot and J. H. Northrop, *ibid.*, **18**: 35, 1934; M. Kunitz and J. H. Northrop, *ibid.*, **18**: 433, 1935; W. M. Stanley, Science, **81**: 644, 1935; Phytopathology, **25**: 305, 1936; F. C. Bawden and N. W. Pirie, Proc. Roy. Soc., **123B**: 274, 1937; Brit. J. Exper. Path., **18**: 275, 1937; *ibid.*, **19**: 56, 251, 1938.

[244] A. Wichmann, Ztschr. f. physiol. Chem., **27**: 575, 1899.

[245] Literature in ref. (246).

intermolecular or interatomic in origin. These are difficult to interpret in detail and have been superseded by the study of single crystals.[246] The unit-cell dimensions and space-group have been established in a number of cases. These provide useful molecular weight values, subject to the usual uncertainty as to the correct value of the partial specific volume and they also impose certain restrictions upon the possible shape of the molecule, if we assume in each case that the molecules present in solution are also present in the crystal. The case of insulin is rather simple and definite, since there is only one molecule in the unit cell, which is a flat rhombohedron approximating to an oblate ellipsoid of diameter 74.8 Å and thickness 30.9 Å.

The water content of the crystals is of both theoretical and practical importance in X-ray work. The wet crystals give much better photographs, with higher resolution, than the dry ones, the smallest observed spacing being around 2 Å for wet crystals and sometimes as high as 20 Å after drying. This limits the value of comparison between wet and dry crystals in the study of the interatomic arrangement and shows that drying is accompanied by a certain loss of order. The enzyme ribonuclease has recently been shown to give unusually perfect photographs down to 2 Å in the dry state.[247] Crowfoot[248] has discussed the effect of water upon the distribution of peaks in Patterson projections of insulin and lactoglobulin. In both cases it seems that the large spacings change a good deal, while the small spacings are shifted in a body when the crystals are dried, and the simplest explanation is that the molecules are merely compacted, with some angular movement, during the process. The same is true of wet and dry gels of tobacco mosaic virus.[249] Naturally these observations tell us nothing about the state of the water other than its intermolecular situation. Katz[250] found the water vapor isotherms of crystalline proteins to be continuous, from which he concluded that the substances contained no water of crystallization. The theory of the stability of concentrated systems of charged particles suggests that the water is probably mostly present in the free state and that protein crystals should be regarded as bipolar coacervates.

The interatomic arrangement has not yet been deduced, even in its main features, from the Patterson projections of crystalline proteins. The most striking feature, in the intramolecular pattern, is the occurrence of important spacings of 4.5 and 10 Å, corresponding to the lateral spacings of packed polypeptide chains.[251] These spacings are prominent alike in the photographs of native, denatured, and fibrous proteins. There is a tendency for the peaks in the Patterson diagrams of insulin, methemoglobin and lacto-

[246] Admirable reviews have been given by D. Crowfoot, Chem. Rev., **28**: 215, 1941; I. Fankuchen, Ann. New York Acad. Sc., **41**: 157, 1941.

[247] I. Fankuchen, J. Gen. Physiol., **24**: 315, 1941.

[248] D. Crowfoot, Chem. Rev., **28**: 215, 1941.

[249] J. D. Bernal and I. Fankuchen, J. Gen. Physiol., **25**: 111, 1941.

[250] J. R. Katz, Ztschr. f. physiol. Chem., **95**: 1, 1915.

[251] E.g., W. T. Astbury, and H. J. Woods, Phil. Tr. Roy. Soc., **232A**: 333, 1933.

globulin to fall on a hexagonal network, and their co-ordinated movement during drying does serve to some extent to identify those which are of intramolecular origin. Attempts have been made[252,253] to interpret the insulin pattern. Wrinch and Langmuir use a cyclol polyhedron[254] as the basis of their interpretation, to which they add the more or less gratuitous assumption that certain slit-shaped spaces, corresponding to edges of the polyhedra, are regions of high electron density caused by the presence, possibly, of particular side-chains. Bernal relates the hexagonal pattern on the basal projection to a body-centered cubic array of scattering centers and concludes that these correspond to submolecules, probably 24 in number. The cyclol interpretation has been freely criticized.[255] Probably both are premature; to the lay reader, J. M. Robertson's remarks carry considerable conviction: "It is quite obvious that fifty-nine relative measurements of amplitude cannot define a structure consisting of several thousand atoms. So far as the measurements go, the structure is effectively a continuous distribution of scattering matter, and every arbitrary assignment of phase constants to the amplitudes will yield a solution. . . . The suggestion that certain concentrations of atoms in the molecule can be treated as point scattering sources does not seem to constitute even a reasonable approximation."

The intramolecular pattern in tobacco mosaic virus can be discussed in this context, although the interparticle arrangement is really more like that of a fiber, with a very perfect hexagonal close packing of rod-shaped particles[255] about 1500 Å long,[257] arranged at random in the axial direction. The small spacings, unlike the large, are independent of the water content of the gel, and their extreme sharpness shows clearly that the particles themselves are crystalline. As in the true protein crystals, the spacings at 11 and 4.5 Å are dominant, with the difference that the 11 Å reflection perpendicular to the particle length exceeds all others in importance. The other spacings are, however, anomalous, in the sense that in order to obtain rational indices it is necessary to assume a unit cell broader than the particles themselves. Considering the small number of repeating units present in the cross-section of each particle (diameter 150 Å), however, it is not to be expected that Bragg's law will hold, and Bernal and Fankuchen have discussed their diagrams in terms of a structure of sub-units of about 11 Å cube, fitted in a hexagonal or pseudohexagonal lattice of dimensions $a = 87$ Å,

[252] D. M. Wrinch and I. Langmuir, J. Am. Chem. Soc., **60**: 2247, 1938; I. Langmuir and D. M. Wrinch, Proc. Phys. Soc., **51**: 613, 1939.

[253] J. D. Bernal, Proc. Roy. Soc., **170A**: 75, 1939.

[254] For reference to papers describing the cyclol fabric and the polyhedra that can be derived from it by folding, see ref. (184).

[255] W. L. Bragg, Nature, **143**: 73, 1939; J. D. Bernal, *ibid.*, **143**: 74, 1939; J. M. Robertson, *ibid.*, **143**: 648, 1939; D. M. Wrinch, *ibid.*, **143**: 768, 1939; J. D. Bernal, I. Fankuchen and D. Riley, *ibid.*, **143**: 897, 1939.

[256] J. D. Bernal and I. Fankuchen, J. Gen. Physiol., **25**: 111, 1941.

[257] G. A. Kausche, E. Pfankuch, and H. Ruska, Naturwissenschaften, **27**: 292, 1939.

$c = 68$ Å. A deeper analysis is scarcely possible without an extension of the theory of X-ray diffraction by thin layers, and for the present the observed reflections have to be expressed in terms of fractional indices which lack quantitative interpretation.

4. Paracrystals, Tactoids, and Coacervates.—One cannot consider the transition from order to disorder without encountering the paradox of symmetry, by which I mean the high degree of symmetry exhibited alike by the most regular and the most irregular arrangements of particles—the crystal and the amorphous body. The spherical symmetry of the latter is statistical, in the sense that no symmetry operation will lead to complete coincidence of all analogous particles, while in a true space lattice the existence of symmetry elements is axiomatic. Now the restriction of symmetry operations to one or two dimensions leads to the definition of various types of partial crystals, but does not by any means exhaust the degrees of ordered arrangement, for order does not necessarily imply periodicity. With a proper definition of statistical symmetry elements, it is possible to arrive at a complete enumeration of the transition types of ordered arrangement analogous to the enumeration of the nonstatistical space-groups of crystallography;[258] these turn out to be 18 in number, of which two—or perhaps 3—are commonly acknowledged to occur in nature. Others will undoubtedly be observed, and should be recognizable from the nature of their X-ray diffraction diagrams.

The existence of types of ordered arrangement other than the strictly crystalline was first recognized by the observation of anisodiametric polar molecules such as *p*-azoxyanisol and potassium oleate, which gave rise to a doubly refracting "mesophase" during melting and again on cooling of the isotropic liquid.[259] This combination of anisotropy, a sure sign of order, with plastic fluid qualities, led to the introduction of terminologies that have since proved misleading; Lehmann used the terms "fliessende Kristalle" and "fluessige Kristalle," the former embracing also the plastic deformability of true crystals, while Friedel referred to the "mesomorphous" state. As Rinne has pointed out,[260] substances exhibiting this characteristic autonomous anisotropy may range in mechanical properties and in morphology from the limpid fluid to the glassy or fibrous solid, the criteria implied in the earlier terminology being thus irrelevant. Ostwald[261] lists a number of experimental criteria which, although apparently selected under the influence of the early definitions, are broad enough to cover the wide range of properties: liquid or plastic character; anisotropy of viscosity and surface tension; absence, or lability, of angular boundaries; spontaneous birefringence referable to "internal" forces of the same order of magnitude

[258] C. Hermann, Ztschr. f. Krist., **79**: 186, 1931.

[259] O. Lehmann, Fluessige Kristalle: Engelmann, Leipzig, 1904; G. Friedel, Ann. Physique (9), **18**: 273, 1922.

[260] F. Rinne, Kolloid-Ztschr., **50**: 288, 1932; Tr. Faraday Soc., **29**: 1016, 1933.

[261] W. Ostwald, Ztschr. f. Krist., **79**: 222, 1931.

as casual external influences. If we insist upon the structural criterion—manifested in spontaneous anisotropy—as the unique one, it is well to choose a term which emphasizes both the resemblance to the crystalline state and the characteristic differences. Rinne's term "paracrystalline" will be used here in this sense.

The subdivision of paracrystals into two distinct types is based upon the observation of characteristic, although extraordinarily varied, patterns under the polarizing microscope. Friedel and Grandjean[262] distinguished between thread-like and conical forms (liquides à fils, liquides à coniques) which were later[263] referred to as "nematic" and "smectic," terms which, notwithstanding reasonable criticism,[264] have been widely adopted. The properties of the nematic state can be accounted for in terms of an array of anisodiametric molecules with a common axial direction and a random distribution of centers of gravity; this type of arrangement shows only diffuse X-ray reflection, corresponding to the absence of true periodicity.[265] It may be observed, for example, in liquid *p*-azoxyphenetol,[266] and in anisalbenzolazonaphthylamine and other resinous solids;[267] as a common characteristic of fiber structure it will be referred to later. The X-ray diagrams of substances in the smectic state, on the other hand, show the existence of reflecting planes like the parallel reticular planes of a crystal, corresponding to a periodic disposition of centers of gravity. The arrangement may be sufficiently regular to give a rotating crystal diagram, as with a smectic drop of ethyl-*p*-azoxybenzoate on a cleavage surface of mica,[268] or it may correspond to a random disposition of planes, as with ammonium and other oleates, which give "powder" diagrams with a characteristic spacing of 43.5 Å,[269] approximately equal to the thickness of the bimolecular layers occurring in the true crystals and in soap films.[270] Thallium stearate and oleate show repeating units of 36 and 32 Å respectively, compared with 42 and 45 Å for the crystals;[271] this may indicate, rather improbably, that the chains are tilted to the reflecting plane, or it may result from a liquid randomness of orientation of the hydrocarbon chains.[272]

Since the number of degrees of freedom in paracrystals is greater than in crystals, isomorphism is far more frequently encountered; one of their most

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[262] G. Friedel and F. Grandjean, Bull. Soc. Min., **33**: 192, 1910.

[263] G. Friedel, Ann. Physique (9), **18**: 273, 1922.

[264] J. W. McBain, Nature, **113**: 534; **114**: 49, 1924.

[265] E. Hueckel, Physik. Ztschr., **22**: 561, 1921; J. R. Katz, Naturwissenschaften, **16**: 758, 1928; cf. S. Van der Lingen, Verhandl. der deutsch. phys Ges., **15**: 913, 1913.

[266] G. Friedel, Ann. Physique (9), **18**: 273, 1922.

[267] D. Vorlaender, Ztschr. angew. Chem. **43**: 13, 1930; Tr. Faraday Soc., **29**: 907, 1933, and several other papers.

[268] E. Friedel, Compt. rend. Acad. d. sc., **180**: 269, 1925.

[269] M. de Broglie and E. Friedel, Compt. rend. Acad. d. sc., **176**: 738, 1923.

[270] Cf. P. V. Wells, Ann. Physique (9), **16**: 69, 1921.

[271] K. Herrmann, Tr. Faraday Soc., **29**: 972, 1933.

[272] Cf. discussion by J. D. Bernal and others, Tr. Faraday Soc., **29**: 1073 ff, 1933.

remarkable properties, indeed, and one of the most important in relation to their occurrence in living systems, is this ability to form complex paracrystalline mixtures.[273] Foreign substances of all kinds—glycerol, olive oil, paraffin oil, water—appear to facilitate dispersion and to increase stability.[274] The profound influence of water is of especial interest to us. The first case to receive much attention was that of 10-bromphenanthrene-3-(or 6-) sulfonic acid, which formed a nematic colloidal phase with relatively large amounts of water, and a more concentrated smectic phase;[275] the latter gave a single spacing 3.47 Å compared to 3.40 for the anhydrous crystals,[276] showing that water must have penetrated between the molecules. Later Rinne[277] drew attention to the extraordinary spherulitic and myelin forms obtained with aqueous emulsions of the phosphatides and cerebrosides, which vary in a most striking manner with increasing water content and are in many respects similar to the myelin forms originally observed by Virchow[278] with nerve substance and other organ extracts. The X-ray diagrams of the emulsions show very clearly the changes in fine structure accompanying dilution. The various substances taken singly do not always form very stable emulsions with water; cholesterol not at all, sphingomyelin rather more readily, and cephalin very easily. The characteristic bimolecular spacings are increased on uptake of water. Mixed emulsions, in which cholesterol can also be stably incorporated, show only a single important spacing, which increases continuously from 63.5 Å for the slightly moist mixture to 150 Å for an emulsion containing 75% of water.[279]

Aqueous suspensions of this type, containing rod-shaped or leaf-shaped particles separated by water layers up to as much as 5000 Å thick, and yet preserving their paracrystalline or even crystalline configurations, are of very general occurrence. They are commonly found in the form of coacervates, phases of high concentration which arise by the spontaneous separation of a suspension into a dilute and a concentrated portion.[280] Spontaneous anisotropy is not invariably found in the concentrated layer; in suspensions of clay, of iron oxide, and of vanadium pentoxide, the anisotropy appears only under certain conditions of concentration or ageing, or when the phase is disturbed by shearing stress. The concentrated phase in suspensions of the clay, bentonite, tends to form an isotropic gel in which,

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[273] E. Friedel, Ann. Physique (9), **16**: 273, 1922; F. Rinne, Kolloid-Ztschr., **50**: 288, 1932, etc.

[274] W. Ostwald, Ztschr. f. Krist., **79**: 222, 1931.

[275] H. Sandqvist, Kolloid-Ztschr., **19**: 113, 1916.

[276] F. Rinne, Ztschr. f. Krist., **82**: 379, 1932.

[277] F. Rinne, Kolloid-Ztschr., **60**: 288, 1932.

[278] R. Virchow, Virchows Arch. f. path. Anat., **6**: 562, 1854.

[279] R. S. Bear, K. J. Palmer, and F. O. Schmitt, J. Cell. & Comp. Physiol., **17**: 355, 1941; F. O. Schmitt and K. J. Palmer, Cold Spring Harbor Symp., **8**: 94, 1940; K. J. Palmer and F. O. Schmitt, J. Cell. & Comp. Physiol., **17**: 385, 1941.

[280] G. Quincke, Ann. Physique (4), **9**: 799, 1902, and numerous later papers by H. Zocher, H. Freundlich, H. G. Bungenberg de Jong, and others.

according to Langmuir,[281] the disc-shaped particles are arranged in an isotropic face-centered cubic lattice. "The axes of the discs are not arranged in random directions, but oscillate about definite equilibrium axes which are related to the crystal axes." In the anisotropic gels which occur at certain concentrations, a mutual orienting effect of neighboring discs causes them to become parallel, with formation of a new kind of uniaxial crystal grain of higher density.

One of the best examples of a truly paracrystalline coacervate is that of tobacco mosaic virus, the elongated particles of which show a strong tendency to form positively birefringent aggregates. Solutions of the virus separate into two phases by formation of tactoids, spindle-shaped regions of low or high density which rise or fall in the solution and coalesce respectively with the upper or lower phase.[282] The X-ray patterns of the lower phase show sharp lines corresponding to reflection from a two-dimensional hexagonal lattice with interparticle distance varying with the water content of the phase from about 100 Å to 450 Å.[283] Absence of reflections corresponding to the length of the particles shows that the particles overlap in an irregular manner, as they do in a nematic paracrystal.

The factors involved in the stability of the concentrated phase and its equilibrium with the dilute phase have been discussed frequently. The phenomenon was at first attributed to long-range Van der Waals forces of attraction,[284] but more recent papers,[285] of which by far the most intelligible is that of Langmuir, have shown that the situation can be analyzed satisfactorily without recourse to any such unsupported hypothesis. The dilute and concentrated phases are obviously in osmotic equilibrium; this would not be possible in any system of uncharged and unassociated particles, for even if the particles themselves were unable to diffuse rapidly, movement of water into the concentrated layer would soon result in uniform dispersion of particles throughout the liquid. Now the profound influence of electrolytes upon the properties of these systems shows, together with a considerable body of other evidence, that we are dealing with charged

[281] I. Langmuir, J. Chem. Phys., **6**: 873, 1938.

[282] F. C. Bawden, N. W. Pirie, J. D. Bernal, and I. Fankuchen, Nature, **138**: 1051, 1936; J. D. Bernal and I. Fankuchen, *ibid.*, **139**: 923, 1937; F. C. Bawden and N. W. Pirie, Proc. Roy. Soc. **123B**: 274, 1937; Nature, **141**: 513, 1938; M. A. Lauffer and W. M. Stanley, J. Biol. Chem., **123**: 507, 1938.

[283] J. D. Bernal and I. Fankuchen, J. Gen. Physiol., **25**: 111, 1941.

[284] H. Kallmann and M. Willstaetter, Naturwissenschaften, **20**: 952, 1932; H. C. Hamaker, Rec. trav., **55**: 1015, 1936; *ibid.* **56**: 3, 727, 1937; *ibid.* **57**: 61, 1938; Tr. Faraday Soc., **36**: 186, 1940.

[285] a. I. Langmuir, J. Chem. Phys., **6**: 873, 1938; b. S. Levine and G. P. Dube, Tr. Faraday Soc., **35**: 1125, 1939; c. S. Levine, Proc. Roy. Soc., **170A**: 145, 164, 1939; d. S. Levine, and G. P. Dube, **36**: 215, 1940; e. S. Levine, *ibid.*, 725, 1940; f. G. P. Dube and S. Levine, *ibid.*, **35**: 1141, 1939; g. B. Derjaguin, *ibid.*, **35**: 203, 1940; h. cf. J. D. Bernal and I. Fankuchen, J. Gen. Physiol., **25**: 111, 1941; k. H. C. Hamaker, Tr. Faraday Soc. **35**: 186, 1940; for short qualitative statement on Levine's papers, see H. Mueller, Ann. New York Acad. Sc., **39**: 111, 1939.

particles with which must be associated an ion atmosphere of opposite sign. Accordingly there are electrostatic forces tending to counteract the dispersing effect of Brownian movement and cause the system to separate into a dense solid phase in equilibrium with its saturated solution, the concentration of which is that at which osmotic and electrostatic forces balance each other. Thus, to account for the fact that the particles actually remain separated by thick atmospheres of water, we require an additional repulsive force instead of the attractive force postulated by Freundlich and others. This repulsive force is identified, in the various theories referred to above, with the electrostatic repulsion between suspended particles with overlapping ion atmospheres; the case resembles the electrostatic problem presented by the interaction of atoms, with the important difference that the size and shape of the micelles have to be taken into account. Theoretical expressions for infinite plane,[285a] spherical[285c] and cylindrical[285b] particles appear to account for the stability of the paracrystalline solutions and for the characteristic swelling of the lattice by continuous penetration of water into the lattice spaces. The phenomenon has its counterpart in the swelling of gels,[285b] in the shrinkage of the unit cell of protein crystals on dehydration,[285a] and doubtless also in changes in the degree of dispersion of chromatin during cell division.

The biological importance of paracrystals in general can hardly be overestimated. It is almost superfluous to mention how much of our knowledge of the fine structure of cells depends upon the ubiquity of this state of matter in protoplasm and upon the very convenient and significant fact that the birefringence of oriented protein happens to be positive, and that of the lipoids and nucleic acid negative.[285] And Rinne has pointed out how singularly well fitted the paracrystalline state is to provide complex forms in which organization and lability can be combined to an unique degree.

5. The Structure of Fibers.—*a*). *General features of crystallization of linear polymers.*—The typical fiber, according to present knowledge, is a linear polymeric system which owes its most characteristic properties to those same peculiarities of elongated molecules which we have seen to be responsible for their behavior in solution: that is, to their length, flexibility, variable size, and the kinetic independence of segments within a single molecule. It follows that in such systems, perfect crystalline regularity is an extreme rarity; invariably there are structural defects and sometimes regions of disorder which impose their amorphous character upon the tangible qualities of the substance. There are several sources of irregularity. Defects arise from simultaneous crystallization at several nuclei within a single covalent chain, and are promoted by the thermal motions

[285] For a discussion of the biological importance of paracrystals and of the value of the polarization microscope in the elucidation of fine structure, the reader cannot do better than to consult the two books by W. J. Schmidt: 1. Die Bausteine des Tierkörpers in polarisiertem Lichte: Cohen, Bonn, 1924; 2. Die Doppelbrechung von Karyoplasma, Zytoplasma, und Metaplasma: Borntraeger, Berlin, 1937.

of the chains, since simultaneous crystallization at several positions may "freeze" a chain in some irregular configuration and result in a polycrystalline material with a nearly random distribution of crystal axes. In extreme cases, as in rubber, thermal motion may result in nearly complete disorder. Added to these sources of defect are the effects of dispersity of molecular weight, or irregularities in the intramolecular pattern (resulting perhaps from copolymerization or other chemical complication), and the presence of amorphous inclusions or foreign bodies. In general, however, despite these numerous influences, there is sufficient interaction between chain segments, and enough periodicity over short lengths of chain, to give a structure with marked orientation of crystal axes and a marked tendency for the chains to assume more or less extended forms. In the crystalline regions, the preferred form of the chains is conditioned by such factors as the nature and size of substituent groups and the presence of ring structures or double bonds.

The mechanical behavior of a fiber is determined largely by the opposing tendencies of crystallization and the random coiling of the chain molecules. The contrast between the brittleness of sucrose and glycine and the flexibility of cellulose and hair[287] illustrates well enough the difference between a perfectly crystalline material and its equally condensed but highly disordered counterpart. The physical basis of other properties of condensed linear polymers will become evident in the ensuing paragraphs.

b). *Peculiarities of X-ray diagrams attendant upon the crystallographic imperfection of fibers, and the resulting difficulties of structure analysis.*—When a polycrystalline specimen is without any orientation of crystal axes, it yields a powder diagram when examined with X-rays. More commonly, examination results in a fiber pattern: one which resembles on the one hand a rotation diagram for a single crystal (although obtained without moving the specimen), and, on the other hand, a powder diagram in which certain arcs of the Debye-Scherrer rings are enhanced. Fiber diagrams result from an approximately parallel alignment of one crystallographic axis of the crystallites, while the remaining axes are disposed at random. Certain peculiarities of the X-ray diagrams of such specimens arise from their polycrystalline nature:

(*i*). Reflections often fall on the meridian (the line through the origin at right angles to the equator); they arise therefore from basal planes tilted with respect to the fiber axis. Such "diatropic" reflections, improbable from an accurately mounted single crystal, result from the imperfect axial alignment of the crystallites, and are often valuable in determining the identity period along a fiber axis. The imperfect alignment may be a random tilt or it may result from special circumstances, such as spiral arrangement of crystallites, or both.[288]

(*ii*). The spots on a fiber diagram usually lack the sharp definition of a single crystal diagram; their more or less diffuse character is a consequence of imperfect resolution by a lattice of limited extent—a phenomenon with a well-known

[287] Cf. E. Sauter, Ztschr. f. physik. Chem., **35B**: 117, 1937.

[288] Cf. T. Kubo, Naturwissenschaften, **27**: 278, 1939.

optical counterpart in line gratings with a small number of rulings.[289] The effect can be put to good use, if the intensity distribution over each spot can be measured, in determining the size of the crystallites; there are, however, alternative explanations, such as the effect of internal stresses on lattice constants, and the somewhat irregular large spacings are perhaps more reliably measured by the observation of diffuse reflections at angles of deviation of about 1° from the origin.

(*iii*). A relatively sharp definition of the layer lines, corresponding to regular axial periodicity, may be accompanied by spreading of the reflections along the layer lines. This indicates a certain scatter of lattice plane separations in the axial zone, and in studies of keratin it has been taken as evidence for the penetration of water into the crystallites, introducing a lattice distortion.[290]

c). *Methods of improving orientation in fibers.*—It is clear that a detailed knowledge of fiber structure may depend upon the possibility of obtaining specimens with an adequate extent of crystallized regions, preferentially oriented, if this be possible, with all three axes parallel. Occasionally, natural sources of highly oriented material are available; the cellulose in the cell wall of *Valonia* or *Halicystis*, for example,[291] and the fiber of natural silk. Such examples are not very common and even natural silk can be made much stronger by causing the silk worm to produce it under tension.[292] Stretching is often effective in producing oriented specimens; fibers[293] or sheets[294] of myosin or of cellulose[295] are more highly oriented if dried under tension, while rubber[296] and certain related synthetic products such as the polyvinyl derivatives[297] and polyisobutylene[298] actually remain oriented only as long as the tension is maintained. The orientation which accompanies viscous or plastic flow of solutions or melts of linear molecules is also made use of in the formation of oriented sheets or fibers by extrusion through a die, the paracrystalline groupings formed during flow tending to persist in the transition to the solid state. Other mechanical devices are known and are of considerable industrial importance, such as the calendering or rolling of a plasticized material, which involves both stretching and lateral compression. In some cases, lateral compression even induces parallelism of the crystallites with respect to a second crystallographic axis in addition to the fiber axis,[299] an occurrence which seems to imply a relation between their morphological and axial dimensions. This

[289] Cf. W. L. Bragg, The Crystalline State: Bell, London, 1933, p. 188.

[290] W. T. Astbury and H. J. Woods, Phil. Tr. Roy. Soc., **232A**: 333, 1933.

[291] D. L. Sponsler, Nature, **125**: 633, 1930; cf. R. D. Preston, Biol. Rev., **14**: 281, 1939.

[292] H. Mark, 1934, cited by R. Houwink, Elasticity, Plasticity and Structure of Matter, University Press, Cambridge, 1937.

[293] G. Boehm and H. H. Weber, Kolloid-Ztschr., **61**: 269, 1932.

[294] W. T. Astbury and S. Dickinson, Proc. Roy. Soc., **129B**: 307, 1940.

[295] E. Sauter, Ztschr. f. physik. Chem., **35B**: 83, 1937.

[296] J. R. Katz, Naturwissenschaften, **13**: 410, 1925; cf. L. Hock, Kolloid-Ztschr., **35**: 40, 1924.

[297] L. Misch and L. E. R. Picken, Ztschr. f. physik. Chem., **36B**: 398, 1937; F. Halle and W. Hofmann, Naturwissenschaften, **23**: 770, 1935.

[298] R. Brill, and F. Halle, Naturwissenschaften, **25**: 12, 1938; C. S. Fuller, C. J. Frosch, and N. R. Pape, J. Am. Chem. Soc., **62**: 1905, 1940.

[299] Cf. O. Kratky and H. Mark, Ztschr. f. physik. Chem., **36B**: 129, 1937.

has been of value in identifying certain spacings in moist keratin[300] and has made possible the production of rotation diagrams of cellulose.[301]

In the case of fusible polymers, crystallization may be promoted by solidification near the melting-point or by annealing after the melt has been quenched. The rate of crystallization varies greatly with the nature of the chains and their degree of interaction,[302] depending in all probability upon rotational freedom;[303] the process is essentially the same, in reverse, as that which produces increased symmetry in crystalline paraffins on heating, and is accompanied by disappearance of diatropic reflections and resolution of certain layer line reflections,[304] while the latter is revealed by their coalescence.[305] The same result is often obtained at room temperature by exposing the substance to the vapor of a suitable solvent, which penetrates between the chains, ruptures hydrogen bonds, and thus promotes free rotation.[306] Undoubtedly the same mechanism plays a part in the formation of an extremely highly oriented native cellulose from cellulose hydrate by heating in glycerol at 250°C,[307] a procedure that has made possible the production of very sharp and detailed X-ray diagrams.[307]

d). *The crystal structure of certain synthetic polymers.*—After all such procedures have been tried, a fiber photograph of natural material is usually still far from perfect. Added to this serious source of difficulty in carrying out an X-ray analysis of the crystallized component is the uncertainty which often surrounds the chemical constitution of naturally occurring substances —a difficulty which, in the past, has perhaps caused true progress to be simulated by mere facile juggling with analogy and numerical coincidence. The study of synthetic materials of known constitution, with its almost infinite scope for the controlled change of parameters, would seem to offer a more solid basis for an understanding of the architecture of natural polymers. Such investigations, impelled by the exigencies of industrial development and issuing largely from commercial laboratories, are in their infancy, still greatly outnumbered by those dealing with rubber and the textile fibers. Although some important principles of fiber structure have been deduced from this more numerous group of early studies, which include the celebrated researches of Meyer and Mark and of Astbury, we shall begin more logically by referring to certain simple synthetic polymers.

It must be remembered that the advantage of a definite chemical constitution is by no means easily achieved in practice; perhaps the lack of any systematic work on structures is due largely to the difficulty of insuring uniformity of molecular size and constitution. We shall refer here only to

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[300] W. T. Astbury and W. A. Sisson, Proc. Roy. Soc., **150A**: 533, 1935.

[301] E. Sauter, Ztschr. f. physik. Chem., **37B**: 161, 1937.

[302] W. O. Baker, C. S. Fuller, and N. R. Pape, J. Am. Chem. Soc., **64**: 776, 1942.

[303] Cf. M. Mathieu, Tr. Faraday Soc., **29**: 122, 1933; C. S. Fuller, W. O. Baker, and N. R. Pape, J. Am. Chen. Soc., **62**: 3275, 1940.

[304] C. S. Fuller, W. O. Baker, and N. R. Pape, J. Am. Chem. Soc., **62**: 3275, 1940.

[305] A. Mueller, Proc. Roy. Soc., **127A**: 417, 1930.

[306] W. O. Baker, C. S. Fuller, and N. R. Pape, J. Am. Chem. Soc., **64**: 776, 1942.

[307] T. Kubo, Naturwissenschaften, **27**; 278, 1939.

polymers formed from reactants and by processes which seem to preclude the formation of branched chains. We shall, moreover, take for granted the coexistence in these preparations of crystalline and disordered states, ignoring those changes in the X-ray pattern, such as improved definition, resolution of fused lines, and so on, which merely provide an index to the degree of orientation, and shall consider only the structure of the crystalline portion.

The most definite information is often obtained from the identity period in the direction of the fiber axis. In a short chain paraffin crystal with n carbon atoms the identity period is determined by the presence of basal planes separated by the length of the extended zig-zag carbon chains, if these are vertical to the basal plane, or by the vertical components of the chain length, if they are tilted, while a secondary period, indicated by an enhanced intensity of the $n/2$ order basal reflection, corresponds to the length of two carbon-carbon linkages resolved in the direction normal to the basal plane. In a high polymer of the polymethylene type, on the other hand, the long spacing is absent because the chains overlap; the only identity period observed is that corresponding to the doubled bond-length. This behavior is readily modified if the uniformity and inertness of the methylene chain is disturbed by almost any means, such as the introduction of a double bond, of a polar group, or a bulky substituent. The simplest case is found when polar groups are introduced with strict periodicity; these provide centers of crystallization or lateral cohesion along the chain-lengths and generate characteristic reflecting planes which, assuming the function of the basal planes in crystals of short chain aliphatic compounds, again give a long identity period. Polyvinyl alcohol and various linear polyamides and polyesters provide interesting examples of such intramolecular identity periods. The chief effect of inert substituents, of double bonds, and of chains linked up by oxygen atoms, seems to be a distortion of the chain from the plane zig-zig form into some kind of helix or spiral, which gives a long identity period corresponding to the pitch of the screw and a decreased interatomic sub-period.

The reasons for this are probably various. In polychloroprene, $(\cdot CH_2 \cdot CH{:}CCl \cdot CH_2 \cdot)_n$, the identity period is 4.8 Å;[308] in rubber, or polysioprene, $(\cdot CH_2 \cdot CH{:}C(CH_3) \cdot CH_2 \cdot)_n$, it is 8.2 Å,[309] rather less than twice the polychloroprene value, corresponding to the distance between alternate methyl groups, with a contraction of the fully extended chain by about 0.35 Å per pair of chain atoms. In polyisobutylene, $(\cdot CH_2 \cdot C(CH_3)_2 \cdot)_n$, the identity period is 18.6 Å,[310,311] with a very strong eighth layer line and a sub-period 2.33 Å;[311] thus

[308] Cited by R. Brill and F. Halle,[310] with an incorrect reference to W. H. Carothers; cf. W. H. Carothers, Ind. Eng. Chem., **26**: 30, 1934.

[309] Cf. K. H. Meyer and H. Mark, Der Aufbau der hochpolymeren organischen Naturstoffe: Akad. Verlagsges., Leipzig, 1930, p. 193.

[310] R. Brill and F. Halle, Naturwissenschaften, **26**: 12, 1938.

[311] C. S. Fuller, C. J. Frosch, and N. R. Pape, J. Am. Chem. Soc., **62**: 1905, 1940.

there are eight isobutylene units in the fiber period, and it has been suggested[311] that successive methyl group pairs are rotated through 45°, completing the cycle within the fiber period. This is presumably a chain distortion arising from steric effects and is reminiscent of the twisting of the C—C bond in ethane. The presence of glycol or ether linkages in the chain likewise causes a helical form to be assumed: the effect is marked in polyoxymethylene, $(\cdot CH_2 \cdot O \cdot)_n$[312] and polyethylene oxide, $(\cdot CH_2 \cdot CH_2 \cdot O \cdot)_n$,[313] and is even to be discerned in the polyethylene esters of dibasic acids, particularly in the succinate.[314]

Returning to the question of extended chains interlinked by polar groups, we find several points interestingly illustrated in recent papers. Polyvinyl alcohol has received a comparatively complete structure analysis,[315] suggesting the presence of double chains associated through hydrogen bonds (Fig. 19) and linked to neighboring pairs only by residual forces. In the linear poly-

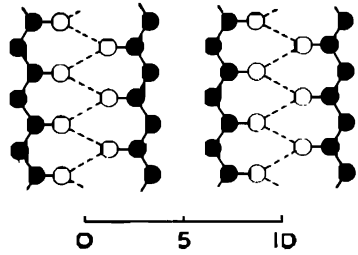

FIG. 19.—Crystal structure of polyvinyl alcohol, showing the part played by hydrogen bonds (dotted lines).

Open circles: oxygen.
Closed circles: carbon.
(Based upon the projections given by R. C. L. Mooney, J. Am. Chem. Soc., **53**: 2828, 1941.)

ethylene esters of carbon chain type 2: $(m + 2)$ (i.e., $\cdot O \cdot (CH_2)_2 \cdot O \cdot CO \cdot (CH_2)_m \cdot CO \cdot)_n$)[316] and polyamides of type p: $(m + 2)$ (i.e., $\cdot NH \cdot (CH_2)_p \cdot NH \cdot CO \cdot (CH_2)_m \cdot CO \cdot)_n$),[317] the same principle operates, but the actual mode of alignment of polar groups is somewhat more complex. In both cases, the fiber period corresponds to the length of the repeating chemical unit if this is assumed to have the form of a slightly contracted or a slightly tilted chain. The polyesters also show a strong reflection of spacing 2.13 Å, attributed to an oblique plane containing two carbon atoms (Fig. 20). In the "even" esters, represented by the suberate ($m = 6$), this reflection is the 6th order of a large pinacoidal[318]

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[312] E. Sauter, Ztschr. f. physik. Chem., **21B**: 161, 1933.

[313] E. Sauter, *ibid.*, **21B**: 186, 1933.

[314] C. S. Fuller and C. J. Frosch, J. Phys. Chem., **43**: 323, 1939.

[315] R. C. L. Mooney, J. Am. Chem. Soc., **63**: 2828, 1941; cf. F. Halle and W. Hofmann, Naturwissenschaften, **23**: 770, 1935.

[316] Ref. (314).

[317] W. O. Baker and C. S. Fuller, J. Am. Chem. Soc., **64**: 2399, 1942.

[318] "Pyramidal" according to Fuller,[314] Pinacoid: denoting "forms consisting of only two parallel faces. . . . Each form would, if indefinitely extended, present the appearance of a board or plank."—W. J. Lewis, A Treatise on Crystallography: University Press, Cambridge, 1897, p. 154.

spacing formed apparently by a longitudinal displacement of carbonyl groups in neighboring chains (Fig. 20), while there are also strong twelfth order layer line reflections, showing that the carbon atoms are disposed in horizontal planes. The azelate ($m = 7$) gives no pinacoidal reflections; apparently here the repeating units are exactly aligned, giving horizontal reflecting planes and an orthorhombic unit cell closely analogous to that of the paraffins (Fig. 20). The situation is rather less definite in the polyamides, but it appears that here

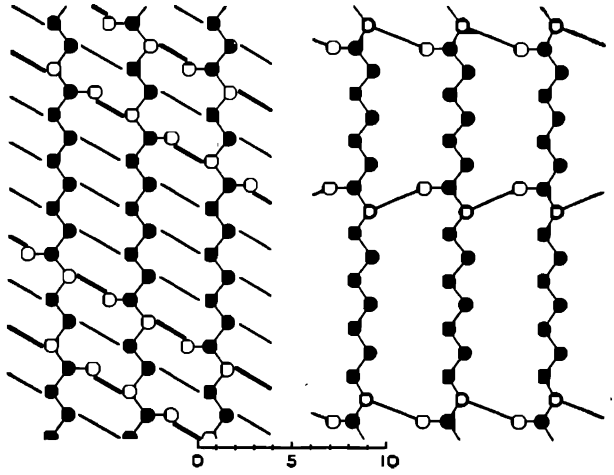

FIG. 20.—Projections illustrating long spacings in synthetic polymers containing polar groups (schematic). Based on the results of Baker, Frosch, Fuller, and Pape. References in text.

Black circles: carbon.
Heavy open circles: nitrogen.
Light open circles: oxygen.

Left: "Even" polyethylene esters, illustrated by polyethylene suberate. Pinacoidal planes with a maximum density of polar groups are indicated by the heavy lines These give rise to long spacings, which are subdivided by the planes of spacing 2.13 Å involving two carbon atoms in each chain. Fiber subperiods of 1.27 Å, the distance between carbon atoms in the axial direction, are also present. The details have not been worked out: in particular, the oxygen-oxygen distance between chains is improbably small-in the arrangement shown.

Right: Showing that the formation of such pinacoidal planes is improbable in "odd" polymers for geometrical reasons. The example given is based upon the properties of the 9-9 copolyamide, but with each methylene chain shortened by two CH_2 groups for convenience in drawing. It is clear that the maximum amount of polar association is obtained when the polar regions are planes perpendicular to the fiber axis.

the interchain association of imido and carbonyl groups may lead again to the formation of inclined planes when p and m are even, while the corresponding planes are horizontal when p and m are odd (Fig. 20).

The polyamide diagrams show a certain amount of disorder resulting probably from chain inversion. A similar effect can be produced when the periodicity of the dipoles is upset by copolymerization; a polymer formed, for example, from an equimolar mixture of the diamines $p = 6$ and 10 and the acids $m = 4$ and 8 will contain segments of the 6:6, 6:10, and 10:10 units distributed at random along the chain. The fiber periods of these copolymers are strikingly

different from those of the several simple polymers and have so far defied interpretation. It is, however, noteworthy that the coincidences of polar groups are sufficiently numerous for crystallization to occur. In other instances, as for example in polyvinyl acetate,[319] the disorder resulting from the irregular sequence in orientation of acetyl groups and the presence of unesterified hydroxyls may destroy any semblance of crystallinity beyond the mere paracrystalline alignment of chains.

e). *The mechanical properties of linear polymers.*—The extent and stability of the crystallized regions in linear polymers are major factors in determining their mechanical properties. The chains possess in varying degree an inherent tendency to assume randomly curled configurations; if unopposed by a considerable density of interacting groups, by the presence of ring systems which lend a certain rigidity to the chains, or by mechanical forces applied externally, this tendency may cause the substance to have the disordered character of a liquid. Upon this property is imposed mechanical toughness arising from the entanglement and overlapping of long curled chains, and a rubber-like extensibility attributable to the ease with which they can be stretched into an extended configuration. The stretching may be accompanied by crystallization, with an appropriate increase in density and evolution of heat, the chains then assuming a final form—fully extended or helical, as the case may be—that is not distorted by any further increase in the applied stress. Although the conditions for rubber-like behavior are not completely understood, there is little doubt that flexible, inert chains with an open structure[320,321] are a primary requisite; the simple polymethylenes do not satisfy this[322] because of the close packing of their chains, which are subject to dispersion forces over a wide area, while polyisoprene, polychloroprene and polyisobutylene are typically rubber-like.

When stretched and crystalline, these substances are more rigid than in the relaxed condition;[323] this is so when the crystallinity is maintained either by continued application of stress or by cooling to a temperature at which the lattice forces exceed the thermal forces of disorientation. The same is true of polymers which, by virtue of their polar character, do not spontaneously revert to a disordered state at ordinary temperatures.[324] It is widely agreed that fibers of these substances owe their flexibility partly to the pliable fibrillar portions, those regions of loose or defective structure that interlink the crystallites, and partly to the loosening of lateral forces within the lattice by the presence of bulky substituents or small molecular pene-

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[319] Cf. L. Misch and L. E. R. Picken, Ztschr. f. physik. Chem., **35B**: 398, 1937.

[320] Cf. H. Mark, J. Phys. Chem., **44**: 764, 1940.

[321] Cf. R. Houwink, p. 65 of ref. (292).

[322] K. H. Meyer and H. Mark, p. 201 of ref. (309).

[323] Meyer and Mark p. 202 of ref. (309).

[324] Cf. W. O. Baker and C. S. Fuller, J. Am. Chem. Soc., **54**: 2399, 1942.

trants which diminish the effects of hydrogen bonding.[325] Such plasticized fibers have something of the toughness and flexibility of rubber without its long range extensibility. Increased crystallinity and increased density of strongly reactive groups, on the other hand, result in more rigid, strong, and inextensible fibers.

f). *Cellulose.*—Of the naturally occurring fibers, cellulose has received the greatest attention. In the various forms in which it has been studied it illustrates most of the known principles of fiber organization, being indeed the parent structure from which these principles were deduced. The properties of cellulose are probably due in the main to a single substance, a linear polymer of cellobiose (4-β-*d*-glucosido-*d*-glucose)[326] the long and comparatively rigid chains of which have a strong tendency, by virtue of hydrogen bonding, to form crystallites; these, often incorporating the ends of several polymer chains, but never extending along their entire length, are interconnected at random by the loose, flexible strings of single chains emerging from them.[327] The effect of stretching fibers and oriented sheets of cellulose are roughly what would be expected from such a structure, being highly dependent upon the general direction of the chains in relation to the direction of the applied stress. In cotton and ramie and in the very highly oriented Lilienfeld rayon, the chains nearly coincide with the fiber axis. These fibers are strong and relatively inextensible; in flax, Young's modulus may be nearly as high as the value calculated for stretching the covalent single bond between carbon atoms.[328] Rayon, on the other hand, with transversely oriented chains, is ductile and can be stretched by as much as 30%. The rupture stress is similarly anisotropic. When stressed in the direction of the chains, a fiber can be broken either by pulling the ends of the chains out from the crystallites, or by breaking covalent bonds. The extremely high tensile strength of well-made fibers bears out this statement of the possible mechanisms of rupture[328,329] without excluding either.

It has been guessed, from the angular width of the X-ray spots[329] and by the observation of diffuse reflections near the origin[329] that the crystallites in cellulose are at least 50 Å wide and more than 600 Å long, but it is improbable that they are as uniform in size and shape as this result might suggest. Concerning their structure, it may be asserted that only the most elementary features are well established.

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[325] Cf. W. O. Baker, C. S. Fuller, and N. R. Pape, J. Am. Chem. Soc., **64**: 776, 1942.

[326] For the chemical evidence see, for example, K. H. Meyer and H. Mark, ref. (309), chap. V, and H. Staudinger, Die hochmolekularen organischen Verbindungen: Kautschuk and Cellulose: Springer, Berlin, 1932.

[327] Cf. A. Frey-Wissling, Protoplasma, **25**: 261, 1936; O. Kratky and H. Mark, Ztschr f. physik. Chem., **35B**: 129, 1937; E. Sauter, Ztschr. f. physik. Chem., **35B**: 117, 1937; H. Mark, J. Phys. Chem., **44**: 764, 1940. According to earlier views, the crystallites or micelles formed discrete units: cf. Meyer and Mark ref. (309), pp. 115 ff; C. Trogus and K. Hess, Ber. d. deutsch. chem. Ges., **68**: 1605, 1935; K. H. Meyer, Kolloid-Ztschr., **53**: 8, 1930.

[328] K. H. Meyer and W. Lotmar, Helvet. chem. acta, **19**: 68, 1936.

[329] Cf. H. Mark, J. Phys. Chem., **44**: 764, 1940.

Most of the observed reflections, which have with recent technical advances become quite numerous,[330] can be referred almost equally well[331] to either of two unit cells with a common fiber axis (b) of 10.3–10.4 Å, the length of one anhydrocellobiose unit. The *a* and *c* axes of the two cells stand in approximately diagonal relationship to each other[332] as shown in Fig. 21, with some uncertainty as to whether they are orthorhombic ($\beta = 90°$) or monoclinic (β around 84°). The larger cell[333] contains four and the smaller cell[334] two

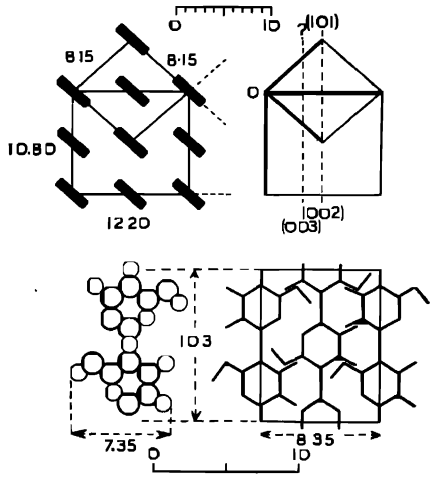

FIG. 21.—Crystal structure of cellulose.

Upper left: basal projection showing the packing of chains in cellulose. The arrangement can be referred to either of two unit cells, the dimensions of which are shown.

Upper right: showing the indexing of certain planes in the two unit cells. The plane (003) in the larger cell obviously cannot be indexed rationally with reference to the smaller cell.

Lower left: projection of the cellobiose molecule according to K. H. Meyer and L. Misch, Helvet. chem. acta, **20**: 232, 1937.

Lower right: cellulose: projection on the plane (001) of the small unit cell, showing the presence of reversed chains as proposed by Meyer and Misch, *loc. cit.*

anhydrocellobiose units. When certain artefacts in the experimental data[335] have been disposed of[336] the only reason for preferring the larger cell is the questionable existence[337] of a reflection parallel to (101) in the small cell which

[330] Cf. E. Sauter, Ztschr. f. physik. Chem., **37B**: 161, 1937.

[331] E. Sauter, Ztschr. f. physik. Chem., **43B**: 294, 1939.

[332] Cf. W. H. Bragg, Nature, **125**: 634, 1930.

[333] Orthorhombic form: O. Sponsler, J. Gen. Physiol., **9**: 221, 1925; *ibid.*, **9**: 677, 1926; Nature, **125**: 633, 1930. Monoclinic form: E. Sauter, Ztschr. f. physik. Chem., **35B**: 83, 1937.

[334] Orthorhombic: M. Polanyi, Naturwissenschaften, **9**: 288, 1921. Monoclinic: K. H. Meyer and H. Mark, ref. (64), pp. 94 ff.

[335] E. Sauter, Ztschr. f. physik. Chem., **35B**: 83, 1937.

[336] H. Kiessig, Ztschr. f. physik. Chem., **43B**: 79, 1939; cf. R. Corey and R. W. Wyckoff, J. Biol. Chem., **114**: 407, 1936; H. Mark and K. H. Meyer, Ztschr. f. physik. Chem., **36B**: 232, 1937.

[337] E. Sauter, Ztschr. f. physik. Chem., **35B**: 83, 1937; H. Kiessig, *ibid.*, **43B**: 79, 1939; H. Mark and K. H. Meyer, *ibid.*, **36B**: 232, 1937; cf. E. Sauter, *ibid.*, **43B**: 294, 1939.

requires a doubling of the axial lengths for its rational indexing. Thus (101) in the small cell becomes (002) in the large, and the questionable reflection can be indexed as (003). The spreading of certain reflections from planes at 45° to (101) (small cell) along the Debye-Scherrer circle containing the disputed reflection is, however, so great that it cannot be certain whether the latter is really present. It is agreed that the arc in question is composite.[338]

The presumed molecular dimensions of cellobiose are consistent with the orientation of the chains in the manner shown in Fig. 21.[339] This arrangement gives rise, however, to certain difficulties, since the cellobiose unit as ordinarily conceived possesses a digonal screw axis,[340] a circumstance that demands systematic extinction of the odd orders of the basal reflection (0k0). Actually, certain of these forbidden diatropic reflections are present, although with very low intensity.[341] It is quite likely that extension of recent work on the structure of α-glucosamine will explain this puzzling fact; for the present we have only the tentative suggestion[342] that the exacting requirements of hydrogen bonding between cellobiose chains distributed statistically in the axial direction b and $-b$ may cause a slight displacement of the oxygen atoms 6 and 6′. This distortion, generating, in effect, a twofold symmetry axis through oxygen atom no. 4 perpendicular to the plane of the rings, will make the direct and reversed chains almost exactly superimposable, and therefore equivalent, from the point of view of molecular packing. At the same time, the digonal screw axis will be abolished.

g). *Protein fibers.*—It may be claimed with little hesitation that the insoluble proteins have the essential properties of linear polymers which occur in characteristic fibrous or laminar habit, the laminar forms being themselves composed of matted fibrils. This is true not only of the well-known thread-like proteins such as silk, hair, wool, and tendon, but also of the contractile elements of muscle.[343] If it is reasonable to link this universal property with the hypothesis of polypeptide chains, it is necessary to attribute the great diversity of properties within the group to the diverse nature of the side-chains, the interaction of which may be supposed to determine the particular form assumed by the peptide backbone under given conditions. Postulating such a structure, it is equally reasonable to expect to find in the fibrous proteins that juxtaposition of order and disor-

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[338] E. Sauter, Ztschr. f. physik. Chem., **43B**: 294, 1939; cf. E. G. Cox, Chem. Soc. Ann. Rep., **34**: 176, 1937.

[339] K. H. Meyer and H. Mark ref. (309), p. 111.

[340] Digonal screw axis: "The operation of an n-fold screw axis rotates the structure through an angle $2\pi/n$ and simultaneously translates it parallel to the axis . . . " W. L. Bragg, The Crystalline State: Bell, London 1933, p. 76.

[341] K. H. Meyer and H. Mark, ref. (309), p. 108; E. Sauter, Ztschr. f. physik. Chem., **35B**: 83, 1937; E. Sauter, *ibid.*, **37B**: 161, 1937; H. Kiessig, *ibid.*, **43B**: 79, 1939; cf. polemic between E. Sauter, *ibid.*, **36B**: 427, 1937 and H. Mark and K. H. Meyer, *ibid.*, **36B**: 232, 1937; *ibid.*, **38B**: 395, 1938.

[342] E. G. Cox, Chem. Soc. Ann. Rep., **34**: 176, 1937.

[343] Cf. A. L. von Muralt and J. T. Edsall, J. Biol. Chem., **89**: 351, 1930; G. Boehm and H. H. Weber, Kolloid-Ztschr., **61**: 269, 1932; and W. O. Fenn's article in this book; Sec. 7.

der, and that correlation between mechanical and chemical properties, that we described in the synthetic polymers.

A limiting case is that in which the polypeptide chains are present in the fully extended form. The X-ray pattern of native silk fibroin, with a unit cell $9.68 \times 7.00 \times 8.80$ Å, $\beta = 75°50'$,[344] containing four alanylglycyl residues, has been interpreted[344] in this sense, the unit translation 7.00 Å representing the length of two peptide linkages. With this is correlated the anticipated very small breaking strain. A similar X-ray pattern, referred to by the prefix β, and somewhat similar mechanical properties, are characteristic of feather and reptilian keratin in the natural state[345] and also of other forms of keratin[346] and of myosin[347] when these are stretched under suitable conditions to the maximum extent; an increased c-translation in the unit cell (9.8 Å) is correlated with the presence of longer side-chains than the methyl groups of silk.

Most native or unstretched keratins[345] and myosin in relaxed dried muscle[347] give almost identical X-ray patterns (α) which are quite different from the β-diagram. The side-chain spacing, 9.8 Å, is retained, but there is a pronounced axial period of about 5.1 Å, while the a-translation in keratin has been given as 27 Å. Although these facts point clearly to a chain folded in some regular manner in the plane perpendicular to the side-chains, the form of the folds is by no means established; recent speculations,[348] however, improve greatly upon the earlier ones. Whatever the structure of α-keratin, it is certain that the intramolecular transformation to the β-form is a complex one involving rupture of several types of linkage, for the mechanical properties of this substance are remarkably intricate. The maximum extension, corresponding to complete transformation to the β-form, is about 100%. The first 20% is without effect upon the X-ray pattern and is attributed to unfolding of the chains in the amorphous regions of the fiber; the next 30% requires the presence of moisture and is accompanied by some change in the X-ray diagram. These changes are reversible; the complete transformation requires heating in steam or immersion in sodium hydroxide solution and is irreversible. The fiber stretched in steam or alkali, having evidently suffered hydrolytic rupture of certain linkages, is in a labile condition, and its behavior depends upon the conditions under which new linkages are permitted to establish themselves. Plunged into cold water, the stretched fiber retains its maximum length and can only be caused to

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[344] R. O. Herzog and W. Jancke, cited by K. H. Meyer and H. Mark, ref. (309), p. 221; R. Brill, Liebig's Annalen der Chimie, **434**: 204, 1923; K. H. Meyer and H. Mark, Ber. d. deutsch. chem. Ges., **61**: 1932, 1928.

[345] W. T. Astbury and T. C. Marwick, Nature, **130**: 309, 1932; W. T. Astbury, Tr. Faraday Soc., **29**: 193, 1933; Cold Spring Harbor Symp., **2**: 15, 1934; Kolloid-Ztschr., **69**: 340, 1934; Compt rend d. trav. du lab. Carlsberg, **22**: 45, 1938.

[346] W. T. Astbury and A. Street, Phil. Tr. Roy. Soc., **230A**: 75, 1932; W. T. Astbury and H. J. Woods, *ibid.*, **323A**: 333, 1933.

[347] W. T. Astbury and S. Dickinson, Proc. Roy. Soc., **129B**: 307, 1940.

[348] W. T. Astbury and F. O. Bell, Nature, **147**: 696, 1941; cf. W. T. Astbury and H. J. Woods, Phil. Tr. Roy. Soc., **323A**: 333, 1933.

contract by being reheated; if allowed to relax in steam or alkali, the fiber becomes much shorter than its original unstretched length ("supercontraction") and after this process, probably one involving principally the noncrystalline regions, and occurring, therefore, without any necessary accompaniment of altered reflecting power of X-rays, it can be reversibly and completely transformed to the β-state by stretching at ordinary temperatures.

The linkages in myosin are considerably more labile than in keratin—a fact that has been associated with the smaller sulfur content of the former protein, and the consequently diminished importance of covalent —S—S— linkages between neighboring chains.[349] Myosin may be caused all the more readily to supercontract, which it may do, indeed, while still in the α-form, without previous stretching.

The remaining proteins which have received attention appear to exist in forms analogous to β and supercontracted keratin, with important differences. The X-ray diagram of collagen, the chief protein of connective tissue, tendon, and cartilage, can be referred to a unit cell 6.5 Å long in the direction of the fiber axis, corresponding to a residue length 3.25 Å and suggesting a somewhat contracted chain.[350] The supercontraction of collagen to about one quarter its original length is well known, being produced by very moderate heating; the substance becomes rubber-like. Gelatin, a partly hydrolyzed collagen, resembles supercontracted collagen in that stretching causes the amorphous X-ray pattern to be replaced by the fiber pattern typical of native collagen. Elastoidin, the material comprising certain supporting fibers in the fins of elasmobranchs, resembles collagen in being transformed on heating to a supercontracted form[351] which, according to the evidence of the X-ray diagram, is amorphous at 62°C. and acquires a certain degree of crystallinity at room temperature.[352] The crystalline picture closely resembles that[353] of the native substance, but the thermoelastic properties indicate that as in other cases the process of supercontraction is one of liberation of flexible chains from a state of association in a lattice.[354] Elastin, present with collagen in connective tissue (elastic fibers), possesses in the native form thermoelastic properties and X-ray scattering powers reminiscent of those of supercontracted collagen.[355]

It is perhaps pertinent to refer to the evidence derived from X-ray studies as to the relationship between crystalline "corpuscular," denatured, and fibrous proteins.[353] The evidence is scanty and no very far-reaching con-

[349] W. T. Astbury and F. O. Bell, Nature, **147**: 696, 1941; J. B. Speakman and M. C. Hirst, Tr. Faraday Soc., **29**: 148, 1933; J. B. Speakman, J. Soc. Dyers and Colorists, Bradford, Jubilee Number, p. 34, 1934; Nature, **138**: 327, 1936.

[350] G. L. Clark and J. A. Schaad, Radiology, **27**: 339, 1936; cf. K. H. Meyer and H. Mark,[301] p. 224. W. T. Astbury, Tr. Faraday Soc., **29**: 193, 1933, finds an identity period 8.4 Å.

[351] E. Fauré-Fremiet and R. Woelfflin, J. Chim. phys., **33**: 801, 1936.

[352] G. Champétier and E. Fauré-Fremiet, J. Chim. phys., **34**: 197, 1937.

[353] W. T. Astbury and R. Lomax, J. Chem. Soc., 846, 1935.

[354] L. E. R. Picken, J. Chim. phys., **34**: 764, 1937.

[355] H. Kolpak, Kolloid-Ztschr., **73**: 129, 1935; W. T. Astbury, Compt. rend. d. trav. du lab. Carlsberg, **22**: 45, 1938; cf. K. H. Meyer and C. Ferri, Pflüger's Arch. f. d. ges. Physiol., **238**: 78, 1936.

clusions should be drawn. Reflections corresponding roughly to the backbone and side-chain spacings of the β-fiber pattern occur quite commonly in crystalline proteins and viruses and may become much sharpened by denaturation.[355] Thus there is some little reason for drawing an analogy between the denatured state and the extended or β-form, but there can be little immediate justification for supposing the α-folding to bear any but the most general resemblance to the configuration of the corpuscular molecule. The supercontracted fibers have something of the amorphous quality to be expected of a collection of randomly coiled chains and the circumstances under which supercontraction is observed are clearly such as would favor the thermal disorientation of chains which under ordinary conditions are subject to powerful constraints. Concerning the manner in which this haphazard coiling can be replaced by the ordered convolutions of the corpuscular protein, there is no information whatsoever. The two processes seem at first sight antithetical. Perhaps a detailed study of the virus proteins will throw further light on the subject; possibly, even a less naïve interpretation of fiber pictures will perform this service, for the observation of axial spacings of several hundred Ångstrom units in collagen and other fibrous proteins[357] suggests an architectural scheme more elaborate than that implied by the β-structure in its elementary form.

h). *Chromosomes.*—The chromosomes, the most important of all natural fibers, are also the least accessible to direct study; consequently, their possible resemblance to one of the types of fiber already discussed is a matter for speculation insecurely based upon cytological observation and held together by a few analogies.

We may crudely represent the prophase chromosome, and perhaps also its precursor in the interkinetic nucleus,[358] as an elongated body perhaps 10^5 Å in length and 10^2–10^3 Å thick,[359] although the latter dimension is open to question;[360] the contracted metaphase chromosome may be 10^4–10^5 Å long and 10^3–10^4 Å thick.[361] Protein (or protamine) and nucleic acid are present at all points along the length of the chromosomes, although their proportions may vary from point to point,[362] a fact that partly underlies the banded

[355] W. T. Astbury, Compt. rend. d. trav. du lab. Carlsberg, **22**: 45, 1938; M. Spiegel-Adolf and G. C. Henny, J. Phys. Chem., **45**: 931, 1941; *ibid.*, **46**: 581, 1942.

[357] R. B. Corey and R. W. G. Wyckoff, J. Biol. Chem., **114**: 407, 1936; G. L. Clark and J. A. Schaad, Radiology, **27**: 339, 1936; C. E. Hall, M. A. Jakno, and F. C. Schmitt, J. Am. Chem. Soc., **64**: 1234, 1942; R. S. Bear, *ibid.*, **64**: 727, 1942.

[358] E.g., T. Boveri, Arch. f. Zellforsch., **3**: 181, 1909; C. H. Waddington, An Introduction to Modern Genetics: Allen and Unwin, London, 1939, p. 41; M. J. D. White, The Chromosomes: Methuen, London, 1937, p. 11; cf. R. Chambers in General Cytology, ed. by E. V. Cowdry: University of Chicago Press, Chicago, 1924, p. 268.

[359] Cf. H. J. Muller, Am. Nat., **69**: 405, 1935.

[360] Cf. C. H. Waddington, An Introduction to Modern Genetics: Allen and Unwin, London, 1939, p. 377.

[361] With very wide variations in absolute and relative dimensions—see C. D. Darlington, Recent Advances in Cytology: Churchill, London, 1937, p. 83.

[362] T. Caspersson, Skandinav. Arch., **73**: Suppl. 8, 1936; cp. A. E. Mirsky and A. W. Pollister, Tr. New York Acad. Sc., Ser. 2, **5**: 190, 1943.

appearance of stained specimens. The observed double refraction of chromosomes is in accord with this result, and although often complicated by the varying contributions of intrinsic and form birefringence which accompany changes in the hydration of chromatic material,[363] is such as to suggest that the nucleic acid molecules are arranged lengthwise in the axial direction of the chromosome.[364] Nothing is known concerning the orientation of the protein component.

The fibrous habit suggested by the linear dimensions of the chromosomes is borne out abundantly by their behavior during cell division and by the results of microdissection. Their extreme flexibility is shown by the remarkable coiled configurations that they temporarily assume during meiosis and cell division, and their marked axial cleavage by the longitudinal splitting undergone during the same processes.[365] At the same time, the formation of chiasmata, apparently in response to local torsional stresses involved in the diplotene resolution of the coiled four strand bivalent,[366] betokens a certain mechanical weakness that at once precludes an extremely long covalent chain as the basis of chromosome structure and is equally at variance, at first sight, with the evidence of extreme extensibility provided by microdissection experiments.[367]

The linear dimensions of the chromosomes are, of course, consistent with a fiber structure based upon polypeptide strands associated laterally with the degree of overlap and disorder characteristic of most fibers; it has indeed been considered that such a structure has a certain "prior probability"[368] in view of the linear structure of the protamines, but little more can be said at present. With 3.5 Å as the length of a peptide residue and perhaps 8 Å as an average lateral spacing, a chromonema $10^6 \times 10^3$ Å would be about 3.10^5 peptide linkages in length and a diameter would include about 120 chains.

It has been suggested that such a polypeptide fiber structure could underlie the genetic linearity of the chromosomes,[368] the specific character of each linear segment being established not only by the axial sequence of polypeptide residues and side-chains within a single strand of the fiber, but also by the varying patterns of neighboring strands within the segment and by their mode of association. In other words, the characteristic disorder of

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[363] W. J. Schmidt, Die Doppelbrechung von Karyoplasma, Zytoplasma und Metaplasma: Borntraeger, Berlin, 1937, Chapter 2.

[364] W. J. Schmidt, Naturwissenschaften, **26**: 413, 1938; cf. W. J. Schmidt, ref. (176); pp. 92 ff; T. Caspersson, E. Hammarsten and H. Hammarsten, Tr. Faraday Soc., **31**: 367, 1935; W. T. Astbury and F. O. Bell, Cold Spring Harbor Symp., **6**: 109, 1938; see also D. M. Wrinch, Protoplasma, **25**: 550, 1936.

[365] This is not due to the presence of a preformed longitudinal plane of symmetry: see W. J. Schmidt, ref. (363), p. 99; cf. U. Dehlinger, Naturwissenschaften, **23**: 558, 1935.

[366] C. D. Darlington, ref. (361), p. 550.

[367] R. Chambers in "General Cytology," ed. by E. V. Cowdry, University of Chicago Press, Chicago, 1924, p. 268; W. R. Duryee, Collecting Net, **13**: 1938: cited by C. H. Waddington, ref. (360); cf. W. R. Duryee, Biol. Bull., **75**: 345, 370, 1938.

[368] D. M. Wrinch, Protoplasma, **25**: 550, 1936.

fibers is tacitly considered to be in abeyance in this case, so that the protein component of a chromosome appears as a linear sequence of highly organized segments perhaps more closely analogous to a particle of tobacco mosaic virus than to a thread of myosin. Aside from the lack of positive evidence in favor of such a hypothesis, it fails to assign a satisfactory role to the nucleic acid component. There is no particular virtue in the fact that it straddles the gap between the two extreme possibilities of chromosome structure: the first of which is represented by the picture of the chromonema as a protein fiber playing the role of a mechanical carrier of specific substances (chromomeres?), just as a clothes line carries a variety of garments, and the second showing the genes as elaborately organized regions of unknown constitution, each of which adheres by some unknown means to two neighbors, like a row of dancers holding hands. It is idle to discuss these possibilities until the linear differentiation of the genetic units shall have become more clearly defined[369] and more closely correlated with the physical linearity of the chromosomes.

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Waddington, C. H., Organisers and Genes: University Press, Cambridge, 1940.

White, M. J. D., The Chromosomes: Methuen, London, 1937.

[369] E.g., C. H. Waddington, ref. (360), p. 398 and elsewhere.

9

SOME PROPERTIES OF FILMS AND MEMBRANES[370]

1. Introduction.—The subdivision or the enclosure of all living matter by membranes, septa, pellicles, or fluid skins is a fundamental fact of biology. Between the extremes of structural rigidity and the easy deformability of the primitive fluid sphere, we encounter all possible mechanical qualities, associated with a great diversity of form and function. It is natural to enquire into the behavior of matter in bulk when it is attenuated in the form of thin sheets, but this is not as a rule the most profitable approach to the problem of biological membranes. All matter becomes anisotropic at its surface, but it is seldom that simple mechanical attenuation can be carried so far that the anisotropy is not masked by the known properties of the material in bulk. This is not to deny the importance of such work as that of Hardy[371] on the mechanism of lubrication, or the suggestiveness of the rigid honeycomb structures that can be formed by soap bubbles,[372] but the boundary state is better studied by starting with a single interface, for here the anisotropy of the surface layer is as clearly exhibited, above all in the phenomenon of surface tension, as it is by the layer of oil between two ground surfaces or by molecular films of soap. It is further possible to introduce other substances into such an interface, and to study the change in properties brought about by their presence. This is probably the first stage in the genesis of all bounding membranes, and in the detailed study of interfacial films it is perhaps possible to find the *in vitro* counterpart of some of the reactions which lead to the differentiation of protoplasmic boundaries—their specialization as sites of functional or metabolic activity, as selectively permeable barriers delimiting the organism from its environment, or as skeletal structures. Interfacial films, for example, readily become rigid or elastic under some circumstances; we need only appropriate localization, therefore, to introduce a new anisotropy into the surface of the primitive sphere and to cause spontaneous deformation. If such influences are sufficiently numerous and complex it is clear that we can eventually get a great variety of cellular structures, although it is admittedly a far cry from

[370] N. K. Adam, The Physics and Chemistry of Surfaces, 2nd ed.: Clarendon Press, Oxford, 1938; see also bibliography at end of chapter.

[371] W. B. Hardy, Collected Papers, Cambridge, 1936. References to separate papers on this subject see ref. (370), p. 227.

[372] E.g., O. Buetschli, Investigations on Microscopic Foams and on Protoplasm, Trans. by E. A. Minchin; A. and C. Black, London, 1894.

our general theoretical statement to a knowledge of the processes leading to the beautiful spiral arrangement of cellulose fibers in the cell wall of *Valonia*,[373] or even to the simple discoidal structure of the mammalian erythrocyte.[374]

In devoting our discussion in this chapter to the properties of oriented interfacial layers, we are attempting—as many others have done—to introduce into the idea of surface activity a new restriction, and therefore a new source of that specificity which is characteristic of vital reactions. Where the mere increase of interfacial area consequent upon subdivision might once have seemed sufficient to explain many of the differences between homogeneous and heterogeneous systems, it now is necessary to attribute an equal importance to the actual molecular configuration of the interface.[375]

2. Gibbs' Equation: Soluble Molecular Layers at an Interface.—Let us consider first the properties of the interface between two immiscible fluids. Its most elementary property is that work must be done in order to increase its area, because this involves the rupture of cohesive forces between molecules, the new surface being composed of molecules which formerly were situated in a symmetrical field of attractive forces and now are subject to a resultant pull in a direction normal to the interface. The work required to increase the area isothermally is γ erg. cm.$^{-2}$, which may be defined as a "surface tension" of γ dyne cm.$^{-1}$ acting over one centimeter.

Substances present in aqueous solution introduce new intermolecular fields of force, either more or less intense than the forces operating in pure water. Their nature is reflected in the surface composition of the solution. Ions or polar molecules, by virtue of their powerful electrostatic interaction with water dipoles, tend to be pulled into the interior of the solution, leaving a surface layer of pure water which, insofar as it is influenced by the solute molecules in the interior, has a higher surface tension than that of pure water. Molecules containing nonpolar groups, conversely, tend to displace water from the interface, causing a decrease in surface tension; in a homologous series, for instance, the surface tension varies in a regular manner with the length of the carbon chain.[375] It is often possible to distinguish by measurement of surface tension[377] between the opposing effects of increasing chain length and of electric moment.[378]

[373] R. D. Preston, Phil. Tr. Roy. Soc., **224**: 131, 1934; R. D. Preston and W. T. Astbury, Proc. Roy. Soc., **122B**: 76, 1937.

[374] Cf. E. Ponder, Brit. J. Exper. Biol., **6**: 387, 1929; Tr. Faraday Soc., **33**, 947, 1937.

[375] Some of the many other aspects of the biophysics of surfaces, with references to early literature, are discussed by R. Höber, Physikalische Chemie der Zelle und der Gewebe, Sechste Aufl.: Wilhelm Engelmann, Leipzig, 1926, Chapter 4; E. K. Rideal, Surface Chemistry, 2nd ed., Cambridge University Press, 1930; H. Freundlich, Kapillarchemie; Vierte Aufl.: Akademische Verlagsges., Leipzig, 1930.

[376] J. Traube, Ann. Chem., **265**: 27, 1891.

[377] Methods: a. ref (370); b. E. A. Hauser in Advances in Colloid Science, ed. by E. O. Kraemer; Interscience Pub. Inc., New York 1942, **1**: pp. 391–415.

[378] E.g., J. R. Pappenheimer, M. P. Lepie, and J. Wyman, J. Am. Chem. Soc., **58**: 1851, 1936; cf. Hauser, ref. (377b).

Generally the surface tension decreases continuously with concentration of a surface-active solute, approaching a limiting value at high concentrations; various irregularities have been reported,[377b] which appear to be connected sometimes with a change in the nature of the solute with concentration, as in the case of the soap solutions studied by McBain, but more frequently with failure to attain a true equilibrium between surface and bulk phases. This may be a very sluggish process in systems containing asymmetric molecules and antagonistic groups.

The basis for quantitative studies of interfacial equilibria is the isotherm derived thermodynamically by Gibbs, an approximate form of which may be expressed by the equation:

$$\sigma RT = -\frac{\partial \gamma}{\partial \ln c} = \frac{\partial F}{\partial \ln c}. \tag{21}$$

Here σ is the surface excess, or amount of adsorbed solute per unit area,[379] and c is concentration of solute in the bulk phase. The quantity F has been introduced because it is often more convenient to use than γ; it is the "surface pressure" and may be defined by considering an imaginary moveable barrier of length l separating the surface of a solution from a surface of pure solvent. Since the surface tension γ of the solution is lower than that of the pure solvent, γ_0, a force F dyne $\cdot$ cm.$^{-1}$ will act on the barrier in such a direction that the surface of the solution will tend to expand at the expense of the solvent surface. If the barrier is allowed to move a small distance dx, the work done will be $(\gamma_0 - \gamma) \cdot l dx$: this must also be $Fl \cdot dx$. Thus

$$F = (\gamma_0 - \gamma). \tag{22}$$

F is equivalent in physical terms to an osmotic pressure; it represents the two-dimensional pressure needed to prevent dilution of the surface phase by pure solvent.

To return to Gibbs' equation (21), we see that it represents a relationship between surface tension lowering, surface excess, and bulk concentration. For ordinary moderately soluble substances, the amount present in the surface layer can be varied by changing the concentration of the solution, and Gibbs' equation can be tested—at least in theory—by measuring the surface excess and the surface tensions for solutions of several concentrations.[370,377] It seems to be agreed that "the verification of Gibbs' equation may now be considered reasonably well established"[380] by measurements on such solutions. Accordingly, it is permissible to use the equation for calculating the surface excess from surface tension measurements, and certain interesting facts concerning the structure of the surface layer have been revealed by such data when used in conjunction with empirical surface tension equations. For very dilute solutions,

$$F = Bc, \tag{23}$$

[379] For exact definition and derivation of Gibbs' equation, see ref. (370).

[380] See ref. (370).

giving with the Gibbs' equation,

$$\sigma = \frac{F}{RT} \qquad \text{or} \qquad FA = RT, \tag{24}$$

where A is the area per molecule, if the amount of solute in the bulk phase can be neglected by comparison with σ. Equation (24) is simply the perfect gas law. For very dilute solutions of short chain fatty acids, this has been confirmed.[381] With increasing length of chain, the values of FA are too small; this is probably due to lateral cohesion between the adsorbed molecules. With increasing concentration, on the other hand, the values become too large, obeying the empirical equation

$$F(A - A') = xRT, \tag{25}$$

where A' is a correction for the area occupied by the molecules, and is analogous to Van der Waals' repulsion forces. For all chain lengths, A' is about 25 Å^2, or equal roughly to the cross-section of the molecules; thus the molecules evidently stand more or less on end in the more concentrated adsorbed films.

The actual value of the constant B (equation (23)) varies regularly with the structure of the solute; this is merely another way of stating Traube's rule. Traube showed the value to increase geometrically from one member of an homologous series to the next; thus we may write empirically

$$\ln B = k + nk', \tag{26}$$

where n is the number of carbon atoms in a given homologue, and k is a constant defining the series. This arithmetical increase in ($\ln B$) amounts to an arithmetical increase in the work of adsorption λ[382] which for very small values of c can be shown thermodynamically to be

$$\lambda = RT \ln \frac{\sigma}{\tau c} = RT \ln B + k'', \tag{27}$$

where τ is the "thickness" of the surface region. Thus, combining (26) and (27), we may write:

$$\lambda = Kn + \Sigma\lambda_0. \tag{28}$$

Langmuir's analysis of the data suggests that the term $\Sigma\lambda_0$, which is negative, represents the effect of the hydrophilic groups of the molecule, and Kn the effect of the CH_2 chain. Values of the constant K and of λ_0 for various groups have been calculated; from these and equation (28) it is thus possible to calculate λ for higher members of homologous series in which no experimental value can be assigned to c because of the infinitesimal solubility of

[381] a. R. K. Schofield and E. K. Rideal, Proc. Roy. Soc., **109A**: 57, 1925; b. *ibid.*, **110A**: 167, 1926.

[382] I. Langmuir, J. Am. Chem. Soc., **39**: 1883, 1917.

the substance. Then if necessary equation (27) can be used to calculate B or $(F/c)_0$.

According to Gibbs' equation, a value can always be assigned to c for any pair of values F and σ. The surface-active substance may, however, be so extremely insoluble that F and σ can be varied over a wide range without any measurable amount of material passing into solution. Such substances form "insoluble" films which are studied by special methods, since the situation imagined in the definition of surface pressure can actually be realized, and the pressure of the film in contact with a clean surface can be measured directly. There is an important intermediate region in which partly soluble films decrease in area upon compression as a result of solution in the bulk phase. For moderately insoluble substances of this type the solubility is given by c of Gibbs' equation, and is a function of the amount of solid substance present in the interface.

Interesting preliminary calculations of the pressure solubilities of homologous polypeptides as a function of surface pressure and molecular weight have been given by Langmuir and Waugh.[383] The surface excess, σ, can be eliminated from equation (1) by introducing an empirical relation between surface tension and concentration such as that of Szyszkowski:[384]

$$F = C\gamma_0 \ln \left(1 + \frac{c}{a}\right) \tag{29}$$

where C and a are constants. Combining with (29) we find

$$\sigma = \frac{c}{RT} C\gamma_0 \frac{1}{a + c} = \frac{\sigma_1 c}{a + c}, \tag{30}$$

where σ_1 is the surface excess when c is very large, corresponding to the value for a close-packed film. We then use equation (10) and integrate equation (21), obtaining

$$F = \sigma_1 RT \ln \left(1 + \frac{c}{a}\right). \tag{31}$$

If c is very small

$$a = \frac{\sigma_1 RT}{(F/c)_0} = \frac{\sigma_1 RT}{B}. \tag{32}$$

Assuming arbitrarily that one mg. of polypeptide covers 0.5 m.^{2} when close-packed on a water surface we find:

$$\sigma_1 RT = \frac{4800}{M}, \tag{33}$$

where M is molecular weight. To apply equation (31) we now only need to know in addition the value of B. This can be calculated by using equation (28), modified to include terms for the side-chain of the polypeptide, and then equation (27). Each peptide unit is assumed to have a residue weight 120,

[383] I. Langmuir and D. F. Waugh. J. Am. Chem. Soc., **62**: 2771, 1940.

[384] B. v. Szyszkowski, Ztschr. f. physik. Chem., **64**: 385, 1908.

the average value obtained for several proteins, and an effective number of carbon atoms estimated from the average number of CH_2 groups and hydrophilic groups in the side-chains. The λ_0 term includes values for peptide CO and NH groups. When the appropriate substitutions are made, equation (31) takes the final form

$$\log [1 + 0.21wB] = \frac{FM}{1100} \tag{32a}$$

and

$$\log B = 1.202 + 0.00533\ M, \tag{32b}$$

where w is weight concentration, 0.001 Mc.

Calculation shows that with polypeptides of the average composition of a protein and molecular weight 2000, pressure solubility would account for so little of the material in the film, and would occur so extremely slowly as to be undetectable. With a reasonably large volume of underlying solution and adequate mixing, measureable changes in area should occur upon compression when M is 1200–1700; when w is greater than 10^{-5} it is found that the substance dissolves very rapidly. The proteins, if they existed as extended polypeptide chains of molecular weight 35,000, should be extremely insoluble, and the fact that they are actually very soluble lends further conviction to the evidence for their globular form in solution.

The theory and technique of pressure solubility measurement promise to become important in the estimation of the amounts and molecular weights of protein degradation products.

3. Insoluble Monolayers.—In subsec. 2 we discussed the transition from the simple Gibbs adsorption of solutes to pressure-soluble films, and finally to films which to all intents and purposes are insoluble. The insoluble films are usually prepared by spreading over the liquid surface; such spreading either occurs spontaneously[385] or can be promoted by dissolving the substance in a volatile solvent.[386] Just as the formation of a Gibbs layer requires the presence of nonpolar groups in an otherwise soluble molecule, so the spreading of an insoluble substance requires the presence of polar groups in a predominantly nonpolar molecule. The long-chain aliphatic hydrocarbons will not spread; the corresponding monobasic acids spread readily, continuing the gradual change in surface properties exhibited by the short-chain acids discussed on page 190.[387] From the observation that the polar group provides a spreading force, and the postulate[388] that only short-range forces, operating between molecules in contact, should be responsible for adsorption, it follows that fatty acid films must be oriented with the carboxyl groups in contact with the water, and that "the . . . amount of the fatty acid that can be spread on a given water surface is limited by the number of molecules that can be packed into a single layer."[388b]

[385] E.g. A. Pockels, Nature, **43**: 437, 1891.

[386] I. Langmuir, J. Am. Chem. Soc. **39**: 1848, 1917.

[387] Ref. (370), Fig. 29.

[388] a. I. Langmuir, J. Am. Chem. Soc., **38**: 2221, 1916.; b. I. Langmuir, Proc. Roy. Soc., **170A**: 1, 1939.

Monolayers can behave as solids, liquids, or gases. The phase relationships shown by a particular substance in the form of a monolayer are not in general those found in bulk, nor would this be expected, for the orientation imposed by the submergence of polar groups in the substrate may interfere with the mode of packing naturally assumed under otherwise analogous conditions in three dimensions.

The state of a monolayer, by definition, must be considered in terms of the mechanical properties of the surface which it occupies. These properties can be determined by special methods: compressibility is usually studied by measurement of surface pressure as a function of area per molecule, the apparatus used being some modification of Langmuir's surface balance, in which the force acting on a floating barrier separating the film from a clean surface is measured.[389] Viscosity can be determined from the rate of flow through a narrow surface channel,[390] or by observing the oscillation of a circular disc or a vane.[391] Measurements of rigidity can also be made with the latter method; rigidity can be detected without special apparatus by observing the mobility of talcum powder placed on the surface. The disposition of polar groups in a film, and its homogeneity, can sometimes be deduced from surface potentials, although the interpretation of these is still in doubt. They are generally measured as the difference in potential between a reversible electrode immersed in the solution, and a second electrode, coated with a source of ionizing radiation, placed near the upper surface.[392]

The types of behavior encountered in relatively simple monolayers can be described by reference to substances with long hydrocarbon chains terminated by a polar group. Among these substances, it is possible to find almost any desired sequence of phase changes upon compression by suitable choice of temperature, chain length, or size and shape of the polar part of the molecule.[393]

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[389] a. Ref. (370), pp. 27–33 for early apparatus. b. W. D. Harkins and R. J. Myers, J. Chem. Phys., **4**: 716, 1936; c. J. Guastalla, Compt. rend. Acad. d. sc., **208**: 973, 1939; d. Mechanical registration of force area curves, see D. G. Dervichian, J. Phys. Rad., (7), **5**: 221, 1935; e. Measurement of surface pressures 0.005 — 0.100 dyne/cm., see J. Guastalla, Comp. rend. Acad. d. sc., **205**: 993, 1938.

[390] E.g., a. W. D. Harkins and W. J. Myers, Nature, **140**: 465, 1937; b. D. G. Dervichian and M. Joly, Compt. rend. Acad. d. sc., **204**: 1318, 1937; c. M. Joly, J. Phys. Rad., (7), **8**: 471, 1937; d. D. G. Dervichian and M. Joly, Nature, **141**: 975, 1938; e. M. Joly, J. Phys. Rad., (7), **9**: 345, 1938; f. M. Joly, Kolloid-Ztschr., **89**: 26, 1939.

[391] E.g., a. K. Schuett, Ann. Physik, (4), **13**: 712, 1904; b. D. Rohde, *ibid.*, (4), **19**: 935, 1906; c. R. J. Myers and W. D. Harkins, J. Chem. Phys., **5**: 601, 1937; d. L. Fourt, Am. J. Physiol., **119**: 310, 1937; e. I. Langmuir and V. J. Schaefer, J. Am. Chem. Soc., **59**: 2400, 1937; f. L. Fourt and W. D. Harkins, J. Phys. Chem., **42**: 897, 1938; g. M. Joly, Kolloid-Ztschr., **89**: 26, 1939.

[392] See ref. (370), pp. 33–35, where other methods also are described.

[393] The possible transformations have been enumerated and discussed by, a. D. G. Dervichian, J. Phys. Rad., (7), **10**: 333, 1939; b. D. G. Dervichian and M. Joly, *ibid.*, 375, 1939; c. D. G. Dervichian, J. Chem. Phys., **7**: 931, 1939; Dervichian's contentions concerning the analogies between the states of monolayers and of three-dimensional matter have been criticized on numerous grounds by, d. A. E. Alexander, Tr. Faraday Soc., **37**: 426, 1941; e. E. Boyd and W. D. Harkins, Bull. Am. Phys. Soc., **15**: 19, 1940; f. W. D. Harkins and E. Boyd, J. Phys. Chem., **45**: 20, 1941.

For many substances at very large areas, the film is gaseous and extremely dilute.[394] On compression, sudden condensation occurs at constant pressure to a very incompressible film of area between 19 and 21 Å per molecule, with which we may compare the values of about 18.3 Å, obtained from X-ray data for the hydrocarbon $C_{29}H_{60}$ and stearic acid at room temperature[395] and about 19.5 Å near the melting point.[396,397] Since the melting point of monolayers on water is almost invariably much lower than that of the crystal, it is probably reasonable to conclude that the hydrocarbon chains in the condensed monolayers are vertically oriented.[398] The compressibility of condensed films is comparable to that of a long-chain paraffin in bulk.[399] Their state of aggregation depends apparently upon the nature of the end groups and their interactions; the film may be a brittle solid, as with stearic acid under certain conditions, a plastic solid, or an extremely viscous fluid. The long-chain alcohols, for example, have viscosities equivalent to about 10^5 bulk poises, comparable to the value for shoemaker's wax.[400] Compression of the condensed film leads to collapse of the film structure at an area around 19 Å.

In other cases condensation may become complete at areas greater than 20 Å. The values show considerable variation, lying in general between 22 and 30 Å, the condensed film being initially more compressible than in the case considered above, but sometimes approaching the same area and compressibility at high pressures.[401] These compressible films are usually liquid. Their area may be determined primarily in many cases by the size of the polar head groups in the particular stable configuration dictated by the positions of the centers of polarity with respect to the surface and to other parts of the molecule. These can often take up a more compact configuration on further compression, as in the case of the methyl ketones and esters; when this occurs, the packing approaches that characteristic of hydrocarbon chains. If the terminal group is a single polar radical, such as hydroxyl, the properties of the film must be determined by those of the hydrocarbon chains, which prevent the polar groups from approaching more closely than 4.5 Å. Larger groups may hinder compression, as we have said; they may also interact. Thus the large areas and rigidity of condensed films of compounds containing the —CO—NH— linkage have been attributed to hydrogen bond formation between neighboring end groups.[402]

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[394] For confirmation of the gas law for insoluble monolayers at pressures of a few millidynes/cm. see J. Guastalla, Compt. rend. Acad. d. Sc. **206**: 993, 1938.

[395] a. A. Mueller, Proc. Roy. Soc., **114A**: 542, 1927; b. *ibid.*, **120A**: 437, 1928.

[396] A. Mueller, Proc. Roy. Soc., **138A**: 514, 1932.

[397] R. Buckingham, Tr. Faraday Soc., **30**: 377, 1934.

[398] a. A. E. Alexander, Proc. Roy. Soc., **179A**: 486, 1942; it has also been noted that polymorphs of the aliphatic acids are known: G. M. de Boer, Nature, **119**: 50, 1927; *ibid.*: 634; F. Francis, H. Piper, and T. Malkin, Proc. Roy. Soc., **128A**: 214, 1930; in one of these the chains are tilted at about 60° to the basal plane. The effective cross-section of such tilted chains would be about 20 Å at temperatures well below the melting point.

[399] See ref. (370), p. 48. The values agree to within 25%; each dyne/cm. corresponds to a lateral compression of about 4 atmospheres.

[400] a. L. Fourt and W. D. Harkins, J. Phys. Chem., **42**: 897, 1938; b. Data for fatty acids and triglycerides, see M. Joly, Kolloid-Ztschr., **89**: 26, 1939.

[401] Cf. Fig. 14 of ref. (370).

[402] A. E. Alexander, Proc. Roy. Soc., **179A**: 470, 1942; b. The possible importance of the

At higher temperatures gaseous films may condense at a certain pressure to a much more compressible liquid, the discontinuity representing complete condensation appearing at progressively higher areas as the temperature is raised.[403] The structure of such "liquid-expanded" films has been discussed in a classical paper by Langmuir,[404] who shows the equation of state for the liquid film to be

$$(F - F_0)(A - A_0) = kT. \tag{33}$$

This would be expected if the film had a duplex structure, consisting of an upper layer of hydrocarbon chains disposed at random with a constant spreading coefficient F_0 and a lower gaseous layer of polar groups in solution. The equation of state for the latter, considered separately, would be

$$F(A - A_0) = kT, \tag{34}$$

If the temperature is not above the critical temperature for solidification, compression of the liquid film leads to a further sharp discontinuity which signals the transformation to the condensed state. This is not a phase change in the simple sense, however, because it does not occur at constant pressure; the surface pressure rises gradually from the nearly horizontal portion immediately following the break, merging imperceptibly into the curve for the condensed state.

Above the critical temperature for liquefaction the gaseous film can be compressed without discontinuity. It shows increasing deviation from the perfect gas law and increasing resemblance to the liquid expanded films, until finally a condensed film may be formed by way of a discontinuity similar to that observed on condensation of liquid expanded films.[405]

The foregoing outline of the properties of long-chain monolayers contains an indication of the kind of information that can be gained concerning the size, shape, flexibility, and fields of force of the hydrophilic and hydrophobic parts of large molecules. Many other substances have been found to form insoluble monolayers, and some have been studied in sufficient detail, as have for instance some of the sterols (including ergosterol and its irradiation products)[406] and various derivatives of oestrin,[407] for useful deductions as to chemical constitution to be made. In numerous other cases the data are at present mainly of descriptive value, and no attempt will be made to review the subject here, despite the existence of several references to substances of biological interest.[408]

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study of such films in connection with the problem of hydrogen bonding in proteins is discussed by A. E. Alexander and E. K. Rideal, Nature, **147**: 541, 1941.

[403] a. Cf. ref. (370), pp. 58 ff. b. N. K. Adam and G. Jessop, Proc. Roy. Soc., **112A**: 362, 1926.

[404] a. I. Langmuir, J. Chem. Phys., **1**: 756, 1933; b. Langmuir reviews his early work very briefly in Proc. Roy. Soc., **170A**: 1, 1939.

[405] Ref. (370), pp. 62 ff.

[406] J. F. Danielli, and N. K. Adam, Biochem. J., **28**: 1583, 1934; N. K. Adam, F. A. Askew and J. F. Danielli, *ibid.*, **29**: 1786, 1935.

[407] N. K. Adam, J. F. Danielli, G. A. D. Haslewood, and G. F. Marrian, *ibid.*, **26**: 1233, 1932; J. F. Danielli, F. G. Marrian, and G. A. D. Haslewood, *ibid.*, **27**: 311, 1933.

[408] The following references are given to supplement those found in Adam,[370] without claim to completeness: Cellulose, cellulose esters and rubber: H. Zocher and F. Stiebl, Ztschr. f. physik. Chem., **148A**: 401, 1930; Lecithin, cephalin: Zocher and Stiebl, *loc. cit.*; A. E. Alexander,

We shall, however, devote some space to a description of protein monolayers in view of the fact that protein films are present at nearly all biological interfaces.

4. Protein Monolayers at an Air-liquid Interface.—*a*). *The spreading of proteins.*—It has long been known that proteins are slowly precipitated when their aqueous solutions are shaken violently.[409] A still surface of a protein solution also becomes coated spontaneously with a film,[410] but the process ceases before a visible coagulum is produced. The rates of diffusion of protein to the surface are such that there may appear to exist equilibrium surface concentrations of protein for different concentrations of solutions.[411] This is illusory, for in the course of 24 hours practically all the protein in a solution of concentration 10^{-8} and 2–3 mm. deep reaches the surface and stays there;[412a] the film shows almost perfect elasticity and complete insolubility, being reversibly compressible to one-fifth of its area,[413] whereas it should pass back into solution if its presence in the surface represented a true equilibrium between dissolved and adsorbed protein. A similar irreversible change often occurs if a drop of protein solution[410,414] or a minute particle of solid protein is applied to a clean water surface;[415] under suitable conditions, most of the substance remains in the surface layer, and the film thus formed can be studied by the methods already described for insoluble monolayers.

The mechanism of spreading is not completely understood. It has been found that the area of the film produced when a given amount of protein is applied to the surface of a solution varies very greatly with the *p*H and the salt content of the solution. The force-area curves are usually linear, or nearly so, over a wide range of areas, and the area found by extrapolation to zero force ("limiting area")[415] gives a convenient measure of the film area per milligram of protein originally applied to the surface. At the

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T. Teorell and C. G. Aborg, Tr. Faraday Soc., **35**: 1200, 1939: Chlorophyll: W. Sjoerdsma, Nature, **138**: 405, 1936; I. Langmuir and V. J. Schaefer, J. Am. Chem. Soc., **59**: 2075, 1937. Phthiocerol: S. Staellberg and E. Stenhagen, J. Biol. Chem., **143**: 171, 1942; Astacene: J. F. Danielli and D. L. Fox, Biochem. J., **35**: 1388, 1941.

[409] E.g. W. Ramsden, Arch. f. Physiol., **1894**: 517; Ztschr. f. physik. Chem., **47**: 336, 1904.

[410] a. W. Ramsden, Proc. Roy. Soc., **72B**: 156, 1903; b. H. Devaux, Bull. Soc. Sc. Phys. et Nat., Bordeaux, Nov. 19, 1903; p. 3; c. W. V. Metcalf, Ztschr. f. physik. Chem., **52**: 1905.

[411] Cf. W. Ostwald, Ztschr. f. physik. Chem., **15**: 704, 1894; see also E. A. Hauser and L. E. Swearingen, J. Phys. Chem., **45**: 644, 1941.

[412] a. H. Devaux, Compt. rend. Acad. d. sc., **200**: 1560, 1935; b. I. Langmuir and D. F. Waugh J. Gen. Physiol., **21**: 745, 1938; c. cf. L. Michaelis, The Dynamics of Surfaces: Spon and Chamberlain, New York, 1914, p. 33.

[413] H. Devaux, Compt. rend. Soc. de. biol., **119**: 1124, 1935.

[414] a. E. Gorter and F. Grendel, Proc. kon. Akad. Wetensch., Amsterdam, **29**: 1268, 1926; b. Tr. Faraday Soc., **22**: 477, 1926.

[415] a. A. H. Hughes and E. K. Rideal, Proc. Roy. Soc., **137A**: 62, 1932; b. H. Devaux, Bull, Soc. franç. de physique, 18 June, 1937, p. 84.

[415] a. E. Gorter and F. Grendel, Proc. kon. Akad. Wetensch., Amsterdam, **29**: 1268, 1926; b. Tr. Faraday Soc., **22**: 477, 1926.

isoelectric point the limiting area is frequently about 1 m.^{2}/mg.[417] In what has been considered a typical case, it falls off sharply, on either side of this point, and may again increase at *p*H values near the stability limit of the protein.[418] These variations are misleading. The very low areas obtained at some *p*H values sometimes increase gradually if the time interval between spreading and compression is extended.[419] Moreover, formation of films of large limiting area is promoted by the presence of electrolytes.[420] These films, however, give very similar force-area curves to those of low area.[421] Finally, a film of limiting area 0.1 m.^{2}/mg. does not expand when the underlying solution is brought to a *p*H at which films of high limiting area are formed[422] nor do films formed at the isoelectric point contract when the *p*H is changed to a value less favorable for spreading.[423] Thus, it is probable that limiting areas represent only variations in the amount of protein which enters the surface, a certain fraction being lost by diffusion into the underlying solution or entangled in the upper surface of the highly viscous film. Since it is very hard to determine how much protein is lost in this way, the numerous studies[424] of limiting area as a function of *p*H and other variables tell us only about the mechanism of spreading, and very little about the real properties of protein monolayers. Probably the maximum of about 1.0 m.^{2}/mg. is not far from the true value of the limiting area, but small variations with *p*H and other properties of the substrate would be very difficult to establish.[425]

b). *Force-area curves of proteins.*—We have referred to the high reversible compressibility of protein monolayers. This statement requires qualification before the shape of the force-area curves can be discussed quantitatively. Firstly, the course of the curves depends upon the rate of compression, having a smaller slope when compression is carried out slowly.[426]

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[417] Some data illustrating this point have been compiled by H. Neurath and H. B. Bull., Chem. Rev., **23**: 391, 1938.

[418] E. Gorter, J. van Ormondt and F. J. P. Dom, Proc. kon. Akad. Wetensch., Amsterdam, **35**: 838, 1932.

[419] E. Gorter and G. T. Philippi, Proc. kon. Akad. Wetensch., Amsterdam, **37**: 788, 1934.

[420] a. E. Gorter, Proc. kon. Akad. Wetensch., Amsterdam, **37**: 20, 1934; b. J. Gen. Physiol., **18**: 421, 1935.

[421] J. B. Bateman and L. A. Chambers, J. Phys. Chem., **45**: 209, 1941.

[422] C. V. Seastone, J. Gen. Physiol., **21**: 621, 1938.

[423] a. J. B. Bateman and L. A. Chambers, J. Chem. Phys., **7**: 244, 1939; b. D. G. Dervichian, Nature, **144**: 629, 1939.

[424] For bibliography of papers by Gorter and others on this subject, see H. Neurath and H. B. Bull, Chem. Rev., **23**: 391, 1938.

[425] Methods for promoting rapid and uniform spreading: (i) by adding surface-active soluble substance to the protein solution: see a. D. G. Dervichian, Nature, **144**: 629, 1939; b. S. Staellberg, Tr. Faraday Soc., **35**: 1416, 1939; (ii) by applying protein along a line instead of at a point in the surface: c. I. Langmuir, Cold Spring Harbor Symp., **6**: 171, 1938; d. I. Langmuir and D. F. Waugh, J. Am. Chem. Soc., **62**: 277, 1940.

[426] a. E.g., L. Fourt and F. O. Schmitt, J. Phys. Chem., **40**: 989, 1936; b. I. Langmuir and D. F. Waugh, J. Am. Chem. Soc., **62**: 2771, 1940.

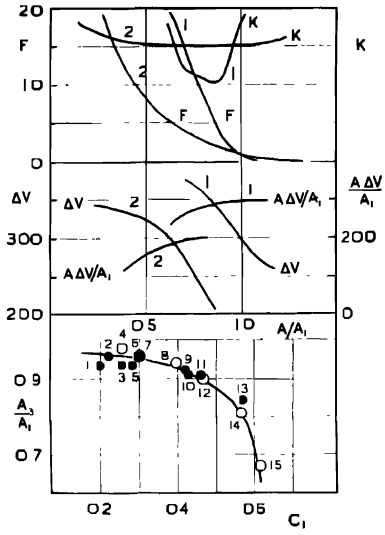

FIG. 22. Top: force-area curves (marked F) and surface compressibility curves (marked K) for ovalbumin (1) and gliadin (2). Abscissa is A/A_1, where A_1 is area of film at a surface pressure of 1 dyne/cm. Surface pressure ordinate is in dynes/cm., and compressibility ordinate is in arbitrary units.

Middle: the corresponding curves of surface potential (marked ΔV) and of $A.\Delta V/A_1$.

Bottom: the ratio A_3/A_1 plotted against the content of relatively hydrophilic amino-acids for several proteins (explanation in text).

Open circles: data of I. Langmuir and D. F. Waugh, J. Am. Chem. Soc., **62**: 2771, 1940.

Closed circles: unpublished data of J. B. Bateman and L. A. Chambers.

1. serum albumin
2. serum globulin
3, 4. insulin
5. acetyl insulin
6, 7. egg albumin
8. edestin
9. rabbit myosin
10. casein
11, 12. zein
13, 14. gliadin
15. gelatin

Secondly, the curves always show some hysteresis upon expansion, and sometimes a permanent decrease in area.[426b] Application of the theory of pressure solubility suggests that these changes are associated with the displacement of substances of molecular weight between 1000 and 2000,[426b] but the evidence does not seem to eliminate the possibility that the effect is due to elution of unspread protein. In either case, reproducible closed hysteresis loops can be obtained from films after "conditioning" at high surface pressures, provided the times of compression and expansion are not altered.

At least two types of force-area curves have been observed. The majority of proteins give curves of the type shown in Fig. 22 for ovalbumin, with a well-marked linear region that changes at the low-pressure end to a curve of gradually decreasing slope. The lower end of the linear portion is a secondary transformation point at which the compressibility, $d \ln A/dF$, has a minimum value.[427] The low pressure region often has a fairly definite area of zero surface pressure, indicating that the film is coherent; in other cases, particularly for films on acid and alkaline substrates, it becomes asymptotic to the A-axis. The second type of curve is roughly hyperbolic, so that the transformation point is absent, and the compressibility is practically constant over a wide range of areas. Such curves have been recorded for gliadin,[128] gelatin,[428d] and edestin.[428d]

Remembering that the true areas per molecule are uncertain, we must select ways of measuring and comparing force-area curves in terms of arbitrary units of

[427] J. B. Bateman and L. A. Chambers, J. Chem. Phys., **7**: 244, 1939.

[428] a. A. H. Hughes and E. K. Rideal, Proc. Roy. Soc., **137A**: 62, 1932; b. J. S. Mitchell, Tr. Faraday Soc., **33**: 1129, 1937; c. E. G. Cockbain and J. H. Schulman, *ibid.*, **35**: 1266, 1939; d. I. Langmuir and D. F. Waugh, J. Am. Chem. Soc., **62**: 2771, 1940.

area. An obvious method is to express the areas, A, in terms of that at a standard pressure of, say, one dyne/cm. For a more detailed analysis, the actual compressibility, $d(\ln A)/dF$, can be used, while special significance should attach to the singular point of minimum compressibility.

Langmuir and Waugh[129] have published $A/A_1 - F$ curves for a number of proteins. The areas A/A_1 at 25 dynes/cm. range from 0.06 and 0.14 for gelatin and gliadin, respectively, to 0.58 for insulin. It is not certain that these curves are suitable for accurate quantitative comparisons. Protein films tend to become more compressible the larger the relative area of free surface available for spreading, so that if varying amounts of protein are spread initially over the whole surface of the Langmuir trough and then compressed, both A/A_1 and the minimum compressibility values vary with the actual area in cm.^{2} of the film at some standard pressure.[130] There is little doubt that the structures of the films under these various conditions differ, and it is obviously necessary to standardize the conditions before comparable curves can be constructed for different proteins, or for the same protein on different substrates. Roughly speaking, the minimum compressibility values for all proteins tend to converge when the area for spreading is very restricted, while they approach individual limiting values, which vary in a highly characteristic manner with pH, when infinite area is available.[431] Closely correlated with these changes is the co-existence of a coherent and an expanded phase at very large areas[432]—the viscous "B" films and the mobile hysteresis-free "A" films of Joly.[433] According to Guastalla[434] the A films are gaseous at areas of about 100 m.^{2}/mg., with apparent molecular weights of 40,000, 27,000, and 12,000 for ovalbumin, gliadin, and hemoglobin—certainly of the right order of magnitude for protein molecules. On the other hand, it is possible that there is some relationship between the pressure-soluble materials found by Langmuir and Waugh in compressed films and this mobile phase, although the latter, compressed separately, certainly gives a characteristic protein curve.[432a] The whole question requires more detailed study.

c). *Other properties of protein monolayers.*—The surface potentials vary during compression in somewhat the manner shown in Fig. 22.[435] The product $\Delta V \cdot A/A_1$ corrects for changes in the surface density. It is evident

[429] I. Langmuir and D. F. Waugh, J. Am. Chem. Soc., **52**: 2771, 1940.

[430] J. B. Bateman and L. A. Chambers, J. Phys. Chem., **45**: 209, 1941, and numerous unpublished measurements; see also J. B. Bateman, L. A. Chambers, and H. E. Calkins, J. Immunol., **39**: 511, 1940.

[431] L. A. Chambers and J. B. Bateman, unpublished experiments, 1940.

[432] a. J. B. Bateman and L. A. Chambers, J. Phys. Chem., **45**: 209, 1941; b. D. G. Dervichian, Compt. rend. Acad. d sc. **209**: 16, 1939; c. M. Joly, J. chim. phys., **36**: 285, 1939.

[433] M. Joly, Compt. rend. Acad. d. sc., **208**: 975, 1939.

[434] J. Guastalla, Compt. rend. Acad. d. sc., **208**: 1078, 1939.

[435] a. J. H. Hughes and E. K. Rideal, Proc. Roy. Soc., **137A**: 62, 1932; b. J. H. Schulman and E. K. Rideal, Biochem. J., **27**: 1581, 1933; c. G. T. Philippi, "On the Nature of Proteins": Thesis, University of Leyden, N. V. Noord-Hollandsche Uitgeversmaatschappi, Amsterdam, 1936; d. M.G. ter Horst, Rec. trav., **55**: 33, 1936.

that considerable rearrangement of polar groups occurs at a certain stage of compression. Large changes occur also at acid and alkaline reactions; these are greatly reduced by the presence of salts in the substrate.[435]

Protein films exhibit remarkable changes of state during compression. The most elementary facts can be demonstrated by very simple observations: the behavior of dust particles;[437] the very characteristic patterns produced when a spreading oil drop displaces the film;[438,439] the passage of film material through a surface channel 5–10 mm. wide.[440] Practically all protein monolayers become gelatinous and elastic when compressed. The pressure at which solidification occurs for isoelectric films, or films on pure water, varies over very wide limits for different proteins, and also depends to a considerable extent upon the history of the film; in many cases the accompanying structural changes occur slowly, and gelation may take a few seconds or a few hours. It can usually be reversed by expansion or mechanical disturbance, the films being markedly thixotropic. Egg albumin and pepsin are characteristically rigid at very low pressures, while insulin and gliadin remain fluid, when freshly spread, up to 10 or 15 dynes/cm. These differences are not correlated in any obvious way with other properties. Gliadin, which forms liquid films, is very compressible; insulin, which also forms liquid films, is almost as incompressible as ovalbumin, which is solid. Aged gliadin films appear to solidify at a lower pressure than fresh films, and at a point coinciding with an inflection of the force-area curve,[441] but no such coincidence can be noted in the case of other proteins. Films of ovalbumin on sodium tungstate may be extremely rigid at 0.1 dyne/cm. and yet show the usual inflection at higher pressures. On the other hand, the changes in compressibility which mark the transition from "B" to "A" films, as the area available for spreading is increased, are accompanied by an increase in the gelation pressure; according to Joly,[442] the pure A films, obtained by spreading at pressures below 0.15 dyne/cm., remain fluid when compressed.

Quantitative studies with the oscillating disc method in its several forms[443] have given values for the absolute viscosities of films in the very limited low pressure region which is free from anomalous effects, and apparent values, at high amplitudes of oscillation, in the plastic region. The enormous range of variation is illustrated by the following values obtained at a surface pressure of

[436] For a detailed, but presumably very tentative, discussion, see G. T. Philippi, ref. (435c).

[437] a. H. Devaux, Bull. Soc. Sc. Phys. et Nat., Bordeaux, 19 Nov., 1903, p. 3.

[438] "Si on met une trace de substance grasse sur le voile on le brise en étoile avec une nétteté remarquable": H. Devaux, Compt. rend. Soc. de biol., **119**: 1124, 1935.

[439] a. V. J. Schaefer, J. Phys. Chem., **42**: 1089, 1938; b. I. Langmuir, Cold Spring Harbor Symp., **6**: 171, 1938.

[440] J. B. Bateman and L. A. Chambers, Nature, **142**: 1158, 1938, and unpublished data.

[441] J. S. Mitchell, Tr. Faraday Soc., **33**: 1129, 1937.

[442] M. Joly, J. chim. phys., **36**: 285, 1939.

[443] a. M. Joly, J. chim. phys., **36**: 285, 1939; b. L. Fourt, J. Phys. Chem., **43**: 887, 1939; c. I. Langmuir, Cold Spring Harbor Symp., **6**: 171, 1938.

6 dynes/cm.:[443c] gliadin, 0.001 C.G.S. units; zein, 0.002; insulin 0.021; trypsin, 0.12; pepsin, edestin, 2.3. Films on acid solutions at pressures below the gelation point are about 30 times less viscous than at the isoelectric point when first spread,[444] and do not at once solidify at higher pressures. The acid reaction does not, however, necessarily abolish the ability to form a gel; it only prolongs the relaxation time from a few seconds to a few hours.[445]

The relation between the various elastic moduli has not been worked out fully. The rather elaborate experiments that might be needed would certainly yield useful information concerning the nature of the binding forces in protein monolayers. The oil expansion patterns show clearly enough that the compressed films are anisotropic, and it is possible that quantitative data could be obtained by a refinement of this technique.

d). *The structure of protein monolayers.*—The insolubility of protein monolayers suggests at once that they have an open polypeptide chain structure. This possibility invites comparison with the molecular arrangement in fibers. If the chains had the β-keratin configuration, with the side-chains lying in the plane of the surface, the area per amino-acid residue would be about 33 Å.^{2} If the side-chains were perpendicular to the surface and spaced so as not to get in each other's way, the area would be 16 Å.^{2} In an α-keratin configuration or something similar, with side-chains perpendicular, the area would be about 6 Å.^{2} The corresponding values in m.^{2}/mg., assuming an average residue weight of 125, would be about 1.6, 0.8 and 0.3 respectively. We have already referred to the difficulty of assigning absolute values to the experimental areas, but the range of areas found for gliadin films when apparently spread without loss of material is from 1.6 to 0.5 m.^{2}/mg.[446] At first sight this suggests a change in side-chain orientation between 1.6 and 0.8 m.^{2}/mg. and some sort of backbone folding at lower areas. As a general explanation this is inadequate. The surface electric moment is constant between 1.6 and 1.0 m.^{2}/mg., so that probably no change in configuration occurs in this range. This must mean that the side-chains are tilted all the time, and it has been suggested that the compressibility at very large areas arises from the elimination of water of hydration.[447]

The disposition of the polar and nonpolar side-chains presents some difficulty. In an extended polypeptide chain composed of *l*-α-amino-acids, neighboring side-chains are on opposite sides of the main chain. In general, however, polar and nonpolar chains will not alternate in this regular manner, so that it will not be possible for all the hydrophilic groups to point downward and the hydrophobic groups up. If the chains are folded in the plane of the surface by rotation about single bonds, any desired arrangement of

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[444] M. Joly, J. chim. phys., **36**: 285, 1939.

[445] L. Fourt, J. Phys. Chem., **43**: 887, 1939.

[446] a. A. H. Hughes and E. K. Rideal, Proc. Roy. Soc., **137A**: 62, 1932; b. J. S. Mitchell, Tr. Faraday Soc., **33**: 1129, 1937; c. E. G. Cockbain and J. H. Schulman, *ibid.*, **35**: 1266, 1939.

[447] G. T. Philippi, On the Nature of Proteins: Thesis, University of Leyden, N.V. Noord-Hollandsche Uitgeversmaatschappi, Amsterdam, 1936.

side-chains can be obtained,[448] but the X-ray pattern of protein multilayers seems[449] to show the presence of parallel chains, while the oil expansion patterns prove that there are lines of weakness at right angles to the direction of compression. It is more probable that the folding occurs in a plane perpendicular to the surface, so that segments of polypeptide chain tend to float up out of the surface if the side-chain is hydrophobic, even if it is oriented downward, while the whole segment tends to be immersed if the side-chain is hydrophilic.

According to this idea, the chain molecules can be regarded to a first approximation as linear polymers capable of acting independently in segments, subject to restraints imposed by the properties of neighboring segments. Langmuir and Waugh,[450] applying the theory of pressure solubility, have sought to explain the differences in compressibility of different proteins in terms of differences in the proportions of hydrophilic side-chains. Dividing the amino-acid constituents of proteins into three groups according to the degree of hydrophilic character of their side-chains, they have shown that the change of area upon compression from 1 to 3 dynes/cm. is closely correlated with the content of acids of the most hydrophilic class (Class I), while the contraction between 1 and 25 dynes/cm. is related to the ratio of the most hydrophobic (Class III) to the most hydrophilic acid contents. The first correlation they interpret as the result of reversible pressure displacement (solubility) of the Class I acids. The area at 25 dynes/cm. is assumed to be a measure of the more strongly hydrophobic residues.

It is significant that the correlation thus established involves the actual proportions of the various acids, or their relative volumes, and not their molecular proportions. This must surely indicate that at low pressures the area occupied by each polypeptide unit is a function of the volume of the side-chain attached to it, although it does not necessarily mean that the side-chains are coplanar with the polypeptide chains; this seems rather improbable at areas less than 1.6 m.²/mg.

The actual relations given by Langmuir and Waugh are

$$\frac{c_3}{c_T} = 1.88 - 1.67\frac{A_3}{A_1} \tag{35}$$

and

$$\frac{c_3}{c_1} = 0.26 + 2.37\frac{A_{25}}{A_1}, \tag{36}$$

where c_T is the total fraction of the protein accounted for by amino-acid analyses and c_1 and c_3 are the proportions of acids of Classes I and III. The use of c_3/c_T instead of c_3 involves the assumption that the acids unaccounted for in the analytical data are distributed in Classes I, II, and III in the same proportions as those already found. This would be hard to justify. Accordingly, it seems

[448] E. G. Cockbain and J. H. Schulman, Tr. Faraday Soc. **35**: 1266, 1939.
[449] W. T. Astbury, F. O. Bell, E. Gorter, and J. van Ormondt, Nature, **142**: 33, 1938.
[450] I. Langmuir and D. F. Waugh, J. Am. Chem. Soc., **62**: 2771, 1940.

that the remarkable exactness of equations (35) and (36) is largely fortuitous, and in presenting Langmuir and Waugh's data in Fig. 22 we have preferred to use mean values of c_3 and c_3/c_T as a better approximation to the true values of c_3. When this is done, with the inclusion of data for a few other proteins, the relationship, although still remarkable, takes the form shown. This is easy to understand, because one would expect that if Class I residues occurred in several neighboring positions along a polypeptide chain, their pressure displacement would occur more readily than that of isolated residues, and might also tend to cause the simultaneous submergence of more hydrophobic residues.

The weak point in the picture is that it leaves the region of compression between 2 and 25 dynes/cm. unaccounted for. Clearly some process must occur here other than the displacement of Class II acids, since it is precisely in this region that the compressibility increases from its minimum value. This minimum value may signal the complete displacement of Class I acids; the increase on the high pressure side is unaccounted for unless, indeed, one again invokes dehydration, as some authors have done already.[451]

5. Properties of Mixed Films and Membranes.—We may readily imagine mixtures which, by virtue of isomorphism or of specific interactions, remain of constant composition upon compression; others in which one component is pressure-displaceable but not soluble. In others we may expect true pressure-solubility of one component, with a Gibbs equilibrium, modified by possible specific interactions, between surface and bulk phases. There may also be adsorption of dissolved substances, in which specific affinities may give the reaction an irreversible character. Within this wide range of possibilities, we shall find important and suggestive analogies with the behavior of biological systems. In the mixed films of insoluble substances we have, on the one hand, a mosaic structure of molecular dimensions; in the pressure-displacement of one component into the substratum we see the formation of a bimolecular layer reminiscent of the lipoprotein membranes that have been so widely discussed as a model for the plasma membrane; and in the possible effects of surface-active solutes we foresee influences which must have a counterpart in the relationship between biological fluids and the interfaces with which they come into contact.

a). *Mixed monolayers of insoluble substances.*—Mixtures of saturated straight-chain compounds show the comparatively simple behavior that would be expected. In general, a mixed film of substances giving liquid films is itself liquid, with a molecular area not greatly different from the mean area of the components, while a liquid-expanded film tends to be condensed by a substance which forms a condensed film.[452] This is what would be expected if the degree of condensation of a film depends upon the time average area of contact between neighboring chains. The evidence for

[451] a. A. H. Hughes and E. K. Rideal, Proc. Roy. Soc., **137A**: 62, 1932; b. R. J. Fosbinder and A. E. Lessig, J. Franklin Inst., **215**: 579, 1933; c. G. T. Philippi, On the Nature of Proteins: Thesis, University of Leyden, N.V. Noord-Hollandsche Uitgeversmaatschappi, Amsterdam, 1936.

[452] W. D. Harkins and R. T. Florence, J. Chem. Phys., **6**: 847, 1938.

head-group interactions is slight in these cases, although an equimolecular mixture of stearyl alcohol and stearic acid gives a solid film of unusually large area, while there is strong evidence for interaction between acids and amines.

The condensation of expanded myristic acid films by tripalmitin and pentaerythritol tetrapalmitate falls into line with the above argument. There are other condensing effects which seem to be more specific. Myristic acid is also condensed by cholesterol; p-nonyl phenol is condensed by cholesterol, but not by tripalmitin.[453,454] The determining factor here might be the smaller number of degrees of freedom of the cholesterol molecule.

Mixed films containing unsaturated chains have also received some attention.[455] Mixtures of oleic acid and triolein follow Harkins' law of averages, as one would expect. Oleic acid and triolein with hexadecyl alcohol give films of larger area than the average of the components; upon compression, the oleic acid is squeezed out from the film. Florence and Harkins[455b,c] attribute this effect to the bending of the carbon chain in oleic acid at the double bond, this steric factor serving to lessen the energy of contact between the oleic acid molecule and its neighbors. In support of this contention they cite experiments with the corresponding *trans*-compound, elaidic acid, which has a straighter chain than oleic acid. In the most striking case, they find that a mixture of calcium elaidate and stearyl amine, which separately form expanded films, gives a highly condensed film, while the corresponding oleate-amine film was more expanded than a film of oleate alone. In the case of mixtures of oleic acid and tripalmitin[455a] there is an effect of condensation. This may be due to favorable steric relationships conditioned by the fixed arrangement of carbon chains in the triglyceride molecule. Upon compression of this film it is the tripalmitin, and not the oleic acid, which collapses.

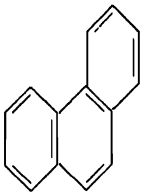
The sensitiveness of the surface method in detecting interactions which might otherwise pass unnoticed has been put to use in an investigation of mixtures of polycyclic hydrocarbons and sterols.[456] These hydrocarbons do not, of course, form pure monolayers. Some, such as phenanthrene (i), also do not form mixed films with sterols; others, by virtue presumably of steric factors favoring close contact, are able to remain associated with the sterol monolayer at certain surface pressures, although they are ejected from the film at high pressures. It seems that two types of film may be formed. The components may be mutually soluble; the course of pressure-displacement is then analogous to the variation of osmotic pressure with concentration. A typical case is that of 10-methyl-1,2-benzanthracene

[453] N. K. Adam, ref. (370), p. 70.

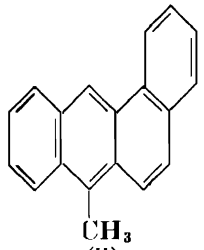
[454] Cf. Leathes, quoted by Adam, ref. (370).

[455] a. J. H. Schulman and A. H. Hughes, Biochem. J., **29**: 1243, 1935; b. R. T. Florence and W. D. Harkins, J. Chem. Phys., **6**: 856, 1938; c. Other differences between *cis*- and *trans*-olefinic chains are referred to by E. K. Rideal, Nature, **144**: 693, 1939.

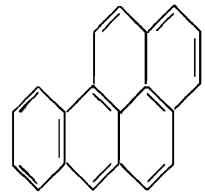
[456] a. G. H. A. Clowes, W. W. Davis, and M. E. Krahl, Am. J. Cancer, **35**: 98, 1939; b. *ibid.*, **37**: 453, 1939; c. W. W. Davis, M. E. Krahl, and G. H. A. Clowes, J. Am. Chem. Soc., **62**: 3080, 1940.



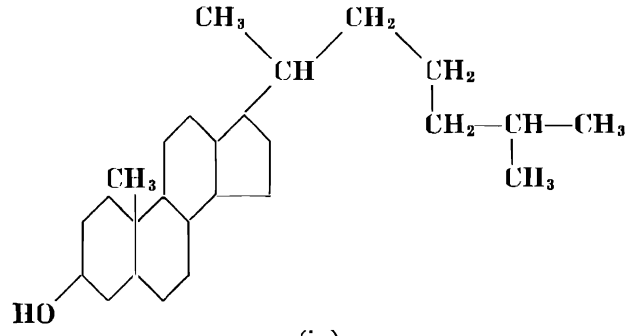
(i)



(ii)



(iii)



(iv)

(ii) mixed with cholesterol (iv). In the second type, well illustrated by 3-4-benzpyrene and cholesterol, there is evidence that complexes are formed. This type of effect is especially pronounced in presence of stearic acid, and tends, on the other hand, to be abolished by high temperatures and by the presence of double bonds or of particular substituents in the sterol molecule.

Both 10-methyl-1,2-benzanthracene and 3-4-benzpyrene are carcinogenic; 10-butyl-1,2-benzanthracene, which also forms mixed films of the "solution" type, is not. Thus there is at first sight no correlation between surface behavior and carcinogenic activity. It has however been suggested that the solution type of interaction may be important "as a mechanism of transport of polycyclic hydrocarbons from the point of administration to the site of action in the animal organism"[455c] while the association type may provide a mechanism for the binding of these substances in oriented biological structures.

Reversible displacement of a component into the aqueous phase has been observed in compression of a mixed monolayer of gliadin and cholesterol.[457] In this submersion of the protein fraction, one is reminded of the pressure displacement of residues in pure protein monolayers, but it must be assumed that in the present case the protein remains as an adsorbed underfilm by virtue of its insolubility, and not necessarily because of any anchorage to the more hydrophobic component. The compressed films have the fluid character of pure cholesterol monolayers. In remarking upon the resem-

[457] J. H. Schulman, Proc. Roy. Soc., **155A**: 701, 1936; Tr. Faraday Soc. **33**: 1116, 1937.

blance of the expanded film to a mosaic lipoprotein membrane[458] and of the compressed film to a stratified membrane[459] one is tempted to suggest that the two theories of membrane structure can perhaps be reconciled.

b). Adsorption and penetration.—The surface tension of solutions of capillary-active substances is determined by the Gibbs equation. In the absence of specific interactions this remains true when the surface of such a solution is covered by an insoluble monolayer. Thus the minimum surface pressure of the monolayer is defined by the nature and concentrations of capillary-active substances present in the substrate. If this equilibrium pressure is low enough, the monolayer and the Gibbs layer will remain in equilibrium; alternatively, injection of surface-active material under a monolayer will result in penetration and dispersion of the latter and rise of pressure until the equilibrium value is attained. Compression of this mixed film will be characterized, first, by a decrease in area at constant pressure until the soluble portion has been completely expelled, and then by the usual compressibility curve of the homogeneous monolayer. This process will be reversible. If the equilibrium pressure of the Gibbs layer happens to be above the collapse pressure of the monolayer, the latter will be completely and irreversibly displaced from the surface.

Irreversible displacement of monolayers by soluble substances has been observed in numerous instances, of which we may mention the penetration and displacement of protein and of tripalmitin monolayers by sodium oleate.[460] Instances of simple reversible squeezing-out of the Gibbs layer at constant pressure are not so easy to find. It seems likely that this effect is nearly always masked by head-group interaction, so that after expulsion of the hydrophobic chains that have penetrated the monolayer, the head groups remain adsorbed with the chains oriented downward. The adsorbed layer may prevent close packing of the monolayer and cause the compressibility curve to be of the expanded type, or it may cause the formation of cross-linkages between head groups and thus decrease the area of an expanded film. This was observed by Askew in his study of films on aqueous butyl alcohol.[461] Expansion of the condensed film has been found with short-chain, capillary-active solutes, such as benzoic acid and resorcinol and monolayers of hexadecylamine.[462] Dicarboxylic acids such as azelaic, $HOOC(CH_2)_7COOH$, are also active. The adsorbed under-layer is best detected by its effect on the phase-boundary potential, which may be considerable.

Penetration occurs more and more readily as the hydrophobic portion of the penetrant is increased. In penetration by octyl alcohol, caprylic

[458] Cf., R. Höber, J. Cell. Comp. Physiol., **7**: 367, 1936.

[459] Cf., J. F. Danielli and H. Davson, *ibid.*, **5**: 495, 1935; J. F. Danielli, *ibid.*, **7**: 393, 1936. Compare also the phase inversion theory of ion antagonism: G. H. A. Clowes, Ztschr. f. physik. Chem., **20**: 407, 1916.

[460] J. H. Schulman, and A. H. Hughes, Biochem. J., **29**: 1243, 1935.

[461] F. A. Askew, cited in ref. (370), p. 98.

[462] E. G. Cockbain and J. H. Schulman, Tr. Faraday Soc., **35**: 716, 1939.

acid, and *n*-ethyl phenol, it has been found that the increment for each additional CH_2 group is greater than that given by Traube's rule, the corresponding free energy decrease being around 1000 cal./mole per CH_2 instead of the value 640 found at an air-water interface.[463] This result may express the influence of Van der Waals forces between the hydrophobic portions of penetrant and monolayer. Penetration is also favored by the presence of suitable polar groups capable of reacting with the polar groups of the monolayer; Rideal[464] considers this to be an essential step in the process of penetration.

The most striking cases of penetration are those in which there is considerable interaction, which may even amount to stoichiometric complex formation. All semblance to the behavior demanded by the Gibbs adsorption equation may be absent in these cases, penetration continuing up to surface pressures far higher than those corresponding to Gibbs adsorption of the penetrant in absence of a monolayer. Injection of a minute amount of sodium hexadecyl sulfate under a hexadecyl alcohol monolayer at a pressure of 16 dynes/cm. causes, in the course of two minutes, a rise of pressure to 40 dynes/cm. and a large rise of phase boundary potential.[465] Hexadecyl alcohol and sphingosine form a very stable, rigid complex, from which the sodium oleate cannot be expelled by compression.[466] Sterol films are penetrated by the hemolytic glucosides saponin, digitonin, and psychosin in very small concentrations[466] and by bilirubin and the capillary-active porphyrins.[467]

Nearly all lipoid penetrants or substances capable of dispersing protein monolayers are hemolytic. A distinction has been made[468] between penetrants for protein and sterol monolayers and some tentative conclusions have been drawn concerning the constitution of certain membranes on the basis of the lytic activity of these two groups of substances. Other analogies suggest that a close study of penetration and adsorption in monolayers must lead to a more unified theory of pharmacological action, and, in particular, to a more sophisticated use of the idea of lipoid solubility. The new molecular point of view should eliminate some of the early anomalies and offer some suggestions for the synthesis of new anesthetics, anticoagulants and sensitizers; it may give us closer understanding of the mechanism of transport and operation of lipotropic and carcinogenic substances, and of vitamins or their precursors. At present, however, there are no sufficiently systematic investigations to show whether these hopes will be justified.

[463] K. G. A. Pankhurst, Proc. Roy. Soc., **179A**: 393, 1942.

[464] E. K. Rideal, Proc. Roy. Soc., **155A**: 684, 1936.

[465] J. H. Schulman and A. H. Hughes, Biochem. J., **29**: 1243, 1935.

[466] a. J. H. Schulman and A. H. Hughes, Biochem. J., **29**: 1243, 1935; b. J. H. Schulman, Tr. Faraday Soc., **33**: 1116, L93, 1937; c. E. K. Rideal, Nature, **144**: 693, 1939; d. I. Langmuir, V. J. Schaefer, and H. Sobotka, J. Am. Chem. Soc., **59**: 1751, 1937.

[467] E. Stenhagen and E. K. Rideal, Biochem. J., **33**: 1591, 1939.

[468] a. J. H. Schulman, Tr. Faraday Soc., **33**: 1116, 1937; b. E. K. Rideal, Nature, **144**: 693, 1939.

Adsorption is not necessarily followed by penetration. There are numerous substances capable of being attached at the under surface of a monolayer, ranging from simple metallic ions to the multilayers of protein presumed by Hauser and Swearingen[469] to be formed beneath the primary monolayer at the surface of an ovalbumin solution. All are capable of influencing profoundly the properties of the monolayer. Tartrazine, by combining with the free NH_2 groups, enables ovalbumin to spread rapidly on acid solutions[470] and causes liquefaction and increased pressure solubility of the resultant monolayer.[471] Spermidine promotes spreading of pepsin on alkaline solutions.[470] By becoming attached to two or more barium atoms of a barium stearate monolayer, or to two NH_3 ions of hexadecylamine, the polymetaphosphate "Calgon" causes a remarkable increase in the rigidity of these films.[472b] Adsorption at several points is also probably responsible for the solidification of some protein monolayers spread on solutions of formaldehyde[473] or of the multibasic substance, tannic acid, at neutral reaction,[474] or of sodium tungstate at *p*H 2.[475] A thorough analysis of these effects, which show considerable variations among different proteins, should contribute to our knowledge of the geometrical distribution of polar groups in monolayers, at the same time presenting an analogy to the action of agglutinins.

The adsorption of metallic ions, which in minute concentrations may profoundly affect the properties of monolayers of stearic acid and other substances containing carboxyl groups, has been studied by Langmuir and Schaefer.[475,477] The di- and tri-valent ions are able in occupying two or more end groups to confer considerable rigidity. Under suitable conditions this effect can be antagonized by monovalent cations, an action which can be expressed in analytical terms by the use of a semiempirical adsorption isotherm with properly chosen constants.[476]

c). *Bimolecular films and membranes: study of the liquid-liquid interface.*— Proteins spread very rapidly at a liquid-liquid interface to form insoluble monolayers of much the same area as those at the air-water interface, and with

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[469] E. A. Hauser and L. E. Swearingen, J. Phys. Chem., **45**: 644, 1941.

[470] a. E. Gorter, H. van Ormondt, and T. M. Meyer, Biochem. J., **29**: 38, 1935; b. E. Gorter, Tr. Faraday Soc., **33**: 1125, 1937.

[471] J. B. Bateman and L. A. Chambers, unpublished data.

[472] a. I. Langmuir and V. J. Schaefer, J. Am. Chem. Soc., **58**: 284, 1936; b. *ibid.*, **59**: 2400, 1937.

[473] I. Langmuir, V. J. Schaefer, and D. M. Wrinch, Science, **85**: 76, 1937.

[474] E. G. Cockbain and J. H. Schulman, Tr. Faraday Soc. **35**: 716, 1266, 1939.

[475] J. B. Bateman and L. A. Chambers, unpublished data.

[476] a. I. Langmuir and V. J. Schaefer, J. Am. Chem. Soc., **58**: 284, 1936; b. *ibid.*, **59**: 2400, 1937.

[477] Compare also R. J. Myers and W. D. Harkins, Nature, **139**: 368, 1937; J. S. Mitchell, E. K. Rideal, and J. H. Schulman, *ibid.*, **139**: 625, 1937; C. Robinson, *ibid.*, **139**: 926, 1937.

much the same mechanical properties.[478,479] The force-area curves resemble those of gliadin and are said to follow closely the behavior suggested by Langmuir's theory of the liquid expanded state.[480] The films can be compressed until the interface takes on an opalescent appearance, resulting from the formation of numerous ripples in the monolayer surface,[478b,481] a phenomenon that can also be observed by spreading a froth of egg white on a sheet of stretched rubber: upon relaxation, the hexagonal cell-walls are thrown into sinous folds.[482] In the former case, it is probable that the folding occurs when the interfacial tension becomes zero; oil drops covered with a folded film of this type can form a thermodynamically stable emulsion. The occurrence of such films also favors the preparation of protein membranes.[481] The film can be folded double by lowering a wire loop vertically through the interface. Membranes made in this way from a film at a pure benzene surface soon break in water, but much more stable ones are produced if the benzene contains a little lecithin. These consist, probably, of two layers of protein separated by one or two of lecithin. Danielli, two years earlier,[483] had described spherical shell oil films of the same type obtained by allowing a heavy drop of a salt solution containing a little protein to fall through the interface between an oil and a dilute protein solution. The tension of these membranes has not been mentioned; it is probably very low. The low surface tension of certain cells, such as the unfertilized *Arbacia* egg, has been attributed to the effect of a protein component of the plasma membrane, and a similar effect has been demonstrated directly in the case of the oil droplet from a mackerel egg, which in its natural environment has an interfacial tension of only 0.6 dyne/cm. In water the value rises to 10 dynes/cm., falling again to the original value on being placed in an aqueous egg extract.[484–494] This implies that the protein component is adsorbed in soluble or elutable form; more work is needed to determine the conditions under which soluble films can be produced *in vitro*.

6. Chemical Reactions of Monolayers.—It is somewhat difficult to make a valid quantitative comparison between reactions involving monolayers and the corresponding reactions as they occur in bulk. In a monolayer, the different parts of the molecule find themselves in widely different environments, and an approximate definition of the analogous conditions in

[478] a. F. A. Askew and J. F. Danielli, Proc. Roy. Soc., **155A**: 695, 1936; b. H. Devaux, Compt. rend. Acad. d. sc., **202**: 1957, 1936; c. I. Langmuir, V. J. Schaefer, and D. M. Wrinch, Science, **85**: 76, 1937; d. J. F. Danielli, Cold Spring Harbor Symp., **6**: 190, 1938.

[479] Phenomena depending upon the adsorption of proteins on oil droplets, etc.: inactivation of staphylococcus toxin, J. M. Johlin and R. H. Rigdon, J. Immunol., **41**: 233, 1941; removal of proteins from solution by shaking with chloroform, M. G. Sevag, Biochem. Ztschr., **273**: 419, 1934, and elsewhere; protective action of adsorbed protein on oil-in-water emulsions, R. L. Nugent, J. Phys. Chem., **35**: 449, 1932; electrophoretic mobility of fat droplets in blood, A. C. Frazer, Physiol. Rev., **20**: 561, 1940.

[480] A. E. Alexander and T. Teorell, Tr. Faraday Soc., **35**: 733, 1939.

[481] I. Langmuir and D. F. Waugh, J. Gen. Physiol., **21**: 745, 1938.

[482] D. A. W. Thompson, On Growth and Form: Macmillan, New York, 1942. Thompson points to the possible analogy between these ripples and those found in the endothelium of blood vessels and elsewhere.

[483] J. F. Danielli, J. Cell. Comp. Physiol., **7**: 393, 1936.

[484–494] Review: E. N. Harvey and J. F. Danielli, Biol. Rev., Cambridge, **13**: 319, 1938.

bulk cannot be made unless the reaction mechanism is known in some detail. The tentative conclusion has been drawn from the study of a number of surface reactions[495] that the absolute velocity, reaction order, effect of *p*H, and activation energy are often nearly the same as for the reaction occurring in bulk. The most interesting difference lies in the possibility of controlling the steric factor in film reactions by making the reactive groups more or less accessible to reactants present in the sub-solution. The possibility also exists that, under the influence of surface forces or cross-linkages in the monolayer, reactive groups will be altered enough in their relative configurations for the activation energy to be significantly changed. The most striking case of this occurs in protein monolayers, which seem to lose a good deal of their specific reactivity when spread. Thus antipneumococcus antibody monolayers will not react with the specific polysaccharide,[496] while spreading completely destroys the biological activity of the interstitial cell-stimulating hormone, metakentrin.[497] At the other extreme are reactions which are said to occur only when one reactant is adsorbed. Haurowitz, for example, finding that the hemin-catalyzed oxidation of linoleic acid occurs only at an oil-water interface, concluded that orientation and deformation of the unsaturated acid are necessary for the reaction.[498]

Despite the loss of their more specific properties, protein monolayers are still attacked by proteolytic enzymes, the kinetics of the reaction apparently agreeing closely with that observed in bulk[499] if care is taken to avoid artefacts produced by penetrating fatty impurities in the enzyme.[500] This only means, presumably, that the bulk reaction is also essentially heterogeneous, and that the first stage consists in the unfolding of the protein molecule. Gorter found that the spreading of myosin is promoted by partial digestion with trypsin,[501] and that of fibrinogen by trypsin or prothrombase.[502] It is concluded from the time course of the change in phase boundary potential[499] that the subsequent reaction in the process of digestion consists in the successive hydrolysis of terminal peptide links, the fragments passing into solution and leaving a residual monolayer of progressively shorter polypeptide chains. This conclusion is based, however, upon a questionable interpretation of the changes in phase boundary potential.

When the reaction rate depends greatly upon surface pressure it is likely that steric factors are of importance. Liquid-expanded films of unsaturated

[495] E. K. Rideal, Proc. Roy. Soc., **155A**: 684, 1936; cf. ref. (370), p. 95.

[496] J. F. Danielli, M. Danielli, and J. R. Marrack, Brit. J. Exper. Path., **19**: 393, 1938.

[497] B. F. Chow, H. B. van Dyke, R. O. Greep, A. Rothen, and T. Shedlovsky, J. Biol. Chem., **140**: xxvi, 1941.

[498] F. Haurowitz and P. Schwerin, Enzymologia, **9**: 193, 1941.

[499] J. H. Schulman and E. K. Rideal, Biochem. J., **27**: 1581, 1933.

[500] J. H. Schulman and A. H. Hughes, *ibid.*, **29**: 1236, 1935.

[501] E. Gorter and H. van Ormondt, Biochem. J., **29**: 48, 1935.

[502] E. Gorter, L. Maaskant, and G. J. van Lockeren Campagne, Proc. kon. Akad. Wetensch. Amsterdam, **39**: 1187, 1936.

acids with a double bond half way along the carbon chain are rapidly oxidized at low pressures by acid permanganate, while removal of the double bonds from the surface by compression greatly decreases the rate of reaction.[503] The rate of digestion of lecithin monolayers by snake venom is similarly extremely dependent upon surface pressure, presumably because the reaction involves attack of the double bond in the oleyl group, which may be lifted by compression.[504] The same thing occurs, however, in the hydrolysis of long-chain esters[505] where the active group remains in the aqueous phase on compression, in the autoxidation of an unsaturated long-chain triglyceride (the maleic anhydride compound of β-elaeostearin), in which the double bond is still accessible to attack by oxygen, even after compression has removed it from the lower interface,[506] and in the photochemical hydrolysis of stearic anilide.[507] In the first case the effect has been attributed[505] to the pressure submergence of the ester group, which thus provides a protective hydrocarbon environment for the point of hydrolytic attack, but the data presented do not give very strong support to this view. In the autoxidation reaction, although the case is complicated by the occurrence of a secondary surface polymerization, it seems certain, from a study of the effects of *p*H and of inhibitors, that water is involved in the oxidation process. The pressure variation of the photochemical reaction rate is attributed in part to the dichroism of stearic anilide, with consequent change in extinction coefficient as the molecules are reoriented.

These few examples show how hard it is to find clear-cut illustrations of the steric effect; in each case the effect exists under circumstances in which, at first sight, it would not be expected, and for each case a different explanation is invoked. This is not to question the correctness, at least in part, of the explanations offered, but only to emphasize the complexity of surface reactions and the difficulties of interpretation when direct analytical methods are precluded. This circumstance removes some of the advantages gained by the possibility of examining the reactions of a single layer of oriented molecules. Nevertheless the study of chemical reactions in monolayers is likely to increase in importance and will contribute greatly to our knowledge of the reactions of polymeric substances.

7. Films Transferred to Solid Surfaces.—Although the amount of useful biological information derived from them has not hitherto been very great, the properties of films at solid surfaces are of some interest. These films may be obtained either in the form of monolayers or multilayers transferred to a solid surface from an air-water interface, or in the form of layers adsorbed directly from solution. Studied with the aid of optical and other methods, they may prove very useful in the quantitative examination of

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[503] A. H. Hughes and E. K. Rideal, Proc. Roy. Soc., **140A**: 253, 1933.

[504] A. H. Hughes, Biochem. J., **29**: 437, 1935; E. K. Rideal, Nature, **144**: 693, 1939.

[505] A. E. Alexander and J. H. Schulman, Proc. Roy. Soc., **161A**: 115, 1937.

[506] G. Gee and E. K. Rideal, Proc. Roy. Soc., **153A**: 116, 129, 1935; G. Gee, *ibid.*, **155A**, 592, 1936; G. Gee and E. K. Rideal, J. Chem. Phys., **5**: 794, 1937; G. Gee, *ibid.*, **5**: 801, 1937.

[507] E. K. Rideal and J. S. Mitchell, Proc. Roy. Soc., **159A**: 206, 1937; cf. J. S. Mitchell, J. Chem. Phys., **4**: 725, 1936.

more or less specific interactions between biological substances available only in minute amounts.

a). *Preparation and structure of transferred multilayers.*—Monolayers of many substances can be transferred from the surface of a solution to a solid surface merely by dipping the plate vertically through the surface and raising it. The conditions under which this transfer occurs have been studied in especial detail by Blodgett in the case of stearic acid and its salts.[508] It has been found, for example, that a clean glass or polished chromium-plated slide rubbed vigorously with ferric stearate[509] takes up part of a compressed monolayer of copper-barium stearate on being dipped through the surface occupied by the monolayer and a second portion on being raised. The decrease in area of the monolayer, held at constant surface pressure during each part of this process, is about equal to the measured area of the plate, notwithstanding the fact that the measured area is only a two-dimensional projection of the true area, which must be very much greater. The plate, therefore, acts merely as a grid upon which the monolayer supports itself at isolated points.[510] A slide thus coated with a basal layer of ferric stearate and two layers of Ba-Cu-stearate, if subjected repeatedly to the dipping procedure, can be coated with a multilayer of any desired thickness, expressed in odd multiples of the monolayer thickness. As would be expected, these built-up films, removed from the influence of restraints operating at the water surface, tend to undergo rearrangement, in the course of a few hours, to the configuration characteristic of the crystal lattice. This change is comparatively slight for the stearates, but rather greater for unsaturated substances.[511] The configuration of multilayers and its relation to those occurring in the crystalline polymorphs has been the subject of X-ray and electron diffraction studies, with somewhat divergent results in various matters of detail.[512]

Multilayers of sterols have been prepared with thickness per molecule about the same as that found for 3-dimensional crystals;[513] chlorophyll also can be obtained in this form.[514] Studies of protein multilayers by X-ray diffraction appear to confirm the polypeptide chain structure of the monolayers from which they were built.[515]

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[508] K. B. Blodgett. J. Am. Chem. Soc., **57**: 1007, 1937; K. B. Blodgett and I. Langmuir, Phys. Rev., **51**: 964, 1937; I. Langmuir and V. J. Schaefer, J. Am. Chem. Soc., **59**: 2400, 1937; cf. L. Denard, J. Chim. Phys., **36**: 210, 1939. Denard claims that multilayers of the types described by Blodgett and Langmuir can be obtained from pure stearic acid on 0.01M HCl.

[509] I. Langmuir, V. J. Schaefer, and H. Sobotka, J. Am. Chem. Soc., **59**: 1751, 1937.

[510] J. J. Bikerman, Proc. Roy. Soc., **170A**: 130, 1939.

[511] A. E. Alexander, J. Chem. Soc., 777, 1939.

[512] E.g., L. H. Germer and K. H. Storks, Proc. Nat. Acad. Si. U.S., **23**: 390, 1937; Phys. Rev. **55**: 648, 1939; G. D. Coumoulos and E. K. Rideal, Proc. Roy. Soc., **178A**: 421, 1941; A. Cameron and G. D. Coumoulos, *ibid.*, **178A**: 415, 1941; G. Knott, J. H. Schulman, and A. F. Wells, *ibid.*, **176A**: 534, 1940; C. Holley and S. Bernstein, Phys. Rev., **52**: 525, 1937.

[513] I. Langmuir, V. J. Schaefer, and H. Sobotka, J. Am. Chem. Soc., **59**: 1751, 1937.

[514] I. Langmuir and V. J. Schaefer, *ibid.*, **59**: 2075, 1937.

[515] W. T. Astbury, F. O. Bell, E. Gorter, and J. van Ormondt, Nature, **142**: 33, 1938. G. L. Clark and S. Ross, Science, **86**: 292, 1937, previously found a long spacing of 73.3Å but failed to observe any at 10Å.

b). *Determination of film thickness by deposition of stepped multilayers.*—Of more immediate interest is the application of this technique to the preparation of stearate multilayers suitable for the optical measurement of film thickness.[515] In the simplest possible case, monochromatic light rays reflected from the chromium and the stearate surfaces of a multilayer will interfere if the optical path difference between the rays is $\lambda/2$ (λ = wave-length). In practice it is found that multilayers built in steps of two show up as a series of bands of different brightness when viewed at large angles of incidence in monochromatic light. If the angle of incidence is 73° the bands of 47 and 49 layers are about equally dark, and the bands on either side of these get progressively brighter. If the whole series of steps is now covered with a film of uniform thickness, the 47 and 49 bands are no longer equally dark, but can be made so by adjusting the angle of incidence to some new value. In this manner the optical thickness of the new layer can be calculated to within a few Å., and its true thickness can be determined if its refractive index be known. In some cases, the refractive index can be determined independently by observing the disappearance of interference fringes formed when the substance is deposited on glass slides of the same refractive index; in other cases more or less plausible assumptions have to be made. Permanent reference blocks of etched lead glass have been described.[517]

Protein monolayers are somewhat thicker when transferred from a water surface at 30 dynes/cm. than at 15,[518] but there is no certainty that their actual thickness does not change during transfer, nor that there is not some rearrangement during the process. Some evidence has been given[518] that the outer surface of a protein film deposited on dipping (*A* film) and the inner surface of that deposited when a plate is withdrawn (*B* film) are both hydrophilic, as one would expect, while the opposite surfaces are hydrophobic. The differences are not, however, very clear-cut, perhaps because of the imperfect segregation of polar and nonpolar groups, or perhaps because of stretching and reorientation[519] during transfer. Such differences as are noticeable tend to become obliterated when the films are dried.

The actual recorded values of the thickness of transferred protein monolayers show wide variation, from 9.5 Å for ovalbumin spread at the isoelectric point[520] to 76 Å for a *B* film of a complex protein fraction obtained from hemolytic streptococci.[521a] Some authors, assuming that the thickness of a protein monolayer must be 5 Å at low pressures and 10 Å at high, tend to

[515] K. B. Blodgett, J. Phys. Chem., **41**: 975, 1937; K. B. Blodgett and I. Langmuir, Phys. Rev., **51**: 964, 1937.

[517] K. B. Blodgett, Rev. Sc. Instr., **12**: 10, 1941.

[518] I. Langmuir, V. J. Schaefer, and D. M. Wrinch, Science, **85**: 76, 1937; H. Neurath, *ibid.*, **85**: 289, 1937; cf., L. A. Chambers, J. B. Bateman, and H. E. Calkins, J. Immunol., **40**: 483, 1941.

[519] Reversal of orientation of sterol molecules in monolayers has been described: I. Langmuir, V. J. Schaefer, and H. Sobotka, J. Am. Chem. Soc., **59**: 1751, 1937.

[520] W. T. Astbury, F. O. Bell, E. Gorter, and J. van Ormondt, Nature, **142**: 33, 1938.

[521] a. J. B. Bateman, L. A. Chambers, and H. E. Calkins, J. Immunol, **39**: 511, 1940; b. cf. E. A. Hauser and L. E. Swearingen, J. Phys. Chem., **45**: 644, 1941.

regard thicker films as imperfectly spread or inhomogeneous. Although some interest attaches to the thinnest and most completely degraded films, particularly from the point of view of protein structure, a dogmatic refusal to regard the less degraded films as legitimate monolayers is unreasonable in our present state of ignorance. It is conceivable that the physical conditions at biological interfaces are such as to prevent complete spreading and to encourage the formation of viscous or plastic partially oriented films. In these may be embedded protein fragments which still retain some of the specific properties of the "native" substance, and which influence in great degree the physical properties of the film. In presence of tissue fluids containing dissolved protein, such a film will also tend to be associated with an adsorbed layer of practically undenatured protein;[521b] the films mentioned above, which on deposition as a *B* layer had a thickness of 76 Å, gave an *A* layer only 24 Å and an *AB* layer 47 Å thick. The former value can be due only to the elution of adsorbed soluble protein while the slide was immersed in water.

c). *Specific reactions of transferred monolayers.*—Closely related to the foregoing remarks is the question of the extent to which transferred protein monolayers retain their biologically specific character. Specific activity has certainly been observed in monolayers of enzymes or in substances associated with the monolayers;[522] egg albumin appears to retain its ability to react with specific antisera after spreading,[523] and so do various antigenic proteins and nucleic acid-protein complexes from hemolytic streptococci.[524] Antipneumococcal antibody, on the other hand, is no longer able to react with specific polysaccharide after spreading.[525] The relation of these observations to the completeness of spreading remains largely an open question; in one case,[521c] however, it appears certain that specific reactivity is retained even by the thinnest films of antigen, and probable[524b] that a certain difference in reactivity of the two surfaces of the antigen monolayer becomes apparent when spreading approaches completion.

d). *Multilayer surfaces as adsorbents for dissolved substances.*—While there remains some doubt as to the survival of specificity in proteins unfolded at a liquid interface, it is very well known that, at solid surfaces, adsorption is rarely accompanied by significant loss of activity; dispersion of the active substance over a considerable area may even cause an apparent potentiation of infectivity of virus particles.[526] A bacterial toxin or a hormone may be

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[522] a. E. Gorter, Proc. Roy. Soc., **155A**: 706, 1936; b. I. Langmuir and V. J. Schaefer, J. Am. Chem. Soc., **60**: 1351, 1938; c. I. Langmuir, Cold Spring Harbor Symp., **6**: 171, 1938; d. H. Sobotka and E. Bloch, J. Phys. Chem., **45**: 9, 1941.

[523] A. Rothen and K. Landsteiner, Science, **90**: 65, 1939.

[524] a. L. A. Chambers, J. Immunol., **36**: 543, 1939; b. L. A. Chambers, J. B. Bateman and H. E. Calkins, *ibid.*, **40**: 483, 1941; c. J. B. Bateman, H. E. Calkins, and L. A. Chambers, *ibid.*, **41**: 321, 1941.

[525] J. F. Danielli, M. Danielli, and J. R. Marrack, Brit. J. Exper. Path. **19**: 393, 1938.

[526] *Inter alia*, R. Doerr and S. Seidenberg, Ztschr. Hyg. u. Infektionskr., **114**: 269, 1932.

inactivated by adsorption at a chloroform-water interface,[527] while it may remain active after attachment to collodion particles.[528] At the same time, the mechanisms of various sensitive agglutination reactions,[529] doubtless depending for their occurrence upon adsorption, remain obscure. It would seem that more detailed information could be obtained, at the cost of some loss of diagnostic sensitivity, if the conditions for adsorption could be simulated at a multilayer surface, and the process followed by measuring thickness increments. Comparatively little has been done in this direction, but preliminary work shows what may be expected. Methods of conditioning surfaces for adsorption have been suggested[530] and the adsorption of egg albumin (50 Å increase in thickness), tobacco mosaic virus (300 Å) and diphtheria toxin (36 Å) observed.[530a] The toxin so adsorbed reacts with antitoxin, giving a further 75 Å increment.[530a,531c] Similar activity is displayed by adsorbed layers of egg albumin,[531a] catalase,[531b] and pneumococcus antipolysaccharide antibody[531a,c] when exposed respectively to anti-egg albumin, anti-catalase antibodies, and to pneumococcus capsular polysaccharide. Nonspecific adsorption is apt to obscure some of these reactions. Quantitative study and interpretation of some of these effects is obscured by the ambiguity of any apparent increase in thickness, which may represent complete covering of a surface with a thin layer of material, or partial covering with a thick layer.[532]

Extremely thick layers (100–500 Å) of salmine, insulin, and casein are adsorbed from isoelectric solutions at the surface of conditioned plates.[533] Isoelectric insulin, but not isoelectric casein, can also adhere to the layers of salmine; on the other hand, salmine causes insulin layers to detach themselves. There is some slight indication that 2,4-dinitro-*o*-cyclohexylphenol, which is known as a respiratory stimulant and inhibitor of cell division,[534] may block the adsorption of insulin by protamine.

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[527] E.g., J. M. Joblin, Proc. Soc. Exper. Biol. & Med., **36**: 523, 1937; J. M. Joblin and R. H. Rigdon, J. Immunol., **41**: 233, 1941.

[528] E.g., J. Freund, Proc. Soc. Exper. Biol. & Med., **28**: 65, 1930.

[529] To mention only recent examples, the agglutination of red cells upon adsorption of influenza virus: G. K. Hirst, J. Exper. Med., **76**: 195, 1942, and other papers by the same author; the agglutination of collodion particles after adsorption of the virus-antibody complex: K. Goodner, Science, **94**: 241, 1941.

[530] a. I. Langmuir and V. J. Schaefer, J. Am. Chem. Soc., **59**: 1406, 1937; b. *ibid.*, **59**: 1762, 1937; c. G. H. A. Clowes, Am. Assn. Adv. Sc., Publication No. 7, p. 61 (undated).

[531] a. M. F. Shaffer and J. H. Dingle, Proc. Soc. Exper. Biol & Med., **38**: 528, 1938; b. W. D. Harkins, L. Fourt, and P. C. Fourt, J. Biol. Chem., **132**: 111, 1940; c. E. F. Porter and A. M. Pappenheimer, J. Exper. Med., **59**: 755, 1939.

[532] J. B. Bateman, H. E. Calkins, and L. A. Chambers, J. Immunol., **41**: 321, 1941.

[533] a. G. H. A. Clowes, Am. Assn. Adv. Sc., Publication No. 7, p. 61, (undated). b. G. H. A. Clowes, M. E. Krahl, and W. W. Davis, Proc. Soc. Exper. Biol. & Med., **38**: 526, 1938.

[534] a. G. H. A. Clowes and M. E. Krahl, J. Gen. Physiol., **20**: 145, 1936; b. M. E. Krahl, and G. H. A. Clowes, *ibid.*, **20**: 173, 1936, and many other papers.

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Section 3

INTRODUCTORY REMARKS REGARDING THE ARCHITECTURE OF PROTOPLASM

By RUDOLF HÖBER

Grateful acknowledgment is due to Dr. Elinor Moore Irvin, to Dr. Margaret Sumwalt McCouch, and especially to Dr. Karl Sollner for reading and commenting on the manuscripts of Sections 3, 4, 5, and 8.

My contributions to this work are dedicated to my wife, Dr. Josephine Höber, who inspired and encouraged me to create a renaissance of my previous book, and passed away too early to see it come to life and, as I hope, to usefulness.

INTRODUCTORY REMARKS

The objective of the last section was a survey and an analysis of molecular structure. The discussion began with atoms and their co-ordination to molecules and proceeded to aggregates, whose structural complexity surpassed the ordinarily accepted limits, up to which the units may be identified as discrete molecular entities. In this regard, the submicroscopical giant virus molecules, the aggregates of single thread-like myosin molecules, or the synthetic polymers, may be remembered. Investigation of these furnished guidance to the study of films, leaflets, coacervates, bundles of fibrils, and others.

Entering now the realm of research upon living systems, which we are accustomed to regard as of unrivaled order of complexity, one may try to continue with the morphological approach to this material, as it was the preferred approach to the nonliving material. In recent years, in an effort to secure a more direct attack upon the constitution of protoplasm, various cells and tissues (liver, kidney, chick embryo, sea-urchin eggs, chloroplasts), often as frozen, dried, and ground-up material, have been extracted with dilute salt solutions (NaCl, KCl). The residue has been dissolved in strong salt solution or in weak alkali, precipitated by dilution, acidification, or strong ammonium sulfate, redissolved, and so forth.[1] Through these procedures, submicroscopic aggregates are obtained, which, according to chemical as well as physical analyses, seem to display a still higher order of aggregation than the units discussed above. By chemical analysis, proteins, nucleoprotein, flavoprotein, fat, lecithin, sterols, cytochrom oxidase, and other substances have been identified. Optical tests have revealed a fibrous structure, evidenced by X-ray diffraction diagrams, with the precipitated material, by intrinsic and flow birefringence with its solutions. But, in order to identify these particles as preformed and discrete units instead of more or less accidental protoplasmic fragments, their composition must be demonstrated to be below a certain degree of randomness, since probably the bonds holding together the diverse chemical constituents are the relatively weak forces of residual valencies.

Among the conditions inducing the aggregation of the various compounds, there must be taken into account particularly protoplasmic movements of any kind, either flow or tension in a certain direction, instrumental in orienting parallel to each other nonspherical units, like polypeptide chains, fibrinogen needles, or gelatin micellæ, to form strands, the submicroscopic

[1] R. R. Bensley and N. L. Hoerr, Anat. Rec., **60**: 251, 1934; A. E. Mirsky, Science, **84**: 333, 1936; R. R. Bensley, Anat. Rec., **72**: 351, 1938; Science, **95**: 389, 1942; L. Banga and A. Szent-Györgyi, *ibid.*, **92**: 514, 1940.

interstices of which may be occupied by both solvent and various solutes, lipoids, carbohydrates, enzymes, and others. Such strands can be shown by optical methods to arise in living protoplasm; for instance, birefringence can be detected in the structureless hyaloplasm of normal cells, and is especially evident in streaming hyaloplasm.

An interesting symptom of [illegible] in protoplasm has been discovered by A. R. Moore.[2] The plasmodia of myxomycetes are able to grow through the pores of silk gauze (or of hard filter paper), the average pore size of which is as small as 1 μ. But, if the plasmodium is pressed through the silk, it dies, whereas the remainder on the surface of the sieve continues to proliferate. Only when the pores of the sieve have a diameter greater than 200 μ, does the filtered material survive.

Another pertinent evidence of a structural organization of protoplasm has been furnished by Harvey and Marsland[3] in centrifuging *Ameba dubia*. When these animals are observed under the microscope during centrifugation, heavy particles (crystals) are seen in the cytoplasm to fall not continuously, but in jerks, even when moving through a visibly clear field.

By observations like these, light is thrown upon the familiar fact that mechanical insult to protoplasm, possibly involving disintegration of an internal architecture, is accompanied by alterations of many metabolic reactions. For instance, respiration of the sea-urchin egg is reduced upon cytolysis to about 10 per cent of the original, while the uptake of oxygen by amphibian gastrulæ under the same conditions is increased five times.[4] Such effects can be considered as being due to the presence of interfaces in heterogeneous systems, which, nearly invariably by the way of adsorption, change the chemical equilibria and the reaction velocities, which otherwise would be consistent in the homogeneous fluid spaces enclosed in the vacuoles and channels of the intraplasmatic framework. Indeed, there are many ways to interpret as effects of adsorption not only the accelerations, but also the retardations. For instance, the reactivity of organic molecules with a nonpolar-polar structure has been found to be sometimes reduced, when the reacting group is attached to the nonpolar part by orientation at the interface in such a way that this part of the molecule is buried by adsorption in the interface, while the chemically inert polar half is turned toward the aqueous phase.[5] More frequently acceleration is encountered as a result of an increase of concentration of the reagents in the adsorption layer. But, since the protoplasmatic structures can be assumed to be

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[2] A. R. Moore, J. Cell. & Comp. Physiol., **7**: 113, 1935.

[3] E. N. Harvey and D. A. Marsland, J. Cell. & Comp. Physiol., **2**: 75, 1932.

[4] O. Warburg and O. Meyerhof, Pflüger's Arch. f. d. ges. Physiol. **148**: 295, 1912; O. Warburg, Erg. Physiol. **14**: 253, 1914; A. Brachet, Arch. de Biol. **45**: 511, 1934. For these and others of the following references cf. the substantial review article of I. M. Korr upon oxidation-reduction in heterogenous systems, Cold Spring Harbor Symp., **7**: 74, 1939.

[5] H. R. Kruyt, Zischr. f. Elektrochemie., **35**: 539, 1929; H. R. Kruyt and van Duin, Rec. trav., **40**: 259, 1921; R. Kuhn, Naturwissenschaften, **11**: 732, 1923.

composed of more than one adsorbent, the effect of adsorption in such a polyphasic system would be extremely variable. However, in addition, it must be considered that the single structural units, instead of showing a differential adsorbing power, also can act as solvents, each of them distinguished by its own distribution coefficient (see Sec. 1, chap. 5, and Sec. 5, chap. 23). Furthermore, it can be foreseen that in such a polyphasic system the effect will depend not only upon the variety, but also upon the mutual location, of the differently acting units. The significance of a suitable arrangement of these is made evident, for instance, in the following experiments. First,[6] a stream of hydrogen is passed through water containing a suspension of platinized asbestos. Methylene blue dissolved in the water will be hydrogenated by the activated hydrogen. But no hydrogenation occurs, if the methylene blue is added after being adsorbed to filter paper, which has been thoroughly washed with water to free it from the unadsorbed surplus of dye. This is because hydrogenation is effected only by hydrogen activated at the Pt surface. However, the dyestuff store in the filter paper is gradually depleted, if a small amount of dye remains dissolved in the water, because the dissolved dye mediates as an electron carrier between the two surfaces (see Sec. 1, chap. 4; Sec. 6, chap. 30; Sec. 8, chap. 38). Second, it can reasonably be inferred that a great number of intra-cellular enzymes are attached to the structural colloids of the cells, but in such a pattern that a certain spatial succession secures a proper sequence of reactions (e.g., the stepwise breakdown of large molecules), or that an immediate linkage provides the association. The latter case is illustrated by the following observation.[7] In lactic acid formation in muscle the two enzymes, muscle-amylase and an esterifying enzyme, appear to be so closely concatenated and interdependent in their activity that they can be considered to form a single colloid aggregate, held together, however, so weakly that even by a short exposure to a moderately elevated temperature the delicate complex is ruptured.

Through many observations like these, the old idea of a "chemical organization" of the protoplasm, as complement to its structural or mechanical organization,[8] has received new and more, and more substantial support. Beyond that, it has stimulated the investigation of the linkage between cell work and cell chemistry. The numerous enzymes can be pictured as adhering to the cell structure and acting as catalysts not only to accelerate the orderly sequence of the metabolic reactions, but, presumably, further to induce the immediate transfer of the liberated chemical energy to those structural components, which can be considered parts of the acting cell machinery. For, unlike the prevailingly thermodynamic technical machines, the physiological machines are representative of the type of

[6] H. A. Abramson and I. R. Taylor, J. Phys. Chem., **40**: 519, 1936; I. M. Korr, J. Cell. & Comp. Physiol., **11**: 233, 1938.

[7] E. M. Case, Biochem. J., **25**: 561, 1931.

[8] F. Hofmeister, Naturwissenschaftl. Rundschau, 1901, p. 581.

chemodynamic machines, which—in one way or the other—transform chemical energy directly into mechanical, electrical, and radiant energy. Moreover, the cell machine excels other chemodynamic machines by the submicropic dimensions of its elementary parts spread to form a huge area for "adsorption catalysis," thus providing an adequate means for prompt and reversible acceleration or retardation of metabolic reactions as the source of energy.[9] By giving increased consideration to the study of the microstructure of large molecules, the old and fundamental problem of how to conceive of the direct interaction between the structural and the chemical events seems to have been attacked with greater success than before on the classical field, where for a long time one is searching to detect a device for coupling muscle work and muscle metabolism. Today, it is generally believed that the myosin molecules, which are folded polypeptide chains, are arranged to form long fibrillar micellæ (see Sec. 2, chap. 8), which under various—either chemical or physical—influences are shortened by an increase or lengthened by a decrease of their folding. These changes in length are reversible. Therefore, the myosin threads are believed to be the contractile machinery. Now, Engelhardt and Ljubimova[10] have discovered that in a sol of myosin, after adding adenosinetriphosphate, which is known as one of the essential components of the metabolic cycle in muscle and as the chief contributor of free energy of contraction (see Sec. 6, chap. 30; Sec. 7, subsec. 13), the micellæ shorten at once, and subsequently, while liberating one phosphate from the triphosphate, i.e., yielding adenosinediphosphate, spontaneously relax. Therefore, one may say that myosin behaves like an enzyme, like an adenosinetriphosphatase, and so, evidently, the coupling between mechanical and chemical action takes place in such a way that the machine itself, as a "contractile enzyme," releases the chemical reaction, by which it sets itself going. Certainly, on the background of this progress, it is inspiring to direct attention to other microstructures possibly suitable, in their physicochemical properties, to demonstrate the direct transformation of free energy of a chemical process into mechanical energy. In this respect, one may refer to models, which have been described by Sollner[11] and will be discussed later (see Sec 8, chap. 38). Sieve membranes, being essential parts of the surface and the interior of each cell, presumably are endowed with pores of various width and of variable electric charge of their walls. Interposed between appropriate electrolyte solutions

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[9] For instance, the surface-active narcotics can sever the contact between the enzymes and their substrates by covering the interior surfaces (see the adsorption theory of narcosis in Sec. 5, chap. 23).

[10] W. A. Engelhardt and M. N. Ljubimowa, Nature, **144**: 668, 1939. See further J Needham, *et al.*, Nature, **147**: 766, 1941; **150**: 46, 1942; A. Szent-Györgyi and I. Banga, Science, **93**: 158, 1941; M. Dainty, A. Kleinzeller, A. S. C. Lawrence, M. Miall, J. Needham, D. M. Needham, and S. C Shen, J. Gen. Physiol., **27**: 355, 1944.

[11] K. Sollner, Ztschr. f. Elektrochemie, **36**: 36, 1930; **36**: 234, 1930; K. Sollner and A. Grollman, *ibid.*, **38**: 274, 1932.

of different concentration, due to the selective ionpermeability of the individual pores, such membranes can perform electroosmotic work by shifting water, cations, or anions against the concentration gradients, until osmotic equilibrium is reached. Metabolic production of ions at the interfaces of the membranes may become effective for initiating or maintaining the electroosmotic work, not only by establishing concentration gradients, but also by altering the width or the charge of the pores of the sieve-membrane.

Following these introductory remarks about some physicochemical aspects of protoplasm as the basic substance in all living things, animals and plants, cells and tissues, the subsequent chapters will deal with the surface of the protoplast, its properties and its architecture, with environmental influences on manifestations of cell life, with cell metabolism and cell energetics, with excitability and contractility, with active transfer as superimposed to passive penetration.

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Section 4

THE SURFACE OF THE PROTOPLAST, ITS PROPERTIES AND ITS ARCHITECTURE

By RUDOLF HÖBER

INTRODUCTION

There is no problem in cell physiology more challenging to investigators than the conditions allowing dissolved substances to enter the protoplasm or to leave it. This problem has occupied the general interest of physiologists since Nägeli's discovery (1855)[1] of the plasmolysis and deplasmolysis of plant cells afforded a number of botanists, Pfeffer, de Vries, G. Klebs, and others, an easy way to measure the osmotic forces of the living cell and the permeability of the protoplasmic surface to solutes. After enough data regarding the osmotic properties of plant cells had been collected, E. Overton in 1895[2] arrived at the result that, for innumerable organic compounds, there are certain general rules correlating their physical and chemical properties with their ability to penetrate protoplasm. (See p. 231.) The picture as drawn originally by Overton has lost much of its plainness, as refined procedures have increasingly revealed the fact that genus- and species-specificity is characterized not only by morphological features and by the elaboration of specific chemical products, but also by physicochemical properties, such as differential permeability. Though many irregularities and deviations from Overton's general statements have been encountered in the course of years, while research on permeability was being extended, by the invention of new methods, over the entire field of plant and animal life, the principles referring to organic nonelectrolytes are still fairly sound.

Overton concluded from his results that the rate of penetration of organic compounds is determined by their solubility in the lipoids assumed to be present in the protoplasmic surface. However, the existence of lipoid insoluble, though readily penetrating, organic compounds, on the one hand, and, on the other hand, the intricate behavior of electrolytes caused by their dissociation into ions and by the electric forces of the latter, have called for complementing Overton's "lipoid theory" by a "pore theory." Both of these concepts have suggested schemes, to represent the architecture of the protoplasmic surface, and experiments on models as well as on natural objects, to test their adequacy.

[1] K. Nägeli and K. Cramer, Pflanzenphysiol. Untersuchungen: Zürich, 1855.

[2] E. Overton, Vierteljahrsschr. der naturforsch. Ges. in Zürich, **40**: 1, 1895.

10

THE PERMEABILITY OF THE CELLS TO ORGANIC NONELECTROLYTES

1. Plant Cells, the Lipoid-Pore Theory.—The basic results regarding permeability to nonelectrolytes have been obtained by studying plasmolysis of plant cells. When the cells are placed in solutions of an indifferent substance, plasmolysis appears if the concentration is increased to a limiting value. Impermeability to the substance is indicated if the plasmolysis can be persistent over a long period of time, and if it can be succeeded by deplasmolysis, when the concentration is diminished below the threshold value, as, for instance, with hexoses or hexites. The limiting concentration c (expressed in mol./1) is a measure of the osmotic pressure of the plant cell, which resembles an osmometer provided with a semipermeable membrane. For a substance to which the cell is permeable a higher molar concentration c_1 is necessary in order to start plasmolysis, which, however, disappears after a shorter or a longer period of time, depending upon the rate of penetration. Some substances enter so fast (for instance, many monohydric alcohols) that plasmolysis cannot be observed even with high concentrations. Thus, it is evident that $\frac{c_1 - c}{c} = \mu$ provides an approximate, but only half-quantitative, measure of permeability (permeability coefficient).[3]

The best procedure for an exact study of permeability is certainly the chemical determination inside the cells of the compounds in question. This can be done with sufficiently large plant cells, where a voluminous cell sap vacuole is surrounded by the mantle of living protoplasm. Collander and Bärlund[4] used single cells of *Chara ceratophylla* (1–4 cm. long, with 10–20 mm.^{3} cell sap), in solutions of a great number of different organic compounds. They followed the entrance into the sap by analyzing samples at intervals with Bang's method of chromic acid titration and by micro-Kjeldahl, until equilibrium was reached, as indicated by a final concentration in the sap 90 to 100 per cent of the outside concentration. If the compounds enter by diffusion, their movement should fulfil Fick's law (see

[3] About useful modifications of this method, also taking into consideration the time of deplasmolysis, see Lepeschkin, Ber. dtsch. bot. Ges., **25a**: 198, 1908; **27**: 129, 1909; A. Tröndle, Jahrb. f. wiss. Bot., **48**: 175, 1910; H. Fitting, *ibid.*, **55**: 1, 1915; **57**: 533, 1917; **59**: 1, 1919; K. Höfler, Ber. dtsch. bot. Ges., **36**: 414, 423, 1918; H. Bärlund, Acta Bot. Fenn., **5**: 1929.

[4] R. Collander and H. Bärlund, Acta Bot. Fenn., **11**: 1933.

Sec. 1, chap. 1) as set forth in the equation: $P = \frac{V}{qt} \ln \frac{C}{C - c}$, where V is the volume, q the surface area of the regularly shaped cylindrical cell, c the inside concentration at the time t, C the equilibrium concentration, and P the amount (in mol.) of the substance, entering in unit time across unit area along unit concentration gradient. P was found to be constant; in other words, the penetration actually appeared to be a passive diffusion across the protoplasmic layer into the sap. By determining the time t for half-saturation of the sap ($c = \frac{1}{2}C$), convenient relative values of the permeation power of various substances can be calculated (Table XI).

TABLE XI.—PERMEABILITY OF *Chara* CELLS TO ORGANIC NONELECTROLYTES
(R. Collander and H. Bärlund)

Substances	Half-saturation, time (in minutes)	Permeation constants
Methylalcohol	1 3	About 0 99
Ethylalcohol	2 3	0 56
Ethylurethan	3 0	0 43
Butyramide	7.6	0.17
Propionamide	9 9	0 13
Monochlorhydrine	14	0 090
Propylenglycol	15	0 087
Ethylenglycol	30	0 043
Methylurea	190	0 0068
Urea	320	0 0040
Glycerol	1700	0 00074
Malonamide	9000	0 00014
Erythritol	28000	0 000046
Mannitol	About 42000	About 0 000030
Glucose	About 42000	About 0 000030
Lactose	About 42000	About 0 000030

This series of the determinations of cell permeability to 45 substances, the most exact so far made, is in good agreement with earlier observations of Bärlund[5] upon plasmolysis of cells of *Rhoeo discolor*, despite the two plants being very different in size, in their physiological anatomy and in their living conditions. Furthermore, the results correspond to the data regarding the permeability of ox erythrocytes, which were obtained by Hedin,[6] applying a freezing point determination (see later p. 238); and to some extent they are comparable to permeability measurements of Stewart and Jacobs[7] upon sea-urchin eggs (see p. 241). Finally, all of them give an illustration of the old general conclusions drawn by Overton[8] from his numerous experiments on plant and animal cells (Table XII).

[5] H. Bärlund, Acta Bot. Fenn. **5**: 1929.

[6] S. G. Hedin, Pflüger's Arch. f. d. ges. Physiol., **58**: 229, 1897; **70**: 525, 1898.

[7] D. Stewart and M. H. Jacobs, J. Cell. & Comp. Physiol., **7**: 333, 1935.

[8] E. Overton, Vierteljahrsschr. der naturforsch. Ges. in Zürich, **44**: 88, 1899; Pflüger's Arch. f. d. ges. Physiol., **92**: 115, 1902.

TABLE XII.—ORGANIC SUBSTANCES FOUND BY OVERTON TO PENETRATE

Rapidly	Monohydric alcohols, aldehydes, ketones, hydrocarbons and their mono-, di-, and trihalogen derivatives, esters, many weak organic acids and bases;
Less rapidly....	Dihydric alcohols and amides of monobasic acids;
Slowly....	The trihydric alcohol glycerol, urea, thiourea;
More slowly .	The tetrahydric alcohol erythritol;
Most slowly .	The hexahydric alcohols, sugars, amino-acids, and many neutral salts of organic acids.

It appears that the introduction of polar groups (hydroxyl, amino, carboxyl) decreases, that of nonpolar groups (halogens, alkyls) increases, the penetrating capacity. Further, in homologous series (e.g., of the aliphatic monohydric alcohols or the aliphatic monocarboxylic acids) the permeation rate rises, as the length of the C-atom chain rises.

It is well known that the experimental results suggested to Overton the idea of referring the differences of permeability to a differential constitution of the surface layer or the plasma membrane. It was obvious that an interpretation cannot be given on the basis of a chemical correlation between the permeant and the membrane components. Hence, the physicochemical hypothesis of a membrane component with selective dissolving power was proposed. An organic fat-like ("lipoid") solvent, e.g., olive oil, seemed to be an adequate model, and it was ascertained by numerous studies that the distribution coefficients oil : water (or even ether : water) correspond remarkably well to penetrating power, as is obvious from the aforementioned studies of Collander and Bärlund, illustrated by Fig. 23.

All those substances which fall between the two parallel lines in Fig. 23 are in fairly satisfactory agreement with the theory. The correlation between penetrating power and relative solubility in oil is evident, but there are important discrepancies which need special discussion.

In objection to the lipoid theory it has been pointed out that a state of *equilibrium* as represented by a distribution coefficient can hardly be conceived of as the crucial factor governing *rate* of penetration. However (see Collander and Bärlund, *loc. cit.*), it must be kept in mind that, according to the lipoid theory, the process of penetration consists in, first, the distribution of the penetrating substance at the external phase boundary of the lipoid "layer"; second, the migration across this layer; and, third, another distribution in the inverse direction at the internal phase boundary bordering the protoplasm, the two distribution coefficients supposedly being identical. Then, the second step of the process, the diffusion through the lipoid layer, can be assumed to be the slowest, but in its speed parallel to the distribution coefficient of the substance.

The more striking discrepancies, which are indicated in Fig. 23 by small dots above the two parallel lines, belong to substances of low molecular volume (MV), expressed in terms of molecular refraction at the wave-length D, MR_D. Evidently, here lipoid solubility is not the dominant factor.

Urea, for instance, can enter the cells, though it is practically lipoid insoluble. Below the lines are compounds of an especially high lipoid solubility, for instance, the strongly alkylated ester triethylcitrate. These pass into *Chara* cells more slowly than could be expected theoretically, possibly due to their high *MV*. A search in the field of plant cells revealed the sulfur alga *Beggiatoa*,[9] in which plasmolytical study showed that the predominant

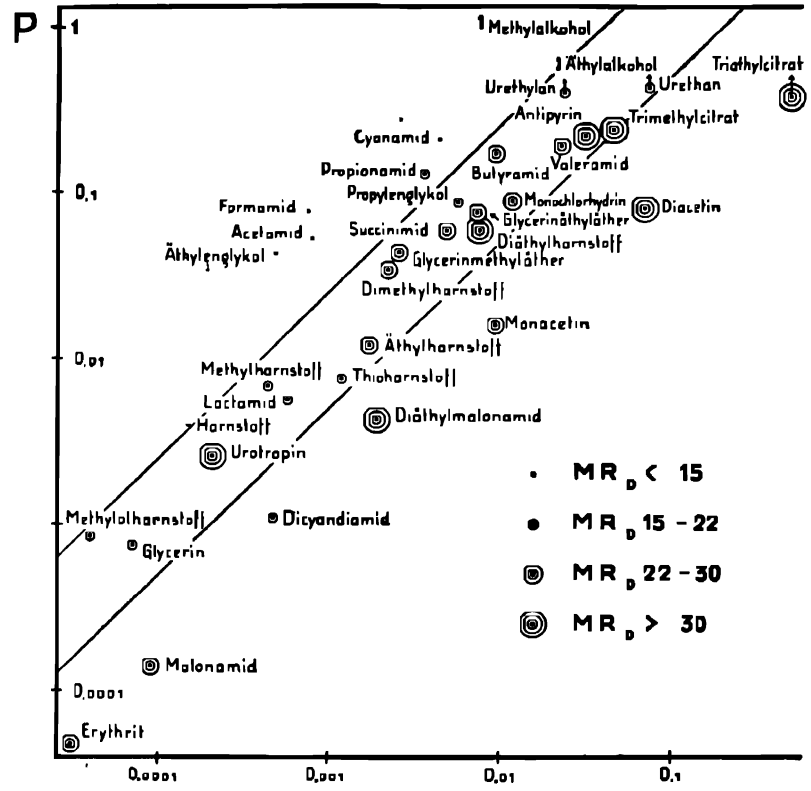

FIG. 23.—Rates of penetration into cells of *Chara ceratophylla* and distribution between olive oil and water. (Collander and Bärlund.)

factor is the *MV* (Table XIII). Thus a second theory of cell permeability has emerged.

The table shows that greater penetrating power, as indicated by higher plasmolytic threshold concentration (see method of Lepeschkin and Tröndle, p. 229) corresponds fairly well with lower values of *MV*. In other words, the plasma membrane resembles a molecular sieve with pores allowing the dissolved substances to penetrate according to their diameter. Plasmolytic threshold concentrations standing in the table in the wrong order, however, often display by their partition coefficient (ether:water) a

[9] W. Ruhland and C. Hoffmann, Planta, 1: 1, 1925; S. Schönfelder, *ibid.*, 12: 414, 1930.

relatively great lipoid solubility. Just as inadequacies in the lipoid theory gave rise to the pore theory, faults in the latter point back to the former.

The pore theory has been based upon various model experiments with sieve-like membranes, such as the copperferrocyanide membrane[10] or collodion membranes varying in average pore size according to their preparation,[11] or membranes of hydrophilic colloids apt to swell or to shrink (gelatin, agar).[12]

TABLE XIII.—PERMEABILITY OF *Beggiatoa mirabilis* TO ORGANIC NONELECTROLYTES (Ruhland and Hoffmann)

Substance	Plasmolysis, threshold conc. in mol./l	MR_D	Distribution coeff. ether: water
Urea	0.35	13.57	0.0005
Ethylenglycol	0.09	14.4	0.0068
Methylurea	0.01	18.47	0.0012
Thiourea	0.075	19.59	0.0063
Glycerol	0.009	20.63	0.0011
Ethylurethan	0.015	21.01	0.6370
Lactamide	0.007	21.13	0.0018
Malonamide	0.007	22.92	0.0003
Dimethylurea	0.005	23.43	0.0116
Butyramide	0.0125	24.11	0.0580
Erythritol	0.001	26.77	< 0.0001
Succinamide	0.0015	27.54	0.0002
Arabinose	0.0008	31.40	< 0.0001
Diethylurea	0.003	32.66	< 0.0185
Glucose	0.00055	37.54	< 0.0001
Mannitol	0.00055	39.06	< 0.0001
Saccharose	0.00020	70.35	< 0.0001

It is of physiological interest to note that the pore size of natural and of most artificial membranes is not uniform, as it is with several porous minerals (e.g., chabasit), which, due to a regular lattice-like crystalline structure, can absorb the molecules of a homologous series of gases only up to a limited MV, (e.g., ethylene), but above this limit are entirely inaccessible (butylene, benzene).[13] In ordinary membranes, an assortment of graded pore diameters seems to be present, the widest pores open only to the largest molecules, which, therefore, traverse only a restricted part of the total sieve area, while many more pores can be utilized by the smaller molecules. This makes it clear why the rates of diffusion for substances of different MV, *i.e.*, the amounts (in mols.) passing along a certain gradient,

[10] R. Collander, Kolloidchem. Beihefte, **19**: 73, 1924.

[11] L. Michaelis, and A. Fujita, Biochem. Ztschr., **170**: 18, 1926; R. Collander, Soc. Sc. Fenn. Comment. **2**: 6, 1926.

[12] See R. Höber, Physiol. Rev., **16**: 53, 1936.

[13] O. Schmidt, Ztschr. f. physik. Chem., **133**: 263, 1928.

can differ more widely than coefficients of ordinary free diffusion or of diffusion through the water-filled channels of a sieve membrane with wide meshes.[14]

Collander and Bärlund have proposed to fuse the two theories into one, the "lipoid-sieve theory." They picture the structure of the plasma membrane as a mosaic, composed of lipoid areas and sieve-like areas, either one or the other exerting the greater influence. For instance, in *Chara*, due to the prevailing effect of lipoid, methylurea enters faster than urea, whereas in *Beggiatoa*, due to the dominance of porosity, urea faster than methylurea. This is no more than a very crude picture, that needs a great deal of modification based on a more intimate study of the spatial relations between the molecules in submicroscopical structures, on the one hand (see Chap. 8), and of the chemical differentiation of the membrane components underlying the species-specific differences in permeability, on the other hand.

a). *Special qualities of the lipoids as factors in permeability.*—The lipoid theory postulates an organic solvent, the dissolving power of which, compared to that of water, parallels the permeating power of the solute. Overton and others have done a great deal of work in the attempt to find a generally appropriate solvent. The most careful investigation of Collander and Bärlund showed that in this respect the classical lipoid model, olive oil, gives a surprisingly good result, but is not an ideal solution of the problem. It became more and more clear that there is no one lipoid which fits all conditions. Rather, the oil-like solvent must be assumed to undergo slight variations, either of a chemical, or of a physicochemical, nature, to account for the exceptional permeability of certain cells.[15] It seems to be of particular importance that the lipoid used for a model, whether it be olein or another oil or a natural lipoid, like lecithin, or another phosphatide, easily hydrolyzes slightly to form a weak acid. This behavior affects their dissolving power toward those so-called nonelectrolytes, which in the narrow sense are actually electrolytes having the character of weak acids or weak bases. This idea is suggested by observations of vital staining in *Paramecium* by Nirenstein,[16] who showed that many dyestuffs enter the living cell, though insoluble in neutral oil, and that a congruence with the lipoid theory can be achieved by referring the staining power of the dyestuffs to their solubility in a mixture of oil + oleic acid + diamylamin, the acid component of the mixture providing solubility of the basic stains; the basic component, of the acidic vital stains. This suggests the interpretation that a similar mixture of several components may be present in the cell surface. A stronger argument for such an assumption follows from the discrepancies frequently observed in the order of the permeation rates of the organic nonelectrolytes,

[14] R. Collander, *loc. cit.*; L. Michaelis, *loc. cit.*; A. A. Weech and L. Michaelis, J. Gen Physiol., **12**: 55, 221, 1928; see, further Sec. 1, chap. 1.

[15] R. Höber, Biol. Bull., **58**: 1, 1930; R. Höber and G. Pupilli, Pflüger's Arch. f. d. ges. Physiol., **225**: 585, 1931.

[16] E. Nirenstein, Pflüger's Arch. f. d. ges. Physiol., **179**: 233, 1920.

when one compares the behavior of different species (Wilbrandt,[17] Collander and Bärlund, *loc. cit.*). These authors have discovered that there are certain species of plants which give preference to the entrance of nonelectrolytes containing NH_2-groups, and other species which in contrast prefer compounds, that lack this basic character. For instance, *Chara* is more permeable to malonamide than to erythritol, more to urea than to glycerol, more to propionamide than to ethylenglycol; but with *Rhoeo* the succession goes in the inverse direction. Correspondingly, the distribution (olive oil + oleic acid) : water compared to olive oil : water is remarkably greater with malonamide, urea, and propionamide than with erythritol, glycerol, and glycol. Following the aforementioned interpretation, this would mean that *Chara* has a relatively acidic surface solvent, *Rhoeo* a relatively basic. Furthermore, Jacobs,[18] studying erythrocytes (see p. 240) from a large number of species of mammals, reptiles, and fishes, with regard to their permeability to urea and to ethylenglycol (and glycerol), has found that, in general, with mammals and reptiles the rate of entrance of urea far exceeds that of glycol, while with fishes the contrary is true. Correspondingly, Höfler,[19] on the basis of numerous observations with plant cells, found urea to penetrate faster than glycerol in most of them, which he accordingly designated the urea-type, while a few, such as *Rhoeo*, were more readily permeable for glycerol, and were designated the glycerol-type. The complexity is still more enhanced by observations that, within the same species, either one or the other type shows up,[20] depending upon the season, or upon the age, or upon the site of the plant body supplying the experimental objects.

It seems reasonable to refer these variations to a more acid or a more alkaline reaction of the protoplasm, according to the metabolic state of the plant, but whether all these phenomena can be summarized alike as due to chemical or physicochemical variation in the lipoid components of the cell membrane, is by no means proved. One point at least should be stressed in this connection, viz., the majority of these experiments, for reasons which will be explained later (p. 237), deal with substances, whose low lipoid solubility and rather poor correlation between lipoid solubility and permeability favor the concept of their passing across the pores. Then one would have to account more for a chemical affinity proper, instead of a solution affinity, between the membrane material and the penetrating substance, both of them reacting with each other as acid and base.[21]

b). *Special kinds of porosity in relation to permeability.*—The *Chara* experiments of Collander and Bärlund have led to the hypothesis that, beside the prominent influence of lipoid solubility, a diffusion through pores

[17] W. Wilbrandt, Pflüger's Arch. f. d. ges. Physiol., **229**: 86, 1931.

[18] M. H. Jacobs, Proc. Am. Philos. Soc., **70**: 363, 1931.

[19] K. Höfler, Ber. dtsch. bot. Ges. **52**: 355, 1934; **50**: 53, 1932; **55**: 133, 1937.

[20] G. Marklund, Acta Bot. Fenn., **18**: 1936.

[21] For oleinalcohol compared with olive oil as lipoid, see Sec. 5, chap. 8.

plays a distinct role, which is evidenced by the fact that compounds with a relatively small volume penetrate the cells faster than could be anticipated from their lipoid solubility. In other words, while the lipoid route is accessible to all the lipoid soluble substances, irrespective of their *MV*, the pores between the lipoid areas afford a second means of entry to substances with a low *MV*. The next question is whether, with other plants than *Chara*, the role of the plasma membrane as a mediator of the intake of nonelectrolytes is divided into two parts in a similar manner. It has been shown before that *Beggiatoa* is, so to speak, the reverse of *Chara*, since its permeability is almost exclusively a pore permeability, though evidently including some lipoid effect (see p. 233). An analogous behavior is displayed by epidermis cells of *Gentiana Sturmiana*[22] and by the diatoms *Melosira* and *Licmophora*.[23] Between *Chara* and *Beggiatoa*, however, there are numerous connecting links, the analysis of which is worthwhile in order to throw light on the many possibilities for satisfying cellular requirements.

Porosity can be expected to influence permeability in several ways. First, a variety of widths of pores has been observed by looking for the limiting *MV* of the lipoid insoluble substances able to penetrate. For example, the threshold substance for *Beggiatoa* is raffinose ($MR_D = 103.7$); for *Rhoeo*, saccharose (70.35); for the mushroom *Psalliota*, malonamide (22.9).[24] Second, the porous areas in one membrane may contain a great number of narrow pores, in another a small number of wide pores. Though the sum of the single pore areas available for diffusion may be the same, this does not mean that the ratio of the penetration constants of two substances, traversing these membranes, will be the same. The ratio will be smaller with the membrane containing the wider pores, larger with the other. This seems to be indicated by the unusually small differences among the penetration constants with the diatom *Melosira*, which, for other reasons as well, is believed to have wide pores (Marklund, *loc. cit.*). Third, the relative extent of lipoid and porous surface in a membrane may contribute to the nature of its permeability. Into the root cells of *Lemna*, the voluminous molecules of methylurea penetrate more slowly than those of urea, as though porous areas were the larger and more influential portion of the membrane. In *Chara* the lipoid effect is predominant (see also p. 235). Fourth, in a study of the electrical properties of the surface membrane of *Nitella*, Curtis and Cole[25] came to the surprising result that only 13 per cent of the surface area is involved in the considerable increase of permeability to ions (decrease of resistance) concomitant with excitation (see Sec. 5). In other words, 87 per cent of the area should be considered a rigid skeleton-like substance.

c). *Comparative physiology of permeability.*—The quantitative analysis of cell permeability as a passive diffusion-like process has resulted, so far

[22] K. Höfler, Ber. dtsch. bot. Ges., **52**: 355, 1934.

[23] G. Marklund, Acta. Bot. Fenn., **18**: 1936.

[24] F. von Hofe, Planta, **20**: 354, 1933.

[25] K. S. Cole and H. J. Curtis, J. Gen. Physiol., **22**: 37, 1938 and **22**: 469, 1939.

as nonelectrolytes are concerned, in the isolation of only two chief factors, functioning jointly to separate solutes from solvent in the fluid surrounding the cell, first, selective dissolving, second, selective mechanical separation. As a third factor, evidently, a chemical separation can be added, referring to basic or acidic properties of the solutes. More factors may be segregated, as will become evident, e.g., in the following section regarding electrolytes. So far, so many variations of each of the factors have already been surveyed as to give a fairly embarrassing picture. However, at least under certain restricting conditions, a rather simple pattern appears, as shown, for instance, in Fig. 24, (see p. 238). Collander[25] has compared the cell permeability of 16 utterly diverse types of plants, (flowering plants, mosses, green algæ, diatoms, brown algæ, red algæ, blue-green algæ, bacteria) toward 7 nonelectrolytes. The plants are arranged according to increasing permeability to erythritol. The chemicals are chosen under the viewpoint that a moderate lipoid solubility and a fairly moderate MV make it possible to migrate along either the lipoid or the porous path, according to the prevalence of one or the other disposition. A graphic presentation of his results looks embarrassing enough, but a certain common trend of the curves cannot be denied; erythritol, for instance, without exception, is the slowest, propionamide, without exception, the fastest, of the compounds. Between their lines the curves are often overlapping, due to the highly varying distances between the corresponding points of the curves, with the result that, instead of the order I to VII of the compounds, as it obtains with the majority of the objects, the cells of *Rhoeo* (9), for instance, follow the order: II, I, V, III, IV, VII, VI. However, this great irregularity is obviously the effect of the third of the aforementioned factors of permeability, since (according to p. 235) the penetration rates of the compounds I, III, IV and VI, owing to their NH_2 groups, can be foreseen to be slow, e.g., in relation to *Spirogyra* (7) or *Chara* (11), Fig. 24.

One may conclude from this analysis, as well as from other points, that an understanding of species-specificity, which often is believed to rely upon morphological or biochemical features, can also be approached by physicochemical study of membrane permeability. This will become even more conspicuous with erythrocytes, where species-specific permeability is manifested to an amazing degree.

2. Permeability of Erythrocytes to Nonelectrolytes.—No other cells have been used more as the object of permeability studies than erythrocytes. They are single cells, available in large numbers, and their behavior, though different from species to species, is on the whole constant if taken from the same sample of blood. In contrast to most of the plant cells mentioned in the last section, they are immersed normally in a well-balanced (Sec. 5) electrolyte solution; but in an isotonic solution of an

[25] R. Collander, Phys.-ökonom. Ges. Königsberg, **50**: 53, 1937; Tr. Faraday Soc., **33**: 985, 1937; see, further, K. Höfler, Ber. dtsch. bot. Ges., **50**: 53, 1932; **52**: 355, 1934; L. Hofmeister, Bibliot. Bot., **113**: 1, 1935.

"indifferent" nonelectrolyte solution they undergo severe alterations of their normal permeability, which, however, can be largely reduced by adding small amounts of electrolyte (e.g., 0.1 per cent NaCl), (see Sec. 5, chap. 16).

The first systematic information about permeability of erythrocytes was contributed, almost contemporaneously with the fundamental work of Overton, by Grijns and Hedin. Grijns[27] observed that the blood cells of chicken or of horse undergo hemolysis, if added to an isotonic solution of

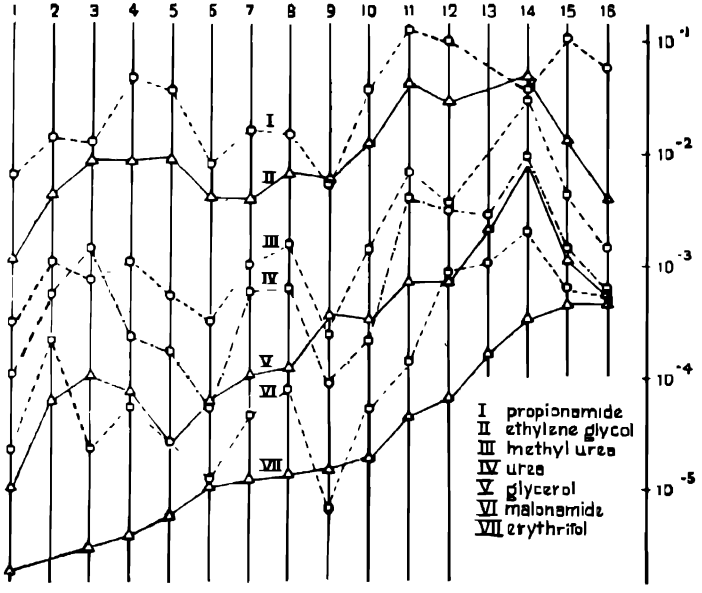

FIG. 24.—Permeability Constants of Plant Cells to Nonelectrolytes. (Collander.)

1. Leaf cells of Plagiothecium Denticulatum; 2. Oedogonium sp.; 3. Root cells of Lemna Minor; 4. Pylaiella Litoralis; 5. Zygnema Cyanosporum; 6. Subepidermal cells of Curcuma Rubricaulis; 7. Spirogyra sp.; 8. Leaf cells of Elodea Densa; 9. Epidermis cells of Rhoeo Discolor; 10. Epidermis cells of Taraxacum Pectinatiforme; 11. "Leaf cells" of Chara Ceratophylla; 12. Internodial cells of Ceramium Diaphanum; 13. Bacterium Paracoli; 14. Oscillatiria Princeps; 15. Melosira sp.; 16. Licmophora sp.

aliphatic monohydric alcohols, ether, esters, glycerol, or urea, but remain intact in saccharose, lactose, mannitol, inositol, dextrose, and asparagin. Hedin[28] determined the freezing-point depression, after equimolar amounts of the organic compounds had been added to a certain volume of blood and to the same volume of the serum; according to whether the freezing-points of the fluids are different or not, and whether they become more different with time, one is enabled to distinguish between impermeability and permeability, and, eventually, to determine even rates of penetration. Hedin, with ox cells, obtained the following series of increasing penetration: saccharose, hexoses < hexites, pentites, pentose < erythritol < glycerol

[27] G. Grijns, Pflüger's Arch. f. d. ges. Physiol., **53**: 86, 1896.

[28] S. Hedin, Pflüger's Arch. f. d. ges. Physiol., **68**: 229, 1897; **70**: 525, 1898.

< ethylenglycol < urea, aldehydes, ketones, esters. The results of Grijns and Hedin obviously are similar to those of Overton regarding plant cells (p. 230). They show that lipoid soluble substances, for instance, amyl- and benzylalcohol, ethylurethane, ethylacetic ester, independent of their molecular size, can readily enter the cells. Hedin, referring to the lipoid theory of Overton, already paid attention to the fact that urea and glycol, though lipoid insoluble, have a high penetration rate.

After these early investigations, many technical improvements toward more quantitative results have been developed, all gauging permeability by osmotic swelling (and its final stage, hemolysis), either by direct measure of the cell volume with the hematocrit,[29] or by the increasing transparency of the cell suspension to light, [30] at best applying a photocell,[31] or by taking the dispersion of light at the cell surface as an indicator.[32] These methods have been used particularly to compare the species-specific permeability of erythrocytes in a similar way to that used with plant cells (see p. 237), comparing the penetration rates of substances characterized by a moderate lipoid solubility and a moderate molecular size.[33] Thus, according to Höber and Ørskov, with blood corpuscles of rat, man, pig, and ox, the permeability appeared to decrease in the order: acetamide < propionamide < lactamide < malonamide, also to decrease in the order: urea < methylurea < thiourea; and, even irrespective of the increase of lipoid solubility, to decrease in the order: methylalcohol < ethylalcohol < propylalcohol < butylalcohol. In other words, the influence of porosity outweighs the influence of the dissolving capacity of the lipoid.

Picturing in Fig. 24 (p. 238), the permeability of various species of plants toward the organic nonelectrolytes, more attention should have been turned to the fact that the great differences actually existing from species to species between the penetration constants may have been obscured by using a logarithmic scale. Differences of a like degree are observed with animal cells, as is shown for glycerol in the following table.[34]

TABLE XIV.—TIME IN SECONDS FOR 75 PER CENT HEMOLYSIS IN 0.3 MOL. GLYCEROL IN PRESENCE OF 0.12 PER CENT NaCl

Rat	3 5	Dog	253
Mouse	12 9	Cat	459
Rabbit	21 8	Pig	340
Guinea-pig	38 2	Ox	612
Man	5 1	Sheep	850

[29] S. Hedin, Skandinav. Arch. Physiol., **2**: 134, 360, 1892; **5**: 207, 1895.

[30] M. H. Jacobs, Biol. Bull., **58**: 104, 1930.

[31] H. Netter and S. L. Ørskov, Pflüger's Arch. f. d. ges. Physiol., **231**: 135, 1932.

[32] S. L. Ørskov, Biochem. Ztschr., **259**: 349, 1934; **279**: 241, 1935; A. K. Parpart, J. Cell. & Comp. Physiol., **7**: 153, 1935.

[33] See M. H. Jacobs, Am. Philos. Soc., **70**: 363, 1931; R. Mond and F. Hoffmann, Pflüger's Arch. f. d. ges. Physiol., **220**: 194, 1928; R. Höber and S. L. Ørskov, *ibid.*, **231**: 599, 1933.

[34] M. H. Jacobs, Ergebn. d. Biol., **7**: 1, 1931.

It appears that two groups of mammals can be sharply distinguished. They differ in other respects, also.[35] The red cells with a high permeability to glycerol are distinguished by a low temperature coefficient of penetration, and their permeability is diminished by CO_2 and other acids and by traces of Cu. A similar correlation has been found with glycol. A hindering effect resembling that of Cu has been found with Ni, Zn, Co, and Hg, and is possibly based upon an acidification of the cell surface due to the formation of undissociated complex salts of the heavy metals.[36]

A series of experiments concerning erythrocytes has been mentioned before with reference to the permeability of plant cells to organic substances containing in their molecule one or two NH_2-groups (p. 235). Jacobs[37] had found that the red cells of mammals and reptiles are more permeable to urea than to glycol, while those of fishes and birds show the reverse behavior. Höber and Ørskov, including in their study some amides and glycerol, arrived at approximately the same result. More experiments are needed, however, to secure this correlation between certain chemical properties of nonelectrolytes and their penetration power. It seems not unlikely, as yet, that the hindering effect of acidification on the permeability of the erythrocytes of mammals is a counterpart of the opposite effect of the NH_2-groups.

Especially fascinating problems are presented in this field by observations dealing with compounds, which, in spite of their lipoid insolubility and their large *MV*, enter the erythrocytes of certain species and thus fit into neither of the main theories of permeability. These problems have already been touched upon in findings of Jacobs[38] that erythritol, which, due to its somewhat smaller *MV*, is enabled to enter very many cells, passes into mammalian erythrocytes with exceedingly diverse speeds—fastest (in less than 5 minutes) into those of mouse, slowest (in more than 24 hours) into those of ox and sheep. Similar studies sprang originally from an interest in the "blood sugar" in human physiology and pathology, and were stimulated by the fact that glucose can enter the erythrocytes of man, but of no other mammal except monkey.[39] Besides glucose, the other hexoses and pentoses likewise enter the human cells, but neither glucoheptose nor methylglucoside; further not disaccharides, hexites, pentites. More recent studies have amplified the knowledge concerning the human cells;[40] first, pentoses enter somewhat faster than hexoses; second, the aldohexoses faster than the ketohexose fructose. Both facts are interesting in contrast with the earlier observations of Wilbrandt on the passage of sugars across the

[35] M. H. Jacobs, H. N. Glassman, and A. K. Parpart, J. Cell. & Comp. Physiol., **7**: 197, 1935.

[36] W. Wilbrandt, Pflüger's Arch. f. d. ges. Physiol., **244**: 637, 1941.

[37] M. H. Jacobs, Am. Philos. Soc., **70**: 363, 1931.

[38] M. H. Jacobs, Ergebn. Biol., **7**: 1, 1931.

[39] S. Kozawa, Biochem. Ztschr., **50**: 291, 1914; R. Ege, *ibid.*, **111**: 189, 1920, and **114**: 88, 1921; for further literature see R. Höber, Physik. Chem. d. Zelle u. d. Gewebe. 6. Aufl., pp. 462 ff: Engelmann, Leipzig, 1926.

[40] W. Wilbrandt, Pflüger's Arch. f. d. ges. Physiol., **241**: 302, 1938.

intestinal membrane of rats, where pentoses have been found to be slower than hexoses, though having the smaller *MV*, and where poisoning with iodoacetate abolishes the characteristic succession of the hexoses, while here it is indifferent (see Sec. 8, chap. 34).

In extending these studies toward the erythrocytes of other mammals, more species-specific variations have been encountered. According to Höber and Ulrich,[41] mouse erythrocytes not only fail to admit the hexoses, but allow the hexites mannitol, sorbitol, dulcitol, to enter freely. Rat cells do not display this characteristic feature.[42] The red cells of the guinea-pig show another differential permeability; they admit amino-acids (glycin, alanin), but exclude hexoses and hexites (Höber and Ulrich). Still another pattern appears with dog cells; pentoses are here the fastest, the ketohexose fructose and sorbose enter more slowly and the aldohexoses are practically nonpermeant (Wilbrandt). Finally, the cells of the ox, pig, rabbit, and rat show the same impermeability as may be expected with lipoid insoluble substances of a moderate *MV*, such as sugars, hexites, and amino-acids (Kozawa).

Certainly, exploration of more species with more substances under more varied conditions, with an additional study of other cells, will disclose more specificities. One would like to know whether such peculiarities of permeability correspond to special functional requirements of an animal or its individual cells. For example, the behavior of certain erythrocytes toward sugars and amino-acids is, perhaps, associated with some role they play as regulators of the normal unbalance between intracellular and extracellular fluids. In the present state of our knowledge, it is impossible to distinguish a true specialization from an incidental feature, a mere "lusus naturæ" (see, further, Sec. 8, chaps. 34 and 35).

3. Permeability of Some Other Objects to Nonelectrolytes.— There is, so far, very scanty knowledge about the permeability to nonelectrolytes of single cells other than erythrocytes and plant cells studied by plasmolysis or osmotic swelling and shrinking. The only equivalent systematic investigation is that of Overton concerning muscle tissue, a study more qualitative than quantitative.

Marine eggs such as those of the sea-urchin, are single cells which appear appropriate for study by osmotic swelling and shrinking, but scarcely more is known than that the permeability rises in the series: acetamide < propionamide < butyramide, and glycerol < ethylenglycol, that methyl- and ethylalcohol enter very rapidly, and that glucose does not.[43] These suc-

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[41] R. Höber and H. Ulrich, Klin. Wchnschr., **13**: 63, 1934. H. Ulrich, Pflüger's Arch. f. d. ges. Physiol., **234**: 42, 1934.

[42] M. H. Jacobs, H. N. Glassman, and A. K. Parpart, J. Cell. & Comp. Physiol., **11**: 479, 1938.

[43] M. H. Jacobs and D. R. Stewart, J. Cell & Comp. Physiol., **1**: 73, 1932; see, further, D. R. Stewart and M. H. Jacobs, *ibid.*, **7**: 333, 1936; B. Lucké, H. K. Hartline, and R. A. Ricca, *ibid.*, **14**: 237, 1939.

cessions are like those found with *Chara* (see Collander and Bärlund, *loc. cit.* 3). In other words, the succession of the amides parallels their lipoid solubility, while the order of the lipoid insoluble glycerol and ethylenglycol suggests their passage through the porous areas of the membrane (p. 235). With a membrane of a more outstandingly porous type the succession of the amides probably would run in the opposite direction (see p. 237); further, the observations of Höber and Ørskov, (p. 239).

Overton's procedure for studying the *permeability of muscle*[44] is analogous to his plasmolytic studies of plant cells (p. 230). Frog sartorius muscles, placed in isotonic Ringer's solution, keep their weight constant over a long period. When a nonelectrolyte is added in a certain molar concentration to the Ringer's solution, impermeability is indicated by a permanent loss of weight. Addition of a substance with a relatively small permeating capacity is followed by a loss of weight, which disappears after some time, differing according to the rate of penetration. With a quickly entering substance, no initial weight change whatever appears.

The results of these experiments are about the same as those outlined in the table, p. 230, for *Chara*;[45] for instance, urea, which is distinguished by the combination of a small lipoid solubility and of a very low MV, is mentioned by Overton as entering especially slowly.

In analyzing the data concerning the permeability of the muscle tissue, one may refer the effects to the muscle fibers themselves, which occupy between $\frac{4}{5}$ and $\frac{5}{7}$ of the total volume in the frog sartorius muscle, depending upon whether they have been soaked in Ringer's solution or in plasma; in other words, the interfibral space is $\frac{1}{5}$ to $\frac{1}{7}$.[46] This space readily communicates with the surrounding fluid by free diffusion, and calculation shows that, if, for instance, the Cl percentage in this fluid is varied, the Cl content of the muscle changes in such a way as to indicate a distribution equilibrium whereby Cl is located practically to completion outside the fibers, i.e., the "chloride space" is equal to the "interfibral" or "tissue space."[47] This justifies the assumption that the aforementioned results concerning permeability or impermeability to the nonelectrolytes are bearing upon the surface membrane of the fibers.[48]

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[44] E. Overton, Pflüger's Arch. f. d. ges. Physiol., **92**: 115, 1902.

[45] See also Overton in Nagel's Handbuch Physiol. des Menschen, **2**: p. 744: Vieweg., Braunschweig, 1906–07.

[46] See W. O. Fenn, Physiol. Rev., **16**: 450, 1936.

[47] See more about the interfibral space later, p. 253.

[48] About some osmotic experiments concerning the permeability of the liver, see R. Höber, Pflüger's Arch. f. d. ges. Physiol., **229**: 402, 1932. For more about passive penetration of the intestine, the kidney, the salivary gland, see Sec. 8.

11

PERMEABILITY TO ELECTROLYTES

1. Plant Cells.—Since Overton's fundamental study, plant cells have often been considered impermeable to inorganic neutral salts, or, at most, only slightly permeable. This conclusion was drawn from observations on cells, which from water were transferred into NaCl or KCl solutions of such a strength that plasmolysis appeared, which seemed to be persistent for some time. Later, with a more refined procedure, it has been shown,[49] that the plasmolysis, sooner or later, is succeeded by deplasmolysis, which, after a while, may be followed by another plasmolysis, often leading to death. This sequence of events has been interpreted as due to an impairment of the cell surface by the solutions, which brings about an abnormal permeability to the salt, indicated by deplasmolysis; the consequent escape of solutes from the cell interior ends in collapse of the protoplast. The concept that in its normal state the membrane is impermeable is supported by the following observations. First, S. C. Brooks,[50] by measuring the conductivity of the surrounding fluid, has stated that *Taraxacum* cells, placed in distilled water, suffer a slight loss of electrolytes, but that this loss is markedly increased in dilute NaCl solution. Second, cells have been shown extremely impermeable to salts or their ions, presented in small concentrations, provided care is taken to keep them under physiological conditions in other respects. For instance, several ions (Li, SCN and others) added to sea water as the normal milieu of the alga *Valonia*, do not appear in the cell sap, even after hours or days; very little Cs was found even after more than a year, Rb somewhat more.[51] Collander[52] analyzed by spectrography samples of the cell sap of two species of *Characeæ*, after salts were added in small subplasmolyzing concentrations to the brackish water in which they live. The result was that, even after 8 to 14 days, Li salt did not reach a concentration within the sap equal to that in the surrounding medium; light accelerated, and anoxia retarded, the movement. Returned to the normal Li-free medium, the cells did not lose the Li previously taken up. Third, the strongest argument for impermeability, or at least dyspermeability,

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[49] W. J. V. Osterhout, Science, **34**: 187, 1911; The Plant World, **15**: 129, 1913; H. Fitting Jahrb. f. wiss. Bot., **56**: 1, 1915; also **57**: 553, 1917; A. Tröndle, Arch. de Sc. phys. et nat., **45**: 38, 1918.

[50] S. C. Brooks, Am. J. Bot., **3**: 483, 1916.

[51] W. C. Cooper, M. J. Dorcas, and W. J. V. Osterhout, J. Gen. Physiol., **12**: 427, 1929.

[52] R. Collander, Protoplasma, **33**: 215, 1939.

relies on comparing the chemical composition of the cell sap with the surroundings of large-sized ("giant") cells, some living in sea water, some in brackish water, and some in pond water[53] (see Table XV).

TABLE XV.—CHEMICAL ANALYSES (MOL.) OF THE SAP AND THE MILIEU OF CELLS
(According to Osterhout, Cooper and Blinks, Blinks and Jacques, Zschiele and Collander)

	Sap of *Valonia macrophysa*, mol.	Sap of *Halicystis*, mol.	Sea water, mol.	Sap of *Nitella clavata*, mol. $\times 10^3$	Pond water bathing *Nitella*, mol. $\times 10^3$	Sap of *Chara ceratophylla*, mol. $\times 10^3$	Brackish water bathing *Chara*, mol. $\times 10^3$
Cl	0 597	0 603	0.580	90 8	0 903	225 0	73 0
Na	0 09	0.557	0 498	10 0	0 217	142 0	60 0
K	0 5	0 0064	0 012	54 3	0 051	88.0	1 4
Ca	0 0017	0 008	0 012	10 2	0 775	5.3	1 8
Mg	trace?	0 0167	0 057	17 7	1 60	15 5	6 5
SO_4	trace?	trace	0 036	8 33	0 323	3.9	2.8

Table XV shows the great variation of the internal milieu from one species to another, even taking into account that individual differences will probably appear when more single cells have been compared. Table XVI is particularly important as it raises the questions how the gradients of concentration can arise, whether they represent a static or a dynamic equilibrium, and in the latter case, by what means this equilibrium can be maintained. The second

TABLE XVI.—RATIOS OF INTERNAL TO EXTERNAL CONCENTRATIONS

	Valonia macrophysa	*Halicystis*	*Nitella clavata*	*Chara ceratophylla*
Cl	1 03	1 04	100 50	3 1
Na	0 18	1 12	46 10	2 4
K	41.6	0 53	1065.00	63 0
Ca	Very small	0 67	13.17	2 0
Mg	Very small	0 29	10 47	2 4
SO_4	0	0	25 80	1 4

alternative, a dynamic equilibrium, is more probable. This would explain the aforementioned observation of Collander (p. 243) and similar results obtained with *Nitella*.[54] Presumably, some active factor is superimposed on a passive distribution, permeability being not so much a matter of simple diffusion as of active transfer, which needs an energy supply. This will be discussed in detail later (see Sec. 8, also Sec. 5).

Fourth, returning to the problem of plasmolysis and deplasmolysis of plant cells under the influence of neutral salt solutions, and to its interpreta-

[53] W. J. V. Osterhout, Biol. Rev., **6**: 369, 1931; Bot. Rev., **2**: 283, 1936.

[54] D. R. Hoagland and A. R. Davis, J. Gen. Physiol., **5**: 47, 1923; see, further, chap. 38.

tion as the consequence of an impairment of the normal surface conditions, it can be shown that a pure Na salt or K salt in contact with a cell creates an abnormal condition of the surface, and that the addition of small amounts of certain other salts restores normalcy. For instance, *Nitella* cells can be shown, by the diphenylamine test, to take up NO_3 from a $NaNO_3$ solution, but NO_3 does not appear in the presence of $Ca(NO_3)_2$.[55] The balancing effect of Ca in concentrations small compared to Na or K has often been demonstrated by the intensified plasmolysis obtained with the mixture, and by the abolition of deplasmolysis. This antagonism is evident to different degrees with different cells; it is more evident with *Rhoeo*[56] or *Lupinus*[57] than with *Lamium*.[58] When permeability is measured by rate of plasmolysis, alkali ions are found to increase permeability according to the series: $Li < Na < K$, anions according to $SO_4 < Cl \leqq NO_3 \leqq Br$.[59] The antagonizing effect of Ca is similar to that of Sr and Ba. Mg resembles more closely the alkali ions. The result of these and other studies is the concept that, applying solutions of appropriate mixtures of salts, or, more precisely, of alkali and alkaline earth salts, for investigating the permeability of plant cells, instead of the unnatural solutions of single salts, the cells appear to be impermeable or fairly impermeable. It will be shown later that this is equally true with numerous animal cells. Since this antagonism in plant, as well as in animal, cells is an antagonism between mono- and poly-valent cations,[60] which was first interpreted by J. Loeb to be an influence upon the colloidal constituents of the cells (see Sec. 5, chap. 16), the conclusion seems indicated that a normal molecular architecture of the cell surface is a prerequisite for normal impermeability or dyspermeability (see chap. 17). This concept does not conflict with the aforementioned postulate that by physiological means, especially by an adequate supply of metabolic energy, the properties of the cell surface can be changed toward a greater permeability.[61]

2. Erythrocytes.—*a*). *Permeability to anions.*—On the background provided by the studies of Hamburger (1891), Gürber (1895), and Koeppe (1897), it has been generally accepted that the erythrocytes differ from other

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[55] W. J. V. Osterhout, Science, **34**: 187, 1911; The Plant World, **16**: 129, 1913; further, H Fitting, Jahrb. f. wiss. Bot **56**: 1, 1915; **57**: 553, 1917; A. Tröndle, Arch. de Sc. phys. et nat., **45**: 38, 1918; W. J. Osterhout, J. Gen. Physiol., **4**: 275, 1922.

[56] H. Netter, Pflüger's Arch. f. d. ges. Physiol., **198**: 225, 1923; A. Kaczmarek, Protoplasma, **6**: 264, 1929.

[57] H. Kaho, Univers. Dorpat. Inst. Bot. Opera, Nr. **18**: 1924.

[58] H. Weixl-Hofmann, Protoplasma, **11**: 210, 1930.

[59] Osterhout, Tröndle, Kaho, *loc. cit.* About contradictory results, see p. 324.

[60] For plant cells see especially H. Netter, Pflüger's Arch. f. d. ges. Physiol., **198**: 225, 1923, who found with *Rhoeo* cells the balancing strength of divalent cations to follow the order: $Ca > Ni > Co > Sr > Ba > Mn$.

[61] For an interesting study of the permeability of *Beggiatoa* to organic anions and the physicochemical viewpoints concerned, see W. Ruhland, H. Ullrich, and G. Yamada, Planta, **18**: 338, 1932.

cells in their selective permeability to inorganic anions and their impermeability to cations. The well-known physiological significance of these facts is their influence upon the acid-base equilibrium in blood, evinced by the alternating exchange of Cl and HCO_3, the so-called "chloride shift," (see also Sec. 8, chap. 37).

In view of this situation, an isotonic solution of an inorganic salt may appear to be osmotically indifferent to the red cells suspended in it, as was found 50 years ago by Grijns and Hedin. Ammonium salts display an exceptional behavior: in their solutions the red cells hemolyze, with chloride and bromide faster than with nitrate, sulfate, and phosphate.[52] This behavior occurs solely with erythrocytes, and is effected not by permeability to the cation NH_4, but by anionpermeability. According to Jacobs,[53] e.g., NH_4Cl is slightly hydrolyzed, and, due to its small *MV*, the free NH_3 enters. Whereas nothing happens after that with common cells, except a minute increase of alkalinity, with erythrocytes there follows an exchange of internal OH and external Cl, and this transfer continues until hemolysis appears. A picture of this progress is given by Fig. 25.

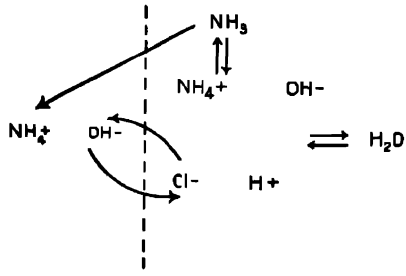

FIG. 25.—Hemolysis in isotonic ammonium chloride. (Jacobs.)

It was mentioned before that the isotonic solutions of inorganic salts are osmotically indifferent to the red cells. This is true only when monovalent anions are concerned in the exchange. In an isosmotic solution of polyvalent anions, blood corpuscles shrink. The explanation is that in order to maintain electroneutrality a SO_4 ion, for instance, exacts 2 Cl or 2 HCO_3 ions in exchange.[54] At the same time the osmotic resistance rises. This presumably is due to several circumstances, among others to the dehydrating effect of the polyvalent anions, sulfate, phosphate, tartrate, citrate and others, which results in a closer packing of the structural components of the surface membrane. This effect, coupled with the greater hydration of these ions (see Sec. 5, chap. 16), accounts for the polyvalent anions penetrating the membrane at a distinctly lower rate than the monovalent do, comparing, for instance, the exchange of Cl against HCO_3, and of Cl against SO_4, as has been shown by chemical analysis,[55] or by applying an Ag, AgCl-electrode in order to follow the exit of Cl.[55]

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[52] G. Grijns, Pflüger's Arch. f. d. ges. Physiol., **63**: 85, 1896; Hedin, *ibid.*, **68**: 229, 1897; **70**; 525, 1898; R. Ege, Biochem. Ztschr., **130**: 160, 1922.

[53] M. H. Jacobs, Am. J. Physiol., **68**: 134, 1924; The Harvey Lectures, **22**: 146, 1927; M. H. Jacobs and D. R. Stewart, J. Cell. & Comp. Physiol., **7**: 351, 1936.

[54] H. Koeppe, Pflüger's Arch. f. d. ges. Physiol., **67**: 189, 1897; see also A. K. Parpart, Cold Spring Harbor Symp., **8**: 25, 1940.

[55] R. Mond and H. Gertz, Pflüger's Arch. f. d. ges. Physiol., **221**: 623, 1929.

[56] K. Timm, Pflüger's Arch. f. d. ges. Physiol., **239**: 286, 1938.

Our understanding of erythrocyte *permeability to organic anions* is so far highly unsatisfactory, for the reason that a great number of factors must be taken into account in order to explain the effects, which are chiefly osmotic changes of the cell volume and hemolysis. For, the effects depend upon whether the organic acids forming the salts are strong or weak, whether they are lipoid soluble and surface active, whether the ions are strongly hydrated and have "hydrotropic" or "solubilization" properties (chap. 20), and whether they are poisonous, acting either on the surface or on the entire protoplast, after having entered freely. It will be helpful to begin with comparing the Na and the NH_4 salts.

(i). *The Na salts of the aliphatic fatty acids,* with a chain of C-atoms not longer than C_5, are fairly indifferent, provided the *p*H of the solutions has been buffered to about 7.3; beginning with valerate (C_5) or capronate (C_6) and proceeding to the higher members of the series, hemolysis occurs.[67] Decreasing the *p*H favors the lytic influence; increasing the *p*H weakens it.[68] For an explanation of these findings, several factors must be taken into consideration. The fatty acids are weak acids; all those from C_2 to C_9 have a *p*K of about 4.8. The lower the *p*H, the higher is the concentration of the free acid in the solution. Their undissociated molecules are lipoid soluble (see chap. 12). So far, the break at C_5 in the graph of their effectiveness seems unexpected. But the same break appears, if one compares the surface activity, either water-air or water-hydrocarbon, of the aqueous solutions of the Na salts; from C_2 to C_5 it rises slowly, but then, from C_5, abruptly.[69] Obviously, the lower members of this series can be classified fairly near to Cl in physicochemical (chap. 15) and in physiological respects (chap. 34), whereas the hemolytic effect of the higher members must be attributed not only to an exchange of anions, but also to the hydrotropic effect, probably exhibited at the exterior as well as at the interior phase boundaries (Sec. 5, chap. 20).[70]

Turning now to the NH_4-salts of the fatty acids, not much remains to be added. The main point is that at *p*H 7.3 the NH_4-salts of the C_2- to C_5-acids cause hemolysis at a speed rather like that with NH_4Cl, but increasing from C_2 to C_5.[71] This increase corresponds closely with the increase of lipoid solubility; hence, these compounds can be supposed to pass through the erythrocyte membrane by the lipoid route as well as the pore route.

(ii). *The Na- and NH_4-salts of aliphatic di- and tricarboxylic and monooxymonocarboxylic acids.* The Na-salts of the C_4-compounds, succinic, tartaric, fumaric, and citric acid, have been known for a long time to be fairly indifferent to various cells and tissues (see the potential measurements). The same is true with the NH_4-salts,[72] which rather resemble $(NH_4)_2SO_4$ (see p. 246). This can be ascribed to several circumstances. We are dealing with rather strong acids,

[67] R. Höber, J. Cell. & Comp. Physiol., **7**: 367, 1936.

[68] Compare the effects on sea-urchin eggs: D. Stewart, Biol. Bull., **60**: 171, 1931.

[69] H. Freundlich and G. V. Slottmann, Ztschr. f. physik. Chem., **129**: 305, 1927; H. Freundlich and D. Krüger, Biochem. Ztschr., **205**: 186, 1929; L. Lascarey, Kolloid-Ztschr. **34**: 73, 1924; A. von Kuthy, Biochem. Ztschr., **237**: 396, 1931.

[70] See, further, R. Höber, M. Andersch, J. Höber, and B. Nebel, J. Cell. & Comp. Physiol., **13**: 195, 1939.

[71] M. H. Jacobs, The Harvey Lectures, **22**: 146, 1927.

[72] R. Höber, J. Cell. & Comp. Physiol., **7**: 367, 1936.

which as such are lipoid insoluble, but also, because they are strongly hydrated due to the polar carboxylic and, eventually, also hydroxyl groups; hence they diffuse slowly and traverse pores with difficulty. In addition, they are strongly hydrophilic toward colloids; in other words, they compete with these colloids for hydration water; they are surface inactive, and lack "hydrotropic" properties (chap. 20). Among the NH_4-salts, the rate of hemolysis is greater in solutions of the C_3-series, lactic, and pyruvic acid, i.e., monooxymonocarboxylic acids, than in solutions of C_4. It is still more increased with the corresponding C_2-compound, ammonium glycolate, which approximately equals ammonium acetate.

(iii). *NH_4-salts of the aromatic monocarboxylic acids* are hemolytic at *p*H 7.3; Na-salts are not. This seems contradictory to the response of muscles, which display an injury current with Na benzoate and salicylate. (Höber, *loc. cit.*) However, it is questionable whether an alteration indicated by the electric behavior of the surface membrane of muscle is equivalent to an alteration which releases hemoglobin. The latter is believed to be a rupture of the membrane, whilst the former can be brought about even by a slight diminution of the lateral adhesion forces in the molecular array of the surface structure (see chaps. 17 and 20). The hemolytic action of NH_4 salicylate is comparable to that of NH_4Cl. In contrast to the salts of the aliphatic monocarboxylic acids (p. 247) NH_4 salicylate is the salt of a rather strong acid ($pK = 3$) and as such not subject to much hydrolysis. On the other hand, the free salicylic acid is highly lipoid soluble. Thus, the rate of hemolysis appears to be the effect of rival factors. Furthermore, denaturation of proteins, including hemoglobin, can be produced by salicylate as well as by the salts of other aromatic acids, probably due to their polarnonpolar molecular configuration (subchap. 16, 2 and chap. 20), and is followed by the escape of solutes from the interior of the cells. This seems to increase the osmotic resistance, and so partly offsets the lytic tendency.[73] Obviously, further study is needed to settle the relative importance of these conflicting influences.

(iv). *Behavior in solutions of nonpenetrating nonelectrolytes.*[74] It has been known for a long time[75] that in nonelectrolyte solutions (saccharose) erythrocytes suffer a leakage of electrolytes, as indicated by chemical analysis and by a rise of osmotic resistance.[76] Jacobs and Parpart[77] have found that this process occurs in two stages, one, which is reversible and rapid and finished after about 1 minute, and one which is irreversible, slow, and extending over a long time. The nature of the second process will be discussed later (see subchap. 16, 3); the first one is due to the steep gradient of anions from the cell interior to the nonelectrolyte solution, causing an exchange of Cl and HCO_3 ions from inside for OH ions from outside, and hence resulting in an increase of acidity outside, of alkalinity inside. The latter brings about an increase of ionization of

[73] R. Höber, J. Cell. & Comp. Physiol., **7**: 367, 1935. For further information about permeability to organic anions, see Maizels, Biochem. J., **28**: 337, 1934.

[74] See also subchap. 16, 3.

[75] I. Bang, Biochem. Ztschr., **15**: 255, 1909; A. Joel, Pflüger's Arch. f. d. ges. Physiol., **161**: 5, 1915.

[76] E. Ponder and G. Saslow, J. Physiol., **73**: 267, 1931.

[77] M. H. Jacobs and A. K. Parpart, Biol. Bull., **62**: 178, 1932; **65**: 512, 1933; W. Wilbrandt, Pflüger's Arch. f. d. ges. Physiol., **243**: 537, 1940.

hemoglobin, i.e., the formation of polyvalent hemoglobin anions Hb^{n-}, replacing the monovalent HCO_3^- anions, according to the equation:

$$n\,B^+ + n\,HHb + n\,HCO_3^- = n\,B^+ + Hb^{n-} + n\,CO_2 + n\,H_2O.^{78}$$

Consequently, the internal osmotic pressure diminishes, as indicated by the increase of osmotic resistance.

b). *Permeability of erythrocytes to cations.*—In Tables XV and XVI (p. 244) it has been shown that various species of plant cells preserve in their cell sap characteristic mixtures of ions with steep gradients, some uphill, some down, toward the outside solution. This is equally true whether the normal medium of the plants is fresh, brackish, or sea, water. This looks like convincing evidence of impermeability to these inorganic ions, unless one takes into consideration that this ionic distribution could also be the result of a dynamic equilibrium which the living cell maintains with metabolic energy, and which gives way only in death to a stable state. Similarly, the species-specific distribution of inorganic ions in erythrocytes, which is apparent from the following table, can be thought of as being essentially the result of activity during an early stage of the life cycle, when the red cells were being generated in the blood-forming organs.[79] However, although erythrocytes have been supposed to be exceedingly inactive biochemically as well as structurally it has been found recently that a slow shift of cations against the concentration gradient compensates a preceding loss (see p. 251).

TABLE XVII.—K AND Na IN MILLIMOL. PER 1000 GR. ERYTHROCYTES (KERR[80])

Animal	K	Na	Animal	K	Na
Man	110		Pig	100	11
Monkey	111		Horse	88	
Rat	100	12	Ox	22	79
Rabbit	99	16	Dog	9	107
Guinea-pig	105	15	Cat	6	104

As appears in Table XVII, K and Na are distributed very characteristically in the red cells of different mammals. First, there are 2 groups of animals, one with a preponderance of K in the cells, the other with a preponderance of Na. Second, this selectivity is independent of the cation concentration in the blood serum, as the ionic composition of the serum is practically identical in all mammals, with Na far in excess of K. Now, this characteristic unbalance, K:Na, is easily upset under many circumstances. Almost any kind of alteration of the normal surrounding medium is followed

[78] See E. J. Warburg, Biochem. J., 16: 153, 1922; D. D. Van Slyke, H. Wu, and F. C. McLean, J. Biol. Chem., 56: 765, 1923.

[79] See V. Henriques and S. L. Ørskov, Skandinav. Arch. f. Physiol., 74: 63, 1936, and 82: 86, 1939.

[80] S. E. Kerr, J. Biol. Chem., 117: 227, 1937.

by a movement toward a more equal distribution, most observations so far referring to a leakage of K. K can be released, for example, by slight hyper- or hypotonicity accompanied by a deformation of the cells, by a small change in the balance of cations in the suspending salt solution, by a rise in temperature, even by mere standing in serum or in Ringer's solution, or by centrifuging. Furthermore, the addition of certain cytolytics or of NaF leads to an escape of K.[81]

Now, disregarding for the moment the effect of cytolytics and of fluoride, selective ionpermeability is likely to be lost, as indicated by the escape of K, as the result of relatively slight changes of the physical properties of the surface, milder changes than those that cause visible leakage of hemoglobin. Presumably, the changes consist in loosening of the linkage of colloidal aggregates. The same assumption has already been proposed (p. 245) in interpreting rates of plasmolysis and deplasmolysis as a measure of permeability to agents as gentle as neutral alkali salts are, where the effects of ions increased gradually in the order: Li < Na < K and SO_4 < Cl < NO_3 < Br, an order well known from colloid chemistry as the Hofmeister's or lyotropic series. Numerous similar observations will be referred to later. In muscles particularly, studied by measuring, for example, the membrane potentials of the fiber surface, a reversible loss of selective ionpermeability follows exposure to various cations and anions of alkali salts, and has been correlated with the state of colloidal components of the surface membranes. The same agents, viz., the isotonic or slightly hypotonic solutions of the alkali salts, when acting upon the red blood corpuscles through hours or days (instead of minutes), increase leakiness so much as to enable hemoglobin molecules to escape. In this phenomenon, also, the strength of the cationic, and still more of the anionic, effect is found to vary according to the lyotropic series, though numerous irregularities have been observed, depending upon species-specific properties of the erythrocytes and upon additional factors connected with the surrounding solution (chap. 16, 2c; further, the studies of Davson regarding cat and dog erythrocytes, *loc. cit*).

Among the various conditions abolishing the normal impermeability to cations, fluoride deserves special discussion, because it suggests an influence

[81] For many details concerning these alterations of the normal ionic distributions see, with respect to hyper- and hypotonic solutions: E. Ponder and Saslov, J. Physiol., **70**: 169, 1930; **73**: 267, 1931; E. Ponder and Robinson, Biochem. J., **28**: 1940, 1934; M. H. Jacobs, H. N. Glassman and A. K. Parpart, J. Cell. & Comp. Physiol., **8**: 403, 1936; H. Davson, *ibid.*, **10**: 247, 1937; **15**: 317, 1940; Biochem. J., 33, 38, 1939; regarding centrifugation in serum, Ringer's, and NaCl: F. Danielli and H. Davson, *ibid.*, **32**: 981, 1938; regarding lytic agents: F. Danielli and H. Davson, *ibid.*, **32**: 991, 1938; regarding photodynamic dyestuffs: H. Davson and E. Ponder, J. Cell. & Comp. Physiol., **15**: 67, 1940; regarding fluoride: W. Wilbrandt, Tr. Faraday Soc., **33**: 956, 1937; Pflüger's Arch. f. d. ges. Physiol., **243**: 519, 1940; regarding temperature: M. H. Jacobs, H. N. Glassman and A. K. Parpart, J. Cell. & Comp. Physiol., **8**: 403, 1936; regarding Na-rich cat and dog corpuscles: H. Davson, *ibid.*, **15**: 317, 1940; J. Physiol., **101**: 265, 1942; Biochem. J., **34**: 917, 1940.

based upon breaking the normal concatenation of reactions participating in glycolysis. According to Wilbrandt,[82] after addition of 0.25 mol. NaF to a suspension of human erythrocytes in NaCl solution, an enormous increase of osmotic resistance occurs. This is not due to precipitation of Ca, since iodoacetate, another agent which interrupts glycolysis, also raises the osmotic resistance by releasing K. The poisoning effect in both cases must be referred to the break-up of the glycolytic sequence of reactions, as the poisoning effect runs parallel to the glycolytic activity of red cells of different species and is nil with nonglycolyzing pig cells, though they are rich in K (see Table XVII). Another argument for normal glycolysis being indispensable to the normal impermeability to K is the fact that the increase of osmotic resistance after addition of NaF is abolished by a proper amount of pyruvic acid (see Sec. 6). This raises the question as to the mechanism of the capacity of the erythrocyte membrane to retain K ions. On the one hand, normal glycolysis may be effective by warranting a structural situation, which retains the cations. On the other hand, glycolysis may maintain ionic equilibrium by releasing energy for the backtransport of K, which is on the way to escape from the interior of the cells. Examination of this alternative[83] so far has led to contradictory findings.[84] According to Wilbrandt, no change of the normal cation distribution occurs, if the temperature is markedly lowered. Harris,[85] however, also working with human erythrocytes, has found that when they were stored at refrigerator temperatures (2° to 5°) under sterile conditions for 5 days, K ion leaked out into the plasma along the diffusion gradient, and reentered when the cells were returned to temperatures of 25° and 37°. Restoration of K was more complete after glucose had been added. These latter facts recall the experiments of Hoagland and Davis[86] with *Nitella* cells, which at a low temperature or in the dark become leaky to the inside Cl, and recapture it at room temperature or during exposure to light (see Sec. 8, chap. 38).

3. Other Animal Cells.—The selective anionpermeability of blood corpuscles is unique. Its existence has been proved directly by chemical means and, indirectly, by observation of osmosis, and can be accepted as indicative of a purely physicochemical property of the cell surface. In addition, there are signs of cationpermeability, which is probably due to an active transfer. Now, before turning to studies concerning other animal cells, the situation in plant cells may be recalled. The conclusion was drawn (p. 245), in discussing them, that the "quiescent" cells are generally impermeable to anions and to cations, but, that the quiescent state, according to manifold convincing evidence, can give way to a state of activity varying

[82] W. Wilbrandt, Pflüger's Arch. f. d. ges. Physiol., **243**: 519, 1940.

[83] Wilbrandt, *loc. cit.*; J. E. Harris, J. Biol. Chem., **141**: 579, 1941.

[84] See also H. Davson, J. Cell. & Comp. Physiol., **18**: 173, 1941.

[85] J. E. Harris, *loc. cit.* See further T. S. Danowski, J. Biol. Chem., **139**: 693, 1941.

[86] D. R. Hoagland and A. R. Davis, J. Gen. Physiol., **5**: 629, 1923; **6**: 47, 1923; D. R. Hoagland, P. L. Hibbard, and A. R. Davis, *ibid.*, **10**: 121, 1926.

in degree from ample permeability to both cations and anions to almost perfect impermeability. This will be shown later (especially in Sec. 8,) chap. 36 by reviewing the studies of Hoagland, Steward, and Brooks, and has been shown by the observations of Osterhout, Collander, and others already discussed (p. 243).

> Another way is known, and will be discussed later, to demonstrate cation (and perhaps anion) permeability in plant cells: the measurement of electrical potentials across the cell surface. Ordinarily, this permeability cannot be proved to exist by chemical analysis, because of the high electrical resistance of the membrane, which prevents a sufficient number of ions being transported by the current. But, this passage possibly can be evidenced by chemical reaction, for instance, with cells of *Halicystis*, where the protoplasmic wall is the seat of an electromotive force of 70 to 80 mV, which is maintained for weeks, and which has been observed to drive a current of 1 to 5 microamperes for several days through a capillary electrode, fixed inside the sap, and connected through the external circuit with the second electrode, attached to the outside of the cell.[87]

a). *Eggs of marine invertebrates* are animal cells, which are single like erythrocytes, and readily available for experimental purposes, but, so far, have scarcely been utilized for studying permeability to the common ions. As was mentioned before (p. 246), other cells, in contrast to erythrocytes, do not cytolyze in NH_4Cl, unless appreciable amounts of bicarbonate are present. Thus, the volume of sea-urchin eggs remains unchanged for a long time in isotonic NH_4Cl or NH_4NO_3 as well as in the corresponding Na and K salts. But, they swell and die in solutions of the ammonium salts of the lower fatty acids.[88] This is due to the strong hydrolysis of these salts, compared to that of salts of strong acids. This has been proved by the observation that increasing the acidity, e.g., from *p*H 7.8 to 6.2 increases the rate of swelling. At this *p*H the rates rise in the order: acetate < propionate < butyrate < valerate. This is the order of increasing lipoid solubility and is an example of the rather general phenomenon that the weak acids enter cells and are disposed to do so, because the undissociated molecules of organic acids (and bases) are often lipoid soluble (see pp. 259ff), though to different degrees. Thus, at *p*H 7.8, swelling of the sea-urchin egg is almost imperceptible in a solution of ammonium acetate, whereas in ammonium valerate the cells are cytolyzed within a few minutes. The reason is the low lipoid solubility of acetic acid.

b). *Single muscle and nerve fibers.*—These objects can be compared with single cells, since their fibers are held together by rather loose connective tissue, the interspaces, as a rule, being open to the surrounding fluids (see p. 242). Information about the distribution of the most common inorganic

[87] L. R. Blinks, Cold Spring Harbor Symp., **8**: 204, 1941.

[88] D. Stewart, Biol. Bull., **60**: 171, 1931; M. H. Jacobs and D. Stewart, J. Cell. & Comp. Physiol., **7**: 351, 1936.

ions, K, Na, H, Cl, and OH is desirable. Studies on plant cells, immersed in water or aqueous solutions such as sea water, led to the concept that a practically static equilibrium between cell and surrounding is the result of impermeability to the ions (see especially Table XV p. 244); though, in addition, with certain cells and under certain conditions, the ionic distribution must be conceived as due to a dynamic state.

Muscles.—In order to reach a corresponding statement for muscle, the entire muscle (ordinarily the frog sartorius) is soaked in isotonic solutions and chemical analyses are made of the ionic contents of the muscle substance, the interfibral fluid being assumed to communicate freely with the outside solution. Urano[89] and Fahr[90] have soaked muscles at a low temperature for several hours in isotonic saccharose with the result that K, which is the predominant cation, here as in so many other cells, escapes only to about 6 per cent into the surroundings; whereas Cl, contained in muscle at a considerably smaller molar concentration, appears during the same period of time up to 90 per cent. The escape of Na is similar to that of Cl, although somewhat slower and more irregular.[91] These facts have been interpreted as indicating that K is retained inside the fiber, while Cl is extrafibral. This conclusion is confirmed by the fact that K escapes as completely as Cl does after injury or death of the muscle. Accepting this idea that Cl is entirely extrafibral, one can calculate, by comparing the Cl content of the total muscle with that of the surrounding fluid, the volume of the so-called chloride space, i.e., the interfibral or extracellular space.[92] It has been found to be about 14.5 per cent of the total volume. This is in satisfactory agreement with the histological tissue space, as determined microscopically in frozen sections of frog sartorius muscles.[93] Regarding the K found in muscle as in diffusion equilibrium with plasma, the concentration of intrafibral K proves to be from 30 to 40 times higher than the extrafibral. This K must be assumed to be largely free to account for osmotic equilibrium (see Sec. 7). However, this ratio 30 or 40:1 is not constant, but varies in both directions, in response to experimental changes of *p*H or of the outside concentration of K. This has been shown by the following observations. Mond, Amson, and Netter,[94] by perfusing frog

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[89] F. Urano, Ztschr. f. Biol., **50**: 212, 1908; **51**: 483, 1908.

[90] G. Fahr, Ztschr. f. Biol., **52**: 72, 1908.

[91] See W. O. Fenn, D. M. Cobb, and B. S. Marsh, Am. J. Physiol., **110**: 261, 1934; W. O. Fenn and D. M. Cobb, J. Gen. Physiol., **17**: 629, 1934; R. Mond and H. Netter, Pflüger's Arch. f. d. ges. Physiol., **224**: 702, 1930; **230**: 42, 1932.

[92] W. O. Fenn, D. M. Cobb, and B. S. Marsh, *loc. cit.*; W. O. Fenn, Physiol. Rev., **16**: 450, 1936; A. B. Hastings and L. Eichelberger, J. Biol. Chem., **109**: xii, 1935; M. G. Eggleton, P. Eggleton, and A. M. Hamilton, J. Physiol., **90**: 167, 1937.

[93] About the interfibral space calculated from analyses of muscles, which had been soaked in solutions containing glucose, inulin, Mg, see, among others: P. J. Boyle, E. J. Conway, F. Kane, and H. L. O'Reylly, J. Physiol., **99**: 401, 1940.

[94] R. Mond and K. Amson, Pflüger's Arch. f. d. ges. Physiol., **220**: 69, 1928; R. Mond and H. Netter, *ibid.*, **224**: 702, 1930; **230**: 42, 1930; H. Netter, *ibid.*, **234**: 680, 1934.

muscle with Ringer's, Fenn and Cobb,[95] by placing the isolated muscles in Ringer's, containing various amounts of K, have found that, at a certain maintenance concentration, K neither enters nor leaves the muscle, but that this equilibrium can be shifted by changing the outside *p*H. For instance, at *p*H 7.2 the equilibrium concentration was found to be 19 mg.%, at *p*H 6.3, 23 mg.% and at *p*H 5.6, 46 mg.%. This means that increasing the H ion concentration raises the tendency of K to escape and must be compensated by a higher K concentration outside. In other words, the equilibrium state is defined by $H_0/H_i = K_0/K_i$. Therefore, at a certain ratio H_0/H_i, if K is added to the outside solution, K_i must rise. Since normally K_i is considerably greater than K_0, K will be shifted against the concentration gradient (see, further, p. 309).

This situation has been illustrated by an interesting model experiment of Netter.[96] Michaelis has shown by measuring the membrane potentials, which are displayed by a dried collodion membrane interposed between different electrolyte concentrations, that this membrane is permeable to H and K, but not to Na nor to Cl and other anions (see p. 317). In this it resembles the surface membrane of muscle fibers, which were considered by Mond and Netter and by Fenn to be characterized by the same kind of selective ionpermeability. Among other experiments, Netter separated a solution *a*, consisting of mol./1400 K_2SO_4 + mol./10 H_2SO_4, from a solution *b*, consisting of mol./1400 K_2SO_4, which was rendered isotonic by addition of a proper amount of glucose in order to avoid a shift of water. Obviously, H in this system tends to pass the membrane, following the steep concentration gradient $10^{-1} \rightarrow 10^{-7}$; but, for electrostatic reasons, such a passage is not possible except by exchange for an equivalent amount of K. Notwithstanding the fact that the concentration of K is initially the same on both sides, such an exchange does proceed and K is transported against an increasing concentration gradient. Netter has interpreted this happening, among others, by the following consideration. H_a and K_b can meet in the pores of the sieve-like membrane, moving in opposite directions, and the chance of their meeting at the same moment is proportionate to $H_a \times K_b$; the same is true for the corresponding cations H_b and K_a, their chance of meeting being proportionate to $H_b \times K_a$. The exchange in the first direction is thus cancelled partially by that in the second, and goes on until equilibrium is attained, when

$$H_a \times K_b = H_b \times K_a \qquad \text{or} \qquad \frac{H_a}{H_b} = \frac{K_a}{K_b}.$$

The experimental results are consistent with this theory. The following numerical example illustrates one of the experiments.

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[95] W. O. Fenn and D. M. Cobb, J. Gen. Physiol., 17: 629, 1934; Am. J. Physiol., 112: 41, 1934.

[96] H. Netter, Pflüger's Arch. f. d. ges. Physiol., 220: 107, 1928.

In the beginning	After 13 days
$\frac{K_a}{K_b} = \frac{1}{1}$	$\frac{K_a}{K_b} = > \frac{25}{1}$
$\frac{H_a}{H_b} = \frac{10^{-1}}{10^{-7}} = \frac{10^6}{1}$.	$\frac{H_a}{H_b} = \frac{52}{1}$.

Because of the thickness and very low permeablility of the membrane, the equilibrium state was not reached even after 13 days, but it is plain that, under the conditions of this experiment, while the concentration gradient of H decreased by diffusion, a strong concentration gradient of K was established. This model elucidates how the high accumulation of K inside muscle fibers may arise, and how, after reaching an equilibrium, each upset of the latter must be succeeded by a redistribution.

Another concept about ion permeability of muscle has been suggested by Boyle and Conway[97] on the basis of experimental results and of theoretical interpretations. They start from the premise that the muscle membrane is permeable for Cl as well as for K, but not for Na, and that the muscle fiber normally contains large amounts of K and small amounts of Na and Cl, all of which are osmotically active.[97a] Under such conditions, after immersion into an isotonic NaCl solution, Cl could not enter for electrostatic reasons; also Cl hardly could be exchanged for equivalent amounts of organic anions from within the fiber, e.g., the large anions of the various phosphorylation products or of colloid-ampholytes. On the other hand, after adding small amounts of KCl to the NaCl solution, the entrance of Cl together with K is hampered by the steep gradient of K from inside to outside. Only after the K concentration outside had been raised in the experiments of Boyle and Conway to 12 milliequiv./1 (corresponding to 0.09% KCl), a distinct entrance of K and Cl in nearly equivalent amounts was observed and was continued up to 300 milliequiv./1 (corresponding to 2.25% KCl). However, even as little as 10 milliequiv./1 (= 0.075% KCl) has been found by Hegnauer, Fenn, and Cobb[98] to create unphysiological conditions for muscle: the excitability is strongly diminished, the oxygen consumption is raised, phosphocreatin is split, and contracture begins. Also swelling of the muscle is often provoked by KCl.[99] Therefore, possibly, permeability to anions is caused in these experiments only by alterations of the normal permeability instead of pre-existing. No mention is made by the authors about functional behavior of the muscle and reversibility of the processes they studied.

The next problem is to investigate whether the behavior of K, Na, and Cl is specific or may be correlated with that of other inorganic salts. This problem has been attacked mainly by [illegible] frog muscles with various

[97] P. J. Boyle and E. J. Conway, J. Physiol., **100**: 1, 1941.

[97a] For Cl see also L. V. Heilbrunn and Hamilton, Physiol. Zool., **15**: 363, 1942.

[98] A. H. Hegnauer, W. O. Fenn, and D. M. Cobb, J. Cell. & Comp. Physiol., **4**: 505, 1934.

[99] W. O. Fenn, and D. M. Cobb, J. Gen. Physiol., **17**: 629, 1934.

alkali ions and by measuring muscle potentials. Mond and Amson[100] have observed by chemical analysis that not only K above a certain concentration level passes over from the capillaries into the muscle, but also Cs and probably Rb, whereas Na, Li, and Ca do not. This is in agreement with the potentiometric measurements (pp. 313ff), and can be interpreted to signify permeability, probably dependent upon an exchange of these cations with hydrogen ion (p. 254). In other words, this entrance should not be accompanied by an osmotic effect.

As a matter of fact, muscles in isotonic solutions of indifferent inorganic salts as well as of indifferent lipoid insoluble nonelectrolytes behave like an osmometer; raising the tonicity diminishes, decreasing the tonicity enhances, the volume or the weight, as known already from Overton's studies.[101] Even in isotonic solutions after a longer period of time, however, minor changes of volume may appear, which are dependent upon both cation and anion, and are probably due primarily to swelling of hydrophilic colloids, rather than to osmotic swelling. This influence is especially evident with K-salts. It has been described by Overton—but needs further investigation—that, in isotonic solutions of K sulfate, tartrate, phosphate, and acetate, muscle shows a perfectly normal appearance over several days, though it is entirely inexcitable (depolarizing effect of the K-ions). It readily returns to a normal physiological behavior after being transferred to Na-salts. Swelling and death, however, occur in the corresponding solutions of K chloride, bromide, iodide, and nitrate. Possibly the stronger hydration by the second group of anions alters the colloidal components of the surface structures so radically as to lead, after some time, to disorganization and disintegration (see Sec. 5).

Further information about the permeability of muscles to ions is obtained by measuring the potential difference between two spots on the surface of an intact muscle, which are in contact with two different electrolyte solutions. The results of such measurements will be presented later (pp. 313ff). They allow the conclusion in fairly satisfactory agreement with the results of the perfusion experiments, that the surface membrane is permeable to K and Rb, less so to Cs, not to Na and Li, and scarcely or not at all to anions. The order of the anionic and of the cationic influences again suggests alterations of the colloidal structure of the surface.

So far, the passage of ions in one or the other direction has been described as a passive process. Even the migration of K against the concentration gradient into muscle, as in Netter's model, seems to depend upon the distinctly smaller penetration power of one ionic component, as in a Donnan equilibrium, and does not demand energy from a metabolic process (see

[100] R. Mond and K. Amson, Pflüger's Arch. f. d. ges. Physiol., **220**: 69, 1928; see, further. R. Mond and H. Netter, *ibid.*, **224**: 702, 1930; also P. H. Mitchell and J. W. Wilson, J. Gen. Physiol., **4**: 45, 1921; P. H. Mitchell, J. W. Wilson, and R. E. Stenton, *ibid.*, **4**: 141, 1921.

[101] E. Overton, Pflüger's Arch. f. d. ges. Physiol., **105**: 176, 1904; **92**: 182, 1902. Further pp. 290ff.

also introduction, Sec. 8). However, as in plant cells and in erythrocytes an active factor, also, must be dealt with in muscles. Heppel[102] has raised rats on a diet deficient in K, with the result that nearly half of the K in the muscle was replaced by Na; there was more intrafibral Na than extrafibral. After restoration of K to the diet, the rats rebuilt the normal content of K in their muscles up to a gradient of 30:1. Steinbach[103] has observed similar supplementary exchanges of K and Na in the isolated frog muscle after soaking it in K-free Ringer's. Fenn and Cobb[104] have found that indirect stimulation causes rat muscle to lose about 15 per cent of K, which is replaced by Na, and that, during recovery, K is regained and Na lost; i.e., during recovery K returns to the fiber, Na to the plasma, both against the gradient. Facts like these suggest that work is required,[105] but the situation is very much unsettled as yet, especially from lack of metabolic study of the problem.

Conditions favorable to the uptake of Mg into the muscle fiber against the concentration gradient have been established by W. O. Fenn and L. F. Haege (J. Cell. & Comp. Physiol., **19**: 37, 1942).

Nerves.—In nerve, as in muscle, the essential units, the fibers, are held together by connective tissue, including fluid-filled spaces, which are more or less open to the surrounding fluid and allow free exchange of their solutes with those in the latter. It has been shown previously that only indirect information about the ionic contents of the muscle fibers can be gained by soaking the muscles in various isotonic solutions and calculating the contents of the fibers from analytical data referring to the entire muscle (p. 253). More reliable data concerning the fibers of the nerves have been afforded during recent years by utilizing the giant nerve fibers of the squid (*Loligo*), which can be isolated from the smaller sized fibers in the stellate nerves of these marine cephalopods. Just as the characteristic distribution of ions between the cell sap and the aqueous surroundings of giant plant cells (*Valonia*, *Halicystis*, *Chara*, and others) has been elucidated by direct chemical determination (p. 244), so analyses of the fluid contents or axoplasm extruded from the axis cylinder of the giant fibers and uncontaminated by such extraneous matter as fiber sheaths, connective tissue, or interfibral fluid have contributed data which are particularly valuable for a better insight into the electrical processes as the most significant signs of nerve activity.

The composition of the squid axoplasm in regard to inorganic ions resembles in a general way that of muscle (see p. 253). The most important difference between inside and outside solutions is in their content of K and Cl. K_i is about 18 times higher than K_0, as found in the blood fluid,

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[102] L. A. Heppel, Am. J. Physiol., **127**: 385, 1939; **128**: 449, 1940. See, further, p. 321.

[103] H. B. Steinbach, J. Biol. Chem., **133**: 695, 1940.

[104] W. O. Fenn and D. M. Cobb, Amer. J. Physiol., **115**: 345, 1936.

[105] For more details concerning this ion transfer, see later (pp. 322ff, and Sec. 8), and see especially R. B. Dean, Biol. Symp., **3**: 331, 1941.

whereas Cl_i/Cl_0 is about $\frac{1}{4}$.[108] It is interesting to find, in contrast to the results with muscle (see, however, p. 255), an appreciable, though low, concentration of Cl inside the nerve fiber, so that Cl here cannot be considered entirely extracellular. Chemical analyses, as well as conductivity measurements, show that there is a remarkable excess of inorganic cations, and that the deficit of inorganic anions probably indicates the presence of organic anions of a rather small ionic weight (about 130) and corresponding ionic mobility, with chemical properties, which suggest amino-acids.[107]

Earlier observations concerning the properties of commoner specimens of nerves (medullated nerves of frog, unmedullated of lobster and crab) are in general agreement with those regarding the squid nerve.[108] Following the procedure of Urano and Fahr (p. 253), Fenn and coworkers have found that, from frog nerves soaked in isotonic sugar solution, Cl and most of the Na readily escape, while K is relatively indiffusible. The amount of Cl present in the nerve varies in proportion to the outside concentration, and can be readily exchanged for other anions. Assuming, on this basis, as in the earlier discussions of ionic distribution in muscle, that K is mainly intrafibral, Cl and Na extrafibral, the extrafibral space in frog nerve has been calculated to be about 64%. The data indicate that K_i is many times higher than K_0. It has been stated that here, as in muscle (p. 254), at a certain limiting K_0 concentration, K neither enters nor escapes from the nerve, that above this threshold value K enters and does so against the gradient, and that this behavior is probably due to an exchange of K and H. Nerve differs strikingly from muscle in that changing the outside *p*H appears not to be followed by a K shift in either direction; for instance, K does not enter the nerve when the outside H concentration falls (see p. 254).

Concerning permeability of the nerve fiber to other inorganic ions than K, very little is known. Frog muscle, as the perfusion experiments of Mond and Amson (p. 256) have shown, is probably permeable to Cs, but impermeable to the cations Na, Li, and Ca, and to the anions Cl, Br, SCN. So far, conclusions regarding the behavior of nerves can be drawn only from determinations of the resting potentials, which may be interpreted, according to the theory of Bernstein, as due to selective or preferential ionpermeability of the surface membranes (see chap. 17, Sec. 5). By this method Netter[109] has found that the permeability of frog nerve to cations decreases in the order: K > Rb > Cs > Na > Li, whereas, at least during a limited period of time, the nerve is not permeable to any anions.[110]

For the permeability of nerves during their electric activity see p. 321, Sec. 5.

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[108] R. S. Baer and F. O. Schmitt, J. Cell. & Comp. Physiol., **14**: 205, 1939.

[107] See also W. O. Fenn, D. M. Cobb, A. H. Hegnauer, and B. S. Marsh, Am. J. Physiol., **110**: 74, 1934; F. O. Schmitt, R. S. Baer, and R. H. Silber, J. Cell. & Comp. Physiol., **14**: 351, 1939.

[108] Fenn *et al.*, *loc. cit.*; further, S. L. Cowan, Proc. Roy. Soc., **B, 115**: 216, 1934.

[109] H. Netter, Pflüger's Arch. f. d. ges. Physiol., **215**: 373, 1927.

[110] See also W. Wilbrandt's experiments on crab nerves: J. Gen. Physiol., **20**: 519, 1937.

(See also p. 314.)

12

PERMEABILITY TO WEAK BASES AND WEAK ACIDS

The group of weak bases and weak acids includes innumerable compounds important to physiology, pharmacology, toxicology, and clinics. The bases are distinguished by the presence of excess OH ions in their aqueous solutions, the acids by excess H; but their characters are often more latent than manifest, since these substances, with decreasing strength, approach the behavior of nonelectrolytes (e.g., urea, acetamide, phenol, glucose). In the wide range between strong and weak electrolytes, the ratio of ions to molecules present in aqueous solution conforms to the mass law, and is represented numerically by the ionization (dissociation) constant (pK). Among the manifold aspects of the biological activity of these compounds, only the question of permeability will be discussed in this section.

Previous sections concerning the permeability of plant and animal cells to nonelectrolytes and to ions contribute a groundwork which may be summarized briefly for the coming discussion. Strong bases and strong acids resemble the strong neutral salts in being ionized to completeness, and in largely lacking—at least primarily—the power to pass into cells.[111] Since the pathway formed by the lipoid components of the surface membrane has very little, if any, dissolving power for ions, what little penetration by strong neutral salts does occur, depends upon whether the membrane affords an adequate pore width (or pore affinity) for the migration of ions (chap. 17, Sec. 5). On the other hand, undissociated molecules, as contained in the solutions of weak bases and weak acids, may penetrate the lipoid layer at a rate correlated with their solubility therein, provided their constitution is suitable, especially provided that their array of polar groups is sufficient to prevent a marked hydroaffinity (p. 231 and Sec. 5, chap. 20).

The early observations of A. Bethe, O. Warburg, and E. N. Harvey upon the umbrella of medusæ, upon the leaves of *Elodea*, upon *Paramecium* and sea-urchin eggs, by applying neutral red as indicator of the intracellular reaction, have shown that strong bases, like NaOH, $Ca(OH)_2$, and $N(C_2H_5)_4OH$, below a certain concentration, paradoxically enter much more slowly than the weaker bases NH_3, $NH_2C_2H_5$, $NH_2{\cdot}C_3H_7$, and others. The strong bases actually penetrate fairly fast also, but this is due to the strongly injurious effect of free OH ion. Corresponding results have been

[111] See pp. 243ff, 245ff, 251ff. further, Sec. 5.

obtained with oxalic, citric, tartaric, and the strong mineral acids, on one hand, the weaker acetic, butyric, and benzoic acids, on the other hand.[112]

The penetrating power of undissociated molecules in the solution of a weak electrolyte has been proved in quantitative experiments of Osterhout with H_2S.[112a] The distribution of H_2S between sea water and the sap of *Valonia* cells was investigated at different *p*H values. The normal *p*H of the sap is 5.8, independent of the outside reaction. The *p*H of the sea water (about 8) was adjusted to a range of values from 5 to 10 by suitable addition of HCl or NaOH. H_2S was introduced into the sea water, and its distribution at equilibrium observed. That at *p*H 5 in the sea water, H_2S is present almost completely as free acid, at *p*H 10 as ions HS and S, can be calculated from pK_{H_2S} and, further, has been determined directly by vapor pressure measurements. The analyses of H_2S in samples of the cell sap (whose *p*H remained constant at 5.8) gave the result that at equilibrium throughout the *p*H range from 5 to 10 the inside concentration of molecular H_2S was practically equal to the outside concentration. In other words, at *p*H 10 the inside concentration was near zero, at *p*H 5 was close to the outside concentration, as shown in the following table (Table XVIII).

TABLE XVIII.—DISTRIBUTION OF H_2S BETWEEN *Valonia* SAP AND SEA WATER AT VARIOUS EXTERNAL *p*H VALUES (OSTERHOUT)

*p*H Sea Water	Sap Conc. of Hydrogen Sulfide Expressed as Percentage of Sulfide Conc. Outside
10	0
8.5	4
7.1	32
6.8	60
5.7	92
5.2	97

It follows that the *Valonia* protoplast is permeable only to the un-ionized H_2S, impermeable to its ions. In order to determine whether this entrance of molecular H_2S is simply a diffusion, cells of *Valonia*[113] were again exposed to various external concentrations of undissociated H_2S, while the rates of entrance of H_2S were followed at intervals of time from 1 to 5 minutes by analyzing samples of sap. It was found that up to 5 minutes the rates were proportional to the concentration of the molecular H_2S in the external solution.

[112] A. Bethe, Pflüger's Arch. f. d. ges. Physiol., **127**: 219, 1909; O. Warburg, Ztschr. f. Physiol. Chem., **66**: 305, 1910; Biochem. Ztschr., **29**: 414, 1910; E. Newton Harvey, J. Exper. Zool., **10**: 507, 1911; Am. J. Physiol., **31**: 335, 1913; Internat. Z. f. physik. chem. Biol., **1**: 463, 1915; see also R. S. Lillie, J. Gen. Physiol., **8**: 339, 1926; **10**: 703, 1927; S. E. Hill, J. Gen. Physiol., **12**: 863, 1929.

[112a] W. J. V. Osterhout, J. Gen. Physiol., **8**: 131, 1925; Regarding NH_3 see W. J. V. Osterhout, Proc. Nat. Acad. Sc., USA, **21**: 125, 1935; regarding CO_2, W. J. V. Osterhout and A. G. Jacques, J. Gen. Physiol., **13**: 695, 1930.

[113] A. G. Jacques, J. Gen. Physiol., **19**: 397, 1936.

So far, only a few weak acids and weak bases have been mentioned as being obviously enabled to enter living cells by the presence of their undissociated molecules; but physiology and pharmacology have afforded a great amount of evidence that the stimulating or paralyzing or other specific effect of weak aliphatic or aromatic acids or amines (the alkaloids, in particular) can be enhanced by the addition of proper small amounts of stronger acid or base, i.e., by shifting the equilibrium toward the undissociated components. This raises the question, which physicochemical properties, or whether in all cases the same properties of the molecules, are acting to cause the enhanced effect. The problem has been attacked by Poijärvi[114] in experiments with several kinds of plant cells, which were immersed in mostly equimolar solutions (0.1 mol.) of a great number of N-bases of a rather diverse chemical structure. The sap of the experimental cells was stained either by their own pigment or by adding neutral red. The entrance of the weak bases rendered alkaline the naturally acid cell sap, and thus their presence became visible by the color change of the indicator-like pigment. This change proceeded with time through a series of intermediary shades of color (each indicating a certain degree of alkalinity) to a final equilibrium notable by a certain definite color, for instance, a yellow color of the cells, initially red-stained by neutral red. This procedure was used to measure the rates of permeation and to calculate permeation constants (see p. 230). Results obtained with cells of *Rhoeo discolor* are pictured in Fig. 26, corresponding to the graph for nonelectrolytes given on p. 232. The permeation constants P have been plotted against the partition coefficients ether: water. In addition, the molecular volumes (or, rather, the molecular refractions MR_D) are indicated. Although, for several reasons mentioned by Poijärvi, the colorimetric method applied provides no more than rather dubious approximations, the following conclusions may be drawn. 1. Over a wide range of partition coefficients (from novocain, cocain, cevadin = 12 to ethylendiamin = 0.00085) the permeation rates of these organic bases are correlated with their lipoid solubility, while the strength of the bases, as also noted by Poijärvi (in his Table XI), varies over a wide range, independent of the partition coefficients. 2. Again, as with nonelectrolytes, the penetration power of bases with low MV (e.g., NH_3, or $(NH_2)_2$, or $NH_2 \cdot CH_3$) is greater, that of bases with high MV (atropin, brucin, cevadin) smaller, than could be expected from their lipoid solubility. The first fact, corresponding similar conclusions previously discussed, may be referred to pore permeation; the second, perhaps to the membrane enclosing the protoplast as being inhibitory to diffusion of the large molecules, or to their slow diffusion in the interior of the lipoid layer (see p. 231).

In order to explain the entrance and accumulation of K so often observed with plant and animal cells, Osterhout, instead of assuming the exchange of K_0 for H_i across a cationpermeable membrane (p. 253, also Sec. 5, chap. 17), has

[114] L. A. P. Poijärvi, Acta Bot. Fenn, 4: 1928.

suggested permeation across an oil-like membrane, (Sec. 5, chap. 19) such as guaiacol, by undissociated molecules of the strong base KOH, which would depend upon whether $K_0 \times OH_0$ exceeded $K_i \times OH_i$ or not. As a matter of fact, with *Valonia* it has been shown that increasing *p*H in the sea water is followed by an increase of the rate of entrance of K, and vice versa,[115] but

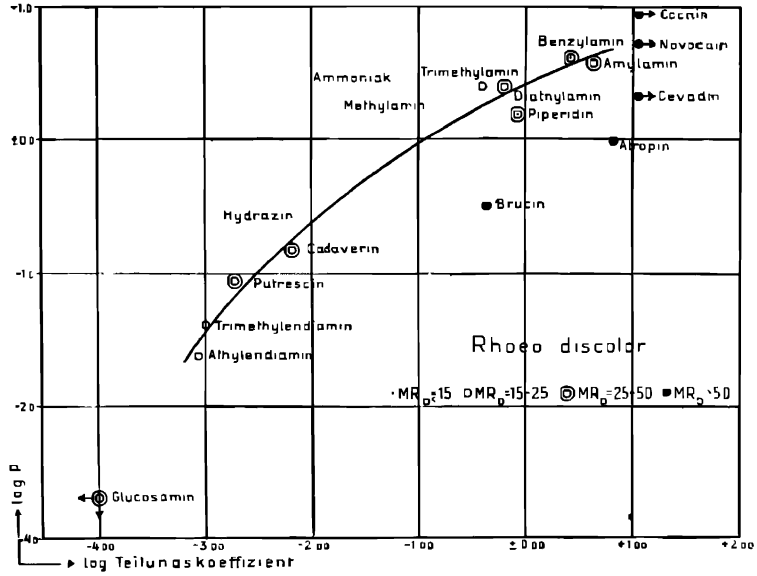

FIG. 26.—Rates of penetration of organic bases into cells of *Rhoeo discolor*. (Poijärvi.)

experiments with *Nitella* conflict with the hypothesis, in that, for instance, the rate of entrance of K is independent of the external *p*H between 6 and 8.[116] (The higher rate of penetration of K compared to Na in Osterhout's model studies upon organic solvents, like guaiacol, is correlated with the greater dissolving power of guaiacol toward KOH than toward NaOH.)

[115] A. G. Jacques and W. J. V. Osterhout, J Gen. Physiol., **17**: 727, 1934.

[116] A. G. Jacques and W. J. V. Osterhout, J. Gen. Physiol., **18**: 967, 1935; A. G. Jacques, Proc. Nat. Acad. Sc., U. S. A., **22**: 191, 1936; see also W. J. V. Osterhout, Bot. Rev., **2**: 283, 1936; W. J. V. Osterhout, Ergebn. d. Physiol., **35**: 967, 1933; Cold Spring Harbor Symp., **8**: 51, 1940.

13

PERMEABILITY TO DYESTUFFS

In this chapter, a special group of electrolytes will be discussed, distinguished by their staining power, which has made them an important tool not only in histology, but also in experimental physiology, where they serve to trace the pathway of dissolved substances across cells and tissues. They are suitably classified as basic and as acidic dyestuffs, the first ones bearing color in the cation, the second in the anion.

1. Basic Dyestuffs.—Many well-known basic dyestuffs, such as the typical "vital stains," like neutral red, toluidine blue, brilliant cresyl blue, rhodamine, methylviolet, resemble the colorless weak bases, which were mentioned in the last chapter, by the fact that the rate of their entrance into cells depends upon the concentration of the undissociated molecules, either present or liberated by adequate amounts of alkali added to the solution of their salts, and that the distribution equilibrium shows equality of the free base inside and outside, independent of the concentration of their free ions. This is evident from the following observations of Irwin.[117] A solution of azur B of a certain *p*H is divided into two portions, *a* and *b*, by interposing a "diaphragm" of chloroform, which has come into equilibrium with the dye. If acid is added to portion *a*, the concentration of free base is decreased, and dyestuff moves from *b* across the chloroform "membrane" to *a*; in other words, dyestuff is accumulated in *a*. This is analogous to the distribution of H_2S in the *Valonia* experiments of Osterhout (p. 260). In another experiment, the sap of *Nitella* cells is stained with brilliant cresyl blue, then the cells are transferred into samples of water with the *p*H varying from 5.4 to 8.2, and the rates of decoloration of the sap are determined. It appears that the rate of exit increases as the external *p*H is decreased. On the other hand, the basic dye being added outside, the rates of entrance increase, when the external *p*H is shifted from *p*H 6.1 to pH 9.3. Other illustrations of this principle of distribution of basic dyestuffs will be mentioned later (Sec. 8, chap. 37, 3) in discussing glandular secretion. One consists in the striking transit of these substances across the gastric mucosa, and accumulation in the acid gastric juice, up to concentrations many times higher than in the blood, into which they have been injected.[118] In these experiments, accumulation is a mere passive phenomenon. Further,

[117] M. Irwin, J. Gen. Physiol., 10: 927, 1927; 10: 75, 1926; 9: 561, 1926.

[118] M. B. Visscher, Federation Proc., 1: 245, 1942.

Chambers and Kempton[119] have compared the passage of two dyestuffs through the tubular epithelium of the frog kidney. One, the basic dyestuff neutral red, in line with other dyestuff bases, responded passively to changes of *p*H brought about inside the tubules. The other, phenol red, a sulfonphthalein, was actively transferred by the special internal mechanism of the tubular cells (Sec. 8, chap. 35*d*), which is unaffected by change of *p*H.

Permeation by colorless weak bases was found in the experiments of Poijärvi (p. 261) to be correlated with their solubility in ether as a lipoid-like solvent. As for dyestuff bases, Nirenstein,[120] in an extensive study, has compared their staining power for *Paramecium* with their relative solubility in lipoid solvents. He observed, on one hand, that the vital staining parallels distinctly the distribution between water and oil, but, on the other hand, that this correlation is intensified by adding some oleic acid to the oil, thus showing that the solvent affinity is to some extent a chemical affinity, relying upon the presence of basic radicals in the dye molecule (see pp. 234ff); see also the model experiments of Beutner, (Sec. 5, chap. 20).

Another factor has to be considered, acting either at the surface of the cell, or after the dye has passed the surface lipoids and has entered the interior. Basic dyestuffs are known to react with cell colloids, and especially with the colloid-ampholytes. These are mainly polyvalent ampholyte proteins, which are assumed to contain in their large structure a number of cationic and anionic centers (Sec. 5, pp. 296ff). The anionic centers are the site of an electrostatic adsorption of basic dyestuffs, which generally exceeds the adsorption of acidic dyestuffs at the cationic centers, because in cell colloids the anionic character ordinarily surpasses the cationic, as indicated by the generally greater effectiveness of cations. The electrostatic adsorption of dyestuffs (including the acidic ones) is rather stable, due to the colloidal or semicolloidal behavior of the dyestuffs themselves, which prevents dissociation and diffusion.

2. Acidic Dyestuffs.—These are the neutral salts of organic carboxylic and mostly sulfonic acids. In contrast to the aforementioned basic dyes, most of them are strong electrolytes, especially the sulfonic acid dyestuffs. In this respect, their penetrating power cannot be expected to be much influenced by additional acidification or alkalinization, as was that of the basic dyes. In general, the acidic dyestuffs are lipoid insoluble,[121] except a few (methylorange, tropeolin 00 and 000, brilliant orange G, Echtrot A). Only a slight increase of lipoid solubility can be attained, even, when the affinity of these acidic compounds for neutral oil is strengthened by the addition of diamylamin, as that of the basic dyes by the addition of oleic acid in the experiments of Nirenstein (*loc. cit.*). This suggests that the large

[119] R. Chambers and R. T. Kempton, J. Cell. & Comp. Physiol., **10**: 199, 1937; R. T. Kempton, *ibid.*, **14**: 73, 1939; see also introduction, Sec. 8.

[120] E. Nirenstein, Pflüger's Arch. f. d. ges. Physiol., **179**: 233, 1920.

[121] E. Overton, Jahrb. f. wiss. Bot., **43**: 669, 1900.

body of nonpolar radicals attached to the chromophoric part of a dye anion provides enough organophilic (lipophilic) strength to overbalance the great hydroaffinity of the sulfonate group (see Sec. 5, chap. 20). In any case, according to Nirenstein, at least a slight capacity to enter *Paramecium* can be observed with those acidic dyes whose entrance into an oily phase can be brought about by diamylamin (see p. 235).

However, in extending these physiological studies of the properties of the sulfonic acid dyestuffs to many different objects, one encounters the embarrassing fact that, while many cells fail to show any color after a long exposure to strong solutions of readily diffusing nontoxic lipoid insoluble sulfonic acid dyestuffs, other kinds of cells accumulate them quickly and copiously. This uptake is to be referred to an active transport brought about by the living cells, since it can be inhibited or diminished in a reversible manner by narcotics, by anoxia, by low temperature, and by other means. It is encountered particularly with animals in gland cells ("adenoid activity" of Overton), in cells with phagocytic, or with strong metabolic, functions. This will be discussed in detail in Sec. 8. Here, it may be mentioned briefly that liver and certain kidney cells, when exposed to the dyestuffs in question, display a homogeneous coloration of their protoplasm distinctly more intense than that of the surrounding solution. Often the color is raised to an especially great concentration in vacuoles inside the cell, or in the secretion expelled by the cells (chap. 35, 2 and 38, 2). Only when the dye offered to the liver or kidney tissue is highly colloidal (suspensoid) in its solution do the cells fail to incorporate it.[122] On the other hand, after injection of colloidal dyes (trypan blue and many others) into an animal, numerous phagocytic cells (fibrocytes, histiocytes) throughout the body appear loaded with the particles.[123] To this class of cells also belong the reticuloendothelial cells of the liver (stellate cells of Küpffer). In plants, the majority of cells remain colorless in solutions of the readily diffusing lipoid insoluble sulfonic acid dyes, but special cells are encountered near the vascular bundles which take up the dyestuffs, except in the presence of ether in narcotizing concentration or during anoxia.[124]

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[122] See R. Höber and A. Titajew, Pflüger's Arch. f. d. ges. Physiol., **223**: 180, 1930; here also older literature.

[123] E. Goldman, Beitr. z. klin. Chir., **64**: 192, 1909; **78**: 1, 1912; W. Schulemann, Biochem. Ztschr., **80**: 1, 1917.

[124] R. Collander, Jahrb. f. wiss. Bot., **60**: 354 1921; R. Collander and A. Holmström, Acta Soc. pro Fauna et Flora Fenn., **60**: 129, 1937.

14

PERMEABILITY TO WATER

Each plasmolysis or deplasmolysis in plant cells, each shrinking or swelling, decrease or increase of volume in animal cells achieved by placing the object in an anisotonic solution, indicates permeability to water. In general, this is manifested by entrance or exit of the water toward an equilibrium state, which, as previously shown, can be attained at a speed that depends upon the penetrating power of the dissolved substances, their oil: water distribution coefficients or their MV. True rates of permeation of water, however, can be determined experimentally and directly only by establishing the osmotic gradient across the cell surface with dissolved substances which are unable to penetrate; for, the simultaneous penetration of solvent and solute necessarily influence each other, since the rate of entrance of water at any given instant depends upon the amount of dissolved substance that has already entered the cell, and vice versa. Taking this into consideration, the following procedures have been employed, dealing with the change of volume of various single cells.

1. Sea-urchin Eggs.[125]—Due to their spherical form, the volume of sea-urchin eggs can be determined by measuring their diameter, either individually under the microscope or, more accurately, by a diffraction method, which gives a statistical average of the diameters of many thousand single cells, simultaneously observed.[126] The rate of volume change can be expressed in a differential equation, $\frac{dV}{dt} = kA(C_i - C_o)$ formally comparable to that describing Fick's law of diffusion (chap. 1),[127] where V is expressed in cubic micra μ^3, the surface area A in square micra μ^2, the concentration difference between inside and outside $(C_i - C_o)$ in atmospheres of osmotic pressure, and where k is the permeation constant. Then k is computed from a convenient integrated form of this differential equation.[128] In

[125] R. S. Lillie, Am. J. Physiol., **40**: 249, 1916; J. H. Northrop, J. Gen. Physiol., **11**: 43, 1927; M. McCutcheon and B. Lucké, *ibid.*, **9**: 697, 1926.

[126] B. Lucké, M. G. Larrabee, and H. K. Hartline, J. Gen. Physiol., **19**: 1, 1935; B. Lucké, H. K. Hartline, and R. A. Ricca, J. Gen. Physiol., **14**: 237, 1930.

[127] $\frac{ds}{dt} = Pq(C - c)$ where ds is the amount of substance, which passes during the period of time dt across the area q. See, further, R. Collander and H. Bärlund, Acta. Bot. Fenn., **11**, 1933.

[128] B. Lucké, H. K. Hartline, and M. McCutcheon, J. Gen. Physiol., **14**: 405, 1931; B

experiments upon unfertilized *Arbacia* eggs, for instance, swelling, observed in 20, 40, and 60 per cent sea water over a period of 15 minutes, indicated that the permeation constant k at 20°C. was 0.1 μ^3 of water per μ^2 of surface per minute for each atmosphere of difference in osmotic pressure; with a longer duration of the experiment or with a greater dilution of the sea water, slight deviations of the constant appear, which probably are accounted for by incipient disintegration of the surface. With other species of echinoderms, nearly the same values were obtained (k = 0.1 to 0.4). It does not make much difference whether the determinations of rate are made by swelling in hypotonic, or by shrinking in hypertonic, sea water.

2. Erythrocytes.—A certain small amount of blood or of a dense suspension of blood corpuscles is rapidly mixed with a certain large volume of distilled water or of several hypotonic solutions of an indifferent substance (sucrose, dextrose), and the increase of transparency of suspension is observed until the osmotic hemolysis has reached a certain arbitrary degree (e.g., 75 per cent).[129] Again, the increase of volume with time is expressed by the differential equation: $\frac{dV}{dt} = kA(C_i - C_o)$, which—provided one neglects, for simplicity, the escape of salts, the osmotic resistance as influenced by the external solution (chap. 16, 3), and other factors,—can be integrated to

$$kAt = -\frac{C_i V_i}{2}\left(\frac{1}{C_o^2} - \frac{1}{C_1^2}\right),$$

where C_i is the initial concentration inside the cells and C_o the concentration at which the arbitrary degree of hemolysis is observed. For certain reasons, only observation times up to about 1.8 seconds are regarded as giving correct figures. Following this procedure, k has been calculated for ox cells to be about 2.5, for human 3.0.

3. Plant Cells.—In corresponding studies on plant cells, the volume changes can be measured by plasmolyzing cells, which have a sufficiently regular (cylindrical) shape (see p. 230).[130] The rates of water penetration have been shown to vary from species to species and from site to site of the plant body over a wide range (more than 50:1). This may be referred to differences in viscosity of the protoplasm, adhesion of the protoplast to the cell wall, and other special conditions.

It has been mentioned before that one can obtain an exact value for the rate at which water leaves cells in a hypertonic solution only when the solute applied to the surface is one to which the cell is impermeable, but if a penetrating solute is applied in the presence of an isotonic solution of a nonpenetrating one (e.g., 0.5 mol. ethylene glycol to sea-urchin eggs in sea water), the rate at which the solute enters, as well as the rate at which

Lucké, M. G. Larrabee, and H. K. Hartline, J. Gen. Physiol., **19**: 1, 1935; see also: B. Lucké and M. McCutcheon, Physiol. Rev., **12**: 68, 1932.

[129] M. H. Jacobs, The Harvey Lectures, **22**: 146, 1927; Biol. Bull., **62**: 178, 1932.

[130] B. Huber and K. Höfler, Jahrb. f. wiss. Bot., **73**: 300 and 351, 1930.

water leaves, can both be calculated from certain measurements. Initial shrinkage appears, which is followed by return to the original volume as the penetrating solute becomes equally distributed between cell and surrounding. This is analogous to plasmolysis and deplasmolysis mentioned earlier in connection with the behavior of plant cells immersed in a hypertonic solution of a penetrating nonelectrolyte (p. 229). In this process the two permeation rates, considered independently of each other, would be described by the two equations, $\frac{ds}{dt} = k_{solute}A(C_o - C_i)$ and $\frac{dV}{dt} = k_{water}A(C_o - C_i)$. Jacobs[131] has shown that three determinations, (1) the initial volume of the cells, (2) the minimum volume, and (3) the time required to attain this volume, are sufficient to yield the separate values of k_{solute} and k_{water} from a chart constructed by solving the differential equations numerically and computing a series of conversion factors. In a similar manner, Jacobs[132] has employed numerical methods to describe the simultaneous penetration of solute and solvent into erythrocytes, and to prepare tables from which both permeation constants can be obtained.

The results of such determinations on animal cells have been listed in the following Table XIX.

TABLE XIX.—SIMULTANEOUS MEASUREMENT OF PERMEABILITY TO WATER AND TO A DISSOLVED SUBSTANCE (JACOBS)

1. From experiments of Lucké, Hartline and Ricca on *Arbacia* eggs:
   k_{water} ..... 0.2–0.3 μ^3/μ^2/minute/atm.
   k_{solute}
   Ethylene glycole ..... 3.2 $\times 10^{-15}$ mols./μ^2/minute/mol. per liter
   Glycerol ..... 0.3 $\times 10^{-15}$ mols./μ^2/minute/mol. per liter
2. From experiments of M. H. Jacobs on ox erythrocytes:
   k_{water} ..... 2.5 μ^3/μ^2/minute/atm.
   k_{solute}
   Urea ..... 108.00 $\times 10^{-15}$ mols./μ^2/minute/mol. per liter
   Ethylene glycole ..... .96 $\times 10^{-15}$ mols./μ^2/minute/mol. per liter
   Glycerole ..... .011 $\times 10^{-15}$ mols./μ^2/minute/mol. per liter

Data mentioned earlier in this section make it clear that the rate of water permeation can differ from one object to another and can vary, depending upon outside conditions. This is especially evident in studies of plant cells where considerable differences up to more than 50:1 have been met. Little is known concerning the nature of these differences. Probably the penetration of water is mainly affected by mechanical properties of either the surface membranes (including in plant cells the vacuolar membrane) or the protoplasm. It must be kept in mind that there are reasons to believe that to a certain extent the plasma membrane is a sieve-like structure (p. 233), so that the pathway of water is like the interstices of a filter. This is more or less true also with the protoplasmic cell body (Sec. 5). The building

[131] M. H. Jacobs, J. Cell. & Comp. Physiol., 2: 427, 1933; D. Stewart and M. H. Jacobs, *ibid.*, 7: 333, 1936; B. Lucké, H. K. Hartline and R. A. Ricca, *ibid.*, 14: 237, 1939.

[132] M. H. Jacobs, J. Cell. & Comp. Physiol., 4: 161, 1934.

material of this sieve structure is partly constituted of various hydrophilic colloids (see Sec. 2 and 3), so that water traverses not only interstices like pores, but also intermicellar spaces between the colloidal aggregates, which build up the substance of the cells. The penetrating aqueous solutions interact with the hydrophilic colloids to bring about a looser or a more condensed architecture, which affects the micro- and submicroporosity so that permeability increases or decreases. In any case, it seems a clarifying viewpoint to consider that the permeation of water driven by osmotic pressure across a protoplasmic membrane, is like a filtration, and to refer variations of the permeation rate, as brought about by solutes such as inorganic ions, to their well-known interaction with hydrophilic colloids (see Sec. 5, chap. 16). Such influences have been observed by Lucké and McCutcheon.[133] Sea-urchin eggs were placed in a solution of 0.38 mol. dextrose, which is approximately isotonic with 40 per cent sea water. The permeation rate was found to be about 0.1. To this sugar solution were added electrolytes in such a small concentration (10^{-2} to 10^{-5} mol.) that their osmotic effect as such was practically zero. It appeared that various ions had a significant effect upon the migration of water, for instance, monovalent cations (Na, K) increased the permeation, divalent ones (Ca, Mg) decreased it, and in appropriate mixtures they antagonized each other in the typical way (see chap 16, 2*a*). In this connection, it should be mentioned that narcotics inhibit the osmotic permeation of water, and it is assumed that the hindrance is due to their adsorption at the micellar surfaces (see Sec. 5, chap. 23, 2), so that the pathways become narrowed or blocked.

In concluding this chapter, attention should be called to some striking physiological phenomena characterized by the fact that water traverses cell membranes in the absence of a hydrostatic or osmotic drive, or even against such a force. Most of the work on this subject has attempted to explain how the isolated frog skin, as long as it survives, develops a force driving a normal Ringer's solution from outside to inside, even against a hydrostatic pressure. This has been found to depend on the supply of energy by certain metabolic reactions, and to be bound up with electric differences of potential maintained by the skin. The situation resembles "anomalous osmosis," in which water by virtue of an electrokinetic charge is transported by a membrane potential resulting from the selective ion permeability of the membrane. For other similar effects and for the theory of anomalous osmosis see Sec. 8.

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[133] M. McCutcheon and B. Lucké, J. Gen. Physiol., **12**: 129, 1928; also **12**: 571, 1929. See, further, M. H. Jacobs and A. K. Parpart, Biol. Bull., **63**: 224, 1932.

15

CHEMISTRY AND PHYSICS OF THE PLASMA MEMBRANE

1. The Identification of a Plasma Membrane.—It has been concluded from experimental study of cell permeability that the surface of protoplasts is formed by a special organ, the plasma membrane. Often, this membrane is covered by one or more protecting or supporting extraneous coats, which seem to be readily permeable to crystalloid solutes, except those with very large molecules. These "hyaline layers" and "jelly-like coatings" can be removed mechanically, leaving the essential protoplasmic surface layer entirely viable and, hence, the natural cell permeability intact.[134] Although the plasma membrane itself is not clearly distinguishable under the microscope, its existence, for various reasons, cannot be denied. One of the most convincing evidences of its reality is provided by the following experiment of Chambers.[135] Starfish eggs, stained with the intracellular indicator, neutral red, are placed in isotonic NH_4Cl solution. The color of the eggs shows a change to a more alkaline reaction, due to the penetration of NH_3, which results from the slight hydrolysis of the salt.[136] If, instead, some of the same NH_4Cl solution is injected with a micropipette into the interior of one of the eggs, immediately the indicator assumes a distinctly acid color, and, due to the escape of NH_3 into the surroundings of the cell, this color spreads in all directions throughout the protoplasm, but not farther than the boundary of the egg has been reached. In a corresponding experiment with an alkaline solution of $NaHCO_3$, which contains a considerable amount of CO_2, neutral red indicates an acid reaction inside the eggs, resulting from the free entrance of CO_2. But, following the injection of the solution into one of the eggs, the acid reaction turns to an alkaline one, with the color again spreading as far as to the barrier at the cell surface.

Furthermore, the existence of a film-like surface is shown by quantitative studies of swelling and shrinking of the cell body when exposed to heterotonic solutions of various concentrations of a nonpenetrating substance, (e.g., glucose or diluted or concentrated sea water). Provided the cell

[134] See R. Chambers, Amer. Nat., **72**: 141, 1938; Cold Spring Harbor Symp., **8**: 144, 1940; M. J. Kopac, *ibid.*, **8**: 154, 1940; Colloid Chemistry (ed. by J. Alexander): Reinhold, New York, vol. 5, p. 864, 1944.

[135] R. Chambers, J. Gen. Physiol., **5**: 189, 1922.

[136] See pp. 246 and 252; see also M. H. Jacobs, J. Gen. Physiol., **5**: 181, 1922.

behaves as a perfect osmometer, the volume changes should follow the Boyle-Van't Hoff law, PV = const., while, if it resembles a membrane-less gel, as sometimes has been asserted, the volume changes produced by the various concentrations would not even remotely follow the law. Many experiments have shown that actually the law is obeyed satisfactorily, if one takes into account the fact that each protoplast must contain a "dead space" b, filled with osmotically inert substances, such as lipoids and proteins which are more or less dispersed in the cell body, possibly forming a kind of stroma, whereas the remainder, $V - b$, holds the "osmotically transferable" free water; in other words: $P(V - b)$ = const.[137]

The well-known Donnan-equilibrium likewise indicates the presence of a membrane. When a membrane is freely permeable to the ions of an electrolyte solution which bathes both sides of it, but is impermeable to a colloidal ion on one side (for instance, intracellular), an unequal distribution of the diffusible ions takes place, expressed by the equation: $x^2 = y(y + z)$, where x and y are the diffusible, z is the indiffusible ion (see Sec. 1, chap. 5). This distribution has been observed frequently, e.g., with red blood corpuscles (see also Sec. 8, introduction).

Even more demonstrative of a special surface barrier is the fact, shown by the data given in this section, that among crystalloid solutes which have been applied to cells, only special groups are permitted to penetrate. It is just this property of *selectivity* that poses the problems of the chemical nature and of the physical structure of the cell surface. It has been found that the two classes of nonelectrolytes which are able to enter cells are, first, those which are soluble in fat-like solvents, and second, those which have a small molecular size. The problem is to find out whether the chemical and physicochemical behaviors of isolated plasma membranes lend support to the postulates of fat-like and sieve-like constitution.

2. The Chemical Composition of Plasma Membranes.—Unfortunately, direct information about this composition is exceedingly poor. Practically the only membrane material available in quantities sufficient for chemical analysis consists in the "stromata" or "ghosts" of the red cells, and possibly these are only partly pieces of the plasma membrane and partly remnants of a "skeleton" or meshwork extending through the cell interior. According to Parpart and Dziemian,[138] the chief building stones of this structure are lipoids and proteins (in the average ratio 1:1.7). Predominant among the lipoids are the phospholipoids, which are hydrophilic colloids; the fairly hydrophobic cholesterol makes up the remainder. The latter and some of the proteins and lipoproteins which are not more than moderately soluble, seem to confer, at least, some of their characteristic consistency upon the stromata, evinced, for instance, under the influence of fairly strong swelling agents, by the formation of threads, globules, and other products

[137] See the review articles of B. Lucké and M. McCutcheon, Physiol. Rev., **12**: 68, 1932; B. Lucké, Cold Spring Harbor Symp., **8**: 123, 1940.

[138] A. K. Parpart and A. J. Dziemian, Cold Spring Harbor Symp., **8**: 17, 1940.

of a gradual disintegration ("stromatolysis") resembling the "myelin forms" of nerves.[139] These results obviously provide an adequate foundation for the theory of permeability. They supplement numerous earlier statements about the dissolving power of cell lipoids,[140] and they allow one to picture protein aggregates as porous structures (chap. 16, 2, and 17) by applying modern concepts of hydration and dehydration of colloids in membranes and fibers. However, no analytical results have so far been brought to bear (a) upon the great variety in relative importance of the solvent-factor versus the pore-factor in permeability (see p. 235), which possibly could be interpreted as due to differences in the relative extent of the lipoid and protein areas in the membrane mosaic, or (b) upon the possibility that the chemical composition of the phospholipoids may vary from plant to plant and from animal to animal with regard to the degree of acidic or basic character (p. 234, ff), or of the ratio unsaturated: saturated fatty acids (Sec. 5, pp. 351 and 359), and other factors. It further should be recalled that the present data of quantitative analysis presented refer to red cell stromata only.

3. Physics of the Plasma Membrane.—These latter remarks call attention to the problems of the architectonics of the plasma membrane. Their study has been stimulated and rendered more accessible by the background investigations of Langmuir, Harkins, Adam, and others into the formation and properties of surface films. In view of the dimensions and the chemistry of the plasma membrane it is important that these recent studies have made it possible to compare natural films with artificial mono- and multilayers of fatty acids, sterols, and proteins, also with "mixed films," the components of which can appear in separate strata or can interpenetrate each other and mutually influence molecular orientation, and with other types of membrane as shown and discussed before (Sec. 2, pp. 203, ff). Some of the results of comparing artificial and natural architectures will be referred to in the following Sec. 5, particularly regarding mixtures of lipoids with proteins and the influence of hydration and dehydration upon their microstructure (Sec. 5, pp. 295 ff). Much effort has been spent in trying to construct models, which portray the natural surfaces with regard to certain features; in this effort, chiefly the following properties have been studied: a. the interfacial tension of protoplasts; b. the thickness of the plasma membrane; c. its electrical conductivity.

a). The interfacial tension of protoplasts.—This can be measured in invertebrate eggs which behave like fluid spherical drops when distorted by external forces. Harvey[141] applied centrifugal force, by which eggs of

[139] R. F. Furchgott, Cold Spring Harbor Symp., **8**: 224, 1940.

[140] E. Overton, Jahrb. f. wiss. Bot., **34**: 669, 1900; H. H. Meyer, Arch. Exper. Path., **42**: 109, 1899; W. Ruhland, Jahrb. f. wiss. Bot., **46**: 1. 1908; E. Nirenstein, Pflüger's Arch. f. d. ges. Physiol., **179**: 233, 1920; H. Bärlund, Acta Bot. Fenn., **5**, 1929; R. Collander and H. Bärlund, *ibid.*, **11**, 1933; K. H. Meyer and H. Hemmi, Biochem. Ztschr., **277**: 39, 1935, and others.

[141] E. N. Harvey, Biol. Bull., **60**: 67, 273, 1931.

Chætopterus or of *Arbacia*, due to the different specific gravity of the oil globules and the yolk granules included in their protoplasma, are elongated more and more, until at a certain force the cylindrical cells break in two. This can be watched or photographed with a microscope-centrifuge. From the force applied and the diameter of the breaking cylinder the interfacial tension (cell surface:sea water) can be calculated. Cole,[142] for the same purpose, pressed a beam upon the top of the sea-urchin egg and measured the two radii of the flattened cell at various degrees of deformation.

By these methods the interfacial tension of the eggs is found to be as low as 0.1 to 0.2 dyne/cm. This low value is unexpected, if one anticipates that the cell surface is formed by a fat-like substance, since fats are known to show a considerably higher tension. As a matter of fact, Harvey and Shapiro[143] found the tension of fish oils (from mackerel and other teleosts), at a *p*H corresponding to that of cells (*p*H 6.8), to be about 9 dynes/cm. To clarify this situation, they determined the tension of the oil droplets, which are often included in the eggs of fishes. Centrifuging flattens the droplets by pressing them against the tough outer membrane of the egg; then, from measurements of the deformation, the interfacial tension (oil:egg fluid) can be calculated. It was found to be much lower than the tension (oil:water), about 0.6 dyne/cm. From this, Danielli and Harvey[144] have drawn the conclusion that, among the contents of the egg plasma, there is some substance which has a great surface activity toward the oil, and they offer reasons for assuming that this substance is an egg globulin which forms a film and covers the surface of the oil (see Sec. 2, pp. 208 ff).

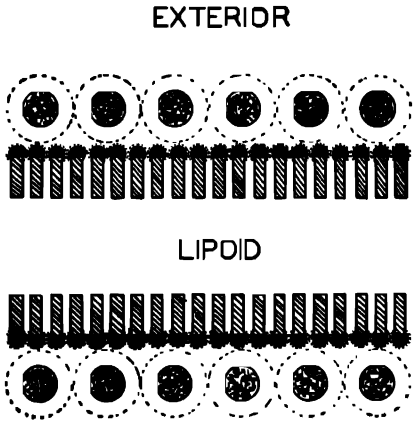

FIG. 27.—Schema of molecular conditions at the cell surface.

These observations have been generalized to propose the following picture (Fig. 27) of the plasma membrane as a mixed film with a stratified architecture.[145]

The picture shows two monomolecular arrays of fatty molecules, each of them stabilized, on one hand, by anchorage of their polar groups in the

[142] K. S. Cole, J. Cell. & Comp. Physiol., **1**: 1, 1932.

[143] E. N. Harvey and H. Shapiro, J. Cell. & Comp. Physiol., **5**: 255, 1934.

[144] J. F. Danielli and E. N. Harvey, J. Cell. & Comp. Physiol., **5**: 483, 1935.

[145] J. F. Danielli and H. Davson, J. Cell. & Comp. Physiol., **5**: 495, 1935; see also E. Gorter and F. Grendel, J. Exper. Med., **41**: 439, 1925; Proc. kon. Akad. Wetensch., Amsterdam, **29**: 314, 1926.

aqueous phases, and, on the other hand, by lateral adhesion forces between the hydrocarbon chains. Each stratum is covered by an adsorption layer of hydrated protein molecules, which thus provide toward the exterior, as well as toward the interior, the characteristic low interfacial tension. The space between the two lipoid layers may be thought of as filled with unoriented lipoid molecules, so as to allow for the variety of the thickness observed among such films, and discussed in the next section.

Some objections may be raised against such a picture representing the aforementioned studies. First, Mudd and Mudd[145] have investigated the wetting properties of cells by observing whether at a saline: oil interface, originating between a microscopic slide and a coverslip, the cells are hydrophobic enough to enter the oil phase preferentially, or hydrophilic enough to enter the aqueous phase. Erythrocytes were shown to be more hydrophobic, leucocytes hydrophilic, and for this reason the former were assumed to bear an external lipoid, the latter a protein, coat. With regard to the erythrocytes, this behavior is in agreement with their electrophoretic properties as the ζ-potential is not affected by adding protein to the rinsed cells, nor by changing the *p*H (see Sec. 5, chap. 18). It is doubtful whether this objection is conclusive, as, according to the discussion of Bateman,[147] the orientation of molecules in mixed films containing different lipoid components (oils, sterols) and protein is exceedingly intricate, due to adsorption and displacement, to mutual dissolution and to steric factors.

Second, the picture in question is probably much too simple. With regard to erythrocytes, this follows not only from chemical analysis of the stromata (p. 272; further, Sec. 5, p. 294), but also from optical analysis with the polarization microscope.[148] The latter reveals a many-layered structure of protein lamellæ with long axes oriented tangentially, i.e., with flat surfaces parallel to the surface, and interposed lipoid micelles, whose optical axes are arranged radially (see Sec. 5, pp. 296 ff). Nevertheless, the natural architecture of the stromata might be crudely represented by the proposed scheme, at least with respect to stratification.

Third, the picture seems to ignore the rather well established concept of a mosaic of lipoid and protein areas, as a background for the solvent-sieve theory of permeability (see p. 234). From the viewpoint of surface films, Danielli[149] has objected to this theory that surface tension forces, acting at the border of the different areas (lipoid and protein) of a mosaic membrane, would tend to disrupt the film, since the lateral adhesion of the hydrocarbon chains, which stabilizes the lipoid area, would not extend to the protein

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[145] S. Mudd and E. B. H. Mudd, J. Exper. Med., **43**: 127, 1926; J. Gen. Physiol., **14**: 733, 1931.

[147] In Sec. 2, chap. 9.

[148] F. O. Schmitt, R. S. Bear, and E. Ponder, J. Cell. & Comp. Physiol., **9**: 89, 1936, and **11**: 309, 1938.

[149] J. F. Danielli, J. Cell. & Comp. Physiol., **7**: 393, 1936.

area. On the other hand, it has been asserted that a lipoid molecular surface layer alone can suffice to account for permeation of a solute through pores as well as by being dissolved. In this regard, Michaelis[150] was the first to point out that, even in a membrane consisting of a layer of liquid, which functions distinctly as a solvent medium, the penetrating molecules must pass through interstices between the molecules of a solvent just as they do in diffusing across a molecular sieve like a dried collodion membrane, although there is a marked difference, since the intermolecular spaces in a liquid membrane can be easily deformed and enlarged when penetrating substances squeeze themselves between, whereas the intermolecular spaces in the collodion membrane are preformed in the rigid structure of a solid phase. However, the contrast between the classic porous membrane of dried collodion and the solvent oil membrane has been overbridged for a long time by Northrop,[151] Michaelis,[152] and Collander,[153] and recently especially by Sollner.[154] They showed that rigidity is not an essential character of the collodion membrane; on the contrary, it may swell by the uptake of water and change its porosity, and it can display selective permeability toward organic compounds not unlike that of an ordinary solvent membrane. In other words, permeability depends upon the molecular volume of penetrating compounds as much as upon their affinity toward the membrane as a solvent (see this chapter, pp. 234, 235). Returning at last to the picture of a plasma membrane (Fig. 27), one should inquire after the possible significance of the superficial layer of protein. According to Harvey, Danielli, and Davson (*loc. cit.*), it may be assumed that adsorbed protein provides the characteristically low interfacial tension of about 0.6 dyne/cm. found for marine eggs, and so protects the membrane against rupture by surface forces. Without it, rupture could be brought about as easily as in those artificial films, the polar groups of which are in immediate contact with the aqueous phase.

b). *The thickness of the plasma membrane.*—Much effort has been spent to solve this important problem, by exploring it with chemical, optical, and electrical means. But, so far, the yield has been rather poor.

Chemical Methods.—For chemical analysis cytolyzed erythrocytes are unique material in being available in sufficiently great amounts, although one has to take into consideration (see p. 272) that the ghosts are possibly an aggregate not only of plasma membranes, but also of a meshwork from inside the cells. The study of the ghosts has appeared especially enticing to those in favor of the rather common assumption that lipoids are the backbone of the cell surface structure, after it has been stated that the entire amount of lipoid contained in the erythrocytes is bound up with the ghosts.

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[150] L. Michaelis, Bull. Nat. Research Council, No. 69, 1929.

[151] J. H. Northrop, J. Gen. Physiol., **11**: 233, 1927, and **12**: 435, 1928.

[152] L. Michaelis and A. Fujita, Biochem. Ztschr., **151**: 47, 1925; also Sec. 5, p. 335.

[153] R. Collander, Soc. Sci. Fenn. Communicationes biol., II: 5, 1926.

[154] K. Sollner and P. W. Beck, J. Gen. Physiol., **27**: 451, 1944; see also Sec. 8.

The following procedure has been chosen to measure the thickness of a supposedly real continuous lipoid layer spread over the surface of the cells.[155] The lipoids of a certain amount of rinsed erythrocytes, the number and the size of which are known, are extracted and dissolved in a small amount of petroleum ether, and are spread on water. A monomolecular coherent film is formed, and its area measured (see Sec. 2, chap. 9). This area is found to be twice as large as the total surface area of the erythrocytes used in the experiment. This proves that, if all the lipoids are spread on the surface of the erythrocytes, the film must be bimolecular. Taking into account the average percentage of each of the different lipoids mixed in the surface membrane, and the length of their molecules, the thickness of the membrane can be roughly estimated to approximate 40 Å.

Obviously, one can make several objections to this interpretation or to the generalization from these experimental data. First, in the structural scheme derived from measurements of the surface tension of cells (Fig. 27), there likewise appears a bimolecular lipoid layer, but, in addition, as components of the plasma membrane, two monolayers of protein, to the thickness of which, according to numerous observations with protein films (see Sec. 2, chap. 9), most variable values could be ascribed. Second, proteins and lipoproteins are regular components of the ghosts (p. 272). Third, it remains an open question whether or not the lipoid molecules are arranged as a discontinuous bimolecular layer with protein molecules interspersed in a mosaic pattern, accounting for the permeability properties of the plasma membrane.

Optical Methods.—These likewise have failed so far to give unequivocal results. From their measurements with polarized light (p. 275) Schmitt, Bear, and Ponder[148] came to the conclusion that the envelopes of the red cells are many molecular layers in thickness. A more direct approach to an exact measure has been made by Waugh and Schmitt,[156] with their analytical leptoscope. Light is reflected from a step film of barium stearate, i.e., from a film which is built up on a glass or metal plate by superimposing one monolayer above the other,[157] and this light is compared with the light reflected from ghosts, which have been dried on another plate. One has to observe, which step of the calibrated step film matches the ghosts in intensity of reflected light. In this manner a thickness of more than 200 Å was measured with the dried ghosts of rabbit erythrocytes, when hemolysis had been produced at *p*H 6 of the hemolyzing buffer solution, the thickness diminishing on either side of *p*H 6. After extraction of the ghosts with a lipoid solvent, the maximal thickness dropped from 200 to about 120 Å. This possibly indicates that proteins share in the thickness.

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[155] E. Gorter and F. Grendel, J. Exper. Med., **41**: 439, 1925; F. Grendel, Biochem. Ztschr., **214**: 231, 1929. See, further, A. J. Dziemian, J. Comp. & Cell. Physiol., **14**: 103, 1939.

[156] D. F. Waugh and F. O. Schmitt, Cold Spring Harbor Symp., **8**: 233, 1940.

[157] K. B. Blodgett, J. Phys. Chem., **41**: 975, 1937; K. B. Blodgett and I. Langmuir, Phys. Rev., **51**: 964, 1937. See also Sec. 2, chap. 9, 7.

Electrical Methods.—Assuming that the erythrocytes are surrounded by a thin layer of nonconducting material, Fricke[158] has measured the impedance of suspensions of erythrocytes and has interpreted the results as due to the presence of a certain static capacity of the condenser-like membrane. From this value and, further, assuming that the nonconducting material is fat-like, and therefore may have a dielectric constant as low as 3, the thickness of the membrane was calculated to equal 33 Å. We will come back to this kind of measurements in the next subsection (p. 280).

It is obvious that the information concerning the thickness of the plasma membrane is far from being final, because even with the same object, according to the measure applied, the numerical values are scattered over a wide range (from 33 to 200 Å), because, for technical reasons, the studies mostly were confined to erythrocytes, and because, even in the study of the erythrocytes, essential features have been disregarded. Whenever active transfer (p. 249 and Sec. 8) is looked at as a matter of permeability conditioned by the plasma membrane controlling at least the entrance of substances, then the question must be raised, whether the concept of the plasma membrane must not be widened to comprise more constituents than have been pictured in many of the proposed schemes. One would have to take into account certain metabolites and enzymes, to explain, for instance, in the case of erythrocytes, why the backtransport of K ions, which have somehow escaped from the interior (p. 249) appears to be coupled with the glycolytic breakdown of carbohydrates (p. 251). It is hard to believe that such a film as the envelope of the red cells, as currently pictured, is capable of this complex action. More likely, essential components have been extracted or destroyed in preparing the ghosts. Further, one should recall that in plant and animal protoplasts there has been observed a more solid cortex of varying thickness, lying directly beneath the plasma membrane.[159]

c). The electrical resistance of the plasma membrane.—For about 100 years, by the investigations of du Bois-Reymond and others, it has been known that living tissue is a poor conductor for the electric current, and that the resistance falls off in death. This process is accompanied by exosmosis of electrolytes. In many plant cells, the loss is chiefly due to the escape of the ions contained in the cell sap vacuoles; in animal tissues the source is the protoplasm, where the ions could be assumed either to be freely present, or to be liberated during its disintegration. Conclusive evidence of one factor or the other being mainly involved would be provided by measuring the "internal conductivity" of the cells. It means, one should determine the electric conduction of a solution, which is contained in a closed vessel of nonconducting material. As a matter of fact, this can be

[158] H. Fricke, J. Gen. Physiol., **9**: 137, 1925; also H. Fricke and H. J. Curtis, *ibid.*, **18**: 821, 1934; J. F. McClendon, J. Biol. Chem., **69**: 733, 1926. Further, J. F. Danielli, J. Gen. Physiol., **9**: 19, 1935.

[159] See, for instance, R. Chambers, Proc. Eighth Am. Sc. Congress, vol. III, p. 25, Dept. of State, Washington, 1942.

done by special procedures, with the result that the interior of normal cells is shown to be a good conductor, and that, therefore, the high electrical resistance of living cells must be referred to their surface.

However, ordinary electrical methods also can avail for measuring the resistance of cells, viz., of plasma membrane + cytoplasm. The electrical conductivity of a brei of centrifuged erythrocytes suspended in serum or in isotonic salt- or sugar-solution has been determined with *low-frequency alternating current* (100 to 200 cycles per second), following the method of Kohlrausch.[160] The conductivity was found to decrease with increase of the percentage volume of cells. According to Clerk Maxwell,[161] the specific resistance of suspended particles can be calculated from the formula:

$$\frac{r_1/r - 1}{r_1/r + 2} = \rho \frac{r_1/r_2 - 1}{r_1/r_2 + 2}$$

where r is the specific resistance of the suspension, r_1 that of the suspending liquid, r_2 the specific resistance of the suspended particles, and ρ the volume concentration of the suspended material. With red blood corpuscles, by varying the ratio cells: suspending fluid, r_2 was found to be *practically infinite;* in other words, the red cells appeared to be practically perfect nonconductors.[162]

The common procedure of measuring resistance with a Wheatstone bridge by leading a *direct current* across a multicellular tissue cannot contribute much information to our problem, as the intercellular spaces as pathways for a part of the electric flow remain uncertain. But this imperfection can be more or less overcome with single giant cells as objects, as e.g., *Valonia*,[163] or *Nitella*,[164] or the axon of the squid.[165] In experiments with the plant cells, the DC has to pass the protoplasm, the plasma membrane, the vacuolar membrane and cell sap, and, moreover, a by-path is open through the cell wall. The squid axon provides more favorable conditions, as the actual resistance of the axoplasm, compared to that of the enveloping membrane, is small. When DC is applied, care has to be taken that the current is not allowed to flow for more than a few seconds; otherwise, polarization of the membranes can be succeeded by alterations of the normal resistance (Sec. 5, chap. 22). Also the current strength must be small enough not to release an excitation wave. The DC resistance with the squid nerve was found to be about 1000 ohm/cm.,[2] with *Valonia* about 10,000, and

[160] See, for instance, St. Bugarszky and F. Tangl, Centralbl. f. Physiol., **11**: 297, 1897; M. Oker-Blom, Pflüger's Arch. f. d. ges. Physiol., **79**: 510, 1900; G. N. Stewart, Centralbl. f. Physiol., **11**: 332, 1897; Am. J. Physiol., **49**: 233, 1919.

[161] Clerk Maxwell, Treatise on Electricity and Magnetism: Clarendon Press, Oxford, 1873.

[162] H. Fricke, J. Gen. Physiol., **6**: 375, 741, 1924; Cold Spring Harbor Symp., **1**: 117, 1933; K. S. Cole, *ibid.*, **8**: 110, 1940.

[163] L. R. Blinks, J. Gen. Physiol., **13**: 361, 1930.

[164] L. R. Blinks, J. Gen. Physiol., **13**: 495, 1930.

[165] K. S. Cole and A. L. Hodgkin, J. Gen. Physiol., **22**: 671, 1939.

with *Nitella* 100,000 to 200,000. The latter value is possibly due partially to the fact that the outer cell wall of this tap water alga is soaked with tap water, while the habitat of *Valonia* is sea water. From these various values it may be concluded that the *plasma membrane*, though not a perfect insulator, actually *offers an extremely great resistance* to ionic penetration, e.g., for the squid axon, when we assume the thickness of the nerve membrane to be 100 Å, the resistance would be about as great as that of glass.

The results are rather the same, when, instead of DC, *low-frequency AC* is employed, as is customary, after Kohlrausch proposed this form of current for measuring the resistance of electrolyte solutions in order to avoid the polarization effects. But the conclusions from all these experiments regarding the resistance of the plasma membrane seem to be entirely upset when one turns over from low-frequency to *high-frequency AC*.

When an electrical potential difference is established across a membrane, which is nearly (if not entirely) impermeable to ions, at the first moment, on one side of the membrane, cations will move from the electrode toward the membrane, anions in the opposite direction, the result is a positive charge. The corresponding events appear on the other side of the membrane. This accumulation of ions proceeds until the excess charge prevents further accumulation, so that a certain potential difference is built up, depending upon the charge density and the thickness of the membrane. The ionic flow is stopped. This is like charging a condenser, the static capacity of which is $C = Q/V$, Q being the charge and V the potential difference. (This static capacity, with a variety of cells—erythrocytes, echinoderm eggs, muscle, nerve, plant cells—has been found to be about 1 microfarad/cm^2.) However, when, instead, a high-frequency alternating potential difference is applied, ions will have hardly begun to be piled up at the membrane, before the direction of the potential is reversed, and they will travel in the other direction. Thus they swing as though no membrane were present in the electrical circuit. But, in addition to these electrostatic effects, which refer to ions unable to pass across the substance of the membrane, there can be more or less ionic shift through a resistance, as, e.g., in a cell membrane, which, due to its architecture, allows a certain amount of real ion permeability. Such a system works like an AC circuit with a static capacity lying parallel to an ohmic resistance. In this system, the maximum current and the maximum potential do not coincide, as they do in the absence of a condenser, but follow each other in a certain phase difference varying with the ratio of condenser effect and resistance effect. In such a system the apparent resistance ("impedance") decreases with increasing frequency.

Turning now to physiological objects, first the behavior of a suspension of red blood corpuscles has been studied. The cells are rinsed with isotonic sugar solution and centrifuged to the highest possible volume concentration. Supplied with low-frequency AC current (100 to 200 cycles/sec.), their

conductivity may be found to correspond to about 0.02 per cent NaCl. The high-frequency conductivity is much greater. This has been determined by the following procedure.[166] An AC generator provides a receiving circuit, by the way of an induction coupling, with the oscillations. This circuit contains, besides a self-induction coil, a variable plate condenser and a second condenser, which is a glass trough with the metal plates as electrodes and filled with water. The dimensions of the self-induction and the capacity are such that the frequency of the oscillations amounts to several millions per second. The two circuits, the generator and the receiver, by slight variations of the condenser capacity in the receiving circuit, are brought to maximal resonance, indicating maximal amplitudes of the oscillations. Then the trough is filled with a certain volume of the cell suspension and, due to the presence of ions in the cells, a certain damping effect of the oscillations appears. This effect can be matched by filling the trough successively with a series of NaCl solutions of different strengths; concentrations between 0.1 and 0.4 per cent were found to be as equally damping as the cells, comparing to the 0.02 per cent NaCl, matching with the cell suspension at the low-frequency oscillations. The conclusion that the high-frequency values are representative of the internal conductivity of the cells, is corroborated by the observation that hemolysis (saponin), in other words, opening of the envelope of the cell contents, does not, or does only moderately, alter the high-frequency conduction, whilst the low-frequency conduction rises considerably (e.g., 1200 ohm → 165 ohm).[167] Evidently, the high-frequency current penetrates the condenser-like plasma membrane and passes through the considerably ionized contents of the cytoplasm. In this manner one can follow, that, starting with about 100 oscillations per second and progressively rising to several millions, the conduction by the interior of the cells increases more and more and finally reaches a maximum, which in this case, and similarly in others, is about ⅓ to ⅔ of the conduction of the outer milieu (serum, Ringer, or sea water). This behavior of the cells is pictured [illegible] in Fig. 28 showing the stream lines on their way from sea water through an unfertilized and a fertilized sea-urchin egg at different frequencies, indicated in kilocycles, according to Cole.[168] It is made clear that at about 1 kc. the current flows entirely around the unfertilized and the fertilized egg, that in the neighborhood of 50 kc. there is still very little current flow through the plasma membrane, but considerable current is passing through the fertilization membrane, and as the frequency is raised to about 1000 kc. and more, the stream lines also penetrate the plasma membrane and spread through the cytoplasm.

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[166] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **148**: 189, 1912; **133**: 237, 1910; **150**: 15, 1913; M. Philippson, Bull. Acad. roy. de Belg. de cl. sc., **7**: 387, 1921; H. Fricke and St. Morse, J. Gen. Physiol., **9**: 137 and 153, 1925; J. F. McClendon, J. Biol. Chem., **69**: 733, 1926; regarding muscle: E. Bozler and K. S. Cole, J. Cell. & Comp. Physiol., **6**: 229, 1935.

[167] Compare also H. Fricke and H. J. Curtis, J. Gen. Physiol., **18**: 821, 1934.

[168] K. S. Cole, Tr. Faraday Soc., **33**: 966, 1937.

However, by the extensive and intensive studies during the last decade, with increasingly refined methods, ample evidence has been provided that, following the passage of alternating currents across the various kinds of cells, a multitude of modifications of the time and strength relations of the current are effected, which reflect differences or alterations of the architecture of the cell membranes during rest and activity, during excitation, narcosis, injury, decay, and death. For example, during rest, either the factor of ion conduction or the factor of condenser charge and discharge can be predominant, as, e.g., shown by comparing sea-urchin eggs and muscles, on one hand, and red cells, on the other, or, during activity, the condenser properties of the membrane may display rather no change, while ion permeability does to a great extent, as, e.g., after stimulation of a squid axon or of a

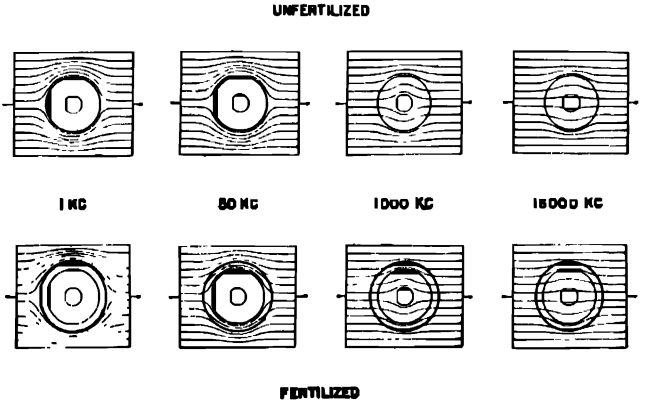

FIG. 28. Alternating current of various frequencies (in kilocycles) flowing through unfertilized and fertilized sea-urchin eggs. (Cole.)

Nitella cell (p. 316). It is beyond the limitations of this chapter to outline the methods and discuss the results in their details.[159] But it is evident that new ways are opening to describe essential features of cell life by the way of the electrical characteristics of the cells in terms of molecular arrangements and rearrangements.

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[159] See chiefly the studies of K. S. Cole and H. J. Curtis, and their co-workers, E. Bozler, R. H. Cole, R. B. Dean, H. Fricke, A. L. Hodgkin, and J. M. Spencer, in J. Gen. Physiol., vols. 1929 to 1940. See further, Sec. 5, and also The Cold Spring Harbor Symp., vols. **1** (1933), **4** (1936), and **8** (1940).

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Section 5

INFLUENCE OF SOME EXTRACELLULAR FACTORS ON CELLULAR ACTIVITY

By RUDOLF HÖBER

INTRODUCTION

Cells under natural conditions are embedded in a fluid medium, the chemical and physical properties of which are rather constant. Deliberate changes of these standard conditions appear to be of essential interest, as usually they bring about functional changes resembling those which are observed under normal circumstances. By such experimental procedures a resting state can be converted into one of activity. Existent activity can be increased or depressed. For instance, one of the most common signs of activity in living things is motion of some kind—protoplasmic streaming, or ameboid movement, or rhythmic shift to and fro in protoplasm, or, most important, contraction and relaxation can be excited or inhibited. Even development and growth can be considered as a kind of motion. Furthermore, changes in electrical behavior are initiated, causing potential differences to appear and disappear. The continuous chemical processes of metabolism, including respiration, can be made to rise or subside. By this way of provoking from the outside artificial changes of activity, which resemble normal functional changes, one may draw conclusions as to the nature of the processes which underlie both the natural and the experimentally produced events.

The extracellular factors which shall be considered here are prevailingly chemical ones. The site of the attack might be expected to be either the cell surface or the cell interior as constituents of the cell body which, according to the preceding chapters, can be differentiated fairly well as to their microscopic and their molecular structure. The chemicals which will be mentioned here can be grouped roughly as follows: (1) chemicals which, at least primarily, are unable to penetrate the cell boundary, and therefore must be assumed to act by changing the properties of the surface layer. Substances of this kind are, first of all, the inorganic salts But there are also many more substances which often fail to enter directly; for example, organic nonelectrolytes like sugars, polyhydric alcohols, aliphatic acid amides, and amino-acids, which seem to be rather inactive toward the cells; (2) chemicals which enter the cells due chiefly to adsorption or to lipoid solubility or to diffusion, and which, for this reason, react primarily in the cell interior, but eventually at the surface as well. Among the substances of this nature will be mentioned here mainly narcotics and cytolytics.

It is not the plan to present in the following section a survey of the entire field of extracellular influences. Instead, main results will be selected and analyzed in order to gain theoretical viewpoints which are useful in the interpretation of increasing and decreasing cellular activity in terms of physicochemical phenomena.

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THE INFLUENCE OF INORGANIC IONS ON CELL ACTIVITY

1. The Influence on the Excitability of Muscle.—No material can be more promising for studying changes of activity due to changes in the ionic medium than muscle, particularly after, some sixty years ago, certain fundamental, pertinent facts were discovered. It was demonstrated by E. Hering, that the frog muscle begins to twitch when it is transferred from its natural milieu into physiological saline solution. Using the heart, S. Ringer found that the rhythmic action is decidedly improved by adding K and Ca to the normal saline, while Claude Bernard noted that K had a paralyzing effect. Further, W. Biedermann reported that K, locally increased at the muscle surface, produces a reversible electronegativity confined to the treated area. With this background, the physiological properties of the alkali and the alkaline earth cations and of the inorganic anions have been compared in numerous experiments.

Overton,[1] studying permeability to organic and inorganic compounds by placing frog muscles in isotonic solutions, was amazed to find that in solutions of sucrose, glucose, or mannitol the muscles, although failing to show any changes in weight or other signs of alteration, lost their electrical excitability, but recovered it after the addition of as little as 0.07 per cent NaCl. Continuing the experiments by varying the kind of salt, he discovered that any neutral Na salt, irrespective of the accompanying anion, restored the excitability, but of the other *alkali cations* only Li was a fairly effective substitute for Na, while Cs restored slightly and for a short time, and Rb and K not at all. Studying the change of electrical excitability in the isotonic solutions of the various alkali chlorides, the result was that the excitability was longest maintained in NaCl and decreased through the series: $Na > Li > Cs > NH_4$, $Rb > K$.[2] However, K has long been known to have a stimulating effect also. For instance, if a small amount of isotonic KCl is added to Ringer bathing a frog sartorius, the muscle makes a number of spontaneous twitches or falls into contracture, which may or may not be reversible. A muscle can be "sensitized" for this effect to various degrees by placing it for a few minutes in an isotonic mixture of Ringer plus

[1] E. Overton, Pflüger's Arch. f. d. ges. Physiol., **92**: 346, 1902; see also L. V. Heilbrunn and E. W. Ashkenaz, Physiol. Zool., **14**: 281, 1941.

[2] E. Overton, Pflüger's Arch. f. d. ges. Physiol., **105**: 176, 1904.

one of the alkali chlorides.[3] In this way it has been found that the stimulating effect of the alkali ions is strongest with K, and decreases in the following series: K > Rb > Na, Cs > Li,[4] which is rather the reverse of the first-mentioned series. Whether the stimulating or the paralyzing effect appears, depends upon the concentration of the salts as well as upon the duration of their action.[5] The stimulating effect is frequently reversible, the paralyzing frequently irreversible.

The *inorganic anions* show a corresponding behavior. Studied by the "sensitization" method of Lillie, the anions appear to raise the excitability in the direction: $SO_4 < Cl < Br < NO_3$, $I < SCN$.[6] But with higher concentrations or after a longer exposure the stimulating influence of I and SCN changes into an inhibitory and irreversible action.[7]

The gradual change of the stimulating or the inhibitory effect of the anions is especially well demonstrated in the experiments of C. Schwarz.[8] Frog muscles, hanging in isotonic solutions of sucrose plus Na-salt equivalent to 0.2 per cent NaCl, are electrically stimulated at intervals of one second. In sulphate, contractions stop after a small number of shocks; with acetate, somewhat more shocks are required; with NaCl, still more; and so on, so that the excitability appears to rise in the order: $SO_4 < CH_3COO < Cl < Br$, $NO_3 < I < SCN$. Moreover, a muscle which is paralyzed in SO_4, recovers in Cl; one paralyzed in NO_3 recovers in SCN; but one whose contractions have been suppressed in Br does not start again in SO_4 or in Cl. However, regarding SCN and I, this is true only with low concentrations; in higher concentration, e.g., equivalent to 0.4 per cent NaCl instead of 0.2 per cent, the muscle becomes fatigued in SCN or I after a considerably smaller number of stimuli than in the lower concentrations, it relaxes more slowly, the single contraction ends in more and more incomplete relaxation, and, after being completely fatigued, it may partially recover even in the presence of those anions which usually are less favorable.

a). *The plurivalent cations.*—The alkaline earth chlorides presented in isotonic concentrations are rather incapable of maintaining the contractility (Mg > Ca, Sr > Ba). But in an infinite number of experiments it has been shown that either an increased or a decreased functional activity, produced by pure NaCl solutions or by solutions containing, in addition to Na, small amounts of K sufficient to raise or to diminish the activity, can be brought back to normal by the addition of small amounts of Ca or Sr, also Mg, and to a lesser degree of Ba. This *balancing or antagonizing action* of the alkaline earth ions appears in many variations in the functioning of

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[3] R. S. Lillie, Proc. Soc. Exper. Biol. & Med., **6**: 170, 1910; Am. J. Physiol., **28**: 197, 1911.

[4] E. Gellhorn, Pflüger's Arch. f. d. ges. Physiol., **219**: 761, 1928.

[5] I. Chao, J. Cell. & Comp. Physiol., **5**: 1, 1935.

[6] R. S. Lillie, *loc. cit.*, I. Chao, Am. J. Physiol., **109**: 550, 1934; E. Gellhorn, *loc. cit.*

[7] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **105**: 599, 1905; **134**: 311, 1910; I. Chao, J. Cell. & Comp. Physiol., **6**: 1, 1935.

[8] C. Schwarz, Pflüger's Arch. f. d. ges. Physiol., **117**: 161, 1907.

plants and animals. The classical example is the composition of Ringer's solution[9] and is favorable action upon a beating frog heart. Extending these investigations beyond the group of the alkaline earth ions, it has been found that the trivalent rare earth ions, e.g., La, Ce, Y, are very toxic,[10] whereas the di- and trivalent (not the univalent) cations of the complex cobalt salts act very much like Ca.[11] Furthermore, the paralyzing effect of KCl (0.05 per cent) in standard saline (0.65 per cent NaCl) can be antagonized by Co and Mn and to a smaller extent by Ni, while Cu, Fe, UO_2, Cd, and Zn were found unable to function in such a balancing action. Also, the spontaneous twitches of frog muscles, induced by pure NaCl, can be abolished by the same divalent ions. These results are analogous to the earlier fundamental findings of J. Loeb[12] concerning the capacity of Ba, Co, Zn, Mn, Ni, Pb, and UO_2 to balance, in appropriate small concentrations, the inhibitory effect of large amounts of NaCl, as exhibited in the development of the fertilized eggs of *Fundulus*.[13]

Before proceeding in the enumeration of the multitude of findings concerning the effects of the neutral salts on the muscle activity, the so-far-mentioned observations will be subjected to an analysis, essentially in terms of physical chemistry, since the neutral inorganic strong electrolytes, at least the predominantly present alkali salts, are not involved by straight chemical reactions in the physiological phenomena under consideration.

Very often and for various reasons the surface of normal cells has been believed impermeable to the neutral salts, mainly because in isotonic solutions the cells keep their volume or their weight fairly constant, but undergo, in anisotonic solutions, corresponding changes of these manifestations of their osmotic conditions. This behavior complies as well with impermeability to cations plus anions, as, for reasons of electroneutrality, with impermeability to either cations or anions.

Muscles placed in an isosmotic solution, likewise, frequently are found to keep their weight constant or, better, to show not more than slight changes. The nature of these latter is very complex.

1. They can be the result of changes in the interfibral tissue space[14] which is open to free diffusion of both cations and anions in either direction; the ionic composition of this space varies according to the previous history of the muscle (preceding work, slight injury) as well as to other factors.

2. Changes can be due to the colloidosmotic pressure of lymph space

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[9] S. Ringer, J. Physiol., **3**: 380, 1882; **4**: 29, 222, 370, 1883.

[10] G. R. Mines, J. Physiol., **40**: 327, 1910; **42**: 309, 1911; R. Höber and R. Spaeth, Pflüger's Arch. f. d. ges. Physiol., **159**: 433, 1914.

[11] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **165**: 531, 1917.

[12] J. Loeb, Am. J. Physiol., **6**: 411, 1902; Pflüger's Arch. f. d. ges. Physiol., **88**: 69, 1901; **91**: 248, 1902.

[13] See, further, R. S. Lillie, Am. J. Physiol., **10**: 419, 1904; **17**: 89, 1906; A. Mathews, *ibid.*, **12**: 419, 1905; R. S. Lillie, Protoplasmic Action and Nervous Action: University of Chicago Press, 1923.

[14] E. Overton, Pflüger's Arch. f. d. ges. Physiol., **92**: 152, 1902; see, further, p. 253ff.

proteins, also to the establishment of a Donnan equilibrium, determined by their presence.

3. The fiber surface is permeable to the univalent cations according to their volume, which decreases along the series: Li > Na > K. This can be inferred from various observations; (a) from a study of the height of the salt potentials produced by various cations, K-salts being found the most effective among the neutral salts (p. 313); (b) from the fact that, under certain experimental conditions, perfusing a frog muscle with a salt solution containing more K than 13 mg. per cent results in the penetration, with less than 13 mg. per cent in the escape of K,[15] and that the entrance of K causes, by ionic exchange, a loss of H (a rise of *p*H) and vice versa.[16] This *p*H change is accompanied by a change of muscle weight.

4. Greater increases in weight than those described above are observed in isotonic KCl (0.83 per cent) or in mixtures of this solution with isotonic NaCl (about 150 mg. per cent KCl). However, this effect is more or less irreversible and is followed by death.[17] The same is true with Rb.[18] But this increase in weight is dependent also upon the concomitant anions. Cl resembles Br, NO_3, and I, whereas with SO_4, HPO_4, CH_3COO, even in isotonic concentrations, the muscle keeps its weight constant for a long time, although its excitability is lost. However, NaCl solutions containing not more than about 60 mg. per cent KCl, though paralyzing the muscle, fail to bring about a gain of weight. Therefore one may conclude that, although permeable to K, the muscle is practically impermeable to KCl, at least for some time, and it is still more so and for a longer time to those K-salts, the anions of which, according to many other observations, have greater difficulty in penetrating cellular membranes (pp. 245ff). This also refers to SO_4, HPO_4, CH_3COO in their behavior toward the intestinal wall, the kidney tubules, the frog skin, and plant roots (Sec. 8). As to the higher concentrations of KCl exhibiting an irreversible increase of weight, this can be perceived on the background of the observation,[19] that the presence of abnormal concentrations of KCl releases several metabolic reactions. Starting with about 16 mg. per cent KCl in a Ringer solution, the maximum excitability is reached with about 45 mg. per cent; then the excitability falls off, and simultaneously the O_2 consumption rises. At about 80 mg. per cent KCl, phosphocreatine is decomposed and a slight contracture appears, and at about 120 mg. per cent KCl lactic acid is being formed.

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[15] R. Mond and K. Amson, Pflüger's Arch. f. d. ges. Physiol., **220**: 69, 1928; R. Mond and H. Netter, *ibid.*, **224**: 702, 1930; **230**: 42, 1932; see further, p. 253.

[16] W. O. Fenn and D. M. Cobb, J. Gen. Physiol., **17**: 629, 1934; A. H. Hegnauer, W. O. Fenn, and D. M. Cobb, J. Cell. & Comp. Physiol., **4**: 505, 1934; H. Netter, Pflüger's Arch. f. d. ges. Physiol., **234**: 680, 1934; see, further, p 254.

[17] E. Overton, Pflüger's Arch. f. d. ges. Physiol., **105**: 176, 1904.

[18] See also E. Gellhorn, Pflüger's Arch. f. d. ges. Physiol., **200**: 583, 1924.

[19] A. H. Hegnauer, W. O. Fenn, and D. M. Cobb, J. Cell. & Comp. Physiol., **4**: 505, 1934; see also p. 255.

5. Lastly, in outlining this intricate picture of the factors, which are pertinent in the osmotic balance of muscles as affected by neutral salts, it should be realized that there are reasons for believing that, in addition to passive penetration of ions across the surface membrane, a slow active transfer of ions takes place, as it does with erythrocytes.[20]

On the basis of these studies of the various effects of the neutral salts we arrive at the final conclusion that the very first changes of excitability can be referred to processes going on in the outermost layers of the fibers, which only secondarily are penetrated by the ions.

2. The Colloid Theory of the Change of Excitability by Ions.—The statement of J. Loeb concerning the balance effect of multivalent cations toward isotonic NaCl in the development of *Fundulus* embryos (p. 291) suggested correlation of this physiological phenomenon with changes in the colloidal structure, because only the dispersion of (inorganic) suspensoid colloids at that time was known to be acted upon by ions according to the sign of their electrical charge and to their electrovalence (rule of Schulze and Hardy).[21]

The fundamentals of such a colloid theory of cellular action have been very much extended, mainly on the basis of the experiments on muscles.[22] These results have indicated, first, that the series of *anions:* SCN, J, NO_3, Br, Cl, SO_4, showing the effect on excitability and on the related salt potentials (see p. 313), is paralleled by a series of anions, known as the Hofmeister series[23] (or the hydrotropic or, more generally, the lyotropic series) which accounts for the dispersity of many hydrophilic organic colloids, like gelatin or egg albumin, with regard to gelation, viscosity, colloidosomotic pressure, and other properties; and, second, they have indicated that the series of *cations* K, Rb, Cs, Na, Li, with its peculiar location of Cs (see pp. 289, 290, 315),[24] likewise is met under certain conditions (*p*H), in the physico-

[20] J. E. Harris, J. Biol. Chem., **141**: 579, 1941; W. Wilbrandt, Pflüger's Arch. f. d. ges. Physiol., **243**: 519, 1940; see more details in Sec. 4, p. 251. It is important also to note that under the particular conditions of nutritional deprivation of K, the muscle fibers of rats which normally are devoid of Na have been found to contain, besides a reduced amount of K, considerable amounts of Na. (L. A. Heppel, Am. J. Physiol., **127**: 385, 1939.) See more about these experiments on p. 257. Furthermore, K is claimed to be bound to the myosin threads, which build up the fibrillæ of the muscle (see Sec. 7).

[21] J. Loeb, Pflüger's Arch. f. d. ges. Physiol., **91**: 248, 1902; **88**: 68, 1901.

[22] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **106**: 599, 1904.

[23] F. Hofmeister, Arch. f. Exper. Path. u. Pharmakol., **24**: 247, 1888; **25**: 1, 1889; **27**: 395, 1890; **28**: 210, 1891.

[24] This location of Cs near to Na and Li, instead of a location beyond K, as it should be corresponding to the scale of the cationic mobilities (see p. 317), also has been observed with nerves (E. Overton, Pflüger's Arch. f. d. ges. Physiol., **105**: 176, 1904; H. Netter, *ibid.*, **218**: 310, 1927; R. Höber and H. Strohe, *ibid.*, **22**: 71, 1929; L. Wassiliew and K. I. Iwanow, *ibid.*, **235**: 184), 1934; ciliated epithelia (R. S. Lillie, Am. J. Physiol., **17**: 89, 1906, and **24**: 459, 1909); red blood corpuscles (R. Höber, Biochem. Ztschr., **14**: 209, 1908; F. Port, Deutsches Arch. f. klin. Med., **99**: 259, 1910; C. Kroetz, Biochem. Ztschr., **137**: 372, 1923); potentials of plant cells (E. B. Damon, J. Gen. Physiol., **22**: 819, 1939; W. J. V. Osterhout, *ibid.*, **23**: 53, 171, 1939); potentials of muscle (R. Höber, Pflüger's Arch. f. d. ges. Physiol., **106**: 599, 1904);

chemical reactions of the organic hydrophilic colloids.[25] These interrelationships have suggested that a dispersing effect upon structural colloids as components of the protoplasm facilitates the action, provided that the dispersion does not exceed a certain range of a swelling or loosening effect on the structure. Otherwise, the effect approaches more and more a dissolution (as a stage of cytolysis), so that the initial favorable influence, for instance, of SCN and I or of K (pp. 290 and 313) is reversed.

Provided the effect of the neutral alkali salts in the beginning is mainly restricted to the surface and cannot extend far beyond the superficial envelope of the fibers, the weight of a muscle as a measure of the dispersity of its structural colloids is not to be expected to change considerably in an isotonic solution, and, further, should show but slight variations on applying for instance, various anions. As a matter of fact, comparing the percentage increase of weight of sartorius muscles, placed for two hours in a buffered Ringer's solution and in corresponding solutions with NaCl substituted by NaBr, $NaNO_3$ and NaJ, the ratios for the percentage increases, referred to NaCl = 1.0, are found for NaBr = $NaNO_3$ = 1.5, NaJ = 1.8.[26]

The process of colloid dispersion in general is dual in its nature; it is either a matter of hydration and dehydration of colloidal micelles or a matter of increase and decrease of their electrical charge (see pp. 299ff). It depends upon quite a number of factors, which of these processes is dominant under various conditions. Certainly one of the main factors is the chemical nature of the colloids. But the information about the chemical components of the plasma surface membranes is very poor as yet. The most satisfactory approach to a better knowledge has been found in the analysis of the stromata (ghosts) of the erythrocytes, which are accessible in great quantity as a relatively uncontaminated material.[27] Their chief constituents are found to be lipoids and proteins in the ratio 1:1.7. Two-thirds to three-quarters of the lipoids are phospholipoids, the rest cholesterol. Cerebrosides are not present in the red cells. Three-quarters of the phospholipoids are made up by cephalin, one-quarter by lecithin. Lecithin is an ampholyte due to the presence in the molecule of glycerophosphoric acid, on one side; of cholin, on the other. Cephalin has an acidic character, since colamin is a weaker base than cholin. The protein is more or less insoluble. This seems to be due partially to the formation of lipoproteins (see p. 297). One such lipoprotein is a reaction product of the acidic cephalin and the basic histone,[28] and globin also has been found to precipitate with cephalin. Products like these may account for the formation of relatively solid cell structures.

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permeability of plant cells (R. Collander, Protoplasma, **33**: 214, 1939); and other biological material.

[25] R. Höber, Beitr. z. Chem. Physiol. u. Path., **11**: 35, 1907.

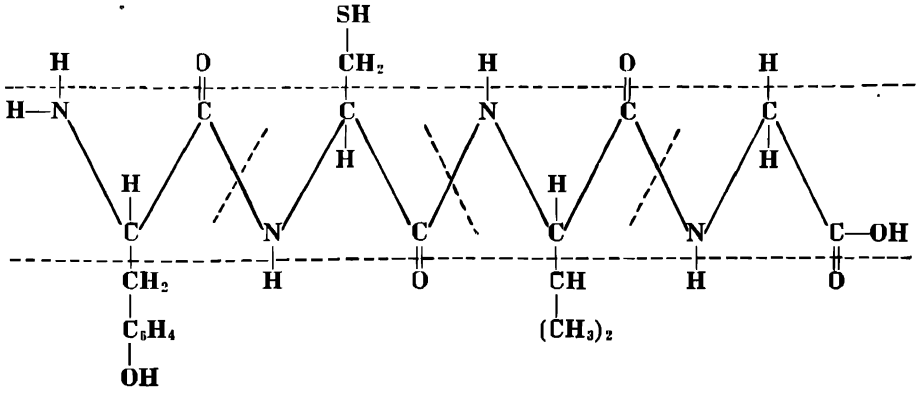
[26] I. Chao and K. T. Chen, Chinese J. Physiol., **11**: 253, 1937.

[27] A. K. Parpart and A. J. Dziemian, Cold Spring Harbor Symp., **8**: 17, 1940.

[28] E. Chargaff and M. Ziff, J. Biol. Chem., **131**: 25, 1939.

The facts concerning the main components of the protoplasmic surface lead to the question of how the building stones are composed to form its molecular architecture, which has to serve the various demands of cell life. This has been partially discussed in the preceding Sec. 4, chap. 15. Here the main problem is how ions are enabled to impose on this structure an increase or decrease in dispersity as it is found with colloidal sols and gels, which are known among proteins, lipoids, and lipoproteins.

a). *Hydration and dehydration of proteins of lipoids, and of lipoproteins.*— *Proteins:* In general, the molecules of proteins or polypeptides are conceived as being shorter or longer chains of amino-acids attached to each other by the peptide linkage between the COOH and the NH_2 groups. Correspondingly, their molecular dimension ranges from about 5 Å units up to about 5000 Å = 0.5 micron. The chain linkage favors a fibrous shape of the molecules, but, as will be seen later (p. 297), it also does not conflict with the shape of leaflets or with that of more rounded particles, due to folding and coiling of the chains. Each moderately long peptide chain resembles a tridimensional rod-like body with a zig-zag axis composed of the repeating —N—C—C— groups, due to the zwitterion character of the amino-acids. To each of these chains there is attached at a regular distance of about 3.5 Å an amino-acid residue as a side chain projecting perpendicularly to the axis and alternately in opposite directions as indicated in the following formula of a tetrapeptide, tyrosyl-cysteinyl-valylglycine. It is obvious that the length of the side chains is variable, thus determining the width of the rod or fibril.



These rods can be identified as such by various optical methods, mainly by studying the birefringence (double refraction) with the polarizing microscope and by measuring the space of the various planes in an x-ray diffraction pattern, as one does in a crystal lattice. The nature of birefringence is dual; it is either a form birefringence or an intrinsic birefringence. The form birefringence is based upon the presence of rods as asymmetric units of an isotropic substance, embedded into an isotropic medium, and upon their more or less regular parallel orientation, whereas the intrinsic birefringence is caused by optically anisotropic crystal-like particles. By either of these methods, changes in the mutual distance and orientation of the submicroscopic units can be

observed and measured quantitatively.[29] Even with single ghosts of erythrocytes, fibrous structures have been detected by the birefringence technique, whereas the layers formed by their cellular envelopes are too thin to allow the detection of x-ray diffraction spacings.[30] Fibers, of course, are constructed of several peptide chains arranged with their axes parallel to each other and parallel to the main axis of the single fibrils.

It is now of particular interest for our problem concerning *hydration and dehydration*, to understand what kind of forces are involved in binding together the individual peptide chains (see Sec. 2, chap. 9). The affinity to water of the single polypeptide chain depends mainly upon the water affinity of the individual side-chains. For instance, side-chains which are composed only of hydrocarbon groups, like the valine residue in the aforementioned formula, have rather no affinity to water, while the N and O containing radicals, $-NH_2$, $-NH$, $-COOH$ and $-OH$, are hydrophilic. This is due to the fact that, in the N and O atoms of these radicals, residual "lone" electrons are available to be shared with residual electrons of adjacent water dipoles, and thus to effectuate a co-ordination linkage with water. In this way, it is understandable that the hydrophilic or hydrophobic character of the polypeptide chains, among others, is bound up, first, with the chain length insofar as this decides the ratio of hydrophilic polar and hydrophobic nonpolar groups.[31] A second factor is the acidic or the basic character of the terminating groups, $-COOH$ or $-NH_2$, which, depending on the *p*H, can form electrically charged centers of co-ordination with water dipoles.

With regard to the fiber proteins, which are constructed by linking together several single peptide chains, additional factors contribute to the hydration. First, there is evidence that fibrils with short side-chains can approach closer together and thus form fibers with a tighter packing (e.g., keratin) than those with longer chains. This certainly is inhibitory to the access of water molecules. Second, the *p*H again is an important point. With a *p*H fairly distant from the isoelectric point, the multivalent ampholyte proteins can be assumed to contain a number of either cationic or anionic centers, which by their electrical charge will repel each other, and thus favor the intrusion of water. Inversely, at the isoelectric point, parts

[29] W. J. Schmidt, Die Bausteine des Tierkörpers im Polarisierten Licht: Bonn, 1924; Die Doppelbrechung von Karyoplasma, Cytoplasma and Metaplasma: Berlin, 1937; F. O. Schmitt, J. Applied Physics, **9**: 109, 1937; F. O. Schmitt, Phys. Rev., **19**: 270, 1939; F. O. Schmitt and R. S. Bear, Biol. Rev., **14**: 27, 1939; A Frey-Wyssling, Submikroskopische Morphologie des Protoplasmas und seiner Derivate: Berlin, 1939; L. E. R. Picken, Biol. Rev., **15**: 133, 1940. Further, Sec. 2, chap. 7.

[30] F. O. Schmitt, R. S. Bear, and E. Ponder, J. Cell. & Comp. Physiol., **9**: 89, 1936; **11**: 309, 1938; D. F. Waugh and F. O. Schmitt, Cold Spring Harbor Symp., **8**: 233, 1940.

[31] D. Jordan Lloyd and H. Phillips, Tr. Faraday Soc., **29**: 132, 1933; D. Jordan Lloyd, Biol. Rev., **7**: 254, 1932; O. L. Sponsler in The Cell and Protoplasm, Publications of the Am. Assn. for Adv. of Sc., No. 14: The Science Press, 1940; L. S. Moyer in The Structure of Protoplasm, State College Press, Iowa, 1942.

of the structure opposite to each other may be oppositely charged and thus, like zwitterions, become linked together by electrostatic forces. Indeed, protein structures ordinarily are found to be more inaccessible to water at the isoelectric point.[32] Third, there have not yet been mentioned the special cross-linkages between individual fibrils, contributing considerably to the solidity of the fibril bundles, as, for instance, the di-sulfide-linkage between two cystein groups, forming a primary valence bond requiring chemical forces for disruption, or salt linkages formed by the reaction between the acidic and basic groups. Taking these various factors together, it is obvious that, in general, an increase of hydration will be indicated by a decrease of the birefringence and by an increase of the spacings in the x-ray diagram, and vice versa.

It has been mentioned (p. 295) previously that the polypeptide chain is deemed to construct not only the fiber proteins, but also leaflets and other aggregates of manifold configurations. From various kinds of measurements (viscosity, ultramicroscopy, ultracentrifuging) it has been concluded that some colloidal micelles are spherical or ovoid in shape. This is considered to be due to folding and coiling of the polypeptide chains and to holding together such a feltwork by the various aforementioned forces of interlinkage of greater or smaller strength. This picture accounts for the possibility of different degrees of hydration and dehydration, corresponding to the ratio between polar and nonpolar radicals, and suggests thinking of these particles as forming a meshwork filled with more or less loosely attached water, as encountered in gels. Such aggregates often are highly soluble, e.g., egg albumin particles, probably because the polar groups are sticking out of the surface toward the water. This concept is corroborated by the finding, among others, that spreading a spherical protein as a film on the surface of water is accompanied by denaturation, probably due to an unrolling of the long chains, attended by the rupture of interlinkages in the framework, the exposure of more hydrophobic groups, and the breakdown of S—S cross-linkages, making SH groups detectable, and other results.[33]

Hydration and dehydration of lipoids and lipoproteins.—By chemical analysis of the red cell ghosts it has been shown (pp. 272 and 294) that about one-third of this structure is formed by lipoids and lipoproteins and that the lipoids are partially phospholipoids, partially cholesterol. Cholesterol possibly does not participate in functional changes of this and of other cell surface layers, or only to a small degree, because it is a complex ring system of C and H which, in the case of cholesterol itself, includes but one O. However, this O may convey to the molecule a weak polarity, as displayed in the

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[32] C. L. A. Schmidt, Chemistry of the Amino Acids and Proteins: C. C. Thomas, Baltimore, 1938; W. Pauli and E. Valkó, Kolloidchemie der Eiweisskörper: Dresden, 1933.

[33] H. Wu, Chinese J. Physiol., **5**: 221, 1931; A. E. Mirsky and M. L. Anson, J. Gen. Physiol., **19**: 427, 1936; W. T. Astbury, S. Dickinson and K. Bailey, Biochem. J., **29**: 2351, 1935; H. B. Bull, Cold Spring Harbor Symp., **6**: 140, 1938; A. E. Mirsky, *ibid.*, **6**: 150, 1938; see further: Sec. 2, pp. 129ff, 139ff, 196ff.

capacity of sterols to form surface films on an air-water interface,[34] and, therefore, cholesterol might be assumed to play a role as a fairly rigid framework for the more labile hydrophilic colloids, which are included in the cell membranes (see p. 323 and Sec. 4). The reactivity of the phospholipoids is much greater, lecithin being an ampholyte, which is ionized to some extent at a *p*H around 7, and cephalin exhibiting a more definite acidic character (p. 294).[35]

In order to gain more direct information about the structural capacities of the lipoids, it has been found useful to investigate mixed lipoids, e.g., benzene-alcohol extracts of the brain, instead of studying the pure chemical individuals.[36] If emulsified with water, x-ray diffraction measurements show the mixed lipoids to form bimolecular layers, each of the two arrays of molecules turning their polar (positive and negative) groups outward, and being attached to each other by the nonpolar endings of the fatty acid radicals (see Fig. 27, p. 274). Between each pair of such bimolecular layers (65 Å in thickness) there is interposed a layer of water, the thickness of which is indicated by the x-ray diffraction spacings to vary according to the percentage of total water present in the emulsion (0 per cent to 75 per cent). Thus, the polar groups of the lipoid phase are anchored in the intercalated water layer, the thickness of this layer varying from 0 to 85 Å.

A still better model is provided by a mixture of lipoids and proteins which are bound to each other as in the aforementioned lipoproteins, arising from a reaction between a basic histone and acidic cephalin (p. 294). The resulting structure resembles the architecture of the emulsified mixed lipoids, but differs by the interposition between the lipoid layers of a monolayer of histone, the polar phosphoric acid groups of cephalin being linked together with the basic groups of the highly hydrated histone, with such strength that the water is expelled by this interaction.[37]

Finally, the molecular organization of *natural lipoid protein surfaces* also has been subjected to an analysis, most thoroughly by combining both the measurements of x-ray diffraction and of intrinsic as well as form double refraction in the study of the sheath of myelinated nerves.[38] Several kinds of investigation have suggested again the interpretation of the findings as due to lipoid-protein layers which are concentrically arranged around the axon, each of them being 170 to 190 Å in thickness and composed

[34] N. K. Adam, Physics and Chemistry of Surfaces, 2nd ed., Clarendon Press, Oxford, 1938.

[35] Concerning the influence of a rigid porous membrane with meshes filled by hydrophilic colloids upon the hydration and dehydration and thus upon permeability, see: K. J. Anselmino, Biochem. Ztschr., **192**: 390, 1928; further, O. Risse, Pflüger's Arch. f. d. ges. Physiol., **212**: 399, 1926, **213**: 685, 1926.

[36] F. O. Schmitt and K. J. Palmer, Cold Spring Harbor Symp., **8**: 94, 1940; see, further, F. O. Schmitt, Physiol. Rev., **19**: 270, 1939.

[37] F. O. Schmitt and K. J. Palmer, Cold Spring Harbor Symp., **8**: 94, 1940.

[38] G. Boehm, Kolloid-Ztschr., **62**: 22, 1933; W. T. Astbury, S. Dickinson and K. Bailey, Biochem. J., **29**: 2351, 1935; W. J. Schmidt, Ztschr. f. Zellforsch. u. mikr. Anat., **23**: 657, 1936; F. O. Schmitt, Cold Spring Harbor Symp., **4**: 7, 1936.

of two double molecular layers of lipoids, which are separated by a thin protein layer. Between these concentric layers a great amount of water is interposed.

A similar structure has been ascribed to the envelope of the red cells.[39] As mentioned previously (p. 294), this is marked by a high content of cephalin.

b). *The influence of inorganic alkali salts upon hydration and dehydration of cell colloids.*—Returning now to the main problem of this section regarding the nature of the ionic influence upon cell activity, we must determine whether the present knowledge about the architecture of the artificial and natural complexes of the colloidal constituents of cells contributes to an understanding of this influence. The bases of such a study are the aforementioned observations of Hofmeister regarding the influence of neutral alkali and of alkaline earth salts upon the hydration and dehydration of hydrophilic colloids, like gelatin and albumin. Two factors are preponderant in the interrelation between ion and colloid: first, the amount of hydration of ion and colloid or salt and colloid (p. 300); and, second, the positive or negative electric charge of each.

The hydration of the ions increases in the order: $SCN < J < Br < Cl < SO_4$ and $Rb < K < Na < Li$. For the halide anions and for the cations this is the order of decreasing atomic radii, and thus probably the order of increasing electrostatic interaction of ion and water dipole. For instance, the relative hydration of cations is estimated: (H = 1), K = 5.4, Na = 8.4, Li = 13.9, (Ca = 22). The water molecules are oriented around the ion as a shell, the strength of fixation diminishing toward the periphery, where the water dipoles can be stripped off with increasing ease.

The hydration of the colloids varies over a wide range. This in general is due to the number and strength of residual valences as hydration centers—as explained before (p. 296); and, thus, the range can reach from the highest grade of hydrophilic behavior to that of the hydrophobic or suspensoid colloids.

The interaction between the colloids and the ions may be conceived as follows: In general the hydrophilic colloids, particularly those which we are mainly interested in, the proteins, are colloid-electrolytes, the colloidal particles being either cationic or anionic. As such they attract the ions bearing the opposite *electric charge* and repel those with like charge. The strength of the charge of the individual colloid varies with *p*H with reference to the isoelectric point of the colloid.

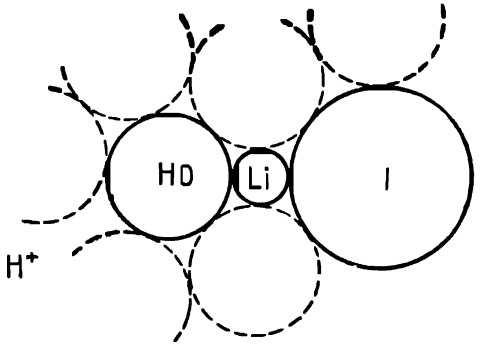
Another factor is the *adsorption* affinity of the colloids toward the ions, cations as well as anions. The adsorption affinity toward the ions differs, increasing with decreasing hydration, and vice versa.[40] In other

[39] F. O. Schmitt, R. S. Bear, and E. Ponder, J. Cell. & Comp. Physiol., **9**: 89, 1936; **11**: 309, 1938; D. F. Waugh and F. O. Schmitt, Cold Spring Harbor Symp., **8**: 233, 1940.

[40] M. Gouy, Ann. de Chim. Phys., (7) **29**: 145, 1903, A. Frumkin, Kolloid-Ztschr., **35**: 340, 1924: Ztschr. f. Physik. Chem., **109**: 34, 1924.

words, the shell of water dipoles surrounding the atoms interferes with their attachment to the colloid. For this reason, the affinity toward K is greater than that toward Li; toward SCN and I greater than toward Cl and SO_4. Further, it must be realized that—as another significant feature of adsorption—with lower salt *concentrations* a relatively greater fraction is attached to the colloid than with higher concentrations.

A third factor is the *competition for water* between the colloids and the "outside" ions surrounding the colloid particles, according to their respective water affinity[41] (including their osmotic influence). The operation of this factor in the case of nonelectrolytes (instead of colloids) is well known



Lithiumiodid

FIG. 29.—Lithium iodide forming in water an aquoacid.

as the "salting out" effect of electrolytes at higher concentrations. It is especially strong if the water affinity of both cations and anions is great. The dehydrating influence toward the colloids is attributable to all ions bearing the same charge as the colloid particles, and in addition to that fraction of the oppositely charged ions, which remains unadsorbed.

A fourth factor seems to be the "ion pair effect," i.e., the joint action of cation plus anion, which form, more or less distinctly, either aquoacids or aquobases, as indicated by Fig. 29, representing the formation of an aquoacid by LiI. Li, as the ion with the smaller atomic diameter attaches more water dipoles than the larger I and orients the dipoles in such a way that OH is firmly fixed on Li, so that H projects as a free ion. Aquoacids are supposed to react more with basic, aquobases with acidic proteins.[42]

It is obvious that the combined effect of electric charge, of adsorption, of ionic concentration, and of hydration can bring about most variable and sometimes embarrassing results and, as a matter of fact, the order of cations or that of anions or that of both has been found to run in one or the other direction or even to turn into a disorderly succession, according to the salt concentration, to the *p*H; further, to the admixture of other colloids or of

[41] See with this regard also P. Rona and H. H. Weber, Biochem. Ztschr., **203**: 429, 1928.

[42] See K. H. Meyer and M. Dunkel, Ztschr. f. physik. Chem., Ergänzungsband 1931, p. 553.

non-electrolytes like sucrose or alcohol, which themselves have a strong dehydrating influence. For instance, a mere *change of salt concentration* can cause a reversal of the order of cations or anions in the following manner: KCl at low concentrations, due to the relatively strong adsorption of K, affects a negative colloid directly by a considerable discharge, compared to the less adsorbable Li. But, at high concentrations the effect of Li is more prominent, due to its strong dehydrating power, affecting more indirectly (from outside) the water attached to the colloid. Or, by changing the sign of the *electric charge* of a colloid ampholyte by modifying the *p*H, a complete inversion of the ionic order is established. With negative particles the dispersity is diminished by cations essentially due to adsorption, in the order: K > Na > Li, and by anions essentially because of dehydration from the outside phase in the order SO_4 > Cl > Br > I > SCN: whereas with positive particles the opposite order: SCN > I > Br Cl > SO_4 and Li > Na > K appears. But, regarding the accomplishment of these inversions of the ionic order, it can be logically expected that by stepwise changing of the ionic atmosphere around the colloidal micelles, one may encounter highly irregular intermediate arrangements which by no means can be referred to experimental error, if carefully checked, especially with regard to the *p*H. As a matter of fact, there are scattered in the literature many examples of this kind.[43]

About the influence of the neutral alkali salts upon the colloid state of lipoids and lipoprotein complexes very little is known as yet. Again, there has been observed the ordinary cation and anion series displaying the gradually changing effect upon flocculation or clarification.[44]

Increase of birefringence and decrease of x-ray diffraction spacings in mixed brain lipoids also were found to be indicative of a rising dehydration after the addition of certain neutral salts in certain concentrations.[45]

From the analysis of the architecture of the colloid complexes attempted here (pp. 295–299) arises a picture of aggregates of many different types of colloids cemented together like stones in a mosaic or disposed in layers, one upon the other. The component colloids in these artificial systems as well as in natural fragments of protoplasm are of varying proportions of hydrophilic and hydrophobic types; therefore their isoelectric points will be well separated and the electrical charges will differ from point to point, creating consequently anomalous effects of the neutral salts. Even greater complexities are to be expected in living material than in these models. Such

[43] Posternak, Ann. Inst. Pasteur, **15**: 85, 1901; W. Pauli, Beitr. z. chem. Physiol. u. Path., **5**: 27, 1903; R. Höber, *ibid.*, **11**: 35, 1907; R. Ehrenberg, Biochem. Ztschr., **53**: 356, 1913; R. Labes, Pflüger's Arch. f. d. ges. Physiol., **186**: 112, 1921; Sh. Dokan (L. Michaelis), Kolloid-Ztschr., **37**: 283, 1925; J. R. Katz, Biochem. Ztschr., **257, 259, 261, 262, 263, 1933; 271, 1934.** See, further, H. Freundlich, Colloid and Capillary Chemistry; Dutton, New York **1922**; W. Pauli and D. Valkó, Elektrochemie der Kolloide; Wien, **1929**.

[44] O. Porges and E. Neubauer, Biochem. Ztschr., **7**: 152, 1907; P. Rona and W. Deutsch, *ibid.*, **171**: 89, **1926**.

[45] F. O. Schmitt, Cold Spring Harbor Symp., **8**: 94, 1940.

considerations as these are chiefly of importance as stimulus and guide for future studies.

c). *The interpretation of physiological effects of ions as due to changes of the state of the hydrophilic colloids.*—It is almost surprising that, in the beginning of this chapter, some relatively simple observations concerning the change of muscle excitability by inorganic alkali salts could be made to suggest a colloid theory of stimulation based upon the appearance, in muscle excitation, of the lyotropic order of anions and cations, well known from the physical chemistry of hydrophilic colloids. Such a theory appeared to be highly plausible for several reasons: 1. Each protoplast is like a gel or a sol of hydrophilic colloids; 2. An important part of the protoplast, the plasma membrane, is built up of various colloids to a structure, which often, as in muscle fibers, appears to be fairly impermeable to many solutes, but which can lose this property under the (reversible) influence of alkali salt solutions; such solutions are known to have a dispersing and eventually a liquefying power toward organic hydrophilic gels; 3. The dispersing effect of, e.g., NaCl can be balanced by the addition of a small amount of alkaline earth salt in the case of physiological objects as well as colloidal models (see pp. 303ff).

However, many objections have been made to such a colloid theory as can easily be observed in reviewing the physiological literature. It is true, the order of anions, $SO_4 < Cl < Br < I < SCN$, and of alkali cations $Li < Na < K$, is met frequently in animal and plant physiology, but it is likewise true that "irregularities," either as inversions of the regular arrangement or as complete disorder, have often been encountered. To mention only one old example: hemolysis has been studied by suspending erythrocytes in the isosmotic solutions of the neutral alkali (Na, K) salts, and adding alcohol, urethane, saponin, vibriolysine, or NaOH, or applying heat to the suspension, or comparing erythrocytes, the natural cationic contents of which differ considerably. Regarding the influence of the anions and the cations upon the order of the hemolytic effect, very great variations are displayed, the ordinary order of anions and of cations running in either the usual or the inverse direction, or being displaced by a random arrangement.[46] This result can be thought of as being due to the presence of various colloids or colloidal complexes and to their combinations with the added hemolytic factors, superimposed on the effect of the inorganic salts. But, from these and similar findings, the conclusion should not be drawn that the colloid theory is to be rejected as a reasonable explanation of ionic action. On the contrary, all the variations in cellular response to the salts should be analyzed from the viewpoint of colloidal behavior, and are likely to be correlated to colloidal processes.

[46] Miculicich, Zentralbl. f. Physiol., **24**: 523, 1910; A. Jarisch, Pflüger's Arch. f. d. ges. Physiol., **192**: 255, 1921; Fr. Port, Deutsches Arch. f. klin. Med., **99**: 259, 1910; Teruuchi, Comm. de l'Inst. Sérothérap. de l'État Danois, **3**, 1909. See, further, H. Davson, J. Cell. & Comp. Physiol., **15**: 317, 1940; Biochem. J., **34**: 917, 1940; further, Sec. 4.

d). *The antagonistic action of pairs of cations.*—So far the effects of single salts, only, have been taken into consideration. An especially strong support of the colloid theory of physiological ion activity comes from the study of pairs of salts issuing from the aforementioned physiological antagonisms (p. 291), discovered by J. Loeb. He suggested this antagonism to be a physiological illustration of the Schulze-Hardy rule, that the flocculating power of an electrolyte depends upon the valence of the ion whose charge is opposite to that of the colloidal particles, and that the influence of valence rises nearly in a geometrical progression.

The Schulze-Hardy rule is derived chiefly from observations with the inorganic supensoid and, in general, hydrophobic colloids. Here the preponderant unstabilizing factor is the electric charge. Thus, according to their valence, the cations in appropriate concentration can bring about an isoelectric precipitation of an anionic colloid; the anions, of a cationic. However, hydrophilic colloids, which in general are the prevailing fraction of the colloids in protoplasm, are not subject to the Schulze-Hardy rule, except with certain modifications. By gradually substituting the water in a solution or in a gel of a hydrophilic colloid, such as gelatin, with polar hydrophilic alcohol or acetone, the hydrophilic colloid is changed into a hydrophobic suspensoid.[47] In the hydrophilic state, high concentrations of salt are required to produce precipitation by dehydration (salting-out effect, p. 300). However, after a certain extraction of water has been accomplished, the colloidal particles attain more and more the character of hydrophobic colloids which are precipitated by small electrolyte concentrations, following the Schulze-Hardy rule, as indicated by the greater influence of the polyvalent ions. Thus, the precipitating power of both $(NH_4)_2SO_4$ and $CaCl_2$ toward gelatin appears to be low—although in agreement with the hydration of their ions—but after transformation into a hydrophobic suspensoid the effect is great with $(NH_4)_2SO_4$ on the acid side of the isoelectric point of the colloid, with $CaCl_2$ on the alkaline side. Further, a colloid, which in the hydrophilic state differentiates between the individual monovalent alkali cations and the monovalent halide anions depending upon their hydration, as a hydrophobic colloid reacts more or less uniformly to the monovalent (or the polyvalent) ions, but differentiates among them according to the electric charge. However, even in the hydrophobic state the colloid can respond somewhat differently toward equally charged ions. For instance, the precipitating power of bivalent cations toward negative suspensoids like As_2S_3 increases generally in the order: Mg < Ba, Ca < Mn < Co < Ni < Pb < Cu. This is about the order of the standard electrode potentials of these metals,[48] and insofar probably the order of their decreasing hydration.

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[47] H. R. Kruyt and de Jong, Ztschr. f. physik. Chem., 100: 250, 1921; J. Loeb, Proteins and the Theory of Colloidal Behavior: McGraw-Hill, New York, 1924.

[48] A. Mathews, Am. J. Physiol., 12: 419, 1905.

Now, as mentioned previously (page 291), phenomena similar to these have been met by J. Loeb in his experiments on the development of fertilized eggs of the marine teleost *Fundulus* under the influence of various salt mixtures. The development is stopped, and the embryos die in isotonic alkali chloride solution, but the deleterious effect can be antagonized by the addition of certain small amounts of divalent cations. Most favorable is the effect of the alkaline earth ions, next are Co and Mn, much more toxic are Ni and Pb.[49] In similar experiments concerning the liquefying influence of NaCl upon the cilia of marine invertebrates,[50] the bivalent cations have been found to protect the cilia in the order: Mg, Ba, Ca, Sr, Mn, Fe, Co, Ni, Cd, Pb, Zn, Cu, UO_2. The last members in this series show the favorable effect only if applied in small concentration and for a short period of time. Otherwise, irreversible impairment occurs, evidenced by a beginning opalescence and flocculation of the protoplasm, and ending with death. Obviously, this series strikingly resembles the aforementioned order of the standard electrode potentials. The results strongly support the hypothesis that a normal function requires the existence of a certain colloidal state, and this necessitates a certain balance of mono- and polyvalent cations. If only monovalent cations are present, the dispersity of the structural colloids is abnormally great; the proper amount of bivalent cations insures the necessary rigidity. This concept is strengthened by the fact that trivalent cations (Al, Cr, Fe) also show an antagonistic effect, but only when applied in concentrations considerably smaller than those most favorable with bivalent ions. These observations are in agreement with the Schulze-Hardy rule, which states that the flocculation power of the ions rises with rising valence nearly in a geometrical progression.

The order of bivalent cations, practically identical with the order of the standard electrode potentials, can possibly be interpreted physiologically as an order of varying hydration capacity. Following the addition of $CaCl_2$ to the gel of a hydrophilic colloid, dehydration, in other words, shrinking or stiffening, occurs, indicating the competition for water by the highly hydrated Ca (p. 299), (but see, further, p. 305). Following the addition of Ni or Cu, flocculation inside the gel occurs, due to the adsorption to, and the discharge of, the colloid aggregates (p. 299). Shrinking is a reversible, flocculation an irreversible, process. Thus again the physiological effects parallel the physicochemical reactions (see also the footnote (58) p. 307).

Our main example, the influence of ions on muscle activity, lends itself particularly to the correlation of the effect of mixtures of ions on muscle excitability and on hydrophilic colloids.[51] 1. After increasing the colloid dispersity in frog muscles by the addition of small amounts of KCl to

[49] J. Loeb, Am. J. Physiol., **6**: 411, 1902; Pflüger's Arch. f. d. ges. Physiol., **88**: 68, 1901; **93**: 246, 1902.

[50] R. S. Lillie, Am. J. Physiol., **10**: 419, 1904; **17**: 89, 1906.

[51] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **166**: 531, 1917.

Ringer's solution so as to inhibit contractility completely, restoration takes place following the addition of appropriate amounts of bivalent cations in the series Ca > Sr > Mg > Co > Ba, Mn > Ni > Zn. Cd, UO_2, Cu, however, are incapable of restoring function. 2. The gelation of a gelatin solution can be accelerated beyond that in the presence of NaCl by the addition of one of the following chlorides in proper amounts: Ca, Sr, Ba, Mg, Co, Mn, Ni and Zn, whereas, evidently due to their precipitating power, the gelation is slowed by Cd, UO_2 and Cu. 3. The complex cobalt and chromium salts furnish an excellent example of the importance of the Schulze-Hardy rule in physiology. Only those complex cations, which are bi- or trivalent, are capable of antagonizing the paralyzing effect of a small excess of KCl in Ringer, whereas the monovalent complex cations fail to do so. The bi- and trivalent complex cations are comparable in the degree and duration of this beneficial action only to Ca and Sr; whereas the simple trivalent cations of the rare earths, Ce, Al, Y in much smaller concentrations are strongly toxic.[52] According to Mines[53] hexammin cobalt salt is rather indifferent toward hydrophilic colloids like hemoglobin, egg albumin, gelatin, and is thus, again, similar to Ca. All this is probably due to the special configuration of these complex cations, the metallic nucleus of which is surrounded by a sphere of molecules or radicals resembling the sphere of water dipoles around alkali or alkaline earth ions like Li, Na, or Ca, and, therefore, probably less adsorbable and less capable of discharging the colloidal aggregates than the simple cations (p. 299).

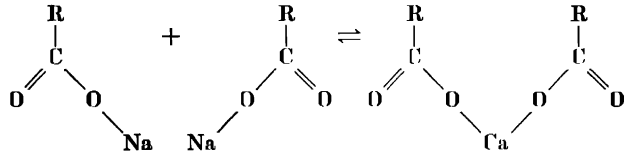
It has been emphasized already (p. 290) that the alkaline earth ions and especially Ca are often much more powerful in balancing the unfavorable dispersing influence of the monovalent cations than are the other polyvalent cations. This seems to be due to the fact that upon their stabilizing, anti-dispersing action there is superimposed a chemical reaction which links together more firmly the colloidal units than could be done solely by the presence of alkali ions. The concept of a solidifying influence of Ca is suggested by innumerable observations, particularly upon tissues, the cellular building stones of which are loosened by lack of alkaline earth salt, e.g., the blastomeres of sea-urchin eggs, the cells of root hairs, the epithelium of mucous membranes, and others.[54] The reason for this is that the cells normally are packed together by a cementing substance with the character of a weak acid, forming rather insoluble Ca-salts, which, in the absence of Ca in the bathing salt solution, dissociate and are transformed

[52] R. Höber and R. Spaeth, Pflüger's Arch. f. d. ges. Physiol., **159**: 433, 1914; K. Yomogita, Ztschr. f. Biol., 96, 487, 1935.

[53] G. R. Mines, J. Physiol., **40**: 327, 1910; **42**: 309, 1911; see, further, B. Lucké and M. McCutcheon, J. Gen. Physiol., **12**: 571, 1929.

[54] For literature see R. Höber, Physik. Chemie der Zelle und der Gewebe, 6th ed., chapter 11; Engelmann, Leipzig, 1926; L. V. Heilbrunn, The Colloid Chemistry of Protoplasm: Borntträger, Berlin 1928; L. V. Heilbrunn, An Outline of General Physiology, 2d ed.: W. B. Saunders, Philadelphia, 1943, especially pp. 462–467; R. Chambers, Cold Spring Harbor Symp., **8**: 144, 1940.

into the softer and less resistant Na-salt. Such compounds are the pectinates, the soaps, the phospholipoids, some proteinates and others. These reactions taking place in the intercellar space and in the cell surfaces can be pictured by the following equation:[55]



Assuming that in this manner a chemical reaction is superimposed upon a physical chemical reaction, the gradually differing effects of the alkaline earth and the other bivalent cations can be interpreted as dependent upon weaker or stronger, upon more reversible or more irreversible, reactions of these ions with the cementing substrata. This concept is supported by the following observations.[56] According to Locke and Overton,[57] a nerve muscle preparation loses its indirect excitability after being transferred from Ringer into NaCl, and regains its activity after the addition of Ca. Ca can be substituted only by Sr, but not by Ba, Mg, Co, Mn, Ni, hexammin-cobaltion. However, the direct excitability, after being abolished by NaCl plus 0.05 per cent KCl, is restored after the addition of any one of these bivalent cations. A similar behavior has been found with the heart and the stomach of the frog. Their contractility, as manifested either by spontaneous pulsation or as the response to an electric shock, is lost in NaCl and reappears only after the addition of Ca, Sr, and Ba. But, if paralyzed in Ringer plus 0.075 per cent KCl, the heart recovers also with Co, Mn, Ni, the stomach with Mn and hexammin-cobalti-ion. Locke has accounted for his observations regarding the loss of the indirect excitability in the absence of Ca as indicating an interruption in the pathway of the impulses at the myoneural junction. In other words, normal conduction requires a "cement" between nerve and muscle tissue which can be loosened or tightened reversibly, but must possess some intermediate degree of stiffness in order to allow the conduction wave to travel across the synapse. In addition, there is needed a certain consistency of the structural colloids of each separate tissue, which, after being abnormally softened by a surplus of K, can be brought back to its normal state, not only by Ca or Sr, but in accordance with the Schulze-Hardy rule more or less by the adsorption of any bivalent cation.

These different kinds of linkage between the colloidal aggregates, a physicochemical linkage on one hand, a more chemical one on the other, are

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[55] J. F. Danielli and H. Davson, J. Cell. & Comp. Physiol., **5**: 495, 1935; I. Langmuir, J. Franklin Inst., **218**: 143, 1934; J. F. Danielli, Proc. Roy. Soc., London, Ser. B, **122**: 155, 1937.

[56] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **182**: 104, 1920.

[57] F. S. Locke, Centralbl. f. Physiol., **8**: 166, 1894; E. Overton, Pflüger's Arch. f. d. ges. Physiol., **105**: 176, 1904.

manifested not only in a concatenation, as that on synapses, but also in many other cases. One particularly illuminating example is the behavior of the *Fundulus* embryo in Loeb's experiments. The predominant influence of valence is displayed only while the embryos are developing inside the egg membrane. This latter is a dead structure, probably keratin-like, and, as such, tough and rather impermeable to water. But a pure NaCl solution renders this membrane permeable, and it is this loosening effect which is antagonized rather indiscriminately by bivalent cations, as stated by Loeb. However, the embryo, liberated from the cover, can be protected against the injuring effect of pure NaCl only by Ca, and the rather closely related Sr and Mg, whereas even Ba is useless as a substitute for Ca. Thus, it becomes understandable why the combination of the cations Na, K, and Ca mostly satisfies the wants of active protoplasm, insuring its physicochemical, as well as its chemical, consistency.[58]

3. Effects of Substituting Nonelectrolytes for Electrolytes.—It was mentioned previously (p. 289) that frog muscle, soaked in isotonic sucrose solution, after a short time (about 15 min.) loses excitability, but regains it after immersion in Ringer or any nontoxic solution containing Na ions.[59] Chemical analyses by Urano[60] and by Fahr[61] showed that, during the paralyzing process, the muscles, if carefully prepared and kept at low temperature, lose considerable amounts of NaCl (up to 90 per cent) by escape from the interfibral space, but of K, though present in large quantities inside the fibers, as little as 6 per cent (p. 253). Further, it was shown[62] that phosphate and lactate appear in the sugar solution, and that these losses, which indicate a rise of permeability, are accompanied by a strong increase of O_2-consumption. In addition, a slight contracture is often observed. According to Overton, similar effects are brought about by the isotonic solutions of other lipoid insoluble, and chemically rather indifferent, substances, like lactose, maltose, glucose, mannitol, alanin, and asparagin, which, obviously, by no means, are physically as harmless as originally assumed.

These striking effects of the isotonic nonelectrolyte solutions can be counteracted by adding small amounts of salt—0.07 per cent NaCl is enough to preserve the normal state. According to Fenn (*loc. cit.*), the abnormal

[58] With respect to the physiological effects of the bivalent metal ions, it should be mentioned that the activating properties of Mn, Fe, Co, Ni possibly can be correlated to some extent with their activating or reactivating power toward certain enzymes, particularly toward those concerned with oxidation-reduction processes. (See Sec. 6, chaps. 27 and 28, also L. Hellerman, Physiol. Rev., **17**: 454, 1937.) Further, it should be borne in mind that amino-acids and proteins are known to form stable complexes with certain heavy metals: see Carl L. A. Schmidt, the Chemistry of the Amino Acids and Proteins: Thomas, Springfield, 1938.

[59] E. Overton, Pflüger's Arch. f. d. ges. Physiol., **92**: 346, 1902.

[60] F. Urano, Ztschr. f. Biol., **51**: 483, 1908.

[61] G. Fahr., *ibid.*, **52**: 72, 1908.

[62] G. Embden and H. Lange, Ztschr. f. physiol. Chem., **125**: 258. 1923; W. O. Fenn, Am. J. Physiol., **97**: 535, 1931; A. H. Hegnauer, *ibid.*, **107**: 667, 1933.

O_2-consumption and phosphate loss are considerably reduced also by the addition of $CaCl_2$ or KCl in low concentration, the latter being known to paralyze in higher concentration and to provoke contracture. Even HCl (0.01 and 0.026 mol.) produces a rapid fall of O_2-uptake after sucrose treatment.

In order to find an interpretation of these effects upon muscle, it seems to be useful to compare the behavior of erythrocytes under similar conditions. As previously described by Jacobs and Parpart,[63] the osmotic resistance of the red cells of ox, as measured in equally hypotonic solutions, is greater in nonelectrolyte than in salt solution. Furthermore, the nonelectrolyte solutions, either hypotonic or isotonic, cause an increase of osmotic resistance, which occurs in two stages; one rapid, which is readily reversible, and one slower, which succeeds and can extend over a long period of time. The rapid process is likely to be due to an anionic exchange through the anionpermeable surface membrane, comparable to the previously (p. 254) described exchange of K and H through a cationpermeable collodion membrane, by which, beginning with equality of K_i and K_o and with $H_i > H_o$, K is shifted from outside to inside against an ever-increasing concentration gradient, while H moves in the opposite direction toward a diffusion equilibrium.[64] In a corresponding manner the plasma membrane of erythrocytes, transferred into a nonelectrolyte solution, is passed by anions (HCO_3, Cl) from inside toward outside in exchange for an equivalent amount of OH, with the result of an increased ionization of hemoglobin, i.e., the formation of polyvalent hemoglobin anions (see Sec. 4, p. 249). In this way the osmotic pressure within the erythrocytes is decreased, and thus the osmotic resistance increased. The slow stage of the increase in nonelectrolyte solution is believed by Jacobs and Parpart to be due to an irreversible loss of electrolytes from the interior of the cells, i.e., not only to an escape of anions, because of the normal anionpermeability, but also to a pathological penetration of cations, mainly of K.[65]

From this comparison of muscle and red cells it appears that a common feature of the nonelectrolyte effects is a leakage of salts. The nature of this leakage will be discussed immediately. But, exchange of ions likewise plays a role in either case. With erythrocytes, due to their anionpermeability, the considerable diminution of ion concentration outside is succeeded by an increase of inside alkalinity; whereas, with muscle, due to its cationpermeability, an increase of inside acidity occurs. For, it previously has been shown that the surface of muscle fibers resembles the collodion mem-

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[63] M. H. Jacobs, Biol. Bull., **62**: 178, 1932; M. H. Jacobs and A. K. Parpart, *ibid.*, **65**: 512, 1933.

[64] H. Netter, Pflüger's Arch. f. d. ges. Physiol., **220**: 107, 1928.

[65] S. E. Kerr, J. Biol. Chem., **85**: 47, 1929; E. Ponder and G. Saslow, J. Physiol., **73**: 267, 1931; M. Maizels, Biochem. J., **29**: 1970, 1935; H. Davson, *ibid.*, **33**: 389, 1939. About special behavior of cat's blood corpuscles see H. Davson, *loc. cit.* See, further, pp. 251 and 257 about active shift of K.

brane in the aforementioned experiment of Netter, and since, according to the theory of membrane equilibria, $K_i:K_o$ equals $H_i:H_o$ (p. 254), the diminution of K_o should be followed by an increase of H_i, and vice versa. As a matter of fact, numerous experimental results fail to give unequivocal support to this theory. However, by changing K_o or H_o artificially, or by trying to influence H_i through metabolic stimuli (anoxia) or metabolic poisons (iodoacetate), so many secondary metabolic reactions are released (production of lactic acid, breakdown of phosphocreatin, dehydrogenations, and others), that even a fairly near approximation to the expected behavior may have a confirmatory value for the theory of exchange of K and H in muscle.[66]

Now, as to the destruction by nonelectrolyte solutions of the normal impermeability to salts, the following hypothesis has been advanced by Danielli.[67] If a long-chain fatty acid is dissolved in an organic solvent (brombenzene), which is immiscible with water, and which is in contact with an aqueous buffer solution of alkaline reaction (Na phosphate or borate), the fatty acid is ionized and a film of soap is formed at the interface, with the long-chain end of the anions adherent to the organic solvent, and with their hydrated carboxyl groups on the other end held in the buffer solution. The interfacial tension is lowered over a *p*H range of 5 to 10, the tension being lower with the more alkaline reaction, as shown in Fig. 30. The tension-*p*H-curves have the same shape as the dissociation curves for the soap in the bulk solution, only differing in that the range of *p*H, over which the change of the interfacial tension occurs, is shifted about 3 units of *p*H toward the alkaline side.[68] This shift is thought to be indicative of a Donnan equilibrium at the interface, the soap anions being retained in the film, the free buffer anions being predominant in the bulk of the aqueous phase, the cations (including H) in the film phase. In other words, the reaction at the interface can be considerably more acid than that in the bulk of the solution.[69] This conclusion is corroborated, among others, by observations upon the influence of ion concentration in the buffer solution. As shown in Fig. 30, the displacement of the curves toward higher *p*H's is greater with 0.001 mol. buffer than with 0.4 mol. But adding neutral salt (0.4 mol. NaCl) (Fig. 31) abolishes the shift, which is brought about by dilution of the buffer. This influence conforms to the usual effect of ion concentration upon the Donnan distribution (see Sec. 1, chap. 5).

According to Danielli, these observations serve to shed light on the

[66] W. O. Fenn and D. M. Cobb, J. Gen. Physiol., **17**: 629, 1934; H. Netter, Pflüger's Arch. f. d. ges. Physiol., **234**: 680, 1934; A. H. Hegnauer, W. O. Fenn, and D. M. Cobb, J. Cell. & Comp. Physiol., **4**: 505, 1934.

[67] J. F. Danielli, Proc. Roy. Soc., London, **B122**, 155, 1937; further, Chapter 21 in H. Davson and J. F. Danielli, The Permeability of Natural Membranes: University Press, Cambridge, 1943.

[68] See R. A. Peters, Proc. Roy. Soc., London, **B133**: 140, 1931.

[69] With this respect, see the critical remarks of S. R. Craxford, O. Gatty, and T. Teorell, London, Edinburgh, and Dublin Philos. Magaz., Ser. 7, **25**: 1041, 1938.

injurious influence upon cells of substituting nonelectrolytes for the electrolytes normally present in their environment. Various effects must be dealt with. As far as surface membranes of protoplasts are concerned,

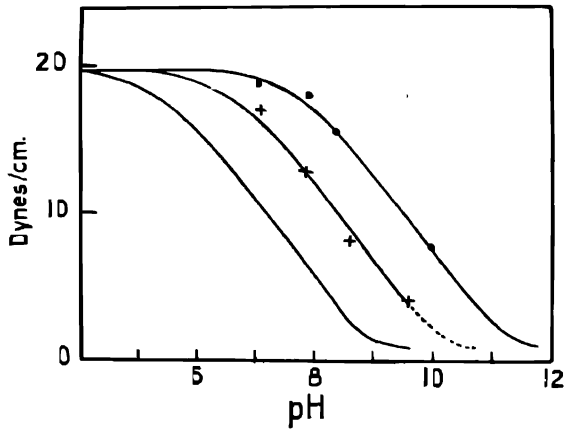

FIG. 30.—Effect of dilution of buffer with distilled water.
10% oleic acid. Buffer 0.4 mol.
+ buffer diluted 20 times
. buffer diluted 400 times

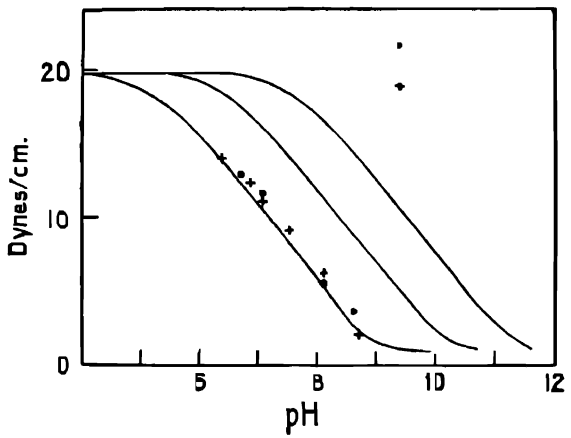

FIG. 31.—Effect of dilution of buffer with 0.4 mol. NaCl.
10% oleic acid. Buffer 0.4 mol.
+ buffer diluted 20 times
. buffer diluted 400 times
Influence of *p*H on the interfacial tension at a soap film.

which are believed to be built up by organic solvents like lipoids, by proteins and enzymes, the lateral adhesion forces between the structural components of the membranes can be assumed to be affected by the change of 3 and more *p*H units in these membranes, the hydration of lipoids will be changed, the proteins can be subject to irreversible denaturation, the enzymes to inac-

tivation, and these disintegrating effects will be succeeded by internal alterations of metabolism, by escape of large amounts of K and of phosphoric acid, and other results.[70]

But, possibly, sugars, as the chiefly applied nonelectrolytes, may produce the functional impairment not only as osmotic substituents, but also by independent direct effect. It has been known for a long time that a certain solidification is induced by sucrose in the plasma membrane of plant cells, preventing, for instance, the separate fragments of the protoplast, which can be formed under the influence of plasmolysis by a hypertonic sucrose solution, from coalescing again, and causing during deplasmolysis a local rupture of the rigid membrane.[71] This solidification may be compared to enhancement of gel formation and of shrinking of gelatin gels by sucrose and glucose, owing to their dehydration power (pp. 301 and 303).[72] The incidentally observed smaller effect of mannitol compared to sugar upon the surface of red cells has been referred to the absence of the aldehyde group, which is thought to react with protein.[73]

[70] The shift toward an acid reaction at the surface of erythrocytes, which is postulated by the theory of Danielli, recently has been shown by W. Wilbrandt, Verhandl. Schweizer Physiologen, 1940; Pflüger's Arch., f. d. ges. Physiol., **243**: 537, 1940.

[71] E. Küster, Ber. dtsch. bot. Ges., **27**: 589, 1909.

[72] I. Traube and F. Köhler, Internat. Ztschr. f. physik. chem. Biol., **2**: 42, 1915.

[73] H. Netter, Pflüger's Arch. f. d. ges. Physiol., **222**: 724, 1929.

17

THE INFLUENCE OF INORGANIC IONS ON FIBER AND CELL POTENTIALS

In the 16th chapter, ions were shown to condition a greater or smaller activity of muscles and nerves or, better, to raise or to diminish their excitability, a normal degree of excitability being met only in the presence of a certain mixture of ions. Another kind of ionic influence becomes obvious in the ability of muscle and nerve to respond to a local change of the ionic medium by disclosing a potential difference or a resting current, comparable to the action current which follows excitation. Muscle and nerve are especially suited for study of these electrical phenomena because of their fibrous structure. There is much less knowledge about the electrical properties of single cells which, as more or less spherical units of small dimensions, are, in general, less suitable for attaching electrodes. The resting potentials, which are produced experimentally by altering the ordinary ionic atmosphere, require particular attention because, by such ionic effects, light is thrown on the nature of the action potentials, the likeness of which to the artificial resting potentials is often denied or doubted.

1. The Salt Potentials of Resting Muscle and Nerve.—If one end of an uninjured frog sartorius muscle is brought into contact with Ringer, and the other end with Ringer the NaCl of which is replaced by another alkali or alkaline earth chloride, and if the two solutions are connected by suitable electrodes in a potentiometric set-up, an "injury" current develops, the strength and the direction of which depend upon the cations replacing Na.[74] The cations appear to follow the order: K > Rb > Cs > Na > Li, K producing the strongest negative pole, Li a weak positivity, compared to Na. This series has been met already (p. 290) as the order of rising excitability, after a short duration of exposure, or after exposure in a fairly low concentration. However, after treatment for a longer period or by a greater concentration, the increase of excitability, as exhibited with K or Rb, changes to a decrease, leaving the electronegativity unchanged (see p. 289). The order of anions is SCN > I ≧ NO_3, Br > Cl, with SCN producing the greatest positivity, which, however, after exposure for a long time or to a relatively high concentration, reverses to negativity. The same is true to a smaller extent with I. The excitability under correspond-

[74] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **106**: 599, 1905; see, further, R. Höber, M. Andersch, J. Höber, and B. Nebel, J. Cell. & Comp. Physiol., **13**: 195, 1939.

ing conditions likewise changes from increase to decrease with SCN and I (p. 290). In general, the effect of cations is greater than that of anions.[75]

Nerves are similar to muscles. With one lead on the intact surface of an excised nerve, the other lead taken from one of the crushed ends dipping into strong KCl and serving as reference electrode, the potential difference is depressed by isotonic alkali chlorides in the order: K > Rb > Cs > Na > Li with frog nerve,[76] in the order Rb > K > Na > Li with the unmyelinated nerve of the spider crab.[77] The effect of the inorganic anions, applied as Na-salts, has been found to be nil or very small, so that it remains questionable whether these effects are significant of the properties of the nerve membranes, or, rather, indicative of diffusion potentials. Measurements on single nerve fibers can be expected to make this clear.[78]

Another interrelation between excitability and resting potential of muscle is encountered in experiments on the antagonistic effect of monovalent and polyvalent cations.[79] Again, (see p. 304) it appears that the injuring influence of pure isotonic NaCl, or NaCl plus small amounts of KCl, is balanced by suitable small amounts of Ca, Sr, Ba, Co, Mn, Ni, but fails to be abolished by Cu, UO_2 and Cd. Furthermore, the trivalent cobalt complex salts, e.g., $[Co(NH_3)_6]Cl_3$, exhibit a balancing effect, while the monovalent, e.g., $[Co(NH_3)_4(NO_3)_2]Cl$, do not. Nerves are subject to the same rule.[80] However, after nerve or muscle has been submitted to isotonic alkaline earth chloride, even KCl, at a strength as high as fully isotonic, is prevented from, or at least delayed in, provoking negativity.[81] This "tanning" action increases in the order: Ca < Sr < Ba. It is protective not only against KCl, but also against various chemicals, which for one reason or another (see later, p. 338) produce negativity, e.g., amylurethane, saponin, veratrine, salicylate. These latter experiments have been done with the nerve of the marine spider crab.[82]

Taking into account these various observations on the salt potentials, and further referring to the aforementioned effects on excitability of muscle and nerve, it is suggestive to interpret the bioelectric phenomena as surface effects taking place in the plasma membrane. Such an idea was presented for the first time 50 years ago by W. Ostwald,[83] in pointing out that precipi-

[75] See also, T. Seo, Pflüger's Arch. f. d. ges. Physiol., **206**: 485, 1924. A markedly different behavior of an invertebrate muscle, the striated portion of the adductor of Pecten, has been found by H. B. Steinbach, J. Cell. & Comp. Physiol., **3**: 203, 1933, and **7**: 271, 1935.

[76] H. Netter, Pflüger's Arch. f. d. ges. Physiol., **218**: 310, 1928; J. van Heuverswyn, Arch. internat. de physiol., **43**: 316, 1936.

[77] W. Wilbrandt, J. Gen. Physiol., **20**: 519, 1937.

[78] See, further, S. L. Cowan, Proc. Roy. Soc., **B115**: 276, 1934; R. Höber and H. Strohe, Pflüger's Arch. f. d. ges. Physiol., **222**: 71, 1929.

[79] R. Höber, Pflüger's Arch. f. d. ges. physiol., **165**: 531, 1916.

[80] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **182**: 104, 1920; also R. Höber and H. Strohe, *ibid.*, **222**: 71, 1929.

[81] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **105**: 599, 1905.

[82] R. Guttman, J. Gen. Physiol., **23**: 343, 1940.

[83] W. Ostwald, Ztschr. f. physik. Chem., **6**: 71, 1890.

tation membranes are the seat of considerable electromotive forces that may be thought of as due to selective ionpermeability. This view was supported by the just-mentioned later observations, according to which the bioelectric potentials are influenced by inorganic ionsin such a way as to recall two of the most familiar rules of ionic effects on colloids, the lyotropic series and the Schulze-Hardy rule. The ionic effects on the potential suggest the presence of a colloidal membrane, which shows both hydrophilic and hydrophobic attributes. The hydrophilic character, on the one hand, for instance, is illustrated by the series of the monovalent cations Li, Na, Cs, Rb, K, which with its characteristic location of Cs has been met in model experiments only with hydrophilic colloids (p. 294) and, in addition, has been encountered frequently in physiology (p. 293); while, on the other hand, the membrane resembles the hydrophobic colloids in the applicability of the valence rule.[84] Finally, a colloidal character is suggested by the fact that in many of the aforementioned experiments the physiological activity of the cations has been found to surpass that of the anions, presumably because the colloidal components of the cells generally carry a negative charge.

2. The Membrane Theory of Biopotentials.—Arguing in this way, the colloid theory of the bioelectric potentials appears as a natural complement of the rather commonly accepted *membrane theory of biopotentials*, which on the basis of Ostwald's conclusion was developed 40 years ago by Bernstein.[85] The characteristic feature of this concept is the assumption of a selective membrane permeability to cations, particularly to K ions, which are present in excess inside the muscle fiber and many other cells, and therefore can establish a concentration potential drop from inside to outside across the membrane, of such magnitude as is otherwise obtained only with metallic electrodes in a concentration chain. The more selective the permeability, in other words, the greater the difference in the ratios of cationic and anionic mobilities inside the membrane structure and the bordering aqueous solutions respectively, the greater is not only the potential drop, but also the ohmic resistance in the system. After disintegration of the structure, both the membrane resistance and the potential drop disappear. Therefore, a local damage is followed by the appearance of a potential difference between the intact and the damaged surfaces, and an injury current starts flowing in the outer circuit from the intact surface as a positive pole to the injured portion as a negative pole, with an electromotive force equal to about 70 mV in a frog muscle. The objection has often been raised to the membrane theory that there are other means to account

[84] With regard to the location of Cs in the hydrotropic series near to Na and not beyond K it should be mentioned that Netter (Pflüger's Arch. f. d. ges. Physiol., **218**: 310, 1928) and others refer this unexpected location to the fact that Cs resembles the alkaline earth ions in some of its chemical properties.

[85] J. Bernstein, Pflüger's Arch. f. d. ges. Physiol., **92**: 521, 1902: J. Bernstein, Elektrobiologie; F. Vieweg, Braunschweig, 1912.

for the persistence of the unequal distribution of ions between inside and outside than selective ionpermeability of the surface membrane. But, particularly by recent experiments on giant cells, conclusive evidence has been advanced in favor of the membrane theory. The procedure is, for instance, as follows:[86] A single cylindrical cell of the fresh-water plant *Nitella* is placed in a groove in such a way that, close to one end, two stimulating electrodes are attached to the cell; two other electrodes, more toward the center of the cell, touch its surface opposite to each other, thus permitting measurement of the transverse resistance, while another electrode is located at the other end, serving to record the action potential wave which travels along the cell after a stimulus has been applied. This arrangement makes it possible to follow at one point of the surface simultaneously the variations of the resistance and the membrane potential. The results show that, fairly coincident with the action potential wave, i.e., the wave of propagated negativity, which has a duration of two to three seconds, the transverse resistance drops and then rises again. In other words, the membrane passes through a short phase of structural breakdown, as indicated by both the rise and fall of electrical conductance and the concomitant loss and gain of selective ionpermeability. The size of the effect of stimulation is such as to bring about, e.g., a decrease of resistance from about 100,000 ohm cm.^{2} to an average value of 500 ohm cm.^{2} Corresponding experiments on the single giant axon of the stellar nerves of the squid (*Loligo*) have shown[87] that, as the negative wave of excitation passes between the resistance electrodes, the resistance falls from the resting value of 1,000 ohm cm.^{2} to about 25 ohm cm.^{2}

It is suggestive to consider that this closely associated temporary drop of potential and of resistance during excitation, indicating a loss of selective cationpermeability, may be accompanied by an escape of electrolyte from the interior of the cell, although, besides other factors, the short duration of the breakdown (i.e., a small fraction of one second with the squid nerve) is unfavorable to proof of this assumption. However, in the experiment with the more slowly reacting *Nitella*, it has been observed that after a single stimulation the conductivity fails to return to its previous value, but remains at a higher value for some time, and that on repeated stimulation the conductivity becomes progressively higher; but that there is an immediate return to the initial value, when water is circulated along the cell. This may indicate the increase of conductivity to be due to a loss of electrolyte from the cell during excitation. This fits in with many statements concerning the increase of permeability involved in experimental or in normal changes of functional activity.[88]

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[86] K. S. Cole and H. J. Curtis, J. Gen. Physiol., **22**: 37, 1938.

[87] K. S. Cole and H. J. Curtis, J. Gen. Physiol., **22**: 649, 1939; see also Sec. 4, p. 278.

[88] See pp. 950ff; further, K. S. Cole, Cold Spring Harbor Symp., **8**: 110, 1940. Older literature in chap. 8 of R. Höber, Physikalische Chemie der Zelle und der Gewebe, 6th ed., Engelmann, Leipzig, 1926.

3. The Nature of Selective Ionpermeability of Physicochemical and of Physiological Membranes.—The concept of a selective ionpermeability of membranes, which at first introduction was considered rather fantastic, but soon was well supported by Koeppe's[89] demonstration of the anionpermeability of the erythrocytes, became a powerful stimulus to a systematic investigation of various simple model substances with respect to their properties as membranes separating different electrolyte solutions. The most important models resulting from such studies are the porous collodion membrane of Michaelis[90] and the oil-like solvent membrane of Beutner.[91]

The dried collodion membrane (see also Sec. 1, p. 70) aids in elucidating the behavior of the muscle and the nerve membranes in the following respects:

1. A wet collodion membrane with sufficiently wide pores, interposed between two solutions of the same alkali salt in different concentrations, yields a low membrane potential, which is equal to a diffusion potential. It is zero with KCl because with this salt the cationic mobility u is the same as the anionic mobility v. Drying the wet membrane raises the potential. It can approach the theoretically maximal value, which at room temperature and with a concentration ratio 10:1 of the two solutions is equal to:

$$E = \frac{RT}{F}\frac{u-v}{u+v}\ln\frac{10}{1} = 58\frac{1}{1}\log\frac{10}{1} = 58\,\text{mV}$$

This is due to v becoming zero, because by drying the porosity of the membrane is decreased in such a way that it becomes anionimpermeable and only the permeability to K persists. The membrane is "polarized" with the positive pole oriented to the lower concentration. With membranes dried only to a lesser degree other cations also can enter the membrane according to the order: Rb > K > Na > Li, whereas the anions have a minor or no effect.

2. Here the order of the cations is not the order of their atomic size, as could be expected with regard to porosity, but it is the order of their ionic mobility signifying that the atomic size is increased by hydration, in other words, by a shell of water dipoles, largest with Li and smallest with K.

3. The preferential cationpermeability depends upon the negative charge of the pore walls of the membrane, which is due to the ionizable acidic groups attached to the collodion (nitrocellulose) molecule. This charge can be converted to positive by impregnating the membrane with adsorbable organic bases, for instance, basic dyestuffs or alkaloids or pro-

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[89] H. Koeppe, Pflüger's Arch. f. d. ges. Physiol., **67**: 189, 1897.

[90] L. Michaelis, Naturwissenschaften, **14**: 33, 1926; L. Michaelis and A. Fujita, Biochem. Ztschr., **159**: 370, 1925; **161**: 47, 1925.

[91] R. Beutner, Am. J. Physiol., **31**: 343, 1912; Die Entstehung electrischer Ströme in lebenden Geweben.: Enke, Stuttgart, 1920.

tamines.[92] The result is a selective anionpermeable membrane, with the negative part of the electric double layer of the diffusion potential oriented toward the more dilute solution and with the potential height indicated by the order of the anions: $SCN > NO_3 > I > Br > Cl > SO_4$.[93] This is, in contrast to the findings with the cationpermeable membranes, not the order of ionic mobility, which would be I, Br > Cl > NO_3 > SCN. Rather it is the lyotropic series of anions (p. 293). Hence it is evident that the ability of the various ions to penetrate the collodion membranes, both the impregnated and the nonimpregnated, does not measure their diameter, including the sphere of water molecules, as at first believed to be the case with the cations, but that it is determined in some other way by hydration; namely, with cations and with anions the *penetrating ability increases* with decreasing hydration or rather *with increasing adsorption.* For, as has been mentioned earlier (p. 299), the least hydrated ions have the greatest adsorbability.[94] When a salt approaches by diffusion the surface of the cationpermeable membrane, the anions act like being repelled; they are restricted in their mobility by the charged negative groups, which are fixed immovably to the collodion. On the other hand, the cations act like attracted, with K more effectively than with Li. This is partially due to adsorption, which is greater with the less hydrated K than with Li, and partially, perhaps chiefly, due to the fact that many more pores are available to the small K than to the big Li ion. Correspondingly, with the anionpermeable membrane the less hydrated SCN is more effective than SO_4 (see p. 299). Thus, it appears that the electrochemical activity of the membranes must be correlated with both the number of ionizable groups per unit of area in the membrane and with the relative size of the movable cations and anions.[95]

4. The same interrelationship between the electrical charge of the membrane substance, the ionic diameter, and the height of the electromotive force obtains with membranes, which are more analogous to the physiological conditions than the collodion membrane, and the charge of which can easily be shifted, as observed with membranes composed of *colloid ampholytes* like protein or gelatin. These membranes are preferentially permeable to the cations on the alkaline side of their isoelectric point, to the anions on the acid side. Correspondingly, their membrane potentials are oriented in

[92] R. Mond and F. Hoffmann, Pflüger's Arch. f. d. ges. Physiol., **220**: 194, 1928; W. Wilbrandt, J. Gen. Physiol., **18**: 933, 1935; I. Abrams and K. Sollner, *ibid.*, **25**: 369, 1943.

[93] Regarding the theory of this influence of charge upon permeability see K. H. Meyer, with J. F. Sievers and H. Hauptmann, Helvet. chem. acta, **19**: 649, 665, 948, 987, 1936; and **20**: 634, 1937; T. Teorell, Proc. Nat. Acad. Sc., U.S.A., **21**: 152, 1935; Proc. Soc. Exper. Biol. & Med., **33**: 282, 1935; W. Wilbrandt, Ergebn. Physiol., **40**: 204, 1938; K. Sollner and C. W. Carr, J. Gen. Physiol., **28**: 1, 1944. Also see Sec. 1, p. 71.

[94] See H. Freundlich and A. Schnell, Ztschr. physik. Chem., **133**: 151, 1928.

[95] See K. Sollner, I. Abrams, and C. W. Carr, J. Gen. Physiol., **24**: 467, and **25**: 7, 1941; **25**: 411, 1942; **26**: 17, 1942; **28**: 119, 1944; also W. Wilbrandt, *ibid.*, **18**: 933, 1935; Ergebn. d. Physiol., **40**: 204, 1938.

directions opposite to each other on either side of the isoelectric point, and display by the potential height the order of the cations and the anions respectively. For instance, measuring the influence of the ions on the electromotive force of the system: 0.1 *N* NaCl | gelatin | 0.1 *N* NaX, the following values (mV) have been observed:[95]

at *p*H 3 with	X =	SCN	I	Cl	SO_4,
		+8.8	+3.2	+0.2	−27.7

NaX being the + pole or the − pole;
with a system: 0.1 *N* KCl | gelatin | 0.1 *N* YCl:

at *p*H 9.4 with	Y =	K	Na	Li
		0	+5.7	+13.2

YCl being the + pole.

A similar behavior is shown by natural animal membranes. In particular, the electric properties of the frog skin have been investigated for various purposes (see Sec. 8). The overall value of this membrane potential is the algebraic sum of at least three separate potentials.[97] One, the skin potential *sensu strictiori*, which is located in the exterior layer of this complex structure and which is displayed, if a freshly excised piece of the skin is bathed on both surfaces by Ringer's solution, is somehow connected with the life state of the epithelia. A second potential is an ordinary diffusion potential, which is brought to evidence if the skin is placed between salt solutions of different concentrations; it probably is bound up to the deeper layers of the skin, the fibrous connective tissue, including blood vessels, smooth muscles, and nerves. The third potential is a membrane potential, which is left after the skin has been killed (e.g., by an acid at about *p*H4). It is signified as such by the fact that its direction can be changed by changing the *p*H, similar to the behavior of the aforementioned colloid-ampholyte membranes; above *p*H4.5 the dead skin is preferentially permeable to cations, below to anions. For this reason, the membrane in question is assumed to consist of a protein located in the tighter exterior layer and having an isoelectric point around 4.5; and, indeed, thus *p*H coincides fairly well with the reversal point for electroosmosis across the skin.

Returning now to Bernstein's membrane theory (p. 315), its main fundament was considered the great surplus of K inside the *muscle fiber*, the inside concentration in frog muscle being about 20 to 40 times greater than the outside concentration.[98] Most of the K is supposed to be free. How much is bound, is so far unknown.[99] It has been mentioned before

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[96] T. Matsuo, Pflüger's Arch. f. d. ges. Physiol., **200**: 132, 1923; R. Mond, *ibid.*, **203**: 247, 1924; A. Fujita (L. Michaelis), Biochem. Ztschr., **152**: 245, 1925; see, further, A. Bethe and T. Toropoff, Ztschr. f. physik. Chem., **88**: 686, 1914, and **89**: 597, 1915.

[97] W. R. Amberson and H. Klein, J. Gen. Physiol., **11**: 823, 1928. M. Sumwalt, W. R. Amberson, and E. Michaelis, J. Cell. & Comp. Physiol., **4**: 49, 1933; M. Sumwalt, Biol. Bull., **64**: 14, 1933.

[98] W. O. Fenn, Physiol. Rev., **16**: 450, 1936; **20**: 377, 1940.

[99] See Sec. 7.

that muscle activity always is accompanied by loss of K to the blood (p. 257) and that K can move from the blood into the muscle against the concentration gradient[100] in agreement with the expectations of a membrane equilibrium (see Sec. 4, p. 254 and Sec. 8, introduction). Thus, a membrane potential dependent upon a selective K permeability can be anticipated. According to chemical analyses of muscle, the fibers appear to be impermeable to Na and to anions (see Sec. 4, pp. 253ff.). With 23 mg. per cent K in the outside Ringer and about 400 mg. per cent inside the fibers, after destruction of one end of the muscle (sartorius) the height of the injury potential could be expected to be

$$E = 59 \log {}^{400}/_{23} = 73 \text{ mV (at } 22°).$$

But in reality it has not been found higher than an average of 41.6 mV.[101] Increasing K_o causes E to fall off—as it should—toward zero. It should even be reversed, if $K_o > K_i$. This postulate of the theory was not complied with in earlier experiments,[102] evidently because the muscle had been soaked or perfused for some time by isotonic KCl and was killed by this treatment. However, the postulate has been fulfilled in recent experiments on the giant squid nerve fibers, where with half or full isotonic KCl a reversal of the potential difference of 15 mV appeared.[103]

The difference between 73 and 41.6 mV is more or less understandable, taking into consideration the following situation: 1. The interfibral fluid and the tissue space between the fiber bundles form short circuits; 2. The various anions do not fail entirely to influence the height of the potential (p. 313) and can be assumed to penetrate the muscle membrane to an individually different degree; 3. The K concentration between the fibers inside the whole muscle may easily be somewhat higher than in the bathing solution, due to slight damage or to twitching; 4. On the other hand, a certain and probably variable fraction of K_i is not free, but bound; 5. Slight up and down effects of anions upon the potential were interpreted earlier as indicating changes in the colloidal state of the membrane components. This was especially obvious from the fact that the most influential anions, SCN and I, provoke at the beginning of their application a positive pole which afterward reverses to a negative pole (see p. 313; see, further, reference No. 74).

By all this, evidence is afforded that a rigid collodion-like porous membrane serving for selective ionpermeability is not adequate to portray the physiological conditions, and that a more flexible colloidal structure is more satisfactory as a model of the muscle membrane. Moreover, the rigid collodion membrane cannot be expected to show the balancing effect of mono- and divalent cations.

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[100] W. O. Fenn, Am. J. Physiol., **112**: 41, 1935; Physiol. Rev., **20**: 377, 1940; W. O. Fenn and D. M. Cobb, Am. J. Physiol., **115**: 345, 1936.

[101] A. H. Hegnauer, W. O. Fenn and D. M. Cobb, J. Cell. & Comp. Physiol., **4**: 505, 1934.

[102] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **106**: 599, 1905.

[103] H. J. Curtis and K. S. Cole, Am. J. Physiol., **133**: 254, 1941; further, S. L. Cowan, Proc Roy. Soc., **B. 115**: 216, 1934; D. A. Webb & J. Z. Young, J. Physiol., **98**: 299, 1940.

Regarding *nerves*, it has already been mentioned (p. 314) that the character of their resting potential resembles that of muscles. It should be added here, as significant for the colloidal properties of their membrane, that the depressant influence of K and Rb upon the potential can be antagonized by Ca.[105]

Moreover, nerves have furnished specimens, which appear to be exceedingly favorable objects for more crucial tests of Bernstein's membrane theory (see p. 315), the excised giant axons of the squid. These cylindrical fibers are large enough to yield at their cut ends enough axoplasm for determining the inorganic and organic constituents (about the results, see p. 257). In studies on the excitation wave of these objects by Webb and Young,[106] $K_i : K_o$ was found to be 29. Assuming that during rest the membrane separating the axoplasm and sea water be permeable only to K, and that during the passage of an excitation wave the membrane be rendered permeable to anions, the calculated value is about 85 mV. The observed value (average of 7 experiments) was 77 mV. In corresponding experiments, Hodgkin and Huxley[107] have obtained values up to 95 mV. With one lead from the intact surface and the second from the injured end, a resting potential of 50 mV has been measured, this lower value probably being due in part to the liquid junction potential between the axoplasm and the fluid surrounding the injured end. Finally, about the same average value (51 mV) has been observed with an "impaled" giant fiber, i.e., with one capillary fluid electrode piercing the axon membrane and pushing inside along the axis, until its tip is opposite to the tip of the second electrode outside the fiber.[108] Under such conditions the impaled axons often remain excitable for many hours. It is obvious that the various, and partially contradictory, results of this group of experiments need further study.

However, in any case it must be realized that there is a considerable number of observations indicating that, at variance to the symptoms of impermeability to ions or also to signs of a slight passive penetration, there is evidence of an ample, though slow and probably active, passage being superimposed and detectable mainly by chemical instead of physical means of analysis. A striking example concerning muscle, which has been mentioned before (p. 257), is the result of feeding rats a K-poor diet for several weeks.[109] The K-deprived rats show a loss of about 50% of K in their muscles and a concomitant rise in Na. The K remaining in the depleted animal is located practically entirely inside the fibers, and Na has passed into the fibers to such an extent that there is more intracellular than extracellular

[105] R. Höber and H. Strohe, Pflüger's Arch. f. d. ges. Physiol., **222**: 71, 1929; S. L. Cowan, Proc. Roy. Soc. **B. 115**: 216, 1934.

[106] D. A. Webb and J. Z. Young, J. Physiol., **98**: 299; 1940.

[107] A. L. Hodgkin and A. T. Huxley, Nature, London, **144**: 710, 1939; further, H. J. Curtis and K. S. Cole, J. Cell. & Comp. Physiol., **19**: 135, 1942.

[108] H. J. Curtis and K. S. Cole, Am. J. Physiol., **133**: p. 254, 1940; J. Cell. & Comp. Physiol., **15**: 147, 1940.

[109] L. A. Heppel, Am. J. Physiol., **127**: 385, 1939.

Na, in some cases even twice as much. This probably must be accounted for as the effect, not of a passive penetration, but of an active transport, compensating for the loss of K during the long period of deprivation. Such a process, which really is a shift of Na against a diffusion gradient, is not unlikely on the basis of many similar observations with various plant cells, with kidney and liver, where the shift of cations and of anions is bound up with metabolic reactions which supply the necessary osmotic energy (see Sec. 8.). That single animal cells also make use of such a mechanism, has been demonstrated with human erythrocytes,[110] which for a long time were assumed to be impermeable to cations under "normal" conditions, but which are now known to show an exchange of K and Na when kept for many hours at body temperature in the presence of glucose. Accompanied by utilization of the sugar, K_i increases, Na_i decreases. Moreover K, which previously was lost, re-enters the cell after addition of glucose. In contrast, at low temperature which stops the metabolic reactions, both K and Na diffuse with the concentration gradient. It often has been conceived a universal phenomenon that cells have to expend energy, even at rest, in order to preserve the dynamic state, which is characteristic of life. Also, the normal polarized state of frog and crab nerve, as indicated by the constancy of their resting potential, cannot be kept on its high level without expenditure of energy. It falls off during anoxia, and rises anew after return to oxygen.[111] These effects upon the resting potential seem to be due to the appearance of lactate and pyruvate in the normal glycolytic cycle of muscle and nerve tissue and to the oxidation of these intermediary products.[112] Only with this cycle intact and with oxygen being present can the membranes keep their normal molecular organization, upon which depends the normal polarized state of rest.[113] Attending failure in this regard, K, along with some anions, escapes, as it may escape also during excitation. This is quite to be expected, since it has been shown definitely, mainly due to the aforementioned observations of Cole and Curtis (p. 316), that the electrical resistance of the surface membrane falls off considerably when the action potential wave travels along a *Nitella* cell or along the giant axon of the squid. Oxygen may then serve not only to rebuild the membrane, but also to cause backtransport of lost contents. However, notwithstanding the high concentration gradient of K from inside to outside, it has often been denied that the decrease of the polarization is associated with a release of K. It is true, muscle activity is always accompanied by the loss

[110] J. E. Harris, J. Biol. Chem., **141**: 579, 1941; W. Wilbrandt, Pflüger's Arch. f. d. ges. Physiol., **243**: 519, 1940; see further: H. Davson, J. Cell. & Comp. Physiol., **15**: 317, 1940; Cold Spring Harbor Symp., **8**: 215, 1940; also Sec. 4, p. 251.

[111] K. Furusawa, J. Physiol., **57**: 325, 1929; R. W. Gerard, Am. J. Physiol., **92**: 498, 1929.

[112] T. P. Feng, J. Physiol., **76**: 477, 1932; A. M. Shanes and D. E. S. Brown, J. Cell. & Comp. Physiol., **19**: 1, 1942; further, **19**: 249, 1942; see also Sec. 8, chap. 38.

[113] About the influence of ions upon respiration as referred to the colloidal structure of the nerve membranes, see T. H. Chang, M. Shaffer and R. W. Gerard, Am. J. Physiol., **111**: 581, 1935.

of K, but this can be an immediate consequence of the contraction process (see p. 257). In nerves there is no conclusive evidence for a release of K either on stimulation or on oxygen lack.[114] However, it must be realized that the chances of obtaining such evidence are rather poor. In myelinated frog nerves the loss probably is restricted to the small area of the Ranvier nods, and recent measurements of the changes of the impedance and of the capacity during excitation have yielded the surprising result that in the giant axon of the squid only 2% of the area of the membrane, in *Nitella* 15%, are involved in the increase of permeability.[115] If, then, during the short duration of an excitation wave, electrolytes should escape, the small amount could evade demonstration, provided the loss was repaired immediately by a backtransport similar to a secretory active transfer. The best chance for a demonstration of such escape might be offered by an object, the excitation reaction of which travels with low speed, and the local depolarization of which is correspondingly of a relatively long duration, as it is in *Nitella*, where, in an experiment referred to before (p. 316), there has been found, indeed, some indication of leakage.[116]

In conclusion, some earlier observations of J. Loeb, Osterhout, and others, correlating colloidal and electrical properties of certain animal and plant membranes, may be discussed. According to Loeb, the *eggs of the brackwater teleost Fundulus* can be kept living in sea water as well as in pure water. When an egg is put into KCl solution, the heart of the embryo, which is covered by a thick egg membrane, the chorion, stops beating, after the salt diffusing across the chorion and the ectoderm of the embryo has reached a certain concentration. Loeb and Cattell[117] have found that the cessation of the heart-beat persists over many hours in distilled water as well as in isotonic saccharose solution, or, more generally, in absence of ions, while the pulsation reappears readily after adding a nonpoisonous salt to the surrounding fluid, the rate of recovery increasing with the salt concentration. Also low concentrations of acid have a recuperating effect.[118]

The surprising influence of the lack of ions seemed to have found a satisfactory explanation by Sumwalt,[119] who ascribes this effect to the resemblance of the chorion to a dried collodion membrane. According to Michaelis, KCl cannot go across this membrane into distilled water because it is impermeable to Cl, but after NaCl has been added to the water, movement of K occurs by exchange of Na. This has been proved to be true mainly by measuring the membrane potentials. The same procedure has been applied by Sumwalt to the chorion of the *Fundulus* egg. One microelectrode was fixed in the sub-

[114] W. O. Fenn, J. Neurophysiol., **1**: 1, 1938; S. L. Cowan, Proc. Roy. Soc., London, **B. 115**: 216, 1934.

[115] K. S. Cole and H. J. Curtis, J. Gen. Physiol., **22**: 37, 1938; **22**: 469, 1939.

[116] Only after an exceedingly prolonged stimulation (60 stimuli per second for one to three hours) the excised myelinated frog nerve has been found to release considerable amounts of K (V. Arnett and W. S. Wilde, J. Neurophysiol., **4**: 572, 1941).

[117] J. Loeb and McKeen Cattell, J. Biol. Chem., **23**: 41, 1915.

[118] Concerning this effect see also M. Sumwalt, Biol. Bull., **54**: 114, 1933.

[119] M. Sumwalt, Biol. Bull., **56**: 193, 1929.

chorionic space filled with m/2 KCl, another electrode was placed in solutions of varied composition outside the egg, and the potentials (during less than a half-hour) were measured across the chorion. It was found that, with K outside at various concentrations, the more dilute solution is positive; that with different anions (Cl, Br, I, NO_3, SCN, acetate) the potentials are equal; and that, varying the alkali cations (in m/2000 solutions), Li solution is the most positive, Cs solution the most negative. By these results it was made evident that the chorion membrane is selectively permeable to cations, or, more correct, is more permeable to cations than to anions.

However, these measurements do not account for some especially significant observations of Loeb and Cattell. It was mentioned before that the heart of the embryo, which had been stopped by KCl, starts beating again in salt solutions of a certain strength. Varying the anions of Na-salts, it appeared that the recovering power is greatest with citrate and falls off in the order: citrate > SO_4 > tartrate > acetate, I > Br > Cl, NO_3. This is unexpected, mainly for two reasons. First, it is at variance with the aforementioned indifference of the anions toward the chorion potentials. Second, the order of anions reminds one of the Hofmeister series, but, disregarding some irregularities, the succession of ions runs inversely to the usual order (p. 302). The following points will contribute to a clearer understanding. First, the indifference of the anions, as stated in the potential measurements, may be due to the brief period of the time of observation, during which the penetration of the cations was predominant. Second, in order to influence the heart-beat, not only the chorion, but also the ectoderm and underlying tissue of the embryo, must be penetrated. Third, especially citrate, but tartrate and phosphate also display their recovering power for a short interval only, which is followed by poisoning.[120]

These experiments of Loeb and Cattell can be compared to some extent with experiments of Raber[121] on the thallus of the *marine alga Laminaria*, studied by the method of Osterhout.[122] One to two hundred discs of the foliage were piled up to form a cylinder and soaked in salt solutions, replacing the normal milieu, sea water. Conductivity measurements served as indicating the ionpermeability of the cells composing the plant tissue. With different Na salts the conductivity rose fairly rapidly with SCN, I, Br, Cl, NO_3, faster with acetate, and fastest with SO_4, tartrate, phosphate, citrate. This behavior reminds one of the inverted Hofmeister series as much or as little as in the experiments of Loeb and Cattell. However, the significance of the experiments is considerably reduced by the fact that the half-molar pure Na salt solutions can be assumed to impair the life of the cells (p. 245) as is evident regarding citrate, phosphate, and tartrate. Furthermore, the subject of this study is a tissue composed of several structures, the cells, the cell walls, and the intercellular substance, each of which offers a specific resistance to the current, contributes to the conduction according to its relative volume, and possesses unknown specific colloidal properties.

Finally, attention also must be turned to the fact that even colloidal material, which is simpler than the double membrane of the *Fundulus* egg or the composite architecture of *Laminaria*, displays the phenomenon of inversion of the

[120] See, further, J. Loeb, J. Biol. Chem., **27**: 339, 353, 363, 1916; **28**: 175, 1916.

[121] O. L. Raber, J. Gen. Physiol., **2**: 535, 1920.

[122] W. J. V. Osterhout, Science, **35**: 112, 1912.

Hofmeister series of anions, for instance, under the influence of higher salt concentrations or after shifting the *p*H in one or the other direction (see pp. 300ff).

Hence, it is obvious enough that a more thorough investigation of these different processes is needed, before they may be quoted as counter-evidence against an interpretation of physiological states or processes, as analyzed in this chapter, on the basis of colloid chemistry.

18

PHYSIOLOGICAL SIGNIFICANCE OF THE ELECTROKINETIC OR ζ-POTENTIALS[123]

The basic principle of the bioelectric membrane potentials can be illustrated by referring to the electromotive force ϵ of a Helmholtz concentration chain of the following type: c_1 $AgNO_3$ | Ag | c_2 $AgNO_3$, where the electric current is led off from c_1 and c_2 by identical fluid electrodes suitably constructed to avoid the establishment of liquid junction potentials at the contact with c_1 and c_2 (Sec. 1, p. 64). Then, if $c_1 > c_2$, $\epsilon = \epsilon_2 - \epsilon_1 = k \log \frac{C'}{c_2} - k \log \frac{C'}{c_1}$, corresponding to the two potentials at either surface of the silver diaphragm, which separates c_1 and c_2. These potentials differ according to the different tendencies of Ag ions to enter into the metallic silver from c_1 and to pass out of it into c_2. It appears that the diaphragm is like a membrane selectively permeable to cations, for instance, like the surface membrane of a giant nerve fiber in contact on one side with high K, on the other with low K (p. 321). It is then conceivable that Nernst's thermodynamic theory of concentration chains (see pp. 63 and 317) likewise applies to the behavior of the physiological structures. But conditions here are generally more or less complicated by the thermodynamic ϵ-potentials being superimposed upon electrokinetic ζ-potentials, which similarly are located at the interface between the membrane material and the adjacent solutions, and are of special interest because of the characteristic sieve-like structure of the physiological membranes, and their great porosity providing a large internal surface for adsorption. But, as outlined in Sec. 1, pp. 83ff. the ζ-potential differs from the ϵ-potential in that its site is not exactly at the phase boundary, but somewhat distant between a thin layer of the solution, which is fixed by adhesive forces to the membrane material and thus quasi-rigid, and the free solution. This interface has been called the "rigidity boundary" or the "surface of shear." Here a "diffuse double layer" of ions[124] is formed, the orientation of which is determined by the electrical charge of the membrane material, which—for one or another reason—is

[123] H. Freundlich, Colloid and Capillary Chemistry: E. P. Dutton and Co., New York, 1926; R. A. Gortner, Outlines of Biochemistry; 2nd ed., J. Wiley, New York, 1938, Chapter VI; R. Höber, Physikal. Chemie der Zelle u. Gewebe: W. Engelmann, Leipzig, 6th ed., 1926, chapters 4 and 12; H. A. Abramson, Electrokinetic Phenomena and Their Application to Biology and Medicine: Chemical Catalog Co., New York, 1934.

[124] A. Gouy, J. de Phys., (4) 9: 457, 1910.

either positive or negative. If an electric field is applied tangential to the internal pore walls of a membrane, which is interposed as a diaphragm into an electrolyte solution (that is, if an external electromotive force is applied to the ends of the capillary pores), then, due to the presence of an excess of ions of one kind in the movable boundary layer, the latter is moved, taking with it the free solution within the core of the pores ("electroosmosis"). According to the formula p. 85, from the rate of the fluid movement u, as brought about by the unit electromotive force (cm./sec. per volt/cm.) the electrokinetic or ζ-potential can be calculated. This potential is somewhat similar to a liquid junction potential (p. 86), but, in contrast to this, is not indicative of a dynamic process like a diffusion, but is a certain small part of the static ϵ-potential of membranes. It is clear that the concentration potential differences measured across a porous membrane must differ parallel with the ζ-potential of the membrane, the mobility of the free cations, while passing a membrane with positive charge, being decreased, as well as in a negative membrane the free anions likewise are decreased.[125] This is particularly evident in experiments with membranes, whose electrical charge is readily changed by the attachment of ions, as observed, for instance, with colloid-ampholytes (proteins, gelatin) by changing the *p*H, as well as with more indifferent substances like collodion or agar, when they have been coated with a film of these colloids. Examples of this influence have been quoted previously (p. 318). Referring to the numerous aforementioned membrane potentials of cellular origin, it follows that all the alterations of their height effected by inorganic salts, or, rather, by the ions, are indicative of adsorption, which modifies the relative mobility of the ions in passing through the charged membranes.

1. Electrophoresis of Cells.—Further information concerning the physiological significance of the electrokinetic potentials is obtained by studying electrophoresis of single cells.[125] According to the formula (p. 85), their ζ-potentials can be calculated from measurements of the velocities of suspended cells moving in an external field. Erythrocytes have been the principal material used. Their surface can be assumed to be the site of an ϵ-potential, since the surface is selectively anionpermeable and since the distribution of ions has been shown to be a Donnan distribution. The ϵ-potential should be determined directly by inserting a microelectrode into a red cell, as previously described for *Nitella* and for giant nerve fibers (p. 321), and taking the second lead from the outside. But such a "transverse" measurement has not been carried out as yet. As a matter of fact, the "tangential" measurement of the ζ-potential shows that this fails to be altered by disintegrating the surface membrane of the erythrocytes; in other words, by hemolysis. The intact red cells and their stromata (ghosts),

[125] A. Bethe and T. Toropoff, Ztschr. f. physik. Chem., **88**: 686, 1914; **89**: 597, 1915. See also p. 318.

[125] See H. A. Abramson, Electrokinetic Phenomena: Chemical Catalog Co., New York, 1934; H. A. Abramson, L. S. Moyer and M. H. Gorin, Electrophoresis of Proteins: Reinhold, New York, 1942. See, further, H. Müller, Cold Spring Harbor Symp., 1, 1933.

suspended in the same medium, and under otherwise identical conditions, have the same mobility.[127]

The ζ-potentials of various suspended particles, either inorganic or organic, like pieces of glass, quartz, collodion, starch, cotton, and droplets of mineral oil, are characteristically different. Most of the particles move toward the cathode; some, e.g., ferric oxide, toward the anode. Size, shape, and orientation of the particles have no influence upon their potential.[128] The differences of direction and velocity of migration disappear in the presence of colloid-ampholytes like gelatin, when coating at the same *p*H the various surfaces. The particles then behave like particles of gelatin.[129] Accordingly, e.g., when glass is suspended in acetate buffer at *p*H 5.6 and coated by globin (is. p. at *p*H 6.6) or serum globulin (is. p. 5.6) or serum albumin (is. p. 4.9), the ζ-potential is found to be positive, zero, and negative, respectively.

The behavior of the erythrocytes is somewhat different. Again, shape and size are irrelevant—single corpuscles, masses held together by agglutination or by rouleaux formation, and ghosts migrate with the same speed. On the other hand, rinsing the cells, which could be supposed to be normally surrounded by an adsorption layer of plasma proteins, with phosphate buffer (*p*H 7.4) has practically no effect upon the mobility. Addition of gelatin to this suspension likewise fails to bring about a distinct change of the potential, whereas it does with suspensions of quartz or cholesterol.[130] This seems rather incompatible with the idea of the ζ-potential of the red cells being due to adsorbed protein. Moreover, *p*H changes have been found to have little effect upon the potential,[131] and this may explain also the rather unexpectedly low value of the isoelectric point, which with sheep and with human cells is found at about *p*H 3.6, provided the cells remain unhemolyzed.[132] Apparently, then, the ζ-potential of the erythrocytes should be referred to their surface proper, and the rather great differences of the electrokinetic potentials found with various animals may be associated with the chemically and physicochemically species-specific surface structures. But this interpretation has not been proved as yet. The following table[133] shows the mean velocity of mammalian erythrocytes $v = \mu$/sec. per volt per cm:

rabbit	<	sloth,	pig	<	opossum	<	guinea pig	<	man	
(0.55)		(0.97)	(0.98)		(1.07)		(1.11)		(1.31)	
		mouse,		cat	<	rat	<	dog		
		(140)		(1.39)		(1.45)		(1.65)		

[127] H. A. Abramson, J. Gen. Physiol., **12**: 711, 1929.
[128] H. A. Abramson and L. Michaelis, J. Gen. Physiol., **12**: 587, 1929.
[129] H. Freundlich and H. A. Abramson, Ztschr. f. physik. Chem., **128**: 25, 1928.
[130] H. A. Abramson, J. Gen. Physiol., **12**: 711, 1929.
[131] H. Netter, Pflüger's Arch. f. d. ges. Physiol., **208**: 16, 1925.
[132] H. A. Abramson, J. Gen. Physiol., **14**: 163, 1930.
[133] H. A. Abramson, J. Gen. Physiol., **12**: 711, 1929.

2. Electroosmosis Across Membranes.—Another kinetic method for determining the ζ-potential of physiological objects is based upon observation of electroosmosis (see p. 328). Serous membranes (mesentery, peritoneum, pericardium) and skin, best placed as diaphragms in buffered solutions, are subjected to a direct electric current. The rate of fluid shift, in contrast to the rate of electrophoresis of red cells, is easily changed by changes of *p*H in one or the other direction, and is zero at a definite *p*H indicating the isoelectric point of the membrane material. This has been found with mammalian membranes to be at about 4.9,[134] probably referable to the type of constituent protein (see p. 319).[135]

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[134] St. Mudd, J. Gen. Physiol., **7**: 389, 1925; **9**: 73, 361, 1926.

[135] Concerning electrokinetic reactions of the human skin, see H. A. Abramson and M. H. Gorin, Cold Spring Harbor Symp., **8**: 272, 1940. About anomalous osmosis involving driving forces of an electrokinetic nature, see Sec. 8, chap. 38.

19

THE OIL-LIKE MEMBRANES AS THE SEAT OF THERMODYNAMIC POTENTIALS

So far the bioelectric potentials of muscle and nerve have been conceived as originating from the presence of sieve-like surface membranes, which are interposed between the intracellular and extracellular electrolyte solutions. However, more than 20 years ago, another concept was proposed, which in many respects is rivaling the former one. This is the concept of solvent-like membranes as envelopes for the protoplasm. Both theories have been proposed and outlined in close contact with the contemporary development of our knowledge of cell permeability, emphasizing for some time the importance of lipoid solubility, and, later, the factor of the molecular volume of the permeating substances. It seems desirable to refer, at least briefly, to the main points of the solvent theory which was built up chiefly by Beutner,[136] who claimed that "oil-like" organic solvents are essential structural elements of the surface of the cells and are the seat of their potentials.

In general, organic liquids which are immiscible with water, are poor solvents for strong inorganic electrolytes, since their low dielectric constant prevents an appreciable dissociation into free ions. This holds true mainly for the solvents of a chemically neutral character, like the neutral hydrocarbon oils. The situation is different in the case of organic solvents having some acid or basic properties, either by their chemical nature, or by impurities, or by additions, such as phenol, guaiacol, salicylic aldehyde, and toluidene, aniline, or solvents like Nirenstein's mixtures (chaps. 10 and 13). They behave as weak electrolytes, and can dissolve small amounts of strong electrolytes chiefly by ionic exchange. Through conductivity measurements, it can be shown that the inorganic alkali salts, upon shaking their aqueous solutions with solvents like guaiacol or benzaldehyde, pass into these liquids in characteristically different amounts.

Now, when two aqueous solutions of the same electrolyte at different concentrations are placed on either side of an intermediate layer of an organic fluid, an electromotive force can be demonstrated, which, e.g., with salicylic aldehyde or with guaiacol in addition to certain amounts of oleic acid as membrane, and with a concentration ratio of KCl 1:100, is equal to about

[136] R. Beutner, Die Entstehung electrischer Ströme in lebenden Geweben: Enke, Stuttgart, 1920.

100 mV, the positive pole being on the side of the lower concentration. If the acidic "oil" is replaced by a basic one (toluidene or aniline), the sign of the poles is reversed. This is like reversing the charge of an ordinary collodion membrane by impregnating it with a basic dyestuff or an alkaloid (p. 317).

The following explanation of the functioning of the "oil-chains" has been proposed. Nernst[137] and Cremer[138] have outlined the theory that at the boundary of two phases, one an electrolyte solution and the other a water immiscible liquid solvent, a phase boundary potential is set up, owing to the individual distribution tendencies of each kind of ion. It follows that in any liquid chain consisting of a solvent membrane, interposed between two different electrolyte solutions, two different phase boundary potentials must be present, the difference between which is a measure of the different tendencies of cations and anions to enter through the phase boundary into the oil phase. Taking as an example the aforementioned concentration chain with the two aqueous solutions containing the same electrolyte at different concentrations, the following explanation can be proposed according to Beutner. At the interface between a KCl solution and salicylic aldehyde, K enters the organic solvent at a higher rate than Cl, because it can be exchanged for H present in the aldehyde in a definite concentration. In the concentration chain, C_1 KCl | salicylic aldehyde | C_2 KCl, with $C_1 < C_2$, in spite of the concentration difference between C_1 and C_2 at either of the phase boundaries, an equal amount of K, dependent on the limited small content of acid within the organic solvent, enters from both sides. Therefore the chain functions according to the following scheme: c_1 K in water | c K in salicylic aldehyde | c_2 K in water, the potential difference being expressed by the Nernst formula $E = 58 \log \frac{c_2}{c_1}$ mV. It is evident that in this case the solvent membrane behaves like a selective cationpermeable membrane. Correspondingly, basic solvents form anion-permeable membranes.

But a quantitative agreement between theory and experiment, as suggested by the formula, cannot be expected because of the just-mentioned interreaction between the electrolyte solutions and the organic solvent. This is evident from careful measurements by Osterhout[139] on a model with guaiacol representing the protoplast of one of the giant plant cells (chap. 21), interposed between K guaiacolate solutions of different concentration which are in equilibrium with the interphase. Physicochemical and chemical analyses of the interphase show that concentration differences inside this interphase, undeniably involving diffusion potentials within it, cannot be neglected.

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[137] W. Nernst, Ztschr. f. Physik. Chem., **9**: 140, 1892.

[138] M. Cremer, Ztschr. f. Biol., **47**: 562, 1906.

[139] W. J. V. Osterhout, J. Gen. Physiol., **25**: 293, 1943; further, Cold Spring Harbor Symp., **8**: 51, 1940.

When different sodium salts are compared, the values of these potentials follow each other in the well-known order of the lyotropic series (p. 293) of anions: $SO_4 < Cl < NO_3$, $I < SCN$, with SCN giving the strongest, SO_4 the weakest, positive pole, irrespective of the oil phase having an acidic or a basic character. This possibly is due to the fact that, in the series of anions, hydration decreases from SO_4 to SCN, and that, therefore, SCN has not only the highest adsorption affinity, but also the highest solubility in the hydrophobic organic solvents (see pp. 299 and 318).

In addition, if the influence of the series of anions and cations upon the potential differences of the oil chains is compared, it appears that in general the cations have the stronger effect in chains with acidic solvents (like salicylic aldehyde or guaiacol), the anions in chains with basic solvents (like toluidene). Therefore, with a basic oil, SCN produces a stronger positive pole than SO_4, as in the case of acid gelatin; while with an acidic oil, Cs calls forth a stronger negative pole than Li does, as with a membrane of alkaline gelatin (see p. 319). Further, the influence of a neutral solvent like olive oil is small, but can be enhanced, for instance, by the addition of oleic acid or of lecithin.[140] Salts with one organic ion often reveal an especially strong effect, either on the positive or on the negative direction of the current, depending upon whether the anion or the cation is organic.[141]

In conclusion, it might be considered desirable to refer briefly to the *surface films* as another kind of models in addition to the porous and the solvent membranes for illustration of the origin of the various bioelectrical potentials. However, this seems premature, since the difficulties of spreading these fragile films between two aqueous solutions, instead of between water and air or water and oil, in order to study the influence of organic (or inorganic) ions under conditions similar to those in living material, have not been overcome as yet.[142]

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[140] J. Loeb and R. Beutner, Bioch. Ztschr., **51**: 288, 1913.

[141] See also R. Beutner, J. Pharmacol. & Exper. Therap., **32**: 101, 1927–28. See, further, the effect of *p*H upon the electrical properties of the membranes, especially with regard to the influences of metabolism, Sec. 8, chap. 38, subchap. 4.

[142] See Sec. 2, chap. 9, subchap. 4 and Sec. 4, chap. 15; J. F. Danielli and E. N. Harvey, J. Cell. & Comp. Physiol., **6**: 483, 1935; J. F. Danielli and H. Davson, *ibid.*, **6**: 495, 1935; J. F. Danielli, *ibid.*, **7**: 393, 1936. About the general properties of films and conditions of their formation, see Sec. 2, chap. 9; see also D. E. Goldman, J. Gen. Physiol., **22**: 37, 1943.

20

THE INFLUENCE OF NONPOLAR-POLAR ORGANIC IONS

In general, the inorganic ions have been found to influence vital reactions according to their adsorption at the interface between water and the physiological object. In other words, the hydrotropic series of anions, $SCN > J\ NO_3 > Br > Cl > SO_4$ and of cations, $K > Na > Li$, which refers not only to organic colloids, but also to cells and tissues, is a series of decreasing adsorption (increasing hydration) or—as one may also say in terms of the theory of Beutner—a series of decreasing distribution between an aqueous and an oil-like phase. In investigating the properties of the cationpermeable collodion membrane, this interrelation first became evident, when, in the experiments of Michaelis, strychnine salt, and even more pilocarpin salt, separated by the membrane from Na salt, gave a positive potential[143] at variance with the rule that the selective permeation of cations decreases with increasing diameter (p. 299). Correspondingly, the permeability of the selective anionpermeable collodion membrane (p. 317) was found to be greatest with SCN, which by no means is the smallest ion in the anion series (p. 318). Later, other alkaloids and dyestuff bases were shown to behave like strychnine.[144] Similarly, various organic anions with relatively large diameters, benzoate, salicylate, aromatic sulfonates, appeared to produce a greater negative potential than Cl.[145] Finally, testing the series of dialkylamine ions, the positive potential was found to decrease from the methyl to the propyl, corresponding to the rising diameter, but to increase considerably from the butyl to the amyl compound, according to the rising surface affinity.[146] All these and other facts can be explained as indicating adsorption to be a factor favoring penetration. This possibly can be accounted for by assuming that the special adsorption affinities alter the homogeneous distribution of the penetrating ions inside the pores in such a way as to increase the rate of passage near the wall. On the other hand, in the absence of special affinities between the solutes and the pore wall—apart from the electric charge of the membrane substance, which in an ordinary collodion membrane repels the anions and

[143] L. Michaelis and A. Fujita, Biochem. Ztschr., 51: 47, 1925.

[144] W. Wilbrandt, J. Gen. Physiol., 18: 933, 1935.

[145] R. Höber, J. Cell. & Comp. Physiol., 7: 367, 1936. See, further, Sec. 4, chap. 11, pp. 245ff.

[146] W. Wilbrandt, J. Gen. Physiol., 20: 519, 1937.

attracts the cations—the solutes would pass the channels in the sieve membrane as in normal diffusion.[145]

Turning now from the rigid collodion membrane to the more flexible physiological membranes, which are composed of various colloids, adsorption may, again, be found effective because, in general, surface active substances, whether electrolytes (of greater or smaller ionization strength) or nonelectrolytes, are lipoid-soluble, and as such can migrate through the lipoid components of these membranes (see chap. 11–13). But, in addition to the influence of the molecular size and of the ordinary adsorption, there can be involved in the penetration of the membranes the dual adsorption affinities of a special class of organic ions, representing, as such, new tools for studying the nature of cell permeability.

Neuberg[147] has called attention to the fact that many water-insoluble or nearly insoluble substances of diverse nature (higher alcohols, aniline, nitrobenzene, oils, Ca-soaps, proteins, starch) become soluble in the aqueous solution of the alkali salts of many organic acids, if the latter are present in high concentration ("solubilization"). Such "hydrotropic" properties are displayed mainly by aromatic carboxylic and sulfuric acids, as well as by higher fatty acids and higher alkylsulfuric acids. Most of the salts are fairly strong electrolytes. All these hydrotropic compounds are related to each other by a certain configuration of their molecules, one molecular half being polar hydrophilic, the other nonpolar organophilic-hydrophobic. Their hydrotropic properties are based upon the adsorption affinity of the organic half, which attaches the molecule to the water-insoluble hydrophobic particles, and upon the affinity of the polar "inorganic" half for the water dipoles (Langmuir-Harkins). If the pull toward the water is strong enough, the insoluble particles can be torn to pieces and thus be dispersed. But, depending upon the greater or lesser firmness of their structure, following the attachment of the organophilic half, the hydrophilic half, with its cloud of water dipoles, can be pulled inside the particles, with the result that an imbibition of water, or even swelling of the particles, occurs.

Corresponding to this theory, coarse suspensions of proteins, of starch, of lipoids are emulsified and dispersed by adding nonpolar-polar ions, while with ions prevailingly hydrophilic, owing to a multitude of polar groups (—OH, —COO) (like citrate, tartrate, succinate, and other plurihydroxy- and pluricarboxylic anions), dehydration and decrease of dispersity is the result.[148] According to this concept, one can expect and really observe that, depending upon the balance between the opposite molecular forces of the added ions, their organophilic half may adhere to a nonpolar-polar structure, e.g., a plasma membrane, with such strength as to produce an irreversible alteration (see p. 338), or that the hydrophilic radicals may be so dominant that the weaker organophilic fail to exhibit their influence (Sec. 8, chap. 35*d*

[147] C. Neuberg, Biochem. Ztschr., **75**: 107, 1916.

[148] I. R. Katz, Biochem. Ztschr., **257**, **259**, **261**, **262**, **263**, **271**, 1933–34; A. von Kuthy, *ibid.*, **237**, **243**, 1931; R. Höber and E. Moore, J. Gen. Physiol., **23**: 191, 1939.

and 37, 5), or that the antagonistic forces of the two halves may be great enough to produce opposing strains capable of mechanically disorganizing or denaturing larger molecules, e.g., hemoglobin. (See pp. 297 and 338.)

For experimental studies, substances of such a variety of nonpolar-polar behavior can be selected, first, from the list given in Neuberg's paper, and, second, among technical products utilized for wetting, dispersing, and emulsifying purposes, the so-called *detergents*.[149] In contrast to these substances, many of the natural products of the metabolism of animals or plants which come into contact with the plasma membrane either from within or from without the cells (lactate, pyruvate, succinate, malate, citrate, various phosphorylation products, and many others) lack the qualities in question. They are prevailingly hydrophilic and, therefore, physicochemically rather indifferent to the physiological membranes, and—although our knowledge in this regard is very poor—they probably fail to pass by simple diffusion, in one or the other direction, except the specifically anionpermeable membrane of the erythrocytes, which, compared to Cl, is penetrated by these ions with remarkable slowness,[150] possibly due to dehydration, to tighter packing, and thus to strengthening of the lateral adhesive forces between the structural units. However, in a survey of the products of cell metabolism, some compounds are encountered which, by their distinct nonpolar-polar configuration, can be expected to be more or less membrane-active in the sense of the aforementioned reactions. Such substances are the salts of the higher homologues of the saturated or unsaturated fatty acids, the salts of the bile acids, and other similar compounds.

In order to exemplify the significance of the nonpolar-polar configuration of organic ions, we turn now to the study of excitation and of production of resting potentials in muscle and nerve.

1. The Influence of Nonpolar-polar Ions on the Resting Potentials and the Excitability of Muscle and Nerve.—*The synthetic nonpolar-electrolytes.*—So far, three groups of nonpolar-polar anions have been investigated, alkylsulfonates $R{\cdot}OSO_3Na$, dialkylsuccinylsulfates $ROOC{-}CH_2$ / $ROOC{-}CH{-}SO_3Na$, and alkylbenzene sulfonates.[151] They are strong electrolytes in water, but, due to the nonpolar organophilic groups, they are more or less soluble in organic solvents, dialkylsuccinylsulfates, for instance, in kerosene, xylol, ether, glycerol, and olive oil. Their organophilic character increases with the number and the chain length of the alkyl groups present. Correspondingly, the interfacial tension water-air and water-organic solvent decreases.[152]

[149] For a general survey about the various factors of technical detergency, see J. W. McBain, Advances in Colloid Sc., **1**: 99, 1942.

[150] M. Maizels, Biochem. J., **28**: 337, 1934; see, further, Sec. 4, chap. 11, p. 247.

[151] R. Höber, unpublished experiments.

[152] C. A. Sluhan, Paper Trade J., 1940; R. Höber and J. Höber, J. Gen. Physiol., **25**: 705, 1942; H. A. Neville, J. Phys. Chem., **37**: 1001, 1933.

Dissolved in Ringer, these substances effectuate with frog muscles (sartorius) a resting potential like K, i.e., a depolarization. The threshold concentration of dialkylsuccinylsulfates, for instance, has been found with dibutyl to be 90×10^{-4} molar, diamyl 14×10^{-4}, dihexyl 8×10^{-4}, dioctyl 0.7×10^{-4}. In general, the reversibility of these bioelectric effects is low and diminishes with rising surface activity. On the basis of the theory, these influences can be accounted for as a dispersing effect brought about by the pull of the interfacial array of the hydrophilic group of the anion toward the water and by the penetration of its organophilic groups into the surface film, with a subsequent widening of the characteristic spacing in the latter and, therefore, eventually, an increase in the content of free water. This disintegration is probably followed by some entrance into the film structure of the anions (e.g., Cl), no more repelled entirely by the negative charge of the membrane material (pp. 299ff). Removal of the active nonpolar-polar ions can result in a complete rearrangement.

Returning to the dispersing effects of K or SCN (pp. 300, 333), there seems not to be a fundamental, but rather a quantitative, difference between these and the effects here dealt with, which are due to the definite opposing forces of the nonpolar-polar compounds. Also there is a certain relationship between surface-active nonelectrolytes and the nonpolar-polar electrolytes. For instance, narcotics, after being brought locally in contact with a sartorius muscle, have a reversible depolarizing effect like that of K. This may be referred to the particular affinities of the narcotics toward the lipoids (see chap. 23) which are structural components in the surface membrane, and which as such can be dislocated by the intruding molecules of narcotics, so that, again, spatial changes in the normal molecular orientation will result. In muscle, a K-like reversible depolarizing effect has been produced, e.g., by 10^{-1} molar amylalcohol, 2×10^{-2} amylurethane, 3×10^{-2} chloral hydrate,[153] and can, like that of K, be balanced by alkaline earth ions.[154] Only in one respect the surface structure of cells and fibers seems to be acted upon specifically by the nonpolar-polar ions, i.e., the denaturing influence upon proteins. Denaturation of proteins[154a] can be brought about by heat, strong acid or alkali, urea, salicylate, ultraviolet light, and by many nonpolar-polar agents, as, for instance, by those mentioned here (p. 336), but not by K, SCN, and narcotics. The denaturation of proteins is easily indicated, for example, by the liberation of SH-and S—S-groups. This liberation has been found concomitant with the loss of the specific effects of enzymes and viruses, with the separation of the protein part from visual purple, from chloroplastin, and others. These reactions occur at low concentration of the nonpolar-polar agents and at neutrality. Certainly,

[153] R. Höber, M. Andersch, J. Höber, and B. Nebel, J. Cell. & Comp. Physiol., **13**: 195, 1939.

[154] R. Guttman, J. Gen. Physiol., **23**: 343, 1940. See also p. 314.

[154a] M. L. Anson, J. Gen. Physiol., **23**: 239, 1939; further, M. L. Anson and A. E. Mirsky, *ibid.*, **17**: 399, 1934; A. E. Mirsky, *ibid.*, **19**: 559, 1935. See also p. 297 and Sec. 2, p. 140.

they contribute to the often observed irreversibility of the electrical phenomena.

With many synthetic nonpolar-polar electrolytes, in contrast to those mentioned so far, the typical activity is located not in the anion, but in the cation. Most frequently the neutral salts of quaternary ammonium bases, particularly salts of the type alkyl-dimethyl-benzyl-ammonium chloride, have been utilized in solving biological problems. Their influence differs mainly from that of the anionic agents by the fact that the dispersing or the splitting influence can be accompanied or succeeded by a precipitation, which is due to a discharge of colloidal protein anions, present or formed in the cell structures, which are susceptible to the nonpolar-polar agents.[155] For instance, at *p*H 5.6 precipitation by a cationic agent occurs with pepsin, but not with trypsin, because the isoelectric point of pepsin is *p*H 2.9, that of trypsin 6.5.

The threshold concentration needed for producing a resting potential with a frog muscle by one of the quaternary ammonium bases has not been found to vary markedly between C_8 and C_{18}, (2×10^{-4}).[156]

In conclusion, one group of simple synthetic organic cations may be dealt with as exemplifying the typical bearing of the organophilic affinity.[157] The dialkylamine ions, comprising the series from CH_3 to C_5H_{11}, have been compared to the inorganic cations Li, Na, Rb, and K with regard to the resting potential produced with crab nerves. The results show that the C_4 and C_5 compounds develop a negative pole like K or Rb, while C_1 and C_3 resemble Li and Na.

2. The Anions of Some Natural Nonpolar-polar Carboxylic Acids. The greatest physiological significance among the natural nonpolar-polar electrolytes can be assigned to the commonly occurring alkali salts of the fatty acids. However, the dual character of their ions, in general, is not so marked as that of the aforementioned compounds, because we are dealing with the carboxylic acids which are weaker electrolytes than the sulfonic acids, and because the products of an intricate hydrolytic reaction of the soaps interfere.[158]

The fatty acids with longer C-atom chains are chiefly effective through their surface activity and their lipoid-solubility. The classical proof of the nonpolar-polar character of their anions is the ability to form, on a water-organic solvent interface, a film held together by lateral adhesion forces and with the polar COO-groups anchored in the water, the nonpolar C-chains oriented more or less perpendicularly toward the organic solvent. The nonpolar-polar character of the lower members of the series, (C_2 to C_4 or C_5), is too weak to show a behavior in the hydrotropic series of anions different

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[155] R. Kuhn and co-authors, Ber. d. Deutsch. chem. Ges., **73**: 1080 to 1113, 1940.

[156] R. Höber, unpublished experiments.

[157] W. Wilbrandt, J. Gen. Physiol., **20**: 519, 1937, see also p. 335.

[158] F. G. Donnan and H. E. Potts, Kolloid-Ztschr., **7**: 208, 1910; L. Lascarey, *ibid.*, **34**: 73, 1924.

from other hydrophilic anions, i.e., they are located in the series between Cl and SO_4 (p. 293), like lactate, pyruvate, tartrate, and others (see Sec. 8, chap. 34, 2). Correspondingly they induce a positive pole with a frog muscle or nerve, relative to Cl, and enter erythrocytes somewhat slower than Cl (p. 337). However, beginning with or beyond valerate, the higher members of this series induce a negative pole, indicating organophilic affinity, in correspondence with which the threshold concentration falls. The reversibility of these effects decreases with the increasing length of the C-chain.[159]

Other natural nonpolar-polar carboxylic acids are the conjugated bile acids, e.g., glycocholic and taurocholic acids. From many reactions they are known to be hydrotropic,[160] they are surface active, and their local application (at 2×10^{-3} molar) to a frog muscle leads to a K-like potential effect. In contrast to this, even a saturated solution (about 10^{-2} molar) of dehydrocholate has no influence, in accordance with its surface inactivity,[161] which probably can be accredited to the presence in the ring system of three keto groups.

Finally, benzoate and salicylate belong to the same physicochemical and physiological category of organic anions,[162] and accordingly have been shown to induce a depolarization of the muscle membrane at very low concentrations (see also Sec. 4, p. 248).

3. The Influence of Nonpolar-polar Ions on Muscle Excitability.[163] Three groups of synthetic nonpolar-polar organic anions (p. 337) have been investigated as to their power to alter the resting potential; two of them have presented a uniform reaction, namely the alkylsulfates, (C_8, C_{10}, C_{12}) and the dialkylsuccinylsulfates ($2C_4$, $2C_5$, $2C_6$ and $2C_8$). As aforementioned, these two groups induce negativity, which may be referred to the strong attachment of the nonpolar half-molecules to the fiber surface, on the one hand, and the strong pull of the polar half-molecules toward the water, on the other (p. 336). The effect can be assumed to be a distortion of the membrane structure. Parallel to these studies, the effect upon excitability has been investigated by stimulating pretreated sartorius muscles with condenser discharges (maximal stimuli, one in 10 to 20 seconds). Again the reaction was uniform; a decline of the height of contraction over a period of time, dependent in its duration upon the concentration and the strength of the contrasting nonpolar and polar forces involved. It seems as though, even with small concentrations, the membranes cannot resist the powerful dual stresses. However, the effect of the third group of

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[159] R. Höber, M. Andersch, J. Höber, B. Nebel, J. Cell. & Comp. Physiol., **14**: 195, 1939.

[160] C. Neuberg, Biochem. Ztschr., **75**: 107, 1916.

[161] R. Höber and J. Höber, J. Gen. Physiol., **25**: 705, 1942.

[162] A. von Kuthy, Biochem. Ztschr., **237**: 381, 396, 1931; I. R. Katz, *ibid.*, **257, 259, 261, 262, 263, 271,** 1933–34; R. Höber and E. Moore, J. Gen. Physiol., **23**: 191, 1939; also H. Freundlich and G. V. Slottmann, Ztschr. f. physik. Chem., **129**: 305, 1927.

[163] R. Höber, unpublished experiments.

these agents, the arylsulfonates, is different. So far, on the one hand, benzene, toluene, xylene, and isopropyl benzene-sulfonates, and, on the other, octyl and decyl benzene sulfonates, have been studied. The last two substances, resembling the alkylsulfonates and the dialkylsuccinyl-sulfates in the longer C-chains of the alkyl rests, are similar to these in their properties, namely, in producing, even in the smallest concentrations, decreasing contractility. However, with the others, at variance with all the last-mentioned compounds, the decrease of contractility is preceded by a more or less marked rise, probably owing to their smaller nonpolar affinity, which is indicated by their lower surface activity.[164] Only after exposing the muscles to high concentrations of these weaker agents (e.g., xylene and isopropyl toluenesulfonate) do they respond from the beginning with decreasing contractions. Evidently these substances can be compared to those earlier-described inorganic ions (pp. 289, 290), which, also, in low concentration have a stimulating, and in high concentrations a depressing, effect. Their influence, again, can be interpreted as a loosening process in the surface membranes, which, if allowed to progress only to a somewhat diminished coherence of the structural units, is correlated with activation, but beyond that results in real disorganization, which is incompatible with function. However, with regard to certain discrepancies, it should be kept in mind that the mechanisms of altering the surface membranes by inorganic ions and by nonpolar-polar ions are different (see pp. 299ff).

Concerning their influence on the potential (see p. 340), the conjugated bile salts, also, may be mentioned. These, with their highly organophilic ring system and their less hydrophilic carboxyl group sticking to the surface membrane, lower the muscle excitability slowly and often irreversibly (10^{-3} molar). Further, the aforementioned quaternary ammonium salts (p. 339), likewise, are inhibitory at and above threshhold concentration.

[164] H. A. Neville, J. Phys. Chem., **37**: 1001, 1933; further, H. Freundlich and G. V. Slottmann, Ztschr. f. physik. Chem., **129**: 305, 1927.

21

THE INFLUENCE OF IONS UPON CELL POTENTIALS IN PLANTS

Bioelectric potentials have been discussed in this section chiefly as providing evidence concerning the surface structure of cells, their sensitivity toward various agents, and the physiological implications of the concomitant alterations. In these respects it seems particularly promising to extend the studies to the giant plant cells (*Valonia*, *Halicystis*, *Nitella*, *Chara*), which offer excellent possibilities for measuring membrane potentials across the cell surface, similar to the potentiometric measurements across the membrane of the giant axon of the squid, which have been mentioned earlier (p. 321). However, the characteristics of the latter object permit drawing more clear-cut conclusions than in the experiment with the plant cells. For, in order to carry out transverse measurements in plant cells, one electrode is pierced through the cell wall into the large sap vacuole, and is thus separated from the outside electrode by the vacuolar membrane, the protoplasmic mantle (about 10 microns in thickness), and the exterior plasma membrane;[155] whereas, in experiments on nerves, the electrode is placed in the axoplasm, the exterior one lying outside the single external axoplasm membrane. The more complex physiological conditions in plant cells become particularly obvious, if the electrolyte concentrations in the two solutions adjacent to the protoplasmic membranes are changed.[156] The normal resting potential of *Halicystis ovalis*, for instance, is equal to 80 mV with the positive pole outside (as with muscle or nerve). After the cell sap has been substituted by sea water, and the outside and inside sea water both brought from the normal *p*H of 8 to *p*H 5, the reaction of the sap, the potential difference is still 80 mV,[157] although there is no concentration gradient whatsoever between the two sides of the protoplasm. This is all the more unexpected, as the natural positive potential could be referred to a preferential membrane permeability to K, which in the sap of *Halicystis ovalis* exceeds the K of sea water thirty times (K_i equal 0.3 molar, K_o equal 0.01 molar). Thus it appears that the two membranes have different properties, and in combination they resemble the artificial asymmetrical "membranes," which have been built up by Beutner, Wilbrandt, and

[155] W. J. V. Osterhout, E. B. Damon, and A. G. Jacques, J. Gen. Physiol., **11**: 193, 1928; E. B. Damon, *ibid.*, **13**: 207, 1929; E. B. Damon and W. J. V. Osterhout, *ibid.*, **13**: 445, 1930.

[156] W. J. V. Osterhout, Ergebn. d. Physiol., **35**: 967, 1933; Physiol. Rev., **15**: 216, 1935.

[157] L. R. Blinks, J. Gen. Physiol., **18**: 409, 1935; further, *ibid.*, **15**: 147, 1932.

others.[168] Wilbrandt, for instance, has pasted an ordinary cationpermeable collodion membrane to one impregnated either with a basic dyestuff or with an alkaloid salt, and hence anionpermeable (p. 318), and thus obtained a double membrane, which with 0.001 *N* KCl on either side yields a potential of several hundred mV.

The next problem is to explain the direction and the height of the potentials of the giant plant cells, each of which has its significant value. The inside K apparently plays here no role. For instance, the potential of *Valonia* is −8 mV, with K_i : K_o equal 42, and, as already mentioned, substituting in *Halicystis ovalis* the normal cell sap (0.3 molar KCl) by sea water (0.01 molar KCl) fails to diminish the positive potential. However, outside K affects the potential in a way similar to that observed earlier with muscle and nerve; in other words, raising K_o decreases the positive potential more and more, eventually to the value expected with a selectively K-permeable membrane, e.g., in experiments with *Nitella*, where raising K_o (as KCl) from 0.001 molar to 0.01 molar is followed by a decrease of electromotive force equal to 56 mV, i.e., nearly the theoretically expected value.[169] However, according to more recent observations with *Halicystis*,[170] this decrease often fails to appear unless the duration of the experiment is short. Whereas, in the corresponding experiment on muscle and nerve, the depolarization by K_o is fairly stable and is correlated in its degree with the concentration gradient (p. 320), here the immediate and rapid decline of the potential is followed by a slower or faster "recovery" during the continued presence of the initial K_o concentration. This reaction is likely to be due, at least partially, to a change of permeability, probably based upon an active reaction of the external plasma membrane, as may be concluded from chemical studies about the ionpermeability of the giant plant cells. But, before discussing this viewpoint, we may turn at first to the influence of other ions upon the potential of these cells. Taking into account that Na and Cl are predominant in the natural surroundings of these cells, and that K_i is fairly indifferent, the normal positive potential of giant cells has been interpreted as due chiefly to the greater membrane affinity to Cl compared to that of Na.[171] This hypothesis has been tested by substituting various anions, inorganic and organic, for Cl.[172] The result is that Br is similar to Cl, but all the other anions tried, NO_3, SO_4, formate, acetate, proprionate, butyrate, lactate, pyruvate, glutamate, diminish the positive potential or even reverse it, according to their concentration.

[168] R. Beutner, Die Entstehung elektrischer Ströme in lebenden Geweben: Enke, Stuttgart. 1920, pp. 119 ff; W. Wilbrandt, J. Gen. Physiol., **18**: 933, 1935. See also R. Höber, Physiol. Rev., **15**: 52, 1936.

[169] A. B. Damon and W. J. V. Osterhout, J. Gen. Physiol., **13**: 445, 1930; also L. R. Blinks, *ibid.*, **16**: 147, 1932; *ibid.*, **18**: 409, 1935.

[170] L. R. Blinks, J. Gen. Physiol., **23**: 49, 1940; Cold Spring Harbor Symp., **8**: 204, 1940.

[171] E. B. Damon and W. J. V. Osterhout, J. Gen. Physiol., **13**: 445, 1929; W. J. V. Osterhout, Proc. Nat. Acad. Sc., **24**: 75, 1938.

[172] L. R. Blinks, J. Gen. Physiol., **23**: 495, 1914; Cold Spring Harbor Symp., **8**: 204, 1940.

When the above anions are introduced into the cell sap of *Halicystis*, the normal positive potential increases, but to a relatively small degree, indicating that the tendency of these anions to enter the protoplasm across the vacuolar membrane is less than that in the opposite direction across the external membrane. These findings are remarkable from two angles: First, the influence of the organic anions is such as could be expected from their place in the Hofmeister-series (p. 247 and Sec. 8, chap. 34, 2). They are physicochemically rather indifferent, but possibly exhibit a dehydration effect upon the membranes. Second, these organic anions are supposed by Blinks to be accumulated in the protoplast as anaerobic metabolites, and their presence and their amount may be reflected by changes in the cell potential, caused by effects upon the metabolic reactions. The plant cells in question differ greatly from muscle and nerve by irregular fluctuations of their potential difference. These are associated with lack and surplus of oxygen, with increase and decrease of CO_2, with rise and fall of *p*H, and all these changes are correlated with the presence and absence of photosynthetic activity. Lack of oxygen depresses the electromotive force, especially in the dark, but light restores it through the liberation of oxygen. In CO_2 also, the potential falls off, but recovers in light insofar as the CO_2 is used-up in the synthetic reactions, and there is a simultaneous rise of *p*H. Lack of oxygen in nerves, likewise, has a reversible depressant effect (p. 322), but it is small compared to that in *Halicystis*, where low oxygen suffices to set down the potential after a few minutes. On the other hand, the potential and the excitability of the fresh water alga *Nitella* were found to resist pure hydrogen and nitrogen for many hours.[173,174]

We now return to the aforementioned observation that exposing *Halicystis* to a surplus of K at first releases a rapid potential fall, which is followed by a "recovery" persistent during the continued presence of K, and interpreted as due to the release of an active change of permeability. We further refer to the aforementioned various fluctuations of the potential, which can be thought of as being indicative of the susceptibility of these structures to a variety of influences. Then, these facts may be correlated with studies of S. C. Brooks[175] about the accumulation of Rb and of radioactive K by *Valonia* and *Nitella*, demonstrating the following intricate behavior. Either of these cations appears in the cell in a concentration surpassing that of the outside fluid (see Sec. 8, chap. 3 subchap. 2.), but the rate of accumulation is rather independent of variations of the outside concentra-

[173] L. R. Blinks, Cold Spring Harbor Symp., **8**: 204, 1940.

[174] Concerning other indications of special electrical reactivity of the giant plant cells to organic substances, see the papers of Osterhout and co-workers regarding the so-called "potassium effect" and the "concentration effect." For the potassium effect, see J. Gen. Physiol. **13**: 715, 1930; **17**: 105, 1933; Proc. Soc. Exper. Biol. & Med., **32**: 715, 1935; J. Gen. Physiol., **18**: 581, 1935; **19**: 423, 1936; **24**: 7, 1940; **24**: 311, 1941; **24**: 699, 1941. Concerning the concentration effect, see J. Gen. Physiol., **20**: 13, 1936; **21**: 707, 1938.

[175] S. C. Brooks, J. Cell. & Comp. Physiol., **5**: 169, 1935; **11**: 247, 1938; **14**: 383, 1939; Cold Spring Harbor Symp., **8**: 171, 1940.

tion. This becomes more or less understandable, if independent analyses of the sap, gained by puncturing the vacuole, and of the cell residue, i.e., the cell wall and the protoplasmic mantle, are carried out. Then, it appears that Rb and K are stored at first inside the protoplasm to a very high level (for instance with Rb 40 times); that only secondarily they are transferred into the sap, eventually long after their entry into the protoplasm, and reaching there a concentration level lower than that of the protoplasm; later, the distribution of these ions varies as due to their escape from the protoplasm into the outside solution or toward the sap. According to the more recent studies of Brooks, these movements can display irregular fluctuations, possibly picturing the double influence of the two barriers, the external plasma membrane and the vacuolar membrane.

22

THE POLARIZATION OF MODEL MEMBRANES AND OF NATURAL MEMBRANES BY ELECTRIC CURRENT

The natural barriers delimiting the protoplasm from its environment have been found, by virtue of their more or less selective permeability to ions, to be the seat of the bioelectric potentials both in animals and in plants. In the following section, the physiological counterpart of this interrelationship will be studied. A membrane, when interposed in a potential originating from an exterior source and falling off through the bordering electrolyte solutions, induces certain changes in the ion concentrations on either interface of the membrane, which are signalled by various effects. The discussion concerning this membrane polarization as a physiological phenomenon will be preceded by the description of some basic observations on models of the two main types, the oil-like and the sieve-like membranes (pp. 331 and 317).

If a direct current is conducted across a system built up by an interphase of, say, nitrobenzene interposed in aqueous hydrochloric acid and at the beginning in distribution equilibrium, at each phase boundary, changes of the ion concentrations take place, which are opposite to each other and which increase with the length of time, until a stationary state is reached. This is due to the fact that the ratios of the cation and anion mobility, (i.e., the ionic transfer numbers) are different in water and in the organic solvent. The result is that a system which at the beginning shows no potential difference, builds up a counter electromotive force of polarization inducing a certain steady state due to back-diffusion and re-distribution at and near to the phase boundaries. After interrupting the polarizing current, the system returns slowly to the initial equilibrium state.[176]

A second type of polarization is bound up with changes of the transfer numbers of anions and cations, which are due to differential adsorption on the walls of porous membranes (see pp. 317ff.), like gelatin, albumin, agar, collodion,[177] or due to the presence of a dissociable pore wall material.[178]

[176] W. Nernst and Riesenfeld, Ann. Physik., (4) **8**: 600, 1902.

[177] A. Bethe and T. Toropoff, Ztschr. f. physik. Chem., **88**: 686, 1914; **89**: 597, 1915. About transfer numbers dealing with collodion membranes, see, for instance, L. Michaelis *et al.*, J. Gen. Physiol., **10**: 671, 685, 1927; Kolloid-Ztschr., **62**: 2, 1933.

[178] K. H. Meyer and J. F. Sievers, Helv. chem. acta, **19**: 649, 665, 1936; K. Sollner and I. Abrams, J. Gen. Physiol., **24**: 1, 1940; K. Sollner, I. Abrams, and C. W. Carr, *ibid.*, **24**: 467, 1941.

If a current is passed, for instance, through a gelatin membrane lying in NaCl solution at a *p*H above the isoelectric point, so that the membrane acquires a negative charge, the anions are relatively retarded (see p. 319). One result of the arising inequality of ion concentrations at the phase boundaries is an accumulation of base at the anode at the point of entrance of the current into the membrane, readily demonstrable by an indicator. Again, a counter-electromotive force of polarization appears, while the current is flowing.

It is of particular interest to study with a sieve membrane the interfacial changes of ion concentrations with regard to the pore size by applying different electrolytes on either side of the membrane. Some results are shown in the following experiment.[179] A collodion membrane is prepared with pores wide enough to allow both cations and anions to pass. It is bathed on one side by a neutral solution of potassium phosphate, on the other by NaCl. A current passed from the K_2HPO_4-side to the NaCl-side is found to be distinctly stronger than one passing in the opposite direction, the membrane being more permeable to K and Cl than to Na and HPO_4 (*rectifier effect*). When the experiment is performed in such a way that a long collodion tubing is filled with K_2HPO_4 and wrapped in gauze wet with NaCl (thus roughly imitating an axon), and when one small electrode as anode, another as cathode, are attached to the gauze some distance from each other, so that the current flows from pole to pole partly inside the tubing, partly outside, the polarization is stronger at the anode, and spreads more around the anode than around the cathode.

Proceeding now from these model membranes to physiological membranes, one new feature of great significance is evident. Due to the constituent hydrophilic colloid ampholytes, the membranes, through polar changes in the concentration and in the composition of their ionic atmosphere, are subject to remarkable alterations of their colloidal structure, to condensing or shrinking effects, and to softening or swelling effects, and even to disintegration, combined with gross changes of the resistance of the membrane as such, because of increased (or decreased) ionpermeability. Particularly, changes in the concentration of H, K, Ca, and of OH and HPO_4 may have a predominant influence upon the structural alterations. Such an effect is proved by a great number of observations.

The various alterations as brought about by sending a direct current along a nerve (or muscle), the so-called *electrotonus*, appears in the following manner.

First, one end of an excised nerve is killed by crushing, an electrode is placed on this end, the other electrode on the intact surface, and the resistance measured in both directions. With the cathode at the living (uncrushed) end (catelectrotonus) the resistance of the preparation is

[179] R. Labes and H. Zain, Arch. f. Exper. Path. Pharmakol., **125**: 1, 53, 1926; **126**: 284. 352, 1927; U. Ebbecke, Ztschr. f. Biol., **91**: 221, 1931. See, further, U. Ebbecke, Ergebn. d. Physiol., **35**: 756, 1933.

lowered, with the anode (anelectrotonus) raised, both changes increasing with time and current strength.[180] In other words, the nerve in this experiment displays a *rectifier effect*. This effect further appears, when alternating current is applied to an intact nerve with electrodes attached to two places of its surface, because one half of the current cycle meets a greater resistance than the other.[181]

Second, with a weak current applied to the nerve of a nerve muscle preparation, catelectrotonus indicates a small resistance and raised excitability, with a strong current an even more lowered resistance, but a decreased or abolished excitability (a "cathodic depression").[182] At some distance from a depressing cathode one can observe also a somewhat lowered resistance, but an increased excitability.[183]

Third, the catelectrotonus probably is due, at least partially, to a surplus of inside K and of outside Cl being shifted to the membrane by the "outgoing" current and thus producing a loosening effect. K-salts, applied directly to a nerve or muscle, are known to raise excitability at small concentrations, to depress it at higher concentrations and to produce simultaneously a depolarization of the membrane (see pp. 289, 320).[184] Therefore, rectification is decreased or abolished by KCl, mainly because the difference of permeability between K and Na disappears (R. Guttman, l.c.).

Fourth, inexcitability by K can be cancelled by an anelectrotonus, although anelectrotonus by itself is a depressant agent, due to its condensing influence. Also Ca produces inexcitability, but not by loosening the membrane structure like K, rather by condensing it (p. 303); for this reason, the depression by Ca can be cancelled by a catelectrotonus.[185]

Fifth, this interpretation is in good agreement with experiments on the fiber-like long giant plant cells (*Nitella*), which respond to an electrical stimulus by releasing an excitation wave (p. 316). The height of this wave is modified as to whether the wave is superimposed upon the regular permanent plasma membrane potential, or upon a potential which is shifted to another height by an "outgoing" or by an "ingoing" polarizing current,

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[180] U. Ebbecke, Pflüger's Arch. f. d. ges. Physiol., **195**: 555, 1922; R. Guttman and K. S. Cole, Biol. Bull., **81**: 277, 1941; Proc. Soc. Exper. Biol. and Med., **48**: 293, 1941.

[181] R. Guttman, J. Gen. Physiol., **28**: 43, 1944, also K. S. Cole and H. J. Curtis, J. Gen. Physiol., **24**: 551, 1941.

[182] B. Wertgo, Pflüger's Arch. f. d. ges. Physiol., **31**: 417, 1883; **84**: 347, 1901. About concomitant changes of the amplitude of the action wave, the conduction rate and the absolutely refractory period which differ according to the direction and the strength of the polarizing current, see: G. H. Bishop and J. Erlanger, Amer. J. Physiol., **78**: 630, 1926.

[183] U. Ebbecke, Pflüger's Arch. f. d. ges. Physiol., **195**: 555, 1922; Erg. Physiol., **35**: 756, 1933.

[184] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **106**: 599, 1905.

[185] D. S. Woronzow, Pflüger's Arch. f. d. ges. Physiol., **203**: 300, 1924; **207**: 279, 1925; **210**: 672, 1925; **215**: 32, 1927; **222**: 159, 1929. Further see: W. Thoerner, *ibid.*, **197**: 159, 187, 1922; **198**: 373, 1923; R. Höber and H. Strohe, *ibid.*, **222**: 71, 1929; R. Guttman, J. Gen. Physiol. **23**: 343, 1940; R. Guttman and K. S. Cole, Biol. Bull. **81**: 277, 1941.

or by application of ions.[185a] For instance, the normal membrane potential of *Nitella* can be depressed by KCl, even to zero with 0.05 M. When separate ingoing electrical stimuli of increasing strength, starting with 2.5 μa/cm.^{2}, are superimposed, no potential wave indicating polarizability can be demonstrated, until 15 μa/cm.^{2} or more are applied; then counter-electromotive forces of polarization reappear. This is analogous to the behavior of the nerve muscle preparation in Woronzow's experiment, where the paralyzing effect of K is antagonized by establishing an anelectrotonus. In another experiment with *Nitella*, the normal membrane potential is brought to zero by a powerful stimulus, and is then rebuilt spontaneously within ten to fifteen seconds to its original height. During this time of recovery, test stimuli show the counter-electromotive force of polarization to grow progressively.

Sixth, finally, on sending an interrupted direct current of moderate strength in one direction along a muscle, the heights of contraction decay gradually until, after some time, the muscle stops twitching entirely and seems to be fatigued. However, after reversing the direction of the interrupted current, the muscle immediately starts contracting to the initial height, but again it is "fatigued" after the same interval. This can be repeated many times ("Wendungseffekt" of F. Scheminzky, *et al*),[186] and thus is proved to be not a real fatigue, but rather a cathodal depression; in this state the muscle loses its excitability, due to the repeated loosening influence of the outgoing current and the concomitant increase of ionpermeability, which can be rapidly counterbalanced by reversing the current, and thus subjecting the loosened area to the condensing influence of the anode. Correspondingly, in experiments upon the giant axon of the squid, which is passed by a direct current of adequate strength, it has been found that the electrical transverse conductivity can be raised at the cathode to the same value as obtained during normal activity, and at the anode diminished to a minimum not far from zero.[187]

Summarizing all these observations, which could be supplemented by many others, fairly conclusive evidence has been afforded in favor of the concept proposed 40 years ago, chiefly on the basis of experiments concerning the influence of inorganic ions on resting potentials of muscles, that excitation is a reversible increase of permeability to ions, brought about by a limited and transitory dispersion of the colloidal structure of the plasma membrane.

[185a] L. R. Blinks, J. Gen. Physiol. **20**: 228, 1936; further, *ibid.*, **13**: 495, 1930.

[186] Z. von. Gulaszy, Pflüger's Arch. f. d. ges. Physiol., **223**: 407, 1929: F. Scheminzky and F. Scheminzky, *ibid.*, **225**: 145, 1930.

[187] K. S. Cole, and R. F. Baker, J. Gen. Physiol., **24**: 535, 1941. Concerning microscopic evidence of the anodal and cathodal alterations of nerve fibers, see A. Bethe, Allg. Anat. u. Physiol. des Nervensystens: Leipzig, 1903; Ztschr. f. Biol., **34**: 145, 1908; S. Katsura, Pflüger's Arch. f. d. ges. Physiol., **217**: 279, 1927; J. Klinke, *ibid.*, **227**: 715, 1931; M. Koll-Schroeder, *ibid.*, **234**: 29, 1934.

This result leads to the question, whether there are not involved, in this loosening effect, additional factors beyond the influence of the inorganic ions which necessarily appear in the vicinity of the membrane during excitation in concentrations other than when at rest. The chemical complexities of the plasma membrane should be considered, and perhaps still more the very characteristic specialized composition of the nerve membranes, including in their constituents split products possibly capable of acting as "precursors," to elicit reversibly, and in small amounts, strong bioelectric effects (see pp. 337, 339). These considerations tempt one to add to the conception of the excitation wave as being, according to the old "Strömchen-theory" of L. Hermann, a mere electrical phenomenon, i.e., a propagated depolarization-repolarization, another feature, namely, a chemical wave, which consists of the liberation of the aforementioned "precursors" and of their re-insertion.[188] The following points may be inferred to be favorable to such a concept. The lipoids of the nervous system consist of: 1. the monophosphatides, lecithin and cephalin (p. 294); 2. as diaminophosphatides the sphingomyelines, containing, besides phosphoric acid and cholin or colamin, an unsaturated bivalent aminoalcohol, sphingosin, and a fatty acid with 24 C-atoms, either saturated as lignoceric acid or unsaturated as nervonic acid; and 3. cerebrosides, which are lacking to the phosphoric acid and the base, but include again lignoceric or nervonic acid, respectively, as their hydroxy-derivatives, cerebronic, and oxynervonic acids. With regard to the monophosphatides, it should be added that the fatty acids included in their molecule, oleic, linoleic, linolenic, and arachidonic acid, likewise, are unsaturated components.[189] Now it is known from the work of Langmuir and of Adam[190] that the presence of double bonds in fatty acids causes the surface films to expand at the interface air-water much more easily than films with saturated chains of similar length, due to the fact that the double bonds attract the water more than a saturated linkage. Further, all the aforementioned acids are significant as electrolytes with a polar-nonpolar configuration of their molecules and are likely, therefore, to display loosening and, finally, disintegrating cytolyzing properties, as has been proved by their depolarizing effect on muscles (see p. 337ff).[191] It is true that so far it has not been proved that, while an excitation wave is running along a nerve, some such substances are liberated. But, on the one hand, a widely distributed enzyme, a lecithinase, has been found in nerve substance. This is able to split the unsaturated fatty acids from the lecithin molecule, leaving

[188] See Hill's discussion about the chemical wave: A. V. Hill, Chemical Wave Transmission in Nerves: University Press, Cambridge, 1932; further, G. H. Parker, A. V. Hill, W. O. Fenn, R. W. Gerard, and H. S. Gasser, Physical and chemical changes in nerve during activity: Science Suppl., 79, 1934.

[189] About unsaturated fatty acids as "essential" growth factors, see G. O. Burr, Federation Proc., 1: 224, 1942.

[190] N. K. Adam, The Physics and Chemistry of Surfaces, 2nd ed.: Clarendon Press, Oxford, 1938, chapter 2.

[191] R. Höber, N. Andersch, J. Höber and B. Nebel, J. Cell. & Comp. Physiol., 13: 195, 1939.

a residue, lysolecithin, which has a strong cytolytic power.[192] On the other hand, von Muralt[193] has succeeded in showing that, when excitation waves are running along a frog nerve, minute amounts of a very labile substance are set free, which is surface active and, therefore, is accumulated in the foam of an extract of the nerve and which, according to various reactions, is similar to acetylcholine. These observations might suggest that during excitation, phosphatides are subjected to a breakdown associated with the liberation of cholin (see, further, p. 359). But probably it will be difficult to reach a definite conclusion since, according to von Muralt, not more than about 0.07 μg./g. nerve of the active substance are produced by a frog nerve of six cm. length and 50 mg. weight, stimulated rhythmically in such a way that six excitation waves are caught on its length by freezing with liquid air. Other indications as to the significance of acetylcholine during excitation of the peripheral nerve have been contributed mainly by Nachmansohn.[194] According to these studies, choline esterase, the catalyzer of acetylcholine, is localized in the nerve sheath rather than in the axoplasm. In chopped or ground peripheral nerve acetylcholine is resynthesized from choline and acetate in presence of adenosinetriphosphate and phosphocreatine.[195] There is also another substance likewise prevailing in the sheath of the nerve fiber, diphosphothiamine (cocarboxylase).[196] Hence, one may assume that, during depolarization and repolarization, breakdown and recovery of the nerve membrane, a complex of chemical reactions comes into action, the individual members of which appear and disappear in a cycle of phosphorylation and dephosphorylation.[195,195a] It seems not unlikely that in such a cycle there are involved also lipophosphatides, which in contact with enzymes like lecithinase would become the source of highly depolarizing chemical agents, the unsaturated fatty acids (p. 351). But up to now this is nothing else but a hypothesis.

Concerning the view that the excitation wave is more than an electrical phenomenon, but includes a series of chemical reactions, the following interesting observations of Osterhout and Hill upon *Nitella* cells may be mentioned. Their ability to respond to an electrical stimulation by producing an excitation wave (p. 316) is lost by exposure to distilled water, but is restored by water, in which the cells have been standing for some time.

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[192] S. Belfanti and C. Arnaudi, Bull. Soc. Intern. Microb., **4**: 399, 1932, quoted from Ergebn. d. Enzymforsch., **5**: 213, 1936; N. Francioli, Fermentforsch., **14**: 241, 493, 1934.

[193] A. von Muralt, Proc. Roy. Soc., London, **B123**: 397, 1937; Pflüger's Arch. f. d. ges. Physiol., **245**: 604, 1942; K. Lissàk, Am. J. Physiol., **125**: 778, 1939; **127**: 263, 1939; K. Brecht and M. Corsten, Pflüger's Arch. f. d. ges. Physiol., **245**: 160, 1941.

[194] D. Nachmansohn, Collecting Net **17**, No. 4, 1942; further J. F. Fulton and D. Nachmansohn, Science, **97**: 569, 1943.

[195] D. Nachmansohn, R. T. Cox, C. W. Coates and A. L. Machado, J. Neurophysiol. **6**, 382 and 397. 1943; W. Feldberg, J. Physiol., **101**: 432, 1943.

[195a] K. Lissàk, L. C. von Muralt, Pflüger's Arch. f. d. ges. Physiol. **245**: 604, 1942; D. Nachmansohn and H. B. Steinbach, J. Neurophysiol., **5**: 109, 1942.

[196a] See further A. Liechti, A. von Muralt and M. Reinert, Helv. Physiol. Acta, **1**: 79, 1943.

This seems to be due to the fact that an organic substance continuously leaves the protoplasm and must be newly produced if irritability shall persist. In searching for the nature of the substance, it was found that the restorative effect is produced by very heterogeneous compounds, guanidine, adrenalin, ephedrin, tetraethylammonium chloride, NH_4Cl. Also addition of blood plasma, saliva, urine, white of egg, and milk re-establishes the irritability.[197]

The conception that chemical events and the electrical processes are linked appears to be favored further by the illuminating experiments of Lillie[197a] regarding the numerous analogies between the nerve process and his well-known "iron nerve" model, displaying an electrochemical wave of alternating polarization and depolarization and alternating oxidation and reduction which on "stimulation" travels along a wire of passive iron coated with an iron oxide film.

[197] W. J. V. Osterhout and S. E. Hill, J. Gen. Physiol., **17**: 87, 1933; W. J. V. Osterhout, *ibid.*, **19**: 423, 1935; **18**: 987, 1935; Physiol., Rev. **15**: 215, 1936.

[197a] R. S. Lillie, Biol. Rev., **11**: 181, 1936; Physiol. Rev., **2**: 1, 1922; J. Gen. Physiol., **13**: 1 1929; **19**: 109, 1935.

23

THE INFLUENCE OF NARCOTICS ON CELL ACTIVITY

Narcotics can be classified as organic nonelectrolytes, acting essentially by the transitory depression of cellular functions. But not every inhibition of this kind is a narcosis. Typically, narcotics do not enter into a chemical reaction with cell components; they are chemically "indifferent." It is not customary to call HCN or CO a narcotic; nor are the depressant barbiturates and many alkaloids (among them, for instance, morphine), which interact with protoplasm as weak acids or weak bases, classified as such. Because of their chemical indifference, in general, narcotics pass the cell unchanged, and this makes it more conceivable that substances of a very diverse chemical nature are found to have narcotizing properties. The reactions they enter into with the cells are of a physicochemical rather than of a chemical nature. They make contact with the cells by secondary valences or Van der Waals forces, changing the surface properties of exterior or interior cellular structures and microstructures, which become apparent as changes of dispersity, of hydration, of colloidal aggregation, of dissolving power, of adsorption affinity. Which of these physicochemical reactions is, in general, the essential one, or is dominant, is still undecided.

It seems useful to analyze this situation from a historical viewpoint. Around 1900, fifty years after its great revival by Claude Bernard, general cellular physiology received exceedingly effective stimuli from physical chemistry. Following the fundamental studies about the osmotic pressure and the ionization of salts, numerous objects of animal and plant life were subjected to a study of their osmotic properties, with the result of increasing knowledge concerning cell permeability. Particularly by applying osmotic methods to plant cells and to muscles, Overton[198] demonstrated that numerous inorganic and organic substances, dissolved in water, can be classified in one of two main groups, the penetrating and the nonpenetrating substances; the first, in general, comprising "unphysiological" compounds, which often are poisonous, especially if the penetration rate is high; the second including, among many others, normal components of the cell interior, like sugars, amino-acids, and inorganic ions. From these facts two conclusions have been drawn by Overton; first, that, considering the highly diverse chemical nature of the first group, there should be detectable

[198] E. Overton, Vierteljahrsschrift der Naturforsch. Ges. in Zürich, **40**: 1, 1895; Pflüger's Arch. f. d. ges. Physiol., **92**: 115, 1902.

a common physical property furnishing the penetrating power by a suitable interaction with the cell surface; second, that the paradoxical impermeability of the cells toward many common cell constituents, as demonstrated by osmotic experiments, suggests the capacity for an "active" uptake, known already at that time in gland cells, and, therefore, named by Overton "adenoid activity." Through this reasoning, general physiology has been confronted with one of its chief problems, the problem of "active transfer," its mechanism, and its thermodynamics. Furthermore, it has acquired the concept of lipoid solubility, which was inspiring in the evolution of the lipoid theory of permeability[199] as well as in that of the lipoid theory of narcosis.[200] The latter made its appearance as a byproduct, so to speak, of Overton's studies on cell permeability insofar as it became obvious that the rate of penetration and the narcotic strength, expressed as the reciprocal value of the minimal narcotizing concentration, increase with the increase of the relative lipoid solubility, measured by the distribution coefficient lipoid: water (see Sec. 1, chap. 5). On the basis of pharmacological considerations, the lipoid theory of narcosis was presented simultaneously and independently by H. H. Meyer.[201] Both these theories necessarily focused attention upon the plasma membrane, its chemical composition and its structure as basis for its properties.

1. The Lipoid Theory of Narcosis.—Lipoids—namely, organic substances resembling fat in their dissolving power—exist in great variety. Overton, H. H. Meyer, and many others used olive oil as a lipoid model, although, from the beginning, they were aware that this neutral fat could hardly exist as a normal component in the plasma membrane. Ether, benzene, mixtures of benzene with cholesterol or lecithin, neutral oil with the addition of oleic acid and (or) diamylamine, and others, also, have been used as model substances, especially in studies of problems of permeability, but none of them was found quite satisfactory, although in numerous experiments there appeared a rough correlation between distribution coefficient on the one side, penetration rate and narcotizing capacity on the other. According to the Meyer-Overton theory, one would expect that, as the outside concentration was raised, the narcotizing effect would become evident with all narcotics at the same molarity in the lipoid inside the cell, irrespective of the chemical formulas of the narcotics applied. For instance, in experiments with tadpoles, the critical concentration in water is reached when the tadpoles just become immobilized by the paralysis of their central nervous system. In the case of propylalcohol, the critical concentration is found to be 0.11 molar, and the distribution coefficient olive oil: water is 0.13. The critical molar concentration in the lipoids of the central nervous system should, then, be $0.11 \times 0.13 = 0.0141$. However, the following Table XX, listing values obtained in this way by K. H. Meyer and H. Gott-

[199] E. Overton, Vierteljahrsschrift d. Naturforsch. Ges., **44**: 88, 1899.

[200] E. Overton, Studien über die Narkose: G. Fischer, Jena, 1901.

[201] H. H. Meyer, Arch. f. exper. Path. u. Pharmakol., **42**: 109, 1899.

lieb-Billroth,[202] shows in the last column not more than a rough approximation to the postulated constancy of the calculated distribution ratio for the lipoid to the central nervous system, although the values in the first and second columns illustrate fairly well the principle of the lipoid theory of narcosis.

TABLE XX

	Narcotic conc. for tadpoles mol./1 water	Distrib. coeff. olive oil: water	Narcotic conc. calculated mol./1 lipoid
Ethyl alcohol	0 4	0 03	0 0120
Propyl alcohol	0 11	0 13	0 0140
Butyl alcohol	0 13	0 18	0 0230
Ethylurethane	0 04	0 03	0 0012
Valeramide	0 05	0 07	0 0035
Ether	0 024	2 4	0 0576
Benzamide	0 005	0 44	0 0022
Salicylamide	0 002	14	0 0028
Carbondisulfide	0 0005	50	0 0250
Phenylurethane	0 0006	150	0 0900
Menthol	0 0001	250	0 0300
Thymol	0 000055	600	0 0330
Average	.....		0 0245

TABLE XXI

	Narcotic conc. for mice, volume per cent in air	Solubility coeff. olive oil 37°C.	Calculated narcotic conc. mol./1 lipoid
Methane	370	0.54	0 08
Ethylene	80	1.3	0 04
Acetylene	65	1.8	0 05
Dimethyl ether	12	11.6	0 06
Methylchloride	6.5	14 0	0 07
Ethyleneoxide	5.8	31	0 07
Ethylchloride	5.0	40 5	0 08
Diethylether	3 4	50	0.07
Amylene	4 0	65	0 10
Methylal	2 8	75	0 08
Dimethylacetal	1 9	100	0 06
Carbondisulfide	1 1	160	0 07
Chloroform	0.5	265	0 05
Average	.....		0 07

It appears that the values of the last column are scattered around an average value of 0.0245, but vary between the wide limits of 0.0012 and 0.0900.

[202] K. H. Meyer and H. Gottlieb-Billroth, Ztschr. f. physiol. Chem., 112: 65, 1920.

However, in another series of experiments by K. H. Meyer and H. Hopff,[203] (Table XXI), where 17 narcotics in vapor form were administered to mice, a much better agreement with the postulates of the theory was attained, as is obvious from the fact that, for the critical molarity in the lipoid of the central nervous system, an average value of 0.07 molar was calculated, with deviations between only 0.04 and 0.10.

This result has been referred by K. H. Meyer to the fact that, at variance with the group of narcotics applied in the first-mentioned series (alcohols, esters, ether, and acid amides), the narcotics of the second series, chiefly hydrocarbons and their chlorine-derivatives, have a molecular configuration free, or nearly free, from polar groups.[204] There are several reasons, which might be discussed in this regard, but one major point seems to be that with apolar-polar solutes, e.g., alcohols, the distribution coefficient lipoid (oil) : water is more variable and more dependent upon the molar concentration applied in the experiment than it is with apolar solutes, e.g., chloroform (see Sec. 1, chap. 5 and Sec. 2).

It has been mentioned previously (p. 356) that olive oil was never assumed to be an ideal model of a cell lipoid, or even to resemble chemically a natural lipoid, and, furthermore, that in all organs and particularly in the central nervous system there certainly is a rather large group of substances, which physicochemically could be regarded as functioning as lipoids, for instance, lecithin, or, more generally, phosphatides, higher fatty acids, and sterols. Indeed, all these have served as lipoid models in various experiments, mainly concerning permeability (Overton, Ruhland, Collander, H. H. Meyer, and others). Considering investigations like these, K. H. Meyer, in continuing his studies on narcosis of tadpoles, has attempted to discover a solvent which does not display the irregularities evident in the experiments with the apolar-polar solutes (Table XVI). As a matter of fact, olein alcohol $C_{18}H_{35}OH$ has been found to have suitable properties, as appears from the following Table XVIII.[205]

In this experiment with 13 different apolar-polar narcotics, the critical narcotizing concentration in the lipoid appears to be reached with 0.03 mol./l on the average, the values scattering only between 0.013 and 0.048, although the critical narcotic concentration in water changes from 0.33 to 0.000047.

In the choice of this model lipoid, K. H. Meyer was guided by the following, or at least by a similar, consideration. As mentioned earlier, the lipoids of the nervous system are monophosphatides, diaminophosphatides, and cerebrosides, all of them containing unsaturated fatty acids with two to four double bonds (see p. 351). Tentatively adopting the idea that the combination of alcoholic OH-groups and of long C-chains may mediate the

[203] K. H. Meyer and H. Hopff, Ztschr. f. physiol. Chem., 125: 281, 1923.

[204] See: N. W. Lazarew, J. N. Lawrow, and A. P. Matwejew, Biochem. Ztschr., 217: 454, 1930.

[205] K. H. Meyer and H. Hemmi, Biochem. Ztschr., 277: 39, 1935.

theoretical behavior, K. H. Meyer decided to try, instead of olive oil, olein alcohol. Furthermore, for somewhat different reasons, the monophosphatides should be dealt with as containing the long-chain acids—oleic, linoleic, linolenic, and arachidonic acids, the unsaturated character of which possibly contributes to the lipoid property in question.[206]

TABLE XXII

	Narcotic conc. for tadpoles mol./1 water	Distrib. coeff. olein alcohol: water	Narc. conc. mol./1 lipoid calculated
Ethylalcohol	0 33	0 10	0 033
Propylalcohol	0 11	0 35	0 038
n-Butylalcohol	0 03	0 65	0 02
Valeramide	0 07	0 30	0 021
Antipyrine	0 07	0 30	0 021
Aminopyrine	0 03	1 30	0 039
Benzamide	0 013	2 50	0 033
Dial	0 01	2 40	0 024
Salicylamide	0 0033	5 90	0 021
Luminal	0 008	5 90	0 048
Adalin	0 002	6 50	0 013
o-Nitranilin	0 0025	14 00	0 035
Thymol	0 000047	950	0 045
Average			0 03

Thus it becomes suggestive to postulate that special lipoids are assigned to special functions. Narcosis appears in the nervous system of the tadpole, when the concentration of the narcotic in the lipoid in question has been raised to 0.03 molar. Evidently, this lipoid is somehow essential for the normal functioning. So far it is unknown why the dissolution of a narcotic in a lipoid to a certain concentration is accompanied by the characteristic functional alteration, viz., an inhibition of excitation, of contraction, of ciliary movement, or of protoplasmic streaming. Perhaps one can assume that the breakdown of the membrane following stimulation (pp. 315ff, 351) is the effect of the liberation of nonpolar-polar unsaturated fatty acids from the fairly unstable molecules of the lecithins, the sphingomyelins, or the cerebrosides, and that this breakdown of a structural lipoid can be inhibited by charging it with narcotic.[207]

If the results of K. H. Meyer and Hemmi compel one to believe that in the natural mixture of many fat-like solvents called lipoids, present in the nervous system (or in other organs), there is one of special functional importance, then, of course, there is no sense in determining the distribution

[206] See K. H. Meyer and H. Hemmi, Biochem. Ztschr. **277**: 309, 1935; R. Höber, M. Andersch, J. Höber, and B. Nebel, J. Cell. & Comp. Physiol., **13**: 195, 1939. See, further, pp. 337, 351, and Sec. 2.

[207] See, for instance, N. K. Adam, chap. II, sec. 31.

coefficients with the mixture extracted from the organ, as frequently has been done in order to test whether the distribution coefficients of the various narcotics are really a measure of the narcotic strength, as postulated by the lipoid theory. However, on the other hand, it will be well worthwhile continuing to study, as in the experiment of K. H. Meyer and Hemmi, the narcosis of various functions, with reference to various individual lipoids, the distribution coefficients of which are well known.[208] There is no other way to decide finally whether Overton and H. H. Meyer were justified in drawing the conclusion that practically all chemically indifferent narcotics are able to produce a certain degree of inhibition, if they have entered into their functionally essential lipoid to the same molar concentration, and, so far, there is only this one remarkable experimental series of K. H. Meyer and Hemmi in confirmation of this original deduction.

2. The Adsorption Theory of Narcosis.—A second physicochemical theory of narcosis, competing for a long time with the lipoid theory appeared after Traube, in 1904,[209] turned attention to the fact that a large group of surface active substances, i.e., substances which lower the interfacial tension between water and a second phase, include many narcotics. He also pointed out that there is an interrelationship between the narcotic strength and the surface activity (adsorbability) like that existing between narcotic strength and lipoid solubility, and, further, that there is a quantitative connection, known as Traube's rule of the homologous series, which at first was assumed not to have an analogue in the lipoid theory. The two theories even seem to exclude each other in that a solute taken up from an aqueous phase into a solvent, like a lipoid, is homogeneously distributed throughout the lipoid phase, whereas adsorption is the accumulation of a substance at the surface between the two phases. Thus, comparing the two theories, one may formulate the adsorption theory—in conformance with the original general formulation of the lipoid theory of H. H. Meyer and Overton—by saying that narcosis occurs when by raising the concentration of a narcotic, irrespective of its chemical nature, the interfacial tension is lowered to a definite point, due to its adsorption affinity toward the substrate of its inhibitory effect. In the following it will be examined whether such a conception is justified.

There are two main objections to such a general formulation of the adsorption theory. First, there are substances which produce narcosis without being adsorbable, as far as is known, upon any cell structure or constituent, and there are also substances which are adsorbable without being able to narcotize. Second, the rule of the homologous series has been

[208] Interesting results can be expected all the more, since (according to Overton) the critical narcotic concentrations in plant cells and protozoa are about six times greater than in vertebrates (E. Overton, Studien über die Narkose: G. Fischer, Jena, 1901). This striking difference may well be related to differences in the functional lipoids.

[209] I. Traube, Pflüger's Arch. f. d. ges. Physiol., **105**: 541, 1904; **132**: 511, 1910; **153**: 276, 1913, **160**: 501, 1915.

found to be significant not only for adsorption, but also for other distribution phenomena.

With regard to the first point, as has been mentioned before (p. 358), a series of hydrocarbons and their Cl-derivatives, like methane, ethylene, acetylene, ethylchloride, chloroform, carbon tetrachloride, are surface inactive, viz., they do not lower the interfacial tension at the phase boundary water-benzene or water-oil,[210] or water-air,[211] although they are narcotics with their narcotic strength increasing in a homologous series with the length of the C-atom chain, as shown, e.g., by the following table.

TABLE XXIII

	Isonarcotic concentrations for mice		
	Mol. weight	Mol./l in air	Q
Pentane	72	0 0052	3 1
Hexane	86	0 0017	2 8
Heptane	100	0 00064	2.0
Octane	114	0 00032	

Further, in a series of 11 narcotic substances examined by Lazarew and co-workers, there are only two which slightly lower the interfacial tension, cyclohexane C_6H_{10}, which has two double bonds, and ether containing oxygen. Evidently, only the apolar or nearly apolar narcotics are surface inactive. It follows that adsorption cannot be an unequivocal preconditioning factor in narcosis. On the other hand, substances which lower the tension of a phase boundary are not of necessity narcotics. Adsorbability is evidently bound up with a nonpolar-polar molecular configuration. It is this configuration, which brings about a surplus concentration of the molecules in the interface by anchoring the polar hydrophilic part in the aqueous phase, while the apolar part has a tendency to enter the second phase. How much this concentration can be effectuated, seems to be a matter of the mutual affinities of the molecular groups. For instance, naphthalene sulfonates,[212] cetylsulfate, and the salts of fatty acids with C-atom chains longer than about C_6, are surface active, but ineffective as narcotics. However, they are cytolytics, e.g., hemolytics, and display this property according to their surface activity; isohemolytic solutions are isocapillary.[213] But, with narcotics like the monovalent aliphatic alcohols and numerous others, isocapillary solutions have been shown to be also "isonarcotic." Possibly this is due to the fact that narcotics are nonelectrolytes, the Van der Waals forces of which, operating in these nonpolar-polar molecules in opposite directions, are not strong enough and not enough

[210] N. W. Lazarew, J. N. Lawrow, and A. P. Matwejew, Biochem. Ztschr., **217**: 454, 1930.
[211] H. Führer, Biochem. Ztschr., **115**: 235, 1921; **120**: 143, 1921.
[212] See K. H. Meyer and H. Hemmi, Biochem. Ztschr., **277**: 39, 1935.
[213] See, e.g., M. Bodansky, J. Cell. & Comp. Physiol., **1**: 429, 1932.

remote from each other to prevent the nonpolar (lipophilic) groups from entering the lipoid phase.[214]

As to the second point, Traube,[215] investigating the capillary activity of organic compounds by the stalagmometric method, was struck by the fact that in a homologous series of surface active nonelectrolytes (e.g., alcohols) the concentrations of isocapillary and of isonarcotic solutions differ from each other by a factor (Q), approximately equal to 3, as is illustrated in the following Table XX, showing the decrease of O_2-consumption of goose erythrocytes and the inhibition of the frog heart.[216]

TABLE XXIV.—CAPILLARACTIVITY AND NARCOSIS

Narcotic	O_2 consumption goose erythr. 50% inhibition by mols.	Rel. capillary depression (water eq. 100)	Q	Inhibition frog heart by mols.	Drop count (water eq. 39)	Q
CH_3OH	5.0	31	3	3 74	50.5	3
C_2H_5OH	1 6	28	2	1 21	51.25	3
C_3H_7OH	0 8	35	5	0 37	51.5	3
C_4H_9OH	0 15	28	3	0 11	51.25	3
$C_5H_{11}OH$	0 045	28		0 039	52.25	
$C_7H_{15}OH$	.		..	0 003	51 25	

This can be interpreted as significant to adsorption. In a homologous series, the addition of one CH_2 group increases the interfacial activity; in other words, increases the distribution coefficient surface : water by a certain factor (Q) in such a way that as the number of CH_2 groups progresses arithmetically, the value of the distribution coefficients progresses geometrically (e.g., $1:3:3^2:$). The numerical value of this factor (Q) is determined by the differences of the secondary valences of the nonpolar and the polar parts of the molecules involved. These account for the energy required to bring the substances in question into equilibrium with the two phases.[217] For this reason, with other distribution phenomena, other factors Q have been encountered,[218] as appears in the following Table XXV.

It is obvious that Traube's rule does not refer specifically to adsorption, but that it is significant for various distribution phenomena, including the distribution organic solvent : water.

In general, it is observed that the amount of the substance adsorbed from a solution to the surface of the second phase, is not proportional to

[214] See also W. D. Harkins, G. L. Clark, and L. E. Roberts, J. Am. Chem. Soc., **42**: 700, 1920.

[215] I. Traube, Liebig's Ann. d. Chem., **265**: 27, 1891; Pflüger's f. d. ges. Physiol., Arch. **105**: 541, 1904; **132**: 511, 1910; **153**: 276, 1913.

[216] O. Warburg, Biochem. Ztschr., **119**: 134, 1921; H. Fühner, *ibid.*, **120**: 143, 1921.

[217] Frumkin, Ztschr. f. physik. Chem., **116**: 501, 1925.

[218] See H. Freundlich, Colloid and Capillary Chemistry: E. P. Dutton and Co., New York, 1926; further, K. H. Meyer and H. H. Hemmi, Biochem. Ztschr. **277**: 39, 1935; A. J. Clark in Handb. Exper. Pharm., vol. 4: Springer, Berlin, 1937.

its concentration, but that an exponential curve, the adsorption isotherm (Freundlich),[218] accounts for the distribution in such a way that the distribution coefficient surface : water decreases with increasing concentration in the solution. So far as the adsorption of a substance to a cell or to a cell component is indicated by a functional alteration, it appears that the concentration action curve is not a straight line, as it would be, if, for instance, a narcotic were taken up by a cell, instead of remaining attached to the interface, and entered a lipoid phase with a distribution coefficient lipoid : water = constant (see, e.g., Table XXVI).

TABLE XXV.—DISTRIBUTION COEFFICIENTS

Distrib. coeff. surface/water (reciproc. molarity of iso-capillary solutions)		Distrib. coeff. benzene: water		Distrib. coeff. cotton seed oil: water	
CH_3OH	0 07	CH_3COOH	0 055	CH_3OH	0.0097
C_2H_5OH	0.20	C_2H_5COOH	0 28	C_2H_5OH	0 0357
C_3H_7OH	0 62	C_3H_7COOH	1 08	C_3H_7OH	0 156
C_4H_9OH	2 20	C_4H_9COOH	3 73	i-C_4H_9OH	0 588
i-$C_5H_{11}OH$	7 00	C_6H_4COOH	16 1	i-$C_5H_{11}OH$	2 13

Distrib. coeff. gaseous phase: water (100°)		Distrib. coeff. enzyme: water (50 per cent inhibition of succino-dehydrogenase)	
HCOOH	0 38	CH_3OH	6
CH_3COOH	0 69	C_2H_5OH	2 2
C_2H_5COOH	1 28	C_3H_7OH	0 8
C_3H_7COOH	2 00	C_4H_9OH	0.4
C_4H_9COOH	3 57		

TABLE XXVI.—DISTRIBUTION OF ACETONE BETWEEN WATER AND TRICHLORETHYLENE

C_W	C_T	$C_T : C_W$
0 160	0 193	1 206
0 350	0 359	1 025
0 654	0 719	1 100
0 940	1 029	1.090
1 389	1.562	1.128

However, in adsorption processes neither a straight line nor an exponential correlation can be expected definitely. There are numerous deviations from the usual isotherm relationship, just as the distribution coefficient solvent : water likewise is by no means with regularity a constant (see, e.g., Table XXVII) (see, further, Sec. 1, chap. 5). It has been pointed out (K. H. Meyer and H. H. Hemmi) that theoretically the straight line relationship is more likely to be observed in adsorption experiments dealing with relatively low concentrations of the adsorbendum, whereas, at higher

concentration ranges, the concentration action curve should resemble more the common isotherm. These theoretical conclusions seem to be justified by observations concerning the "narcosis" of enzymes. Here frequently the poisoning of an enzyme with various concentrations of the inhibitory agent has given evidence of the typical exponential curve of the adsorption isotherm. But, on the other hand, e.g., the concentration action curve of invertase in water, inactivated progressively by raising the concentration of ethyl alcohol, approximates at lower concentrations a linear relation, and only at high concentrations does the exponential relation appear[219] as the maximal effect is approached.

TABLE XXVII.—DISTRIBUTION OF ACETONE BETWEEN WATER AND CHLOROFORM

C_w	C_c	$C_c : C_w$
0 032	0 168	5 26
0 078	0 399	5 11
0 145	0 676	4 65
0 263	1 17	4 44
0 493	1 98	4.01
1 010	3 06	3 02

Thus we reach the conclusion that between the two main rival theories of narcosis brought about by the typically "indifferent" narcotics (p. 355) there is no definite choice except in the case of the nonpolar-polar narcotics, which seem to be effective exclusively on the basis of a solubility in certain lipoids.[220] But so far this has been shown only by experiments upon the central nervous system.

On the other hand, in reconsidering that the polar groups confer to narcotic molecules surface activity or interfacial activity, a special role is assigned to these which was indicated already by applying the often-rejected term of "narcosis of enzymes." The narcotics in this case, as in many others, are believed to inactivate the enzymes, as microheterogeneous structures or any other cellular structures, irrespective of any lipoids being present or absent, by their attachment to the surface, so that the substrate present in the fluid parts cannot reach the site of reaction.[221]

From the viewpoint of adsorption, this is quite a clarifying conception, which, by various modifications, allows an approximation to the usual manifestations of narcosis. For instance, the enzyme invertase can be

[219] See O. Meyerhof, Pflüger's Arch. f. d. ges. Physiol., **157**: 251, 1914. Concerning serum lipase, see P. Rona and A. Lasnitski, Biochem. Ztschr., **163**: 197, 1925. Further, H. Winterstein, Neuere Untersuchungen zur Theorie der Narkose: Advances in Modern Biology, **5**: No. 6, 1936. A. J. Clark in Handb. Exper. Pharm., Bd. 4: Springer, Berlin, 1937; Tr. Faraday Soc., **33**: 1057, 1937.

[220] See also the discussion between K. H. Meyer and I. Traube, Biochem. Ztschr., **282**: 444, 445, 447, 1935.

[221] O. Warburg, Biochem. Ztschr., **119**: 134, 1921.

inactivated by appropriate amounts of narcotics.[222] But this is due to the fact that the ordinary invertase preparations are contaminated by proteins which present a colloidal phase for the adsorption of the narcotics. For, after careful purification of the enzyme, the sensitivity to narcotics disappears, but is re-established by the addition of albumin or globulin.[223] Further, it has been demonstrated by many experiments that the inhibitory effect of narcotics upon cell reactions decreases progressively upon destroying the structural constituents of the cells by cutting, grinding, macerating, and expressing the protoplasm, as it has been shown particularly with regard to cell respiration, or, rather, to the oxygen uptake. This has been interpreted as caused by the diminution of adsorbing areas. Also, it has been observed that, in such a mass, narcotics of low solubility, like thymol, toluene, phenylurethane, which are known to stop growth and development of intact cells, appear to leave some enzymatic activity intact, instead of blocking it.[224]

However, in experiments of this kind it must be borne in mind that the refractory behavior toward narcotics could be referred also to the striking specificity of the reactions of enzymes. For instance, the uptake of O_2 by minced brain is inhibited by luminal with glucose, lactate, or pyruvate present as substrate, but not with succinate;[225] and the oxygen uptake of brain is inhibited by ethylurethane, but not that of yeast (Quastel).

TABLE XXVIII.—THRESHOLD CONCENTRATIONS FOR IMMOBILIZING ARENICOLA LARVÆ

		*p*H = 7	8	9
Alcohols				
i-Propylalcohol	cc./100 cc.	2.5	2 5	2 5
i-Amylalcohol	cc./100 cc.	0 1	0 1	0 1
Chloretone	cc./100 cc.	0.025	0 025	0 025
Alkaloids				
Cocaine	gr./100 cc.	0 01	0 003	0 0025
Procaine	gr./100 cc.	0.002	0 001	0 0005
Barbituric acids				
i-Amyl ethyl	gr./100 cc.	0 006	0 025	0 05
Propyl methyl carbinyl ethyl	gr./100 cc.	0 003	0 006	0 012

Finally, attention has been given to the influence of *p*H, as it is illustrated, e.g., by experiments on a low animal, the larva of *Arenicola*.[226] The above Table XXVIII indicates the minimum concentration of a series of

[222] O. Meyerhof, Pflüger's Arch. f. d. ges. Physiol., **157**: 251, 1914.

[223] A. Schürmeyer, Pflüger's Arch. f. d. ges. Physiol., **208**: 595, 1925; see, further, O. Warburg and O. Meyerhof, *ibid.*, **148**: 295, 1912; O. Warburg and R. Wiesel, *ibid.*, **144**: 465, 1912.

[224] O. Warburg and O. Meyerhof, Pflüger's Arch. f. d. ges. Physiol., **148**: 295, 1912; O. Warburg, *ibid.*, **154**: 599, 1913; **158**: 19, 1914.

[225] J. H. Quastel, Physiol. Rev., **19**: 135, 1939; also F. A. Fuhrman and J. Field, J Cell. & Comp. Physiol., **19**: 1942.

[226] G. H. A. Clowes and A. Keltch, Proc. Soc. Exper. Biol. & Med., **29**: 312, 1931.

compounds at three different *p*H-values, which immobilize the larvæ within five minutes.

It is obvious that the threshold concentrations of the alcohols are independent of the *p*H, that the paralyzing effect of the alkaloids increases with rising *p*H, and that of the barbituric acids decreases with rising *p*H. This is in agreement with both the lipoid theory and the adsorption theory of narcosis, as the undissociated acid is increased at the lower, the undissociated base at the higher, *p*H, and as the undissociated compounds are more adsorbable and more lipoid soluble than the ionized salts.[227] But, by mentioning these experiments, we already cross the border line confining this discussion (see p. 355) to studies concerning the "indifferent" narcotics, which react with the cells only by secondary valences.

3. Permeability and Narcosis.—The most common aspect of narcosis is that of abolishing excitability and of blocking the excitatory process. Since, on the broadening basis of an increasing amount of experimental data, it has been rather generally accepted that excitation is accompanied by a rise of permeability, particularly of permeability to ions, it is suggestive to believe that narcosis is dependent upon the adsorption of the interfacially active narcotics to those structures, in which the excitatory process takes place, and the resultant exclusion of these from the complex of chemical and physicochemical reactions, constituting the process of excitation. In this respect, narcosis is a decrease of permeability, which, considering the electrical manifestations of muscle and nerve, may be thought of as being a decrease of permeability to ions, but also may include permeability to other solutes, and to water.

The adequacy of such a concept can be tested in model experiments on sieve-like membranes, the pore diameter of which, due to the fairly great rigidity of the building material, is stable and approaches molecular dimensions, as in the dried collodion membrane of Michaelis.

The following observations[228] are especially pertinent. With membranes of a certain porosity, the rate of diffusion of glucose during the first hours or days decreased progressively to a low constant value. This can be considered as due to the fact that these artificial membranes, instead of having pores with a uniform diameter, contain an assortment of pores of different widths, so that only a small percentage of the pores is available to the relatively large glucose molecules, and that, gradually, more and more of these channels become clogged by the sugar molecules.

Correspondingly, one may assume that interfacially active substances, such as many narcotics, form an adsorption layer upon the pore walls and, by narrowing the aperture in this way, obstruct, or even completely block, the passage through the channels to a degree dependent upon the molecular volume of the penetrating substances. Even small molecules, such as water, could be prevented from entering.

[227] See, further, G. H. A. Clowes, A. K. Keltch, and M. B. Krahl, J. Pharm., **68**: 312, 1940.

[228] A. A. Weech and L. Michaelis, J. Gen. Physiol., **12**: 55, 221, 1928.

Some experiments of Anselmino[229] seem in favor of this assumption. In one set of experiments with test-tube-shaped collodion vessels filled with 0.5 molar sucrose solution, the rate of osmosis of water was studied with and without urethanes (C_3 to C_5) or alcohols (C_4 to C_7) in isocapillary concentrations on both sides of the sac. The osmosis was markedly and reversibly retarded by the narcotics. In another set of experiments on the rate of diffusion of SCN and Cl ion, similar results were obtained. One membrane was found completely impermeable to SCN after the addition of 0.1 mol. butylurethane.[230] Furthermore, there are quite a number of experiments on physiological objects, the results of which resemble the experiments on the artificial membranes. Some of these are concerned with the entrance or the escape of substances across the surface of red blood corpuscles. The slight spontaneous loss of electrolytes, which follows rinsing the corpuscles with sucrose solutions, and which is indicated by conductivity measurements, is diminished by a proper amount of narcotic.[231] Likewise, the exchange of SO_4 and Cl across the selectively anionpermeable surface is retarded by various narcotics (urethanes, substituted ureas, and alcohols).[232] The volume increase following the suspension of (human) erythrocytes in solutions of slowly penetrating nonelectrolytes (glycerol, erythritol, xylose, arabinose) (see Sec. 4, p. 240) also is slowed down in isocapillary concentrations of alcohols and urethanes.[233]

To the interpretation of all these observations one principal objection may be made: the observed changes refer to rates, whereas narcosis is, in essence, an equilibrium state in which time does not play a role. With a particular drug concentration one can establish a certain state of either complete or partial narcosis for an indefinite length of time. However, under certain conditions, rate measurements, also, are doubtless indicative of narcosis in the ordinary sense of being an equilibrium state. For instance, in the experiments of Anselmino and Hoenig, swelling of erythrocytes in glycerol solution is due to

[229] K. J. Anselmino, Pflüger's Arch. f. d. ges. Physiol., **220**: 525, 1928; also **220**: 633, 1928.

[230] These experiments have been repeated by E. Ponder and J. C. Abels (Proc. Soc Exper. Biol. & Med., **36**: 551, 1937) with results conflicting with the conclusion which may be drawn from the experiments of Anselmino. Urethanes again were found to retard the diffusion rate of SCN, but the diffusion of other anions (including Cl) and cations remained quite unaffected. In nearly all the model experiments with collodion membranes, the membrane material has been considered to present a rigid sieve structure (Sec. 5, pp. 317ff). However, recently, evidence has been provided (see K. Sollner and P. W. Beck, J. Gen. Physiol., **27**: 451, 1944) that certain organic nonelectrolytes and electrolytes can enter the membrane substance as an organic solvent, they can be accumulated in the substance and can cause the membrane to swell, or they can be accumulated by adsorption at the internal interfaces. But, hydrophilic organic compounds, like sugars, polyhydric alcohols, and anions of hydroxyl carboxylic acids (p. 247) are inert; organophilic ("carbophilic") compounds (e.g., valeric acid, valeramide, amylalcohol) are active. These processes, at least beyond a certain concentration range, must reflect upon the permeability.

[231] A. Joel, Pflüger's Arch. f. d. ges. Physiol., **161**: 5, 1915.

[232] R. Siebeck, Arch. f. Exper. Path. u. Pharmakol., **95**: 93, 1922.

[233] K. J. Anselmino and E. Hoenig, Pflüger's Arch. f. d. ges. Physiol., **225**: 56, 1930.

the entrance of glycerol, accompanied by the entrance of water, until hemolysis occurs. This swelling is retarded more and more by increasing concentrations of alcohols according to the length of their C-chains (C_3 to C_7). Thus very small concentrations of heptylalcohol suffice to prevent the hemolysis for a long time. It is probable that nearly the entire assortment of pores of the sieve-like membrane is occluded by the adsorption to their walls of these large molecules, and a nearly complete "narcosis" of the ordinary exchange is similar to that mentioned before concerning the diffusion of SCN across a collodion membrane, and its blocking by butylurethane. Thus, the decrement of rates in these series of experiments can be interpreted to mean an approach to narcosis as an equilibrium.[234]

Additional evidence about the increase of the resistance to the passage of solutes caused by the adsorption of narcotics in the pathways which extend from the exterior to the internal surface structure of cells and tissues is furnished by studies concerning the bioelectrical properties. Old observations[235] on the resting potential of sartorius muscles, which arises during the local treatment with inorganic neutral salts (p. 313), and which to some extent can be compared to the normal reversible electric reaction after excitation, have shown that the curve of its gradual development takes a more flattened course in the presence of a narcotic. This, again, can be accounted for by assuming that adsorption of the narcotics decreases the porosity of the structures in question. The same problem has been studied by measuring the high frequency resistance (impedance) of sartorius muscles with and without the presence of narcotics.[236] It was found that low concentrations of a narcotic bring about a reversible increase of the electric resistance; higher concentrations, an often irreversible decrease. These alterations can be referred to effects upon the membrane of the fibers, because the increase or decrease of the resistance is accompanied by only a slight change of the electrical capacity; this corresponds to the changes during excitation (*Nitella*, squid giant nerve fiber), where the breakdown of the membrane resistance is, again, accompanied by not more than a slight decrease of the capacity.[237]

In this connection it should be mentioned that permeability is affected by narcosis in a special way in the case of active transfer (see Sec. 8). Thus, certain substances fail to penetrate the surfaces of cells or organs,

[234] Concerning a more thorough discussion of the distinction between "rate" and "equilibrium," see M. H. Jacobs and A. K. Parpart, Biol. Bull., **62**: 313, 1932. Cf. further, in regard to decrease of permeability, the experiments on fish eggs by J. F. McClendon, Am. J. Physiol., **38**: 173, 1915, and on plant cells by Lepeschkin, Ber. dtsch. bot. Ges., **29**: 349, 1911, A. Tröndle, Biochem. Ztschr., **112**: 259, 1920 and H. Lullies, Pflüger's Arch. f. d. ges. Physiol., **207**: 8, 1925.

[235] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **120**: 492, 1907; further, T. Seo, *ibid.*, **205**: 485, 1924.

[236] R. Guttman, J. Gen. Physiol., **22**: 567, 1939. See also W. J. V. Osterhout, Science, **37**: 111, 1913; also Botan. Gazette, **61**: 148, 1916; J. Gen. Physiol., **1**: 299, 1919. Further, H. Winterstein and E. Hirschberg, Pflüger's Arch. f. d. ges. Physiol., **217**: 216, 1927.

[237] K. S. Cole and H. J. Curtis, J. Gen. Physiol., **22**: 37, 1938, **22**: 469, 1939. For blood corpuscles, see Sec. 4, p. 278.

unless energy is made available for the transfer by metabolic reactions. These sometimes, but not regularly, are initiated by nervous excitation. It was mentioned earlier (p. 366) that in nerves it is the self-propagating complex of chemical and physicochemical reactions, the excitation wave, released upon a stimulus, which is possibly stopped by the layer of the adsorbed narcotic molecules covering the nerve membranes. In a corresponding manner, the energy-providing aggregates of molecular structures in actively absorbing and secreting organs may be thrown out of action by narcotics.

In contrast to the so far referred ample and various evidence that permeability is decreased by narcotics, there are several observations displaying increase under conditions where there is no reason to assume that the results are due to impairment by excessive concentrations. Thus, according to Jacobs and Parpart,[238] the permeability to glycerol of the erythrocytes of certain animals (man, rat, rabbit, guinea-pig) is decreased by n-butyl alcohol in a certain range of concentrations, whilst that of ox, sheep, pig, dog, cat is increased (see also regarding cat's corpuscles, H. Davson, J. Cell. & Comp. Physiol., **15**: 317, 1940); and, according to Bärlund,[239] the permeability of cells of *Chara* to ethylene glycol, urea, trimethylcitrate, and hexamethylentetramine, substances which can enter the cells by simple diffusion, is increased by ether applied in such a concentration that the protoplasmic streaming in the cells is reversibly slowed (though not more than 10 to 50 per cent), whereas the permeability to Li ion under the same conditions is unchanged, or possibly slightly decreased. These conflicting effects need further investigation.

In conclusion, although in this chapter the adsorption theory of narcosis has been emphasized, the lipoid theory must be given due consideration inasmuch as certain lipoids appear to lose their ability to take part in the excitatory process through preferential dissolution of the narcotics (p. 359).

Furthermore, it should be mentioned that the concept of narcosis is sometimes applied in a much broader sense. For instance, in speaking of *Mg narcosis*, or of *electronarcosis* of the central nervous system, one is confronted with the question, whether the associated phenomena of reversible paralysis can be paralleled to those treated in this chapter. From the viewpoint of traditional classification, these effects certainly cannot be recognized as narcoses. The classical types of narcotics are organic nonelectrolytes, which, according to the studies of the last 40 years, have been found to act in the polyphasic system of cells and tissues, either by adsorption at the phase boundaries or by distribution to the lipoid phases. But, should an extension of the conventional concept of narcosis appear useful, it would be adequate to supplement the adsorption and the lipoid theories of narcosis by a colloid theory, taking into account that, for example, Mg ions or other inorganic ions, which, for the purpose of electronarcosis, are pushed by an

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[238] M. H. Jacobs and A. K. Parpart, Biol., **73**: 380, 1937.

[239] H. Bärlund, Protoplasma, **30**: 70, 1938.

electric current through a living tissue, are very likely to act upon the colloidal constituents of the cellular interphase, where adsorption or solution in lipoids likewise takes place. However, the result would be an excessive generalization of the term "narcosis," since many other reversible inactivations by ions, the paralysis by K or by nonpolar-polar organic ions, or the cathodal depression of nerve excitability, and others, also, would have to be called narcoses.

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Section 6

THE RESPIRATION OF CELLS AND TISSUES

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INTRODUCTION

Most of our knowledge of the mechanism of cellular respiration has been obtained during the last twenty years. One of the most striking features of the modern picture of cellular respiration is the similarity of its mechanism, for, in spite of minor variations which occur among different organisms, the major theme is common to all. A priori, one has no reason for expecting essentially the same mechanism of respiration in bacteria, yeasts, the higher plants, and man. Increasing knowledge may at first seem to result only in increasing complexity, but, as the picture nears completion, its universality results in a profound simplification.

During an earlier period, biochemists searched for an enzyme which would directly catalyze the complete oxidation of glucose by molecular oxygen. No such enzyme has been discovered, nor is it likely that such an enzyme exists. The studies of the last two decades have shown with great clarity that the oxidation of organic foods occurs within living cells by a series of graded steps, involving many distinct enzymes, each reacting in a cyclical manner, and the whole constituting an integrated reaction system, smoothly oxidizing sugars to carbon dioxide and water. These studies have shown that many intermediate compounds are formed along the pathway of glucose oxidation, and that these intermediate molecules are rapidly formed and rapidly degraded, with specific enzymes controlling the rate of formation and degradation of each substance. Each partial reaction of formation and of degradation must have a velocity at least equal to the velocity of the over-all reaction of respiration. Not only is the life of an intermediate molecule short, but its concentration may be very low.

The series of graded chemical reactions results in a stepwise release of the potential energy of the organic foods, rather than its release in a single explosive reaction. The stepwise liberation of energy results in efficient utilization, for the transfer or coupling of the energy will be most efficient when the reactions are operating at or near their equilibrium points. Though this ideal situation will be rarely attained, it is much closer of realization in a series of graded steps, than in one over-all reaction.

Living cells are unique among the engines known to man, for the living engines are constructed of the same kinds of materials that are used for fuel. If the fires of life burned too brightly, not only the fuel, but the engines themselves, would be consumed. If the potential energy of a sugar molecule were to be released in a single reaction, the released energy might frequently activate the structural molecules of the cell, resulting in their destruction by oxidation. The series of graded reactions never

liberates enough energy in any one molecular reaction to surpass the activation energy of the structural molecules, and their stability is not endangered.

Physical chemistry furnishes a theoretical basis for understanding the apparent complexity of cellular respiration, its kinetics, and its mechanism of energy transfer. Perhaps, physical chemistry has served even more in furnishing powerful tools to probe within the living cell and to separate and study the individual enzymes. Such tools as the spectroscope, spectrophotometer, ultracentrifuge, and electrophoretic apparatus have been used in conjunction with the more usual biochemical methods, and, as a result, the physicochemical data and interpretations are so intertwined with the biochemical that any attempt to unravel them would destroy the fabric, leaving isolated threads with little meaning. In the pages which follow, an attempt will be made to present the general pattern of cellular respiration, with an effort to emphasize the physicochemical approach, whether its contributions be theoretical or as tools in the hands of biochemists.

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THE NATURE OF RESPIRATION

1. The Rate of Respiration.—The rate of respiration is normally measured by following the uptake of oxygen per unit time. Some typical examples are shown in Table XXIX. Considerable disagreement exists as to the best method of comparison between organisms. Frequently the comparison is made on the basis of the O_2 consumed/mg. or gm. dry weight/hour. Reference to dry weight is fairly satisfactory, but the amount of non-protoplasmic material, such as cellulose walls, hair, shells, etc., is very variable between organisms. In many cases wet weight is equally satisfactory and is sometimes preferable. For some purposes, the amount of oxygen consumed is referred to unit total N or protein N. This is usually a matter of convenience, though at times it is more reliable than dry or wet weight.

It is observed that in mammals the respiration per unit weight of small species is much higher than that of large species. Brody[1] has proposed that the basal metabolism of adult mammals is related to the weight as follows:

$$M = aW^{0.734}.$$

When M = Cal.[2a]/24 hrs. and W = weight in kg.,
then $a = 70.5$.
When M = liters O_2/kg./hr.,

and the conversion factor of 4.825 Cal./liter O_2 is used, then

$$a = 0.609.$$

This equation appears to be valid to about ± 11 per cent for all mammals from 0.02 kg. mice to 600 kg. cattle and horses. This is illustrated in Fig. 32, taken from Brody, and shows two curves, the increasing metabolism with increasing weight on a double log plot, and the falling metabolism per kg. weight. Thus the metabolism of a mouse is 229 Cals./kg./24 hrs.; and using Benedict's[2] figure, it is 13 Cals./kg./24 hrs. for a 3672 kg. elephant.

The metabolism of birds likewise decreases per unit weight with increasing size. Their metabolism is not represented by the same curve as for

[1] Samuel Brody, *et al.*, Univ. Missouri Agric. Exper. Sta. Res. Bull., No. **166**: 1, 1932; *ibid.*, No. **335**: 8, 1941.

[2] F. Benedict, The Physiology of the Elephant: Carnegie Inst. of Washington, 1936.

[2a] In this Section, Cal. = kgm. cal.

TABLE XXIX.—RESPIRATORY RATES FOR DIFFERENT ORGANISMS

Organisms	Temp.	mm.^{3} O_2/gm. wet wt./hr.	mm.^{3} O_2/gm. dry wt./hr.
Azotobacter[1]	28°C.		200,000 to 4,000,000
Chlorella[2] pyrenoidea			
Endogenous	25°C.		2,200
Exogenous			17,000
Euglena gracilis[3]	25°C.	800 to 1,000	
Paramecium[4]	20°C.	500	
Baker's yeast[5]			
In glucose	28°C.		40,000 to 80,000
Baker's yeast[5]			
Endogenous	28°C.		400 to 800
Neurospora[6]			
Spores, dormant	25°C.	90	250
Spores, germinating	25°C.	7,056	19,600
Carrot root[7]	25°C.	25 to 30	
Carrot leaves[8]			
Young	22°C.	1,133	
Mature	22°C.	439	
Barley seeds[9]			
Dormant	25°C.	0 06	
Germinating	25°C.	108 00	
Pike[4]	20°C.	350	
Frog[10]			
Muscle at rest	22°C.	39 7	
Contracting	22°C.	150	
Mouse[4]			
At rest	37°C.	2,500	
Running	37°C.	20,000	
Cat[11]			
Soleus muscle, at rest	37°C.	230	
Soleus muscle, active	37°C.	4,752	
Man[4]			
At rest	37°C.	200	
Maximal work	37°C.	4,000	
Elephant[12]			
Standing at rest	37°C.	148	

[1] O. Meyerhof, Biochem. Ztschr., **256**: 35, 1932.

[2] L. Genevois, *ibid.*, **186**: 461, 1927.

[3] D. R. Goddard. Unpublished.

[4] A. Krogh, Comparative Physiology of Respiratory Mechanisms: Univ. Pennsylvania Press, 1941.

[5] C. A. Elvehjem, J. Biol. Chem., **90**: 111, 1931.

[6] D. R. Goddard and P. E. Smith, Plant Physiol., **13**: 241, 1938.

[7] S. J. Turner, Australian J. Exper. Biol. Med. Sc., **18**: 273, 1940.

[8] P. Marsh and D. R. Goddard, Am. J. Bot., **25**: 724, 1939.

[9] J. D. Merry and D. R. Goddard, Proc. Rochester Acad. Sc., **8**: 28, 1941.

[10] J. N. Stannard, Am. J. Physiol., **125**: 196, 1939.

[11] G. A. Millikan, Proc. Roy. Soc., London, **B123**: 218, 1937.

[12] F. Benedict, Physiology of Elephant: Carnegie Inst., Washington, 1936.

mammals. Brody and Proctor[3] give the relation between metabolism for 24 hrs. in cal. and weight in kg. for birds as:

$$M = 89W^{0.73}.$$

Rubner[4] and Voit[5] proposed that for mammals the basal metabolism is constant for unit surface area. The historical background has been sum-

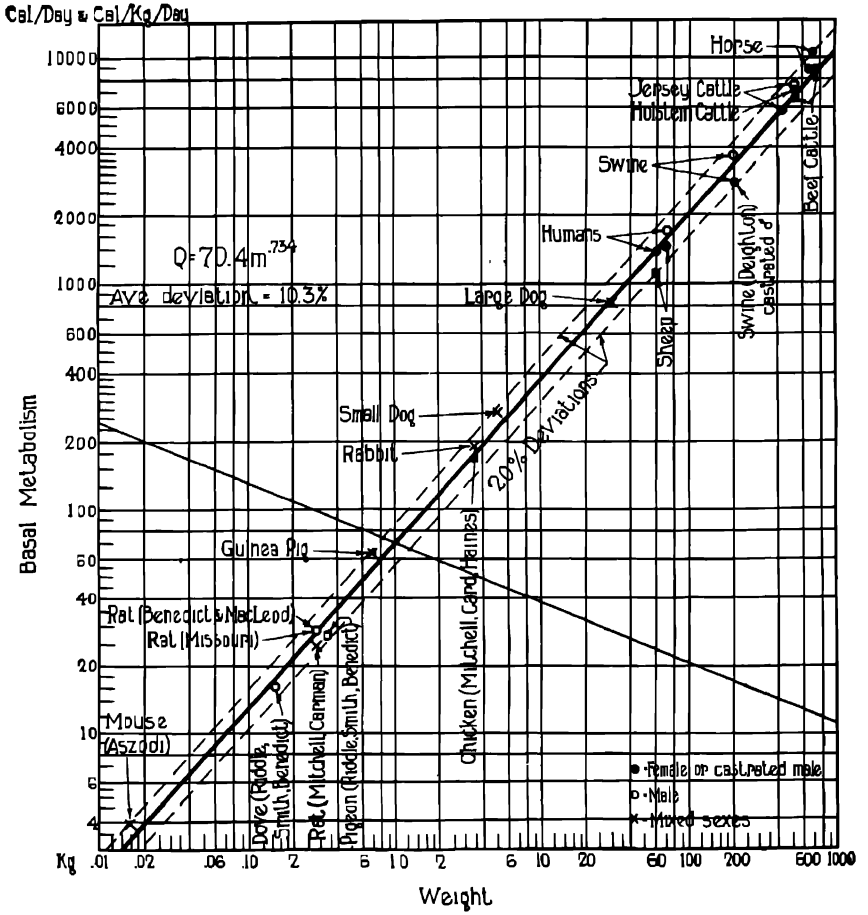

FIG. 32.—Double log plot of the basal metabolism of adult mammals against body weight, rising curve. Falling curve, basal metabolism 1 kgm. body weight/day. (From S. Brody and R. C. Proctor, Univ. of Missouri Agric. Exper. Stat. Res. Bull., No. **166**: 89, 1932.)

marized by Du Bois[6] and Lusk.[7] The Du Bois height/weight formula gives excellent results in predicting the metabolism for men, and the surface area formula is of greatest value as a basis of comparison of the respiration

[3] S. Brody and R. C. Proctor, Univ. Missouri Agric. Exper. Sta. Res. Bull., No. **166**: 89, 1932.

[4] M. Rubner, Biol., **19**: 549, 1883.

[5] E. Voit, *Ibid.*, **41**: 120, 1901.

[6] E. F. Du Bois, Basal Metabolism in Health and Disease: Philadelphia, 1924.

[7] G. Lusk, Science of Nutrition: Philadelphia, 1924.

of large and small animals within a species. For comparisons between adult individuals of different species, surface area seems to be no better than Brody's weight formula.

The surface area comparison is only valid for related organisms; the metabolism of cold-blooded vertebrates may not be predicted by an equation used for mammals. The surface area concept has been used uncritically in an attempt to explain the high respiration of some microorganisms, without the realization that different microorganisms with similar surface areas per cell may differ in their metabolism by 1000 times. For lower organisms which do not store food, there is a real question of what constitutes the basal metabolism, for the metabolism may be 100 times as great in the presence of an exogenous substrate as is the endogenous rate.

TABLE XXX.—RESPIRATORY RATE OF DIFFERENT TISSUES

Organism	Temp.	Tissue	mm.^{3} O_2/gm. wet wt./hr.	mm.^{3} O_2/gm. dry wt./hr.
Ash[1]	25°C.	Phloem	167	
		Cambium	220	
		Outer xylem	78	
		Inner xylem	31	
Barley[2]	22°C.	Endosperm	77	
		Embryo	715	
Frog[3,5]	22°C.	Nerve	48	
		Muscle	39 7	
		Kidney	276	
		Liver	269	
		Heart	143	
		Brain	500	
Rat[4,5]	37°C.	Kidney	.....	21,000
		Brain	2900	14,000
		Liver	.....	9,000
		Lung	.....	6,000
		Large intestine	.....	3,000
		Nerve	180	

[1] R. H. Goodwin and D. R. Goddard, Am. J. Bot., **27**: 234, 1940.
[2] J. D. Merry and D. R. Goddard, Proc. Rochester Acad. Sc., **8**: 24, 1941
[3] A. Rotta and J. N. Stannard, Am. J. Physiol., **127**: 281, 1939.
[4] H. A. Krebs, Tabulæ Biologicæ, **9**: 200, 1939.
[5] R. W. Gerard, Proc. Assn. Res. Nervous Mental Dis., **18**: 316, 1937.

a). *Different tissues.*—Striking differences in respiratory rate are found in different tissues in the same organism. For example, Table XXX shows the differences between xylem and phloem or cambium in stem tissues, or of muscle and nerve of frog in comparison with kidney and liver.

b). *Activity.*—Even more striking changes in respiratory rate occur in the same tissue upon changed activity. Dormant cells, tissues, or organisms usually have a low rate of respiration, and when growth occurs there is a sudden burst of respiration. This is illustrated by the increased respira-

tion upon fertilization of sea-urchin eggs,[8] or the breaking of diapause in grasshopper embryos.[9] A striking illustration occurs when dormant seeds germinate. Merry and Goddard[10] found a rate of 0.06 mm.^{3}/gm./hr. for dry barley seeds. One hour after adding water the rate had increased 386 times, and in 24 hours to 6500 times the dormant rate.

Perhaps the best-known example of explosive changes in respiratory rate are those associated with muscular activity. The rate in frog muscle increases 10 fold in tetanus, while the overall O_2 consumption of man may increase from a rate of 240–250 ml. per minute at rest to over 4000 ml. and, in one case, to 5400 ml.[11] in high activity.

2. Oxygen Supply and Diffusion.—The rate of respiration depends not only upon internal factors, such as the concentration of respiratory substrates and enzymes, but also upon the oxygen supply. It is probable that for the majority of land organisms oxygen supply is rarely limiting. This is not always the case for organisms in deep ponds, at great depths of the ocean, in marshes or where decay is rapid, or perhaps in some soils. In these environments the oxygen tension may determine the respiratory rate.

The oxygen content of water in equilibrium with the air depends upon the partial pressure of oxygen in the atmosphere, the temperature, and the salt content of the water. The relation is shown as:

$$\text{Vol. } O_2 = \frac{\alpha P}{760},$$

where $P = O_2$ pressure in mm.

α = Vol. O_2 dissolved in 1 ml. at 760 mm. of O_2.

The values of alpha are shown in Table XXXI, and the volumes of O_2 dissolved in 1 l. in equilibrium with air ($pO_2 = 155$ mm.) are also shown.

When the respiratory rate falls off with decreasing pressure of oxygen, it is difficult to determine whether the result is due to failure of diffusion to furnish an adequate supply of O_2, or to the effect of the O_2 tension on the rate of oxidation of the cellular oxidases. However, it is generally assumed that the cellular oxidations are independent of oxygen tension except at the very lowest values (less than a few mm.). Though this assumption is probably not fully justified, it may be made as a simplifying case.

The problems of O_2 supply and diffusion have been reviewed by Krogh,[12] Gerard,[13] Hill,[14] Tang,[15] and particularly by Jacobs.[16] The reader is

[8] O. Warburg, Pflüger's Arch. f. d. ges. Physiol., **158**: 189, 1914.

[9] J. H. Bodine, J. Cell. & Comp. Physiol., **4**: 397, 1934.

[10] J. D. Merry and D. R. Goddard, Proc. Rochester Acad. Sc., **8**: 28, 1941.

[11] S. Robinson, H. T. Edwards, and D. B. Dill, Science, **85**: 409, 1937.

[12] A. Krogh, J. Physiol., **52**: 391, 1919.

[13] R. W. Gerard, Biol. Bull., **60**: 245, 1931.

[14] A. V. Hill, Proc. Roy. Soc., London, **B104**: 39, 1928.

[15] P. Tang, Quart. Rev. Biol., **8**: 260, 1933.

[16] M. H. Jacobs, Ergebn. d. Biol., **12**: 1, 1935.

referred to these references for details; a few of the applications are presented here. A general discussion of diffusion is found in Sec. 1, chap. 1, of this book.

TABLE XXXI. -BUNSEN'S SOLUBILITY COEFFICIENT, AND TOTAL GAS DISSOLVED WHEN IN EQUILIBRIUM WITH AIR (pO_2 = 155 MM.)

T°C.	Alpha H_2O	ml. O_2/1	Sea water[1]		Blood	
			Alpha	ml. O_2/1	Alpha	ml. O_2/1
0	0 04889	9 97	0 03908	9.13		
10	0 03802	7 75	0 03113	7.19		
15	0.03415	6 96	0 02839	6 50		
20	0.03102	6 32	0 02603	5 95		
25	0 02831	5 77				
30	0 02608	5 32	0 02187	5 01		
37	0 02386	4 87	. .	.	0 019[2] 0 022[3]	3 87*[2]

* Physically dissolved.

[1] From A. Krogh, Comparative Physiology of Respiratory Mechanism: Philadelphia, 1941

[2] Calculated from Peters and Van Slyke, Quantitative Clinical Chemistry, Vol. I, p. 525: Baltimore, 1932.

[3] J. Barcroft, The Respiratory Function of the Blood, Part II: Cambridge, 1928; gives the value at .022 at 38°C.

We will first consider oxygen diffusion into an aquatic organism which is a plain sheet of tissue like the marine alga, *Ulva*. *Ulva* is 2 cells thick, with an average thickness of 34 μ. We will assume that the oxygen consumption of the tissue is uniform throughout its mass. We may ignore any gas diffusion from the edges, and realize that O_2 may enter from either surface. If the oxygen in the water is in equilibrium with that of the air, and the water is replaced sufficiently rapidly at the surface, the external pressure of O_2 will remain constant. We may use Warburg's[17] equation for determining the O_2 pressure at the center:

$$U = C - \left(\frac{a}{2D}\right)(Hx - x^2),$$

where U = pO_2 in atmos. at x,
a = ml. O_2/min./ml. of tissue,
H = thickness of the tissue,
x = distance from the surface,
D = diffusion coefficient; we will use Krogh's value for muscle = 1.4×10^{-5} ml. O_2/cm.^{2}/min. with 1 atmosphere pressure difference.
C = external pO_2 in atmosphere.

In this equation we have used the atmospheric pressure of O_2 and not the molar concentration of O_2 dissolved in the liquid. Krogh has shown that

[17] O. Warburg, Biochem. Ztschr., **142**: 317, 1923.

this is justified, and the solubility constant is then included in the diffusion coefficient.

When we apply this equation to *Ulva*, using Watambe's[18] value for the respiratory rate (2.6 mm ^{3}/mg. dry wt./hr.) and assuming dry weight is $\frac{1}{5}$ of wet weight, we obtain a pO_2 at the center of 0.199 atmospheres, or very nearly the outside pressure.

The equation may be given in a form to determine at what depth a given pressure, U, may obtain. The equation is then:

$$x = \frac{H}{2} \pm \sqrt{\frac{H^2}{4} - \frac{2D}{a}(C - U)}.$$

For the particular case of the center of the sheet, $x = \frac{1}{2}$ of the thickness, or $H/2$, and the equation simplifies to:

$$H = \sqrt{\frac{8D}{a}(C - U)}$$

If we then take $C = 0.2$ atmos., the respiratory rate the same as *Ulva* (8.7×10^{-3}ml.^{3}/min./ml. tissue), and pO_2 at the center = 0.01 atmos., the maximum thickness = 0.35 mm. This would be the maximum thickness of a plain sheet of tissue with the respiratory rate of *Ulva*, and without any circulating mechanism, if the O_2 tension at the center is to remain above 0.01 atmos. If the tension just at the center is to fall to zero, the limiting thickness is increased to only 0.36 mm. If the respiratory rate were $\frac{1}{10}$ that of *Ulva*, the thickness would be increased to 1.1 mm. Cytoplasmic streaming might allow a somewhat greater limiting thickness. We find, in fact, that aquatic organisms which have no blood transport have greatly irregular surfaces, and are branched, or hollow, or circulate water through tissue openings. With the higher respiratory rates of mammalian tissues, the limiting thickness, when the circulatory system is nonfunctional, is even less, about 0.2 mm.

Similar equations can be derived for cylindrical respiring tissues and for spheres. The equation derived for cylinders by Fenn[19] and by Gerard[20] is:

$$U = C - \frac{a}{4D}(R^2 - r^2),$$

R = radius,

r = radial distance from the center at which U is wanted; other symbols as above.

For a frog nerve of 1 mm. radius, Fenn calculated that when in air, with a respiratory rate of 1.23×10^{-3} ml. O_2/min./ml. of tissue, the pO_2 at the center is just about zero.

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[18] A. Watambe, Acta phytochim, **6**: 315, 1932.

[19] W. O. Fenn, J. Gen. Physiol., **10**: 767, 1927.

[20] R. W. Gerard, Biol. Bull., **60**: 245, 1931.

The equation for a respiring[21] sphere is:

$$U = C - \frac{a}{6D}(R^2 - r^2).$$

When the pressure of O_2 at the center is just zero ($U = 0; r = 0$) the external pressure necessary to supply the oxygen is:

$$C_0 = \frac{aR^2}{6D},$$

or, the limiting radius, for a given external pressure with zero pressure reached just at the center, is:

$$R = \sqrt{\frac{6DC}{a}}.$$

For a spherical organism without internal circulation, and in water in which $pO_2 = 0.2$ atmos., with a respiratory rate of 100 mm.^{3}/gm. wet wt./hr., the radius must be just under 1 mm., or diffusion cannot supply the necessary oxygen.

Land organisms usually allow the entrance of air into tissue or intracellular spaces of leaves, tracheæ of insects, etc. Since the diffusion of O_2 in air is nearly 300,000 times as rapid as in water, considerably greater thickness is possible. Where lungs with forced ventilation and blood transport are involved, the diffusion path may be reduced to a few microns; and great size is possible. The problems of tracheal respiration have been discussed by Krogh[22] and Wigglesworth.[23] The problem of CO_2 diffusion into leaves of higher plants have been discussed by Brown and Escombe.[24] Since the rate of CO_2 absorption in photosynthesis is usually at least 10 times the rate of O_2 absorption in respiration of the same tissue, and since the $pO_2 = 155$ mm. and the pCO_2 is 0.3 mm. it is apparent that photosynthetic tissues should not normally have respiration limited by O_2 diffusion. It has been calculated[25] that, for a barley leaf, the water diffusion path is only $\frac{1}{5}$ the leaf thickness, due to the intracellular spaces. With an internal pCO_2 of 0.1 mm. (in the chloroplast), the calculated steady state for diffusion would be set up in barley leaves in 0.9 sec. In the trunks of large trees, where tissues are present which are potentially able to respire,[26] either some mechanism of longitudinal O_2 transport may exist,[27] or these tissues may obtain their energy by fermentation with an export of the end products to the well-aerated leaf. Further investigation of this problem is indicated.

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[21] R. W. Gerard, Biol. Bull., **60**: 245, 1931.

[22] A. Krogh, Comparative Physiology of Respiratory Functions: Philadelphia, 1941.

[23] V. B. Wigglesworth, The Principles of Insect Physiology: Methuen, London, 1939.

[24] H. T. Brown, and F. Escombe, Phil. Tr. Roy. Soc., London, **193**: 223, 1900.

[25] E. D. McAlister, Smithsonian Misc. Coll., **95**: 1, 1937.

[26] R. H. Goodwin and D. R. Goddard, Am. J. Bot., **27**: 234, 1940.

[27] D. T. MacDougal and E. B. Working, The Pneumatic System of Plants, Especially Trees: Washington, D. C., 1933.

Oxygen Tension and the Respiratory Rate.—There are few data in the literature on the effect of pO_2 on the respiratory rate, where the factor of diffusion can be eliminated. Some of the problems involved have been discussed by Gerard.[28] Warburg and Kubowitz[29] have investigated the problem in the bacterium, *Micrococcus*. They set up conditions in which the rate of invasion of the liquid by O_2 from the atmosphere was not limiting, and they found that the respiratory rate at 1°C. was independent of pO_2 above 10^{-5} atmos. (7.6×10^{-3} mm.). Winzler[30] used the dropping mercury electrode to investigate the relation between pO_2 and respiratory rate for baker's yeast. In the absence of substrate, the rate was independent of pO_2 at all tension above 0.2 mm. at 20°C. In the presence of glucose, the rate was independent of pO_2 above 2.5 mm. at 20°C. Temperature has a marked effect on the pO_2-respiratory relation.[31,32] Winzler found that, for yeast in glucose, the respiratory rate is independent of pO_2 at 0.25 mm. at 5°C., while at 34.3°C. the rate becomes independent only above 4 mm. Winzler, by a clever use of the known inhibitory effect of CO on respiration of yeast, has shown that the respiratory rate is being limited at low O_2 tensions, not by the diffusion of O_2 into the yeast cells, but by the rate of reaction of O_2 with an enzyme.

In mammalian muscle, the O_2 is brought to the muscle as oxyhemoglobin. The pO_2 of arterial blood is approximately 100 mm., while in venous blood it is approximately 40 mm. We may assume that the average pressure in the capillaries is 70 mm. In mammalian muscle, the distance from the muscle cells to the nearest open capillary probably varies from 50 μ in rest to 5 μ in active muscle.[33] Krogh has calculated the tension difference necessary between the capillary (he assumes venous pO_2) and the muscle cell to maintain the observed O_2 consumption. He arrives at a maximum difference in tension of 46 mm. and a minimum of 1.8 mm. If the oxygen tension in the capillary is 70 mm. and in the muscle cell is 3 mm., an adequate supply of O_2 is available by diffusion.

Many muscles contain myoglobin. The dissociation curve of myoglobin has been shown[34] to differ from hemoglobin, since myoglobin is approximately 50 per cent saturated with oxygen at 3.26 mm. at 37°C., and a pH of 7.4; and 90 per cent saturated at 20 mm. O_2 pressure. At a lower pH the saturation occurs at lower O_2 tensions, and since the muscle pH is probably nearer 6.8 than 7.4, the above values are probably too high. Thus, a marked gradient may exist between the capillaries and the muscle cell, and so long as the myoglobin is more than 50 per cent saturated, the intracellular pO_2 must be at least 3 mm.

[28] R. W. Gerard, Biol. Bull., **60**: 220, 1931.

[29] O. Warburg and F. Kubowitz, Biochem. Ztschr., **214**: 5, 1929.

[30] R. J. Winzler, J. Cell. & Comp. Physiol., **17**: 263, 1941.

[31] W. Kempner, *Ibid.*, **10**: 269, 1937.

[32] R. J. Winzler, *Ibid.*, **17**: 263, 1941.

[33] A. Krogh, The Anatomy and Physiology of Capillaries: New Haven, 1941.

[34] H. Theorell, Biochem. Ztschr., **268**: 73, 1933.

25

ENERGETICS AND KINETICS

Living cells receive their energy supply from the potential energy stored in the organic foods absorbed, or, in the case of green plant cells, from the foods produced in photosynthesis. The diet, or photosynthesis, furnishes only a few of the organic compounds required by a cell, and, for a cell to survive and grow, many different kinds of substances must be synthesized from the few foods of the diet. For these syntheses a continuous energy supply is essential, and, in addition, energy is necessary for movement, osmotic work, transport, and the maintenance of many steady states. Cellular respiration is the fundamental mechanism by which the potential energy of the organic compounds is released as kinetic energy and made available for the work of the cell.

The organic foods are oxidized in a stepwise manner by a series of catalysts, the respiratory enzymes, and the potential energy is likewise released in a series of graded steps. Much of the kinetic energy is in the form of heat, and this energy is useless for the cellular work, and is wasted except as it serves to maintain body temperature in the mammals. However, the cell has various contrivances for utilizing some of the liberated energy.

In Sec. 1, chap. 3, is found a general discussion of thermodynamics; here we wish to consider a few aspects of the energetics of respiration. The quantity of heat formed upon the oxidation of a compound may be determined by burning it in a bomb calorimeter. Some typical results are shown in

TABLE XXXII.—HEATS OF COMBUSTION OF SOME ORGANIC COMPOUNDS

Substance	R.Q.	Cal./gm.[1]	$-\Delta H$ Cal./mole
Starch	1 0	4 20	680.4[2]
Cane sugar	1 0	3.96	1354.3
Dextrose	1 0	3 74	674
Lactic acid	1 0	3.62	284.8
Animal fat	0.711	9 50	
Protein	0 85	4 40[3]	
Ethyl alcohol	0.667	7.08	325 7

[1] From J. M. Carpenter, Tables, Factors and Formulas for Computing Respiratory Exchanges: Carnegie Inst., Washington, 1924.

[2] Calculated for $C_6H_{10}O_5$.

[3] For biological oxidation in the mammal; if the N was oxidized to nitrate the value would be 5.65.

Table XXXII. The change in heat content per mole is referred to as ΔH, and may be represented as follows:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$
$$\Delta H = -674 \text{ Cals.}$$

By convention, heat liberated is negative; heat absorbed, positive.

One of the great achievements of physiology was in showing that the amount of heat liberated in the oxidation of food in the body is the same as in the external burning of the same foods. From a knowledge of the amount of oxygen absorbed and the ratio of CO_2 liberated to O_2 absorbed (the respiratory quotient or R.Q.), it is possible to predict the heat formed. These results are ably summarized by Lusk[35] and DuBois.[36]

However, it is the free energy change per mole, ΔF, rather than the change in heat content, ΔH, which is the more useful term, for two reasons: 1. It is the value of ΔF which determines whether a particular reaction will occur spontaneously. 2. The maximum amount of work which may be obtained from a reaction is related to ΔF, and not ΔH. When there is a decrease in free energy (ΔF is negative), a spontaneous reaction is possible; when there is a large increase in free energy (ΔF large and positive), a spontaneous reaction is impossible. If ΔF is positive to the extent of 3–4 Cal. per mole, a spontaneous reaction may be possible at physiological temperatures by absorbing heat from the environment. For a reaction of larger positive ΔF to occur at physiological temperatures, the reaction must be energetically coupled to a reaction of $-\Delta F$ so that:

$$\Delta F_1 + (-\Delta F_2) \leqq 0.$$

It will be recalled, from chap. 3 of Sec. 1, that the relation between ΔF and ΔH is:

$$\Delta F = \Delta H - T\Delta S,$$

where ΔF, ΔH, and ΔS are in terms of Cals. per mole
and T is the absolute temperature.
ΔS is the change in entropy.

For a discussion of the physical meaning of these terms, reference is made to chap. 3 of Sec. 1 However, it may be recalled that the entropy is a measure of the randomness of a system; an increase in entropy means decrease in organization; a decrease in entropy, an increase in orientation.

The entropy term also determines the maximum work which may be derived from a chemical system. When $\Delta S = 0$, as it does at equilibrium of all reactions, the maximum work equals $-\Delta F$. For a thermodynamically reversible system, $\Delta S = 0$. Such chemical reactions probably rarely occur in biological systems, but the reversible oxidation-reduction systems approach theoretical reversibility and ΔS approaches zero. Such reactions will be the most efficient for the transfer of cellular energy, and the nearer

[35] G. Lusk, Science of Nutrition: Philadelphia, 1924.
[36] E. F. DuBois, Basal Metabolism in Health and Disease: Philadelphia, 1924.

they operate to the equilibrium point, the more efficient they will be. Thus, we find another reason for the stepwise oxidation in cells and the stepwise liberation of energy.

Though the oxidation of organic foods occurs in many separate steps, the total free energy change is the same as if the oxidation occurred in one step, for the value of ΔF is determined only by the initial and final states, and not by the pathway. A knowledge of the value of ΔF of each proposed partial reaction in cell metabolism is useful in predicting which are possible and impossible steps, but it does not tell us which of the possible steps actually occur. For a knowledge of the actual pathways of cellular metabolism, it is essential that we determine the actual intermediate substances which are formed; the enzymes which catalyze their formation and disappearance, and the kinetics of their appearance and disappearance.

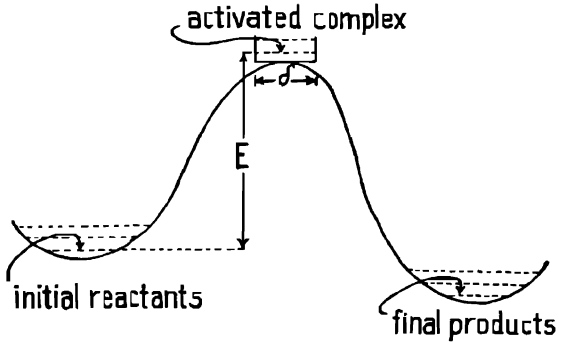


FIG. 33.—Diagram of the formation of activated complex. (From S. Glasstone, K. J. Laidler, and E. H. Eyring, The Theory of Rate Processes: McGraw Hill Co., New York, 1941.)

The general aspects of chemical kinetics are presented in chap. 2 of Sec. 1. Here we will discuss a few aspects in relation to enzymes. When glucose is oxidized to CO_2 and H_2O, there is a large decrease in free energy (ΔF is about -704 Cals.); thus glucose in the presence of oxygen is thermodynamically unstable. However, in the absence of the appropriate enzymes, glucose is not oxidized measurably by molecular O_2 at [illegible] temperatures.

The rate of a chemical reaction is determined by the frequency with which the reacting molecules may surmount an energy barrier. This is illustrated in Fig. 33. The energy barrier may be thought of as the energy essential for the rearrangement of the atoms in the molecule to form an unstable configuration, the reactive molecule (activated molecule or complex); or the energy necessary to form a reactive compound with a second molecule. This may be illustrated as follows:

$$A + \text{activation energy} \rightleftharpoons A^* \rightleftharpoons B + \text{activation energy} \qquad (1)$$
$$+$$
$$\text{free energy of reaction}$$

$$A + X + \text{activation energy} \rightleftharpoons AX \rightleftharpoons B + Z + \text{activation energy} \qquad (2)$$
$$+$$
$$\text{free energy of reaction}$$

Then, A^* is the reactive molecule and AX the reactive compound. The value of ΔF for the conversion of A to B is the same in both reactions, but the value of the activation energy is not necessarily the same. If $X = Z$, and the energy of the activation for equation 2 is less than for equation 1, X is a catalyst.

A molecule may gain the energy for climbing the energy barrier by molecular collisions, by chemical reaction with a catalyst, or by a combination of the two methods. In Sec. 1, chap. 2, the mechanism of evaluating the energy of activation is given; it is determined from the Arrhenius equation which is given below:

$$k = Ae^{-\frac{E_a}{RT}},$$

where k = velocity constant,
A = constant,
e = base of natural logarithms,
E_a = energy of activation,
R = gas constant = 0.00199 Cals. mole,
T = absolute temperature.

An inspection of the equation shows that the rate of the reaction depends upon exponential functions of the activation energy, and the reciprocal of the absolute temperature. At any given temperature, the kinetic energy of translation of the molecules is given simply by $\frac{3}{2}RT$, and at 25°C. this equals 0.890 Cals. per mole. However, at any moment, the energies of the individual molecules will vary considerably. The fractional number of molecules with energies equal to or greater than any particular value of E_a is given by the relation:

$$\frac{N^*}{N} = e^{-\frac{E_a}{RT}},$$

where N^* = no. of molecules with energies $\geqq E_a$.
N = total no. of molecules.

The values of N^*/N are shown in Table XXXIII for two temperatures for various values of E_a. It will be found by calculation that increasing the temperature from 25° to 37°C. causes an increase of but 3.97% in the average energy of the molecules (0.890 to 0.925 Cals.) but that the number of molecules with high energies has increased considerably, at 25 Cals. by 4.97 times.

Table XXXIII also shows some calculated velocity constants at different activation energies, and the times for ½ completion of monomolecular reactions with the given velocity constants. The times for ½ completion of bimolecular reactions depend upon the concentrations of the reacting molecules, but, if both reactants are at 10^{-3} M, the times for ½ completion are 697 times as long as those given.

From the values in Table XXXIII, one would not expect reactions to occur in living cells for which the energy of activation was much larger than 20 to

25 Cal. Another relation which follows from the Arrhenius equation is that a small decrease in activation energy means a large increase in rate; for example, decreasing the activation energy from 20 to 15 Cals. results in an increase of the rate by 1300 times, while halving the activation energy results in an increase of the rate by 500,000 times.

TABLE XXXIII

Energy of activation E_a Cal./mole	$e^{-\frac{E_a}{RT}}$		Monomolecular k_1 25°	$t_{1/2}$ at 25°C. monomolecular
	25°C	37°C.		
0 5	0 431	0 445		
1 0	0 185	0 198		
2 0	$3\ 42 \times 10^{-2}$	$3\ 90 \times 10^{-2}$		
5 0	$2\ 15 \times 10^{-4}$	$2\ 94 \times 10^{-4}$		
10 0	4.71×10^{-8}	$8\ 97 \times 10^{-8}$	$7\ 71 \times 10^{5}$	9×10^{-6} sec.
12 5	$6\ 95 \times 10^{-10}$	$15\ 1 \times 10^{-10}$	$1\ 15 \times 10^{+4}$	$6\ 2 \times 10^{-5}$
15 0	$1\ 05 \times 10^{-11}$	$2\ 69 \times 10^{-11}$	1.72×10^{2}	.004 sec.
20.0	$2\ 33 \times 10^{-15}$	$8\ 05 \times 10^{-15}$	.133	5 2 sec.
25 0	$4\ 84 \times 10^{-19}$	$24\ 1 \times 15^{-19}$	7.98×10^{-4}	145 min.
30 0	$1\ 05 \times 10^{-22}$	$3\ 87 \times 10^{-22}$	$1\ 97 \times 10^{-9}$	112 yrs.
50 0	$2\ 59 \times 10^{-37}$	$42\ 0 \times 10^{-37}$	$4\ 26 \times 10^{-24}$	5×10^{17} yrs.

$$t_{0.5} = \frac{0.693}{k_1}$$

$$k_1 = e\frac{kT}{h}e^{-\frac{E_a}{RT}}$$

$t_{0.5}$ for a bimolecular reaction, each reactant at 1×10^{-4} M = 700 times as long as $t_{0.5}$ of the table.

Thus catalysts which may only decrease the activation energies by a few Calories may result in tremendous increases in rate.

TABLE XXXIV

Enzyme	Substrate	*p*H	Temp. range °C.	E_a in Cal. per mole
Tyrosinase	Cresol, Catechol	6 2	2–30	2 7
Catalase	H_2O_2		0–20	6.4
Carbonic anhydrase	CO_2	7 4	1–13	8.9
Urease	Urea	7 0	0–45	8 7 to 11.7
Yeast invertase	Sucrose	5 2	0–40	11.0
Cytochrome oxidase-cytochrome-succinate dehydrogenase	Succinate and O_2	7.4	20–40	11.2
Xanthine oxidase	Xanthine, Acetaldehyde	6.2	0–60	16.0
Pepsin	Casein		1–30	17 7

Data from I. W. Sizer, Advances in Enzymology, **3**: 35, 1943.

The activation energies of some catalyzed reactions are collected in Table XXXIV. It would be interesting if one could compare the energies of activation of the enzymatic reaction with the same reaction without a catalyst—but in most cases the uncatalyzed reaction is so slow that these values may not be determined.

From the previous discussion, it would appear that reactions in which the energy of activation was high, 50 Cal. or over would not occur at a measurable rate. In fact, some such reactions are known, as the denaturation of proteins and the inactivation of enzymes. Here the energies of activation are very high, 50 to 100 Cals. per mole, yet the reactions occur with high velocity. Also, some reactions, with low energies of activation occur much more slowly (even 10^{15} times) than predicted by the data of Table XXXIII.

Eyring[37] and others have pointed out that the energy of activation of the Arrhenius equation is really a heat of activation, and not the free energy of activation. The free energy of activation is related to the heat of activation as follows:

$$\Delta F^* = \Delta H^* - T\Delta S^*,$$

where ΔF^*, ΔH^*, and ΔS^* are the free energy, heat, and entropy change of activation.

Now, ΔH^* may be calculated from E_a of the Arrhenius equation by the following relation:

$$\Delta H^* = E_a + RT$$

The rate of the reaction depends upon ΔF^* rather than upon heat of activation. In many reactions, the ΔS^* term is small, and no great error is made in using the Arrhenius equation. However, if ΔS^* is positive and large, ΔF may be smaller than E_a, and a rapid reaction may occur even when $E_a = 50$ to 100 Cals. per mole. This is the case in protein denaturation, where, at 65°C. and at *p*H of 5.0, $E_a = 131.43$; $\Delta H^* = 132.1$ and $T\Delta S^* = 166.7$, giving a ΔF^* of 25.4, and a rapid rate of denaturation. Eyring, note (37), has pointed out that the correct formulation of the Arrhenius equation is:

$$k_1 = e\boldsymbol{k}\frac{T}{h}e^{-\frac{\Delta F^*}{RT}}$$

or

$$k_1 = e\boldsymbol{k}\frac{T}{h}e^{-\frac{\Delta H^*}{RT}}e^{\frac{\Delta S^*}{R}}.$$

where $\boldsymbol{k}$ = the Boltzman constant = 1.37×10^{-16} ergs/sec.
T = absolute temperature
h = Planck constant = 6.57×10^{-27} ergs/sec.
other constants as above

$$\frac{\boldsymbol{k}T}{h} = 4.65 \times 10^{12} \text{ at } 25°\text{C.}$$

For a discussion of the derivation of this equation, the reader is referred to

[37] S. Glasstone, K. J. Laidler, and E. H. Eyring, The Theory of Rate Processes; McGraw Hill, New York, 1941.

Glasstone,[38] and for its application to protein denaturation to Eyring and Stearn.[39]

Mechanism of Enzymatic Catalysis.—An enzyme increases the rate of a reaction by decreasing the energy barrier over which a substrate molecule must pass. This may be due either to a decrease in the heat of activation, ΔH^*, or an increase in the entropy of activation, ΔS^*; the net result is the same, a decrease in ΔF^*. The detailed mechanism is unknown, but certainly the critical step is the formation of an enzyme-substrate molecule:

$$E + A \rightleftharpoons EA \rightleftharpoons E + B$$

In most cases the evidence for the intermediate molecules comes from kinetic data, and this has been discussed in Sec. 1, chap. 2. However, in a few cases, spectroscopic evidence exists for the presence of EA, such as the compound between catalase and ethylhydrogen peroxide,[40] or between peroxidase and H_2O_2.[41]

It should be noted, above, that the enzyme actually enters into the reaction. The conventional definition of catalysts says that the enzyme remains unchanged, and the equilibrium point is unchanged by the presence of the enzyme, and for a complete enzymatic cycle this is true. However, if the partial reactions are studied, we find that in all oxidation or reduction reactions the enzyme itself undergoes oxidation or reduction. For each partial reaction, the enzyme does undergo chemical change. However, since one enzyme molecule may react several hundred to several thousand times a minute, an extremely low concentration of enzyme may catalyze a large amount of substrate, and for practical purposes the equilibrium point will not be shifted by the concentration of enzyme present.

[38] S. Glasstone, K. J. Laidler, and H. Eyring, The Theory of Rate Processes: McGraw Hill, New York, 1941.

[39] H. Eyring, and A. E. Stearn, Chem. Rev., **24**: 253, 1939.

[40] K. G. Stern, J. Biol. Chem., **114**: 473, 1936.

[41] D. Keilin and T. Mann, Proc. Roy. Soc., London, **B122**: 119, 1937.

26

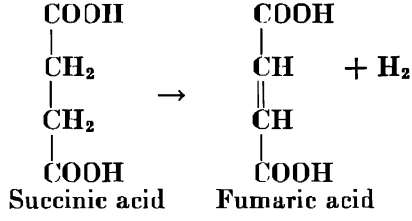
THE NATURE OF OXIDATION

Classically, we think of oxidation as the addition of oxygen to a molecule, and this may be illustrated by the oxidation of hydrogen to water:

$$H_2 + \tfrac{1}{2}O_2 \rightleftharpoons H_2O$$

This type of oxidation is one of the least common methods of oxidation.

Two other types of reaction are also considered as oxidations. One of these is the removal of hydrogen from a molecule, and may be illustrated by the oxidation of succinic acid:



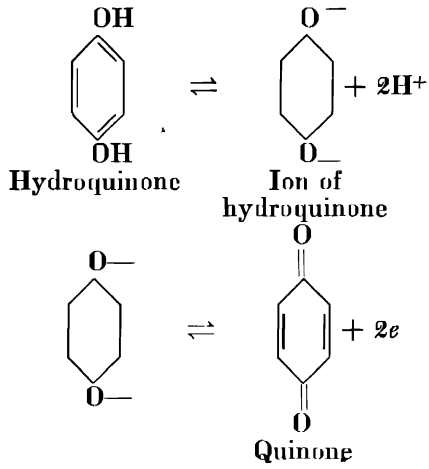
We say that succinic acid has been oxidized to fumaric acid, even though no oxygen is involved. The other is the removal of electrons from an atom or molecule, as follows:

$$Fe^{++} \rightarrow Fe^{+++} + \text{electron}$$

We say that ferrous ion has been oxidized to ferric ion. In oxidations of these two latter types, free hydrogen or free electrons are not formed; hence, there must be present some substance which will combine with either the hydrogen or the electrons; these are designated as either hydrogen or electron acceptors. The reverse of oxidation is reduction, and the removal of oxygen or the addition of hydrogen or electrons is reduction.

Michaelis and Schubert[42] have developed the idea that, basically, all oxidations are the removal of electrons from the molecule oxidized. In the case where hydrogen is removed from a molecule, we can think of the process as occurring in two steps, the liberation of protons (or acid dissociation) and the liberation of electrons (or oxidation):

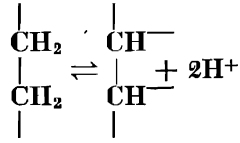
[42] L. Michaelis and M. P. Schubert, Chem. Rev., **22**: 437, 1938.



or

$$AH_2 \rightleftharpoons A^{--} + 2H^+$$
$$A^{--} \rightleftharpoons A + 2e$$
$$\overline{AH_2 \rightleftharpoons A + H_2}.$$

This concept of Michaelis, that all oxidation is the removal of electrons, may seem strange at first sight, particularly because many of the hydrogen atoms which are removed are not those which we are in the habit of thinking of as dissociating, such as:

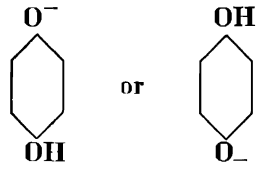


However, Michaelis' interpretation has the advantage of unifying the concepts of oxidation and of electron removal, and of pointing out that the removal of electrons and protons from an organic molecule need not occur simultaneously.

Univalent Oxidation.—Organic compounds usually have an even number of electrons, so that in their oxidation or reduction we are accustomed to think of two electrons added or removed simultaneously. In many, perhaps the majority, of the instances of oxidation, this is not the case, as only one electron is added or removed at a time:

$$AH_2 \rightleftharpoons A^- + 2H^+$$
$$A^- \rightleftharpoons A^- + e.$$

A^- has an odd number of electrons and is known as a free radical, or a semiquinone. As an example of a semiquinone, we may take the case of quinhydrone.



This particular substance is insoluble in water, and precipitates as a double (or dimeric) molecule of quinhydrone. However, many organic dyes, such as pyocyanine, may exist in solution in the half-oxidized state as semiquinones. The evidence for the existence of half-oxidized molecules, or semiquinones, is based on three lines of inquiry: 1. results of potentiometric oxidation studies; 2. absorption spectra; 3. magnetic properties. Michaelis and Schubert[43] have reviewed the evidence for the existence of semiquinones and their wide occurrence.

The semiquinone may react in one of several ways:

$$\begin{aligned} A^- &= A + e \text{ (oxidation)} \\ A^- + e &= A^{--} \text{ (reduction)} \\ 2A^- &= A + A^{--} \text{ (dimerization).} \end{aligned}$$

The concentration of semiquinone ions in biological oxidation systems is often 10 to 50 per cent of the total concentration of a particular substance, or it may be very much less. Their significance probably lies in the fact that they may speed up the rate of cellular oxidations, because an oxidizable substance, which exists as a semiquinone, may accept or donate one or two electrons at a time, and thus they may function between a compulsory univalent system like ferro-ferri cytochrome, and a bivalent system like succinate-fumarate (where the concentration of semiquinone is extremely low). In the absence of an intermediary semiquinone, a trimolecular reaction would be necessary:

$$2EnzFe^{+++} + AH_2 \rightarrow EnzFe^{++} + A + 2H^+.$$

We know that trimolecular reactions are too slow to account for the observed rate of biological oxidations. In the absence of a catalytic semiquinone, the rate of the reaction would depend upon the extremely low concentration of the free radical of the substrate, rather than upon the concentration of the total substrate. If an intermediary substance, R, is present, in which R^- represents its semiquinone, we may diagram the reaction as follows:

$$\begin{aligned} EnzFe^{+++} + R &\rightarrow EnzFe^{++} + R^- \\ EnzFe^{+++} + R^- &\rightarrow EnzFe^{++} + R^{--} \\ AH_2 + R^{--} &\rightarrow A + R + 2H^+. \end{aligned}$$

We may expect that some of the enzymes of cellular respiration may exist temporarily as semiquinones, and this is true for those intermediary enzymes like the flavin and di- and tri-phosphopyridine nucleotides.

[43] L. Michaelis and M. P. Schubert, Chem. Rev., **22**: 437, 1938.

27

RESPIRATORY ENZYMES

Biochemists had long searched for a respiratory enzyme which would catalyze the oxidation of glucose to CO_2 and H_2O. The "indophenol oxidase" and catechol oxidase were discovered in the last century. However, they did not act on glucose, and their significance was unknown. Definite progress in this field came in the 1920's from two different sources, Warburg's studies on the effect of CO and HCN on the respiration of intact cells, and Keilin's spectroscopic observations on cytochrome and its correlation with cellular respiration.

At the present time, no worker in the field holds the view that there is a unique respiratory enzyme bringing about the reaction between cell metabolites and molecular oxygen. Rather, respiration has been demonstrated to be a complicated process, catalyzed by a series of enzymes functioning together in a cyclical manner, to form a connected series of reactions, a reaction system. Several such reaction systems have been identified, and a few of the component enzymes well studied. In a few cases, the quantitative data indicate that most of the respiration may pass over one or another of these systems. In other organisms, several systems may occur even in the same cell. It is almost certain that other respiration systems, as yet little studied, exist.

1. Nature of Respiratory Enzymes.—The respiratory enzymes which have been isolated are all conjugated proteins, that is, proteins which on hydrolysis yield amino-acids, and, in addition, nonamino-acid residues, such as heme, riboflavin phosphate, thiamine diphosphate, etc. Proteins do not readily undergo reversible oxidation and reduction, and, in all cases which have been studied, it is the nonprotein part of the enzyme which is readily oxidized and reduced, such as the iron of the heme or the pyridine ring of triphosphopyridine nucleotide. Some oxidation enzymes may be separated by dialysis into two parts, a specific protein and a coenzyme. The specific protein, which, because of its high molecular weight, remains within the dialysis sac, and the coenzyme which dialyzes out. It is found that neither the specific protein nor the coenzyme has catalytic activity by itself, but if the coenzyme is added to the specific protein, the catalytic activity is regenerated. The protein is usually heat-labile and the coenzyme heat-stable. In some cases, as the flavin enzymes, dialysis alone will not separate the enzyme into two parts, but dialysis against 0.01 *M* HCl does, while the separation of copper from catechol oxidase requires dialysis against HCN.

Whether a particular enzyme is classified as one with or without a coenzyme, is only a question of the value of the dissociation constant. For example, certain dehydrogenases readily separate into two parts:

$$\text{enzyme} \rightleftharpoons \text{specific protein} + \text{triphosphopyridine nucleotide.}$$

Some enzymes, as cytochrome, have not been reversibly separated into a specific protein and coenzyme, i.e.,

$$\text{cytochrome} \rightleftharpoons \text{protein} + \text{heme C,}$$

the equilibrium must be far to the left, that is, the dissociation constant must be very small. In this case, the heme is referred to as a prosthetic group rather than a coenzyme.

The same coenzyme or prosthetic group may occur in several different enzymes, for example, diphosphopyridine nucleotide (cozymase) occurs in several different dehydrogenases, and protoheme is a constituent of the enzymes catalase, peroxidase, and cytochrome peroxidase, as well as the oxygen carrier, hemoglobin. The coenzymes and prosthetic groups are found to be reversibly oxidized and reduced apart from the protein, but usually the oxidation and reduction are sluggish. Combination of the coenzyme with the protein not only determines specifically with which substrate the coenzyme may react, but tremendously speeds up the rate of the reaction. In some cases, the protein stabilizes the semiquinone form of the coenzyme, for example in the flavin enzymes.

We will retain the name enzyme for the catalytically active molecule, and refer to the dissociated molecules as specific protein and coenzyme. Euler refers to the enzyme as holoenzyme, and the dissociated products as apoenzyme and coenzyme.

A respiratory system usually consists of the following components:

1. *An oxidase.*—Oxidases are enzymes which are directly oxidized by molecular oxygen (autoxidizable), and in turn reduced by some component of the respiratory mechanism. The best-known oxidases are: cytochrome oxidase, polyphenol oxidase, d-amino-acid oxidase, and xanthine or aldehyde oxidase. The oxidase is usually reduced by a carrier.

2. *A carrier.*—A carrier is a substance (or enzyme) oxidized by one component of the respiratory system and reduced by a second component, which is not the final substrate, usually another carrier or a dehydrogenase. Occasionally an autoxidizable substance may function as a carrier, if the rate of autoxidation is slow, compared to the rate of catalyzed oxidation. Thus, some flavoproteins may function either as oxidase or as carriers. Cytochrome, cytochrome reductase, and the catechol compounds are the best-known respiratory carriers. Two or more carriers may function between an oxidase and a dehydrogenase, for example, cytochrome and cytochrome reductase. The dehydrogenase systems may be connected by a carrier in which one system oxidizes another of lower potential through a carrier. Coenzyme I and probably coenzyme II may function as carriers.

3. *Dehydrogenases.*—Most respiratory metabolites will not react directly with either a carrier or an oxidase. The metabolites are oxidized by substrate-specific enzymes, the dehydrogenases. The metabolite donates two hydrogen atoms to the dehydrogenase, which is reduced. In exceptional cases, a single enzyme may react with both a cell metabolite and molecular oxygen, for example, xanthine or aldehyde oxidase.

4. *Substrates* (metabolites).—The substrates respired in cells, usually, are either carbohydrates or products derived from carbohydrates. Often the substrates may be phosphorylated compounds, such as glyceraldehyde phosphate. The substrate which has become oxidized by the dehydrogenase-carrier-oxidase system may react with a second dehydrogenase and be further oxidized, or it may react in a cyclic system as outlined below. Amino-acids and fatty acids may constitute respiratory substrates.

5. *Decarboxylation system.*—In the initial oxidation of a substrate, no CO_2 is produced, and only two hydrogen atoms of the substrate are oxidized at a time. Most of the CO_2 of respiration has its origin in the decarboxylation of keto-acids, either directly by the decarboxylation of such keto-acids as pyruvic acid by the carboxylase system, through oxidative decarboxylation, or through a system such as the Krebs cycle, where a cyclical condensation, oxidation, decarboxylation system functions and results in the complete oxidation of three-carbon compounds to CO_2 and H_2O.

6. *Inorganic ions.*—The activity of many respiratory enzymes depends upon the presence of inorganic ions. Among the ions known to be essential for cellular respiration are:

$$Fe^{++},\ Mg^{++},\ Mn^{+++},\ H_2PO_4^-,\ Ca^{++},\ \text{and}\ HCO_3^-.$$

2. Mobilization of Oxygen.—*a*). *Cytochrome oxidase.*—Warburg[44] showed that the respiration of many cells and tissues is partially, or nearly completely, inhibited by low concentrations of cyanide or sulfide, and Keilin[45] has shown that azide acts similarly. Since these compounds form firm complexes with iron and its compounds, Warburg suspected that iron was the catalytically active material, and, since it was known that CO forms compounds with ferro iron, and particularly with heme compounds like hemoglobin, Warburg[46] investigated the effects of CO on cellular respiration. He found that the respiration of yeast was inhibited by CO, and the inhibition depended upon the ratio of CO to O_2, and not upon the partial pressure of CO alone, while the cyanide inhibition was independent of O_2 tension. He suggested that HCN combined with the ferri enzyme, while CO and O_2 competed for the ferro enzyme. Since the inhibition of respiration was the same, whether the yeast was respiring glucose, alcohol, or acetate, Warburg assumed that a single enzyme was undergoing inhibition. Warburg[47]

[44] O. Warburg, Biochem. Ztschr., **151**: 479, 1924.

[45] D. Keilin and E. F. Hartree, Proc. Roy. Soc., London, **B 125**: 187, 1938.

[46] O. Warburg, Biochem. Ztschr., **177**: 471, 1926.

[47] O. Warburg and E. Negelein, Biochem. Ztschr., **244**: 9, 1932.

named the enzyme the "sauerstoffübertragende Ferment der Atmung" or the oxygen-transporting enzyme of respiration. In England and America the enzyme is commonly known today as cytochrome oxidase.

Warburg made the simplifying assumption that, when respiration is independent of O_2 tension and substrate concentration, the rate of respiration is directly proportional to the concentration of the enzyme combined with O_2 (today we would say upon the concentration of ferri enzyme). He then formulated his results as follows:

$$\mathrm{EnFe} + \mathrm{O_2} = \mathrm{EnFeO_2}$$

$$\frac{(\mathrm{EnFeO_2})}{(\mathrm{EnFe})(\mathrm{O_2})} = k_{\mathrm{O_2}} \tag{1}$$

$$\mathrm{EnFe} + \mathrm{CO} = \mathrm{EnFeCO}$$

$$\frac{(\mathrm{EnFeCO})}{(\mathrm{EnFe})(\mathrm{CO})} = k_{\mathrm{CO}}, \tag{2}$$

and, dividing 1 by 2,

$$\frac{(\mathrm{EnFeO_2})}{(\mathrm{EnFeCO})} \cdot \frac{(\mathrm{CO})}{(\mathrm{O_2})} = \frac{k_{\mathrm{O_2}}}{k_{\mathrm{CO}}} = \mathrm{K}, \tag{3}$$

where K is the relative affinity constant for CO and O_2. Now, if the maximal rate of respiration without CO is 1, and the residual respiration in the presence of a particular ratio of CO/O_2 is n; the inhibited respiration is $1 - n$.

On the basis of the above assumption, n should be dependent upon $(\mathrm{EnFeO_2})$; and $1 - n$ on (EnFeCO); so that:

$$\frac{(\mathrm{EnFeO_2})}{(\mathrm{EnFeCO})} = \frac{n}{1-n}, \tag{4}$$

and substituting in (3)

$$\frac{n}{1-n} \cdot \frac{(\mathrm{CO})}{(\mathrm{O_2})} = \mathrm{K}. \tag{5}$$

Since n and $1 - n$ may be directly determined by measuring the respiration with and without CO, and (CO) and (O_2) are known, K may be evaluated. Warburg found in yeast at 20°C. that K lay between 9–10, when the substrates were glucose, alcohol, or acetate. When $n = 1 - n$, or at 50 per cent inhibition, K is the ratio of CO/O_2. For yeast this means that the concentration of CO must be 9 times that of O_2 for 50 per cent inhibition.

The dimensions of K should be expressed in terms of the molar concentrations of CO and O_2; it is in fact given in terms of the partial pressures of the gases in the gas space, and since the solubilities of CO and O_2 are not identical, K includes the ratio of their solubilities.

Warburg's formulation is only correct if the rate of respiration depends only upon the concentration of $\mathrm{EnFe^{+++}}$. This is probably never completely true; it may be approximately true in some cases. Where the substrate concentration, oxygen tension, or other factors limit the rate, the above formulation is not valid. Warburg attempted to correct the equation for substrate concentration, and, as Fisher[48] has pointed out, his formulation is mathematically incorrect. Though one may derive a mathematically correct formulation, so

[48] K. C. Fisher, J. Cell. & Comp. Physiol., 15: 1, 1940.

many simplifying assumptions must be made, it is doubtful whether any simple equation presents the kinetic picture with any validity.

Cyanide, azide, and sulfide all combine with the ferri form of the enzyme. Stannard,[49] and Armstrong and Fisher[50] have studied the relation between

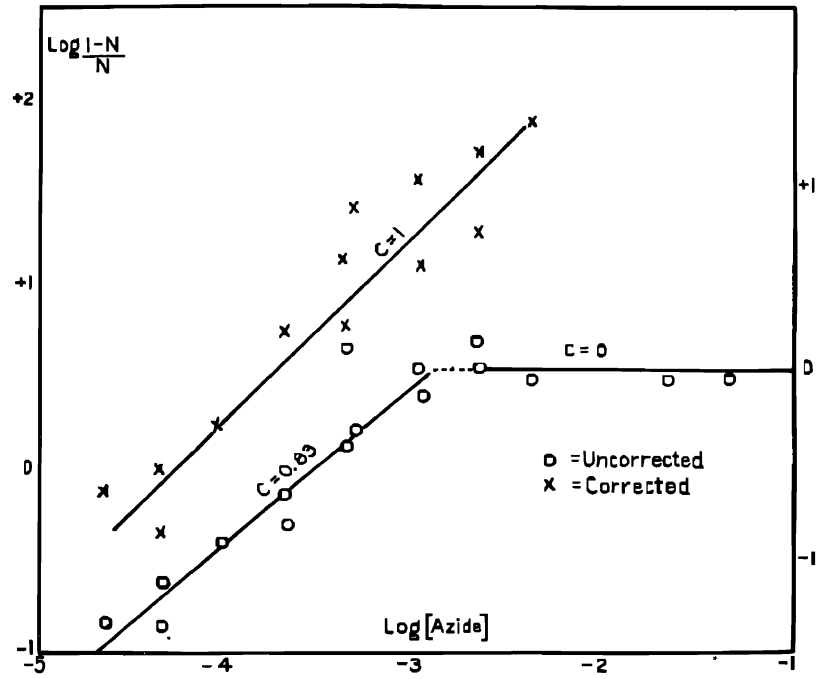


FIG. 34.—Log ratio inhibited respiration/residual respiration against log azide concentration for stimulated frog muscle. "Uncorrected"—values directly from the experiments, "corrected"—the experimental values minus the azide-resistant respiration at maximal concentration. (From J. N. Stannard, Am. J. Physiol., **126**: 196, 1939.)

the concentration of these poisons and the respiratory rate. It must be clearly realized that, in many cells and tissues, only part of the respiration is inhibited by HCN or NaN_3, regardless of the concentration; see Fig. 34. We may formulate the inhibition as follows:

$$EnFe^{+++} + cN_3^- = EnFe^{+++}(N_3^-)_c$$

$$\frac{[EnFe^{+++}(N_3^-)_c]}{(EnFe^{+++})(N_3^-)^c} = K.$$

If the maximal respiration = 1, and the respiration at any particular azide concentration is n, the inhibited respiration is $1 - n$. Making the assumptions used above,

$$\frac{n}{1-n} \cdot (N_3^-)^c = K,$$

or

$$\frac{1-n}{n} = K(N_3^-)^c,$$

and

$$\log \frac{1-n}{n} = c \log (N_3^-) + \log K$$

[49] J. N. Stannard, Am. J. Physiol., **126**: 196, 1939.

[50] C. W. J. Armstrong and K. C. Fisher, J. Cell. & Comp. Physiol., **16**: 103, 1940.

If the log $1 - n/n$ be plotted against log (N_3^-), a linear curve should result, the slope of the line giving c, or the number of molecules of azide inactivating 1 molecule of enzyme. For the caffeine-induced respiration of frog muscle, the azide inhibition depends upon 1 molecule of azide combining with each molecule of enzyme (see Fig. 34), and K lies between 10^{-4} and 10^{-5}. A similar formulation may be given for cyanide. Armstrong and Fisher[51] and Fisher and Ohnell[52] have given a summary of the results. For cyanide, c lies in different organisms between 0.52 and 1.6 and K between 10^{-3} and 10^{-8}; c should theoretically be a whole number; deviations may be caused by inhibition of more than one metabolic step, or due to the fact that the kinetics of respiration do not depend only upon the concentration of the enzyme.

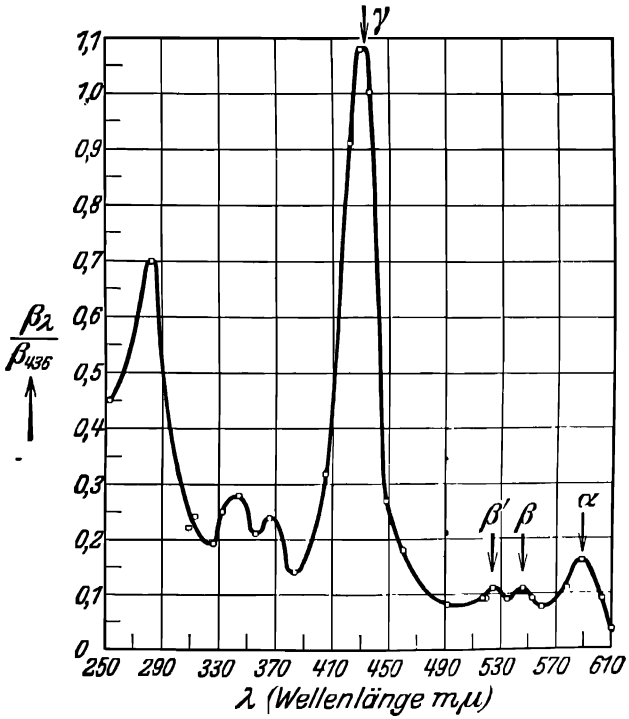


FIG. 35.—Relative photochemical absorption coefficient of the carbon monoxide compound of the oxygen transporting enzyme in *Bacterium Pasteurianum* at 10°C. The absorption at 436 mμ is taken as 1.0. (From F. Kubowitz and E. Haas, Biochem. Ztschr., **205**: 247, 1932.)

Warburg and Negelein[53] investigated the effect of light of various known wave-lengths and of measured intensity on the reversal of the CO inhibition of respiration in wild yeast, *Torula utilis*, measuring the respiratory rate manometrically. From the results obtained, they could plot the relative photochemical absorption spectrum for the coenzyme. The absolute absorption spectrum was also determined by Warburg and Negelein[54] and

[51] C. W. J. Armstrong and K. C. Fisher, J. Cell. & Comp. Physiol., **15**: 103, 1940.

[52] K. C. Fisher and R. Ohnell, *ibid.*, **16**: 1, 1940.

[53] O. Warburg and E. Negelein, Biochem. Ztschr., **193**: 339, 1928

[54] O. Warburg and E. Negelein, Biochem. Ztschr., **202**: 202, 1928.

by Kubowitz and Haas[55] for *Bacterium pasteurianum.* The results obtained by Kubowitz and Haas are shown in Fig. 35. The absorption curve appeared to be similar to a CO-heme compound, but it was not identical with that of any heme pigment known at that time. Warburg and Negelein[54] investigated the absorption spectra of the CO compounds of *Spirographis* heme coupled with the globin of hemoglobin and with phæohemin *b* prepared from chlorophyll *b* (by reduction with HI and replacement of the Mg with Fe). The spectra were similar to that shown in Fig. 35. Warburg, therefore, felt that his enzyme was a phæohemin, and this view is generally accepted.

Recently Melnick[56] has determined the photochemical spectrum of CO-cytochrome oxidase in extracts of rat heart, and he has obtained a marked band at 450 mμ, and lower bands at 510 and 589 mμ. Philips[57] has determined the similar bands of cytochrome oxidase in a membrane from the chick embryo, and obtained bands at 435–445, 553, and 589 mμ. Though neither of these spectra is identical with that of Kubowitz and Haas, they are very similar and are typical of the spectra of heme compounds. It is now certain that cytochrome oxidase is a component of Warburg's oxygen-transferring enzyme. However, it is to be realized that cytochrome oxidase is a generic term, and one must not expect identical spectra for the enzymes in different organisms.

It has been generally believed that cytochrome oxidase is firmly anchored to the structure of the cell, or to cell particles, or to macromolecular constituents. The heart oxidase preparations of Keilin and Hartree[58] lose most of their activity on repeated filtration through Seitz filters. Stern[59] has found that oxidase activity is partially in the sediment when centrifuged at 1000 g. (gravity), and completely so at 16,000 g. He believes that the oxidase is linked to a macromolecular particle of a diameter in the range of 50–200 mμ.

Haas[60] has obtained, for the first time, water-clear solutions of cytochrome oxidase, in which 80 per cent of the activity remains in solution after centrifuging at 10,000 g. for 100 minutes. If the initial material is centrifuged at 10,000 g., the solution contains 20–40 per cent of the activity; by treating with ultrasonic waves at 30°C., he obtains active water-clear solutions.

b). *Cytochrome.*—Keilin[61] rediscovered, in 1925, a class of heme pigments in living cells, which had first been seen by MacMunn[62] in 1886. The significance of MacMunn's discoveries had been lost until Keilin extended MacMunn's observations and correlated these heme pigments with cellular respiration. Keilin named these pigments the cytochromes, and in his original publication recognized three separate pigments, cytochromes *a*, *b*,

[55] F. Kubowitz and E. Haas, Biochem. Ztschr., **205**: 247, 1932.
[56] J. L. Melnick, Science, **94**: 118, 1941.
[57] F. S. Philips, Federation Proc., **1**: 129, 1942.
[58] D. Keilin and E. F. Hartree, Proc. Roy. Soc., London, **B125**: 171, 1938.
[59] K. G. Stern, Cold Spring Harbor Symp., **9**: 312, 1939.
[60] E. Haas, J. Biol. Chem., **149**: 481, 1943.
[61] D. Keilin, Proc. Roy. Soc. London, **B98**: 312, 1925.
[62] C. A. MacMunn, Phil. Tr. Roy. Soc., London, **167**: part I, 267, 1886.

and *c*. Keilin observed that in a contracting muscle of bee's wings, a sharp 4-banded spectrum appeared, that at rest the bands became weak and diffuse. Under anaerobic conditions, in activity, or in the presence of cyanide, the dark bands reappeared. Similar observations were made on yeast under aerobic and anaerobic conditions. Keilin demonstrated that organisms from many phylogenetic groups contained cytochrome, though the relative concentration of the three pigments varied, and in some cases one or another of the pigments was absent.

Keilin proposed, and in later studies[63] has demonstrated, that the cytochromes represent a new class of respiratory enzymes, the carriers, intermediate between an oxidase and other respiratory components. Cytochromes *a* and *c* are not autoxidizable, but are catalytically oxidized from a ferro state with distinct bands to a ferri state in which the absorption is more diffuse. Cytochrome *b* is sluggishly autoxidizable. Keilin[64] correlated his findings with those of Warburg, and proposed that the cytochromes were oxidized by a cyanide-sensitive CO-inhibited oxidase, which was probably identical with Warburg's enzyme.

Recently, several additional cytochromes have been identified; cytochrome a_1, a_2, and b_1 occur in some bacteria lacking cytochrome *a*, such as *Bacillus coli*.[65] Cytochrome a_3 occurs in heart muscle and in yeast and will be discussed more fully below. Cytochrome b_2 has been found in preparations from yeast.[66,67] There is possibly a cytochrome c_1 in heart.[68] The

TABLE XXXV.—ABSORPTION BANDS OF THE FERRO-CYTOCHROMES IN THE VISIBLE SPECTRUM IN $M\mu$

Cytochrome	α	β	γ	Source
a	605	.	452	Heart, yeast
a_1	590	..	...	*Bacillus coli*
a_2	628	...	...	*Bacillus coli*
a_3	600	...	448	Heart, yeast
b	564	530	432	Heart, yeast
b_1	560	..	..	*Bacillus coli*
b_2	557	530	420	Yeast
c	550	521	415	Heart, yeast, wheat

positions of the absorption bands of the cytochromes are shown in Table XXXV. The absorption spectrum of ferro cytochrome *c* is shown in Fig. 36.

Theorell,[69] and Keilin and Hartree,[70] have described methods for the

[63] D. Keilin, Proc. Roy. Soc., London, **B104**: 206, 1929.
[64] D. Keilin, Proc. Roy. Soc., London, **B104**: 206, 1929.
[65] D. Keilin, and C. H. Harkley, Biochem. J., **35**: 688, 1941.
[66] S. J. Bach, M. Dixon, and D. Keilin, Nature, **149**: 21, 1942.
[67] E. Haas, B. L. Horecker, and T. R. Hogness, Science, **95**: 406, 1942.
[68] H. Theorell, Biochem. Ztschr., **279**: 463, 1935.
[69] H. Theorell, Biochem. Ztschr., **285**: 207, 1936.
[70] D. Keilin and E. F. Hartree, Proc. Roy. Soc., London, **B122**: 298, 1937.

preparation of concentrated cytochrome *c*, and Theorell[71] has purified such preparations. Cytochrome *c* is a water-soluble red heme protein of relatively low molecular weight (13,000), fairly stable to heat, and non-autoxidizable, nor does it form compounds with CO, NaF, HCN, in the *p*H range of 4 to 9. This is probably due to the fact that, over this *p*H range, the iron is attached by 6 co-ordination bonds, while at higher or lower *p*Hs, it is attached at only 4 positions and can thus form compounds. At physiological *p*H, cytochrome *c* is readily oxidized by molecular oxygen only in the preence of a specific catalyst, cytochrome oxidase. It is reduced by cytochrome

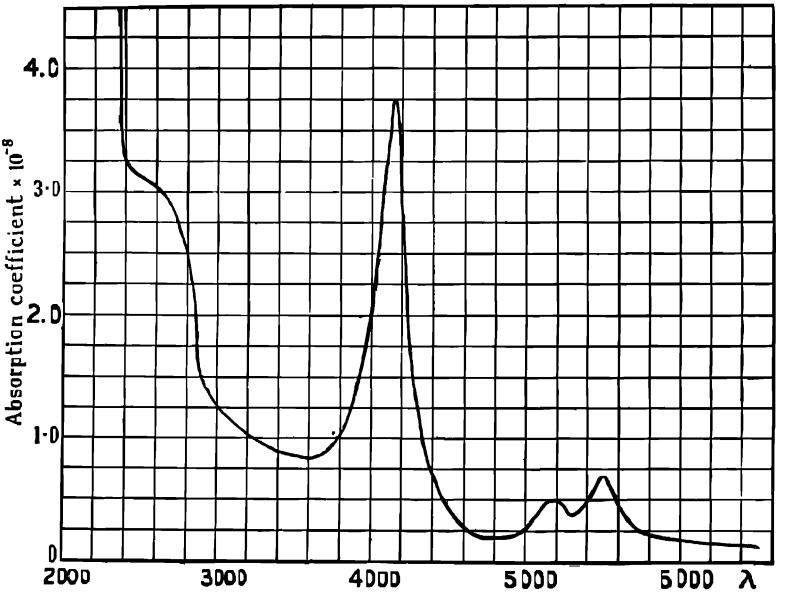


FIG. 36.—Absorption spectrum of yeast ferrocytochrome c. (From M. Dixon, R. Hill, and D. Keilin; Proc. Roy. Soc., London, **B109**: 29, 1931.)

reductase, hydroquinone, cysteine, p-phenylenediamine, and ascorbic acid, but not directly by the respiratory substrates or their dehydrogenases, with the possible exception of succinic dehydrogenase.

The catalytic activity of cytochrome *c* may be easily demonstrated by the methods of Keilin and Hartree.[72] Cytochrome oxidase preparations may be made from heart muscle, which are practically free of cytochrome *c*. These preparations have scarcely any activity in the oxidation of cysteine, p-phenylenediamine, or hydroquinone. Cytochrome *c*, alone, will not catalyze the oxidation of these substrates by molecular oxygen. A complete system gives a rapid O_2 uptake, as is shown in Fig. 37. This catalyzed oxidation is heat-labile and is inhibited by HCN, NaN_3, and photoreversibly by CO. The reactions may be diagrammed below for the oxidation shown in Fig. 37.

[71] H. Theorell and A. Åkesson, J. Am. Chem. Soc., **63**: 1804, 1941.

[72] D. Keilin and E. F. Hartree, Proc. Roy. Soc., London, **B125**: 171, 1938.

$$\begin{array}{l} \begin{array}{l} CH_2SH \\ | \\ 2CHNH_2 \\ | \\ COOH \end{array} + 2\ \text{ferri cytochrome} \rightarrow \begin{array}{l} CH_2S{-}SCH_2 \\ | \quad | \\ CHNH_2\ CHNH_2 \\ | \quad | \\ COOH\ \ COOH \end{array} + 2\ \text{ferro cytochrome} + 2H^+ \\ 2\ \text{ferro cytochrome} + 2\ \text{ferri oxidase} \rightarrow 2\ \text{ferri cytochrome} + 2\ \text{ferro oxidase} \\ 2\ \text{ferro oxidase} + \tfrac{1}{2}O_2 + 2H^+ \rightarrow 2\ \text{ferri oxidase} + H_2O \end{array}$$

$$2\ \text{cysteine} + \tfrac{1}{2}O_2 \rightarrow \text{cystin} + H_2O$$

From the cytochrome concentration determined by Haas and from the rate of O_2 uptake of yeast in glucose at 20°C., Warburg[73] calculates that the

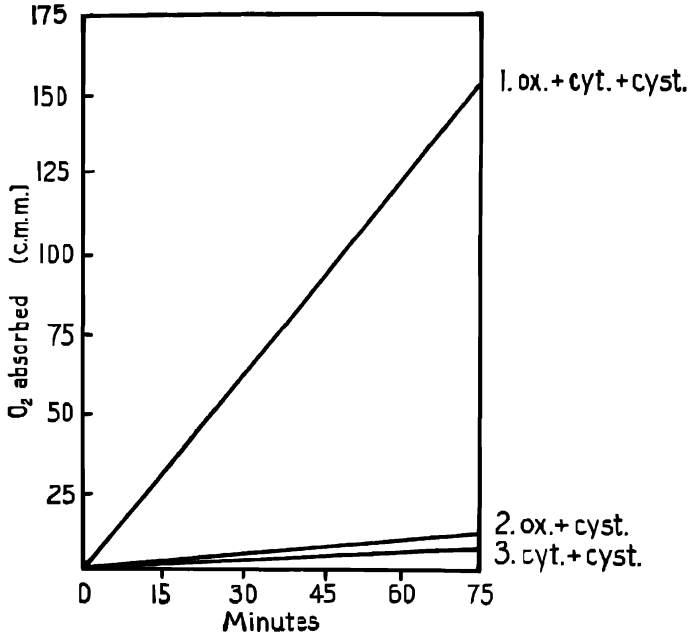


FIG. 37.—The oxidation of cysteine by cytochrome and cytochrome oxidase. (From D. Keilin, Proc. Roy. Soc., London, B106: 418, 1930.)

cytochrome in living yeast must be oxidized and reduced about 4000 times a minute.

It has been known for a long time that many tissues could oxidize α napthol and dimethyl-p-phenylenediamine to indophenol blue, and this property was ascribed to an indophenol oxidase. Recent work[74,75] has made it clear that the indophenol reaction is not due to a single enzyme, but to cytochrome oxidase and cytochrome *c* functioning in a manner similar to that diagrammed above for the oxidation of cysteine.

[73] O. Warburg, Naturwissenschaften, **22**: 441, 1934.

[74] D. Keilin and E. F. Hartree, Proc. Roy. Soc., London, **B125**: 171, 1938.

[75] E. Stotz, E. Sidwell, and T. R. Hogness, J. Biol. Chem., **124**: 733, 1938.

Haas[76] has determined the rate of respiration in baker's yeast by following the rate of cytochrome reduction. This was done by measuring with a photoelectric spectrophotometer[77] the difference in absorption of aerobic and anaerobic yeast at 550 mμ. His conditions (0°C., well-washed yeast, no substrate) were such that in air all the cytochrome was in the ferri form and in a nitrogen atmosphere in the ferro form. He found the concentration of the cytochrome = 1.43×10^{-5} mM./ml. cell suspension. Then, preventing the reoxidation of cytochrome by adding cyanide at $t = 0$, he determined the rate of cytochrome reduction. The results may be expressed by the relation:

$$k = \frac{1}{t} \ln \cdot \frac{C_0}{C},$$

where t = time in minutes,
C_0 = conc. of ferri cytochrome at $t = 0$,
C = conc. of ferri cytochrome at t,
k = velocity constant.

He found that $k = 4$. To convert this into terms of oxygen, we may recall that one mol. of cytochrome is equivalent to ¼ mol. of oxygen, and that ¼ mM. of oxygen is equal to ¼ × 22,400 mm.³ Then: Vol. of O_2 in mm.³ $= k \times \frac{1}{4} \times 22{,}400 \times 1.43 \times 10^{-5}$ mM. = 0.32 mm.³ O_2/ml. cell suspension /min. Direct determination of the oxygen consumed gave, for the same yeast at 0°C., 0.34 mm.³/ml. cell suspension/min. Thus, at least under these conditions, all the respiration could pass over cytochrome.

The exact seriation of the cytochrome actions in respiration is still unclear. It is possible that cytochrome a_3 can oxidize cytochrome *c*, and the latter may have a higher potential than cytochrome *b*.

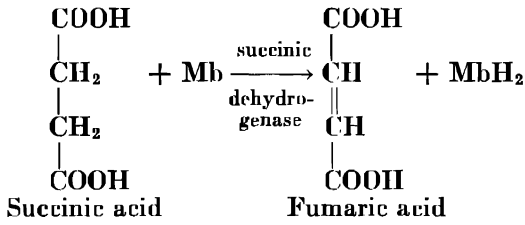
c). *Cytochrome a_3 and cytochrome oxidase.* There is some reason to believe that cytochrome a_3 may be cytochrome oxidase. The evidnce for this view has been ably summarized by Keilin and Hartree[78] and may be briefly given below. Cytochrome a_3 is thermolabile, insoluble; it is rapidly reduced by biological systems, and it forms compounds with HCN, NaN_3, and H_2S. In the ferro state it forms a compound with CO, absorbing at 590 mμ and 432 mμ, which is similar to results obtained by Warburg for the oxygen-transporting enzyme. Cyanide forms compounds with both ferro and ferri a_3, but the cyanide compound is not readily reduced. The CO-ferro-a_3 appears not to be light-sensitive, and this is contrary to what would be expected of cytochrome oxidase.

Straub[79] and Yakushiji and Okunko[80] have prepared from heart muscle colloidal solutions containing cytochrome *a* and a_3, and Straub's preparation was free of *b* and *c*. Straub finds that his preparation forms a compound with CO with a shift of the gamma band from 443 mμ to 430 mμ. He observed that p-phenylenediamine is oxidized only after the addition of cytochrome *c*, and

[76] E. Haas, Naturwissenschaften, **22**: 207, 1934.
[77] M. V. Ardenne and E. Haas, Ztschr. f. physik. Chem., **A174**: 115, 1935.
[78] D. Keilin and E. F. Hartree, Proc. Roy. Soc., London, **B127**: 167, 1939.
[79] F. B. Straub, Ztschr. f. Physiol. Chem., (Hoppe-Seyler's), **268**: 227, 1941.
[80] E. Yakushiji and K. Okunko, Proc. Imp. Acad. Tokyo, **17**: 38, 1941.

Yakushiji reports cytochrome *a* is partially reduced by *c*. No data are available as to light reversal of CO inhibition and inadequate evidence as to the catalytic activity of these preparations is available to decide whether cytochrome a_3 is really the elusive cytochrome oxidase.

3. Hydrogen Mobilization and Transport.—*a*). *Dehydrogenases.*—The cytochrome oxidase-cytochrome system is primarily concerned with the transfer of electrons from the intermediate enzymes to oxygen. Wieland[81] has emphasized the fact that the respiratory substrates normally lose two hydrogen atoms upon oxidation (or two protons and two electrons). Thunberg[82] discovered a class of enzymes, the dehydrogenases, which catalyze the oxidation of specific substrates by the transfer of two hydrogen atoms to a hydrogen acceptor. Thunberg used methylene blue as an artificial hydrogen acceptor, and followed the course of the reaction by observing the decolorization of the methylene blue to its reduced or leuco form. Such a reaction may be illustrated as follows:



Succinic acid Fumaric acid

Today a large number of dehydrogenases are known, which catalyze the oxidation of a wide variety of specific substrates. Some of these are shown in Table XXXVI. As dehydrogenase preparations, washed bacteria or yeast, toluene-treated bacteria, phosphate extracts of seeds, or animal tissues may be used. Several dehydrogenases have been concentrated, and a few are known in essentially pure form. Most dehydrogenases consist of two parts—a specific protein and a coenzyme. At the present time, two coenzymes for dehydrogenases are known; each coenzyme may act with several different specific proteins (see Table XXXVI).

b). *Phosphopyridine nucleotides.*—Harden and Young[83] discovered cozymase, a coenzyme essential for alcoholic fermentation by yeast juice. Its chemical nature remained unknown until the 1930's. In 1932 Warburg and Christian[84] were studying O_2 consumption of an artificial system, consisting of glucose-6-phosphate, a yellow protein from yeast, and a protein fraction (Zwischen ferment) from yeast, and they found that this system required a coenzyme which differed from the cozymase of Harden and Young, and therefore they called it coenzyme II. The Warburg group[85]

[81] H. Wieland, On the Mechanism of Oxidation: Yale University Press, 1932.

[82] T. Thunberg, Quart. Rev. Biol., **5**: 18, 1930, and Ergebn. d. Enzymforsch., **7**: 163, 1938

[83] A. Harden and W. J. Young, Proc. Roy. Soc., London, B77: 405, 1906.

[84] O. Warburg and W. Christian, Biochem. Ztschr., **254**: 438, 1932.

[85] O. Warburg, W. Christian, and W. Griese, *ibid.*, **282**: 157, 1935.

isolated this coenzyme from horse blood and showed that it was a dinucleotide containing one molecule of nicotinic acid amide, one of adenine, two of

TABLE XXXVI. – SOME REPRESENTATIVE DEHYDROGENASES

Substrate	Product	Coenzyme	Occurrence
Triosephosphate	Phosphoglyceric acid	I	Crude preparations from animal tissues
Lactic acid	Pyruvic acid	I	Crystalline form from heart
Alcohol	Acetaldehyde	I	Crystalline form from yeast
Malic acid	Oxalacetic acid	I	Crude preparations from yeast and tissues
β-hydroxybutyric acid	Acetoacetic acid	I	Crude preparations from animal tissue
Formic acid	CO_2	I	From seeds and *Bacillus coli*
Glucose-6-monophosphate	Phosphohexonic acid	II	From yeast in purified state From horse red cells
Isocitric acid	Keto β-carboxy-glutaric acid	II	Crude preparations from seeds, animal tissues and yeast
Glutamic acid	Iminoglutaric acid	II	For yeast specific protein
Glutamic acid	Iminoglutaric acid	I or II	For animal specific protein
Glutamic acid	Iminoglutaric acid	I	For plant specific protein

Data from F. Schlenck in A Symposium on Respiratory Enzymes: Univ. Wisconsin Press, 1942.

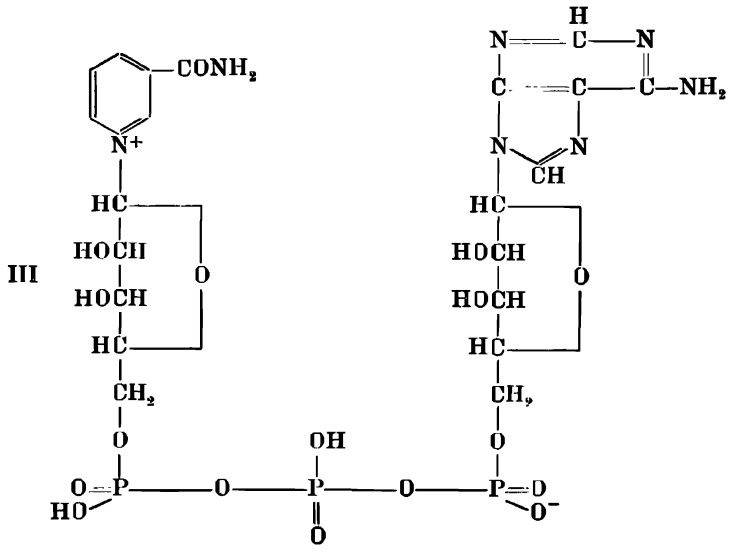


FIG. 38.—Probable structure for triphosphopyridine nucleotide.

ribose, and three of H_3PO_4. A probable structure of this coenzyme is shown in Fig. 38. Nicotinic acid amide had not previously been recognized as occurring in nucleotides, nor had any physiological role been recognized

for it. Warburg showed that, when glucose-6-phosphate was oxidized, the coenzyme was reduced by the acceptance of two hydrogen atoms, and that it was the pyridine ring which underwent reduction. The reduced coenzyme could be reoxidized by the flavin enzyme.

Coenzyme I (cozymase) was soon obtained in pure form, and it was shown to be identical with coenzyme II, except that it had but two phosphorus atoms. Warburg[86] renamed coenzyme I, diphosphopyridine nucleotide; and coenzyme II, triphosphopyridine nucleotide. Both coenzymes are widely distributed, for they are known from yeast, bacteria, and higher plants and animals.

The evidence is very strong that many of the dehydrogenases dissociate into two parts, the protein and coenzyme; it is equally evident that the catalytic activity is a property of the complete dehydrogenase molecule. We may formulate this as follows:

$$\text{dehydrogenase} \rightleftharpoons \text{specific protein} + \text{coenzyme}$$

$$\frac{(\text{specific protein})\,(\text{coenzyme})}{(\text{dehydrogenase})} = K_e,$$

where K_e is the dissociation constant of the enzyme.

In practice, K_e may be determined by using a small amount of specific protein in the presence of an excess of substrate (in the example used here, glucose-6-phosphate) and with increasing amounts of coenzyme follow the velocity of the reduction of the coenzyme. It is essential that conditions are such that the velocity of the reaction depends upon the concentration of the enzyme, and that the concentration of the specific protein is sufficiently small, and its dissociation constant sufficiently large, so that the concentration of total coenzyme is essentially that of free coenzyme. Then the Michaelis-Menten equation (see Sec. 1, chap. 2) may be used:

$$\frac{V}{V\ \text{max.}} = \frac{S}{K_e + (S)}.$$

V = velocity,
V max. = maximum velocity,
(S) = coenzyme concentration,
K_e = dissociation constant of the enzyme.

When V/V max. = 0.5, then $(S) = K_e$.

The rate of the reaction may be followed spectrophotometrically at 340 mμ, for both coenzymes I and II absorb strongly at this wave-length in the reduced form and but slightly in the oxidized form (see Fig. 39.) The value of K_e for glucose-6-phosphate dehydrogenase has been determined by Negelein and Haas[87] and found to be 1.1×10^{-5} mols./liter at 38°C.

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[86] O. Warburg, Ergebn. d. Enzymforsch., 7: 210, 1938.

[87] E. Negelein and E. Haas, Biochem. Ztschr., 282: 206, 1935.

Similarly, the alcohol dehydrogenase has been studied by Negelein and Wulff.[88] The protein has been isolated and shown to be coenzyme I-specific. The dissociation constant has been determined, and, in this case, is different for the reduced and oxidized enzyme:

$$\text{enzyme} + \text{alcohol} \rightleftharpoons \text{enzyme}\cdot H_2 + \text{acetaldehyde}.$$

We may formulate the equation for the dissociation of enzyme into specific protein + coenzyme I and enzyme. H_2 into specific protein + coenzyme $\cdot$I$\cdot H_2$ just as was done above. The K_c of oxidized enzyme was found to be 9.5×10^{-5} and of the reduced 3.2×10^{-5}.

The activity of the specific proteins may be followed by adding them to coenzyme I or II and following the oxidation of the coenzyme spectrophotometrically at 340 mμ. This is illustrated in Fig. 40, where phosphoglyceraldehyde-specific protein is added at zero minutes to coenzyme I, phosphoglyceraldehyde and acetaldehyde. The coenzyme is reduced, then acetaldehyde-specific protein is added and the coenzyme is oxidized. The addition of further protein shifts the steady state to a new value.

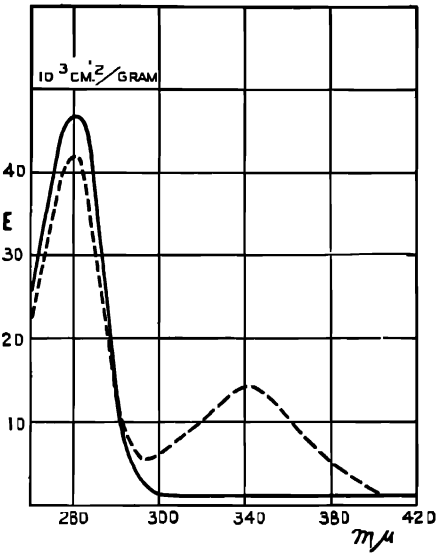


Fig. 39.—The absorption coefficient for reduced triphosphopyridine nucleotide (dashed curve) and for the oxidized form (continuous curve). (From F. Schlenk, in A Symposium on Respiratory Enzymes: Univ. of Wisconsin Press, 1942.)

c). *Succinic dehydrogenase.*—One of the most active and widely distributed enzymes of animal tissues is succinic dehydrogenase. This enzyme occurs in most cytochrome oxidase preparations, and is oxidized by cytochrome oxidase—cytochrome *c* system. So far the enzyme has not been obtained in solution, nor has it been separated into a coenzyme and a specific protein. No intermediate between the dehydrogenase and cytochrome *c* has been established. Dialyzed preparations lose much of their activity, which can be restored by the addition of calcium ions[89,90] and aluminum or chromium ions.[90] This enzyme seems of particular importance, because it may serve to link other dehydrogenases to cytochrome *c*. It is not certain whether succinic dehydrogenase is widely distributed in plants. The observations of Hill and Bhagvat[91] would indicate that it occurs in bean seeds.

[88] E. Negelein and J. J. Wulff, Biochem. Ztschr., **289**: 436, 1937; **293**: 351, 1937.
[89] B. L. Horecker, E. Stotz, and T. R. Hogness, J. Biol. Chem., **128**: 251 (1939).
[90] V. R. Potter and W. C. Schneider, J. Biol. Chem., **142**: 549, 1942.
[91] R. Hill and K. Bhagvat, Nature, **143**: 726, 1939.

d). *The flavin enzymes.*—It is apparent that a substrate may become oxidized by its dehydrogenase, and the dehydrogenase become reduced, but if the dehydrogenase is to act catalytically, it, in turn, must become oxidized. The great majority of dehydrogenases cannot be oxidized by molecular oxygen, nor directly by ferri cytochrome. Some intermediate carriers must be present, or some oxidase which reacts not only with the reduced dehydrogenase, but also with molecular O_2.

Warburg and Christian,[92] in 1932, isolated a yellow protein from bottom yeast, which could be separated into two parts, a colorless protein and a

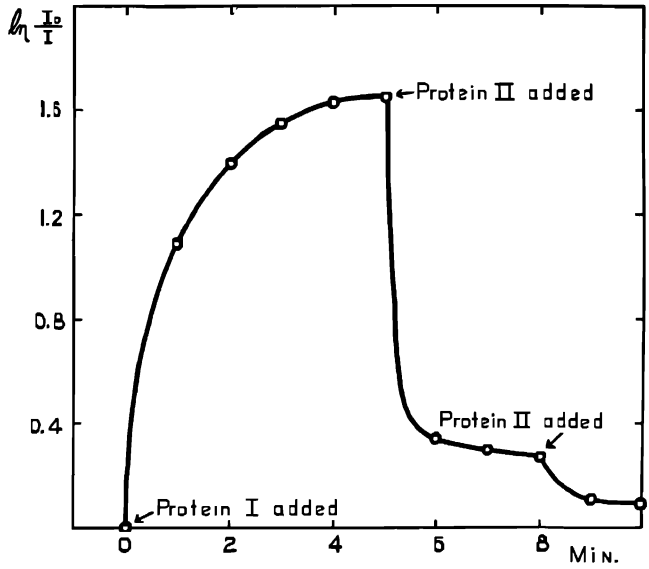


FIG. 40.—The reduction of diphosphopyridine nucleotide by phosphoglyceraldehyde upon addition of the specific protein (I); and the oxidation of the diphosphopyridine by acetaldehyde in the presence of its specific protein (II). (From F. Schlenk, in A Symposium on Respiratory Enzymes: Univ. of Wisconsin Press, 1942.)

mononucleotide. The latter is now known as riboflavin-phosphate (or the phosphate ester of vitamin G). This protein could oxidize the reduced form of coenzyme II, and in the presence of O_2 the flavoprotein was reoxidized, and H_2O_2 was formed. The structure of the flavin is shown in Fig. 41. Warburg and Christian reconstructed a respiratory system using glucose-6-phosphate, coenzyme II, a specific protein (Zwischen enzyme) and flavoprotein, which is diagrammed below:

$$\text{glucose-6-phosphate} + \text{en·coen.} + H_2O \rightarrow \text{phosphogluconic acid} + \text{en·coen·}H_2.$$

$$\text{en·coen·}H_2 + \text{flavoprotein} \rightarrow \text{en·coen.} + \text{leuco flavoprotein.}$$

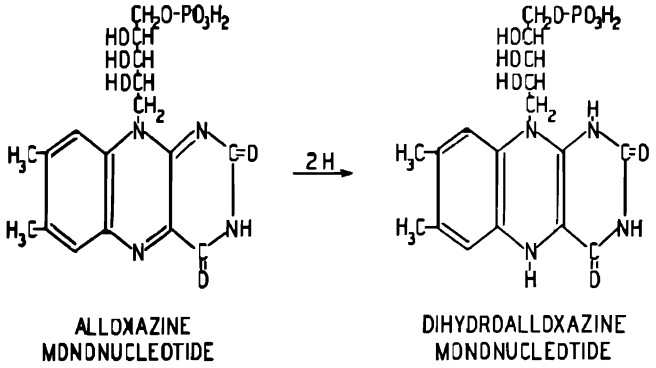
$$\text{leuco flavoprotein} + O_2 \rightarrow \text{flavoprotein} + H_2O_2.$$

The reaction with O_2 is slow, particularly at low O_2 tensions, and there is

[92] O. Warburg and W. Christian, Biochem. Ztschr., **254**: 438, 1932; **263**: (1933); **287**: 299, 440, 1936; **266**: 375, 1933.

some real question whether the particular flavoprotein isolated by Warburg and Christian can function physiologically with O_2.

That a flavin enzyme may act as an oxidase in intact cells is shown by results obtained by Haas, and by Warburg and Christian[93] with *Lactobacillus delbruckii*. This bacillus has no heme pigments and normally grows under anaerobic conditions; however, in the presence of oxygen, it respires and pro-



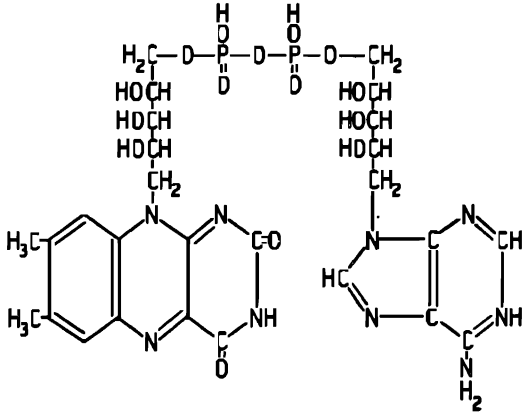


FIG. 41.—Structures of prosthetic groups of flavin enzymes.

duces H_2O_2.[93,94] Unfortunately, Haas has never published his experiments, but Warburg[95] has reported some of Haas' results. Haas determined the difference in the absorption spectrum of the bacteria under aerobic and anaerobic conditions, and he found that no bands of heme pigments were present,

[93] O. Warburg and W. Christian, Biochem. Ztschr., **266**: 375, 1933.
[94] J. G. Davis, *ibid.*, **265**: 90; **267**: 357, 1933.
[95] O. Warburg, Naturwissenschaften, **22**: 441, 1934.

but that aerobically a broad band appeared in the blue, with its peak at 460 mμ; anaerobically the band disappeared. This behavior and the structure of the band were typical of a flavoprotein. He determined the total concentration calculated as flavin, and the rate of oxidation of the flavin by adding, anaerobically, methylene blue to the bacteria. The flavin concentration was 6.02×10^{-4} mM. per ml. of suspension and the velocity constant 0.9 min.$^{-1}$ This gives an oxygen equivalent of 1.2 mm.^{3}/ml. suspension/min. He observed in direct measurements an O_2 consumption of 1.0 mm.^{3}/ml. suspension/min. Thus, all the O_2 consumption could be accounted for by the flavin enzyme. Warburg and Christian have calculated the turnover number of the flavin that would be essential for the observed oxygen uptake in this bacteria at 38°C., and they obtain a value of 30, and since they found a turnover number for flavin enzyme in cytolyzed horse red cells of 50, all the respiration of *Lactobacillus* at 38°C. may be catalyzed by a flavin acting as an oxidase. (The turnover number = 2 moles of enzyme/moles of O_2 consumed per minute.)

The results of Haas, and Warburg and Christian do not allow us to determine what flavin enzyme is involved, since several have similar absorption spectra.

It has frequently been suggested that a flavin enzyme is responsible for the cyanide-resistant respiration of animals and higher plants. These organisms do not accumulate H_2O_2, as is typical of flavin oxidation by molecular O_2, but the nearly universal presence of catalase would prevent that. The low turnover number of cytochrome reductase and the original flavin enzyme of Warburg and Christian, which are respectively 2.2 and 27 at 25°C. in air, and their strong dependence upon O_2 pressure, make them appear unlikely as oxidases. The O_2 consumption of cyanide-resistant leaves is frequently 400 mm.^{3}/hr./gm. wet wt., and this requires a flavin concentration of approximately 1×10^{-5} molar if the flavin enzyme is the oxidase. However, some as yet undiscovered flavin enzyme may well be active in this catalysis, for d-amino-acid oxidase which is a flavo-adenine-protein has a turnover number of 1400 at 100 per cent oxygen and 38°C.[96]

e). *Cytochrome reductase.*—Haas, Horecker, and Hogness[97] discovered in 1940 a new flavin protein which has the same prosthetic group as the flavo-protein of Warburg and Christian, but which is rapidly oxidized by cytochrome *c*. This enzyme, which they have named cytochrome *c* reductase, rapidly oxidized dehydrogenase systems which are coenzyme II-specific. It thus furnishes a connecting link for these systems with cytochrome *c*, cytochrome oxidase and O_2. The new enzyme has but slight reactivity with molecular O_2 or with coenzyme I.

Artificial systems have been reconstructed[97] and their kinetics followed by the rate of reduction of cytochrome *c* spectrophotometrically. A reconstructed system is shown in the diagram below.

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[96] O. Warburg and W. Christian, Biochem. Ztschr., **298**: 150, 1938.

[97] E. Haas, B. L. Horecker, and T. R. Hogness, J. Biol. Chem., **136**: 747, 1940; E. Haas, C. J. Harrer, and T. R. Hogness, *ibid.*, **143**: 341, 1942.

$$\text{glucose-6-phosphate} + \text{coen. II} + H_2O \xrightarrow[\text{protein}]{\text{specific}} \text{phosphogluconic acid} + \text{coen. II } H_2$$

$$\underset{\text{leuco}}{\text{coen. } H_2} + \underset{\text{ferri}}{\text{cytochrome reductase}} \rightarrow \underset{\text{ferri}}{\text{coen.}} + \underset{\text{ferro}}{\text{leuco cytochrome reductase}}$$

$$\underset{\text{ferro}}{\text{cytochrome reductase}} + 2\ \underset{\text{ferri}}{\text{cytochrome } c} \rightarrow \underset{\text{ferri}}{\text{reductase}} + 2\ \underset{\text{ferro}}{\text{cytochrome}}$$

$$2\ \underset{\text{ferro}}{\text{cytochrome}} + 2\ \text{oxidase} \rightarrow 2\ \underset{\text{ferri}}{\text{cytochrome}} + 2\ \text{oxidase}$$

$$2\ \text{oxidase} + 2H^+ + \tfrac{1}{2}O_2 \rightarrow 2\ \text{oxidase} + H_2O$$

$$\text{glucose-6-phosphate} + \tfrac{1}{2}O_2 \rightarrow \text{phosphogluconic acid}$$

By making one constituent after another limiting in the reaction system, the velocity constants of the partial reactions have been determined,[98] and the fact that cytochrome reductase forms intermediate molecules with the coenzyme and with cytochrome *c* has been established. The velocity con-

TABLE XXXVII.—SOME CONSTANTS OF A FEW FLAVIN ENZYMES

k = liter × min.$^{-1}$ × moles^{-1}

Enzyme	Prosthetic group	Velocity constants at 25°C.			Dissociation constant K
		Reduction by coenzyme k	Oxidation by O_2 k	Oxidation by cytochrome c k	
Flavin enzyme of Warburg and Christian.	Alloxazine-mononucleotide	6×10^6	10×10^4	0.3×10^5	60×10^{-9}
Flavin enzyme of Haas	Alloxazine-adenine di-nucleotide	22×10^6	14×10^4	0	
Cytochrome reductase	Alloxazine-mononucleotide	170×10^6	0.8×10^4	$53{,}000 \times 10^5$	1×10^{-9}

Data from E. Haas, C. J. Harrer, and T. R. Hogness, J. Biol. Chem., **143**: 341, 1942; Warburg and Christian, Biochem. Ztschr., **298**: 368, 1938.

stants for several flavoproteins are shown in Table XXXVII. The system outlined in the diagram above is probably more completely worked out than any other reconstructed oxidation scheme involving several steps.

Cytochrome reductase: coenzyme I. The cytochrome reductase of Haas, *et al*, is not reduced by coenzyme I, and therefore cannot link coenzyme I dehydrogenases to cytochrome *c*. There is present[99] in liver dispersions

[98] E. Haas, C. J. Harrer, and T. R. Hogness, J. Biol. Chem., **143**: 341, 1942.

[99] E. E. Lockart and V. R. Potter, *ibid.*, **137**: 1 (1941).

an insoluble enzyme which reduces cytochrome *c* and oxidizes reduced coenzyme I. The nature of this reductase is still unknown.

f). *Other flavin enzymes.*—Warburg and Christian[100] isolated a protein which had flavin-adenine dinucleotide as the prosthetic group. This protein is very active in the oxidation of many d-amino-acids to NH_3 and the corresponding α keto acid. Since the naturally occurring amino-acids are l-amino-acids, the physiological function of this enzyme is unknown.

It has long been known that an enzyme existed in tissues and milk which would oxidize xanthine and hypoxanthine to uric acid, and acetaldehyde to acetic acid; this enzyme is known as xanthine oxidase or Schardinger enzyme. Ball[101] has purified xanthine oxidase, and his preparations contained a flavin-adenine dinucleotide.

Several additional flavo-proteins have been isolated,[102,103] but their physiological function has not been established.

TABLE XXXVIII.—CLASSIFICATION OF CERTAIN OXIDASES

HCN	Inhibited by		Light-reversible CO inhibition	P.P.D.[3] Test	Cytochrome	Enzyme
	NaN_3	CO				
+	+	+	+	+ presence of cytochrome *c*	a_3, *a*, *b*, *c*	Cytochrome oxidase
+	+	+	−	+, if catechol present	. . .	Catechol oxidase
+	+	−	−	+ (without catechol)	.	Laccase
+	+	+	−	−	a_1, a_2, b_1	*Bacillus coli* type
−	−	−	−	−	. . .	*Lactobacillus delbruckii* type (flavin enzyme)
±[1]	+	+[2]	+[2]	+ only with H_2O_2	. .	Dioxymaleic acid oxidase (Peroxidase)

[1] Peroxidase I inhibited, peroxidase II not inhibited.

[2] On plant extracts.

[3] Paraphenylenediamine.

4. Non-cytochrome Systems.—Respiration catalyzed by the cytochrome oxidase-cytochrome system is widespread throughout living organisms, but is not universal. There are organisms known whose respiration is not inhibited by the poisons, cyanide, azide, sulfide, or photoreversibly by carbon monoxide, and which do not have cytochrome. We know less of the mechanism of the respiration in such organisms than in the cytochrome oxidase organisms, but several features are known. It must also be realized

[100] O. Warburg, and W. Christian, Biochem. Ztschr., **295**: 291; **298**: 150, 1938.

[101] E. Ball, J. Biol. Chem., **128**: 51, 1939.

[102] E. Haas, Biochem. Ztschr., **298**: 378, 1938.

[103] F. B. Straub, Biochem. J., **33**: 387, 1939.

that cytochrome oxidase organisms may contain additional oxidative mechanisms, so that several respiratory mechanisms may exist side by side in the same organism and even in the same cell.

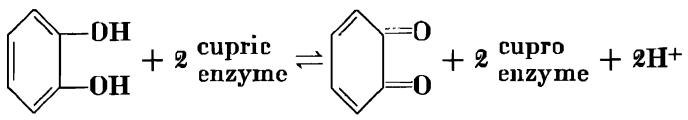
The use of a respiratory poison is a useful, even though fallible, tool for a first classification of respiratory systems, and the responses of several to respiratory inhibitors are shown in Table XXXVIII.

a). *Cyanide-resistant respiration.*—Commoner[104] has reviewed the effects of cyanide on cellular respiration. In many cells and tissues, a residual respiration exists in the presence of cyanide, and in some plants or parts of plants[105,106] and in certain bacteria,[107] no inhibition occurs. The nature of the oxidases responsible for this respiration is unknown. It has frequently been proposed that the flavin enzymes are responsible, and though this suggestion may be probable, it is not established.

b). *Azide-resistant respiration.*—Stannard[108] has shown that the respiration of stimulated or contracting frog muscle is inhibited by azide, cyanide, and photoreversibly by CO, but that the respiration of resting frog muscle is not sensitive to azide or CO, but is inhibited by cyanide. The nature of the oxidase active in resting muscle has not been established.

c). *Catechol oxidase.*—The polyphenol oxidases have been known since the work of Bertrand in 1894,[109] and these have been recently reviewed by Nelson and Dawson.[110] However, it is only recently that they have been purified and their role established. In 1929 Keilin[111] showed that the catechol oxidase of potatoes was inhibited by HCN, H_2S, and CO, and that, unlike cytochrome oxidase, the CO inhibition was not reversed by light. Kubowitz[112] has isolated a copper protein from potatoes which has high catechol oxidase activity and has shown that the activity depends upon the copper content. He was able[113] to remove the Cu by dialysis against HCN and to show that neither the Cu nor the protein was active alone, though he could regenerate the enzymatic activity of the protein upon addition of Cu.

The catechol oxidase of potatoes oxidizes orthodiphenols such as catechol to orthoquinones:

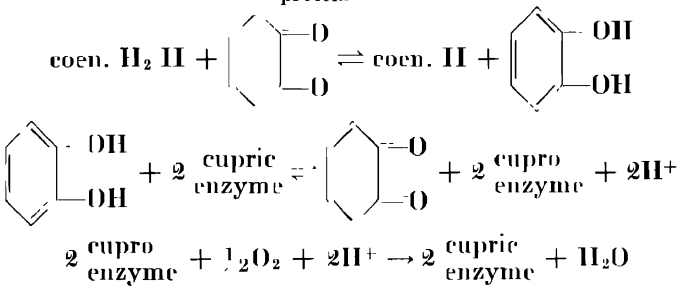


[104] B. Commoner, Biol. Rev., **15**: 168, 1940.
[105] P. B. Marsh and D. R. Goddard, Am. J. Bot., **25**: 724, 1939.
[106] J. Merry and D. R. Goddard, Proc. Rochester Acad. Sc., **8**: 24, 1941.
[107] S. Yamagutchi, Acta phytochim., **8**: 157, 1934.
[108] J. N. Stannard, Am. J. Physiol., **126**: 196, 1939.
[109] C. R. Bertrand, Acad. Sc., Paris, **118**: 1215, 1894.
[110] J. M. Nelson and C. R. Dawson, Advances in Enzymology, **4**: 99, 1944.
[111] D. Keilin, Proc. Roy. Soc., London, **B104**: 2066, 1929.
[112] F. Kubowitz, Biochem. Ztschr., **292**: 221, 1937.
[113] F. Kubowitz, *ibid.*, **299**: 32, 1938.

Meta and para compounds such as resorcinol and hydroquinone are not oxidized, and monophenols, such as phenol and p-cresol, are only oxidized after a lag period.

Catechol may itself act catalytically as a carrier, for a small amount, say 0.01 mg., may be used to oxidize several mgs. of p-phenylene-diamine or glucose-6-phosphate. In the latter case coenzyme II and the specific protein for glucose-6-phosphate are essential. The whole reaction may be diagrammed below:

$$\text{glucose-6-phosphate} + \text{coen. II} \xrightarrow[\text{protein}]{\text{specific}} \text{phosphogluconic acid} + \text{coen. } H_2\text{ II}$$



Thus, any system which functions through triphosphopyridine nucleotide could be oxidized over catechol oxidase. It is probable that similar activity would occur with diphosphopyridine nucleotide. The naturally occurring diphenols are probably not catechol but glucoside derivatives of catechol. Kubowitz's preparations were very active, 1 mg. of enzyme catalyzing the uptake of 34,500 mm.^{3} O_2 per hour at 20° C., which means that each molecule of enzyme went through over 3,000 complete cycles of oxidation and reduction per minute.

Catechol oxidase is widely distributed in higher plants and in fungi.[114] That catechol oxidase functions catalytically in cellular respiration appears to be very probable from the results of Baker and Nelson,[115] who have shown that the respiration of potato tissue is largely inhibited by HCN and 4-nitro-catechol, and the latter is a specific catechol oxidase poison. Further, they showed that the addition of small amounts of protocatechuic acid produced a marked stimulation in respiratory rate (R.Q. of 1.0), which continued for several hours, and the total extra O_2 consumed was several times greater than would account for the oxidation of protocatechuic acid. Protocatechuic acid or other substituted catechols are probably the natural carriers which function in cell respiration.

d). *Other copper enzymes.*—Several additional polyphenolases are known for which no respiratory role has been established; for example, laccase[116] and

[114] D. Keilin and T. Mann, Proc. Roy. Soc., London, **B125**: 187, 1938.

[115] D. Baker and I. M. Nelson, J. Gen. Physiol., **26**: 269, 1943.

[116] D. Keilin and T. Mann, Nature, **143**: 23, 1939; **144**: 442, 1940.

tyrosinase[117] have been isolated and shown to be copper proteins. Polyphenol oxidase has also been reported [118] to occur in animal tissues. Ascorbic acid is widely distributed in plant tissues,[119] and is in some cases rapidly oxidized in injured tissues. A copper protein which specifically oxidizes ascorbic acid has been isolated[120] from summer squash.

e). *Other heme enzymes.*—Cytochrome and, presumably, cytochrome oxidase are heme enzymes in which the hemes are not identical with the heme of hemoglobin. However, a series of heme enzymes are known which have the same heme for their prosthetic group as occurs in hemoglobin. Some of the

TABLE XXXIX.—VISIBLE ABSORPTION SPECTRA FOR SOME HEME PIGMENTS AND ENZYMES

Substance	Prosthetic group	Source	Spectrum mμ
Ferri-hemoglobin	Protoferriheme IX	Mammalian blood	542, 578, 634
Ferro-hemoglobin	Protoferroheme IX	Mammalian blood	430, 555–570
CO-hemoglobin	Protoferroheme IX	Mammalian blood	420, 540, 570
Catalase			
Animal	Protoferriheme IX	Horse liver	409, 505, 540, 622
Plant	Ferriheme	Pumpkin seed	500, 540, 629
Peroxidase			
I (paraperoxidase)	Protoferriheme IX	Horse radish	548, 583
II	Protoferriheme IX	Horse radish	498, 640
Animal	Ferriheme	Milk	355, 600, 640
Verdoperoxidase	Unknown heme	Blood of man	500, 570, 625, 630
Cytochrome peroxidase	Protoferriheme	Yeast	410, 500, 620
Chlororuorin	*Spirographis* heme	*Spirographis*	440, 570, 605
CO-chlororuorin	*Spirographis* heme	*Spirographis*	440, 600
CO-cytochrome oxidase	Phæohemin	*Torula utilis*	430, 510, 540, 591
		Baker's yeast	430, 510, 560, 589
		Acetic bacteria	430, 524, 546, 589
		Chick embryo	435–445, 553, 589
		Extract—rat heart	450, 510, 589
Pasteur enzyme			
Animal	Phæohemin	Rat retina	450, 515, 578
Plant		Yeast	430, 510, 589

heme enzymes and their spectroscopic properties are referred to in Table XXXIX. Each enzyme has, however, its specific properties, none of which is identical with that of hemoglobin—that is, the different proteins impart different catalytic activities to the separate enzymes. Typical of these enzymes are catalase, crystallized by Sumner and Dounce.[121] Catalase forms a compound with the substrate ethylhydrogen peroxide of sufficiently long life to be demonstrated spectroscopically,[122] and this is the first case of the physical demonstration of an intermediate molecule between substrate and enzyme. The enzyme is

[117] H. R. Dalton and I. M. Nelson, J. Am. Chem. Soc., **51**: 2946, 1939.
[118] I. F. Cadden and L. V. Dill, J. Biol. Chem., **141**: 105, 1942.
[119] A. Szent-Györgyi, Biochem. J., **22**: 1387, 1928.
[120] P. L. Lovett-Janison and J. M. Nelson, J. Am. Chem. Soc., **52**: 1409, 1940.
[121] J. B. Sumner and A. L. Dounce, J. Biol. Chem., **121**: 417, 1937.
[122] K. G. Stern, *ibid.*, **114**: 473, 1936.

extremely active, for one molecule may decompose 10^5 molecules of H_2O_2 per second at 0°C. No clearly established function of catalase in cellular metabolism has ever been shown except to get rid of H_2O_2 produced in some oxidations.

Cytochrome *c* peroxidase is a heme protein, isolated[123] from yeast, that oxidizes cytochrome *c* at the expense of H_2O_2. Thus, the H_2O_2 may be used to bring about respiration by way of the cytochrome system.

Peroxidase has long been recognized as an enzyme present in higher plants, which will catalyze the oxidation of some organic compounds (phenols, phenylenediamines, etc.) by H_2O_2. It has been isolated by Theorell[124] from horse radish, and by electrophoresis resolved into two crystalline fractions, both heme proteins, which he has named peroxidase and paraperoxidase. Further, he has separated peroxidase into a white protein and heme, and then has regenerated 93% of the original catalytic activity by combining the protein with heme prepared from hemoglobin. Ferri peroxidase forms a compound with H_2O_2 which is green in color; in the presence of an oxidizable substrate, this intermediate is ephemeral.

The physiological role of peroxidase may really be due to its oxidase activity, for Swedin and Theorell[125] have shown that peroxidase has catalytic activity in the oxidation of dioxymaleic acid. It had been shown[125,127] that many plants contain an enzyme oxidizing dioxymaleic acid to dioxysuccinic (dioxytartaric) acid. Dioxymaleic acid occurs[128] in plants and if dioxysuccinic acid is rapidly and reversibly reduced by dehydrogenases, the peroxidase dioxymaleic acid system may constitute an oxidase carrier system. Paraperoxidase oxidizes dioxymaleic acid only in the presence of phenols, but unlike peroxidase, this oxidation is not inhibited by HCN. Thus, either cyanide-sensitive or cyanide-resistant respiration could be catalyzed by peroxidase, functioning as oxidase.

Verdoperoxidase is a green heme protein isolated[129] from leucocytes of man which has peroxidase activity, but is distinct from the horse radish enzyme.

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[123] A. M. Altschul, R. Abrams, and T. R. Hogness, J. Biol. Chem. **136**: 777, 1940.

[124] H. Theorell, Ark. Kemi. Miner. Geol., **14B**: No. 20, 1940; **15B**: No. 24, 1942; **16A**: No. 1, 1942; No. **16A**: No. 8, 1942.

[125] B. Swedin and H. Theorell, Nature, **145**: 71, 1940.

[126] J. Banga and A. Szent-Györgyi, Ztschr. f. physiol. Chem. (Hoppe Seyler), **255**: 57, 1938.

[127] J. Banga, and E. Philippot, *ibid.*, **258**: 147, 1938.

[128] L. Gatet, Enzymologia, **6**: 375, 1939.

[129] K. Agner, Acta physiol. Scandinav., **2**: supp. 7, 1, 1941; Recent Adv. Enzymology, **3**: 137, 1943.

28

THE ORIGIN OF CARBON DIOXIDE

One of the characteristic features of the respiration of living cells is the production of carbon dioxide. However, the oxidation of many substrates by the dehydrogenase-oxidase system does not directly result in CO_2 formation. Less is known concerning the mechanism of the formation of CO_2 than is known concerning the oxidation of hydrogen to water. Both processes are, however, equally important to an understanding of the mechanism of cellular respiration.

Carboxylase is an enzyme discovered by Neuberg[130] in yeast, and is widely distributed in higher plants. It catalyzes the decarboxylation of α-keto-acids to the corresponding aldehydes and CO_2:

$$\begin{array}{l} CH_3 \\ | \\ C{:}O \\ | \\ COOH \\ \text{Pyruvic acid} \end{array} \rightleftharpoons \begin{array}{l} CH_3 \\ | \\ CHO \\ \text{Acetaldehyde} \end{array} + CO_2$$

The enzyme prepared from yeast,[131,132] is a diphosphothiamine magnesium protein, which in alkaline solutions dissociates into a specific protein and diphosphothiamine (pyrophosphate ester of vitamin B_1). The coenzyme was discovered by Auhagen,[133] and Lohmann and Schuster[134] demonstrated its vitamin character. Divalent manganese or cobalt can replace[135] magnesium without loss of activity. The yeast enzyme is active with pyruvic and oxalacetic acids, but is inactive with α-ketoglutaric acid.

The plant type of carboxylase has been considered absent from animal tissues, but Evans and his colleagues[136] have shown that pigeon liver contains an enzyme which decarboxylates oxalacetic acid to pyruvic acid and CO_2:

$$COOH{\cdot}CH_2{\cdot}C{:}O{\cdot}COOH \rightleftharpoons CH_3{\cdot}C{:}O{\cdot}COOH + CO_2$$

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[130] C. Neuberg and L. Kerczag, Biochem. Ztschr., **36**: 68, 1911.

[131] D. E. Green, D. E. Herbert, and J. F. Subrokmanycam, J. Biol. Chem., **135**: 327, 1941.

[132] F. Kubowitz and W. Luttgens, Biochem. Ztschr., **307**: 107, 1941.

[133] E. Auhagen, Ztschr. f. physiol. chem. (Hoppe Seyler's), **204**: 140, 1932; **209**: 20, 1932; Biochem. Ztschr., **257**: 92, 1932.

[134] K. Lohmann and P. Schuster, *ibid.*, **294**: 188, 1937.

[135] M. G. Vorhaus, R. R. Williams, and R. E. Waterman, J.A.M.A., **105**: 1580, 1935.

[136] E. A. Evans, Jr., B. Vennesland, and L. Slotin, J. Biol. Chem., **147**: 771, 1943.

The ΔF for this reaction, for ionic reactants, is 5.25 Cal. and the equilibrium constant is 4.9×10^3. This means that the equilibrium is far to the right, but the reaction is reversible, as they have shown with the use of CO_2 which contained radioactive carbon of atomic weight 11. One may calculate that at 0.01 M pyruvate and 0.02 M $NaHCO_3$, the equilibrium concentration of oxalacetate is 1×10^{-5} M.

That other types of decarboxylating enzymes are present in animal tissues is clear from Green's preparations[137] with pig heart. He found a diphosphothiamine protein which is inactive with oxalacetic acid but acts on α-ketoglutaric acid forming succinic semialdehyde:

$$COOH \cdot CH_2 \cdot CH_2 \cdot C:O \cdot COOH \rightleftharpoons COOH \cdot CH_2 \cdot CH_2 \cdot CHO + CO_2.$$

With pyruvic acid, CO_2 and acetylmethylcarbinol were formed:

$$2CH_3 \cdot C:O \cdot COOH \rightleftharpoons CH_3 \cdot CO \cdot CHOH \cdot CH_3 + 2CO_2$$

Pyruvic acid is formed in nearly all cells and tissues, and may be formed in many cases either aerobically or anaerobically. There are probably several mechanisms for the formation of pyruvic acid, but the scheme shown in the diagram below is probably a fairly well established mechanism. This scheme is the result of the work of many people, notably Embden, Parnas, Meyerhof, Cori, Lohmann, and Warburg. Several recent reviews of this scheme have been published,[138] and here the scheme will be presented diagrammatically.

1. Oxidative Decarboxylation.[138a]—Barron,[139] working with *Gonococci*, had discovered that pyruvic acid could undergo aerobic decarboxylation:

$$CH_3 \cdot CO \cdot COOH + \tfrac{1}{2}O_2 \rightleftharpoons CH_3COOH + CO_2.$$

Lipmann[136] has shown that *Lactobacillus delbrückii* carried out the same reaction, and that active extracts could be prepared from vacuum-dried bacteria. Lipmann showed that the complete system required at least the following: 1. Specific protein or proteins; 2. diphosphothiamine; 3. Mg^{++}, Mn^{++}, or Co^{++}; 4. inorganic phosphate; 5. alloxazine-adenine dinucleotide.

The mechanism of the oxidation of acetate has long been a mystery. Recently, Slade and Werkman[139a] have shown, by the use of the heavy carbon isotope, that the bacterium, *Aerobacter indologenes*, can condense two acetic acid molecules to succinic acid:

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[137] D. E. Green, W. W. Westerfield, B. Vennesland, and W. E. Knox, J. Biol. Chem., **145**: 69, 1942.

[138] C. F. Cori, Cold Spring Harbor Symp., **7**: 260, 1939; H. M. Kalckar, Chem. Rev., **28**: 69, 1942.

78, 1941; F. Lipmann, Advances in Enzymology, **1**: 99, 1941; O. Meyerhof, Biol. Symp., **5**: 141, 1941.

[138a] For an excellent review, see S. Ochoa, in E. A. Evans, The Biological Action of the Vitamins: Univ. of Chicago Press, 1942, and F. Lipmann, Cold Spring Harbor Symp., **7**: 248, 1939.

[139] E. S. G. Barron and D. C. Miller, J. Biol. Chem., **97**: 691, 1932.

[139a] H. D. Slade and C. H. Werkman, Arch. Biochem., **2**: 97, 1943.

Degradation of Carbohydrates

$$\text{glucose} + H_3PO_4 \rightleftharpoons \text{glucose-6-phosphate}$$

$$\text{glycogen or starch} + H_3PO_4 \rightleftharpoons \text{glucose-1-phosphate} \rightleftharpoons \text{glucose-6-phosphate} \rightleftharpoons \text{fructose-6-phosphate}$$

$$\text{fructose-6-phosphate} + H_3PO_4 \rightleftharpoons \text{fructose-1,6-diphosphate} \rightleftharpoons \begin{cases} \text{glyceraldehydephosphate} \\ \rightleftharpoons \\ \text{dioxyacetone phosphate} \end{cases}$$

$$\text{3-glyceraldehydephosphate} + \underset{H_3PO_4}{\overset{\text{coen. I}}{+}} \rightarrow \text{1,3-diphosphoglyceric acid} + \text{coen. I}H_2 \rightleftharpoons \underset{\text{acid}}{\text{3-phosphoglyceric}} + H_3PO_4$$

$$\text{3-phosphoglyceric acid} \rightarrow \text{phosphopyruvic acid} + H_2O \rightarrow \text{pyruvic acid} + H_3PO_4$$

Aerobically

$$\text{coen. I}\cdot H_2 + \tfrac{1}{2}O_2 \xrightarrow[\text{system}]{\text{oxidase}} \text{coen. I} + H_2O$$

$$\text{pyruvic acid} + 2\tfrac{1}{2}O_2 \longrightarrow 3CO_2 + 2H_2O$$

Anaerobically

Muscle

$$\text{pyruvic acid} + \text{coen. I}\cdot H_2 \rightleftharpoons \text{lactic acid} + \text{coen. I}$$

Yeast

$$\text{pyruvic acid} \rightarrow \text{acetaldehyde} + CO_2$$
$$\text{acetaldehyde} + \text{coen. I}\cdot H_2 \rightleftharpoons \text{alcohol} + \text{coen. I}$$

Some steps omitted; adenylic acid system and enzymes omitted.
See O. Meyerhof, Biol. Symp., **5**: 141, 1941.

$$\begin{array}{ccc} CH_3COOH & \xrightarrow{-2H} & CH_2COOH \\ + & & | \\ CH_3COOH & \xleftarrow{+2H} & CH_2COOH \end{array}$$

The detailed mechanism is unknown, and may involve intermediates. The acetate may, then, be oxidized as succinate by one of several mechanisms.

Peters[140] had shown that in vitamin B_1-deficient pigeons, pyruvic acid accumulated in the blood stream, and that upon administration of vitamin B_1, the pyruvate disappeared. Further, he showed that vitamin B_1 (thiamine) would maintain or increase the respiratory rate of preparations

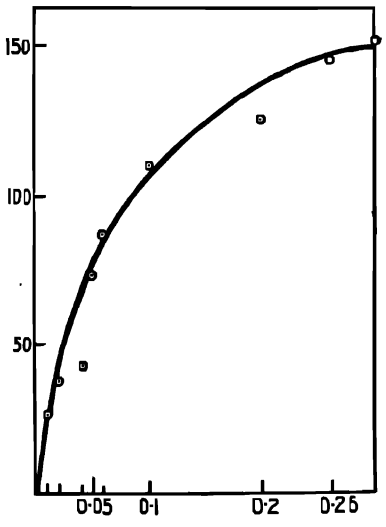

FIG. 42.—Effect of increasing concentrations of added cocarboxylase on the rate of oxidation of pyruvic acid in dispersions of brain from thiamine-deficient pigeons at 38°C. (From I. Banga, S. Ochoa, and R. A. Peters, Biochem. J., **33**: 1109, 1939.)

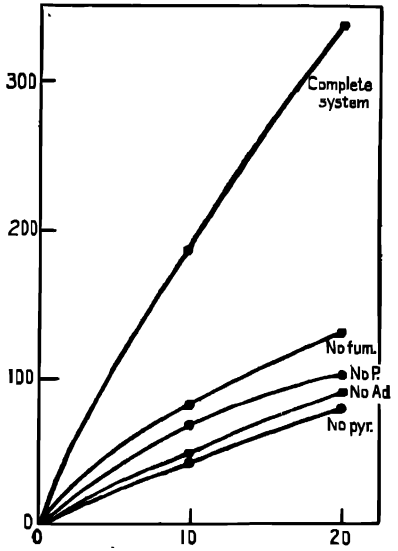

FIG. 43.—Effect of phosphate (0.05 M), fumarate (0.005 M), and adenylic acid (0.00014 M) on the oxidation of pyruvic acid by dialyzed dispersions of normal pigeon brain, with optimal concentrations of Mg^{++} and diphosphothiamine. (From I. Banga, S. Ochoa, and R. A. Peters, Biochem. J., **33**: 1140, 1939.)

of pigeon brain from vitamin B_1-deficient birds, when pyruvate or lactate was the respiratory substrate. Ochoa and Peters[140a] showed that the pigeon brain would synthesize diphosphothiamine from H_3PO_4 and thiamine, and that it was the diphosphoderivative which was active in the enzyme systems (see Fig. 42). In pigeon brain preparations, the oxidation of pyruvate is not complete, but proceeds beyond acetate. The activity of this system requires not only specific proteins, inorganic phosphate, diphosphothiamine, Mg^{++}, or Mn^{++}, but also a dicarboxylic four-carbon acid and adenylic acid or its phosphate esters. This is illustrated in Fig. 43.

[140] R. A. Peters, Lancet, **230**: 1165, 1936.

[140a] S. Ochoa and R. A. Peters, Biochem. J., **136**: 637, 1938.

2. Four-carbon Dicarboxylic Acids.—Szent-Györgyi[141] proposed that dicarboxylic four-carbon acids played a catalytic role in cellular respiration. The evidence upon which this was based consisted of two parts:

1. Malonic acid is known to inhibit succinic dehydrogenase, the enzyme which oxidizes succinic acid to fumaric acid; and the respiration of many tissues, particularly pigeon breast muscle, is inhibited by malonic acid. Fumaric, but not succinic, acid can overcome the inhibition of malonic acid.

2. Low concentrations of fumaric acid will cause a marked increase or maintenance of respiration of pigeon breast muscle, and the extra oxygen consumed is much greater than would account for complete oxidation of the fumarate to CO_2 and H_2O

In the Szent-Györgyi scheme a four-carbon acid (oxalacetate) at one end of the cycle was presumed to be reduced to malate by receiving hydrogen from a substrate, and the hydrogen was then passed through the cycle of four-carbon acids by way of succinate to cytochrome *c* and the cycle was regenerated. There is considerable question about the validity of the Szent-Györgyi cycle, and it has been reviewed by Stare and Baumann,[142] Elliott,[143] and adversely by Ball[144] and Potter.[145] The significance of Szent-Györgyi's work has not been in the particular cycle he proposed, but in his pointing out the catalytic activity of the four-carbon dicarboxylic acids. The recent experiments from Cori's laboratory[146,147] seem to show clearly that succinic acid acts catalytically in the oxidation of glucose and pyruvic acid. A particular mechanism in which these acids may act catalytically is the Krebs cycle.

3. Krebs Tricarboxylic Acid Cycle.—We have seen that neither the carboxylase system of yeast, nor the aerobic decarboxylation mechanism of bacteria will account for the complete oxidation of pyruvic and lactic acids, or of trioses to CO_2 and H_2O. Yet complete oxidation of these compounds does occur in virtually all cells. The most original and fruitful approach to this problem has been that of Krebs, in what he calls the tricarboxylic acid cycle. While some of the details of the original Krebs cycle may not be generally valid, the fundamental idea, that pyruvic acid is oxidized by synthesis to a larger molecule, and then by stepwise oxidation and decarboxylation to CO_2 and H_2O, is probably valid. For reviews of the Krebs cycle, refer to Evans[148] and Krebs.[149]

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[141] A. Szent-Györgyi, On Oxidation, Fermentation, Vitamins, Health and Disease: Williams and Wilkins, Baltimore, 1939.

[142] F. J. Stare and A. Baumann, Cold Spring Harbor Symp., **1**: 227, 1939.

[143] K. A. C. Elliott, Physiol. Rev., **21**: 267, 1941.

[144] E. Ball, Cold Spring Harbor Symp., **7**: 100, 1939.

[145] V. R. Potter, Medicine, **19**: 441, 1940.

[146] S. P. Colowick, M. S. Welch, and C. F. Cori, J. Biol. Chem., **135**: 359, 1940.

[147] S. P. Colowick, H. M. Kalckar, and C. F. Cori, *ibid.*, **137**: 343, 1941.

[148] E. A. Evans, in A Symposium on Respiratory Enzymes: Univ. of Wisconsin Press, 1942; Bull. Johns Hopkins Hospital, **69**: 225, 1941.

[149] H. A. Krebs, Recent Adv. Enzymology, **3**: 191, 1943.

Krebs and Johnson,[150] in a study of pigeon breast muscle, discovered a reaction between fumarate and pyruvate which occurred in the presence of 0.025 M malonate:

1. $\text{Pyruvate} + \text{fumarate} + 2O_2 \rightleftharpoons \text{succinate} + 3CO_2 + H_2O.$

Since malonic acid inhibits the succinic dehydrogenase, the succinate could not have been produced through the reduction of fumaric acid. Further, the oxidation of pyruvate was inhibited when malonic acid was added in the absence of added fumarate; but, upon the addition of fumarate, each molecule brought about the oxidation of one molecule of pyruvate. If malonate is absent, succinate does not accumulate, but is oxidized to fumarate, and the reaction continues:

2. $\text{Succinate} + \frac{1}{2}O_2 \xrightarrow[\text{cytochrome system}]{\text{succinic dehydrogenase}} \text{fumarate} + H_2O.$

Adding equations 1 and 2 gives

3. $\text{Pyruvate} + 2\frac{1}{2}O_2 \rightarrow 3CO_2 + 2H_2O.$

This is the equation for the complete oxidation of pyruvate.

In muscle poisoned with arsenite, Krebs found that pyruvate and fumarate gave rise quantitatively to α-ketoglutarate. Since it was known that the oxidation of citrate stops with α-ketoglutarate in the presence of arsenite, Krebs proposed that citrate is an intermediate in the oxidation of pyruvate. The evidence is as follows:

1. Fumarate may be readily converted to oxalacetate by the enzymes fumarase and malico-dehydrogenase, which are known to be present in muscle.
2. Oxalacetate and pyruvate react anaerobically in pigeon muscle to form citrate and CO_2.
3. Low concentrations of citrate act catalytically in the respiration of carbohydrates and pyruvate.
4. The rate of synthesis of citric acid, its conversion to α-ketoglutarate, and the rate of the degradation of the latter to succinate, are all high enough to account for the observed respiration.

Krebs then summarized his cycle as follows:

4. a. $\text{pyruvate} + 2 \text{ oxalacetate} + H_2O \rightarrow \text{citrate} + CO_2 + \text{malate}$
   b. $\text{citrate} + \frac{1}{2}O_2 \rightarrow \alpha\text{-ketoglutarate} + CO_2 + H_2O$
   c. $\alpha\text{-ketoglutarate} + \frac{1}{2}O_2 \rightarrow \text{succinate} + CO_2$
   d. $\text{succinate} + \frac{1}{2}O_2 \rightarrow \text{fumarate} + H_2O$
   e. $\text{fumarate} + H_2O \rightarrow \text{malate}$
   f. $2 \text{ malate} + O_2 \rightarrow 2 \text{ oxalacetate} + 2H_2O$

Adding 4a through 4 gives equation 3, above. The oxalacetate pro-

[150] H. A. Krebs, and W. A. Johnson, Enzymologia, 4: 148, 1937.

duced in 4f can react with another molecule of pyruvate and go through the cycle again. Each turnover of the cycle will then completely oxidize one molecule of pyruvate to CO_2 and H_2O.

Krebs has emphasized that he has omitted coenzymes and intermediates. Inspection of the formulæ of the compounds would suggest intermediates in reactions 4a and 4b, particularly. It is very likely that some of the compounds are phosphorylated during the course of the reactions. Krebs has shown that all the above reactions do occur in pigeon muscle, and at sufficiently high rates to account for the rate of respiration.

The chief criticisms of the Krebs cycle revolve around two points: 1. Its general validity; 2. the role of citric acid.

Smythe[151] has shown that the above scheme will account for the respiration of pyruvate in pig heart muscle. For other tissues, direct evidence is not available.

Evans[152] has shown that, in pigeon liver, pyruvic acid can be oxidized in the presence of malonate without the addition of four-carbon-dicarboxylic acids. The probable explanation of this reaction is the origin of oxalacetic acid from pyruvic acid and CO_2 by the Wood and Werkman reaction:

5. $$CO_2 + CH_3{\cdot}CO{\cdot}COOH \rightleftharpoons COOH{\cdot}CH_2{\cdot}CO{\cdot}COOH$$
Oxalacetic acid

Krebs and Eggleston[153] have shown that pyruvate is oxidized more rapidly in a bicarbonate/CO_2 medium, than in a saline/phosphate medium. They proposed that oxalacetate was produced according to equation 5.

Evans and Slotin,[154] using the radioactive carbon isotope ${}_6C_{11}$, have shown that pigeon liver may synthesize α-ketoglutarate from pyruvic acid and CO_2, for the isolated α-ketoglutarate contained radioactive carbon, when the latter was initially present only as CO_2. Since a similar reaction did not occur with pigeon muscle, the radioactivity of the α-ketoglutarate could not be attributed to exchange reactions. Evans and Slotin consider it probable that CO_2 is fixed by carboxylation of pyruvate. If, however, the α-ketoglutarate is formed from oxalacetate and pyruvate by way of symmetrical citrate, $\frac{1}{2}$ of the radioactivity should be in the carboxyl adjacent to the carbonyl group and $\frac{1}{2}$ in the distal carboxyl group. When α-ketoglutarate is degraded to succinic acid, it is the carboxyl group adjacent to the carbonyl which is lost as CO_2, and Evans found that all the radioactivity came off in the CO_2; the succinate had none. Thus, in pigeon liver, α-ketoglutarate is not formed by way of citrate.

Wood and his colleagues[155] have studied the same problem using the heavy carbon isotope ${}_6C_{13}$. In every detail they have confirmed Evans and

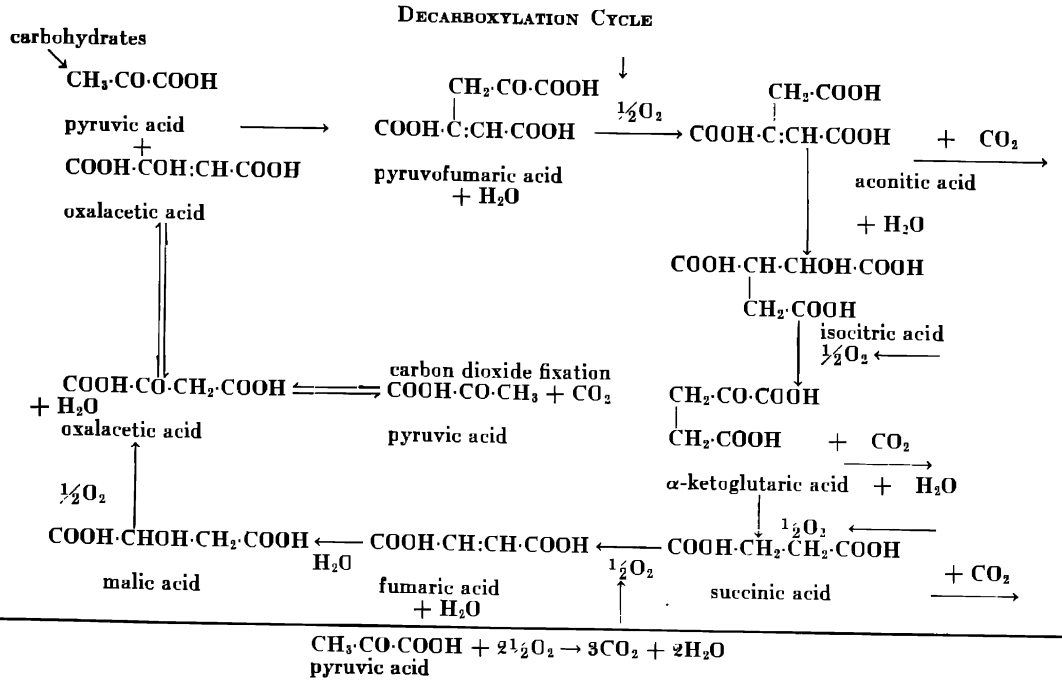
[151] D. H. Smythe, Biochem. J. **34**: 1046, 1940.

[152] E. A. Evans, *ibid.*, **34**: 829, 1940.

[153] H. A. Krebs and L. V. Eggleston, *ibid.*, **34**: 1383, 1940.

[154] E. A. Evans and L. Slotin, J. Biol. Chem., **141**: 439, 1941.

[155] H. G. Wood and C. H. Werkman, A. Hemingway and A. O. Nier, J. Biol. Chem., **142**: 31, 1942.


Decarboxylation Cycle
carbohydrates
CH3·CO·COOH
pyruvic acid
+
COOH·COH:CH·COOH
oxalacetic acid
CH2·CO·COOH
COOH·C:CH·COOH
pyruvofumaric acid
+ H2O
½O2
CH2·COOH
COOH·C:CH·COOH
+ CO2
aconitic acid
+ H2O
COOH·CH·CHOH·COOH
CH2·COOH
isocitric acid
½O2
COOH·CO·CH2·COOH
+ H2O
oxalacetic acid
carbon dioxide fixation
COOH·CO·CH3 + CO2
pyruvic acid
CH2·CO·COOH
CH2·COOH
+ CO2
α-ketoglutaric acid + H2O
½O2
COOH·CHOH·CH2·COOH
malic acid
H2O
COOH·CH:CH·COOH
fumaric acid
+ H2O
½O2
COOH·CH2·CH2·COOH
succinic acid
+ CO2
CH3·CO·COOH + 2½O2 → 3CO2 + 2H2O
pyruvic acid

Slotin. They have proposed a mechanism of the decarboxylation cycle for pigeon liver, which is reproduced here (as a diagram). This mechanism is provisional, and may not be correct in all its details. It is interesting to note that Krebs and Johnson[156] have shown that isocitric and cis-aconitic acids are as rapidly oxidized by muscle as is citric acid. It is possible that citric acid is not on the main path of the cycle, but is produced by side reactions. Krebs and Johnson have pointed out that *Bacillus coli* and yeast do not oxidize citric acid.

Even though it may turn out that citrate is not an intermediate for pigeon muscle, Krebs' experimental results stand, and to Krebs should go the credit for initiating this field of research.

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[156] H. A. Krebs and W. A. Johnson, Enzymologia, **4**: 148, 1937.

29

THE RELATION BETWEEN FERMENTATION AND RESPIRATION

1. The Common Pathway.—It has long been believed that the first steps of carbohydrate degradation were common to respiration and fermentation. In part, this is certainly true. The phosphorylation of starch or glycogen is a process common to both types of metabolism. In addition, such substances as glyceraldehyde phosphate, pyruvic acid, and in plants acetaldehyde, are rapidly oxidized; the first two of these are intermediates in lactic acid fermentation and all three in alcoholic fermentation. However, glucose may be respired without previous degradation in fermentative reactions, for Warburg and Christian[157] have shown that glucose-6-phosphate may be oxidized to phosphogluconic acid, and that the phosphogluconic acid can be oxidized with the absorption of 2½ mols. of O_2 and the liberation of 2.7 mols. of CO_2, presumably to a three-carbon compound. Thus, a mechanism must exist for the degradation of glucose to three-carbon compounds by stepwise oxidation.

The view that glucose may be oxidized by a nonfermentative pathway was strengthened by the results of Lundsgaard[158] with iodoacetic acid-poisoned muscle and yeast, where the glycolysis or fermentation was poisoned, but respiration continued. Shoor[159] and Stannard[160] have shown that with low concentrations (10^{-4} to 10^{-5} M) of iodoacetic acid, glycolysis may be inhibited without a decreased rate of carbohydrate oxidation. Since glyceraldehydephosphate dehydrogenase is poisoned by low concentrations of iodoacetic acid, the respiration in the presence of the poison may not proceed by the Meyerhof scheme shown on p. 423.

2. The Pasteur Effect.—The cells of many organisms contain complete enzymes for the fermentative and oxidative degradation of sugars. However, Pasteur discovered that in yeasts the rate of fermentation was much lower in air than in nitrogen. Pfeffer and Pflüger believed the lower rate of fermentation in air was due to the oxidative removal of the end products. This explanation is inadequate, because in many organisms the enzymes of fermentation may produce the end products far more rapidly than the

[157] O. Warburg and W. Christian, Biochem. Ztschr., **254**: 438, 1932; **257**: 291, 1935; **292**: 287, 1937.

[158] E. Lundsgaard, *ibid.*, **217**: 162, 1930; **220**: 1, 1930.

[159] E. Shoor, Cold Spring Harbor Symp., **7**: 323, 1939.

[160] J. N. Stannard, Am. J. Physiol., **119**: 408, 1937.

maximum rate of their oxidation. In other words, the presence of air, or a normal respiration, serves to suppress or completely inhibit the fermentative mechanism, and this is known as the "Pasteur effect."[151]

The effect may be seen clearly when animal tissues are poisoned[152] with ethyl carbylamine, for this poison does not inhibit the oxygen consumption or the fermentation, but does poison the Pasteur mechanism, so that, in poisoned cells, both fermentation and respiration occur simultaneously at their maximum rates.

That the Pasteur effect can be considered a regulatory mechanism may be seen, when it is realized that the fermentation of one mole of glucose to lactic acid liberates 54 Cal., while the oxidation of one mole of glucose to CO_2 and H_2O yields about 700 Cals. Thus, far more energy is available from a given amount of sugar by respiration than from fermentation, and the suppression of fermentation, when oxygen is available, is an economy for the organism. The Pasteur effect is widespread, for it occurs in yeast, some bacteria, some higher plants, and in vertebrates.

a). *Oxidative resynthesis.*—There have been several explanations of the mechanism of the Pasteur effect. The one proposed by Meyerhof[153] is known as the oxidative resynthesis theory. Meyerhof initially proposed that lactic acid produced in nitrogen is removed aerobically, partially by resynthesis to carbohydrate and partially by oxidation to CO_2 and H_2O.

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3; \qquad \Delta F = -54 \text{ Cal.}^{154a}$$
$$C_3H_6O_3 + 3O_2 \rightarrow 3CO_2 + 3H_2O; \qquad \Delta F = -316 \text{ Cal.}$$
$$2C_3H_6O_3 \rightarrow C_6H_{12}O_6; \qquad \Delta F = +54 \text{ Cal.}$$

It is clear that sufficient energy is available by the oxidation of 1 molecule of lactic acid to resynthesize about 12 molecules of lactic acid to carbohydrate, provided some mechanism exists for coupling the energy liberated in oxidation to the resynthesis. Meyerhof found that between 4 and 5 molecules of lactic acid are synthesized for each molecule oxidized. Meyerhof[154] has pointed out that it need not necessarily be lactic acid which is oxidized, since the oxidation of an equivalent amount of carbohydrate would be equally as consistent with the data, and the material resynthesized may be a precursor of lactic acid. There is a considerable amount of experimental data which supports the resynthesis theory, but perhaps none of it is absolutely critical.

In brewer's and baker's yeast, the rate of CO_2 production is greater in nitrogen than in air, and resynthesis of the alcohol to carbohydrate could not explain the decreased CO_2 production in air. Meyerhof proposed that in yeast, some three-carbon intermediate is resynthesized. Black-

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[151] K. C. Dixon, Biol. Rev., **12**: 431, 1935.

[152] O. Warburg, Biochem. Ztschr., **172**: 432, 1926.

[153] O. Meyerhof, Chemische Vorgänge im Muskel: Springer, Berlin, 1930.

[154] O. Meyerhof, in A Symposium on Respiratory Enzymes: Univ. of Wisconsin Press, 1942.

[154a] Includes the free energy of neutralization of the lactic acid.

man[165] proposed essentially the same scheme to explain the relation between fermentation and respiration in apples.

Meyerhof has proposed a quotient to measure the effect of respiration in inhibiting fermentation:

$$\text{Meyerhof quotient} = \frac{\text{mols. of lactic acid in } N_2 - \text{mols. of lactic acid in air}}{\text{mols. of } O_2 \text{ respired}}$$

The quotient for many tissues lies between 1 and 2. The quotient is often expressed in terms of glucose, and then each molecule of glucose oxidized prevents the fermentation of 3–6 molecules of glucose.

If respiration is inhibited by HCN, NaN_3, or CO, fermentation occurs in air, and, often, at very nearly the maximum rate. In peas,[166] 10^{-5} M HCN does not inhibit respiration, but does inhibit the Pasteur effect, so that aerobic fermentation occurs. Similarly, ethyl carbylamine[167] poisons the Pasteur effect in mammalian cancer. The Pasteur effect in yeast[168] and in mammalian tissues[169] is more sensitive to CO than is respiration. Laser[169] has also found that lowered O_2 tensions which do not inhibit respiration, induce fermentation. The situation outlined above need not be universal, as Marsh and Goddard[170] were unable to induce aerobic fermentation in carrot root tissue (which has a good Pasteur mechanism), with HCN, NaN_3, or lowered O_2 tensions, unless a respiratory inhibition of over 45 per cent occurred. Kempner[171] has shown that, in goose erythroblasts, the respiration may fall to $\frac{1}{3}$ the aerobic rate in 3.8 volume per cent O_2, without increase in the rate of glycolysis. Similar results have been obtained with bone marrow by Warren.[172]

b). *Pasteur enzyme.*—Lipmann[173] does not accept Meyerhof's resynthesis theory. Lipmann believes that fermentation is inhibited by oxygen, but not through the respiratory mechanism. Stern,[174] realizing that the relative affinity for CO in competition with O_2 of the Pasteur mechanism was greater than that of cytochrome oxidase, decided to demonstrate the existence of a Pasteur agent or enzyme. Working with rat retina, he has poisoned the Pasteur effect with CO, and then determined the relative photochemical absorption spectrum by relieving the inhibition with various known wave-lengths of light. The respiration of rat retina is not inhibited by CO, so he compared the spectrum he obtained with that which Melnick[175] obtained on extracts of rat

[165] F. F. Blackman, Proc. Roy. Soc., London, **B 103**: 412, 1928.

[166] L. Genevois, Biochem. Ztschr., **191**: 10, 1927.

[167] O. Warburg, Biochem. Ztschr., **172**: 432, 1926.

[168] O. Warburg, *ibid.*, **189**: 354, 1927.

[169] H. Laser, Biochem. J., **31**: 1671, 1677, 1932.

[170] P. B. Marsh and D. R. Goddard, Am. J. Bot., **26**: 767, 1939.

[171] W. Kempner, J. Cell. & Comp. Physiol., **10**: 339, 1937.

[172] C. O. Warren, *ibid.*, **19**: 193, 1942.

[173] F. Lipmann, Biochem. Ztschr., **265**: 133, 1933, and in A Symposium on Respiratory Enzymes: Univ. of Wisconsin Press, 1942.

[174] K. G. Stern and J. Melnick, J. Biol. Chem., **139**: 301, 1941.

[175] J. Melnick, Science, **94**: 118, 1941.

heart cytochrome oxidase. These spectra are given in Table XXXIX. It will be seen that the Pasteur enzyme differs from cytochrome oxidase in the position of two bands. Then, evidence for the existence of the Pasteur enzyme is: 1. It combines with ethyl carbylamine, HCN, and CO; 2. its spectrum differs slightly from that of cytochrome oxidase; 3. its relative affinity for CO in relation to O_2 is greater than that of cytochrome oxidase. Stern believes the Pasteur enzyme is a phæohemin. Melnick,[176] in Stern's laboratory, has similarly determined the photochemical absorption spectra of both CO-cytochrome oxidase and the Pasteur enzyme in baker's yeast. The spectra are identical at 3 of the bands, but there is an absorption at 560 mμ in the oxidase which is a little greater than in Pasteur enzyme. The evidence for the existence of a Pasteur enzyme is not too strong. If such an enzyme exists, the mechanism by which it prevents fermentation is not known, except that it requires molecular oxygen for the effect. These results of Stern and Melnick need not necessarily be inconsistent with Meyerhof's resynthesis theory.

[176] J. Melnick, J. Biol. Chem., **141**: 269, 1941.

30

THE UTILIZATION OF LIBERATED ENERGY

1. Assimilation.—It is clear that the synthetic powers of living cells are very great, and this becomes strikingly illustrated when one realizes that many bacteria and fungi may grow rapidly on inorganic salt solutions to which a single organic carbon compound has been added, such as acetate, propionate, pyruvate, glucose, etc. In many of the synthetic reactions of growth an increase in free energy occurs, and such reactions may only occur if they are coupled with an oxidative reaction furnishing the energy deficit. Thus, one carbon compound must then furnish the energy source as well as the carbon for the synthesis. Oxidative assimilation may seem much more striking in plants than in animal cells, but animal cells also possess such synthetic ability. This may be illustrated by the conversion of carbohydrates to fats, for the potential energy of the fat is much higher than that of the starting material. The difference must be furnished by energy liberated in cellular oxidation of part of the carbohydrate.

The efficiency of the assimilation may at times be high. Goddard[177] found that the fungus *Trichophyton* degraded 0.625 mg. of protein and 1.0 mg. of glucose for each mg. increase in dry weight over a period of 6 days. The Japanese workers have conducted many experiments on the ratio of the carbon source oxidized to amount assimilated for the fungus *Aspergillus*. These results have been summarized by Tamiya.[178] Barnell[179] has shown that, during the germination of barley seedlings, $\frac{1}{3}$ of the stored glucose is oxidized while $\frac{2}{3}$ is assimilated.

It had long been known that oxidative assimilation occurred in growing cells, but it had been generally assumed that such assimilation did not occur in nonproliferating cells. Barker[180] first clearly showed that oxidative assimilation may occur in nongrowing cells, with a fixed ratio between the substrate, oxidized to CO_2 and H_2O, and that assimilated. Working with the colorless alga, *Prototheca*, he showed that, in short-period manometric experiments, the O_2 consumption fell to the low endogenous rate when the total O_2 uptake and CO_2 liberation were only $\frac{1}{2}$ of the calculated value for

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[177] D. R. Goddard, J. Infect. Dis., **54**: 149, 1934.

[178] H. Tamiya, Recent Adv. Enzymology, **2**: 183, 1942.

[179] H. R. Barnell, Proc. Roy. Soc., London, **B 123**: 321, 1937.

[180] H. A. Barker, J. Cell. & Comp. Physiol., **8**: 231, 1936.

the complete oxidation of acetate, and he recovered the rest of the carbon in the cells. He has been able to show that a large variety of substrates are assimilated in fixed ratios with their oxidation; in all cases the assimilated material corresponded to CH_2O, and is probably glycogen. The acetate experiment may be summarized as follows:

$$6CH_3COOH + 6O_2 \rightarrow 6CO_2 + 6H_2O + (CH_2O)_6.$$

Clifton and Logan[181] have also found that *Escherichia coli* likewise assimilates fixed ratios of the various carbon compounds furnished it. In unpoisoned cells, the coupling between oxidation and synthesis seems to be obligatory, and the ratio of material oxidized to that synthesized is constant over a wide range of concentrations. Clifton,[182] and Clifton and Logan,[181] have been able to separate this coupling by poisoning the cells with 0.0025 M NaN_3 or 0.0005 M dinitrophenol. The poisoned cells oxidize the substrate completely to CO_2 and H_2O. Schade and Thimann[183] have shown that the fungus *Leptomitus* assimilated $\frac{1}{3}$ of its acetate and oxidized $\frac{2}{3}$. Benoy and Elliott[184] have shown that slices of rat liver synthesize carbohydrates from added pyruvate and lactate, while rat kidney cortex forms carbohydrates in addition from alanine, fumarate, succinate, and maleate.

Winzler and Baumberger[185] have followed the heat production by yeast in a microcalorimeter. They have found that aerobically, when the rate of heat production falls to the endogenous rate, the glucose has all disappeared from the medium, but only $\frac{3}{4}$ of the expected heat has been liberated; $\frac{1}{4}$ of the glucose has been assimilated. Similar results were obtained with Na acetate, but here $\frac{2}{5}$ of the acetate has been assimilated. They have also shown that glucose may be assimilated under anaerobic conditions. They have calculated the efficiency of the synthesis on the assumption that the synthesized product is glycogen, using the relation:

$$e = \frac{\text{free energy used in synthesis}}{\text{total free energy made available in breakdown}}.$$

For the aerobic assimilation of glucose they find an efficiency of 2.88 per cent, for anaerobic assimilation 4.62 per cent, and for the aerobic assimilation of sodium acetate 12.2 per cent. Van Niel[186] has measured the anaerobic assimilation in yeast by the manometric technic and obtained results in excellent agreement with Winzler and Baumberger.

This widespread oxidative assimilation must be at the very basis of the chemistry of growth, and the fundamental problem of how the energy coupling occurs has not been solved. However, two suggestive mechanisms may now be examined.

[181] C. E. Clifton and W. A. Logan, J. Bact., **37**: 523, 1939.

[182] C. E. Clifton, Enzymologia, **4**: 246, 1937.

[183] Schade and K. U. Thimann, Am. J. Bot., **27**: 659, 1940.

[184] M. P. Benoy and A. C. Elliott, Biochem. J., **31**: 1268, 1937.

[185] R. J. Winzler and J. P. Baumberger, J. Cell & Comp. Physiol., **12**: 183, 1938.

[186] C. B. Van Niel and E. H. Anderson, J. Cell. & Comp. Physiol., **17**: 49, 1941.

2. Energetic Coupling.—The statement is sometimes made that the outlines of the problem of cellular respiration are clearly understood. This statement has a limited validity if one refers only to the mechanism of the oxidation of carbohydrates. The central problem of cellular respiration—the mechanism of using the energy liberated in oxidation for the work of the cell is a virtual mystery. Cells use the energy in many ways, in contraction, cyclosis, synthesis, secretion, ion accumulation, transmission of nerve impulses, etc. In no single case has an adequate explanation of the mechanism of the energetic coupling between the energy liberating reaction and the cellular machinery, been given.

a). *Oxidation-reduction coupling.*—It will be recalled that the equation for the potential of a reversible oxidation-reduction system is:

$$E_h = E_o + 2.303 \frac{RT}{n\mathbf{F}} \log \frac{(\text{Ox.})}{(\text{Red.})} - 2.303 \frac{RT}{\mathbf{F}} p\text{H} \qquad (1)$$

where E_h is the observed potential; R = gas constant; T = absolute temperature; $\mathbf{F}$ = Faraday constant; and n = number of electrons involved in the oxidation (Sec. 1, chap. 4).

$E_h = E_o$ under standard conditions (25°C.: unit activities; pH = 0.0) when (Ox.) = (Red.); that is, E_o, is a constant for each substance. Table XL summarizes some E_o' values of biologically important systems.

TABLE XL.—THE E_o' VALUES OF SOME BIOLOGICALLY IMPORTANT SYSTEMS AT pH = 7.0

	System	E_o', volts	T°C.
H_2O	$\frac{1}{2}O_2 + 2H^+ + 2e$	0.815	25
Ferro oxidase	Ferri oxidase + e	?	
Ferro cytochrome a	Ferri cytochrome a + e	0.29(?)	25
Ferro cytochrome c	Ferri cytochrome c + e	0.26	25
Ferro cytochrome b	Ferri cytochrome b + e	−0.04(?)	25
Succinate	Fumarate + $2H^+ + 2e$	−0.026	25
Reduced flavin enzyme	Flavin enzyme	−0.063	38
Malate	Oxalacetate + $2H^+ + 2e$	−0.102	37
Lactate	Pyruvate	−0.180	35
Alcohol	Acetaldehyde + $2H^+ + 2e$	−0.190	30
β-hydroxybutyrate	Acetoacetate + $2H^+ + 2e$	−0.293	38
Reduced coenzyme I	Coenzyme I + $2H^+ + 2e$	−0.29	30
H_2	$2H^+ + 2e$	−0.414	25
Formate	$CO_2 + H_2$	−0.420	38

Data from E. S. G. Barron, Physiol. Rev., **19**: 184, 1939; Ann. Rev. Biochem., **10**: 1, 1941. D. E. Green, Mechanism of Biological Oxidation: University Press, Cambridge, 1941.

At a fixed pH, say 7.0, the equation simplifies to:

$$E_h = E_o' + 2.303 \frac{RT}{n\mathbf{F}} \log \frac{(\text{Ox.})}{(\text{Red.})}. \qquad (2)$$

For biological purposes the value of E_o' at pH of 7.0 should be used in comparing different systems; since, if the oxidation-reduction system is an acid with a pK_a between -1 and 8, the E_o' may not be directly calculated by equation 1, the more involved calculation is given by Michaelis.[187]

Unfortunately, the sign of the potentials used by the biochemists is opposite to the sign used by many physical chemists. In the biochemical terminology, a system positive to one below it will oxidize the lower system.

The free energy change, ΔF, may be readily calculated from a knowledge of the potential:

ΔF = n$\mathbf{F}\Delta E$, where n = the number of electrons involved in the process.
$\mathbf{F}$ = the Faraday, or 23.066 Cals. per volt. (3)
ΔE = the change in potential in volts, where a decrease in potential is considered negative; an increase, in potential positive.

The free energy under standard conditions is given by

$$\Delta F_o = n\mathbf{F}E_o, \tag{4}$$

We may illustrate the use of these equations, as follows:

$$\begin{aligned} &\text{lactate ion} \rightleftharpoons \text{pyruvate ion} + 2\mathrm{H}^+ + 2e \\ &E_o' = -0.180 \text{ volt } (p\text{H } 7.0) \\ &\Delta F = 2 \times 23.066 \times -0.180 = -8.304 \text{ Cal.} \end{aligned} \tag{5}$$

This means that the oxidation of 1 mol. of lactate ion to 1 mol. of pyruvate ion, in dilute aqueous solution at pH 7.0, and under such conditions that the potential does not change, yields -8.304 Cals. In practice, the potential would change, unless the volume were infinitely large, or the lactate were constantly replaced and the formed pyruvate removed, as would occur under steady state conditions in living organisms. The reverse reaction, the conversion of pyruvate to lactate, will not occur spontaneously, since ΔF is positive.

However, Borsook and Schott[188] were able to reverse this reaction with toluene-treated *Bacillus coli*, using the energy furnished by the oxidation of formate:

$$\begin{aligned} \text{formate ion} + \mathrm{H_2O} &= \mathrm{HCO_3^-} + 2\mathrm{H}^+ + 2e \\ E_o' &= -0.41 \text{ volt } (p\text{H } 7.0) \end{aligned} \tag{6}$$

The bacteria contain enzymes which catalyze reactions (5) and (6). Without the bacteria, no reaction between formate and lactate would occur: in addition a dye had to be added which would be oxidized by pyruvate and reduced by formate, and such dyes as janus green or methylene violet, whose E_o''s $= -0.26$, were satisfactory. The free energy changes are:

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[187] L. Michaelis, Oxidation Reduction Potentials: Lippincott, Philadelphia, 1930.

[188] H. Borsook and H. F. Schott, J. Biol. Chem., 92: 535, 539, 1931.

For the oxidation of formate $\Delta F = -18.90$ Cal.
For the reduction of pyruvate $\Delta F = +8.304$ Cal.

Sum of the reactions $\Delta F = -10.60$ Cal.

Thus, the energy liberated in the oxidation of the formate was coupled to the synthesis of lactate.

How frequent this type of energy transfer is in the living cell is not known, but Green, Dewan, and Leloir[189] have been able to link the reduction of several substrates to the oxidation of β-hydroxybutyrate, using diphosphopyridine nucleotide as the carrier:

$$\beta\text{-hydroxybutyrate} + \text{coenzyme I} \xrightarrow[\text{dehydrogenase}]{\beta\text{-hydroxybutyrate}} \text{acetoacetate} + \text{coenzyme I}\cdot H_2. \quad (7)$$

$$\text{coenzyme I}\cdot H_2 + \text{pyruvate} \xrightarrow[\text{dehydrogenase}]{\text{lactic}} \text{lactate} + \text{coenzyme I}. \quad (8)$$

It is improbable that all steps in the oxidation of glucose by oxygen are equally able to furnish energy for the work of the cell. The energy liberated by those steps which are irreversible will be degraded as heat. Though we do not know the potential of cytochrome oxidase, we may suppose it is intermediate between oxygen and cytochrome *c*. The reaction between oxygen and the oxidase is presumably irreversible, since, if it were not, its reversal would mean the decomposition of water and the liberation of free oxygen.

$$2 \text{ ferro-oxidase} + \tfrac{1}{2}O_2 + 2H^+ \rightarrow 2 \text{ ferri-oxidase} + H_2O. \quad (9)$$

If we assume that the reaction of molecular O_2, cytochrome oxidase, and cytochrome *c*, is either irreversible or not coupled, we can calculate the amount of energy degraded as heat, as follows:

$$\tfrac{1}{2}O_2 + 2H^+ + 2c = H_2O; \qquad E'_o = 0.815 \text{ volts } (p\text{H } 7.0). \quad (10)$$

$$\text{ferrocytochrome} = \text{ferricytochrome} + e; \qquad E'_o = 0.260 \text{ volt}. \quad (11)$$

$$2 \text{ ferrocyto.} + 2H^+ + \tfrac{1}{2}O_2 \rightarrow 2 \text{ ferricyto.} + H_2O. \quad (12)$$

$$\Delta E' = -\frac{2 \times 0.821 + 2 \times 0.260}{2} = -0.561.$$

$$\Delta F = -25.88 \text{ Cal.}$$

This calculation is valid only at 1.0 atmos. O_2 pressure, and with the ratio of ferricyto. to ferrocyto. = 1.0. Under more physiological conditions, say 0.01 atmos. of oxygen and a ratio of ferricyto. to ferrocyto. of $\frac{1}{10}$, the potentials are shifted as follows:

For eq. 10, $E_h = 0.785$; $\left(E_h = E'_o + 2.303 \dfrac{RT}{n\mathbf{F}} \log (pO_2)\right)$

For eq. 11, $E_h = 0.201$ (by equation 2).

For eq. 12, $\Delta E = -0.502$; $\Delta F = -23.16$ Cal.

[189] D. E. Green, J. G. Dewan, and L. P. Leloir, Biochem. J., **31**: 934, 1937.

Now the oxidation of a glucose molecule means the repetition of reaction (12) six times, or 138.9 Cal. are degraded as heat, and not available for useful work in the cell. Undoubtedly, other irreversible processes intervene below the cytochrome level and the complete oxidation of glucose to CO_2 and H_2O.

b). *Transphosphorylation.*—It has long been recognized that the phosphorylation of sugars is an essential step in fermentation and glycolysis. More recently, it has been recognized that phosphate transfer occurs between various compounds in cells; and that phosphate transfer may play a fundamental role in energetic coupling, the reader is referred to the stimulating articles of Kalckar[190] and Lipmann.[191] They have suggested that there are two groups of phosphate compounds, those in which the phosphate is bound in an ester linkage to an alcohol or aldehyde, and in which the $-\Delta F$ of hydrolysis is only 1 to 3 Cal., and those in which phosphate is bound to N, or to another P atom (pyrophosphates), or to a carboxyl group. We do not know the $-\Delta F$ of the hydrolysis of these bonds, but Lipmann and Kalckar estimate it at about 10 to 11 Cal. (Lipmann classifies this latter group as compounds with energy-rich phosphate bonds. The bond energy is not known, and need not equal the ΔF of hydrolysis.) Both authors believe it probable that the energy liberated in oxido-reductions is stored up in phosphorylated compounds, and transferred from compound to compound by the transfer of the phosphate. This suggestion is extremely interesting, and may well become established. A word of caution here seems not out of place. The extremely stimulating ideas of Kalckar and Lipmann will have served their purpose if they stimulate further work, particularly to evaluate the free energy changes involved in these phosphorylations and dephosphorylations, and in tying these processes into specific loci of cellular metabolism. The recent results obtained on muscle energetics, presented below, illustrate specifically how transphosphorylation functions in energy transfer.

When muscles contract, phosphocreatine is converted to creatine and, the phosphate is bound to adenosine diphosphate, as follows:

$$\text{creatine phosphate} + \text{adenosine diphosphate} \rightleftharpoons \text{creatine} + \text{adenosine triphosphate.} \quad (13)$$

This reaction is reversible, and, since the equilibrium is near 1.0, the free energy change must be small. No enzyme is present in muscle which will hydrolyze creatine phosphate to creatine + H_3PO_4; but the adenosine triphosphate may return to diphosphate in either of two ways:

a). adenosine triphosphate + H_2O $\rightarrow$ adenosine diphosphate + H_3PO_4

b). glucose + adenosine triphosphate $\rightarrow$ glucose-6-phosphate + adenosine diphosphate (14)

[190] H. M. Kalckar, Chem. Rev., **28**: 71, 1941.

[191] F. Lipmann, Advances in Enzymology, **1**: 99, 1941.

Thus, a small amount of adenosine diphosphate may function catalytically in dephosphorylating creatine phosphate. According to Meyerhof and Schultz,[192] the ΔH of 14a is about -11.0 Cal., and 14b proceeds with a considerable evolution of heat. In recovery of the muscle, under either anaerobic or aerobic conditions, the creatine phosphate is resynthesized.

The mechanism of this resynthesis has long been in doubt, since the reversal of 14a or 14b seems to be impossible from energetic considerations. However, the recent work of Negelein and Brömel,[193] and of Warburg and Christian,[194] has gone far toward answering this question. They have shown that adenosine triphosphate is synthesized from inorganic phosphate in a coupled reaction, in which phosphoglyceraldehyde is oxidized to phosphoglyceric acid:

a). phosphoglyceraldehyde + H_3PO_4 + coen. I $\rightarrow$ diphosphoglyceric acid + coen. I·H_2. (15)

b). diphosphoglyceric acid + adenosine diphosphate $\rightarrow$ phosphoglyceric acid + adenosine triphosphate.

There is still some question as to the detailed steps of reaction 15a (see Meyerhof,[195] but not of the reaction itself. Thus, the energy furnished in the oxidation is used in the synthesis of adenosine triphosphate, and the latter can transfer its phosphate by the reversal of reaction (13). The coenzyme may be reoxidized either aerobically or anaerobically:

a). *Aerobically:*

$$\text{coen I}\cdot H_2 + \tfrac{1}{2}O_2 \xrightarrow[\text{unknown carrier}]{\text{cytochrome system}} \text{coen. I} + H_2O. \qquad (16)$$

b). *Anaerobically:*

$$\text{coen. I}\cdot H_2 + \text{pyruvate} \xrightarrow[\text{dehydrogenase}]{\text{lactic}} \text{coen. I} + \text{lactate}.$$

There is reason to believe that phosphorylation is coupled to several other oxidations, particularly pyruvate to acetate;[195] and probably succinate to fumarate.[197,198] That phosphorylation occurs vigorously during respiration of glucose and pyruvate seems clear from the experiments of Colowich, Welch, Kalckar, and Cori[198] on minced kidney cortex.

In the conversion of adenosine diphosphate to triphosphate by reaction with creatine phosphate, there is a transfer of energy. The only way this energy can be liberated is by the loss of the phosphate from adenosine triphosphate. This reaction is catalyzed by the enzyme adenosine tri-

[192] O. Meyerhof and W. Schultz, Biochem. Ztschr., **281**: 292, 1935.

[193] E. Negelein and H. Brömel, Biochem. Ztschr., **303**: 132, 1939.

[194] O. Warburg and W. Christian, *ibid.*, **303**: 40, 1939.

[195] O. Meyerhof, Biol. Symp., **5**: 141, 1941.

[196] F. J. Lipmann, Biol. Chem., **134**: 463, 1940.

[197] V. A. Belitzer and E. T. Tsibrakova, Biokhiminga, **4**: 518, 1939.

[198] S. P. Colowich, H. M. Kalckar, and C. F. Cori, J. Biol. Chem., **137**: 343, 1940; S. P. Colowich, P. M. Welch, and C. F. Cori, *ibid.*, **133**: 359, 641, 1940.

phosphatase. If the hydrolysis were to occur in a random manner in the cell, we should expect the energy to be dissipated as heat. Now, Engelhardt[199] has shown that myosin, the contractile protein of muscle, is itself the enzyme which brings about the hydrolysis of adenosine triphosphate. In this extremely interesting case, it appears from Engelhardt's results that the catalyst accepts the energy liberated by its substrate. Further, he has shown that when myosin threads are placed under tension, the addition of adenosine triphosphate results in a relaxation of the myosin, and an increase in length of 1.5 to 2.0 times. He has found no other substance which will replace adenosine triphosphate in this reaction. It now appears that the first biochemical reaction has been discovered which directly converts the potential energy of a chemical compound into mechanical work (see, further, Sec. 7, chap. 33).

[199] W. A. Engelhardt, Yale J. Biol. Med., **15**: 21, 1942.

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SUMMARY

As one surveys our knowledge of cellular respiration, one is impressed by the number of different enzymes, coenzymes, and intermediates which are involved. The complexity of the metabolic engine, even in a tiny bacterial cell, is very great. The essential similarity in the mechanism from organism to organism is, however, striking and leads to a real unification of our knowledge.

The stepwise degradation of the carbohydrate molecule with degradation of energy, by means of a series of reversible oxidation-reduction catalysts appears to be universal. The liberated energy is probably transferred and made available for cellular work, in a series of small packages. The mechanism of energy transfer is far from settled, but two suggestive schemes have been proposed, by oxidation-reduction coupling and by energy transfer in transphosphorylation.

The picture of cellular respiration is far from complete; certain areas are only roughly sketched in; certain regions are blank; and, in places, the picture as outlined may need repainting, for mistakes may have occurred in the penciled framework, and fine details still have to be added, even to the most finished parts of the work. There would appear to be plenty of opportunity for future research along many lines before our knowledge will approach that definiteness of major outline, fine detail, and clear perspective that constitutes the completed picture of a scientific solution.

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Section 7

CONTRACTILITY

By WALLACE O. FENN*

* The manuscript of this section was received for publication on September 1942.

32

GENERAL SURVEY OF CONTRACTILE TISSUES

Of all types of protoplasm, the skeletal muscles epitomize best the phenomenon of contractility, and it is the study of muscles which has in the past extended, and is most likely in the future to extend, our quantitative knowledge of this protoplasmic phenomenon. Nevertheless, it must not be supposed that muscles operate by any unique mechanism not represented in other contractile tissues. The fundamental mechanism of contraction is presumably the same in all tissues, but in muscles it is less obscured by other functions, such as digestion, absorption, and excretion, and it is easier to measure the forces developed, easier to observe the physical changes which occur, and easier to determine the chemical nature and quantities of the reactants and end products of the chemical processes involved. From a qualitative point of view, however, the nature of contractility is very much illuminated by a preliminary survey of the phenomenon as it appears elsewhere in living cells.

From a practical point of view, it is ridiculous to propose contractility as one of the fundamental characteristics of all living cells, when no obvious contraction can be demonstrated in many of them even by the most delicate means. Nevertheless, the type of physicochemical structure which is required for contractility is so similar in principle to the type of structure which is described for protoplasm in general, that the two subjects can hardly be discussed separately. Indeed, the best introduction to the study of contractility is to be found in recent discussions of the structure of protoplasm,[1,2] and this subject is presented elsewhere in this volume. It is sufficient here to point out that the foundation of these present concepts is a network of protein chains held together by cross linkages of hydrogen bonds or Van der Waals forces, if not by stronger valences. This network may make either a sol or a gel, depending upon the degree of folding of the protein chains and the strength of the cross linkages. Where the chains are all oriented together in a more orderly array, the structure becomes birefringent and may show visible fibrillæ. Any stimulus which causes a folding of the protein chains or an increased aggregation will decrease the

[1] W. A. Seifriz, The Structure of Protoplasm. Monograph of Am. Soc. Plant Physiol., Ames, Iowa, 1942.

[2] W. J. Schmidt, Die Doppelbrechung von Karyoplasma, Zytoplasma und Metaplasma: Berlin, 1937.

birefringence and cause a rearrangement of the material, or change of shape, which may be described as a contraction. The only truly essential requirement for contraction, according to this point of view, is the partially unfolded protein chain. The presence of visible fibrillæ and double refraction proves only increased degrees of differentiation of the contractile machinery. These appear to represent merely the means whereby more and more contractile units can be packed into a given space. Finally, it may be that the development of cross striations is the last step in the same direction. Bernal[3] has argued that it is a manifestation of an alternately reversing spiral arrangement of the fibrillæ, resulting from the tightness with which they are packed together in the fiber.

From the point of view of this general concept of contractility, the various types of contractile cells may now be examined.

1. Protoplasmic Streaming.—This survey may well begin with those phenomena which are least definitely related to contractility, as we know it in muscles. Protoplasmic movement may be regarded as a common property of all cells, although it is not equally obvious in all. Some show protoplasmic streaming as in the stamen hairs of *Tradescantia* or the cyclosis in *Elodea*. In the slime moulds (*Myxomycetes*) the whole protoplasmic mass is naked and unrestrained by a cell wall, and the streaming is accompanied by progression of the whole organism. In other respects the phenomena are very similar. The direction of streaming reverses at regular intervals. This becomes most evident when the movement is speeded up by moving pictures. Then the streaming takes on the appearance of a rhythmical contraction (Seifriz[4]), similar in its time relations to that of smooth muscles. This contraction has been recorded by an ingenious method by Kamiya,[5] who opposed the movement by air pressure in a special apparatus and recorded thus the pressure developed by the contraction. It is not impossible, then, to attribute this phenomenon to the folding of long protein molecules forming the cytoskeleton (Peters[6]), or fibrillar reticulum of the plasmodium. These molecules would also account for the elasticity of these organisms as measured by Norris.[7]

For the phenomenon of protoplasmic streaming in *Tradescantia*, Kühne,[8] 1864, advanced a similar explanation. According to him, the protoplasm is to be regarded as "creeping" about inside the cell wall like a slime mould. It is apparently not always easy, however, to establish the change of shape

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[3] J. D. Bernal, Chapter in Perspectives in Biochemistry: University Press, Cambridge, England, 1938, p. 45.

[4] W. A. Seifriz, Science, **85**: 397, 1937.

[5] N. Kamiya, Chapter in The Structure of Protoplasm, ed. by W. A. Seifriz, p. 199. Monograph of Am. Soc. Plant Physiol., Ames, Iowa, 1942.

[6] R. A. Peters, Perspectives in Biochemistry, ed by J. Needham and D. E. Green: Cambridge, England, 1938. Also, Proc. Roy. Soc., London, B., **121**: 587, 1937.

[7] C. H. Norris, J. Cell. & Comp. Physiol., **16**: 313, 1940.

[8] W. Kühne, Unters. über das Protoplasma und die Contractilität: Leipzig, 1864.

which is necessary for this contraction theory. Marsland and Brown,[9] for example, describe protoplasmic currents occurring in amebæ when the animals are entirely stationary and are exhibiting no external change of shape. This observation does not, perhaps, entirely exclude the contractile theory, since the internal boundary between sol and gel layers may show rhythmical variations in position which are easily overlooked. Nevertheless, Marsland and Brown attributed this streaming to sol and gel changes largely because of the effects of high pressure in stopping the movement. Later, Marsland[10] concluded that this theory was untenable.

According to Rashevsky[11] and Young,[12] protoplasmic streaming could theoretically occur from energy derived from a continuously maintained diffusion gradient between two points in the cell. If so, then this phenomenon is fundamentally different in nature from contractility. Such a theory, however, would not explain very well the rhythmic reversals which are observed in some cases, and it does not seem to be inherently very probable. This difficulty also applies to the electroendosmotic theory of streaming. In general, it may be said that the problem of protoplasmic streaming is harder to reconcile with contractility than is ameboid movement.

2. Ameboid Movement.—Possibly the movement of the plasmodia of the slime moulds is not to be identified with the protoplasmic streaming of stationary cells. If so, it still remains comparable to the movement of true amebæ, where most writers agree that it is the contractility of the superficial plasma gel which causes the movement: Lewis,[13] Schaeffer,[14] Dellinger,[15] Mast,[16] Pantin.[17] At the posterior end of the animal, the protein chains in the gel contract in response to some stimulus. This raises the internal pressure in the endoplasm or plasmasol, which bursts through the ectoplasm to "lunge" forward. At the tip of the pseudopod the sol becomes a gel again and recovery takes place. Chemical differences between the anterior and posterior ends have been established by differential staining (Okada,[18] Pantin[17]). As in muscle, therefore, chemical changes of contraction may occur in the posterior end, which are reversed during the recovery in the anterior end. The change from gel to sol has been regarded as an exothermic process, because protoplasm tends to solate or decrease in viscosity (Marsland[10]) with increased pressure, and gels which do this increase in

[9] D. A. Marsland and D. E. S. Brown, J. Cell. & Comp. Physiol., **8**: 167, 1936.

[10] D. A. Marsland, Chapter in The Structure of Protoplasm, ed by W. A. Seifriz: Am. Soc. Plant Physiol, Ames, Iowa, 1942.

[11] N. Rashevsky, Bull. Math. Biophysics, **1**: 47, 1939.

[12] G. Young, Bull. Math. Biophysics, **1**: 177, 1939.

[13] W. H. Lewis, The Structure of Protoplasm, ed. by W. A. Seifriz, Monograph of the Am. Soc. Plant Physiol., p. 163: Ames, Iowa, 1942.

[14] A. A. Schaeffer, Ameboid Movement: Princeton, 1920.

[15] O. P. Dellinger, J. Exper. Zool., **3**: 337, 1906.

[16] S. O. Mast, J. Morphol. and Physiol., **41**: 347, 1926; Protoplasma, **14**: 321, 1931; J. Exper. Zool., **51**: 97, 1928.

[17] C. F. A. Pantin, J. Mar. Biol. Assn., United Kingdom, **13**: 24, 1923.

[18] Yô K. Okada, Arch. f. Protistenk, **70**: 131, 1930.

volume and absorb heat during gelation (Freundlich, 1937).[19] If so, a corresponding absorption of heat must take place at the anterior end when the gel forms again, and the energy associated with this change of state must be comparable to the thermoelastic effect in muscle (see p. 486). Anyhow, the energy for the ameboid movement must come from some other energy-rich reactions, as it does in muscle.

Still another view regarding the sol to gel change is possible, however. The solation resulting from an increase of pressure, which was observed by Brown and Marsland,[20] may be due to some secondary effect, and the protoplasmic gel may form nearly isothermally like most thixotropic gels (Freundlich).[21] In that case, it is simplest to consider that the change from gel to sol during contraction is merely the result of movement which mechanically breaks up the gel, only to permit it to reform as soon as the movement ceases at the tip of the newly formed pseudopod. In either case, some stimulus is required in the posterior part to initiate contraction. There is no way to decide whether this merely releases some previously stretched "spring," the shortening of which is the contraction, or whether the protein chains unfold again passively in relaxation, and reestablish their side linkages to form a gel, the active liberation of energy being confined to the contraction phase.

One of the most respected arguments against the contractility theory of ameboid movement has been the observation that in the formation of a new pseudopod, granules are seen to move in the new pseudopod before they start to flow out of the old ones. It was argued, therefore, that old pseudopods collapsed because they were "pumped out," and not because of inherent contractility. Movement, in other words, was initiated in the new pseudopod itself, which was not, therefore, pushed out passively by a force from behind. In reply to this, it may be pointed out that movement may become *visible*, first, in the pseudopod, because elsewhere the movement is of fluid only (without granules), or because this is the narrowest place on the route where the velocity would be at a maximum.

If the contractility theory of ameboid movement is correct, then it is a primitive form of contractile structure with widely separated and rather disoriented protein chains, because there seems to be no sign of birefringence in either the Mycetozoa or the Rhizopods (Norris,[7] Schmidt,[22] Schmitt[23]). In the pseudopods of the Heliozoa and the Radiolaria, however, birefringence is well established. These pseudopods are characterized by an axial skeleton surrounded by contractile protoplasm. It is not certain whether

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[19] H. Freundlich, J. Phys. Chem., **41**: 901, 1937.

[20] D. E. S. Brown and D. A. Marsland, J. Cell. & Comp. Physiol., **8**: 159, 1936.

[21] H. Freundlich, Chapter in Structure of Protoplasm, ed. by W. A. Seifriz, p. 85: Ames, Iowa, 1942.

[22] W. J. Schmidt, Die Doppelbrechung von Karyoplasma, Zytoplasma und Metaplasma: Berlin, 1937.

[23] F. O. Schmitt, Physiol. Rev., **19**: 270, 1939.

the birefringence is due to the axial skeleton, or to the protoplasm surrounding it, or to both. When the pseudopod (or axopod) is withdrawn on stimulation, the birefringence disappears, there being no sign of it in the body of the animal (Schmidt[22]). The fibrillar protoplasm, or myoids, in these axopods can shorten on stimulation to $\frac{1}{5}$ or less of their relaxed length, and many forms are said to show cross striations (Spek[24]). Contraction can be initiated by induction shocks; they represent, therefore, a more highly developed contractile element than is found in the ameba.

3. Other Contractile Tissues.—According to Dellinger,[25] the cilium is a better example of contractile tissue than the ameba. Both have a fibrillar structure, but it is better developed in the cilium, which shows birefringence. The cilium consists of a contractile fibril working against an elastic structure, or two antagonistic fibrillæ working against one another with a pliable skeleton in between. The close parallelism between ciliary and muscular activity has been emphasized by J. Gray,[26] to whom we owe some beautiful stroboscopic pictures of the beat of the cilium, and the most extensive study of the subject so far available.

Flagellæ are in general like cilia, but they function mechanically, more like a screw propeller than like an oar. They also are birefringent and Dellinger[25] has shown that they can be separated into several fibrillæ twisted together. The flagellæ are also birefringent, as are the tails of spermatozoa (Schmidt[22,27]).

Another striking example of a well-developed nonmuscular contractile myoid structure is found in the Vorticellidæ. In the lumen of the hollow stalk is a long contractile fibrillar birefringent strand (Schmidt,[28] Spek,[24] Biedermann[29]). On stimulation, this can shorten to 40 or 50% of its length and then slowly recover. This structure shows both form and intrinsic birefringence. On contraction, the birefringence diminishes as it does in skeletal muscle. According to Lapicque[30] this myoid, when separated from the body of the animal, can still be stimulated electrically to contract, but in that condition it no longer relaxes. This observation suggests that relaxation is the active phase of the process, and takes place slowly against the elasticity of the stalk and at the expense of energy mobilized in the body. The extremely rapid contraction is, then, to be regarded as the sudden release of a previously stretched spring. While this interpretation is reasonable enough, it is not thereby proved correct, and other observations will be mentioned later which appear to support the theory that relaxation

[24] J. Spek, Hand. d. norm. u. path. Physiol., VIII, 1: 31, 1925.

[25] O. P. Dellinger, J. Morphol., 20: 170, 1909.

[26] J. Gray, Proc. Roy. Soc. London, B, 93: 104, 1922.

[27] W. J. Schmidt, Die Bausteine des Tierkörpers in polarisiertem Lichte p. 381: Bonn, 1924; see also articles by E. Gellhorn, p. 27, and F. Alverdes, p. 57, in Handbuch der norm. u. path. Physiol., Nov., 1940.

[28] W. J. Schmidt, Protoplasma, Nov., 1940.

[29] W. Biedermann, Electrophysiology, tr. by F. A. Welby. Vol. 1, p. 4: London, 1895.

[30] L. Lapique, Traité de physiol., norm. et pathol., Tome VIII, p. 108.

is passive. Movements of plants depend either upon differential growth rates in different parts of the stems or other structures, or upon local changes in turgidity or moisture contact. They must be regarded, therefore, as dependent upon a mechanism which is totally different from the contractility of animal tissues (Bose,[31] Sierp,[32] Stern[33]).

[31] J. C. Bose, The Motor Mechanism of Plants: London, 1928.

[32] H. Sierp, Hand. d. norm u. path. Physiol, VIII, 1: p. 72, Berlin, 1925.

[33] K. Stern, Hand. d. norm. u. path. Physiol., VIII, 1: p. 94, Berlin, 1925.

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MUSCLES

In the muscles proper the fibrillar protein reticulum as found in the undifferentiated cell reaches its highest degree of specialization for contractility. Similarly, the nerve fiber represents the highest specialization of that same reticulum for the process of conduction. In smooth muscles, individual cells are still involved. In the still more highly developed striated muscles, many cells have fused together into a large multinucleate fiber, thus bringing the protein chains of many cells under the co-ordinated control of one end plate. Likewise many fibers are brought under the control of one axon which branches to reach an equal number of end plates.

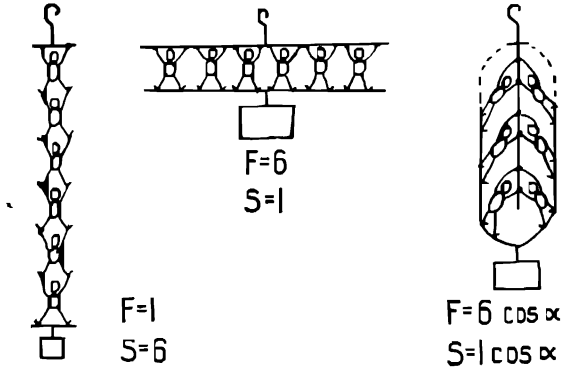

FIG. 44.—Diagram to illustrate different types of arrangement of fibers in muscles. Each man in the diagram represents an equal length of a unit number of muscle fibers. Two parallel-fiber muscles are illustrated on the left and a penniform muscle on the right. The relative force (F) and shortening (S) of the different arrangements are indicated. In the penniform muscles the fibers do not all have the same angle and the calculation of the shortening of the individual fibers is far more complicated than the formula indicates. (See Beritoff.[36])

The multinucleate arrangement permits the development of longer fibrillæ without interference from cell boundaries.

1. Macroscopic Structure.—A muscle consists of individual contractile units arranged in parallel or in series according to the amount of force and shortening required. The principles involved are illustrated by two types of muscles, the parallel fibered muscles (long and short), and the diagonal fibered or penniform muscles (see Fig. 44).

For greater shortening, more units are arranged in series, or the fibers are long, as in the sartorius muscle. For greater force with minimum shortening, many shorter fibers are arranged in parallel. Where a broad

short muscle is anatomically possible, as in the gluteus or rectus abdominis muscle, the latter arrangement is suitable, but in other localities, as in the calf of the leg, the contractile units pull somewhat diagonally on a central tendon like men pulling on a rope. This provides large force and small shortening in a muscle of approximately the same convenient shape as the sartorius.

The length of the fibers bears a fairly close relation to the amount of shortening demanded of the muscle. It seems likely that this relation is automatically adjusted by the mechanical conditions of the situation, so that muscle can be transformed into tendon, or vice versa, until the fiber, when stimulated in the body, can shorten to something less than half its resting length (Haines[34]). According to the figures of Zchakaia,[35] the absolute shortening of any particular muscle in the body is equal to the absolute shortening of any one of its fibers. In the parallel fibered muscle, like the sartorius, the maximum shortening of the whole muscle and of its fibers, when tetanized *in situ* with insertion cut and a very small load, is 55%, and the fibers are nearly as long as the whole muscle. In the rectus femoris muscle, the fibers are only ¼ or ⅕ as long as the whole muscle, and the muscle shortens only 23% of its length. If a sartorius muscle is cut in half, each half will shorten half as much as the whole muscle. In the penniform muscles, however, the absolute shortening of each half, or of each muscle bundle, is the same as that of the whole muscle (Zchakaia[35]). In general, a muscle will not shorten beyond the length permitted by the anatomical situation in the body, even if its tendon is cut. Further shortening is evidently prevented by the connective tissue. Further information on structure and the arrangement of muscle fibers in different muscles may be found in papers by Beritoff,[36] Jansen,[37] and Lindhard.[38]

2. Microscopic Appearance.—The striking appearance of the cross-striated muscles with their alternating light isotropic (*I*) and dark anisotropic (*A*) bands leads to an excessive preoccupation with the details of that particular type of structure. The cross striations, however, seem to be peculiar only to the rapidly shortening fibrils and the more rapid the muscle, the shorter the interval between the bands.[39,40] Likewise, the faster the muscle, the more nearly equal are the anisotropic and the isotropic discs (Brenner[39]). From this point of view, it is tempting to regard the smooth muscles as those in which the tempo became so slow and the sarcomeres so long that the cross striations disappeared. Such a classification, however, is purely artificial, because intermediate forms are not known, and it offers no explanation of the origin of the cross striations from the presumably more

[34] R. W. Haines, J. Anat. u. Physiol., **65**: 578, 1932.

[35] M. Zchakaia, Pflüger's Arch. f. d. ges. Physiol., **209**: 753, 1925.

[36] J. Beritoff, Pflüger's Arch. f. d. ges. Physiol., **209**: 753, 1925.

[37] M. Jansen, J. Anat. u. Physiol., **47**: 319, 1912 13.

[38] J. Lindhard, Physiological Papers, dedicated to A. Krogh, p. 188: Copenhagen, 1926

[39] J. Brenner, Zschrs. f. Zellforsch. u. Mikr. Anat., **29**: 251, 1939.

[40] H. H. Jasper and A. Pezard, Compt. rend. Acad. d. Sc., **198**: 499, 1934.

primitive plain muscles. It does emphasize, however, the important correlation between speed of contraction and the presence of the cross striations.

According to Bozler,[41] the classification of muscles as smooth or striated is not a particularly happy or significant one, except for the muscles of vertebrates, where smooth muscles are, in general, specialized for tonus, and striated muscles for quick movements. In a survey of all the muscles of the animal kingdom as a whole, the classification breaks down, because it fails to maintain its correlation with function. The muscle cell, according to Bozler, is composed of fibrillæ embedded in sarcoplasm. The fibrillæ are of two kinds: 1. The large, darkly staining tetanus fibrils arranged around the periphery of the cell and attached to the sarcolemma either continuously or at intervals (*Z* lines); 2. the small, lightly staining tonus fibrils, either distributed throughout the cell or arranged inside the peripheral layer of tetanus fibrils. A cell which contains both types of fibrillæ will serve the function of both tetanus and tonus. The more rapid tetanus fibrillæ are striated and are attached only at intervals to the sarcolemma. Where the attachment is continuous, the resistance to shortening is greater and movement is slower. The difference in function, however, between tetanus and tonus fibrils is far greater than that between striated and unstriated. In any event, the essential features of a contractile mechanism must be and are present in both smooth and striated muscles; these essentials are the birefringent fibrils and the myosin chains.

It has frequently been questioned whether the striated muscle fibers are composed of a series of discs, or whether what appears to be discs is really a series of layers arranged around a central axis, like a spiral staircase.[42] One frequently sees boundary lines between the sarcomeres, the *Z* lines, which appear to branch; or one sees that on one side of a given length of fiber there are n bands and on the other side $n + 1$ bands. This is known as a "nonius period" or vernier type of banding and is sometimes interpreted as a section through the central axis of a spiral structure.[43] It has even been supposed that the excitation wave, in passing down the muscle, has to pass along this spiral pathway. Some of the pictures of these spiral structures are rather convincing, especially in insect muscles. On the other hand, it may be argued that the same appearance might be produced in other ways, as by a true branching of the *Z* line. A certain measure of skepticism concerning this alleged spiral structure is, therefore, well justified.

On microdissection, the sarcolemma is found to be a tough membrane surrounding the whole fiber. It is composed of interlacing collagenous birefringent fibers embedded in a thick colloidal matrix,[44,45] which has been likened to a knitted stocking. The fibers of the sarcolemma are continuous

[41] E. Bozler, Ztschr.vergl. Physiol., **8**: 371, 1928.

[42] O. W. Tiegs, Trans. Roy. Soc. (So. Aus.), **46**: 222, 1922; **47**: 142, 1923.

[43] L. von Boga, Ztschr. f. Zellforsch. u. mikroskop. Anat., **27**: 568, 1937–38.

[44] A. Bairati, Ztschr. f. Zellforsch. u. mikroskop. Anat., **27**: 100, 1937–38.

[45] A. Nagel, Ztschr. f. Zellforsch. u. mikroskop. Anat., **22**: 694, 1935.

with the fibers of the internal perimysium in places. The sarcolemma stretches reversibly over a considerable range, and its stretch is believed to be due to the displacement of the fibrils or to a change in shape of the meshwork, rather than to true elasticity of its components. The muscle substance itself is a sticky elastic gel with fibrils embedded in it. The fibrils can be pulled out with some success by micromanipulation.[45] The fibers of the *Z* line, which form the boundaries of the sarcomeres, are attached to the sarcolemma and differ in composition from the myofibrils.[47] In most muscles, particularly the insect muscles, these *Z* lines are clearly distinguished, and they serve to keep the myofibrils aligned with one another during shortening, so that the cross striations of the fiber as a whole remain undisturbed.[43]

In the middle of the sarcomere, between the *Z* lines, is the anisotropic or *A* (or *Q*) disc, which usually appears darker than the isotropic *I* disc adjoining the *Z* lines. The *A* discs are definitely anisotropic or birefringent, and are often regarded, on rather meager evidence, as the contractile part of the muscle. This belief depends chiefly upon the fact that, in contraction, the *Q* discs shorten, while the *I* discs lengthen slightly.[48,49,50] The contrary finding of Frank[51] has been attributed to artefacts due to fixation of the muscle, but Studnitz[52] has found that the *I* disc does eventually shorten if the shortening of the *A* disc exceeds 50%. In the Arthropod muscles with which he worked (*Astacus fluviatilis*), a shortening of the whole fiber to 30% of its resting length may occur. It seems possible, therefore, that there is some power of contractility in the *I* discs, and this is confirmed by Schmidt,[53] who insists that the *I* disc is slightly birefringent. This is consistent with the idea that the myofibrils pass continuously from one *Z* line to the next. Indeed, the diagrams of the fine structure of muscles offered by v. Muralt[54] and Hürthle[49] show such continuous fibrils, but with less regular orientation of the myosin chains, which make up the fibrils in the isotropic regions. Without some such continuity, it is difficult to see how the force of contraction of the *A* discs could be applied to the shortening of the fiber as a whole. It may be concluded, therefore, that the *A* discs are possibly stronger than the *I* discs, but that both are contractile. According to Buchthal, Knappeis, and Lindhard,[48] the *A* discs are more easily stretched in the resting muscle than the *I* discs, while the reverse is true in the stimulated muscle.

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[45] H. Ensinger, Ztschr. f. Zellforsch. u. mikroskop. Anat., **28**: 614, 1938.

[47] G. Ciaccio, Ztschr. f. Zellforsch. u. mikroskop. Anat., **27**: 764, 1937–38.

[48] F. Buchthal, G. G. Knappeis, and J. Lindhard, Skandinav. Arch. Physiol., **73**: 163, 1936.

[49] K. Hürthle, Pflüger's Arch. f. d. ges. Physiol., **227**: 610, 637, 1931.

[50] B. Holz, Pflüger's Arch. f. d. ges. Physiol., **230**: 246, 1932.

[51] G. Frank, Pflüger's Arch. f. d. ges. Physiol., **218**: 37, 1927.

[52] G. von Studnitz, Ztschr. f. Zellforsch. u. mikroskop. Anat., **23**: 1, 1935.

[53] W. J. Schmidt, Ztschr. f. Zellforsch. u. mikroskop. Anat., **21**: 2, 1934; *ibid.*, **23**: 201, 1935–36.

[54] A. L. v. Muralt, Kolloid-Ztschr., **63**: 228, 1933.

We have as yet no real understanding of the significance of the cross striations, except that they seem to be important for rapid contractions. One theory is that the *I* discs are specialized for the process of recovery, while the *A* discs are predominantly contraction sites. The only experimental confirmation for such a theory comes from histochemical analyses. In the resting fiber, glycogen is found predominantly in the *A* discs, chiefly between the fibrils in the sarcoplasm. During recovery, especially when the total glycogen is small in amount, there is more in the *I* discs, where it is supposedly being rebuilt from lactic acid diffusing out of the *A* discs.[39]

It is, perhaps, difficult to know just how literally to interpret these measurements of the dimensions of the *A* and *I* discs, and the separation of their functions. According to Jordan,[55] there is, in contraction, an actual reversal of striations in wasp muscle, the I discs becoming dark, and vice versa. This could be accomplished by a movement of granules from one disc to another. Many writers have preferred to regard the striations as dynamic, rather than static, structures. Carey,[56] for example, regards them as standing waves of some sort. More plausible is the intriguing theory of Bernal[57] that they owe their origin to the reversed spiral arrangement of the parallel-packed protein chains. According to him, substances with long molecules tend to show this structure when they are allowed to set in long tubes. The more highly birefringent A bands, then, represent places where the spirals reverse, so that the molecules lie parallel to the long axis of the fiber. At intermediate regions, the molecules are diagonal, some to the right and some to the left. It must be admitted, however, that the fibrillar structure of microscopic dimensions, which has been described for muscle, always appears to be parallel to the longitudinal axis, rather than diagonal. On the whole, this theory seems to be based upon very little evidence. One thing which seems certain is, that the protein chains or the contractile units must extend from end to end of the fiber, and, this being so, there is grave danger of placing too much emphasis upon the *A* discs as the contractile units.

There is considerable evidence to show that, during embryonic development, the fibers become capable of contraction, and show double refraction before they become cross striated.[58] Ettisch[59] has studied muscles in the dark field, using light directed either parallel or perpendicular to the long axis. Parallel illumination brings out the cross striations, while perpendicular illumination extinguishes the cross striations, but brings out the fibrillar structure. Some fibers are found to have lost their cross striations, but

[55] H. E. Jordan, Am. J. Anat., **27–28**: 1, 1920–21. Physiol. Rev., **13**: 301 (1933).

[56] E. J. Carey, Arch. Path., **30**: 1041, 1940.

[57] J. D. Bernal, Chapter in Perspectives in Biochemistry, p. 45: Cambridge, England, 1938.

[58] For discussion, see A. Frey-Wyssling Submikrosk. Morphol. des Protoplasmas, p. 259: Berlin, 1938; and W. J. Schmidt, Die Bausteine des Tierkörpers in polarisiertem Lichte: Bonn, 1924.

[59] G. Ettisch, Pflüger's Arch. f. d. ges. Physiol., **232**: 754, 1933.

they still show uniform double refraction throughout their length. Similarly, Liang[60] has found that muscles put into rigor with caffeine, quinine, or chloroform, lose their cross striations, but retain their fibrillæ, which become uniformly birefringent. Conversely, treatment with ammonia destroys the fibrillar appearance, but preserves cross striation. The conclusion seems to be that the longitudinal and cross striations are due to different structure. The cross striations are believed to be due to periodic accumulation of granular material outside the fibrillæ in the sarcoplasm. The true contractile unit is the birefringent fibril, which is continuous and uniformly birefringent from one sarcomere to the next. From this point of view, smooth and striated muscles differ only in secondary details, the fundamental structure being the same in both. This view confirms, therefore, the theory of Bozler,[61] already discussed. It also may be said to support in some measure the ideas of Carey,[62] who regards the sarcomere as a "morphological myth," because, according to him, the number of sarcomeres in a given muscle can be increased from 397 to 1124 by 10 seconds' treatment of the fiber at 45°C. It has, apparently, never been possible for anyone else to confirm this finding as yet (Speidel and Jordan[63]). With this exception, all the evidence indicates that the sarcomere is a fixed structure.

Not so long ago, particularly under the leadership of Bottazzi,[64] it was fashionable to consider that the sarcoplasm was contractile as well as the myofibrils. The situation in muscle was considered analogous to that described by Engelmann[65] in *Stentor*, where he saw longitudinal somewhat striated fibrils which were responsible for the quick shortenings of the body. The animal was also capable, however, of slow turnings of the body which left these fibrils folded and slack. These slower movements were attributed to submicroscopic fibrils in the protoplasm. From this point of view, a muscle might be described as a striated muscle imbedded in a smooth muscle, and this idea was advanced in explanation of all the slow movements which muscles can make under proper conditions. Biedermann[66] has also championed the idea of contraction without visible fibrillæ, and presented the evidence at some length. It seems unnecessary to suppose that all the myosin chains are packed together into bundles or fibrils; some of them may well be scattered around more or less by themselves in the viscous sarcoplasm, where shortening would be more difficult, and hence slower. The fibrillar arrangement of such chains would facilitate rapid contractions, just as the movement of large numbers of persons is facilitated by some military order in their ranks, thus preventing one individual from interfering with

[60] T. Y. Liang, Chinese J. Physiol., **10**: 327, 1936.

[61] E. Bozler, Ztschr. vergleich. Physiol., **7**: 407, 1928.

[62] E. J. Carey, Arch. Path., **30**: 1041, 1940; also E. J. Carey, W. Zeit., and L. Massopust, Am. J. Anat., **70**: 119, 1942.

[63] C. C. Speidel and H. E. Jordan, Anat. Rec., **82**: 470, 1942.

[64] P. Bottazzi, J. Physiol., **21**: 1, 1897.

[65] Engelmann, Pflüger's Arch. f. d. ges. Physiol., **11**: 433, 1875.

[66] W. Biedermann, Electrophysiology, tr. by F. A. Welby, Vol. 1, p. 4: London, 1896.

the movements of his neighbor. Most investigators at the present time discount this assumption of sarcoplasmic contractility (see Lapique, for example[57]) as unnecessary, but, in our desire to make only the simplest necessary assumptions, we may sometimes forget that it is not difficult for Nature to do things in ways which seem unduly complicated to physiologists. In many ways this would seem to be a very "natural" arrangement, even though a complex one. Among others, Bozler[61] would discard completely the idea of contractile sarcoplasm, and he explains many of the phenomena of slow and fast contractions, occurring in the same muscle, by the evidence which he presents for the presence of both tonus and tetanus fibrils in the same fiber. The former are smaller and less darkly staining, and are arranged usually in the interior of the fibers, and contract slowly, while the latter are larger, contract more rapidly, and are arranged around the periphery of the fibers.

In spite of the validity of this idea, it is perhaps unnecessary to discard altogether the theory of sarcoplasmatic contraction, although the direct evidence for it is very scanty. The best evidence available comes from the work of de Renyi and Hogue[58] on the behavior of cardiac muscle fibers in tissue cultures. Some of the cells contained visible fibrillæ, and some did not, but both kinds contracted equally well. Moreover, some cells were figured which contained fibrillæ on one side, but not on the other. The nonfibrillar parts of such cells were found to be more quickly injured by toxic substances, such as water. In such a case, the cell contracted on the fibrillar side only, and became concave on that side, whereas, previous to this injury, the contraction was symmetrical on both sides. The authors conclude that "both the sarcoplasm and the myofibrillæ are forms of contractile substance which, in the embryonic state and under normal conditions, act synchronously."

3. Visible Diffraction Spectra.—It has been known since 1874 (Ranvier[59]), that a muscle, when traversed at right angles to its long axis by a small beam of light, will act like a diffraction grating, and will produce on a screen, placed beyond the muscle, a series of spectra of orders I, II, III, etc., extending indefinitely in the longitudinal axis of the muscle. From the positions of the series of spectra which are so produced, it is possible to calculate the grating constant, or the distance between the bands. Recent measurements (Sandow,[70] Buchthal and Knappeis,[71]) give values which agree closely with the distance between the anisotropic bands of the same muscles, measured microscopically. When the bands move farther apart, as when the muscle is stretched, the first-order spectrum moves closer to the direct image. The percentage increase in the calculated width of the

[57] L. Lapique, Traité de physiol. norm. et path., VIII, p. 101: Paris, 1929.

[58] G. S. de Renyi and M. J. Hogue, Anat. Rec., **70**: 441, 1938.

[59] L. Ranvier, Arch. Physiol. norm. path., ser. 2, part 1, 774, 1874.

[70] A. Sandow, J. Cell. & Comp. Physiol., **9**: 37, 55, 1936.

[71] F. Buchthal and G. G. Knappeis, Skandinav. Arch. f. Physiol., **83**: 281, 1940.

bands is the same as the percentage increase in the length of the muscle, at least after the initial stages, when the slack in the muscle was being taken up (Sandow[70]). When the muscle shortens in contraction, the bands also become closer together. At the same time, the intensity of the spectra of the first, second, and third orders increases. This is interpreted as indicating a decrease in the width of the dark bands, and a relative increase in the width of the more transparent *I* bands (Buchthal and Knappeis[71]). Some indication has also been found of a fainter diffraction pattern at right angles to the main one, and caused by longitudinal surfaces in the fiber. Since these were obtained in single isolated fibers, they were attributed to bundles of fibrils or "Cohnheim bundles." These diffraction patterns are surprisingly sharp, even when taken from a whole muscle, and this bespeaks possibly some alignment of the anisotropic bands of one muscle fiber with those of the neighboring ones, so that the whole muscle is able to act like a single grating.[70]

Of special interest is the finding of Nicolai,[72] who photographed the positions of the first-order spectrum during a tetanus of the muscle, and reported fluctuations in its position, and, therefore, fluctuations in the width of the individual sarcomeres which were coincident with the frequency of the individual shocks in the stimulating current. Thus, even at frequencies of 60 per second, when the tetanus appears externally to be "complete," it is possible to show that internally it is really still "incomplete." This finding is of importance in the interpretation of some of the data on heat production during a tetanus. The diffraction spectra so far considered are those due to the microscopically visible cross striations, or the first-order grating. Evidence of visible diffraction spectra from a still finer, or second-order, grating has been presented by Langelaan,[73] who has prepared a model of the finer structure of the anisotropic discs based upon dark field observations with the Shierer lens. The model consists of two systems of fine wires at an angle of 60° with each other. These wires themselves form a third-order grating, but, when viewed at different angles, the overlap of successive layers produces the appearance of a coarser, or second-order, grating, the diffraction spectra of which are just visible. All the structure observed in this way is confined, according to Langelaan, to the anisotropic disc, the isotropic discs being completely dark in the dark field of the microscopic. Other evidences of the submicroscopic structure of muscles are, however, more convincing and must now be considered.

4. Submicroscopic Structure.—However important it may be to know the microscopic structure of muscle, none of the histological details offers any very reliable clue as to the nature of the contraction process. The wealth of detail which can be seen in some muscle fibers, especially those of the insects, has sometimes proved too tempting, and some writers have been over-venturesome in applying their imagination to the visual

[72] L. Nicolai, Pflüger's Arch. f. d. ges. Physiol., **237**: 399, 1936.

[73] J. W. Langelaan, Arch. néerl. de physiol., **21**: 6, 1936.

images which they gain from the microscope. The newer knowledge of the subject ascribes the process of contraction rather to the submicroscopic structure, and the unfolding of this field has provided an effective restraint for the previously unbridled imagination. An excellent recent review of this subject has been given by Fischer.[74]

a). *Birefringence.*—Two methods have been in use for the elucidation of the molecular structure, the measurement of birefringence and the x-ray diffraction pattern. From the study of the birefringence of muscle, support has been obtained for the idea originally suggested by Brücke, that within the myofibrils the protein chains are not uniformly distributed, but are arranged in micelles, or small bundles. The existence of double refraction requires some orderly arrangement of the molecules, like that found in a crystal. It can also result from an orderly arrangement of larger units, or giant molecules, such as those found in a solution of myosin. It is believed, therefore, that the birefringence (D.R.) of muscle is due to (1) the orientation of molecules within micelles (intrinsic D.R.), and (2) to the orientation of micelles in the long axis of the myofibrils (form D.R.). The form D.R. disappears if the refractive index of the micelle is the same as that of the surrounding solution and increases in proportion to the difference between the refractive indices of these two phases. It depends also to some extent on the relative volumes of micelles and solution.

In order to measure the form double refraction, it is necessary to vary the refractive index of the solution around the micelles without modifying the volume of the micelles or the arrangement of the molecules within them. Strange as it may seem, the results appear to indicate that this is possible. The muscle is fixed in formaldehyde and is then immersed in solutions of varying refractive index. The fixation process itself has little or no effect on the double refraction, and, in spite of the use of different substances for the variation of the refractive index of the solution, the double refraction of the muscle falls along a smooth curve in the shape of a parabola, indicating that the refractive index of the solution is the only variable. This is shown in Fig. 45, where Fischer's curve[74] for smooth muscle and Weber's curves for striated muscle and myosin threads are represented. The curves are all very similar, and pass through a minimum at the point where the refractive index of the solution equals that of the micelles.[75] The D.R. remaining at this minimum is the intrinsic D.R. due to the molecular orientation inside the micelles, while the increase on either side of the minimum is due to form D.R. This experiment must, therefore, be regarded as strong evidence that there are micelles in muscle. The fact that artificial myosin threads give similar results is evidence that the micelles in muscle are also composed of myosin.

The volume of the micelle has been estimated by Weber from its molecu-

[74] E. Fischer, Biol. Symp., **3**: 211, 1941.

[75] E. Fischer, Cold Spring Harbor, Symp., **4**: 214, 1936.

lar weight as determined in the ultracentrifuge as 1100 mμ^3.[75] From the Wiener theory for form double refraction, the fraction of the muscle volume occupied by micelles can be calculated. One can guess, further, that the rods are long compared to their diameter, because they show double refraction of flow, and only rod-shaped particles would be oriented by the shearing stresses set up in the solution during flow. Since, however, the rods are anisotropic themselves, they must contain enough polypeptide chains in parallel to show this intrinsic double refraction. Thus it is possible to esti-

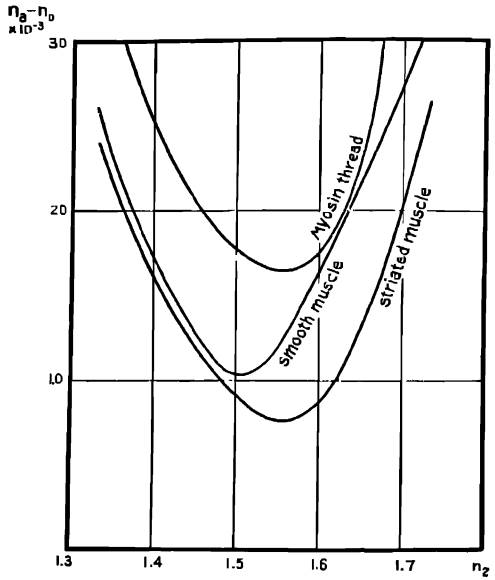

FIG. 45.—Birefringence as ordinates against the refractive index of the impregnating medium as abscissæ. Birefringence is expressed as the difference in the index of refraction of the ordinary and the extraordinary rays. (After E. Fischer.[75])

mate that the micelles may be 5 mμ in diameter and 55 mμ long; Weber calculates that a micelle of this size would contain about 20 myosin chains, the distance of one micelle from its neighbor being 11.5 mμ. Whether the micelles are arranged end to end or with a space between, is only a matter of guesswork. The most uncertain part of this whole estimate of the size of the micelle seems to lie in the figure for the volume. It is based upon a molecular weight of myosin of 1,000,000, which is a very large value and one which is likely to vary widely with changes of *p*H and electrolyte environment. It is known for example, that urea lowers the molecular weight to 100,000. How constant in size the micelles are is unknown, but since the calculated size is based upon the only figure available for the molecular weight of myosin, the micelles might equally well be termed giant myosin molecules.

The double refraction of muscle has been observed by all workers to

[75] H. H. Weber, Pflüger's Arch. f. d. ges. Physiol., **235**: 205, 1934.

decrease in an isotonic contraction, due to a diminished orientation of the myosin chains in the micelles.[78] Likewise, in an isometric contraction, there is usually a decrease, although at large extensions there may be no change, or even an increase.[77] Such an increase may be attributed to the effect of the increased tension *per se*, the photoelastic effect, rather than to a better orientation of micelles.

In smooth muscle there is an increase of birefringence in stretching,[77] but no change in frog striated muscle.[78,79] The effect of stretch on a whole muscle is certainly a complex process. The rate of metabolism increases, for example,[80] and an increase in lactic acid, which may also occur, leads to a decrease of birefringence.[78] In addition, the *A* bands stretch more than the *I* bands, and the effect of this is difficult to estimate. Correction is, of course, made for any decrease of the thickness of the muscle due to stretch. Stretching *per se* should cause a better orientation of micelles and myosin chains, and hence an increase in double refraction.

From the birefringence studies, therefore, we learn that there are micelles in the muscle, and, furthermore, we learn that they may lose, to some extent, their regularity or the completeness of their orientation during contraction. In addition, the micelles themselves may lose some of their intrinsic birefringence. The behavior of the micelles as a whole seems to leave us still unsatisfied in our search for the fundamental nature of contraction.

b). *X-ray diffraction.*—Further information must be sought in the change of the ultrastructure of the micelles. Evidently, the molecules of which they are composed are less regularly arranged when the micelle shortens. Further information concerning this change is derived from the diffraction patterns produced by x-rays. Here the wave-lengths used are so much shorter than they are in visible light, that the dimensions of the grating which produces the pattern are of molecular, rather than microscopic, size. The molecular diffraction patterns tell us more concerning the probable nature of contraction than do the *A* band diffraction patterns previously considered. When a muscle is dried, it is perhaps surprising to find that the x-ray diffraction pattern does not change. Evidently, the removal of the water has not changed the intermolecular distances. The water must, therefore, have been contained between micelles, and this may be regarded as an additional confirmation of the micellar structure. Furthermore, the side-chain spacings of the protein molecules, as given by x-ray analysis, are so small that they would be inconsistent with the high water content found in muscles if this same close packing were continuous throughout the muscle substance. The micellar theory reconciles these two observations.

Many investigators have presented data on the ultrastructure of muscle

[77] E. Fischer, J. Cell. & Comp. Physiol., **8**: 503, 1936; **12**: 85, 1938; **23**: 113, 1944.

[78] F. Buchthal and G. G. Knappeis, Skandinav. Arch. f. Physiol., **78**: 97, 1938.

[79] E. Bozler and C. L. Cottrell, J. Cell. & Comp. Physiol., **10**: 165, 1937.

[80] T. P. Feng, J. Physiol., **74**: 441, 1932.

as it is shown by x-rays.[81] It is, however, to the extensive papers of Astbury[82] on this subject that we owe the most at the present time. Since his work will be discussed later in this chapter in connection with various other manifestations of contraction it will be sufficient here to give only a very brief outline. The principles of the x-ray method have already been described.[83]

When a beam of x-rays is passed through a muscle perpendicular to its long axis, a diffraction pattern is formed on the screen, which shows a striking resemblance to that caused by other fibrous proteins, such as the keratin of hair. In this particular form it is known as α-keratin, and is composed of long polypeptide chains in a partially folded form. In water at ordinary temperatures the α-keratin can be stretched out to about twice its original length, until the chains are unfolded and the pattern changes to the form indicative of β-keratin. In this form it is similar in structure to fibroin, the protein of natural silk. With further treatment in water at elevated temperatures in steam or in caustic soda at ordinary temperatures, the hair may be made to shorten to about 70% of its original length. This treatment, apparently, removes certain of the side-chain linkages between adjacent polypeptide chains, and it is the nature and firmness of these linkages and the length of the side-chains which determine the mechanical properties of the muscle or hair. With these linkages in hair broken down, the protein chains are able to fold still further, and eventually the hair passes into a supercontracted state. Thus, both the hair and the muscle show both contractility (or ability to shorten) and extensibility. It is apparently not a simple matter to stretch a muscle into the β-keratin form, but in Astbury's latest paper[82] it is stated that this can be done, and that the details of this procedure will be presented in a later paper. It has likewise proved impossible hitherto to cause a hair to shorten like a muscle to 30% of its initial length. If, however, Astbury is right in proposing that a globular protein is simply a fibrous protein folded up into globular form, then still further folding of the myosin chains might be expected, continuing at last under proper conditions until they were reduced to the shape of a sphere. A sartorius muscle, shortened until spherical, would have a length not far from 20–25% of its initial length. The sphere-length of a single fiber of the sartorius would be a still smaller percentage of the initial value. To realize a shortening of this magnitude in hair, would presumably require further breakdown of side linkages. The nature of these side linkages

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[81] R. O. Herzog and W. Jancke, Naturwissenschaften, **14**: 1223, 1926; G. Boehm and K. F. Schotzky, Naturwissenschaften, **18**: 282, 1930; G. Boehm, Ztschr. f. Biol., **91**: 203, 1931; Kolloid-Ztschr., **62**: 22, 1933; K. H. Meyer and L. E. R. Picken, Proc. Roy. Soc., London, **124**: 29, 1937.

[82] W. T. Astbury and S. Dickinson, Proc. Roy. Soc., London, B, **129**: 307, 1940; W. T Astbury, Ann. Rev. Biochem. **8**: 113, 1939.

[83] Sec. 2, chap. 8, subsec. 2, p. 149.

has been discussed[84] (see also Huggins).[85] Speakman[86] showed in keratin that the development of the supercontracted state depended upon the breaking of the S-S cystine linkages. This suggests, therefore, that hair is a sort of vulcanized fibrous protein or a vulcanized form of myosin which thus has its extensibility much reduced. In conformity with this idea, hair has a far higher sulphur content than myosin.

There seems to be little doubt that this picture of the fundamental machinery of contractility is correct so far as it goes but it leaves much of the story untold. When one tries to explain what forces actually cause the folding, how these forces are released by stimulation and how the changes in myosin fit into the simultaneous transfers of energy, the uncertainties of our present position become increasingly clear.

The chief disadvantage inherent in the x-ray diffraction method is the prolonged exposure which is necessary to obtain a readable photographic image. Even with the latest improvements described by Spiegel-Adolph, Henny, and Ashkenaz,[87] an exposure of 3–6 minutes is needed. With this technique, however, it was possible to show a change of patterns on contraction indicating a disorientation of the myosin chains. A similar change is caused by shortening in rigor mortis or contractures due to chloroform or caffeine.

5. The Physicochemical Structure of the Muscle—(myosin).—In a striated muscle, 18–20% of the weight is protein, and about 60% of this is the globulin myosin.[88] Of these proteins the myosin is peculiar in that it shows double refraction of flow as was shown by von Muralt and Edsall.[89] It, therefore, occurs in the form of long molecules, which become oriented by velocity gradients in the solution. Weber[90] has extracted myosin from muscle and has made myosin threads by forcing this solution out of a capillary tube into distilled water. These threads can be dried until they contain the same amount of water as skeletal muscle. Such threads show double refraction, like the original muscle, and when stretched they develop as much tension per unit of cross-section area as an intact resting muscle. Astbury and Dickinson[91] have also made artificial muscles by drying thin films of myosin on glass. Such myosin films give x-ray patterns similar to those of intact muscle, and when stretched they change from the α to the β pattern like other fibrous proteins and like muscle. It is generally considered, therefore, that the myosin represents the contractile machinery proper and is responsible for most of the double refraction

[84] In Sec. 2.

[85] M. L. Huggins, Chem. Rev., **32**: 195, 1942.

[86] J. B. Speakman, Nature, **132**: 930, 1933; further, Sec. 2, chap 8, pp. 181ff.

[87] M. Spiegel-Adolf, G. C. Henny, and E. W. Ashkenaz, J. Gen. Physiol., **28**: 151, 1944.

[88] Bate-Smith, Proc. Roy. Soc., London, **124**: 136, 1937.

[89] A. L. von Muralt and J. T. Edsall, J. Biol. Chem., **89**: 315, 1930.

[90] H. H. Weber, Pflüger's Arch. f. d. ges. Physiol., **235**: 205, 1934.

[91] W. T. Astbury and Sylvia Dickinson, Proc. Roy. Soc, London, **129**: 307, 1940.

of the intact fiber. Some double refraction remains even after all possible myosin has been removed by KCl.

When a muscle is dried, it shrinks in the lateral dimensions, but not in length.[92] The water appears to find space for itself largely between longitudinal fibers or between the myosin polypeptide chains. These chains have side-chains which are in general of a highly solvated type. This provides a mobile protein grid rather than one of more rigid properties, and gives a watery medium suitable for the maintenance of a high metabolic rate.[93] When water is removed from between the fibers, the myosin molecules unite with one another rather than with the water, and the mobility is lost.

The myosin is more soluble in potassium salts than in an equal concentration of sodium salts.[94] According to Greenstein and Edsall, myosin solutions tend to lose, with time, their birefringence of flow, this being taken as a sign of denaturation of the protein. As judged by this test, the solutions are much more stable in potassium solutions than they are in NH_4Cl or NaCl.[95] There seems to be, therefore, for the muscle cell, at least, some reason for the high concentration of potassium which is found in the interior.

In spite of this high concentration of potassium inside the muscle fiber, it appears that the myosin, under natural conditions inside the fiber, is readily changed from sol to gel. Other factors contributing to its instability are the low *p*H and its own high concentration. Bate-Smith has studied the solubility of myosin, and states that at *p*H 6.5 it will begin to dissolve if the KCl concentration is 0.25 M, while at *p*H 7.0 a concentration of 0.17 M will suffice.[96] Assuming a *p*H inside the fiber of 6.9,[97] it is evident that little or no myosin would be in solution, since the potassium concentration inside the fibers is at most 0.17 M. Furthermore, if all the myosin were uniformly distributed inside the fibers, its concentration would be 8%, or even higher, if the myosin is confined to the fibrils, and Mirsky[98] has shown that at high concentrations of the order of 10%, myosin tends to become denatured. Inside the fibers, therefore, the myosin may be regarded as on the verge of denaturation or gelation, relatively little being in true solution.

Proteins of cells are of both the globular and the fibrous types. The former offer advantages of greater mobility as in the plasma proteins while the latter serve better for structural purposes. Myosin is the particular fibrous protein responsible for the structure and contractility of the muscle cell. There is evidence that fibrous proteins play a role either for contractility or conduction or, in less highly differentiated cells, merely structure,

[92] K. Hürthle, Pflüger's Arch. f. d. ges. Physiol., **227**: 610, 1931.

[93] F. O. Schmitt, Physiol. Rev., **19**: 270, 1939.

[94] J. Hensay, Pflüger's Arch. f. d. ges. Physiol., **224**: 44, 1930; J. T. Edsall, J. Biol. Chem., **89**: 289, 1930.

[95] J. P. Greenstein and J. T. Edsall, J. Biol. Chem., **133**: 397, 1940.

[96] Bate-Smith, Proc. Roy. Soc., London, B, **114**: 494, 1934, and **124**: 136, 1937.

[97] W. O. Fenn, and F. W. Maurer, Protoplasma, **24**: 337, 1935.

[98] A. E. Mirsky, Cold Spring Harbor, Symp., **6**: 150, 1938.

but little is known at present of their nature. Recently, however, Banga and Szent-Györgyi[99] have isolated a fibrous or structure protein from kidney, which they have called renosin. This protein shows double refraction of flow and forms viscous solutions and thixotropic gels. All these properties indicate that the molecules are long and closely tangled with their neighbors, or connected to them by side-chains or hydrogen bonds. Renosin resembles myosin, therefore, in many respects. A protein similar to renosin was found in liver, brain, lung, and other tissues. It is extracted by the use of urea, which has the property of breaking hydrogen bonds. After extracting with water, the globular proteins are removed, but the microscopic appearance of the tissue has not been seriously altered because the fibrous proteins remain. A subsequent extraction with a urea salt solution removes the renosin, but still leaves the microscopic structure largely intact. The remainder of the structural protein is removed by urea-NaOH solution. Renosin differs from myosin in showing negative, rather than positive, double refraction of flow. It also contains nonextractable phosphorus, and is a nucleoprotein, although not derived exclusively from the nucleus. Likewise, Mirsky[100] has extracted a protein from sea-urchin eggs which closely resembled myosin, and Bensley[101] has given the name plasmosin to a structural nucleoprotein extracted from liver by 10% NaCl, which has somewhat similar functions and properties. It may be mentioned, also, that myosin can be extracted from smooth muscles as well as from striated muscles (von Muralt and Edsall[102]). In any event, it is evident that myosin or myosin-like proteins are of rather widespread occurrence in cells.

Soon after the death of an animal, the myosin of the muscles becomes insoluble or denatured, so that less of it is extracted by KCl.[103,104] Likewise, contraction and fatigue of the muscle are related fundamentally to a coagulation of the protein. According to Mirsky,[105] the type of coagulation that occurs in rigor mortis is similar to that caused by dehydration, and it differs from other types of denaturation, such as those caused by heat, acid, and alkali, in that it is not accompanied by any increase in the number of detectable SH groups. For some reason, the myosin behaves differently in this respect when it is *in situ* in the muscle than after it has been extracted.

There is considerable evidence, therefore, that the act of contraction is intimately concerned with changes in the condition of the myosin. Just what these changes are and how they are brought about is still very uncertain, but a suggestive clue is furnished by the recent indication that the myosin itself is concerned, somehow, with the phosphate changes in muscle.

[99] I. Banga and A. Szent-Györgyi, Enzymologia, **9**: 111, 1942.
[100] A. E. Mirsky, Science, **84**: 333, 1936.
[101] R. R. Bensley, Science, **96**: 391, 1942.
[102] A von Muralt and J. T. Edsall, J. Biol. Chem., **89**: 351, 1930.
[103] H. J. Deuticke, Pflüger's Arch. f. d. ges., Physiol., **224**: 1, 1930.
[104] A. E. Mirsky, J. Gen. Physiol., **19**: 571, 1935.
[105] A. E. Mirsky, J. Gen. Physiol., **20**: 455, 461, 1937.

This might be, therefore, the method by which the physicochemical changes of myosin are coupled with the energy yielding chemical reactions which are known to occur during contraction. When Deuticke[103] noted that there was a decrease in the solubility of muscle protein during rigor, he observed also that there was a simultaneous decrease in the power of the muscle to esterify hexose with phosphate. More recently, it has been shown by Engelhardt and Ljubimowa[105] and confirmed by Szent-Györgyi and Banga[107] that the myosin fraction of muscle is able to split phosphate from adenosinetriphosphate. If the effect is not due to the myosin itself, it is due to some other substance or enzyme with which it is in intimate association. That myosin itself is the active agent is suggested by the experiments of Needham, *et al.*,[108] who reported a decrease in the birefringence of flow of myosin solutions after the addition of adenosinetriphosphate. These results suggest that the phosphate may be split off from the adenosinetriphosphate because of its tendency to combine with the myosin. According to the theory of contraction proposed by Kalckar,[109] stimulation involves the removal of phosphate from combination with side-chains of the myosin, thus permitting a further folding of the polypeptide chains. This may be the method by which chemical potential energy is transformed into mechanical energy in the myosin.

Other points concerning the physicochemical structure of the muscle fiber might be mentioned, although there is no evident reason for connecting them specifically with the contractile mechanism. The interior of the cell contains what is practically an isotonic solution of potassium, most of which, at least, must be free enough so that it can exert an osmotic pressure. If this solution were brought into contact with the outside of the muscle fiber, the result would be the immediate death of the fiber. On the inside, however, the potassium appears to serve a useful purpose in keeping the myosin in solution. On stimulation, the fiber loses some fraction of its potassium in exchange for sodium, while the reverse occurs in recovery.[110] It is not known whether this is related to a change in state of the myosin, which then combines with less of the potassium, or whether the metabolism of organic phosphate fractions is involved. There is some reason to believe that both processes are concerned. In the blood stream, potassium and carbohydrate frequently rise or fall together, suggesting that carbohydrate metabolism is, somehow, connected with the movements of potassium.[110] The adrenal cortex seems to be concerned with both potassium and carbohydrate metabolism. In yeast cells, Pulver and Verzár[111] have shown that the

[106] W. A. Engelhardt and M. N. Ljubimowa, Nature, **144**: 668, 1939.

[107] A. V. Szent-Györgyi and I. Banga, Science, **93**: 158, 1941.

[108] J. Needham, Shih-Chang Shen, D. M. Needham, and A. S. C. Lawrence, Nature, **147**: 766, 1941.

[109] H. M. Kalckar, Chem. Rev., **28**: 71, 1941.

[110] W. O. Fenn, Physiol. Rev., **16**: 450, 1936.

[111] R. Pulver and F. Verzár, Helvet. chem. acta, **23**: 1087, 1940. Also Nature, **145**: 823, 1940.

addition of sugar to starved yeast, suspended in a dilute solution of potassium, causes a sudden intake of large amounts of potassium. Szent-Györgyi[112] has marshalled the evidence in favor of the view that muscle contraction is caused by the shift of water resulting from the sudden liberation of ionized potassium from myosin at the moment of stimulation. Potassium, as well as adenosinetriphosphate, does apparently cause a decrease in the double refraction of flow of myosin, but what prevents this from occurring before the stimulation is not clear. In any event, it seems not unlikely that the secret of the contraction of muscles may lie in the reactions between myosin, potassium, and adenosinetriphosphate.

6. Mechanics of Muscle.—Since judgments of the activity of muscles depend in such large measure upon the tension developed or the amount of shortening produced, it is important to examine the fundamental basis of these observations.

a). *Resting muscle.*—When a muscle is stretched without stimulation, the tension increases, not in accordance with Hooke's law, but in such a way that equal increments of length cause progressively greater increments of tension. Over a considerable range of lengths, this resting tension is an exponential function of the length.[113] This suggests that, as the stretch increases, new structures within the muscle are called upon to offer resistance. This resting tension is probably due entirely to the connective tissue of the muscle, the endomysium and perimysium and the sarcolemma surrounding each individual muscle fiber.

Banus and Zetlin[114] have succeeded in removing the sheath from the frog gastrocnemius muscle and have found that the sheath alone gives the same tension on stretch as the whole muscle. It seems probable, however, that the muscle fibers themselves must contribute something to this tension, for, after dissection free from the connective tissue of the muscle, they show the same exponential type of stretch curve as the whole muscle (Asmussen[115]). Ramsey and Street[116] obtained a similar result with single muscle fibers, but they showed that this tension is almost entirely due to the sarcolemma, because the continuity of the muscle substance itself could be broken by pinching the fiber without altering the resting tension, and the sarcolemma alone gave the same percentage stretch for a given tension as the intact fiber. From histological study, the sarcolemma has been likened to a knitted stocking composed of a network of fibers, and as such it seems well suited to its task of resisting injurious stretches[117] (see p. 455).

This idea that the contractile mechanism itself, the myosin chains, exerts no resting tension on stretching is somewhat at variance with the finding of Weber,[118] who made artificial myosin threads containing, like muscle, 20%

[112] A. Szent-Györgyi, Enzymologia, **9**: 98, 1942.

[113] W. F. Brisbin and F. Allen, Can. J. Research A, **17**: 33, 1939.

[114] M. G. Banus and A. M. Zetlin, J. Cell. & Comp. Physiol., **12**: 403, 1938.

[115] E. Asmussen, Skandinav. Arch. f. physiol., **74**: 129, 1936.

[116] R. W. Ramsey and Sibyl Street, J. Cell. & Comp. Physiol., **15**: 11, 1940.

[117] A. Nagel, Ztschr. f. Zellforsch. u. mikroskop;. Anat., **22**: 694, 1935.

[118] H. H. Weber, Pflüger's Arch. f. d. ges. Physiol., **235**: 205, 1934.

protein, and found that they resisted stretch with the same tension as would be expected from whole muscle of the same cross-section. Presumably the molecular configuration of the myosin chains differed in these two cases, those in the muscle fibers being perhaps more folded. Below a certain critical length, the artificial myosin threads showed plastic, rather than elastic, stretch. It may be concluded tentatively that in the resting muscle the myosin chains offer little resistance to stretch. If these myosin chains are really capable of shortening spontaneously (with release of considerable previously stored energy) as soon as some stabilizing influence is removed by the stimulus, this easy extensibility seems remarkable. If the protein grid is too stable to shorten, it might well be too stable to stretch without the application of an appreciable force. If, however, contraction involves the development of new "shortening linkages," the high extensibility of the resting myosin is readily understood.

When a resting muscle is stretched, other changes occur in addition to the mechanical increase in tension. Most important of these are the increase in threshold of stimulation,[119] a slight (10%) increase in the velocity of conduction of the excitation wave (Wilska[120]), an increased alkalinity (Margaria[121]), and an increase in the rate of resting metabolism (Feng[122]). Apparently the stretch of the muscle has some sort of stimulating effect, and this stimulation is diminished in amount by previous electrical stimulation of the muscle, even if the muscle twitch is not diminished in magnitude.

b). *Stimulated muscles.*—A plot of muscle tension against muscle length constitutes a length-tension diagram upon which can be represented all the various types of mechanical behavior of the muscle.[123] The nature of the length-tension diagram for resting muscle has already been described. The type of curve obtained for stimulated muscle varies with the nature of the stimulation.

Many authors have confined their attention to the tension developed with single twitches. Thus Banus and Zetlin[114] reach the conclusion that the tension of the contractile mechanism increases linearly with the length of the muscle until a maximum is reached, after which the tension increment caused by stimulation *remains constant* in spite of further stretch. These results were obtained with single twitches of mammalian muscles stimulated *in situ*. This, however, does not measure the maximum tension which the contractile mechanism is really capable of developing at each length. It measures rather the *rate* of development of tension at each length, or the amount developed in the brief time of a single shock.

To obtain information concerning the nature of the contractile mechanism, it is necessary to use the maximum tension developed in a tetanus of

[119] F. Buchthal and J. Lindhard, Det. Kgl. Danske Videnskabernes Selskab. Biol. Meddelelser, XIV 6 1–77 1939. See p. 71.

[120] A Wilska, Skandinav. Arch. f. Physiol., **82**: 258, 265, 1939.

[121] R. Margaria, J. Physiol., **82**: 594, 1934.

[122] T. P. Feng, J. Physiol., **74**: 441, 1932.

[123] O. Beck, Pflüger's Arch. f. d. ges. Physiol., **193**: 495, 1921–22.

indefinite duration and optimal frequency. Only in this way can the muscle be kept more or less continuously in a state of contraction. The tension will increase to a maximum with increase of both duration and frequency, and it is this maximum which may best be regarded as an index of the fundamental tensile capacity of the contractile mechanism. After deducting the resting tension due to the connective tissue, it is found that the increment of tension due to the tetanus increases linearly with length until the unloaded resting length is reached, after which it decreases more or less linearly until the length is about twice the resting length.[115] (See Fig. 46.)

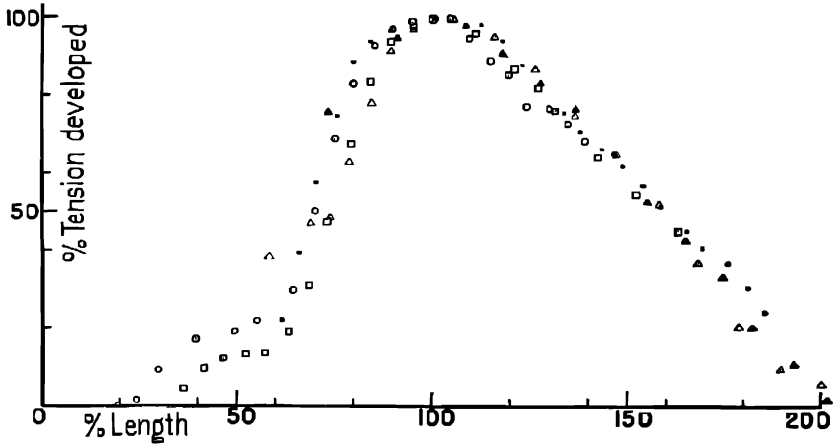

FIG. 46.—Single isolated muscles fibers. Ordinates: tension developed (total minus resting) in percentage of the maximum developed. Abscissa: length in percentage of the resting length. Each symbol represents a separate fiber. Measurements of R. W. Ramsey and Sibyl F. Street.[116] At lengths less than 60% the shortening is irreversible and the fiber is in the "delta state."

This property of the contractile mechanism is one of great importance as a clue to the nature of contractility. It is easily confirmed in human muscle so far as the lengths below the resting length are concerned.[124] Over this range, the longer the muscle the greater the tension. Greater lengths cannot be investigated in man, since the resting length and the maximum extended lengths in the body are approximately coincident.

The length-tension diagram of the stimulated muscle indicates an increase of tension with increase of length, at least over the range of lengths which are physiologically useful. This is by no means an obvious relationship, and, when pushed to its logical conclusion, becomes a point of some importance in any attempt to formulate a theory of muscular contraction. If, for example, one imagines that a muscle consists of n cylinders of radius r arranged in parallel, which are caused to shorten as a result of a sudden increase in surface tension $\Delta\sigma$, then it is evident that the pull will be equal to $2\pi nr\Delta\sigma$. The radius will have to be nearly molecular in dimensions, and n correspondingly large in

[124] W. O. Fenn, J. Applied Physics, 9: 165, 1938.

order to explain the force. Now, if the volume of the fibers remains constant as the length decreases, the value of r will increase, and, therefore, the total pull will increase so long as the change in surface tension remains the same. Hence, a length-tension diagram would be obtained in which greater forces were exerted at shorter lengths, or, conversely, if a muscle could lift a load at all it would lift it as much as if the load weighed little or nothing. This theory, therefore, does not work very well. According to another theory, the force exerted by a muscle is due to an attraction between opposite electric charges. Oppositely charged metal plates attract each other with a force which varies inversely as the distance between the plates. According to this theory, also, the force should increase as the muscle grows shorter, and we are confronted by the same type of difficulty as before.

However, both of these theories (so far as this objection is concerned) can be salvaged by a supplementary hypothesis. As the muscle shortens, the connective tissue is pulled out of shape. Just as it resists an undue amount of displacement by stretching, so also it offers elastic resistance to increasing shortening, which stretches progressively the transverse connective tissue elements. In the absence of a load, the tension of the contractile mechanism comes into equilibrium with the transverse elasticity of the connective tissue. In this condition, a small load will stretch the muscle a little, while a large load will stretch it more in accordance with the length-tension diagram. The muscle will, therefore, behave as if it were an ordinary elastic body manifesting a stress more or less in proportion to the strain.

Whole unloaded isolated muscles, when stimulated, do not shorten to more than 50 or 60% of their resting length. In the body, the antagonistic muscles, as well as the anatomical limitations of the joints, offer still other barriers to further shortening. The individual muscle fibers may shorten more in percentage than the muscle as a whole, but even so they do not shorten in the body to more than 50% of their length. When individual fibers are isolated from frog muscles, they do not readily shorten to less than 66% of their resting length, but with continued stimulation they do proceed to shorten more slowly down as far as 20% (Ramsey and Street[118]). After shortening over this unaccustomed range, the fiber is no longer capable of relaxation, and passes into what the authors call a "delta state." (See also Nageotte.[125])

In this new state, the fiber can be re-extended as before, and it will give the same resting tension as the normal muscle. This means that the longitudinal fibers in the sarcolemma have not been changed in their elastic properties. When stimulated, however, the delta state fiber develops less tension than before, and, at all lengths below the resting length, it fails to relax. The diminution in the contractile force may indicate that some of the myosin chains folded up so much that they remained folded when the rest of the myosin chains were re-extended. There is, however, no evidence of this in the microscopic appearance of the fiber, which shows all the usual cross striations after it has been stretched out again to the resting length. Before such stretching,

[125] J. Nageotte, Compt. rend. Acad. d. sc., **180**: 761, 1925.

it shows typical contracture bands. The failure to relax might be due to excessive bulging of the sarcolemma in the highly shortened muscle, so that the circular fibers were stretched beyond the elastic limit and broke. The elastic return would then fail to occur. If the shape of the length-tension diagram is to be attributed to the elasticity of the sarcolemma, then this same elasticity may be responsible for the active relaxation of the muscle. It is evident, however, that the sarcolemma alone is not strong enough to resist undue shortening when it is deprived of the added support offered by the connective tissue of the whole muscle. When stimulation is continued, the sarcolemma gradually bulges. Its resistance, however, makes it difficult for the myosin chains to find room enough in which to fold up, and the shortening process is, therefore, much retarded. In the process of folding, some of the myosin chains or the micelles may break loose from their terminal attachments. This may contribute to the delay of relaxation, and it may also eliminate a certain number of contractile units from future participation in the development of external tension.

The phenomenon of the delta state offers some support to the theory that shortening of the stimulated fiber, and, therefore, the shape of the length-tension diagram, is limited by the elastic resistance of the sarcolemma and the connective tissue to bulging of the sarcolemma. Over the range of lengths between 65% and 160% of resting length, the fiber acts in a reversible way. Beyond these limits, stretching or shortening becomes an irreversible process. It is reasonable to suppose that this irreversibility is due in part at least to rupture or tearing of the longitudinal or the transverse components of the sarcolemma. Some submicroscopic damage may also be involved.

In spite of these arguments, it is quite possible that the role of the sarcolemma in the length-tension diagram has been exaggerated. It is necessary, therefore, to consider another explanation for the decrease in shortening with increasing loads. This explanation, which is perhaps more in accord with recent thinking on this subject, attributes contraction to the folding of myosin chains or to the closing of successive links in these chains. If, when the muscle shortens, all links do not close with the same tension, then, when the muscle subsequently lengthens again, some links will pull out with a small load, and others will resist extension until a large load is applied. Thus, a statistical distribution of various elements in the shortening mechanism will explain the type of curve obtained.

Both of these explanations of the length-tension diagram appear to be reasonable, and both may contribute to the actual behavior. Consideration of the second explanation would be much facilitated if an experimental length-tension diagram of pure myosin threads in the supercontracted state were available anywhere in the literature, but nothing of the sort appears to have been published. How uniform the various links of these chains may be in their mechanical properties, remains a matter of conjecture.

From the work of Astbury, it is well established that the contractile mechanism consists of myosin polypeptide chains, which in the resting

muscle resemble the partially folded α-keratin. The myosin differs from the α-keratin in its side-chains, which are less firmly united, so that it is capable of a wider range of length changes. By heating in steam or immersion in caustic soda, the side linkages (*S-S* groups, perhaps) are broken down so that the keratin can be readily extended or unfolded into the β-keratin form, which has twice the natural length or can be made to shorten into the supercontracted form which has about 70% natural length. Thus, when the keratin is "devulcanized" it comes to resemble myosin. Astbury was properly impressed with this similarity between muscle and myosin, which was brought out most strikingly by the similarity between the x-ray diffraction patterns given by the two structures. It leads to the idea that the resting muscle, which has the same configuration as myosin, is held in that position and prevented from shortening by side-chain linkages similar to those in α-keratin. On stimulation, side-chain linkages may be loosened, thus permitting shortening to occur. On the assumption that the tension of the resting muscle is due to connective tissue the increment of tension due to stimulation at different lengths (see Fig. 46) must be due to "stimulated" myosin, and, in accordance with Astbury's picture, must be similar to the length-tension diagram or force-elongation curve of supercontracted or devulcanized alpha keratin. It is hardly likely, however, that the force of an elastic myosin thread would decrease when stretched beyond a certain point, as seems to be the case in muscle. The interpretation of the muscle curve at extensions greater than the resting length is, therefore, difficult. Possibly it means that at a certain degree of extension the points of mutual attraction on the polypeptide chains become too far apart for further interaction, or the force exerted might represent merely the number of links still existing in the alpha form.

c). *Time relation and latent period.*—It is not proposed to discuss the time relation of muscle contractions in any detail. Both the development of tension and the rate of relaxation are diminished by lowering the temperature and by fatigue. In the twitch, the isometric tension reaches its maximum before the isotonic shortening. This is natural, since shortening can continue so long as the muscle is capable of developing a tension in excess of the load. The duration of the latent period between the moment of stimulation and the beginning of the mechanogram is also diminished by an increase in temperature. It is suggested that this represents some chemical reaction which supplies energy to the myosin. There are those who believe that the true latency is immeasurably short, or less than 0.4 milliseconds (Roos[126]), while others find an interval of about 3.3 milliseconds in the frog sartorius muscle at 20°C. (Snyder[127]), (Sandow[128]). It is still reasonable, however, to doubt whether any amount of increase in mechanical sensitivity

[126] J. Roos, J. Physiol., 74: 17, 1932.
[127] C. D. Snyder, Am. J. Physiol., 115: 441, 1936.
[128] A. Sandow, Federation Proc., 1: 77, 1942.

can detect the first curling of the myosin chains. The mechanogram begins asymptotically, so that the true point of onset appears to depend upon the sensitivity of the apparatus. Recently, however, Sandow,[128] using a piezoelectric crystal and a cathode ray oscillograph for recording, has reported an initial relaxation of the muscle before the first detectable increase in tension. The same observation has been reported previously by Rauh[129] and Fischer,[130] and appears to indicate an increase in the extensibility of the myosin chain just before actual shortening begins, as if certain stabilizing side-chain linkages were broken before new "shortening" linkages could be established. During this brief interval, the weight is able to stretch the muscle slightly before the contraction begins. In the light of these results, the question of the reality of the latent period loses some of its interest, and it becomes of more importance to learn what evidence of stoichiometric changes in the myosin may be detected during the apparent latent period.

d). *Types of contraction.*— Contractions are conveniently classified into two groups: (1) those accompanied by shortening, and (2) those accompanied by lengthening.

An alternative classification is that proposed by the distinguished pioneer in this field, Adolph Fick,[131] to whom we owe so many important contributions to the mechanics and energetics of muscle contraction; he it was who coined the familiar terms, isotonic (constant load) and isometric (constant length). Each of these, however, is only an abstraction and seldom occurs in practice without very special precautions. On the other hand, all contractions observed in practice are accompanied by more or less shortening or lengthening. Moreover, the differences between these two types of contraction, particularly the differences in rates of energy supply, are of fundamental importance for an understanding of the nature of the contractile process; and the progressive change in the contraction, which occurs as the rate of shortening diminishes, and the rate of lengthening increases, provides a quantitative relationship of great importance as a clue to the nature of contractility.

Theoretically, between these two types of contraction, there stands a third type, the isometric.[132] With this addition, the following scheme will be helpful.

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[129] F. Rauh, Zeitschr. f. Biol., **76**: 25, 1922.

[130] E. Fischer, Pflüger's Arch. f. d. ges. Physiol., **213**: 352, 1926.

[131] A. Fick, Mech. Arbeit und Wärmeentwick l. bei der Muskeltätigkeit; Leipzig, 1882.

[132] Wherever tension is steadily maintained without movement, we may conveniently speak of an isometric contraction, although the length certainly changes during the onset of tension; and even while external tension is apparently constant, the internal tension within the individual sarcomeres or inside the muscle fibers themselves is undoubtedly fluctuating with the arrival of each successive nerve impulse (Nicolai[133]). Some internal shortening, therefore, is undoubtedly involved.

[133] See ref. (72).

Type of contraction	Work	Application	Effect on tension of increasing velocity	Rate of energy supply
Shortening	Positive	Acceleration	Decreases	Increases
Isometric	Nil	Maintenance		
Lengthening	Negative	Deceleration	Increases	Decreases

Some points illustrated in this scheme will require amplification later. It is only necessary to add here that a contraction accompanied by lengthening is in no sense an artefact of the laboratory, but a phenomenon which occurs wherever muscles operate in antagonistic pairs. In such a case, the agonist accelerates a limb at the beginning of its stroke and the antagonist decelerates it at the end of the stroke. During the latter process the muscle lengthens during contraction.

e). *Equation of movement.*—A part of the schema given above may be expressed more generally and more completely in the form of an equation

$$T = w + ax + b\frac{dx}{dt} + c\frac{d^2x}{dt^2}$$

Tension = (load or elasticity) + viscosity + acceleration
(Energy) = (potential) + (heat) + (kinetic)

$x = l - l_o$ and l_o is the length at which $T = 0$. a is a coefficient of elasticity and is constant only over a range of lengths where Hooke's law applies. This states that the total tension T of which the muscle is capable (at the observed length and under continuous optimal stimulation) will be utilized either to balance a load (w) or an elastic force (ax) or to overcome viscous resistance $\left(b\frac{dx}{dt}\right)$ or to accelerate a mass $\left(\frac{cd^2x}{dt^2}\right)$. The energy will be in the process of conversion into potential energy (elastic or gravitational) or heat (viscosity) or kinetic energy (acceleration).

Such an equation applies of course to all moving objects. Since wave motion can be analyzed in terms of moving objects, it applies also in its essential to the transmission of sound waves and mechanical waves, and to electric circuits. (In electric circuits, of course, T is potential, a is capacitance, b is resistance, and c is impedance.)

A great variety of experiments on muscle have been analyzed with the help of this equation and with the assumption that T is constant for a given muscle at a given length. The object in all cases was the accurate description of the mechanical properties of the muscle in both the resting and the stimulated condition. Thus, a small hammer is allowed to strike against a muscle and the extent and rate of the rebound are measured (Steinhausen,[134]

[134] W. Steinhausen, Pflüger's Arch. f. d. ges. Physiol., **212**: 31, 1926.

Richter[135]) or a suspended muscle is set into a to-and-fro rotation around its longitudinal axis (Weber,[136] Lindhard and Möller[137]) or alternately stretched and released by attachment to a vibrating spring, and the period and damping of this oscillating movement were recorded (Gasser and Hill,[138] Hogben and Pinhey[139]).

The results of these and similar investigations are not in good agreement. So far as the elasticity is concerned, some have reported a decrease in elasticity due to contraction, [136,137] some an increase,[138,140] and some no change.[139,134]

This confusion is due chiefly to difficulties in interpretation which may be illustrated by one of the experiments of Gasser and Hill.[138] They found that, for a given sudden stretch of the muscle, the rise of tension was much greater if the stretch was applied immediately after stimulation than it was if applied at later or earlier periods. The experimental result is very clear and is confirmed by the work of Marceau and Limon.[140] Since elasticity is proportional to ΔT (tension)/ΔL (length) this result may be said to indicate an increase in elasticity. This maximum elasticity was obtained, however, at a time when the tension (at least, the internal tension, if not that manifested externally) was increasing anyway, just because the muscle had been stimulated, and not because of the stretch. Two factors contribute therefore separately to increase the T at this moment. The interpretation is accordingly complicated and it might well be argued that no change in the fundamental mechanical properties was indicated.

Similar difficulties exist in the attempts to measure changes in viscosity of muscles during contraction. Gasser and Hill[138] have reported an increase, but Hogben and Pinhey,[139] with essentially the same method, found no certain change. Petit[141] found also an increase, but Winton,[142] in smooth muscles, found an increase with direct current stimulation, and a decrease during actual contraction with alternating current stimulation (followed by an increase just after contraction). The writer has long maintained that what has been called viscosity in muscles is largely a delayed shortening, due to limitations in the rate with which energy for shortening can be mobilized, and Hill[143] has more recently accepted essentially this view. The story about muscle viscosity, therefore, has become outmoded at just about the time when it succeeded in making its first appearance in the textbooks of physiology.

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[135] F. Richter, Pflüger's Arch. f. d. ges. Physiol., **218**: 1, 1927.

[136] E. Weber in Wagner's Handwörterbuch. d. Physiol., III, part 2, 110: Braunschweig, 1846.

[137] J. Lindhard and J. P. Möller, Skandinav. Arch. f. Physiol., **54**: 41, 1928.

[138] H. S. Gasser and A. V. Hill, Proc. Roy. Soc., **B 96**: 398, 1924.

[139] L. Hogben and Pinhey, Brit. J. Exper. Biol., **4**: 196, 1926.

[140] F. Marceau and M. Limon, J. de physiol. et de path. gen., **22**: 793, 1924.

[141] Jean-Louis Petit, Arch. internat. de physiol., **34**: 113, 1931.

[142] F. R. Winton, J. Physiol., **84**: 47P, 1935; **88**: 492, 1937.

[143] A. V. Hill, Proc. Roy. Soc., B. **126**: 136, 1938.

7. Chemical Changes Associated with Contraction.—*a*). *Aerobic.* The rate of oxygen consumption is enormously increased as a result of contraction. Using a thin frog sartorius muscle in a respirometer, this increased oxidation persists for some thirty minutes.[111] The increased oxygen intake apparently begins before the increased excretion of carbon dioxide. This could be due, in small part, to the smaller diffusion constant of the carbon dioxide, or possibly to deficiency in carbonic anhydrase, but it is chiefly due to the increased alkalinity or CO_2 binding capacity of the muscle, resulting from phosphocreatine breakdown.

Millikan[145] has shown by spectroscopic observations of the muscle hemoglobin bands during stimulation, that oxygen is used almost simultaneously with the contraction, and it has been argued (Sacks[116]) that oxidative energy is primarily available for direct use during contraction, and that it is not purely a phenomenon of recovery. According to this theory, it is only in the absence of free oxygen that lactic acid is formed which must be removed in recovery. It has been shown, however, by D. K. Hill,[147] using a thin muscle in a respirometer at low temperatures, that even when free oxygen is continuously available, it is not used until the contraction is over. At low temperatures, the rates of reaction are so much slowed down that the rates of diffusion have very little effect upon the rate of oxygen consumption, and correction can be made for this delay. The analysis shows that the usage of oxygen coincides in time with the appearance of the recovery heat, even under these special conditions. Since recovery heat always follows the contraction, it can be concluded that the use of oxygen also is a recovery process, even at higher temperatures. The studies of oxygen consumption show, therefore, that the contractile machinery is not coupled directly, but only indirectly, with an oxidative reaction.

Measurements of the respiratory quotient indicate that it is carbohydrate which is used directly for the ultimate supply of energy for recovery. When the R.Q. indicates a burning of fat or protein, these substances are used only indirectly after conversion to carbohydrate (Gemmill[148]). Even in isolated frog muscles, the excess respiration resulting from activity has an R.Q. somewhat less than 1.0.[148]

There is evidence that the resting respiration of muscle differs in nature from that initiated by contraction. The latter can be inhibited by a concentration of sodium azide, which leaves the resting metabolism unaffected (Stannard[149]). It is proposed that the latter is not mediated by the cytochrome-oxidase enzyme system.

Energy measurements made simultaneously by direct and indirect

[144] W. O. Fenn, Am. J. Physiol., **83**: 309, 1927.

[145] G. A. Millikan, Proc. Roy. Soc., London, B. **123**: 218, 1937.

[146] J. Sacks, Physiol. Rev., **21**: 217, 1941.

[147] D. K. Hill, J. Physiol., **98**: 207, 1940.

[148] C. L. Gemmill, J. Cell. & Comp. Physiol., **5**: 277, 1934; see also W. O. Fenn, *ibid*., **2**: 233, 1932.

[149] J. N. Stannard, Am. J. Physiol., **126**: 196, 1939.

calorimetry have shown that (within the limits of error of the method used) all the heat developed in an isolated muscle during contraction can be accounted for in terms of the oxygen used (Fenn[150]). This is, of course, to be expected. The disposition of this total excess oxygen which is used in recovery from contraction is shown in Fig. 47. In the diagram, it is assumed that, after contraction, the metabolic products (chiefly lactic acid) requiring oxidative removal are equivalent to 1 gram of glycogen. The complete combustion of this would require 3800 calories, but Meyerhof has shown that only $\frac{1}{5}$ of this need be burned for complete recovery. The total oxidative energy is therefore 760 calories. But if oxygen is admitted to the

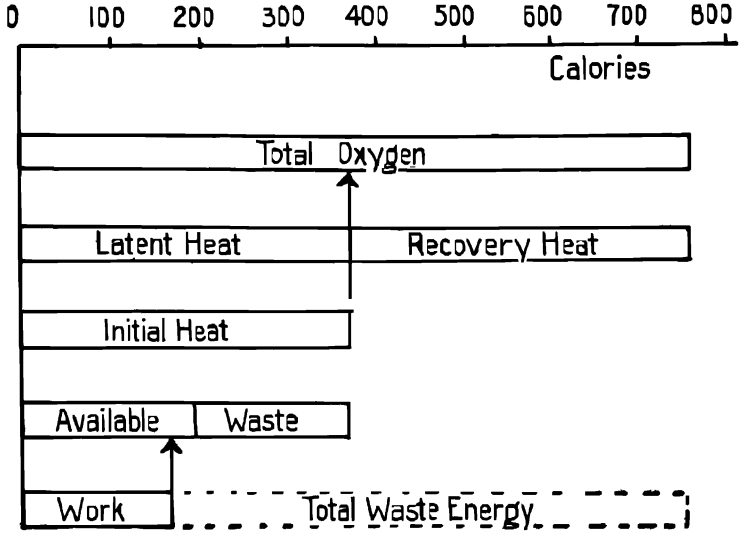

FIG. 47.—Energy diagram calculated for a muscle in which 1 gram of glycogen has been turned into lactic acid as a result of stimulation. All absolute values are approximate and vary with the conditions.

muscle after an anaerobic contraction, only about 380 calories will appear as heat. The remainder, described as latent heat, serves to reverse the initial heat or the anaerobic processes, which, all together, liberated during contraction about 380 calories per gram of lactic acid formed, the exact figure depending on the amount of phosphocreatine breakdown, the fatigue of the muscle, and other conditions of the experiments.[151] This 380 calories of initial heat is equal to the latent heat of recovery, and is the algebraic sum of the heats of all the anaerobic processes of contraction, including the formation and neutralization of the lactic acid, phosphocreatine breakdown, etc. Only about half of the energy which appears as initial heat can be used for work, the remainder being wasted as maintenance heat, or as unexpended mechanical potential energy (i.e., relaxation heat). The maximum amount of energy which can appear as work under optimum con-

[150] W. O. Fenn, The Harvey Lectures, **23**: 115, 1927–28; or Medicine, **7**: 433, 1928.

[151] O. Meyerhof, Naturwissenschaften, **19**: 923, 1931.

ditions will be equal to the free energy of the reaction which is indirectly coupled energetically with the shortening of the myosin chains. Since myosin has been identified as the enzyme essential for dephosphorylating adenylpyrophosphate, it seems most probable that it is the free energy of this reaction, which appears as work.[152] In general, the work amounts to only about half of the initial heat, or 25% of the total oxidative energy. The efficiency of isolated muscles is less than these figures from human experiments, but Hill[153] has recorded an initial efficiency of 40%; or 20% for the whole cycle.

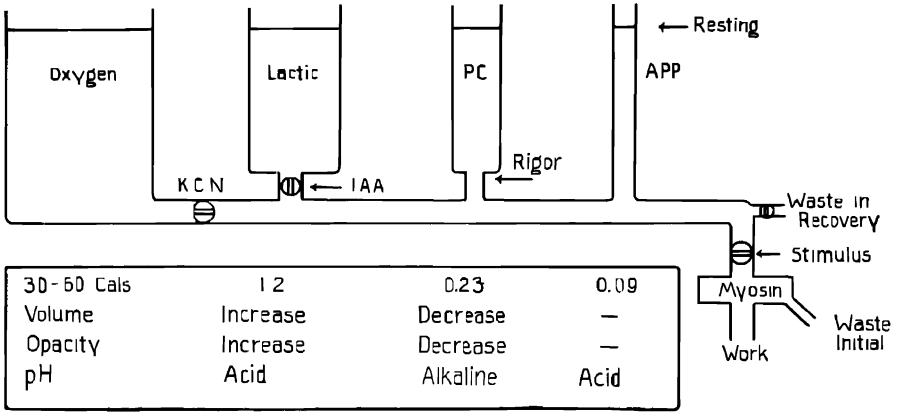

FIG. 48.—Diagram representing various energy reservoirs in muscle. Energy is derived from oxidation, glycolysis (lactic), phosphocreatine breakdown (PC), or adenylpyrophosphate breakdown (APP).

b). *Anaerobic changes.*—There is so much complexity to the anaerobic changes which occur in muscle contraction, that no attempt will be made to give a thorough review of the subject. It will be sufficient to present the theory of Lohmann[154] and Meyerhof,[155] which illustrates the probable relation between the three most important of the chemical reactions concerned. This can best be done, perhaps, by the use of a simple model illustrated in Fig. 48. The four chief energy reservoirs are represented, and their capacities in calories per gram of muscle are indicated. The stimulus releases a flow of energy, which is transformed by the myosin machinery into mechanical work, with some waste heat. In this process, the energy levels in the various reservoirs fall below their resting levels. In some way, any such displacement of level "calls" for increased oxidation for recovery. When energy is drawn from the first reservoir by the breakdown of adenylpyrophosphate (APP), this is replenished at the expense of phosphocreatine (PC). A breakdown of PC, in turn, is replaced from the reservoir which represents the energy to be derived from lactic acid formation. The coupling between

[152] D. M. Needham, Biochem. J., **35**: 113, 1942.

[153] A. V. Hill, Proc. Roy. Soc. London, B, **127**: 434, 1939.

[154] K. Lohmann, Naturwissenschaften, **22**: 409, 1934.

[155] O. Meyerhof, Ergebn. d. Physiol., **39**: 10, 1937.

glycolysis and PC resynthesis occurs again through a phosphate transfer from 1-3 diphosphoglyceric and phosphopyruvic acids.

The schema indicates that all these reservoirs could be returned to their resting levels at the expense of oxidative energy alone, and there is good evidence for this, at least in the case of lactic acid and phosphocreatine. The flow of oxidative energy can be blocked by KCN or sodium azide (NaN_3), and the energy from glycolysis can be stopped by iodoacetic acid (IAA), which inhibits the conversion of triosephosphate to phosphoglyceric acid, this being the first step of great energy yield in the process of lactic acid formation. When this block is applied, the phosphocreatine is readily lowered by stimulation to the level at which rigor mortis sets in. In the absence of lactic acid formation, this is an alkaline rather than an acid rigor.

The breakdown of 2 mols. of phosphocreatine yields just enough energy to cause the resynthesis of 1 mol. of adenylpyrophosphate from adenylic acid. The matter is still further complicated, however, by the fact that adenylpyrophosphate reacts also with glycogen to give hexosediphosphate, and, eventually, the adenylic acid formed can also be phosphorylated once more by reaction with subsequent intermediary products, such as triosephosphate and phosphopyruvic acid. The more recent evidence, moreover, indicates that myosin also combines with phosphate at some point in the cycle, probably receiving it from adenylic acid (see pp. 485 and 521). Thus it may be said that phosphoric acid is passed from adenylic acid through myosin to hexose, which forms lactic acid and gives it to creatine, which finally returns it to adenylic acid. The reactivity of all these compounds is, therefore, much facilitated by the phosphoric acid. Recent discussions of the subject are given by Needham[156] and Meyerhof.[157]

The evidence that adenylpyrophosphate is the first source of energy to be called upon has been incomplete, because of the difficulty in showing that the amount of this substance present in muscle is appreciably diminished after activity.[158] In the light of this scheme, it would have to be assumed that it is rebuilt about as rapidly as it is broken down. Studies with radioactive phosphate show evidence of the expected turnover in APP phosphate if due allowance is made for extracellular phosphate (Furchgott and Shorr[159]). Indirect evidence of the correctness of this view, as illustrated in Fig. 48, has been supplied by measurements of the changes in the volume, opacity, and *p*H of the muscle.

c). *Volume changes.*—A decrease in volume on contraction was first reported by Ernst,[160] and was extensively studied by Meyerhof and Möhle,[161]

[156] D. M. Needham, Chapter in Perspectives in Biochemistry, ed. by J. Needham and D. E. Green: University Press, Cambridge, 1938, p. 201.

[157] O. Meyerhof, Biol. Symp., **3**: 293, 1941.

[158] J. Sacks, Physiol. Rev., **21**: 217, 1941.

[159] R. F. Furchgott and E. Shorr, J. Biol. Chem., **151**: 63, 1943.

[160] E. Ernst, Pflüger's Arch. f. d. ges. physiol., **209**: 618, 1925.

[161] O. Meyerhof and W. Möhle, Biochem. Ztschr., **260**: 454, 469, 1933; **261**: 252, 1933; **284**: 1, 1936.

Meyerhof and Hartmann,[162] and Hartmann.[163] Meyerhof and Hartmann[162] have measured the volume change which occurs with each of the chemical reactions pictured in Fig. 48. Lactic acid formation causes an increase of 24 cc. per mol., while the dephosphorylation of phosphocreatine and adenylpyrophosphate causes decreases in volume of 14 and 21 cc. per mol., respectively (Hartmann[163]). Using these figures, it was possible to account quantitatively for the observed decrease in volume on the basis of chemical analyses for lactic acid, APP, and PC. This was true, however, only when muscle was prevented from engaging in spurious reaction with the surrounding Ringer's solution, by immersing it in paraffin oil. With this refinement,

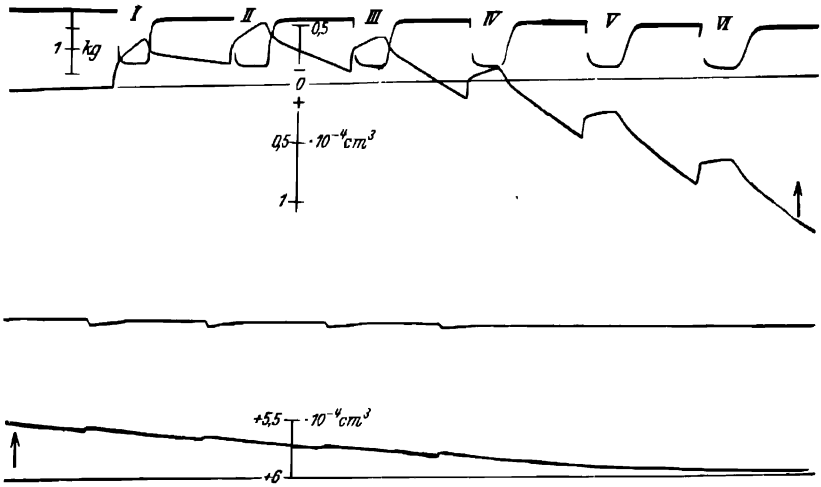

FIG. 49.—Upper curve represents tension developed in successive 2-second tetani of a frog gastrocnemius muscle under paraffin oil. Corresponding decreases in volume (up stroke) in the next lowest curve. Record taken later in the same series of tetani (with altered calibration) is shown in the two lower records. (After Hartmann.[163])

the method provides a valuable means of determining the time relations of the chemical reactions involved in the contraction of muscle. In later studies, however, by Meyerhof and Möhle, in which the muscle volumes were followed over longer periods of time, the agreement was less good and, in general, some further factor causing a decrease in volume had to be postulated. This discrepancy might possibly be due to increase in intracellular water, decreased solubility of the myosin, or liberation of the potassium.

An interesting experiment of Hartmann's, using this method, is illustrated in Fig. 49, which shows the tension (upper curve) and the volume changes (lower curve), in a series of 2-second tetani in a gastrocnemius muscle, immersed in the dilatometer under paraffin oil. In each tetanus, as the tension rises, the volume decreases rapidly, indicated by a rise in the curve, and more slowly as the tension is maintained at its plateau level. In

[162] O. Meyerhof and H. Hartmann, Naturwissenschaften, **21**: 661, 1933.

[163] H. Hartmann, Biochem. Ztschr., **270**: 164, 1934.

relaxation, the formation of lactic acid predominates, and the volume begins to increase. At first, the volume remains smaller, and the curve higher, after each successive relaxation, than it was originally, but, as lactic acid accumulates, the volume begins to increase more and more, and this continues until the point of complete fatigue. The last tetani of the series are illustrated (with altered calibration values) in the lower two curves. A slight decrease of volume with each tetanus still remains, suggesting that no tension can be developed unless some volume-decreasing reaction, like dephosphorylation of APP or PC, is available. Since the first change in volume which occurs on stimulation is a decrease, this supports the idea that PC or APP breakdown occurs nearly simultaneously with the actual contraction.

Thus, it appears that the volume changes of a muscle can be used to some extent as an instantaneous index of the chemical reactions occurring inside the muscle fibers. The method must be used, however, with caution, for Fischer[164] has observed an increase in volume of the sartorius muscle when stimulated at high initial tensions. This matter deserves further investigation, for it suggests that all the factors have not been successfully elucidated.

d). *Opacity.*– At the moment of stimulation, a muscle becomes more transparent, due to phosphocreatine breakdown, and this effect is more slowly reversed in relaxation (von Baeyer and von Muralt[165]). Lactic acid formation causes an increase in the opacity if the muscle is in Ringer's solution, where swelling and an increase in the volume of the colloidal particles can occur. Thus, in heat rigor, lactic acid forms, and the muscle becomes opaque, while in iodoacetate-poisoned muscle the lactic acid no longer forms, and rigor causes a decrease in opacity. This method confirms, therefore, the conclusions obtained by other means.

It should be pointed out, however, that the opacity of the muscle will vary greatly with the wave-length of the light that is used. The result described was obtained with white light, and may depend upon a change in the amount of light scattered by the muscle proteins. A more specific effect is obtained if a wave-length is used which corresponds to the absorption band of a special chemical constituent of muscle, such as those of myoglobin,[166] the yellow ferment, or cytochrome.[167] In this way, it can be shown that, within 50 milliseconds after stimulation, the yellow ferment and cytochrome are reduced as is indicated by increases in the opacity of the muscle to the particular wave-lengths used. The reduction of the myoglobin begins 0.22 seconds after contraction.

e). *pH.*—Many investigations indicate an initial increase of *p*H on stimulation, which is followed later by a decrease. In a respirometer, the alkalinity can be shown by the absorption of CO_2, and the acidity by an

[164] E. Fischer, Biol. Symp., **3**: 211, 1941.

[165] E. von Baeyer and A. L. von Muralt, Pflüger's Arch. f. d. ges. Physiol., **234**: 233, 1934.

[166] G. A. Millikan, Proc. Roy. Soc., **B. 123**: 218, 1937.

[167] F. Urban and H. B. Peugnet, Proc. Roy. Soc., **B. 125**: 93, 1938.

elimination of CO_2 (Lipmann and Meyerhof[168]). With a glass electrode, similar changes have been demonstrated in human arm muscles (Maison, Orth, and Lemmer[169]). With a somewhat modified glass electrode technique, Dubuisson[170] has obtained confirmatory results in isolated muscles. In general, a muscle shows an increased alkalinity early in contraction which gives way to an acidity from lactic acid formation in recovery. The time relations are more accurately analyzed in a slow, smooth muscle (frog stomach), from which the data for Fig. 50 were obtained. Similar results were obtained, however, in gastrocnemius muscles. The dotted line shows the tension as a function of time in seconds. The other curves show heat production in cal. $\times 10^{-5}$ per gram of muscle, as calculated from the observed

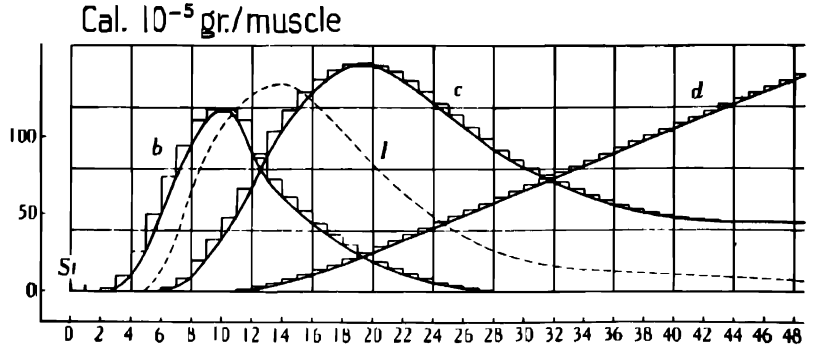

FIG. 50.—Ordinates: Energy made available from 3 different chemical reactions as a function of time. Abscissæ; time in seconds. The dotted line represents the tension developed as a result of a stimulus at time 0. Data estimated from the changes in *p*H recorded as a function of time in frog stomach muscle. Adenylpyrophosphate breakdown represented by *b*, phosphocreatine breakdown by *c*, and lactic acid formation by *d*. (After Dubuisson.[170])

*p*H changes. The earliest curve, *b*, represents the breakdown and resynthesis of adenylpyrophosphate. Curve *c*, representing the breakdown and resynthesis of phosphocreatine, begins to rise as *b* falls. Finally, curve *d* represents the heat produced by the formation of lactic acid, which is the only metabolic product remaining after 30 seconds. In a later publication, Dubuisson[171] discusses a still earlier phase of increased acidity, which is observed in short isometric or in isotonic contractions of the sartorius muscle. When a muscle is stretched, he finds, in agreement with Margaria,[172] that it becomes alkaline. Hence, when it shortens isotonically it should become acid, as Dubuisson found. This change is attributed to a change in the isoelectric point of the myosin as a result of the stretch. Myosin would, therefore, be expected to liberate base when it is elongated. This curious reaction may play some role in the electrolyte changes of muscle,

[168] F. Lipmann and O. Meyerhof, Biochem. Ztschr., **227**: 84, 1930.
[169] G. L. Maison, O. S. Orth, and K. E. Lemmer, Am. J. Physiol., **121**: 311, 1938.
[170] M. Dubuisson, Annales de Physiol., **15**: 443, 1939.
[171] M. Dubuisson, Arch. internat. de physiol., **50**: 203, 1940.
[172] R. Margaria, J. Physiol., **82**: 496, 1934.

and, because of it, the initial *p*H change varies in different muscles according to the load and length of the muscle.

It is not certain just where, in this phosphate cycle, the contractile machinery comes in, or in what particular form the energy has to be presented in order to cause contraction. An interesting suggestion made by Kalckar[173] is that myosin itself combines with phosphate, which might serve as one of the side-chain linkages in the myosin grid. If this phosphate stabilizer were removed on stimulation, it could then start the energy cycle going by reaction with one of the other components in the phosphate cycle, and the protein chain, freed of the stabilizing influence of the phosphate, would be able to release its previously stored potential energy. Finally, after the end of stimulation, the phosphate could recombine with myosin, thus pulling the protein chains back to their previous resting length and storing up potential energy for another contraction. This postulate has at least the advantage that it illustrates for the first time a possible method of energetic coupling between the anaerobic reactions and the contractile mechanism proper.

The most recent development in muscle chemistry is the discovery that myosin itself acts as a dephosphorylating enzyme, and therefore participates directly in the phosphate cycle. The importance of this for the theory of muscle contraction has already been mentioned under myosin, and will be discussed further at the end of this chapter.

f). *Impedance.*—For the measurement of the impedance during contraction, the muscle is placed between electrodes and balanced electrically in a Wheatstone bridge circuit, using a high-frequency alternating current. In strictly isometric contractions, such as can be obtained in a sartorius muscle, there is an increase of impedance (Bozler[174]). A shortening of the fibers, however, is accompanied by a decrease of impedance (Dubuisson[175]). The greater the shortening, the greater the change of impedance. In the isometric contraction, the increase in impedance is analyzed into a quick component, which reaches its maximum before the tension maximum, and a slower process, which is approximately coincident with relaxation.[174,175] The interpretation of these two processes is difficult, but they correspond, respectively, fairly well to the myosin-adenylpyrophosphate reaction and the phosphocreatine breakdown, and have been so interpreted by Dubuisson. In time relations, they also correspond to the spike and the after potential of the electrical change of muscle.[176,177] It is usual to think of action potentials as reflecting the potential difference across the surface membranes of the cells. A similar explanation could also apply to the high-frequency

[173] H. M. Kalckar, Chem. Rev., **28**: 71, 1941.

[174] E. Bozler, J. Cell. & Comp. Physiol., **6**: 217, 1935.

[175] M. Dubuisson, J. Physiol., **89**: 132, 1937.

[176] H. Schaefer and H. Göpfert, Pflüger's Arch. f. d. ges. Physiol., **238**: 684, 1936; **239**: 597, 1937.

[177] G. H. Bishop and A. S. Gilson, Am. J. Physiol., **82**: 478, 1927; **89**: 135, 1929.

impedance. It is not impossible, however, that the effect is an expression of a change in the state of aggregation of the myosin, which would not be an exclusively surface process. This idea is confirmed by the parallelism between the decrease in double refraction, which likewise occurs in two peaks (von Muralt[178]). Fischer[179] has indicated theoretical reasons why a change in double refraction of myosin should result in a change in impedance.

8. The Thermoelastic Effect.—The mechanical behavior of muscle, as well as its ultrastructure, has already been considered. We must now deal with another method of attack, which is concerned with the interpretation of the mechanical behavior of muscle in terms of its ultrastructure; it represents an approach to the problem through the principles of thermodynamics. Unfortunately the method is really applicable only to the resting muscle, although some attempts to extend the application to the stimulated muscle have also been made.

The general principle of the method may be illustrated by a concrete example. It is known that a muscle, over certain ranges of length, gives off heat when it is stretched, and cools when it is released. This was originally shown by Heidenhain[180] and was confirmed by Hartree and Hill[181] and by Feng.[182] Conversely, this same reaction should be reversible, so that the addition of heat to the muscle or an increase in its temperature should cause it to shorten or to increase its tension. This deduction from the principles of thermodynamics was worked out by Lord Kelvin, and the relationship, as expressed by Hill, is represented by the equation

$$Q = \alpha T \frac{(\text{increase of tension})(\text{mean length})}{4.26 \times 10^4},$$

where Q is the heat in calories, α the coefficient of thermal expansion T the absolute temperature, and where tension is expressed in gms. weight and the length in cm. In this respect, muscle behaves like rubber and unlike most other elastic bodies. If now we can tell, from thermodynamic principles, what kinds of ultramicroscopic structures behave like muscle, and what kinds become cooler when they are stretched, then we shall learn something about the ultrastructures in muscle, which are responsible for the manifestation of tension.

The theoretical treatment of this subject has been presented by Meyer and Ferri[183] and Meyer and Picken.[184] Only an abbreviated presentation will be attempted here. Two fundamentally different mechanisms for the development of tension on stretching may be distinguished. These may

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[178] A. von Muralt, Pflüger's Arch. f. d. ges. Physiol., **230**: 299, 1932.

[179] E. Fischer, Biol. Symp., **3**: 211, 1941.

[180] R. Heidenhain, Zentralbl. f. med. Wiss., **1**: 545, 1863.

[181] A. V. Hill and W. Hartree, Phil. Tr., **210**: 153, 1921.

[182] T. P. Feng, J. Physiol., **74**: 455, 1932.

[183] K. H. Meyer and C. Ferri, Pflüger's Arch. f. d. ges. Physiol., **238**: 78, 1936.

[184] K. H. Meyer and L. E. R. Picken, Proc. Roy. Soc., London, B **124**: 29, 1937–38.

be illustrated by (*A*) a steel spring, and (*B*) a gas put under pressure by the stretch (Fig. 51). In the first case, the molecules of the steel are pulled apart and displaced from their positions of minimum energy. As work is done on the spring, an equal amount of potential energy is stored in it, and there is no exchange of heat. In the case of a stretch which is resisted by the compression of a gas, as in *B*, the work which is done against the gas in compressing it appears as heat in the gas, and is not stored as potential energy. Conversely, when pressure is released, the work the gas does in expanding back to its original position is derived from heat energy, and appears as a cooling of the gas. The resisting force is due to the bombardment of the molecules against the piston. The movement is merely the statistical result of the larger concentrations of molecules which are present below the piston.

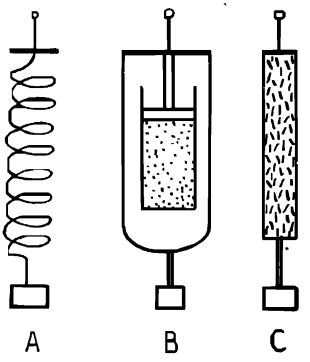

FIG. 51.—Three different types of elastic structure. A, a steel spring. B, compression of a gas. C, muscle or rubber.

A third type of structure, *C*, is really a modification of type *B*; it consists of long molecules fastened together in chains. The movements of these molecules are not quite random, because they are somewhat restrained by the chain structure. When the structure is stretched, the restraint is increased, just as the molecules of a gas are more restrained when the piston in *B* is pulled down and the gas is compressed. It is the tendency of the molecules to revert to a more random position, or to increase the entropy of the system, which is responsible for the restoring force in both cases.

The classical example of type *C* is rubber. A useful lecture table demonstration can be set up with a steel spring and a rubber band, each supporting a weight, and so attached to a light lever that changes in length are greatly magnified. If each of these structures is warmed slightly with a match, the steel spring lengthens while the rubber band shortens (unless the load is too small). Likewise, if the rubber is suddenly stretched it will feel perceptibly warm if placed against the upper lip. Similarly the gas in type *B* warms up when it is compressed. Rubber is composed of long primary-valence chains which can fold and unfold to give long-range elasticity, and which, by their regular orientation when stretched, cause birefringence. It is possible to account quantitatively for the elasticity of rubber by the entropy changes.[185] Since muscle shows the same type of thermoelastic behavior as rubber, it is natural to transfer to muscle the theoretical concepts which have been developed so thoroughly for rubber, and to explain the elasticity of muscle by the thermal agitation of the myosin molecules which tend to resist the forced longitudinal orientation.

Some samples of rubber show a short Hooke's law region in the begin-

[185] E. Wöhlisch, Kolloid-Ztschr., **89**: 239, 1939; Naturwissenschaften, **28**: 305, 1940.

ning of a stretch, and over this range they belong to the A system (Guth[186]). Meyer and Picken[187] found the same thing for resting muscle, but Wöhlisch and Renk[188] do not find this when most of the connective tissue is eliminated. All observers agree that both rubber and muscle behave like the *A* type at large extensions, where molecules are pulled apart out of their positions of minimum potential energy. In the muscle, this is attributed to the connective tissue elements which begin to resist further stretch at this point. Likewise, at such lengths, muscles begin to lengthen instead of to shorten with increase of temperature;[189] they become cooler instead of warmer on stretching.[190] This interpretation of the reversal of the thermoelastic behavior at large extensions is confirmed by the behavior of the connective tissue itself, which definitely belongs to the *A* type of elasticity, which always cools on stretching.

This analysis of the resting muscle according to the thermoelastic theory appears to show that the tension of the muscle during extension is at first due to the decrease of entropy of the myosin chains, and, at greater extensions, to the elastic potential energy of the connective tissue.

Some attempts have also been made to extend the theory of elasticity of rubber to cover the *active* contraction of muscle. This is the thermokinetic theory of Wöhlisch.[185] A test of this theory might be found if it could be shown that the force exerted by the contractile machinery increases in proportion to the absolute temperature. According to Bernstein,[191] the force in a muscle twitch decreases with rise of temperature, and has, therefore, a negative temperature coefficient, like surface tension. This, however, can easily be shown to be due to the fact that the heat production is also increased by a slowing of the reactions which accompanies the fall in temperature.[192] The amount of tension produced per unit of energy liberated is independent of the temperature. The constancy of the T/H (tension to heat) ratio in twitches at different temperatures would seem to indicate that temperature had little effect upon the contractile machinery itself. This is not in conflict with the thermokinetic theory, which demands only an increase of about 10% in tension for a rise of 30°C. in temperature. It would seem that more reliable evidence could be obtained from the effect of temperature on the isometric tetanus tension. It would be necessary to choose a maximal stimulus and a frequency which is sufficiently high to produce maximal economy (Bronk[193]), even at the highest temperatures. It might be argued, then, that the tension so produced at each temperature would represent the maximum of which the myosin machinery was capable. On this basis, it appears that, in striated muscle, temperature increases the tension produced

[185] E. Guth, J. of Applied Physics, **10**: 161, 1939.
[187] K. H. Meyer and L. E. R. Picken, Proc. Roy. Soc. London, **B, 124**: 29, 1937–38.
[188] E. Wöhlisch and F. Renk, Naturwissenschaften, **27**: 370, 1939.
[189] E. Wöhlisch and H. G. Clamann, Ztschr. f. Biol., **91**: 399, 1931.
[190] T. P. Feng, J. Physiol., **74**: 455, 1932.
[191] J. Bernstein, Pflüger's Arch. f. d. ges. Physiol., **122**: 129, 1908.
[192] W. Hartree and A. V. Hill, J. Physiol., **55**: 133, 1921.
[193] D. W. Bronk, J. Physiol., **59**: 306, 1930.

(Hartree and Hill;[192] Gad and Heymans[194]). In smooth muscle, however, the tension decreases under similar conditions (Winton[195]). It is doubtful whether this can be taken as a fundamental difference between these two types of muscle. It seems more likely that the interpretation is incorrect, and that there is no certainty that the supply of energy can be made optimal at all temperatures. In striated muscle, the energy liberated at different temperatures increases more rapidly with temperature than the tension developed, and the increased tension may reasonably be attributed to the increased amounts of energy available. The experiment of Winton with smooth muscle may then be attributed to the fact that the heat did not increase with temperature sufficiently to compensate for the more rapid dissipation of the tension. These difficulties of interpretation make it impossible to obtain a satisfactory test of the thermokinetic theory by temperature effects.

It should be emphasized at this point that it is the resting muscle which resembles rubber in its thermoelastic effect. In the stimulated muscle, exactly the opposite behavior is encountered—shortening liberates more heat, while stretching decreases the heat production.[196] Taken at its face value, this does not support the idea that the active contraction of a muscle is merely a matter of forcefully oriented molecules seeking a more probable position by random heat movements. To apply this theory, some excuse must be found for the heat. Nevertheless, Karrer[197] has supposed that the long chain molecules are held taut in the resting muscle by some stabilizing influence which is suddenly removed at the moment of stimulation by the excitation process. The resulting contraction should be accompanied by cooling, so the observed heat must be attributed to some other purpose.

The analogy between rubber and muscle is also illustrated by the fact that an increase of external pressure causes a shortening in both, as was first shown by Ebbecke and Hasenbring.[198] Both structures have a high compressibility and a low extensibility in the direction of the fibers, and vice versa in the direction across the fibers. In other words, they show elastic anisotropy. From this point of view, the shortening is to be regarded as a purely physical effect rather than a physiological response. It leads Wöhlisch[199] to suppose that, at the moment of contraction, some change occurs in the muscle, which is equivalent to an increase in the external pressure and causes a shortening. Ebbecke[200] has a somewhat similar idea in proposing that the contraction of muscle is a heat contraction, since heat also causes both rubber and muscle to shorten. In the muscle, the heat is supposed to act in some ill-defined way, not by doing the work itself as in a

[194] J. Gad and J. F. Heymans, Arch. f. Anat. u. Physiol., Suppl., p. 59, 1890.

[195] F. Winton, J. Physiol., **63**: 28, 1927.

[196] W. O. Fenn, J. Physiol., **58**: 175, 373, 1923; R. Azuma, Proc. Roy Soc. London, B. **96**: 338, 1924.

[197] E. Karrer, Protoplasma, **18**: 475, 1933.

[198] U. Ebbecke and O. Hasenbring, Pflüger's Arch. f. d. ges. Physiol., **236**: 405, 1935.

[199] E. Wöhlisch, Naturwissenschaften, **27**: 678, 1939.

[200] U. Ebbecke, Pflüger's Arch. f. d. ges. Physiol., **240**: 458, 477, 1938.

heat engine, but by liberating previously stored energy. Again, the theory seems to work well enough for a resting muscle, but the stimulated muscle does not lend itself readily to this simple interpretation.

The contraction of a muscle produced by high pressure has been thought to be related to the Ernst effect,[201] according to which the muscle decreases in volume when it contracts. Thus a high external pressure which would cause it to decrease in volume should also cause shortening. According to Hartmann,[202] the decrease in volume is due merely to the breakdown of phosphocreatine, since that reaction *in vitro* gives a similar decrease in volume of the right order of magnitude. In that case, the compression shortening of Ebbecke could be due to a breakdown of phosphocreatine caused by the pressure, rather than to an effect of pressure directly upon the myosin fibers. This interpretation seems to agree better with the view of Cattell,[203] who regards it as similar in every way to any chemical contracture (see p. 511), there being the usual energy change, etc., associated with it. Moreover, if the pressure is maintained, the contracture undergoes relaxation after a short time, and the amount of tension developed is not directly proportional to the pressure, but is instead an S-shaped function of pressure. Finally, it has been stated by Hasenbring[204] that there is a latent period of 0.03 seconds between the onset of pressure and the beginning of the shortening. This is taken to indicate that the compression shortening is a physiological response, rather than a true physical effect.

Taking all these matters into consideration, it is difficult to believe that this thermokinetic theory of Wöhlisch[199] offers much help in explaining the mechanism of active contraction, although it appears to explain certain phases of the behavior of resting muscle fairly well.

a). *The effects of pressure.*—In this connection it is appropriate to mention other effects of increased pressure upon muscle. A thorough discussion of the general problem has been given by Cattell.[203] The essential facts which seem to bear upon the problem of contractility are as follows: 1. ameboid movement,[205] protoplasmic streaming,[206] and ciliary activity (after initial acceleration) are inhibited or stopped by pressures of the order of magnitude of 300 atmospheres. This is regarded as due to a solation of the protoplasmic gel the contraction of which is believed to be responsible for the movement.[207]

2. When a pressure of 100–200 atmospheres is applied during the early part of a contraction, at which time the muscle is still in the process of being activated, it causes an increase in the tension developed as well as an increase

[201] E. Ernst, Pflüger's Arch. f. d. ges. Physiol., **209**: 613, 1925.

[202] H. Hartmann, Biochem. Ztschr., **270**: 164, 1934.

[203] McK. Cattell, Biol. Rev., **11**: 441, 1936.

[204] O. Hasenbring, Pflüger's Arch. f. d. ges. Physiol., **243**: 96, 1939.

[205] D. E. S. Brown and D. A. Marsland, J. Cell. & Comp. Physiol., **8**: 159, 1936.

[206] D. A. Marsland, J. Cell. & Comp. Physiol., **13**: 23, 1939.

[207] D. A. Marsland, p. 127, in The Structure of Protoplasms, ed. by W. A. Seifriz: Ames, Iowa, 1942.

in the amount of activation, as judged by the measurements of the heat production. In other words, the tension is increased because more energy is liberated by the same stimulus when the pressure is high.[208,203]

3. When still higher pressures of 300 to 400 atmospheres are used, the muscle may be activated without the application of any other (electrical) stimulus, thus causing the "compression shortening" of Ebbecke[198] which has already been discussed.

4. The direct effect of pressure upon the contractile machinery is to cause a diminution of the tension developed, just as it causes a diminution in the contraction of ameba, etc.[209] Thus, pressures applied after the initial stages of the contraction cause a decrease in the tension developed, and a sudden release of pressure causes a sudden increase in the tension as this inhibiting influence is removed. In fatigued muscles and muscles at very low temperatures, the increased activation due to the high pressures appears to be absent, and the inhibiting effect only remains.[210] The latter effect may be purely physical, while the former involves more physiological processes, such as, possibly, the breakdown of phosphocreatine.

b). *The alpha process.*—The effects of pressure have been utilized by Brown[211] to study the time course of what he calls the "alpha process" and what Gasser and Hill[212] have called the "fundamental process" of muscle contraction. During the early stages of contraction, the application of high pressures, as already mentioned, results in an increase in the tension developed. This effect soon decays to zero, and in its time relations it coincides roughly with the action potential spike, the rate of development of tension, and the rate of initial heat production. If the pressure is applied after the first 0.01 seconds of the contraction period, it causes no augmentation of tension, because after that time the amount of energy liberated by the stimulus is "settled" and is no longer subject to modification. Gasser and Hill[212] found that a sudden stretch of the muscle, causing a sudden increment in the tension, was much more effective in that respect when applied immediately after stimulation, than later in the course of the muscular response, and they interpreted this as indicative of an increase in muscle viscosity at that time.

While these two effects are similar in their time relations, they appear to differ in one important particular. The application of pressure results in an increase in the energy liberated (Cattell and Edwards,[213]) while the sudden stretch tends to decrease the energy liberated (deducting, of course, the work done on the muscle by the stretch itself.[214]) The effectiveness of

[208] D. E. S. Brown, J. Cell. & Comp. Physiol., **4**: 257, 1934; **8**: 141, 1936.

[209] The mechanisms of these two effects need not be identical. In the ameba it has been attributed to a decrease in viscosity,[205] and in the muscle to an increase in viscosity.[203]

[210] McK. Cattell and D. J. Edwards, J. Cell. & Comp. Physiol., **1**: 11, 1932.

[211] D. E. S. Brown, J. Cell. & Comp. Physiol., **8**: 141, 1936; Biol. Symp., **III**: 161, 1941.

[212] H. S. Gasser and A. V. Hill, Proc. Roy. Soc., London, **B, 96**: 398, 1924.

[213] McK. Cattell and D. J. Edwards, Am. J. Physiol., **86**: 371, 1928.

[214] W. O. Fenn, J. Physiol., **58**: 373, 1924.

the sudden stretch depends upon the fact that it takes up the internal slack in the submicroscopic contractile elements, thus accelerating the transfer of the energy into tension. The application of pressure, on the other hand, appears to increase the amount of energy available for transfer into tension. Both effects are additive to the normal rate of tension development, and therefore have the same time relations—small differences being of no general significance. The time relations of these effects can be expressed as a curve which has a peak about 0.01 seconds after the stimulus and reaches zero at about the time when the twitch tension is at a maximum.[211] It does not seem necessary to signalize this by calling it an "alpha process," as if it were some new and different reaction. It does not detract from the importance of these observations to consider that it indicates merely the time when the rate of the initial breakdown, or the rate of the development of tension, is at a maximum. It does show, however, that the internal tension is developed much more rapidly than it is manifested externally in the record of the ordinary isometric twitch.

9. Heat Production.—The first measurements of the heat production in muscles were made by Helmholtz, who wished merely to demonstrate the principle of the conservation of energy. After him came many others, the most outstanding being Heidenhain,[215] Fick,[216] and Hill.[217] Even with the slow thermopiles which were available in the early days, the most important of the facts known today were soon outlined. Both Heidenhain and Fick, for example, discovered that the amount of energy liberated depended upon the mechanical conditions of the contraction, and they recognized the general principle that, the greater the work performed, the greater the energy liberated. The imperfections of the technique were such, however, that it was impossible to make this into a clear and quantitative story.

The entrance of A. V. Hill into the field was marked by improvements in the technique, which continued with few interruptions until the outbreak of the present war.[218] In his first work, attention was focussed particularly upon the isometric contraction which was technically the simplest, because it did not involve the complications due to the movement of the muscle against the thermopile. If the part of the muscle beyond the thermopile is, for example, slightly warmer than the part touching the thermopile, then a contraction will bring warmer material against the junctions of the dissimilar metals and will cause spurious effects. It was in the early study of the isometric contraction, that the classical work on heat production was completed, and the time relations of the heat production, its various phases, and the variations in the magnitude of total heat under different conditions

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[215] R. Heidenhain, Mechanische Leistung, Wärmentwickelung und Stoffumsatz bei der Muskeltätigkeit: Breitkopf u. Härtel, Leipzig, 1864.

[216] A. Fick, Mechanische Arbeit und Wärmentwickelung bei der Muskeltätigkeit: Brockhaus, Leipzig, 1882.

[217] A. V. Hill, Physiol., Rev., **2**: 310, 1922.

[218] A. V. Hill, Proc. Roy. Soc. London, **B, 125**: 136, 1938.

of temperature, duration of stimulation, and length were all described in terms of the isometric twitch.

Until recently it has been supposed that the isometric contraction was essentially simpler than any other type, but now it has become evident that no contraction is ever really isometric as far as the internal mechanism is concerned, even though the two ends of the muscle are absolutely fixed in their relative positions. The result is that complications of internal shortening enter into the interpretation of the isometric contractions as well as the isotonic, and they are the more dangerous because they are not realized, and, if they are realized, they are not measurable. The "isotonic era," in which proper attention was paid to the heat production during shortening, was ushered in with the measurement of the isotonic contraction in Hill's laboratory in 1922.[219] In that work, the finding of Heidenhain was rediscovered. When a muscle shortens and does work, it liberates an extra amount of heat, which is quantitatively equal to the work performed. It is for this reason that the subject of the heat production of muscles can now be better presented from the point of view of the isotonic contraction than from that of the isometric contraction. The former enables one to deal with shortening, while the latter manifests internal shortening in appreciable amount, without permitting one to deal with it in a quantitative way.

The heat production may be discussed under the following headings:

1. Contraction heat	0.6
a. shortening heat	
b. work heat	
c. maintenance heat	
2. Relaxation heat	0.4
3. Recovery heat	1.0
a. aerobic	1.0
b. anaerobic	0.08

a). *Initial heat.*—The contraction heat and relaxation heat together make up the initial heat. In a single twitch, this fraction is given off during the twitch itself, which lasts only about $\frac{1}{10}$ of a second in the frog sartorius muscle. The initial heat is followed by the recovery heat, of nearly equal magnitude. Since the latter lasts perhaps 600 times as long (10 minutes), the average *rate* of recovery heat production is 600 times smaller than the average rate of initial heat production. Consequently, the initial heat appears explosively and determines the magnitude of the initial deflection of the galvanometer which is recording the potential difference developed in the thermopile. In 1923 A. V. Hill[219a] shared with Meyerhof the Nobel Prize in Medicine, chiefly for his success in working out the time relations of these various phases of the heat production. The recovery heat is largely due to oxidation, and most of it disappears if the muscle is in an atmosphere of nitrogen. The small fraction which persists in nitrogen, amounting to

[219] W. O. Fenn, J. Physiol., **58**: 175, 373, 1923.

[219a] A. V. Hill, Muscular Activity; William and Wilkins, Baltimore, 1926.

about 8% of the initial heat,[219b,220] is due to a formation of lactic acid after the contraction is over, which provides energy for the synthesis of phosphocreatine. The amount of this anaerobic delayed heat varies considerably with different conditions. It is complete in 20 sec. at 17°C. It occurs in oxygen and in equal amount, but is then merged with the true oxidative recovery heat.

The *relaxation heat* appears coincidentally with the mechanical relaxation, and is due to the degradation to heat of the elastic potential energy stored in the tensed muscle during the contraction phase. If the muscle lifted a load during contraction and lowered it during relaxation, then the work done in raising the load is added quantitatively to the relaxation heat. In a supposed isometric twitch, the relaxation heat amounts to 0.4 of the total initial heat. In a truly isometric contraction, it is possible that there would be no relaxation heat, because there would be no internal shortening of the anisotropic discs, or folding of myosin chains, and no possibility of stretching any other internal structure, such as the isotropic discs. Hence, there would be no elastic potential energy stored during contraction to be degraded to heat in relaxation.

b). *Contraction heat, shortening heat.*—Likewise, if there were no internal shortening, i.e., if a true isometric contraction could be realized, it is probable that there would be no *shortening heat* but only *maintenance heat.* This is shown particularly well by the galvanometer deflections recorded in Fig. 52, from A. V. Hill.[221] The insulation on the thermopile was so thin, and its heat capacity so low, that with a rapidly responding galvanometer he could record muscle temperature directly without serious error. The graphs show, therefore, heat plotted against time from the beginning of a tetanic stimulus. In Fig. 52a, curve *A* represents the heat production in an isometric tetanus. It shows a quick rise at the beginning, which was formerly called an outburst of heat accompanying the development of tension in the muscle. The slower, more or less constant subsequent rise is attributed to the energy for maintaining tension. The isometric maintenance heat rate could, therefore, be measured from the slope of this line at different temperatures and muscle lengths. If now the muscle is not held at fixed length, but is allowed to shorten freely for increasing distances under a fixed load, the galvanometer gives deflections *B*, *C*, and *D* for distances 3.4, 6.5, and 9.6 mm. respectively. From these records, it can be said that the initial upswing of the galvanometer in *A* could have been caused by an initial internal shortening, corresponding in its heat equivalent to about 3 mm. of external shortening. Here, then, is the evidence that all the true isometric heat is really maintenance heat. This also is the evidence for *the shortening heat, a fraction which is proportional to the amount of shortening, and independent of the load.* The relationship to the load is shown in Fig.

[219b] W. Hartree, J. Physiol., **75**: 273, 1932.

[220] W. Hartree and A. V. Hill, Proc. Roy. Soc., London, B, **103**: 207, 1928.

[221] A. V. Hill, Proc. Roy. Soc., London, B, **126**: 136, 1938.

52b, in which E is isometric, while F, G, H, and J represent isotonic contractions for a fixed distance under progressively decreasing loads. With all these loads, the same total heat is liberated, provided the weight is removed from the muscle after shortening is complete. With light loads the shorten-

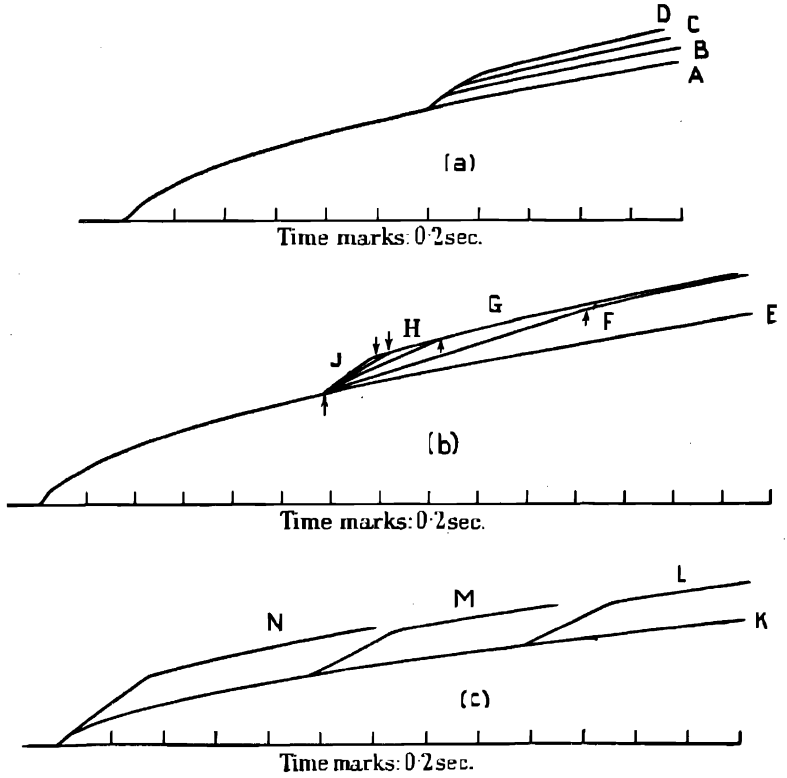

FIG. 52.—Galvanometer deflections as given by A. V. Hill[221] by use of a rapid thermopile and galvanometer. In aA, bE and cK, the muscle is isometric. When the muscle is released and begins to shorten the galvanometer gives a sudden deflection upward as in the other curves. In (a) the muscle after release is allowed to shorten under the same load to increasing distances represented by B, C, and D. In (b) it is allowed to shorten the same distance under increasing loads represented by J, H, G, and F. In (c) the muscle is released at different times during the isometric tetanus, shortening each time under the same load and to the same distance.

ing heat is given off quickly in proportion to the high velocity of shortening, and with large loads it is given off slowly, but the total amount is the same.

The total energy, of course, is not the same, because varying amounts of energy have been taken out of the system in the form of work. This work fraction may be as large as, or larger than, the shortening heat. The curves of Fig. 52 are not continued long enough to show the relaxation heat, but, if the muscle has to lower the weights in relaxation, then the energy which goes into the weights in contraction reappears quantitatively in the muscle

as heat in relaxation. If the loads are not lowered in relaxation, then the relaxation heat will be the same in F and G as in H and J.[222]

According to Hill,[221] the shortening heat is not due to frictional resistance, because an equal amount of heat does not appear as heat during the reverse movement in relaxation. There seems to be some doubt whether, in relaxation, there is any heat either given out or absorbed which is equivalent to the shortening heat in contraction. If there is an equal absorption of heat in relaxation, then the shortening heat may be regarded as a sort of thermoelastic effect for the stimulated muscle. If so, it is opposite in direction to the thermoelastic effect of the resting muscle. The fact that the shortening heat is proportional to the amount of shortening, but independent of load and speed, certainly suggests this interpretation. The answer to this problem will come from a more careful study of relaxation heat.

The relation between the shortening and the maintenance heat can also be shown in mathematical form. The heat (H) of an isometric contraction was described by Hartree and Hill[223] by the equation

$$H = A + Bt,$$

where A was the heat expended in setting up the tension in the muscle, and Bt was the maintenance heat, B being a constant, and t the duration of the stimulation. In this equation, the moiety A includes the relaxation heat, because the H refers to the total initial heat of the contraction. If, however, the relaxation heat be subtracted from both sides, the same equation still applies.[224]

$$H' = ax + Bt.$$

In this form, the H' refers only to the heat liberated during contraction, and the A has become ax, the shortening heat, x being the amount of shortening, and a being a constant. If the external shortening is zero (isometric), then x represents entirely internal shortening. Energy stored as potential energy in the myosin chains, which will appear as heat in relaxation, is not included. In an ideal case, never realized in practice, it may be supposed that x is zero, in which case only the maintenance heat remains.

Since the heat varies with the size of the muscle and its ability to develop tension, it is usual to write the equation

$$\frac{H}{Tl} = A + Bt,$$

Here again H refers to the total initial heat, and the absolute values of A and B are correspondingly changed. The ratio H/Tl is equivalent to the ratio (heat per gm. of muscle)/(tension per cm.^{2} cross-section), since length is approximately weight divided by cross-section. Where t is very short, as in single

[222] W. Hartree, J. Physiol., **60**: 269, 1925.

[223] W. Hartree and A. V. Hill, J. Physiol., **55**: 133, 1921.

[224] A. V. Hill, Proc. Roy. Soc., London, B, **127**: 434, 1939.

twitches, the maintenance heat Bt approaches zero, and the value of H/Tl is more constant. It is found to be independent of temperature, and has a minimum value at resting length, increasing at both longer and shorter muscle lengths.[225] Its value also varies with the arrangement of the fibers in the muscle. In the long parallel-fibered sartorius muscle its value is 1/6.8, while in the short diagonal-fibered gastrocnemius muscle its value is 1/13.8. The difference would be expected merely from the arrangement of the fibers, if the individual fibers in the two muscles were similar in nature, because the total shortening per cm. of length is less in the gastrocnemius muscle.[225] The T in the equation is unduly large for the gastrocnemius muscle, because of the diagonal arrangement of the fibers and the large "physiological cross-section" of the muscle.

Because the ratio H/T is constant, it is usual to suppose that each unit of energy liberated provides for one unit of tension. This idea has been extended by Reichel[227] to apply to the resting heat and tension. When a resting muscle is stretched, its oxygen consumption (Meyerhof, Gemmill, and Benetato[228]) and its resting heat production (Feng[229]) are increased along with the increase in tension. According to the figures given by Reichel, the ratio H/T of the stretched resting muscle is the same as that for the stimulated muscle. The inference seems to be, that a given amount of tension, however it is produced, leads to the liberation of a corresponding amount of heat. In the resting muscle, however, the tension is in the connective tissue, while in the stimulated muscle it is in the myosin. Moreover, the ratio H/T in both resting (stretched) and stimulated muscle increases with increasing duration of the tension so there is something arbitrary in the comparison. In addition, the heat measurements were made at different muscle lengths without separate calibration of the muscle. The interesting relation brought out by Reichel must be regarded, therefore, as probably fortuitous.

If a muscle stimulated at resting length can develop an isometric tension T, or can shorten without load to a length $l/3$, then the area of this length-tension diagram is approximately $1/2(T \times l/3) = Tl/6$ and in the sartorius muscle this is approximately equal to the observed heat, which was found to be $H = Tl/6.8$. Thus, it was supposed that the heat represented the degraded potential energy of the length-tension diagram. If this had been strictly true, all the heat should have appeared in relaxation, when this mechanical potential energy disappeared. According to more recent findings, it must be supposed that this equality between the heat of a single isometric twitch and the area of the length-tension diagram of the muscle was largely accidental. Not over 0.4 of this heat is actually due to stored potential energy, and this appears as heat only in relaxation. The remainder is partly due to maintenance heat, and partly to the internal shortening heat, which is produced when the internal potential energy is stored.

[225] W. Hartree and A. V. Hill, J. Physiol., **55**: 389, 1921; A. V. Hill, *ibid.*, **60**: 237, 1925.
[226] A. V. Hill, Proc. Roy. Soc. London, B, **109**: 267, 1931.
[227] H. Reichel, Ztschr. f. Biol., **98**: 510, 1937.
[228] O. Meyerhof, C. L. Gemmill, and G. Benetato, Biochem. Ztschr., **258**: 371, 1933.
[229] T. P. Feng, J. Physiol., **47**: 441, 1932.

c). *Maintenance heat.*—With stimuli of longer duration, the maintenance heat becomes increasingly important and is in a sense the most characteristic quantity which can be measured in an isometric contraction, because it is free of complications due to internal shortening. This maintenance heat is supposed to represent a constant rate of energy production, which maintains the muscle in an excited or contracted state. It is independent of the frequency of stimulation, provided the frequency is sufficient to maintain maximum tension.[230] Theoretically, it begins with the beginning of contraction and continues until shortly after the end of the stimulus. It is difficult to measure it until after the tension has reached its maximum, because otherwise the galvanometer deflection is complicated by the shortening heat which is being liberated at the same time due to internal shortening. The maintenance heat can, therefore, be measured most simply by measuring the heat production during a short and a long tetanus. The difference between these two quantities of heat, divided by the difference in time, represents the rate of production of the maintenance heat. The shorter tetanus should, of course, be long enough to permit maximum tension to be developed. In Hill's measurements, the maintenance heat[231] was found to be increased at high temperatures, with a temperature coefficient between 0° and 15°C. of 2.8. Bozler[232] suggested that the ratio between the rate of heat production per gram of muscle and the tension maintained per unit cross-section area be called the "Economy" of the contraction. Thus

$$\text{Economy} = \frac{\text{(Tension-time in gm. sec.)} \times \text{muscle length}}{\text{Heat in gm. cm.}} = \frac{Ttl}{H}$$

The economy decreases with rise of temperature[233,230] and with fatigue; it is greater in slow smooth muscles than in the rapid striated muscles.[232] In tonus muscles it is so great that it has been argued that such muscles possess a "catch mechanism" which enables them to maintain tension without any expenditure of energy. Actually, in such muscles the maintenance energy is probably too small to detect readily in the presence of the resting energy production, but there seems to be no necessity for postulating any special mechanism. It is reasonable to consider that the tension in a muscle is spontaneously disappearing at a certain rate, and that this must be replenished at the same rate in order to keep the tension constant. On this basis, any condition which accelerates the rate of disappearance of tension, such as a high temperature, would be expected to increase also the maintenance heat. Whether the tension is maintained by electrical stimulation or by chemical contracture, the maintenance heat and economy seem to be of the same order of magnitude.[234]

[230] T. P. Feng, Proc. Roy. Soc., London, **B, 108**: 522, 1931.

[231] W. Hartree and A. V. Hill, J. Physiol., **55**: 133, 1921.

[232] E. Bozler, J. Physiol., **69**: 442, 1930.

[233] W. Hartree and A. V. Hill, J. Physiol., **55**: 147, 1921.

[234] W. O. Fenn, J. Pharmacol. and Exper. Therap., **42**: 81, 1931.

From a theoretical point of view, the variation of maintenance heat with length of the muscle is a matter of special importance when isotonic and isometric contractions are to be compared. The effect of length has been tested by measuring the heat production during isometric tetanic contractions at different lengths. Using this method, Fenn and Latchford[235] found that the maintenance heat, like the heat to develop tension or the heat in a single twitch,[236] passes through a maximum at a length equal to about 90% of the length at which the maximum tension is developed. Similar results were also obtained by Fischer[237] in turtle muscle.

In these experiments, the heat was calculated for a constant length of the muscle. When the muscle shortened, a correspondingly larger fraction of the total muscle weight was to be found between the electrodes. If the heat is calculated for equal weights of muscle or for the whole muscle, then the heat falls off even more rapidly as the shortening increases, but less rapidly on the other side of the maximum where the length increases. With this method of calculation, the heat maximum and the tension maximum both occur at the resting length.[238] Of course, the cross-section of the muscle also increases as it shortens, and this tends to increase the tension. If the heat is to be compared with the tension which it maintains, then the heat per gram of tissue should be compared with the tension per unit cross-section area (or the heat per unit of length with the tension uncorrected for cross-section area) as in the original report.

The maintenance heat can also be measured by a sufficiently quick thermopile from records similar to those in Fig. 52. Here the slope of the curve represents the maintenance heat. Using this method, Hill[221] has found values increasing at first with decreasing lengths, and then decreasing. This would seem to be a confirmation of results described above and obtained by older methods, but it is difficult to make exact comparison without exact definitions of resting length.

There is a possibility that the shortening heat should be regarded as a fraction of the maintenance heat, for Brown[239] has shown that the sum of these two moities is remarkably constant in a series of contractions, with increasing shortenings under decreasing loads. As the shortening heat increased, the remainder left for maintenance heat decreased. If this is the case, then the observed decrease in maintenance heat at a sufficiently diminished muscle length may be attributed to the increase in the shortening heat liberated in shortening to that length. With this possible exception, the maintenance heat is regarded as independent of work done, and of shortening, but varying with length and total tension.

d). *Work heat.*—The records in Fig. 52 illustrate the important fact that the initial heat production is independent of the load if the muscle is not

[235] W. O. Fenn and W. B. Latchford, J. Physiol., **80**: 213, 1933.

[236] A. V. Hill, J. Physiol., **60**: 237, 1925.

[237] E. Fischer, J. Cell. & Comp. Physiol., **5**: 441, 1935.

[238] R. W. Ramsey, personal communication.

[239] D. E. S. Brown, Biol. Symp., **3**: 161, 1941.

required to lower the load in relaxation, i.e., if the extra energy which was liberated for work was left as potential energy in the load, and was not restored to the muscle as heat. At the end of the stimulation, regardless of load, the galvanometer reaches the same point of deflection. This is a confirmation of the experiment first performed by Fenn[240] when he permitted a muscle to lift a series of increasing loads a fixed distance, and measured the heat production. The loads were caught by an electromagnet after they had been lifted the required distance, so that the muscle relaxed under a constant small load just large enough to pull it back precisely to its original length. Without any elaborate calibration of the muscle to permit the heat to be expressed accurately in work units, this experiment proved that the *extra energy liberated was just equal to the extra work done.* The result has since been duplicated in the writer's laboratory by J. B. Hursh (PhD thesis), with some indication of a slight decrease of heat at the larger loads. Hartree[241] also repeated the experiment in the same way, but added an analysis of the heat production to show the distribution with time. He compared an isotonic and an isometric contraction, both of which gave the same amount of total heat (minus work). The two differed, however, in the fact that the isometric gave less heat during contraction, and correspondingly more during relaxation. The excess heat in relaxation of the isometric muscle represented the greater elastic potential energy of the tensed muscle, while the excess in the isotonic muscle during the contraction represented shortening heat. This experiment, as well as the more recent ones of A. V. Hill,[221] illustrated in Fig. 52, shows that the *extra energy for contraction is liberated during the contraction rather than during the relaxation.*

This liberation of extra energy for work is all the more remarkable when it is recalled that the extra energy is exactly equal in amount to the work which is performed. Indeed a muscle may be likened to a taxicab which charges (1) so much per hour for the rent of the machine (maintenance heat); (2) so much per mile (shortening heat, proportional to distance); (3) so much for each unit of actual work done (force × distance); and (4) so much for overhead (recovery heat).

It will be convenient at this point to consider some of the theories which have been proposed to account for this peculiar phenomenon.

According to the classical elastic body theory, the muscle is transformed by the stimulus into a new elastic body which develops more tension at the same length and has a shorter natural length. This is similar to the theory of active relaxation, in that both picture a certain amount of mechanical potential energy thus made available for muscular work, and predict that this amount of potential energy can be determined from the area of the length-tension diagram. Certainly, the work which the muscle does can be represented on the length-tension diagram, and the work bears some relation to the heat produced, but this does not mean that there is any direct

[240] W. O. Fenn, J. Physiol., **58**: 175, 373, 1923–24.

[241] W. Hartree, J. Physiol., **60**: 269, 1925.

relation between area of the length-tension diagram and the heat. This theory was discredited by the discovery of the importance of work in the muscle economy.[240] The energy liberated seemed to depend not so much upon the amount of the length-tension diagram "covered" by the shortening, as on the amount actually used for work, and those areas of the length-tension diagram which were used for work represented energy which otherwise would never have been developed at all. The energy for shortening was liberated or transformed from chemical potential energy at the moment of shortening, not beforehand.

A perfectly reasonable attempt was made by Hill[242] and by Wyman[243] to salvage the elastic body theory by the suggestion that the energy corresponding to these unused portions of the length-tension diagram failed to appear as heat, not because it had never been developed, but because it reverted to chemical potential energy at the end of the contraction, in case it had not been utilized as work. The end result, however, was the same in either case. The muscle had taken what energy it wanted for work and left the remainder for another time, and the heat production could not be predicted from the length-tension diagram.

Some writers have been unwilling to accept the idea that the muscle really does discriminate in some way between the performance of work and the mere development of tension. Tiegs[244] has argued that the extra energy for work is nothing but internal friction, as if it could for that reason be neglected. Likewise, Lindhard and Möller[245] have argued that it is nothing but "deformation heat" and derives from a change in the shape of the muscle fiber which loses more potential energy at the big end where it shortens than it gains at the small end where it is stretched. This argument loses its point, however, when it is recalled that the reverse process must occur in relaxation. Moreover, all the energy ultimately comes from chemical breakdown in the muscle, whatever it may have been used for. By any argument, therefore, more work requires more chemical breakdown. This is clearly shown by the chemical studies which have been made. Fischer[246] showed that a muscle uses more oxygen when it does work than when it contracts isometrically. Both Rothschild[247] and Nagaya,[248] who measured lactic acid formation, found variations of lactic acid with work in the gastrocnemius muscle, which might have been predicted quite well from the heat production figures already available.[249] However one may

[242] A. V. Hill, J. Physiol., **60**: 237, 1925.

[243] J. Wyman, Jr., J. Physiol., **61**: 337, 1926.

[244] O. W. Tiegs, Australian J. of Exper. Biol. & Med. Sc., **1**: 47, 1924.

[245] J. Lindhard and J. P. Möller, Det. Kgl. Danske Videnskabernes Selskab. Biol. Meddelelser, **VIII**: 8.

[246] E. Fischer, Am. J. Physiol., **96**: 78, 1931.

[247] P. Rothschild, Biochem. Ztschr., **222**: 21, 1930.

[248] T. Nagaya, Pflüger's Arch. f. d. ges. Physiol., **221**: 720, 1929.

[249] In reading papers on the gastrocnemius muscle, it is important to remember that the arrangement of the fibers is such that the internal work due to change of shape in isometric

wish to explain it, the extra energy for work remains a reality which does not fit with the elastic body theory.

A point of particular importance in connection with the work heat is the fact that stretching the muscle during the period of contraction has the opposite effect, i.e., it causes a diminution in the amount of heat energy liberated[240] (after deducting the heat due to the work done on the muscle by the stretch). The whole phenomenon, therefore, has some sort of a thermodynamic basis, and from this point of view it is very similar to a thermoelastic effect. It is, however, opposite in direction to the thermoelastic effect found in the resting muscle. As an analogy, one may think of an electric motor which uses more current when it is loaded than when running idly, and which will reverse the process and deliver current if it is turned backward. In addition, it has been found that it is only during the contraction phase that a stretch causes a decrease, and a shortening an increase, in the heat production. During relaxation, the reverse is true. Thus Fenn[240] found that the lengthening associated with ordinary relaxation caused (usually) an additional heat production, and, conversely, if the muscle was released and allowed to shorten during relaxation the heat production was (usually) diminished. These results were also confirmed by Azuma,[250] but they should be repeated with modern improved methods.

10. Rate of Heat Production, Viscosity, and the Speed of Shortening.—It seems very natural that a muscle should lift a light load higher than a heavier load, and likewise lift it more rapidly. The more one tries to explain these simple facts, however, the less obvious do they seem to be. The explanation of the first has already been discussed in connection with the length-tension diagram. It remains now to explain, if possible, the greater speed with which lighter loads are lifted.

The original observation on muscle viscosity was derived from experiments of A. V. Hill[251] with human subjects pulling against an inertia device. He observed that the work performed decreased linearly with increase in the speed of the movement. This suggested that at high speeds the subjects were really working against a viscous resistance which increased in proportion to the speed. In other words, the second term of the equation of movement[252] was of predominant importance under these conditions. The importance of viscosity was shown, however, to be overestimated in this theory by the measurement of the work done in sprint running.[253] Using the work measured in this way, the efficiency turned

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contractions is much larger than it is in the sartorius muscle. Hence the isometric heat is larger than the isotonic heat, except for certain intermediate loads where the external work is at a maximum. For a discussion of this point and further references, see W. O. Fenn, Cold Spring Harbor Symp., **4**: 233, 1936.

[250] R. Azuma, Proc. Roy. Soc., London, B, **96**: 338, 1924.

[251] A. V. Hill, J. Physiol., **56**: 19, 1922.

[252] See p. 476.

[253] W. O. Fenn, Am. J. Physiol., **92**: 583, 1929; **93**: 433, 1930.

out to be just as great as in slower movements. If much of the work had been expended against internal viscosity, the externally measured efficiency would have been correspondingly low.

Finally, measurements were made of the speed with which various loads were lifted by isolated muscles, or, in other words, the force exerted at different speeds of shortening.[254] The results of these measurements over a wide range of forces and speeds showed that the force did not decrease linearly with increase in speed, as the theory demanded. This is shown

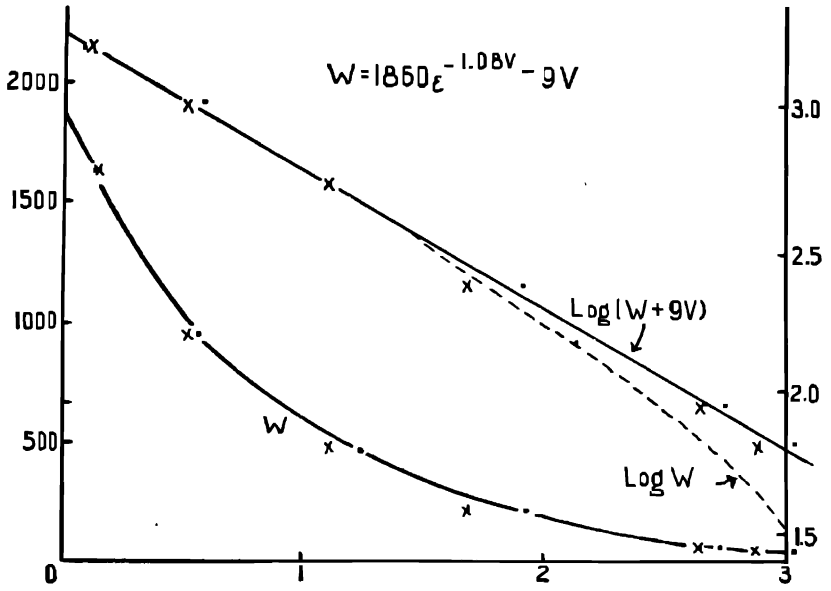

FIG. 53.—Load, W, as ordinates, and speed of shortening as abscissæ. Upper graph shows the same data plotted logarithmically. The difference between the dotted line and the straight line represents the small correction for viscosity. The points are experimental and the lines are drawn according to the empirical equation (Fenn and Marsh[254]).

by the lower graph in Fig. 53, representing the loads plotted against the speed of shortening. The upper graph shows that this curve can be represented by an exponential equation after making a small correction for a hypothetical amount of viscosity. This equation leads to no particular interpretation of the data, but the authors expressed the opinion that it was not viscosity, but *a chemical delay in the liberation of the extra energy for shortening*, which was of chief importance in limiting the speed of movement.

It is not necessary, of course, that the viscosity of a muscle should remain constant. Myosin probably belongs to the category of thixotropic gels which give a lower viscosity the more rapidly they are stirred.[255] Thus, in rapid movements the muscle may have less viscosity than in the slower movements. This by itself, however, will not explain why smaller loads

[254] W. O. Fenn and B. S. Marsh, J. Physiol., 85: 277, 1935.

[255] See p. 450.

are lifted more rapidly, because the viscosity is just as high at the beginning of the contraction, whether the load is small or large. If, for some other reason, a smaller load can be made to move more rapidly than a larger one, then it may be able to continue with still greater rapidity because of the diminishing viscosity of the rapidly stirred myosin gel. Bouckaert and Delrue[255] have made a model muscle with a concentric cylinder viscosimeter, with a thixotropic gel between the two rotating cylinders. With this model they have been able to duplicate very nicely the curves of lengthening and shortening of a sea-anemone muscle. It seems likely at present, however, that viscosity does not play so large a role in striated muscle as it does in smooth muscle.

From the work described it was concluded, therefore, that large weights are lifted by muscles more slowly than smaller ones because they demand more excess energy liberation in the process, and this requires more time. This conclusion, however, was reached largely by intuition, and quantitative support for the theory was lacking. Stevens and Metcalf[257] had tried to show that a muscle contracted with constant power, or constant rate of performance of work, or constant Pv (force, P, $\times$ velocity, v). This quantity was constant, however, only over a very narrow range of loads. The quantity $(P + f)v$, where f was frictional resistance, gave a better constancy (Fenn and Marsh[254]), this being the sum of the rates of internal work against viscosity and external work; but even this did not fit at the larger loads where v approached zero.

More recently, the requisite mathematical formulation of this theory has been submitted by A. V. Hill[258] who proposed that the rate of expenditure of energy for work and shortening is not constant, but is proportional to $P_o - P$ or to the difference between the isometric tension P_o and the tension of the load, P. This idea was expressed in his equation of muscular motion

$$(P + a)v = b(P_o - P). \tag{1}$$

In this equation frictional force f is replaced by the constant a. This constant has the dimensions of force and is found to be a rather constant fraction of the isometric tension, so that a/P_o is constant and has an average value of 0.257, while av is the rate of expenditure of shortening heat. The constant b has the dimensions of velocity and an average value at 0°C. of $l/3$ per second where l is the length of the muscle. The value of b increases rapidly with temperature, the temperature coefficient for 10°C. being 2.05. The equation states that the rate of expenditure of energy in excess of the maintenance heat is proportional to $P_o - P$. The constant a is not frictional resistance, because an equal amount of heat apparently does not appear during the reverse change of shape in relaxation. Instead, it is a sort of thermoelastic effect presumably accompanied by an equal absorption

[255] J. P. Bouckaert and G. Delrue, Arch. internat. de physiol., **38**: 108, 1934.
[257] H. C. Stevens and R. P. Metcalf, Am. J. Physiol., **107**: 568, 1934.
[258] A. V. Hill, Proc. Roy. Soc., London, **B, 126**: 136, 1938.

of heat in relaxation, although there seems to be no certain evidence of such a reversal.

Hill's equation is tested as follows. In the first place, the value of a is determined from heat production measurements similar to those illustrated in Fig. 52. Here the added heat due to shortening x cm. is ax gm. cm. Knowing x, the value of a is calculated. With this constant determined, the muscle is allowed to shorten while lifting various loads, P, and the corresponding velocities of shortening v are determined. Thus, $(P + a)v$ can be calculated and plotted against P. The graph, according to equation (1), should be a straight line of negative slope $= b$. Hill gives a number of such plots in which a was determined by heat measurements on the same muscle used for the measurements of P and v, and it is evident that satisfactory straight lines were obtained.

While this is, to be sure, a very nice theoretical confirmation, its real value as a test of the theory is to some extent limited, because a cannot be determined with great accuracy from the heat measurements, and, unfortunately, even a 50% variation in a does not affect the linearity of the graph very seriously.

Now if a straight line is obtained in a plot of this sort, then the equation will also necessarily give an equally good fit to the usual force-velocity plot like that shown in Fig. 53. To show this, Hill's equation (1) may be rewritten by adding ab to both sides and rearranging:

$$\begin{aligned} P(v + b) + a(v + b) &= bP_o + ab \\ (P + a)(v + b) &= b(P_o + a) = \text{constant.} \end{aligned} \qquad (2)$$

Since this equation is of the form $xy = \text{constant}$, it may be concluded that the force-velocity or P-v curve is a section of a rectangular hyperbola with asympototes at $-b$ and $-a$. Thus, both b and a may be determined by fitting force-velocity data to equation (2); b has the dimensions of velocity and a is a force. The values are often not very precisely determined in this way, because a rather wide variation of values will give a good fit. The value of b cannot be determined from heat measurements alone, and the critical test of the equation depends upon a comparison of the values of a obtained thermally with those obtained on the same muscle by velocity measurements. No survey of just this sort is available, but it seems unlikely that any discrepancy in excess of the experimental errors will be found. On the other hand, the experimental error in the measurement of a by either method is certainly fairly large.

While some further testing is, therefore, desirable, this theory is, nevertheless, good enough to justify further consideration of its implications. In the first place, it should be emphasized that this does not represent the total rate of energy liberation, for the equation says nothing about the maintenance heat which proceeds at a rate determined, presumably, by the length of the muscle, and apparently serves the sole purpose of keeping the muscle in a state of continuous excitation. So far as the performance of work is

concerned, the maintenance heat is pure loss. Hill's equation also neglects relaxation and recovery heat, and applies only to the shortening heat and the work heat, or the *excess energy* fraction.

In theory, the equation should apply to muscles undergoing stretch as well as to muscles which are shortening. The only difference is that the values of the velocity would be negative instead of positive. The evidence for a negative extra heat production in lengthening muscles has already been discussed (p. 502). This is in accord with the theory, because $P > P_o$ so that $b(P_o - P)$, which is equal to the rate of liberation of excess energy, would also be negative. In trying to verify the relation between P and v in the stretch, difficulties are encountered due to the danger of injuring the muscle by too rapid a stretch, and the tendency of the muscle to "give" or "slip" while it is being stretched. No precise application of the equation to lengthening muscles is, therefore, possible at this time (Katz[259]).

The essential feature of Hill's theory is that the rate of liberation of this excess energy is proportional to $P_o - P$. As an explanation of this peculiar relation, Hill suggests that during shortening under a load, P, a certain fraction P/P_o of the total number of active points or myosin linkages are occupied in maintaining tension, only the remainder $P_o - P$ being available to serve as centers for the liberation of energy for shortening and work. It is peculiar, however, that these shortening linkages must then be supposed to liberate energy at a more or less constant rate, so that the longer the time taken in shortening, the greater the amount of energy liberated.

This conclusion results from the assumptions which were made, for the total rate of extra energy liberation is $b(P_o - P)$, and the number of linkages engaged in liberating this extra energy is $(P_o - P)n/P_o$, where n is the total number of linkages. Then the rate of extra energy turnover in each linkage is equal to the ratio of these two quantities, or bP_o/n, which is constant. But the total extra energy for a constant amount of shortening, x, is $(P + a)x$, i.e., it increases with increase in the load P. The only way in which a smaller number of linkages, each working at a constant rate, can liberate more energy is to spend more time doing it. Thus, the extra energy liberated by each linkage depends upon the time during which it is kept at the business of shortening, rather than being directly a function of the load. It is also, of course, proportional to the amount of shortening, but this is because it takes longer to shorten a greater distance under the same load.

Each shortening linkage must be regarded, therefore, as a center for the continuous liberation of extra energy. If this made a reasonable and plausible hypothesis, it would provide an explanation for the fact that a muscle liberates extra energy for work. This concept is, however, a difficult one to entertain, and it is made no easier when one realizes, in addition, that the linkages or active points of the muscle which are supposedly engaged in maintaining tension P (i.e., the fraction P/P_o) are also acting as centers of continuous energy turnover, the rate of turnover being independent of the

[259] B Katz, J. Physiol., **96**: 45, 1939.

tension maintained. This is evidently true, because the rate of liberation of maintenance heat does not appear to change when an isometrically stimulated muscle is suddenly released and allowed to shorten, either under a large load or a small one.[259] We evidently know too little about the submicroscopic contractile mechanism to make speculation of this type profitable, but it seems that *Hill's theory does not provide an adequate explanation for the excess energy. If, however, one assumes the necessity for the liberation of extra energy for shortening and work, then the theory provides a good explanation for the force-velocity data.*

Hill's theory has also supplied a good explanation for the shape of the isometric tetanus.[258] The muscle is regarded as a contractile element, governed by the characteristic equation $(P + a)(v + b) = \text{constant}$, and an undamped noncontractile elastic element composed of tendon, sarcolemma, etc. The contractile state is assumed to be set up instantaneously, and, as shortening begins, the elastic component is stretched so that the tension P rises. At first, however, P is low, and the velocity of shortening is high. As the elastic element stretches, P becomes larger, and the velocity of shortening becomes less, finally reaching zero when $P = P_o$. According to Hill, the values of a and b obtained from force-velocity data and heat measurements explain satisfactorily the shape of the isometric tetanus of the same muscle at the same temperature. The explanation introduces one new quantity, which depends upon the elasticity of the free elastic component of the muscle, but methods for determining this are proposed. The theory applies rather well to the frog sartorius muscle, but not so well to tortoise muscle.[259] Various other equations can be suggested which fit the experimental isometric tetanus curves as well as, or in some cases perhaps better than, the equation of Hill, but the latter must be credited with offering, in addition, a rational basis for the equation. In any event, this work of Hill's must be regarded as a great step in advance. Henceforth, it is not enough to describe the shapes of isotonic and isometric curves; now it is necessary to explain why they have these particular shapes, and the constants of the mechanogram must be related to the constants of the thermogram. Muscle physiology is advancing, therefore, to the status of a science!

Before leaving the problem of the explanation for the velocity of shortening, one further theory may be mentioned which has been advanced by Dr. Ramsey (personal communication). Consider a muscle at resting length OL_o, which can develop an isometric tension L_oP_o, Fig. 54. This muscle is allowed to shorten against various loads, L_oP, isotonically until it strikes a stop at length OL. The velocity is measured over this short range of lengths between L and L_o. For the smaller loads, at least, the velocity at any load is reasonably constant over this whole range. For loads greater than L_oD, the velocity will become zero before the length OL is reached.

[259] The maintenance heat does vary to some extent as a function of P_o and of length, as already mentioned.

Without the stop at OL, the muscle would shorten to the line BP_o, and it approaches this line with a velocity which is more or less proportional to the distance which it still has to go. Further shortening is impossible beyond BP_o, because of the internal elasticity of the muscle connective tissue, and it may be supposed, somewhat arbitrarily, that if this extraneous factor did not enter in, the myosin chains themselves would be able to shorten further perhaps to the line OCP_o. With continued stimulation, Ramsey and Street have actually observed a further shortening of single muscle fibers to a rather similar line (see Fig. 46). At lengths less than OB, the muscle is in the condition described as the "delta state." The initial

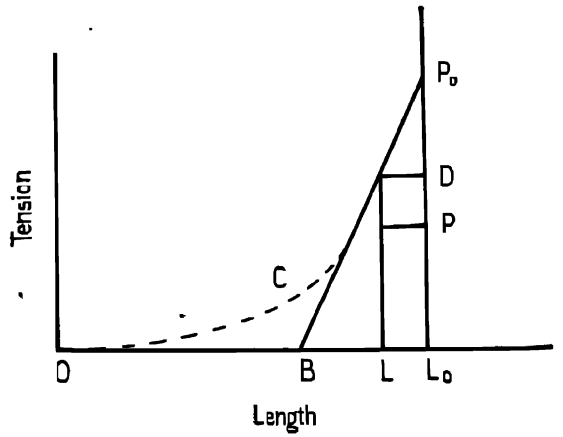

FIG. 54.—Length-tension diagram. BP represents the tension developed in a tetanus (ordinates) at different lengths (abscissae). Dotted line shows additional shortening which can be realized in part in single muscle fibers when connective tissue does not interfere.

velocity of shortening may be assumed to be proportional to the distance the muscle must go before it reaches the line OCP_o. If this line is properly drawn, it will of course provide an explanation of the velocity of shortening at different loads. For the larger loads, the line OCP_o may nearly coincide with the line BP_o, but at lower loads the experimental basis is somewhat less adequate. Actual force velocity curves can be fitted to such a curve very satisfactorily. (In this case, distances to the left from L_o represent velocities, and vertical distances represent force.) Although this theory is rather arbitrary, it does emphasize the fact that the velocities which have been measured at different tensions are all initial velocities, and Hill's equation has been verified only over this narrow initial range between L and L_o. The rules which govern the heat production and the velocities, as the muscle approaches equilibrium positions along the line BP_o, have not been worked out.

Summary.—Hill proposed that rapid movements of muscles were limited by the viscous resistance of the muscles. Fenn cast doubt on this interpretation by showing that the force did not fall off linearly with increase in the speed. He proposed, instead, that the muscle contracted more slowly

under large loads because it needed more time to liberate the extra amount of shortening energy and work energy required by these large loads. Hill then accepted this idea, but gave it quantitative expression in his equation of muscular motion. While this theory is very ingenious and successful, it requires further and more precise verification. Some of its logical conclusions, as applied to the myosin chains, are unfortunate. Another simpler, but less quantitative, theory is proposed (From Ramsey).[261]

11. Is Relaxation Active or Passive?—The answer to this question is fundamental to any theory of contractility. On the one hand, the resting muscle may be regarded as a loaded gun containing stored potential energy ready for delivery on stimulation. Contraction is, then, purely passive in the sense that it is analogous to the release of a previously tensed spring, the mechanical performance of which is fixed in advance. During relaxation, the spring must be stretched back to its original position, and this is, then, the active phase where work is done at the expense of chemical potential energy. If, on the other hand, contraction is an active process, then chemical potential energy is transformed directly into work during shortening, and during relaxation the muscle returns to its original resting condition by its own internal elasticity, simply because the linkages which cause shortening are no longer energized, the supply of chemical energy having been turned off.

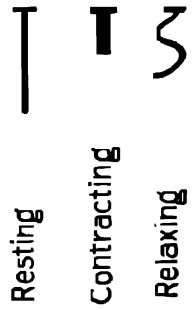

FIG. 55.—Experiment of Ramsey and Street on single isolated muscle fibers suspended from one end in Ringer's solution and tetanized. The fiber relaxes more rapidly than it can fall to the vertical position.

There can be no question about the fact that a muscle will tend to return to its resting length after contraction, even in the absence of any external force, such as that provided by a weight. An unloaded muscle, or one floating on mercury, will do this fairly well. This active relaxation was more convincingly demonstrated to me by Dr. Ramsey with one of his single muscle fibers, skilfully dissected by Mrs. Ramsey and stimulated while suspended freely by one end in Ringer's solution (see Fig. 55). The weight of this tiny contractile thread in water was so small that the fiber only slowly fell to a vertical position under its point of support. The relaxation process, however, was too fast for this fall, and the much-shortened fiber seemed to be so violently extended by some internal force at the moment when the stimulus ceased, that the fiber was thrown sideways into loops and then slowly floated back into its original position. In this sense, therefore, relaxation is undoubtedly active. The origin of this relaxing force, however, is uncertain. Two possible forces causing relaxation may

[261] Personal communication. In writing this chapter, the author has received much help and stimulus from frequent discussions with Dr. Ramsey, to whom grateful acknowledgement is made. His own views differing somewhat from those here reported, are included in the article published in The Handbook of Medical Physics, p. 784, 1943, edited by Otto Glaser: The Yearbook Publishers, Chicago, Ill.

be mentioned: 1. The elastic bulging of the sarcolemma of the shortened muscle, or some similar elastic resistance to shortening; and 2. some chemical reaction yielding free energy which is used to re-establish side-chain linkages between myosin chains, thus forcibly pulling the polypeptide grid back into its resting configuration.

The elasticity of the sarcolemma has already been considered. Some force of this sort certainly exists, but it is a question whether by itself it is sufficient to explain the amount of "activity" which has been observed in relaxation. As to the second suggestion, it is sufficient to point out that it is just as easy to imagine linkages between neighboring myosin chains, which when established will pull the chains out into more or less straight lines, as to imagine linkages between different parts of the same chain, which will cause shortening or kinking of the chain. Thus, we could postulate relaxation linkages as well as contraction linkages. Until we know exactly what the chemistry of the folding process may be, either of these postulates has equal a priori validity. The combination of phosphate with myosin, as proposed by Kalckar,[252] behaved as a relaxation linkage, but it might equally well have been suggested as a contraction linkage without, apparently, violating any known facts.

In most respects, there is little to choose between these two theories. The active relaxation theory has the advantage that it fits better with the all-or-none law, particularly as it applies to single muscle fibers. These studies (Ramsey and Street) suggest that there is no fatigue of the contractile mechanism proper—if an individual fiber responds at all, it develops the same tension or shortening, whether it is fatigued or not. (There are some exceptions, or apparent exceptions, to this rule, which need not be considered here.) After a certain number of stimulations, the fiber may suddenly fail to respond, and it is this progressive dropping out of the individual fibers in the multifibered muscle which accounts for the phenomenon of fatigue as it is ordinarily encountered. In terms of the active relaxation theory, this means that the myosin machinery does not fatigue, but only the ability to respond or the excitability. On the other hand, there is no evidence that the relaxation (assuming it to be active) ever fatigues, either, except after the fiber has been allowed to shorten excessively (when it goes into the "delta state" and thus fails to relax), or under conditions which induce rigor. Rigor itself may well be regarded as a sign of failure of an active relaxation process.[253] When a single fiber fails to respond, however, it remains inactive in a state of relaxation (not in contraction). It has recovered from its last contraction and energy has been

[252] H. M. Kalckar, Chem. Rev., **28**: 71, 1941.

[253] This theory was very nicely stated in 1871 by Radcliffe, whose ideas on the subject developed from seeing a rabbit in the death spasms from strychnine poisoning. "Is it possible, I asked myself, that life may show itself, not in causing contraction, but in keeping the muscle relaxed, and that the doctrine of muscular motion may need reforming in accordance with this idea?"—Dynamics of Nerve and Muscle; Preface: MacMillan, London, 1871, by C. B. Radcliffe.

restored to the myosin chains, which are ready to "contract" again. But contraction has become temporarily impossible because the fiber can no longer be stimulated. For this theory, therefore, it is necessary to suppose that the process of excitation is something quite apart from the process of contraction, which, apparently, does not fatigue.

On the other hand, the active contraction theory is favored by the heat production data. In the first place, Hill[264] has found no heat in relaxation other than that derived either from the lowering of the weight in relaxation or from the potential energy of the tensed muscle. With his improved technique, he did not confirm a moiety of the relaxation heat described by Fenn,[265] which apparently came from some active relaxation process. More important than this evidence, however, is the fact, already mentioned, that extra energy for work is liberated during contraction rather than relaxation. If contraction were merely passive, the energy (heat plus work) liberated during contraction should depend upon the amount of shortening or upon the area of the length-tension diagram "covered" by the shortening, but should be independent of the load or the tension under which the shortening took place. Since, in the last analysis, the total initial heat is a measure of the total chemical breakdown, and since the variation in the heat (plus work) due to load occurs in contraction, it is reasonable to conclude that the variations in the amount of chemical breakdown also occur during contraction. The number of myosin linkages which are activated, therefore, by a given stimulus, or the amount of energy which each linkage liberates, must be determined by the load or other mechanical conditions after the stimulus is over. Contraction, in other words, is not merely the removal of some "stop" which prevents shortening of a previously stretched spring, but involves some other process which discriminates between different mechanical conditions and liberates more energy in proportion to the work done in addition to the energy which might be calculated from the length-tension diagram and the maintenance heat.

12. Contractures.—Thus far in our consideration of the mechanical activity of muscles we have dealt only with the typical all-or-none twitch and the tetanus built up by a series of twitches. This presents, however, a very limited view of the actual situation, for there is a great variety of muscles not only in the different parts of the vertebrate body, but also in the various species of animals, and the nature of their activities differs widely. Even in one and the same muscle, there may be different kinds of fibers with different characteristics, and, finally, the same fiber under suitable conditions may exhibit mechanical activities which differ markedly from the all-or-none contractions usually used for student demonstrations. These abnormal responses are conveniently classified as contractures, meaning that they lack some of the usual features of a muscle contraction. Generally, contractures are not conducted from one end of the muscle to

[264] A. V. Hill, Proc. Roy. Soc., London, **B, 126**: 136, 1938.

[265] W. O. Fenn, J. Physiol., **58**: 373, 1923–24.

the other, and are diminished in tempo. They differ from tetani in lacking the repetitive action potential which is the inevitable sign of the all-or-none mechanism.

The most careful and critical analysis of the nature of contractures is to be found in the classical review by Gasser,[266] which is as good today as when it was written. Contractures are usually elicited by exposure to chemical agents, which are supposed to act directly upon the contractile mechanism without involving the conducting action potential mechanism. The same effect, however, can be produced by electrical stimulation. Gerard and Gelfan[267] arranged to stimulate, in this way, the isolated single muscle fibers in the retrolingual membrane of the frog, and were able to observe local responses in the vicinity of the electrodes which were not conducted, and which were graded in intensity in proportion to the intensity of the stimulus. When the stimulus intensity reached a certain critical point, however, the all-or-none mechanism was excited, and the whole fiber gave the familiar twitch response. Gelfan and Bishop (1936)[268] showed that their local contractures were not accompanied by any action potential, but represented the response of the contractile mechanism alone.

Pricking a muscle fiber with a fine pin point may initiate either showers of normal impulses accompanied by action potentials[269] or, on occasions, local contractures lacking altogether in any electrical sign.[270] According to Gelfan and Bishop, it is even possible to initiate, in this way, conducted contractures without action potentials,[270] which are difficult to distinguish from normal twitches in other respects. Thus the contractible mechanism is by itself conductile. Once the myosin threads begin to shorten in one spot, adjacent chains may also become involved until the whole fiber is contracted. The fact that this does not always occur, and that localized contractures are often observed, indicates that the all-or-none action potential mechanism is to be regarded as something superimposed upon the contractile machinery, which makes its responses more certain and predictable. The nature of conduction in the contractile machinery is not known, and the process is even more complicated by the finding of Steiman and Pratt,[271] that nonconducted contractures may occur at some distance from the point of electrical stimulation without any detectable mechanical activity in the intervening tissue. Nevertheless, something has been conducted which can be blocked by mechanical compression between the point stimulated and the point of response.

Some of these curious facts are difficult to reconcile with any current theories. They do serve to show, however, that the contractile mechanism

[266] H. S. Gasser, Physiol. Rev., **10**: 36, 1930.

[267] S. Gelfan and R. W. Gerard, Am. J. Physiol., **95**: 412, 1930.

[268] S. Gelfan and G. H. Bishop, Am. J. Physiol., **101**: 678, 1932.

[269] A. Wilska and K. Varjoranta, Skandinav. Arch. f. Physiol., **82**: 276, 1939; *ibid.*, **83**: 82, 88, 1939.

[270] S. Gelfan and G. H. Bishop, Am. J. Physiol., **103**: 237, 1933.

[271] S. E. Steiman and F. H. Pratt; Am. J. Physiol., **122**: 27, 1938.

can be separated from the action potential. There is usually, to be sure, a close correlation between the magnitudes of the mechanical and the electrical responses of muscles, but this is usually attributable to variations in the number of fibers involved, and does not prove a parallelism within a single fiber. Even with indirect stimulation through the nerve, localized contractures can be produced, as is shown by the contracture which was observed by Feng[272] in the neighborhood of the end plate at certain frequencies of stimulation and coincident with the appearance of an inhibition of myoneural transmission. If the hormonal theory of myoneural transmission is valid, it must be supposed that acetylcholine or similar agents liberated in an end plate can stimulate both the muscle action potential and the contractile machinery, and, probably, under some conditions, may produce only contractures similar to those caused by immersing an isolated muscle in a solution of acetylcholine. To what extent such local nonpropagated contractures occur *in vivo* must remain for future investigation to elucidate, but it is not improbable that further instances of this sort will be encountered. In any event, the mere existence of contractures focuses our attention upon the obscure processes which intervene between the initiation of the action potential and the onset of the mechanical response itself.

From the point of view of an inquiry into the fundamental mechanism of muscle activity, the contractures offer as good a starting point for observations as the more typical contractions. Qualitatively, at least, the two types of activity are identical, the same physical, chemical, and biochemical changes being involved. In some cases, these are better studied in contractures, which are more amenable to analysis because of their slowness. Since, however, muscles in contracture readily pass into irreversible rigor, the responses cannot be so readily repeated for further observations. On account of this similarity between contractures and ordinary twitches, no special consideration of the mechanism of the former is warranted.

13. Theories of Muscle Contraction.—Throughout the time of recorded history man has been inventing machines for transforming energy into useful mechanical work. In many of these machines, the source of energy is in the form of chemical potential energy. To transform this into work, it is first transformed into heat, or the original material is burned to gaseous products which occupy an expanding volume. Various of these man-made machines have served as models for explaining the muscle machine. Thus, the muscle has been supposed to be a heat machine (Engelmann[273]), and the shortening has even been attributed to an increase in volume, due to accumulation of CO_2 in the form of a gas (Wacker[274]). Others have attributed the contraction to electrical forces, as in an electric motor. No one, apparently, has reversed this process and used the muscle as a model of a

[272] T. P. Feng, Chinese J. Physiol., **14**: 209, 1939.

[273] T. W. Engelmann, Proc. Roy. Soc., London, **57**: 411, 1895

[274] L. Wacker, Pflüger's Arch. f. d. ges. Physiol., **168**: 147, 1917.

machine for industrial use, although such a machine, shorn of its painful afferent impulses, would be more efficient, and hence more economical, than any machine now in use. Even if the muscle could be imitated in the laboratory, however, its practical usefulness would probably not be very great, because of the delicateness of the parts, its susceptibility to bacterial invasion and to temperature changes, and the fact that it could not utilize chemical energy in any natural form, such as crude oil, without first elaborating it into very special chemical compounds.

The contraction of muscles is so commonplace a phenomenon to all of us that it seldom arouses our conscious curiosity. Yet nothing could be more dramatic than to witness a muscle being "galvanized into activity" by a modern galvanic cell. A mechanical force suddenly leaps forth from a viscid, transparent, inert jelly. Whence comes this miraculous transformation? We have a conviction that the fundamental idea is no more complicated than any gas engine, and the essential moving parts are probably far simpler. So we find ourselves faced with one of the most tantalizing of biological problems. It is tantalizing just because it looks so absurdly simple and easy. It is, nevertheless, a problem which has challenged man's scientific ingenuity for centuries, and in every age the most recent advances have deluded investigators into thinking that ultimate success was almost within reach. So it is today. The necessary methods of study seem to be ready for use, and much of the necessary information is at hand to complete the theory.

It has now become apparent that the contractile machinery of muscles has developed from the protein structure of the undifferentiated cells. The fibrous, rather than the globular, proteins of the cell have become mobilized in the vertebrate muscle into a close-order array of contractile units. There is probably nothing essentially new in the mechanism which is not already present in the ameba.

Muscle physiology, like the ameba, has advanced through the ages by a series of "lunges." The pressure of new discoveries gradually builds up in the endoplasm of the research laboratories until it suddenly bursts through the gelated ectoplasm of preconceived notions, letting loose a flood of new ideas and new theories which advance like a pseudopod into new fields of knowledge.

a). *Historical development.*—The first noteworthy advance of this type was initiated by the great discovery of animal electricity by Galvani, which laid the foundation for the electrical theories of contraction. Even today, one hears suggestions that a striated muscle is a series of plates with alternating positive and negative charges.[275] A second great advance came from the introduction of the kymograph into physiology by Ludwig. This let loose a host of papers in which the length and tension of the muscles were recorded under every imaginable condition and situation and with every

[275] H. R. Procter, Kolloid-Ztschr., **10**: 281, 1912; G. Pusch, München. med. Wchnschr., **71**: 1164, 1924.

imaginable device—inertia levers, tension levers, isotonic and auxotonic levers, free shortenings and after-loaded shortenings, and muscles held tight and released with a snap. All these helped to provide precise observations, but provided no important suggestion as to the mechanism of contraction.

As rapidly as the science of thermodynamics developed, it found fertile field for its application in muscle. It was Helmholtz,[276] working on the problem of the conservation of energy, who first sought to find out where the energy for a contraction came from. He measured the rise in temperature of a contracting muscle, and satisfied himself that chemical reactions of substantial energy value were responsible for the dramatic mechanical effects observed. Later, Fick[277] went farther in the same direction, and actually established an early value for the mechanical equivalent of heat by comparing the heat generated in a muscle with the energy lost simultaneously by the fall of a weight which stretched it. Fick was a teacher of both physics and physiology, and he was quick to see that the mechanical efficiency of a muscle was too high for any heat engine, which was limited for its highest temperature to the relatively low value recorded in stimulated muscle. Except for the fact that it was contrary to the newly announced second law of thermodynamics, Engelman's[278] piece of catgut or violin string, heated with a coil of wire, made an excellent model of a muscle contraction.[278] Later, the same model was made to contract by the application of acid, and served a better purpose as experimental support for a colloid chemical theory (Strietmann and Fischer[279]). The impetus derived from heat production measurements has not yet spent itself, and the results have formed a substantial guiding framework into which all contraction theories must be made to fit. But the results seem to be far removed from the actual mechanism of the contraction.

Another of the great advances dates from the work of Fletcher and Hopkins,[280] who did not discover lactic acid in muscle, but who learned to control it. They chilled the muscle before grinding it up for analysis, thus eliminating the traumatic formation of the substance, and so they revealed clearly, for the first time, the fact that lactic acid is formed in contraction and anoxia, and disappears in recovery in the presence of oxygen. Thereafter, up-to-date teachers were no longer satisfied to demand of their students technically perfect kymograph records of after-loaded muscle

[276] H. von Helmholtz, Arch. f. anat. Physiol., p. 144, 1848.

[277] A. Fick, Mech. Arbeit und Warmentwick. bei der Muskeltatigkeit; Leipzig, 1882.

[278] The efficiency of a heat engine at an absolute temperature of T_o is given by $\frac{T_1 - T_o}{T_o}$ so that at a resting temperature of 19°C. the stimulated temperature would have to be 92°C. to give an efficiency of 25%.

$$\frac{92 - 19}{273 + 19} = 0.25.$$

[279] W. H. Strietmann and M. H. Fischer, Kolloid-Ztschr., **10**: 65, 1912.

[280] W. M. Fletcher and F. G. Hopkins, Proc. Roy. Soc., London, **B, 89**: 444, 1917.

twitches, but they began, instead, to devise experiments on muscle metabolism. Thus began the "lactic acid era" which was continued by the classical heat production measurements of A. V. Hill[281] and the chemical experiments of Meyerhof,[282] which culminated in the Hill and Meyerhof theory. The contributions of these two investigators, working independently, left the whole matter so well worked out in 1924 that one knew instinctively that it could not last. While Hill and Meyerhof did not commit themselves to any particular theory of the actual mechanism of shortening, the prestige of their chemical and thermal analyses of the muscle contraction readily provided a basis for innumerable theories which all regarded lactic acid as the causative agent. The general fault in all these theories was that no provision was made for the transfer of energy derived from the formation of lactic acid into the work to be done. The lactic acid was formed, and the resulting energy was degraded into heat, after which the lactic acid might migrate to, or find itself in, a "contraction spot," where it could, perhaps, raise the osmotic pressure or ionize some protein, solidify a liquid crystal (Clark [283]), or form a solid surface film on the fibrils (Garner[284]), and so cause contraction. The energy for such a contraction, however, would have to come from the lactic acid protein reaction, the energy of which would be quite inadequate to explain the high efficiencies observed; and the osmotic pressure produced was also inadequate to explain the work, even if all the lactic acid appeared in one spot in the form of a 90% solution (Meyerhof[285]). The problem of an energetic coupling between the products of glycolysis and the products of contraction in other words was not properly taken into account.

The lactic acid theories were also deficient in a far simpler way because it soon transpired that the lactic acid did not appear in the muscle anyway until after the contraction was all over. So there came another revolution[285] which ushered in the "phosphate era." Now the first detectable change was a breakdown of the newly discovered phosphocreatine, and, later, the loss of one phosphate from adenylpyrophosphate. Little by little the biochemists began to take over the teaching of muscle chemistry, while the physiologists seemed to be turning their attention elsewhere. More and more chemical reactions, more enzymes and co-enzymes appeared on the muscle scene, and they all appeared to revolve about the phosphate molecule. If a molecule of adenylpyrophosphate lost a phosphate on stimulation of the muscle, it collected another from a phosphocreatine neighbor. And the creatine, in turn, picked up one from phosphopyruvic acid, which (as hexose) had received it originally from adenylpyrophosphate.

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[281] A. V. Hill, Muscular Activity: Williams and Wilkins, Baltimore, 1926.

[282] O. Meyerhof, Die Chemische Vorgänge im Muskel: Julius Springer, Berlin, 1930.

[283] Janet H. Clark, Am. J. Physiol., **82**: 181, 1927.

[284] W. E. Garner, Proc. Roy. Soc., London, B, **99**: 40, 1925.

[285] O. Meyerhof, Handb. d. norm. u. path. Physiol., VIII, **1**: 530, 1925.

[285] A. V. Hill, Physiol. Rev., **12**: 56, 1932.

Stimulation started the phosphate gears grinding, and somewhere in the chain the energy of a phosphate bond was thrown out into the contraction machinery. The phosphate era was one of marked progress, not unaccompanied by bewilderment, for it seemed to lead to no clean-cut theory of contraction which an elementary student could comprehend. The theories of contraction were left to the pictorial powers of histologists, and students of physiology were to be found struggling with multitudinous details of muscle chemistry.

With the work of Meyer[287] and Astbury,[288] aided by many others, "the myosin era" dawned. Once again the physiologist had some machinery to talk about in connection with muscle. The flame has spread into the remotest corners of biology. The newer knowledge of the configuration of protein structure has illuminated innumerable problems, from the structure of protoplasm to the nature of viruses. Muscle physiology also has advanced greatly under the impetus of these new ideas. Every aspect of the subject must now be scrutinized again to see how it will fit into the submicroscopical picture. It is from this point of view that this chapter has been written. Now, at last, we seem to have not only the chemical cycle which provides the energy for work, but also the machinery for doing the work. The phosphate cycle provides the energy; the partly folded molecules of myosin cause the contraction by superfolding. And even more recently, the myosin itself seems to have been fitted into the energy cycle to give us an outline of the modern theory.

b). *Surface tension theory.*—To some extent it is true that the prevalent theories of muscular contraction do not change as the subject progresses—they are merely revised as to details and brought up to date without alteration of the fundamental idea. An example of this is the surface tension theory. When first presented, the main experimental support was found in the fact that the tension developed in a twitch, like surface tension, is decreased by a rise of temperature (Bernstein[289]). Further consideration showed, however, that this evidence was of no value. The difficulties encountered in the interpretation of temperature effects of this sort have already been discussed. In general, it may be said that the tension produced under a given set of conditions may be regarded as dependent upon the amount of energy available, and cannot be regarded as a measure of the true force of the contractile machinery.

Originally, the surface tension theory was conceived of as applying to cylindrical sarcomeres, which increased in surface tension at the moment of stimulation, thus rounding up and shortening. When it became evident that the surfaces involved were too small to provide the required forces, smaller units were considered. But, even if the surfaces of the fibrils are

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[287] K. H. Meyer, Biochem. Ztschr., **214**: 253, 1929.

[288] W. T. Astbury, Science Progress, **133**: 1, 1939; Ann. Rev. Biochem., **8**: 113, 1939.

[289] J. Bernstein, Pflüger's Arch. f. d. ges. Physiol., **122**: 129, 1908.

considered, it is necessary to assume a surface tension of 110 dynes per cm. in order to explain the force exerted by muscle.[290] Considering that each fibril is made up of micelles and assuming, with Fischer,[291] that each micelle has a diameter of 5 mμ, it may be calculated that the distance along the edges of all the micelles (numbering about 3.7×10^9) in a square millimeter cross-section of muscle is 5800 cm. If the muscle force is taken as 22,000 dynes per mm^2 (Hill[290]), then it is necessary to assume a change of surface tension of only 22,000/5800 or 3.8 dynes per cm. to explain the force. The interfacial tension between oil and water is about 20 dynes per cm., so that this is a reasonable figure. Surface tensions against air are much higher and cannot properly be used for comparison, since no water-air interfaces can be involved in muscle. If, instead of using the micelles, one calculates on the basis of myosin molecules 10 mμ in diameter, there would be 20 chains in each micelle, and the total circumference in each $mm.^2$ of muscle would be 23,200 cm., so that a change of surface tension of a little less than 1 dyne per cm. would suffice to explain the force. A somewhat similar calculation was recently made by Lange.[292] Granting that it is permissible to *formulate* the contraction process in this way, it still remains necessary to explain what change in the myosin molecule has resulted in the calculated change in surface tension. It certainly seems probable that all the myosin molecules must be involved in the contraction, rather than only those which are confined to the surface. In a micelle of 5 mμ diameter, however, nearly half of the myosin chains (each 1 mμ diam.) would be on the surface, so that the reaction of myosin with the soluble products in the solution would be rapid.

A greater difficulty with the surface tension theory is in explaining the change of length. If a sphere is stretched out into a cylinder without change in volume, it will become unstable and break into two when the length of the cylinder becomes equal to its circumference,[293] or when the length is about 3.8 times the diameter of the original sphere. Since muscles or contractile tissues in general can shorten to say $\frac{1}{4}$ or less of their resting length,[294] and can be stretched reversibly to nearly twice their resting length, an over-all change of 8 fold or more is required. In any event, it hardly seems possible or profitable to regard a myosin molecule, or even a stack of 20 such molecules, as a fluid cylinder. When reduced to these dimensions, the surface tension hypothesis has largely lost its usefulness as a guide to investigation, but it has nevertheless attained thereby a certain measure of truth.

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[290] A. V. Hill, Proc. Roy. Soc., London, B, **98**: 506, 1925.

[291] Ernst Fischer, Cold Spring Harbor Symp., **4**; 214, 1936.

[292] E. Lange, Sitzungsb., d. phys.-med. Soc. zu Erlangen, **71**: 257, 1940.

[293] D. W. Thompson, Growth and Form, p. 227: University Press, Cambridge, 1917.

[294] According to Engelmann (Proc. Roy. Soc., London, **57**: 411, 1895), the muscles of insects can shorten to $\frac{1}{20}$, and the myopodia of Acanthocystis to $\frac{1}{50}$, their resting length.

c). *Colloid-chemical theory.*—Another group of theories may be classed as colloid-chemical (Lillie,[295] McDougall,[296] Strietmann and Fischer,[297] Meigs[298]), or osmotic pressure theories (Roaf,[299] Wacker,[300] Berg[301]). Both relate contraction to movements of water resulting either in asymmetric (lateral rather than longitudinal) swelling of colloidal particles, or in an inflation by osmotic pressure of cylindrical structures (sarcomeres), which become spherical and thus shorten. In the latter form, the theory is certainly untenable: 1. Because it does not apply to unstriated muscle; 2. because the known chemical changes in muscle could not produce enough osmotic energy to do the work of a muscle; and 3. because a shortening of only 33% is explained in this way. It seems unfortunate, therefore, that the theory should have been revived in recent years. (Szent-Györgyi[302].) In this latest application of the idea, however, the theory has also been extended into submicroscopic dimensions, and in this form the distinctions between the colloid-chemical theory, based on the behavior of catgut or gelatin in electrolyte solutions, the myosin theory based on protein structure, and the osmotic theory based on changes in the hydration of myosin molecules, largely disappear. All these theories now appear to have been reduced to a single least common denominator—the myosin chain.

Indeed, the folding of the myosin chain seems to be the inevitable terminus of any theory, once it is granted that a fibrous protein is responsible for the contraction. Then, when the whole muscle shortens, the long molecules of which it is composed must also inevitably roll up, or kink themselves into some more compact structure. Speaking very generally, it may be said that the fibrous protein coagulates or aggregates in a reversible manner. Now, coagulation can be explained in a variety of ways. An increase of surface tension, correlated with a change in potential, may cause an aggregation of individual particles and a resulting decrease of surface area. Or there may be a decrease in hydration, resulting in loss of the solvate layer, closer approximation of neighboring molecules, and the establishment of new hydrogen bridges. Finally, there may be more specific reactions which permit neighboring points on a chain to combine together, thus drawing the long chain into loops. Thus, in the original theory of Meyer,[303] a change in *p*H was supposed to bring the myosin to its isoelectric point and cause a sort of intramolecular zwitterion formation, whereby adjacent COOH and NH_2 groups became COO^- and NH_3^+ and, so combined together, causing the molecular chain to coil upon itself.

[295] R. S. Lillie, Science, **35**: 247, 1912.
[296] W. McDougall, Quart. J. Exper. Physiol., **3**: 53, 1910.
[297] W. H. Strietmann and M. H. Fischer, Kolloid-Ztschr., **10**: 65, 1912.
[298] E. B. Meigs, Ztschr. f. allgem. Physiol., **8**: 104, 1908.
[299] H. E. Roaf, Proc. Roy. Soc. London, B, **88**: 139, 1915.
[300] L. Wacker, Pflüger's Arch. f. d. ges. Physiol., **168**: 147, 1917; **169**: 492, 1917.
[301] W. N. Berg, Pflüger's Arch. f. d. ges. Physiol., **149**: 195, 1912.
[302] A. Szent-Györgyi, Enzymologia, **9**: 98, 1940.
[303] K. H. Meyer, Biochem. Ztschr., **214**: 253, 1929.

Other stereochemical suggestions have been proposed by Astbury[304] to account for the various degrees of folding of the fibrous proteins, as illustrated by the extended β-keratin, the partially folded α-keratin, and the supercontracted keratin which results from the treatment of hair with steam. The work of Mark[305] on the stereochemistry theories of the stretching of rubber are of interest in this connection, for they suggest the great range of lengths which results from molecular rearrangement caused merely by rotation of a molecule about its free C—C bonds. The problem of coagulation, therefore, has reached the molecular stage, and so have theories of muscular contraction. In some measure it is true that many of the older theories have in consequence become one. Present discussion is not concerned with a theory of contraction, but rather with the chemistry of the folding of myosin molecules.

d). *Myosin folding.*—In the older theories, the folding of the myosin was due to the formation of lactic acid, and, later, to the alkalinity resulting from the breakdown of phosphocreatine. According to Szent-Györgyi,[302] it is due to the liberation of potassium. In support of this he can show that artificial myosin threads will shorten when exposed to KCl. It is known, furthermore, that K is liberated from muscles on stimulation, and that muscles will contract when exposed to KCl. It is hard to believe, however, that the myosin is not exposed to the same amount of K before stimulation as afterward, and it is quite as likely that the loss of K from stimulated muscles is the result, rather than the cause, of contraction. It is asserted by Ernst and Morocz,[306] to be sure, that previously bound K becomes unbound or ionized at the moment of stimulation, but this conclusion is nothing more than a description of his experiment in which it was found that more K can be extracted from muscle when stimulated than when resting. The decrease in volume which occurs in contraction is no proof of this theory, even though it be true that the solution of K in water leads to a decrease in volume, and even though the decrease in volume is approximately what might be expected if the amount of K lost from the muscle were suddenly dissolved. There is still no reason to believe that all the K in muscle is not dissolved or ionized, even before it is stimulated. Indeed, it is impossible to make up the necessary osmotic pressure in the fibers unless nearly all the potassium is in ionized form.[307] Most of the decrease in volume is quantitatively explained by the breakdown of phosphocreatine (see p. 482), so that there would seem to be little volume change remaining which could be attributed to K. The crucial experiment which elucidates the relation of K to muscle contraction still awaits discovery.

Muscle contraction has also been attributed to the liberation of calcium, but the evidence for this theory is even more fragmentary than the evidence

[304] W. T. Astbury, Science Progress, **133**: 1, 1939.

[305] H. Mark, Chem. Rev., **25**: 121, 1939.

[306] E. Ernst and E. Morocz, Enzymologia, **9**: 133, 1940.

[307] W. O. Fenn, Physiol. Rev., **20**: 377, 1940.

for the K theory. The theory is based largely upon the contraction which results when a muscle is immersed in isotonic solution of $CaCl_2$.[308] Such a concentration is far beyond physiological limits. Some evidence for a liberation of Ca in contraction has been advanced,[309] but other attempts to demonstrate such a change were unsuccessful.[310] Ca has been found to serve as a co-enzyme in adenylpyrophosphatase reactions,[311] but this by itself means little. Mg is also a co-enzyme in other reactions of the energy cycle. Further evidence concerning the role of Ca is therefore required, particularly some better proof that the concentration of free or ionized Ca is increased by stimulation.

e). *Myosin as an enzyme.*—The most recent theory of myosin shortening originated in the discovery that myosin can act as or is an enzyme for the removal of PO_4 from adenylpyrophosphate (APP).[311,312,313,314] Moreover APP when added to a myosin solution causes a shortening of the molecules as indicated by a decrease in the double refraction of flow. (Needham *et al.*[315,316].) In this respect the APP is highly specific and produces its effect in a much lower concentration than is required by other sustances. Thus a 60% decrease in birefringence is caused by 1.5 M urea, 1.0 M monovalent cation, 0.2 M bivalent cation or a *p*H of 10, while APP has the same effect in a concentration of 0.004 M and can cause 15% decrease in birefringence in a concentration of 5×10^{-5} M.

These are striking facts but it is still not clear just how they explain the mechanism of contractility. It was suggested by Needham, *et al*,[315] that the combination of APP with myosin was the immediate cause of the shortening and that relaxation occurred at the expense of free energy derived from the splitting off of one PO_4 from APP. It may be, however, that this energy is released during contraction and is the immediate source of the muscular work which is performed. Furthermore, the actual shortening may result from a phosphorylation of the myosin by the PO_4 donated by APP as suggested by Kalckar (see p. 485 above). Mention has already been made also of the theory that the shortening is due to the effect of K, which also causes a decrease in birefringence.[315,302] Likewise,

[308] L. V. Heilbrunn, p. 188 in The Cell and Protoplasm; Symposium of the A.A.A.S.: Science Press, 1940; also Physiol. Zool., **13**: 88, 1940.

[309] E. Weise, Arch. f. exper. Path. u. Pharmakol., **176**: 367, 1934.

[310] W. O. Fenn, Doris M. Cobb, Jeanne F. Manery, and W. R. Bloor, Am. J. Physiol., **121**: 595, 1937; see also A. Keyes and L. Adelson, *ibid.*, **115**: 539, 1936.

[311] K. Bailey, Biochem. J., **36**: 121, 1942.

[312] W. A. Engelhardt and M. N. Ljubimowa, Nature, **144**: 668, 1939; also W. A. Engelhardt, Advances in Contemp. Biol., **14**: 2, 1941.

[313] A. Szent-Györgyi and I. Banga, Science, **93**: 158, 1941.

[314] D. M. Needham, Biochem. J., **36**: 113, 1942.

[315] J. Needham, Shi-Chang Shen, D. M. Needham, and A. S. C. Lawrence, Nature, **147**: 766, 1941. See also M. Dainty, A. Kleinzeller, A. S. C. Lawrence, M. Miall, J. Needham, D. M. Needham, and S. Shen, J. Gen. Physiol., **27**: 355, 1943.

[316] J. Needham, A. Kleinzeller, M. Miall, D. M. Needham, and A. S. C. Lawrence, Nature, **150**: 46, 1942.

Ca has been implicated because it catalyzes the dephosphorylation of the APP by myosin. Bailey[311] has supposed that the stimulus to the muscle liberated Ca, which causes the contraction by assisting in the transfer of PO_4 from APP to myosin. Possibly there is truth in all these theories and contraction should be regarded as intimately dependent upon K and Ca as well as APP, PO_4, and myosin. In any event, the discovery of Engelhardt and Ljubimova[312] seems to be the starting point of a new era in muscle physiology. In this new era, the coupling between myosin and the energy cycle will be clarified. This is now easy, for myosin itself has become a component in the phosphate cycle. At last the contractile machinery has become more than a structure which is passively acted upon by lactic acid or some other metabolic product. It is as if steam had at last been admitted to the previously empty cylinders of the muscle machine.

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Section 8

PASSIVE PENETRATION AND ACTIVE TRANSFER IN ANIMAL AND PLANT TISSUES

By RUDOLF HÖBER

INTRODUCTION; THE PROBLEMS

In this section, once more, permeability of cells and tissues will be the principal subject, as it was in Section 4. Here, however, the term "permeability" will be used chiefly in a sense different from that prevailingly used in the earlier discussion. As repeatedly mentioned before, permeability means the capacity of a physiological boundary to allow the transit of solute or solvent molecules from the surroundings into or across a cell or a layer of cells. In many cases the process can be fully accounted for as a "spontaneous" diffusion or osmosis. But in other cases the passage appears to be "enforced" by energy ordinarily derived from cell metabolism and indispensable for the process. Thus, it has become customary to distinguish between passive penetration and active transfer, both as being aspects of permeability, but which are essentially different in nature, and which have sometimes been designated as "physical" or "physicochemical permeability" and as "physiological permeability."[1]

Investigation of the former was initiated in intimate association with the beginning of the physicochemical interpretation of physiological phenomena. It was the era of Van't Hoff, Pfeffer, Arrhenius, and Nernst. The present section will deal mainly with the problems of the physiological permeability, a concept originating in the earlier physiology at a time when one still attempted to describe the action of entire organs in terms of mechanics, so successfully applied to the physiology of circulation, of respiration, and of lymph formation, but then increasingly found to be inadequate in the physiology of urine formation, of intestinal absorption, of glandular action, and of others. In pursuing this line of research, it became obvious that in analyzing the activity of those organs, which accomplish a shift from one place to another of water and of certain dissolved substances in characteristic concentration ratios, one was compelled to differentiate between that part of the functional result which might be due to passive penetration, and that part which might be due to active transfer. This problem has been solved, as yet, in only a few cases, partially owing to the simple fact that sometimes it is difficult to define what is active and what is passive.

Active transfer usually is manifested by the establishment of an unbalance in concentrations. Solute or solvent molecules are shifted "up-hill" against a diffusion gradient; they are "accumulated" as the result of osmotic work, enabled by the liberation of metabolic energy. However, concentration gradients may also arise without metabolic intervention, for

[1] R. Höber, Physikalische Chemie der Zelle u. der Gewebe, 6th ed.: W. Engelmann, Leipzig, 1926.

example, when a Donnan equilibrium is established. Consequently, the simplest way to distinguish between active transfer and passive penetration seems to be to cut off the source of energy by a metabolic poison, by anoxia, by a narcotic, or by other means. However, it will be seen later that this procedure can fail to give a clear-cut result. Another criterion is based upon the fact that the Donnan distribution applies to ions, but not to nonelectrolytes. Let us consider some examples:

1. The classical example is the distribution of ions arising when a solution (i) of a colloidelectrolyte NaR is separated from a solution (o) of NaCl by a membrane, which is permeable to Na and Cl, impermeable to R. The resulting equilibrium can be expressed by the equation: $Na_i : Na_o = Cl_o : Cl_i$, i and o indicating the "inside" and the "outside" solution, $Cl_o > Cl_i$, $Na_i > Na_o$ (pp. 68 and 79). The corresponding inequality of cation and anion distribution has been ascertained in a great number of physiological systems, where two body fluids were separated by a physiological membrane, e.g., the fluid content of the red blood corpuscles and the serum separated by the plasma membrane, or serum (s) and edema fluid (f) separated by the capillary wall. This latter system has been analyzed, among others, by Hastings, Salvesen, Sendroy, and Van Slyke,[2] with the following result: $(BHCO_3)_s : (BHCO_3)_f = 0.980$; $(Cl)_s : (Cl)_f = 0.976$; $(Na)_f : (Na)_s = 0.922$; $(H)_f : (H)_s = 0.91$. The calculated ratio r is 0.969.

2. A somewhat different form of Donnan distribution is illustrated by the following experiment[3] (see also p. 254). A dried collodium membrane separates two solutions, one (i) containing $m/1400$ $K_2SO_4 + m/10$ H_2SO_4, the other (o) $m/1400$ K_2SO_4. The two solutions are kept in osmotic balance by addition to o of nonpenetrating glucose. The membrane is permeable to K and H, impermeable to SO_4; in other words, at the beginning of the experiment K_i and K_o are equal, H_i about 10^6 times greater than H_o. Since H and OH are reciprocal to each other, the final ionic distribution should be:

$$K_i : K_o = OH_o : OH_i = H_i : H_o$$

After 14 days of ionic exchange across the membrane the following concentration ratios were found:

$$K_i : K_o = 10:1 \text{ (instead of } 1:1); \qquad H_i : H_o = 80:1 \text{ (instead of } 10^6:1).$$

In other words, K was shifted from outside to inside against an increasing concentration gradient, whereas in the opposite direction H_i fell to a value below the initial value. Thus, the two ratios, which at the beginning were highly different, approached each other toward the same theoretical value.

This experiment has suggested interpretation of the conditions in muscle fibers as a Donnan distribution, because K_i in muscle is about 30 to 40

[2] A. B. Hastings, H. A. Salvesen, J. Sendroy, and D. D. Van Slyke, J. Gen. Physiol., **8**: 701, 1926.

[3] H. Netter, Pflüger's Arch. f. d. ges. Physiol., **220**: 107, 1928.

times greater than K_o, and H_i greater than H_o. As a matter of fact, this concept is substantiated by the fact that the fibers have appeared to be selectively permeable to K and to H, impermeable or at least much less permeable to anions and to Na, and that by changing H_o (and OH_o respectively) the ratio $K_i : K_o$ can be displaced in one or the other direction.[4] However, the quantitative agreement between theory and experiment is not very satisfactory, as might have been anticipated on account of a number of complicating circumstances, caused mainly by metabolic reactions, which are released by unphysiological concentrations of H and K (p. 309 and Sec. 7).

3. Another variety of Donnan distribution, due to a selective ion permeability of a membrane, is shown in an experiment of Teorell.[5] A layer of carbontetrachloride is placed between a solution of 0.2 norm. sodium benzoate (*i*), which is kept at a fairly neutral reaction by the addition of buffer, and a solution of 0.1 norm. NaCl (*o*). The membrane is impermeable to Na, and behaves as though permeable to H and benzoate, since the undissociated free benzoic acid is soluble in the organic solvent. After two hours, pH_o was found to be 3.80, pH_i 6.90. In other words, the concentration of H outside has increased *several hundred times*, whereas that of benzoate has fallen with the concentration gradient to a lower value.

This model was investigated by Teorell with the idea of correlating the process governing the accumulation of H ions to that involved in the production of gastric juice. Although this model was found useless, since the gastric glands are able to raise the H concentration about three million times above the blood level (p. 599), attention may be drawn to the fact that the formation of the slightly alkaline secretion of the pancreas has been attributed tentatively to Donnan distribution across the epithelial cells of the gland (p. 605).

It follows that, with regard to physiology, the cases of Donnan equilibria mentioned under 1, 2, and 3 can be identified as such—at least in principle—first, by the distribution of the penetrating ions, the ratios of the various cations being identical and being reciprocal to those of the anions, and, second, by the failure to show a relationship between the establishment of the unequal distributions and metabolic reactions. In other words, the Donnan distribution of ions belongs to the category of passive penetration.

Finally, another form of passive penetration should be described, which likewise leads to an unequal distribution, but, although dependent upon a metabolic reaction, is essentially the result of a passive penetration. As mentioned previously (p. 260), Osterhout[6] has investigated the distribution of the weak acid H_2S ($K = 5.7 \times 10^{-8}$) between the acid cell sap of *Valonia*, which by means of the cell metabolism is maintained at

[4] H. Netter, Pflüger's Arch. f. d. ges. Physiol., **234**: 680, 1934; W. O. Fenn and D. M. Cobb, J. Gen. Physiol., **17**: 629, 1934.

[5] T. Teorell, Skandinav. Arch. f. Physiol., **66**: 225, 1933.

[6] W. J. V. Osterhout, J. Gen. Physiol., **8**: 131, 1925.

*p*H 5.8, and the surrounding sea water, the *p*H of which, by the addition of HCl or NaOH, was varied between 5 and 10. The result was that, e.g., at *p*H 5.2 the outside concentration (c_o) of H_2S was found to be approximately the same as the inside ($c_o : c_i = 100:97$), whereas, e.g., at *p*H 8.5 it was about 25 times higher than that inside ($c_o : c_i = 100:4$). The explanation is as follows: at *p*H 5.2 outside, H_2S is almost entirely present in the form of undissociated molecules, which, like those of many weak acids, enter and pass as such by diffusion across the protoplasmic wall, whereas the ions cannot penetrate. At *p*H 5.2 outside, the concentration of undissociated H_2S is slightly higher, the concentration of the ions slightly lower, than at *p*H 5.8. However, at *p*H 8.5 outside, H_2S will be prevailingly ionized, and only the small remainder of molecular H_2S can distribute freely until diffusion equilibrium. This interpretation is supported by the fact that, for the entire scale of *p*H, the concentration of total H_2S inside (unionized + ionized) was found to be approximately the same as the concentration of undissociated H_2S outside, as can be calculated from the concentration of added H_2S and its *p*K. It follows that *de facto* we are dealing with a passive penetration. However, provided that the natural difference of *p*H between the cell sap of *Valonia* and the surroundings is based upon the metabolic activity of the protoplasmatic wall, as it probably is, each change in the rate of this activity will be followed by a redistribution of the weak electrolyte in one or the other direction, and will produce the illusion of an active transfer.[7]

A corresponding behavior can be demonstrated with the salts of weak bases, e.g., of alkaloids and of basic dyestuffs.[8] For instance, neutral red delivered to the outside of the proximal tubules of the frog kidney in a neutral saline solution by way of the renal portal vein, while an acid fluid flows from the glomeruli, appears highly accumulated in the tubular lumina, thus displaying the picture of an active transfer. In contrast, an alkaline fluid, passing from the glomeruli along the lumina, prevents the basic dyestuff from appearing concentrated.[9]

On the basis of these introductory remarks, an analysis of what—in a broader sense—has been called *absorption* and *secretion* will now be attempted. For this purpose we shall continue to distinguish between the life processes described under *passive penetration* and *active transfer*. In this way, the simpler facts can be made to serve as a foundation for understanding the more complex and less accessible phenomena. In order to facilitate the classification of the findings, the following criteria will be set up. A movement against the concentration gradient will in general be considered as indicating active transfer (1) if the movement is inhibited by stopping the concomitant chemical reactions, which may provide the

[7] M. H. Jacobs, Cold Spring Harbor Symp., **8**: 30, 1940.

[8] See E. Overton, Ztschr. f. physik. Chem., **22**: 189, 1897.

[9] R. Chambers, and R. T. Kempton, J. Cell. & Comp. Physiol., **10**: 199, 1937; R. T. Kempton, J. Cell. & Comp. Physiol., **14**: 73, 1939; see further, p. 603.

energy for effecting the up-hill shift; (2) if, in contrast to the Donnan distribution, cations and anions are transported simultaneously and in approximately equivalent amounts and in the same direction; (3) if the shift takes place with nonelectrolytes; (4), finally, if an unequal distribution of a weak electrolyte appears to be due to a *p*H difference originating from cellular metabolism.

The subsequent analysis of "absorption" and "secretion" will be made with reference to: (1) intestinal absorption; (2) urine formation; (3) permeability of the body surface; (4) the elaboration of digestive juices; (5) some remarks about the energetics of the active transfer, the transferring devices, and their action, including remarks concerning the action of the chorioidal plexus and the ciliary body.

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INTESTINAL ABSORPTION

For a long time, intestinal absorption has been the favorite subject for studying absorption. The intestine of higher animals offers for this purpose an extensive layer of epithelium, covering many folds, each of them often providing space for a great number of villi. This epithelial membrane bounds the wide cylindrical cavity of the lumen, which in an experiment can be easily filled with a solution, the absorption of which is to be investigated. This procedure would approximate the normal conditions, since, in the higher animal, absorption deals with solute plus solvent. The delicate epithelial layer, about 25 micra in thickness, is interposed between the solution and the subepithelial tissue, which to a large part can be considered fluid, since it includes the blood plasma in the capillaries and the lymph in the interstitial spaces. This subepithelial fluid, though small in volume, can be thought of as representing a much larger volume, because of the continuous circulation which sweeps away substances entering this space by absorption, and thus may keep the composition of the subepithelial fluid fairly constant. Local variations of concentration in the experimental solution when it comes into immediate contact with the epithelial membrane, in filling the narrow spaces between the folds and between the villi, are diminished by the stirring effect of the movable villi.

These complicating conditions raise the question whether absorption experiments are capable of permitting a definite decision concerning the relative importance of diffusion and osmosis, or whether superimposed processes may cloud the interpretation.

Regarding the methods proposed for studying intestinal absorption, the nearest approach to a scheme suitable for fruitful physicochemical analysis seems to be the "loop experiment," although more than one obvious objection can be made even to this. In conducting such an experiment, the body cavity is opened and a section of the intestine is isolated by ligatures, rinsed with Ringer, and filled with a measured amount of the experimental solution. The conditions resemble those in a diffusion experiment, in that one can follow by chemical methods the passage of substances across the constant wall area of the loop during a definite length of time at the fairly constant temperature of the circulating blood. And, if one compares isosmotic solutions introduced successively at the beginning of each experimental period, the concentration gradients across the separating wall are identical, since the concentrations beyond the membranes are near zero or

rather constant, providing the solute in question is not one of the normal constituents of the body fluids and possibly subject to metabolic concentration changes. Absorption under the above conditions can be expected to obey Fick's law of diffusion (see Sec. 1, chap. 1) and to show the following characteristics: 1. The rate of absorption, i.e., the amount of solute leaving the loop contents in a certain time, is in a linear relation to the concentration; in other words, the percentage of absorption is constant. 2. When several substances are compared under identical conditions, individual differences in the rates of absorption should appear to correspond to Fick's diffusion coefficients.

However, it should be anticipated that the method described does not permit an exact confirmation of Fick's law to be obtained. Impairment of the vitality of the delicate epithelium, through mechanical insults following the repeated filling and emptying of the loop, uncontrollable changes of the blood circulation, the effect of the narcotics, and other factors, are unavoidable causes of irregularity. For many special problems it would be very useful to change the loop method in such a way as to provide the isolated loop with its own artificial circulation of an adequate perfusion fluid. This procedure has been tried by Öhnell.[10]

Additional methods have been proposed, which avoid some of the faults of the loop method, but introduce others. The procedure of Cori[11] has often been the method of choice in comparing the absorption of several substances, or absorption under other varying conditions. Groups of rats, which by their anatomical and their physiological behavior resemble each other as much as possible, are fasted, then fed the solutions by stomach tube, and killed after a suitable time. The total gastrointestinal tract is excised and the unabsorbed contents analyzed.

TABLE XLI.—ABSORPTION BY THE RAT INTESTINE OF TWO NONELECTROLYTES AT DIFFERENT CONCENTRATIONS

Erythritol[12]			Sorbose[13]		
At the beginning, molar conc.	After 25 minutes		At the beginning, molar conc.	After 60 minutes	
	Millimol. abs.	Per cent abs.		Millimol. abs.	Per cent abs.
0 06	0 130	54 1	0 14	0 10	23 3
0 09	0 235	65 0	0 29	0.19	21 9
0 12	0 207	62 2	0 46	0 26	19 4
0 18	0 393	54 4	0 60	0 42	23 3
0 24	0 585	55 5	0 76	0 54	23 9

[10] R. Öhnell, J Cell. & Comp. Physiol., **14**: 155, 1939.

[11] C. F. Cori, Proc. Soc. Exper. Biol. & Med., **22**: 495, 1925; J. Biol. Chem., **66**: 691, 1925.

[12] R. Höber and J. Höber, J. Cell. & Comp. Physiol., **10**: 401, 1937.

[13] F. Verzár, Biochem. Ztschr., **276**: 17, 1935. Average values of experiments with seven animals.

In the following, some experimental results are listed in an effort to describe the absorption of nonelectrolytes, of electrolytes, and of colloids, in terms of physical chemistry.

1. The Absorption of Nonelectrolytes.—There are not many observations concerning nonelectrolytes, although they are generally "foreign" substances and, as such, especially suitable for experiments because 1. their concentration in the body fluids outside the epithelial layer is 0; and 2. metabolic reactions supposedly do not interfere with the course of diffusion. Table XLI refers to experiments on rats, where the influence of concentration on the rate of absorption was studied.

Obviously, the course of these experiments, in at least a rough approximation, follows the predictions of Fick's law, even though—irrespective of the various aforementioned interfering factors—the regular diffusion probably was partially obscured by osmosis across the intestinal wall, due to the varying osmotic unbalance between the experimental solutions and the body fluid.

Another group of experiments deals with the rate of absorption of different nonelectrolytes presented in equimolar concentrations. Comparing such experiments to the corresponding observations with artificial membranes, the results provide a clue to the "physiological diffusion coefficients." Table XLII gives an example of the absorption of various aliphatic acid amides.[14]

TABLE XLII.—ABSORPTION BY THE RAT INTESTINE OF DIFFERENT NONELECTROLYTES AT THE SAME MOLAR CONCENTRATION

Exper. period	Substance	Per cent abs.	Mol. vol.	Remarks
a	Succinamide	3 4	126 4	Solutions 0.06 molar in ⅔ Ringer each
b	Lactamide	42.5	98 5	period (*a*–*f*): 25 min.
c	Acetamide	78 2	68.7	
d	Lactamide	36 7	98 5	
e	Succinamide	6.3	126 4	
f	Acetamide	68 4	68 7	

As explained in Sec. 1, chap. 1, in agreement with Einstein's law, the diffusion coefficients can be correlated with the molecular volume (M.V.)[15] They are the same whether determined in free diffusion or measured by diffusion through the pores of a sieve-like membrane, provided the pore diameter exceeds markedly the size of the molecules in question. The table shows that, on the whole, the observations with the acid amides meet the theoretical requirements. However, the differences of absorption rates observed are far greater than the differences in the rates of free diffusion or of diffusion through membranes with wide pores. Diffusion coefficient

[14] R. Höber and J. Höber, *loc. cit.*

[15] In particular they roughly vary inversely as the square roots of the molecular weight (L. W. Öholm, Ztschr. f. physik. Chem., **50**: 309, 1904).

ratios as great as 1:2 are considered very large in free diffusion. For instance, typical values obtained for mannitol (MV 189.2) = 0.55, for glycerol (MV 87.8) = 0.79, or a ratio 1:1.4. According to Michaelis and Collander, this can be interpreted as being due to the fact that in sieve-like membranes, e.g., in the dried collodion membranes, the pores fail to have a uniform diameter, but rather show a variety of sizes. It follows that, for larger molecules, only a small number of pores may be available, i.e., a small portion of the total pore area, while the smaller molecules penetrate the narrower pores as well.[16]

It is obvious that, below a certain minimum pore diameter, a sieve membrane will appear to be impervious to certain substances, which among others may be present in an experimental solution. In this case, they will prevent the absorption of the solution to a degree depending upon their partial osmotic pressure, by inducing an osmotic current to flow into the lumen of the intestine. To a smaller extent, medium-sized molecules will produce the same result. This effect is the chief factor in the purgative action of a copious administration of such water soluble substances as disaccharides (sucrose, lactose) in contrast to monosaccharides. The osmotic increase in volume is accompanied by a distention of the intestinal wall, which initiates a peristaltic wave, causing expulsion of the liquid contents.

But there are many observations regarding the absorption rate of nonelectrolytes in equimolar solutions, which by no means show a correlation to the molecular volume. Two examples are given in the following table, referring again to the absorption of aliphatic acid amides.[17]

TABLE XLIII.—ABSORPTION BY THE RAT INTESTINE OF DIFFERENT NONELECTROLYTES AT THE SAME CONCENTRATION AND WITH DIFFERENT LIPOID SOLUBILITY

Experiment		Substance	Per cent abs.	Mol. vol.	Distrib. coeffic. oil: water	Remarks
A	*a*	Succinimide	84.3	102 9	0 0049	Solutions: 0.1 mol. in ⅓ Ringer each period: 25 min.
	b	Malonamide	13 2	104 4	0.00008	
	c	Succinimide	79.3	102.9	0 0049	
B	*a*	Lactamide	65	98.5	0.00058	Solutions: 0.06 mol. in ½ Ringer each period: 25 min.
	b	Valeramide	81	134 7	0.023	
	c	Lactamide	69	98 5	0.00058	

It is evident from this table that two substances of nearly equal molecular size (succinimide and malonamide) can differ considerably in their absorption rate (*A*) and even that, as in the case of lactamide and valer-

[16] A. A. Weech and L. Michaelis, J. Gen. Physiol., **12**: 55, 221, 1928; R. Collander, Soc. Sc. Fenn. Comment. Biol., **2**: 6, 1926; see also R. Höber, Physiol. Rev., **15**: 52, 1935; further, Sec 4, p. 238.

[17] R. Höber and J. Höber, *loc. cit.*

amide, larger molecules can penetrate the absorbing membrane with greater speed than smaller (*B*). The cause apparently is relative lipoid solubility, which with two of the experimental substances (lactamide and malonamide) is extremely low, and with the other two (succinimide and valeramide), although still very small (compared to Fig. 23, p. 232), is about fifty times greater. It may be concluded that the factor of lipoid solubility plays the same role in tissue permeability as in cell permeability, discussed in extenso in Sec. 4. No systematic investigation into this interrelation has been carried on as yet. However, pharmacology records a great number of separate pertinent observations, such as the rapid action of narcotics, many alkaloids, hydrocyanic acid, and others following oral administration.

Another factor besides molecular volume and lipoid solubility, in determining the rate of nonelectrolyte absorption, seems to be of a more chemical nature. On several occasions it has been mentioned that the concept of lipoid solubility is rather vague. But just this vagueness is of great interest and will possibly become more and more so if each slight shade of lipoid solubility can be correlated with certain chemical or physicochemical variations, and can be shown to reflect certain structural or functional manifestations of living entities. Already Overton, when he developed his theory, has stressed that cellular lipoid is not fat, but fat-like, since it appears to be a mixture of a group of substances, each individual component of which is capable of conveying to the mixture, according to its relative amount, special characteristics, either as a solvent or as the substrate of some kind of chemical reaction (see pp. 234ff). Today, there is reason to believe that the same cells, or the same physiological unit, may harbor a number of lipoids, each of them involved in a certain function of life. For instance, it was mentioned earlier, in discussing the lipoid theory of narcosis, that the depression of excitability by a narcotic seems to be due to the presence of a substance resembling olein alcohol (p. 358). Another example of the significance of small variation in lipoid solubility appears in some further experiments concerning the intestinal absorption of nonelectrolytes, that are only worth mentioning here because they throw light on the significance of such light differences.

Referring to the findings shown in Table XLIII, the conclusion can be drawn, that substances which are practically lipoid insoluble, as well as of nearly the same molecular volume, can differ enough in lipoid solubility to cause definite differences in the rate of absorption. As a matter of fact, Jacobs,[18] with erythrocytes of different species, Wilbrandt,[19] Höfler,[20] and others with many kinds of plant cells, have observed a contrasting behavior regarding the penetration of aliphatic acid amides, on the one hand, and of polyhydric alcohols, on the other. Under the same conditions, one group of erythrocytes or of plant cells is found to be more permeable to the acid

[18] M. H. Jacobs, Proc. Am. Phil. Soc., **70**: 363, 1931.

[19] W. Wilbrandt, Pflüger's Arch. f. d. ges. Physiol., **229**: 86, 1931.

[20] K. Höfler, Ber. Dtsch. botan. Ges., **50**: 53, 1932; **52**: 355, 1934.

amides, the other to the polyhydric alcohols (see pp. 234ff). Possibly this can be accounted for as being due to differences in the individual cell lipoids (Höber[21]). As a matter of fact, Collander and Bärlund[22] have corroborated this idea by model experiments showing that, for instance, the preferential penetration of a number of acid amides into cells of *Chara* (compared to *Rhoeo*) is imitated by the greater relative dissolving power of olive oil plus oleic acid in contrast to pure olive oil. This is possibly due to the amide radical, although the basic character of the aliphatic acid amides in aqueous solution (as well as in organic solvent solution[23]) is very weak. The reverse physiological effect can be expected to be produced by adding to the olive oil an organic substance, which increases the basic affinities of the lipoid (see Nirenstein[24]).

Returning to intestinal absorption, the acid amides were found to pass the absorbing membrane markedly more slowly than the polyhydric alcohols (Höber and Höber[25]).

So far absorption has appeared to be like a passive penetration. We now turn to the absorption of salts.

2. The Absorption of Salts.—Upon reviewing the history of studies upon intestinal absorption, one is immediately struck by the fact that during the more than forty years succeeding the enlightening research of R. Heidenhain about absorption, secretion, and lymph formation, and his endeavor to differentiate between physical and physiological factors involved in these properties, the major part of the work in the field of absorption was devoted to the study of salt absorption. This historically understandable fact has hampered progress since, the time being immature for the useful application of physicochemical concepts, great complications inherent in an analysis of the penetration of a membrane by electrolytes could not be perceived.

These complications grow out of the following facts: 1. In the case of diffusion of a salt, we are dealing with diffusion of at least two substances, the anions and the cations, the mobilities of which influence each other through their electric charges (see Sec. 1, chap. 1). 2. In solutions of weak electrolytes, for instance, the salts of fatty acids (see later), hydrolysis takes place which may affect membrane diffusion either by shifting the *p*H in one or the other direction, and thus altering the membrane structure, or by the fact that the undissociated molecules of the free acids or the free bases are able to pass through the substance of the membrane, which is impervious to the ions. 3. A colloid cation or a colloid anion, present on one side of the membrane and too large for penetration (absolute impermeability),

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[21] R. Höber, Biol. Bull., **58**: 1, 1930.

[22] R. Collander and H. Bärlund, Acta Bot. Fenn., **11**: 1, 1933.

[23] W. M. Clark, The Determination of Hydrogen Ions, 3rd ed.: Williams & Wilkins, Baltimore, 1928, chap. 29.

[24] E. Nirenstein, Pflüger's Arch. f. d. ges. Physiol., **179**: 233, 1920.

[25] R. Höber and J. Höber, J. Cell. & Comp. Physiol., **10**: 401, 1937.

will cause a Donnan distribution (see p. 79). 4. As has been shown, especially by Teorell,[26] some sort of Donnan distribution ("diffusion effect") also will appear, if two salt solutions are separated from each other by a sieve membrane, the pores of which are wide enough to allow the passage of each of the ions, but the passage of one of which is retarded by a relatively low mobility ("relative impermeability"). 5. The picture of salt absorption frequently is much obscured by the entrance of salt from the tissues. This is due, first, to diffusion of salt into the lumen from the body fluids, particularly from the blood plasma circulating through the capillaries of the mucosa; second, to the secretory action of the intestinal glands, the product of which contains salts, especially NaCl. The existence of the first process has often been denied, for the reason that there is evidence that various normal absorbing or secreting membranes exhibit only unilateral permeability, even to substances as highly diffusible as are, in general, the inorganic salts of the plasma. As a matter of fact, it will be shown later (p. 542) that, in the intestinal wall, forces are evidently at work shifting particularly the highly diffusible salts from the lumen to the body fluids. On the other hand, there is also reason to believe that the entrance of salts into the intestinal fluid is, partly at least, attributable to diffusion. For instance, Goldschmidt and Dayton[27] have introduced into the colon of a dog, NaCl solutions of increasing strengths (0.04 to 0.19 per cent). The amount of NaCl passing into these solutions from the surroundings was found to be smaller, the higher the concentration, and to become zero at a certain concentration level (about 0.16 per cent NaCl, $\Delta = 0.134°$). Above this level NaCl passes into the blood. Most obvious is the bilateral movement of ions in recent experiments of Visscher and associates,[27a] showing that radio-active sodium ion Na^{24}, present in the intestine of dogs in a mixture of isotonic NaCl and isotonic $MgSO_4$ (see p. 542), is shifted against the Na gradient from the intestine to the blood, but that Na^{24}, after intravenous injection, simultaneously passes (along the concentration gradient) into the intestinal fluid. Accordingly, Pendleton and West[28] have found that, after the intestine has been filled with normal saline, urea appears in the solution and rapidly rises to the blood level. Furthermore, after an intravenous injection of urea, the intestinal fluid again comes to a concentration equilibrium with the blood urea, and, as the urea percentage declines in the blood, the urea level in the intestine falls off correspondingly.[29] 6. Finally, in trying to unravel the multitude of physicochemical factors

[26] T. Teorell, Proc. Nat. Acad. Sc., U. S., **21**: 152, 1935; Proc. Phys. Soc., **78**: 11, P. 1933; J. Gen. Physiol., **21**: 107, 1937.

[27] S. G. Goldschmidt and A. B. Dayton, Am. J. Physiol., **48**: 419, 1919.

[27a] M. B. Visscher, R. H. Varco, C. W. Carr, R. B. Dean, and D. Erickson, Am. J. Physiol., **141**: 488, 1944.

[28] W. R. Pendleton and F. E. West, Am. J. Physiol., **101**: 391, 1932.

[29] Concerning this question of diffusion of solutes into the intestinal fluid, see further, O. Cohnheim, Ztschr. f. Biol., **36**: 129, 1899; E. Knaffl-Lenz and S. Nogaki, Arch. f. Exper. Path. u. Pharmakol., **105**: 109, 1925.

hampering the effort to clarify the distinction between passive penetration and active transfer, osmosis must be mentioned, although it is by no means specific to salt absorption, but plays a role in each kind of exchange across the absorbing membranes. The significance of the association of osmosis with salt movement is obvious, e.g., in the following experiments of McDougall and Verzár[30] regarding absorption by the rat's intestine of nearly equimolar solutions of glucose and xylose, both strongly hypertonic to the rat's blood (about 0.3 molar) (Table XLIV).

TABLE XLIV.—DIFFUSION AND OSMOSIS DURING ABSORPTION BY THE RAT INTESTINE OF GLUCOSE AND XYLOSE

Substance	Solution introduced	After 60 min. found	Osmotic pressure equal to NaCl
I glucose.. ..	In 3 cc. 320.4 mg. = 0.59 mol.	In 3.7 cc. 121.6 mg. (= 38% unabs.) + 9.9 mg. NaCl	0 87%
II xylose.....	In 3 cc. 251.7 mg. = 0.56 mol.	In 5.0 cc. 165.7 mg. (= 65% unabs.) + 15.8 mg. NaCl	1 07%

Three cc. of nearly equimolar hypertonic solutions were introduced into the intestinal loops. After one hour, they had become approximately isotonic with the blood (the osmotic pressure of the remainder in the loop being expressed in per cent NaCl). This was evidently due, partly to an influx of water, partly to the entrance of NaCl. Glucose appears to be considerably more rapidly absorbed than xylose. This difference between the two sugars will be discussed later (p. 544). It explains the greater increase of volume as well as the greater amount of NaCl entering the xylose solution, both contributing to establish osmotic equilibrium with the blood. This final isotonicity between blood and intestinal fluid has been observed very frequently and regularly, disregarding whether the experiment is started with a hypertonic, an isotonic, or a hypotonic solution.[31]

Concluding this discussion of factors to be considered in order to account fully for the great variety of findings in absorption experiments, including such factors as, so far, have not received much attention (see 2 to 4), we turn to experiments approaching the problem of passive diffusion versus active transfer, by way of a procedure similar to that which appeared to be useful in studying the absorption of nonelectrolytes. This procedure was distinguished by the avoidance of great osmotic gradients, and the use of short absorption times.

Comparing the *absorption of anions*, administered in isotonic solutions

[30] E. J. McDougall and F. Verzár, Pflüger's Arch. f. d. ges. Physiol., **236**: 321, 1935.

[31] See, among others, R. Heidenhain, Pflüger's Arch. f. d. ges. Physiol., **55**: 579, 1894; R. Höber, *ibid.*, **70**: 624, 1898; O. Cohnheim, Ztschr. f. Biol., **36**: 129, 1898; S. Goldschmidt and A. B. Dayton, Am. J. Physiol., **48**: 459, 1919; S. Goldschmidt, Physiol. Rev., **1**: 421, 1921.

as sodium salts, the absorption rates follow roughly the order of: Cl, Br, I ≧ formiate, acetate, propionate, butyrate, valerate, capronate (C_6) > NO_3, lactate, heptoate (C_7), caprylate (C_8), sulfate, phosphate, ferrocyanide, tartrate, citrate, malate > oxalate, fluoride.[32]

This series looks, at least with respect to the inorganic anions, as though diffusion were the predominant factor, since the ionic mobilities decrease in a corresponding succession. This interpretation also contributes to the understanding of the purgative action of sulfates and phosphates and other members of this group, which, like the disaccharides, in higher concentration and because of their low diffusibility, produce a large osmotic influx of water into the lumen (see p. 534). But there are several other factors involved in determining the absorption rate of the salts. First, they may damage the tissues. Thus, oxalate, and still more fluoride, often are visibly injurious to the intestinal mucosa, and produce an irreversible functional disturbance, probably caused by a complete precipitation of Ca, which is accompanied by a loss of adhesion between the epithelial cells, so that this layer is readily detached and disintegrated (see p. 305). Also the milder reversible purgative action, which is observed not only with sulfates and phosphates, but also with malate, tartrate, and citrate, has been referred by Wallace and Cushny to the relatively low solubility of their Ca-salts, or to the deionization of Ca by these anions. Indeed, some lack of Ca is not unlikely to contribute to the laxative action by increasing the permeability of the cells themselves to water. Another viewpoint referred to by Wallace and Cushny is suggested by the striking resemblance of the aforementioned physiological order of anions and the Hofmeister series of anions (p. 293, also 234). This may be thought of as being connected with the well-known reversible swelling and shrinking effect of salts with univalent and bivalent anions upon hydrophilic colloids, owing to hydration and dehydration (see pp. 295ff) and possibly localized in the intestinal wall at the colloids serving as cementing material in the interepithelial pathways of the ions (see p. 540). Finally, it is known, mainly from the studies of Donnan and Potts and of McBain[33] that in the series of the alkali salts of fatty acids, starting with acetate, the surface tension of their solutions rather abruptly falls off beyond valerate or capronate, so that an injurious cytolyzing effect can be expected to start with heptoate and caprylate, as is, in reality, indicated in the detrimental influence on absorption (see, further, Sec. 4. p. 247).

With regard to the *absorption of cations*, there is not much difference in the absorption rate of the chlorides of Na, K, NH_4, Ca. Markedly slower is the absorption of $MgCl_2$ and $MgSO_4$, both well known as purgatives.

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[32] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **70**: 624, 1898; **74**: 246, 1899; G. B. Wallace and A. R. Cushny, Am. J. Physiol., **1**: 411, 1898; Pflüger's Arch. f. d. ges. Physiol., **77**: 202, 1899.

[33] F. G. Donnan and H. E. Potts, Kolloid-Ztschr., **7**: 208, 1910; J. W. McBain, Ztschr. f. physik. Chem., **76**: 179, 1911; Kolloid-Ztschr., **12**: 256, 1913; L. Lascarey, *ibid.*, **34**: 73, 1924.

Their diffusion rate is low, due partially to the low mobility of the Mg ion, partially to incomplete dissociation and formation of molecular complexes.

Concerning the absorption of uni-univalent, chemically indifferent neutral salts, as influenced by the presence of certain other solutes, see pp. 542ff.

a). *Intercellular or transcellular absorption.*—This problem first became evident when lipoid soluble substances were found to be absorbed by the intestine at a higher rate than lipoid insoluble ones, even though their diffusion rate was lower. This presumably indicated a direct passage across the lipoid components of the cell body. In order to solve the problem, dyestuffs were chosen[34] which as basic dyestuffs (usually the hydrochlorides of dyestuff bases) in general are "vital stains"[35] (i.e., they enter the living cell because they are lipoid soluble[35]) in contrast to acid dyestuffs (mostly sulfonic acid dyestuffs), which, in general, are indifferent toward the cells. (chap. 13). Dogs and frogs (best as tadpoles) are fed the dyestuffs. In the intestinal epithelium of the tadpoles, the basic dyes are found either within intensely stained granules, or distributed more homogeneously throughout the protoplasm, or both. Acid dyes, in general, fail to be visible in the cell body as well as in the intercellular cement, but are proved to pass the intestinal wall, since they appear in the urine, although often slowly and scantily. Is it permissible to conclude that the entire group of lipoid insoluble substances, nonelectrolyte as well as electrolyte, do not pass the epithelial walls, but, rather, the minute spaces between the cells? Do not even foodstuffs, like sugars, amino-acids, inorganic salts, enter the cell body? These questions have been approached in experiments with dyestuffs in the following way: Fresh excised epithelial membrane of tadpoles, vitally stained, e.g., by toluidene blue, was placed in a solution of ammonium molybdate, which precipitates basic dyestuffs. The effect was that the color faded in the granules and escaped from the cell to its surface, and formed a dark blue envelope around the cell body. This indicates that, like many other salts, molybdate cannot enter the cell, but sucks the dye into the intercellular space. This microscopic picture fails to appear, if mercuric chloride or picric acid is substituted for the molybdate. The reason is that, in contrast to molybdate, these two compounds are lipoid soluble and are precipitants of the dye, so that the dye can be fixed *in loco* inside the granules. Furthermore, after the vitally stained epithelium has been killed by osmic acid or by formaldehyde, molybdate can enter and preserve the original picture with the dyestuff stored inside the granules.

Now, it certainly should be decided whether the behavior of molybdate can be generalized and accepted as a proof that the absorption of the lipoid insoluble substances is restricted to the small intercellular spaces. In this respect, it is rather well established that some lipoid insoluble food stuffs, i.e., certain alkali salts and some sugars, pass across the intestinal

[34] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **86**: 199, 1901; J. Arnold, Sitzungsber. d. Akad. Wissensch., Heidelberg, No. 14, 1911.

[35] See Table I in E. Nirenstein, Pflüger's Arch. f. d. ges. Physiol., **179**: 233, 1920.

membrane not by diffusion, but by active transport, which is associated with cell metabolism. This will be shown more convincingly to take place with certain single cells which, sufficiently supplied with energy, are able to shift inorganic salts selectively and against a concentration gradient (see chap. 36 and 38). Thus, we leave so far unanswered the question of the importance of intercellular absorption,[36] but we will return to it later.

Only one point must be added, which has been neglected as yet. There is a group of lipoid insoluble substances which can be assumed to undergo transcellular absorption by mere diffusion, because their molecular volume is small enough to allow them to pass the sieve-like cell surface. This may occur with acetamide, ethylenglycol, glycerol, urea, and others (see p. 233), but has not yet been proved.

b). *The part played by colloids in intestinal absorption.*—Assuming that in many respects intestinal absorption may resemble diffusion across a membrane, absorption of colloids cannot be expected to occur, except to a very small extent. Therefore, the apparent absorption of common food colloids can be referred to a preparatory breakdown, mainly effected by the splitting power of enzymes, which even after careful rinsing of the intestine with normal saline can hardly be entirely excluded. On the other hand, there is clear evidence of absorption of genuine proteins in all those cases, where the intake of "foreign" protein is detected by specific immunological reactions, as in food allergy. However, in these absorption processes, where often extremely small amounts are involved in the poisoning effect, the uptake is probably of another nature than hitherto considered, e.g., the result of phagocytosis, of an unusual leakiness of the mucosa, and of other factors.

This nearly total incapacity of the colloidal protein to penetrate the absorbing membrane is of interest in another connection. It has been suggested that the colloidosmotic pressure of the blood of about 30 mm. Hg, due to the plasma proteins, may under appropriate conditions play a part as a driving force for the fluid contained in the intestine. Rabinovitch[37] and Nasset and Parry[38] have tested this hypothesis in the following way: They tried to balance the colloid-osmotic pressure of the plasma by filling the intestine with a solution of a non-protein colloid, 6% gum acacia in 0.9% NaCl. According to earlier work, the 6% acacia was expected to be osmotically equivalent to the normal percentage of plasma protein. The experimental result showed no difference between the rates of absorption of the saline with and without acacia. However, this does not disprove the influence of colloidosmotic pressure upon absorption. For, according to Dodds and Haines,[39] NaCl has a profound depressing effect upon the osmotic pressure of the gum, which decreases to only little more than one-third of that of the blood colloids. Thus the problem remains unsettled.

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[36] See R. Chambers, Cold Spring Harbor Symp., **8**: 144, 1940.

[37] J. Rabinovitch, Am. J. Physiol., **82**: 279, 1927.

[38] E. S. Nasset and A. A. Parry, Am. J. Physiol., **109**: 614, 1934.

[39] E. C. Dodds and R. T. M. Haines, Biochem., J., **28**: 498, 1934.

c). *Absorption of inorganic salts in presence of slowly diffusing substances.*—In the introduction to this section dealing with physicochemical aspects of absorption and secretion of dissolved substances, it was proposed (p. 525) to divide the processes, which are co-ordinated in the physiological functions, into spontaneous and enforced movements, the first appearing to be mainly diffusion-like. In this special part dealing with intestinal absorption, we turn now to observations which conflict with the course that could be anticipated for spontaneous penetration. Whether the criteria of active transfer (p. 528) apply to their course or not, this has to be tested.

After a hypotonic solution of NaCl has been placed in the intestine, its concentration rises until isotonicity has been attained (see p. 538).

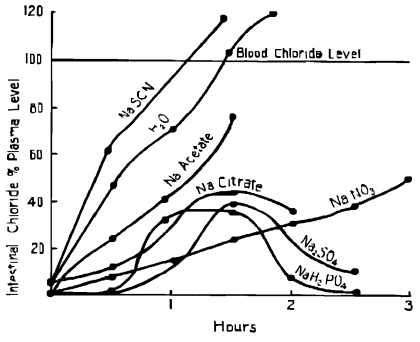

Fig. 56.

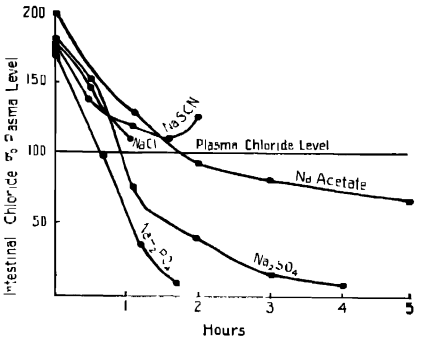

Fig. 57.

Fig. 56 and 57.—Influence of solutions of various Na salts upon the distribution of Cl between intestinal fluid and blood.

In experiments with the colon of dogs, Goldschmidt and Dayton[40] have found that, if Na_2SO_4 and NaCl are introduced simultaneously, the concentration of NaCl decreases rapidly, and even can reach the zero point, whereas the concentration of Na_2SO_4 rises so that the solution approaches the osmotic value of the blood. The absorption of NaCl is increased by increasing the concentration of Na_2SO_4.

Burns and Visscher[41] have extended these observations by comparing the behavior of anions other than SO_4. When isotonic solutions of the various Na-salts are placed in intestinal loops, Cl enters, and its concentration varies during the experiment in a characteristic fashion, as shown in Fig. 56, where the Cl concentration in percentage of the plasma chloride is plotted against time. It appears that phosphate and citrate inhibit the initial entrance of Cl as much as does SO_4, and that, following the entrance, the chloride is more or less reabsorbed. Also nitrate and acetate diminish the ingress of Cl, but to a smaller extent than do the bivalent ions. The same is shown in a somewhat different way in the experiments listed in

[40] S. Goldschmidt and A. B. Dayton, Am. J. Physiol., **48**: 459, 1919.

[41] H. S. Burns and M. B. Visscher, Am. J. Physiol., **110**: 490, 1934.

Fig. 57. Here the experiments are begun with sodium chloride present in the loop at a concentration above the plasma chloride level and, in addition, the various sodium salts in half isotonic concentration. The result is that, again, sulfate and phosphate strongly force the chloride from the intestinal lumen, and acetate does the same to a smaller extent.[42]

The question arises how to account for this peculiar distribution. Evidently, the chief factor is the difference in the diffusion rate of the ions in question, acetate being slower than chloride, and sulfate, phosphate, and citrate still slower. For a number of reasons (see later), it is suggestive to believe that the intestinal epithelium is able to shift Cl together with Na up hill against the concentration in the tissue fluids. But this cannot become evident, unless a second substance of lower diffusibility is present. For, provided nothing but NaCl in isotonic concentration were present, and NaCl were shifted from inside to outside across the cell layer, then the now hypotonic solution would become isotonic again by osmosis, and so on; a simple complete absorption of the salt solution would be the end result without showing any indication of the special driving factor involved. However, e.g., with the rather indiffusible, and therefore rather inabsorbable, Na_2SO_4 present in isotonic solution, in addition to a small amount of NaCl, hardly any osmosis would occur, and the NaCl removal would become evident and would continue until the zero concentration had been reached.

This interpretation is in line with older experiments of Katzenellenbogen,[43] which dealt with the absorption of slightly hypertonic solutions containing 0.4% NaCl in addition to various nonelectrolytes. The result was that, e.g., in the presence of the almost inabsorbable mannitol the NaCl concentration, though from the beginning below the blood level, further decreased, but increased slightly with erythritol or glycerol, due to their smaller molecular volume. A corresponding result has been obtained by Ingraham and Visscher,[44] who investigated the absorption of NaCl in the presence of sucrose and found the salt concentration to fall to one-third of the plasma level, although the relative absorption rate of sucrose was only moderately slow.

Furthermore, the same authors have continued this work on absorption of mixtures by adding to NaCl chlorides with polyvalent cations. The likelihood of an effect analogous to that with polyvalent anions is not great, since the diffusibility, as determined from the ionic mobilities, is not very low, and since, in addition, many polyvalent cations are poisonous. Indeed, the results gathered so far with the bivalent Mg, Ca, Mn, and the trivalent $Co(NH_3)_6$ prove the Na to fall below the plasma level, but not so much as Cl in the corresponding experiment with polyvalent anions.

[42] About the identical behavior of Cl and Br, see R. Ingraham, Proc. Soc. Exper. Biol. & Med., **33**: 453, 1935.

[43] M. Katzenellenbogen, Pflüger's Arch. f. d. ges. Physiol., **114**: 522, 1906.

[44] R. C. Ingraham and M. B. Visscher, Am. J. Physiol., **121**: 771, 1938.

Thus, according to these three sets of experiments, under special and understandable circumstances, i.e., in presence of substances with low diffusibility, NaCl or, better, Na and Cl, can be shown to move against a considerable concentration gradient from the intestinal lumen into the surroundings.

For an explanation of this phenomenon, two viewpoints may be considered. First, for several obvious reasons, it seems suggestive to refer for interpretation to the Donnan distribution or to Teorell's "diffusion effect" (see Sec. 1, chap. 1). However, provided that a more thorough study of the influence of nonelectrolytes confirms the existing results, it will be impossible to explain them on the basis of the purely ionic phenomena, studied by Donnan and Teorell.[45]

Second, the conclusion might be drawn that an active transfer was demonstrated by the special experimental conditions. This primarily postulates the liberation of energy, which is linked with the shift of ions (see p. 528). Following this line of thought, Ingraham and Visscher[46] have studied the influence of metabolic poisons, like cyanide, hydrogen sulfide, fluoride, or arsenic, upon characteristic changes of concentration of Cl, SO_4 and Na in the intestinal fluid. One typical result is reproduced in Figs. 58 and 59. There it appears that, in the presence of the poison, the impermeability to sulfate disappears, that the normal rapid fall of Cl to zero is reversed to a rise toward the plasma level of 100 m mol., and that the sodium concentration, which at the beginning is as high as 200 m mol., likewise approaches the plasma level. Thus, the picture has been completely changed. However, the results permit no more than the conclusion that evidently the poison has abolished the normal cellular limitation of ion permeability, so that unrestricted diffusion can occur.

This is not, then, a direct demonstration of active transfer in this complex system. However, it will be shown in the following chapters that there are systems, in which the relative rates of diffusion of concomitant substances do not play such a decisive role in identifying the active transfer, and in which the correlation between this transfer and the liberation of metabolic energy is unambiguous. It may be that substances will be discovered which are specific inhibitors of the energy providing reactions, for instance more like phlorizin in its specificity than like iodoacetate in its general impairment of cell structure (see p. 548).

3. The Absorption of Sugars.—Sugars are lipoid insoluble nonelectrolytes. It can be inferred, therefore, that in their rate of absorption the molecular volume will be a determining factor. However, the fact that we meet here, for the first time, physiologically important organic substances, may suggest a special behavior. As a matter of fact, more than forty years ago it was pointed out that, on the one hand, the lower absorption rate of disaccharides compared to monosaccharides can be correlated

[45] See also R. C. Ingraham, Am. J. Physiol., **114**: 676, 1935.

[46] R. C. Ingraham and M. B. Visscher, Am. J. Physiol., **114**: 681, 1935.

with the greater molecular volume and the lower diffusion rate respectively, but, on the other hand, glucose is absorbed at a higher rate than other

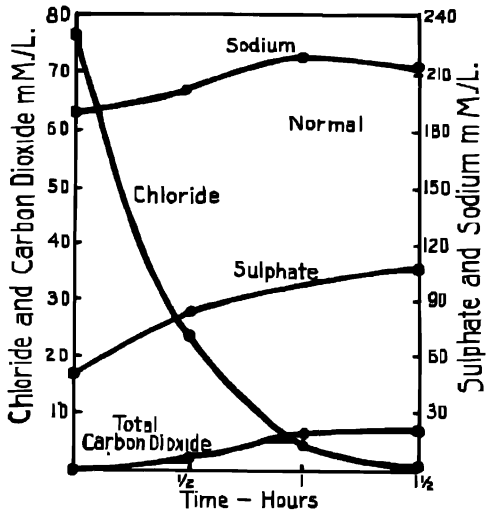

FIG. 58.—Normal intestine.

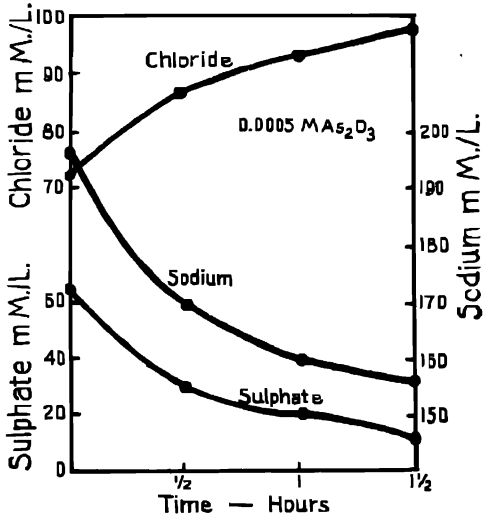

FIG. 59.—Intestine after poisoning.

FIGS. 58 and 59.—Influence of poison upon the distribution of Cl, SO_4, and Na between intestinal fluid and blood.

hexoses.[47] Studying, then, a series of monosaccharides with various methods and different animals, it has been found that, apparently incompatible with the diffusion law, not only hexoses fail to be absorbed with

[47] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **74**: 246, 1899; E. Hédon, Compt. rend. Soc. de Biol., **52**: 29, 41, 87, 1900.

equal speed, but pentoses pass even more slowly than the hexoses. With different methods, Nagano,[48] working on dogs, Cori,[49] and Wilbrandt and Laszt[50] on rats, have obtained the following relative values: galactose 115, glucose 100, fructose 44, mannose 33, sorbose 30, xylose 30, arabinose 29. These observations have been supplemented, and more light has been thrown on the physiological nature of these differences, by the studies of Macleod, Magee, *et al.*,[51] using the excised loops of cats and rabbits, submerged in normal saline and filled with solutions of sugars, the penetration of which was measured by analyzing the outside solution. This simple procedure has yielded the following results: From surviving loops of equal size, glucose passes faster than xylose, but after the mucosa has been injured by heat or by fluoride, the contrary is true. Even lowering the temperature to 0° has the effect that xylose leaves the inside fluid slightly faster than glucose; but the normal ratio can be re-established by warming the loop to 40°. Further, the temperature coefficient of penetration of glucose between 0° and 20° is about the same, irrespective of whether the intestine is alive or dead; whereas, between 20° and 40°, this coefficient is much greater in the living (see p. 31). All these facts point to the conclusion that the absorption of xylose is a nonselective physical process, whereas in that of glucose the peculiar properties of the living structures of the intestinal wall are involved. Another contribution to this interpretation is the observation of Cori and Goltz[52] that the different sugars are absorbed at equal rates from the peritoneal cavity, the walls of which, in this regard as well as in others, behave like an inert physical membrane.

Another remarkable result of the experiments of Auchinachie, Macleod, and Magee (*loc. cit.*) with excised loops is the following. Two loops, one surviving, the other killed, are filled with glucose-saline and placed in saline, and the rates of penetration are measured. From the fluid in the dead loop there appears more glucose outside than from the living. This is in agreement with the fact that the permeability of all living cells increases in death. But the more important point is that, while during several hours the amount of glucose escaping from the killed loop diminishes progressively, the longer the penetration is allowed to proceed, the rate of absorption through the living loop remains practically constant over several hours. This same difference, shown by the surviving and the killed excised loop, appears under normal conditions in comparing the absorption from the peritoneal cavity, on the one hand, and from the intestinal cavity, on the other. This constant shift of glucose or, more generally, of certain hexoses, is the most conspicuous indication in intestinal absorption of

[48] J. Nagano, Pflüger's Arch. f. d. ges. Physiol., **90**: 389, 1902.

[49] C. F. Cori, Proc. Soc. Exper. Biol. & Med., **22**: 497, 1925; J. Biol. Chem., **66**: 691, 1925.

[50] W. Wilbrandt and L. Laszt, Biochem. Ztschr., **259**: 398, 1933.

[51] D. W. Auchinachie, J. J. R. Macleod, and H. E. Magee, J. Physiol., **69**: 185, 1930; J. J. R. Macleod, H. E. Magee, and C. B. Purves, *ibid.*, **70**: 404, 1930.

[52] C. F. Cori and H. L. Goltz, Proc. Soc. Exper. Biol. & Med., **23**: 122, 1925.

transfer through some intravital activity, as contrasted with passive penetration. It is convincingly demonstrated by showing that, over a certain range of concentrations (about 6 to 12 per cent with rats), the same amount of sugar leaves the intestinal fluid[53] in the same period of time. This is evident, e.g., from experiments of Verzár, which are listed in Fig. 60. The average amounts of sugar absorbed within one hour are plotted against the amounts introduced into the loops in 3 cc. of water. It is evident that glucose (*a*) and galactose (*b*) form one group, xylose (*d*), sorbose (*e*), and

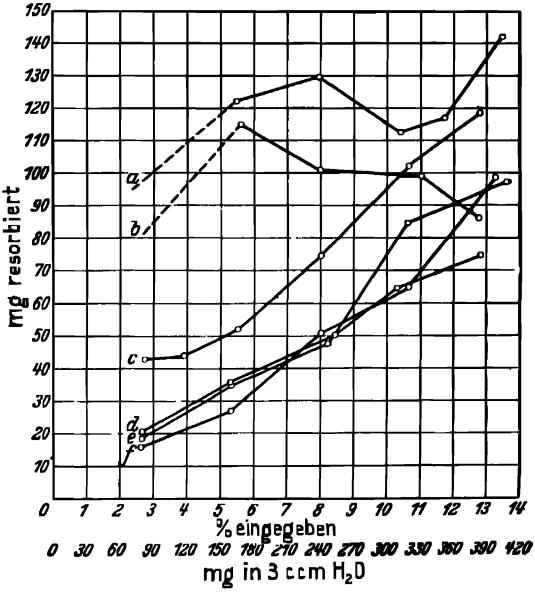

FIG. 60.—Intestinal absorption of sugars: *a*. glucose, *b*. galactose, *c*. fructose, *d*. xylose, *e*. sorbose, *f*. mannose.

mannose (*f*) a second group, whereas fructose (*c*) takes an intermediary position. Corresponding to physical diffusion, the rate of absorption rises in the second group rather regularly with rising concentration, whereas glucose and galactose are considered by Verzár to show a fairly constant absorption rate between 5.5 and 13 per cent. The behavior of fructose approaches that of the second group. In the concentration interval of 2.5 to 5.5 per cent, the absorption rate of glucose and galactose increases like those of the sugars of the second group. Below 2.5 per cent within the one hour of duration of the experiments, the entire amount of these sugars present is absorbed.

These results suggest the belief that the selective absorption of glucose and galactose is due to some kind of carrier system, which is seated in the

[53] C. F. Cori, G. T. Cori, and H. L. Goltz, Proc. Soc. Exper. Biol. & Med., **25**: 433, 1929; F. Verzár, Biochem. Ztschr., **276**: 17, 1935; Sz. Donhoffer, Arch. f. Exper. Path. u. Pharmakol., **177**: 689, 1935.

intestinal wall and which is unable to shift more than the maximum load of these sugars at one time. Since it is unfit to carry the other sugars, it leaves them to an unselective physical diffusion. This hypothesis can be supported by various observations. On the basis of the well-known work of Lundsgaard on the severe alterations of carbohydrate metabolism by iodoacetate, Wilbrandt and Laszt[54] have found that the preferential absorption of glucose and galactose is markedly impaired by this poison, so that the characteristic difference between these sugars and mannose, sorbose, and the pentoses almost disappears. Further, postulating that phosphorylation, as a regular intermediary reaction in carbohydrate metabolism, may be involved in the preferential absorption of sugars, they have found that, on the one hand, addition of phosphate (*p*H 7) to the intestinal solution seems to speed the rate of absorption of glucose, not of xylose.[55] On the other hand, extracts of intestinal mucosa, normally able to link inorganic phosphate with glucose, galactose, and fructose, but not mannose and xylose, lose this phosphorylizing power in the presence of iodoacetate[56] (see later). Thus, phosphorylation may be connected somehow with the carrying function. About the different behavior of human erythrocytes toward sugars, see Sec. 4, chap. 10.

An especially strong argument for an intermediary phosphorylation is the action of another poison, phlorizin. This glucoside has been known for a long time to prevent reabsorption of glucose in the kidney. Its inhibitory effect is much milder than that of iodoacetate. This latter substance very easily brings about irreversible damage of many cell functions. It interferes with many transport reactions, e.g., the secretion of dyestuffs by the kidney and the liver, the reabsorption of Cl and the absorption of amino-acids. In general, these reactions are definitely stopped, and the effect is often manifested even by gross pathological changes.[57] Phlorizin, however, inhibits phosphorylation and dephosphorylation in muscle brei, in kidney, and in brain extracts, and, furthermore depresses in a fully reversible manner the intestinal absorption of glucose.[58] Phlorizin also interferes with the absorption of galactose and fructose, much less with that of xylose and arabinose. It does not affect the absorption of glycine, glutamic acid, and asparagine.[59]

The effect of phlorizin is illustrated by the following experiment of

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[54] W. Wilbrandt and L. A. Laszt, Biochem. Ztschr., **259**: 398, 1933.

[55] See, further, H. E. Magee and E. Reid, J. Physiol., **73**: 163, 1931; L. Laszt, Biochem. Ztschr., **275**: 40, 1935.

[56] L. Laszt, Biochem. Ztschr., **275**: 44, 1935.

[57] K. A. Klinghoffer, J. Biol. Chem., **126**: 201, 1938; R. Öhnell and R. Höber, J. Cell. & Comp. Physiol., **13**: 161, 1939; R. Öhnell, *ibid.*, **13**: 155, 1939. See also chap. 382.

[58] E. Lundsgaard, Biochem. Ztschr., **264**: 209, 221, 1933; also E. Wertheimer, Pflüger's Arch. f. d. ges. Physiol., **233**: 514, 1933; further, Nakazava, Tohoku J. Exper. Med., **3**: 288, 1922. About substances resembling phlorizin, see E. Abderhalden and G. Effkemann, Biochem. Ztschr., **268**: 461, 1934.

[59] Öhnell and R. Höber, *loc. cit.*

Donhoffer[60]: Under identical conditions, loops of the intestine of a rabbit are filled with equal volumes (10 cc.) of 2.5% (equal 250 mg.) and of 15% (equal 1500 mg.) glucose, either with or without phlorizin. Of the 250 mg. glucose present in the first loop, 123 mg. are absorbed in the absence of phlorizin, 19 mg. in presence of phlorizin. Correspondingly, of the 1500 mg. in the loop containing the stronger solution, 338 mg. disappeared without phlorizin, 231 mg. with phlorizin. This means that 123 − 19 = 104 mg. and 338 − 231 = 107 mg., i.e., practically identical amounts, are absorbed from the two loops through phosphorylation, whereas 19 and 231 mg., respectively, must pass the intestinal wall without phosphorylation; in other words, by simple diffusion. From the viewpoint of the carrier hypothesis, this can be interpreted as indicating that, irrespective of the great concentration difference in the two solutions, the carrier is filled to the same maximum load (104 to 107 mg.), and that, in addition, 15.4% of the amount absorbed from the weaker solution and 68.3% absorbed from the stronger solution pass by diffusion. But these conclusions should be substantiated by further study.

Possible influence of two sugars, administered simultaneously to the loop, upon each other's absorption, has also been investigated.[61] Such a mutual influence has been found to exist in the case of glucose and galactose, which are absorbed from a mixture of equal parts of these sugars in such a way that the rate of absorption of each is reduced so that the total amount absorbed is not greater than if glucose alone or galactose alone were absorbed. This, too, fits in with the idea of a carrier system which cannot transfer more than a maximum load. However, it has been found also[62] that not only glycine and alanine, but also glycine and glucose, influence each other in the same way.

The opposite effect than with phlorizin, an ample increase of glucose absorption, has been recently observed[63] with rats, which were given for several days thyroxine. Certainly, there is a number of factors which might be considered responsible for this effect. But depletion of carbohydrates, rise in the velocity of intestinal blood circulation, increased peristalsis, generally increased permeability of the mucosa, and others can be ruled out. On the other hand, evidence has been provided that the rats, after being treated with thyroxine, display a strong increase of absorption of glucose, galactose, oleic acid, i.e., of substances which are readily phosphorylized, but fail to show the increase in presence of phlorizin, whereas thyroxine has no influence upon the absorption rate of alanine and of xylose.

Concerning the nature of the influence of phosphorylation upon absorption, more recent work has shown that this means much more than a simple

[60] Sz. Donhoffer, Arch. f. Exper. Path. u. Pharmakol., **177**: 689, 1935.

[61] C. F. Cori, Proc. Soc. Exper. Biol. & Med., **23**: 290, 1926.

[62] C. F. Cori, Proc. Soc. Exper. Biol. & Med., **24**: 125, 1926.

[63] T. L. Althausen and M. Stockholm, Amer. J. Physiol., **123**: 577, 1938.

intermediary reversible reaction with sugars. As in muscle metabolism, phosphorylation is rather a sequence of interlocked transphosphorylations involving the intracellular action of a group of enzymes as mediators between the adenylic acid system and the carbohydrate system and as such including an oxidative breakdown of a fraction of the available sugar, and thus a liberation of energy, which is likely to be utilized for the transfer mechanism.[64] This will be discussed in more detail later (chap. 38). Correspondingly, cyanide has been proved to inhibit the absorption of glucose.[65]

Regarding this situation, one may hesitate to use the term "carrier system" to represent such a concatenation of chemical reactions. However, it will be shown later (p. 617) that the enzymes concerned are localized in the body of the absorbing epithelium in such a way as to suggest the functional participation of certain structural components, thus providing perhaps a vague idea about the histochemical nature of the transporting machinery.

There is another mechanism that co-operates in the absorption of the physiological sugars and is analogous to the conditions found to support the absorption of salts (pp. 542ff). It has often been observed that glucose is absorbed along the concentration gradient from a higher concentration level in the intestine to a lower level in the blood.[66] But, Bárány and Sperber[67] have been able to demonstrate that glucose may also be shifted against the gradient by applying the principle of adding a slowly diffusible compound to the hypotonic intestinal sugar solution, as was found to be successful in demonstrating the marked up-hill transfer of NaCl. For this purpose, they have used sodium sulfate or sorbose. After filling, for instance, a loop of a rabbit intestine with a blood-isotonic solution of a mixture of glucose and sorbose, the concentration of sorbose was observed to remain high throughout the experiment (more than 2 hours), while the glucose concentration rapidly dropped down toward zero, although the level of the rabbit's blood sugar was about 100 mg. per cent.

4. The Absorption of Amino-acids.—Since, among the hexoses, the physiological sugars are subject to a preferential absorption, and, among the salts, the most physiological one, NaCl, seems to undergo a corresponding treatment, it has been inferred that special cellular devices insure a speedy absorption of the regular components of food. In this respect, particularly, amino-acids must be considered.

The problem is simpler in the case of the hexoses, which are more nearly related to each other than are the amino-acids, especially in the

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[64] S. P. Colowick, H. M. Kalckar, and C. F. Cori, J. Biol. Chem., **137**: 343, 1941; C. F. Cori, Biol. Symp., **5**: 131, 1941; C. F. Cori, Symposium on Respiratory Enzymes: University of Wisconsin, 1942; C. F. Cori and G. T. Cori, Ann. Rev. of Biochem., **10**: 151, 1941; see, further, Sec. 6 and 7.

[65] K. Kjerulf-Jensen and E. Lundsgaard, Ztschr. f. Physiol. Chem., **265**: 217, 1940.

[66] See, e.g., O. Cohnheim, Ztschr. f. Biol., **36**: 129, 1898.

[67] E. Bárány and E. Sperber, Skandinav. Arch. f. Physiol., **81**: 290, 1939.

identity of their molecular volume. The amino-acids possibly could be best compared to the acid amides, although the rate of their absorption has been shown to be inferior to that of the polyhydric alcohols of a corresponding molecular volume, for reasons that so far are not clear (p. 535). Nevertheless, absorption experiments with amino-acids appear promising for our problem in two respects.

It is conspicuous from old observations of Overton[68] concerning the osmotic properties of frog muscle, that amino-acids, like glycine or alanine, enter with great slowness, if at all, although, because of their smaller molecular size, one could expect them to exceed, for instance, erythritol. Still more unexpected is their inertia in penetrating the cell surface of the sulfur alga *Beggiatoa*,[69] which is remarkable for its outstanding behavior as a molecular sieve (Sec. 4, chap. 10) and in which the permeation rates of several amino-acids have been found to be abnormally low. The reason for this slowness is not of a physiological nature, since the slowness is evident also in diffusion experiments with collodion membranes,[70] and can be explained as being due to the ampholyte character of the amino-acids (Sec. 1, chap. 5), which, probably due to the formation of a shell of water dipoles around the ampholyte ions, brings about an enlargement of the molecular volume. Consequently, a porous membrane, such as the intestinal wall (see p. 534), would be expected to be passed comparatively slowly also. But the contrary is true. In comparison with acid amides, with erythritol, with xylose, the amino-acids actually pass the intestinal wall much faster than was anticipated from their diffusion rates.[71]

TABLE XLV.—ABSORPTION BY THE RAT INTESTINE OF AN ACID AMIDE AND AN AMINO-ACID

Malonamide		Asparagine	
Mol. conc. at the beginning	Percentage absorbed after 35 min.	Mol. conc. at the beginning	Percentage absorbed after 20 min.
0 066	16 9	0 03	80 4
0 132	16 8	0 06	57.7
0 198	16 5	0 09	53 2
0 264	18 4	0 12	39 2

Another aspect of the problem is the following: According to Fick's law, when a substance is administered in a loop experiment at different concentrations, the absolute amount entering by passive penetration would bear a linear relationship to the concentration, and the relative amount would be constant, as shown in Table XLI (p. 532). But in a preferential

[68] E. Overton, Pflüger's Arch. f. d. ges. Physiol., **92**: 115, 1902.

[69] W. Ruhland and C. Hoffmann, Planta, **1**: 1, 1925; S. Schönfelder, *ibid.*, **12**: 414, 1930.

[70] F. Schmengler, Pflüger's Arch. f. d. ges. Physiol., **232**: 591, 1933. See also C. Schmidt, The Chemistry of Amino Acids and Proteins; C. C. Thomas, Springfield, 1938.

[71] R. Höber and J. Höber, J. Cell. & Comp. Physiol., **10**: 401, 1937.

absorption of glucose, on the contrary, the absolute absorption within a certain concentration range turns out to be fairly constant, and the percentage absorption decreases (see Fig. 60, p. 547). Now, according to a series of preliminary experiments, the amino-acids appear to resemble glucose. In Table XLV, the behavior of malonamide is compared to that of asparagine.[72] These results concerning the absorption of amino-acids are in agreement with others indicating an active reabsorption by the kidney tubules (see p. 562).

5. The Absorption of Water.—So far, no conclusive evidence has been presented that, aside from osmotic, colloidosmotic, and hydrostatic forces (page 270), there is a special driving force for water within the living intestinal membrane. Evidently, such a mechanism has been proved to exist within the excised surviving frog skin transporting Ringer solution in one direction from outside to inside (p. 628).[73] But the observation of a corresponding action of a diaphragm formed by a piece of fresh mucosa of a rabbit intestine as described by Reid, could not be confirmed by other investigators.

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[72] R. Höber and J. Höber, J. Cell. & Comp. Physiol., **10**: 401, 1937.

[73] W. Reid, British Med. J., **1**: **323** 1892; J. Physiol., **26**: 236, 1901; E. Huf, Pflügers' Arch. f. d. ges. Physiol., **236**: 1, 1935; **237**: 143, 1936.

35

THE FORMATION OF URINE

Comparable to the intestine, the kidney is like a very extended membrane of epithelial cells which separates the blood from the urine. This membrane is arranged into a multitude of tubes, the nephrons. In the kidney, one end of each of these nephrons is closed by a Malpighian corpuscle, all nephrons run practically side by side and end in a common collecting structure. All are supposed to be essentially identical as far as their anatomy and physiology are concerned.

Our task, in studying kidney function, resembles the analysis of intestinal absorption, since it must be decided whether the permeation of water and solutes across the membrane is a passive penetration or an active transfer. But the task here is much more diverse, and thus more interesting, than with the intestine, as each nephron is divided into a series of segments, each of them endowed with special functions, which mostly, but not always, appear to be indicated by a visible characteristic structure of the epithelial cells. We can distinguish, as histophysiological units of each nephron, the Bowman capsule in the Malpighian body, the proximal tubules, the distal tubules (perhaps better, the proximal distal and the distal distal tubules) and, in addition, the thin segment of the loop of Henle, a special feature of the kidney of mammals. Thus, the contents of the nephron pass along the active epithelial wall like a technical product which moves on the assembly line, and which, by the addition of certain compounds and by the subtraction of others, is transformed into the final product, the urine. Thus the kidney accomplishes its main function of eliminating the waste products of metabolism, which are circulating in the blood, without abandoning valuable components, which are on the way to get lost, after having entered the nephron through Bowman's capsule. The final goal is the maintenance of a normal internal medium surrounding the cells in the body and consisting of water and of certain solutes in proper concentrations. In view of such a goal, it is tempting to advocate a teleological point of view, and to foresee a selective and active transport for those substances that are either useful or useless to the body as a whole—although many a recent finding is contradictory to this viewpoint, perhaps only owing to our meager ability to judge what is useful and what is not. The selective behavior of the intestine would be likewise in agreement with such a teleological concept (see p. 550). On the other hand, taking into consideration the customary experimental procedure for examining the behavior of nonphysiological

foreign substances, e.g., dyestuffs, it will appear that a certain correlation between the physical or physicochemical properties of these substances and the selectivity of their transport can be detected, which eventually may serve to eliminate the mystery of a teleological concept. For instance, a toxic foreign substance is expelled from the body by an active process in the kidney, and, in addition, proves to possess such physicochemical properties as will precondition the release of the secretory mechanism (see p. 571).

In the analysis of kidney functions, it has for a long time been recognized as the primary task to attribute to the different portions of the nephrons the various functions, which become obvious even in a superficial view of the urine formation. Thus, merely on the basis of the histological picture, the glomeruli have been surmised to be a place of filtration. Further, a comparison of the various ratios of concentration in the blood fluid and in the urine has shown the tubules to control this interrelationship either by allowing a passive penetration or enabling an active transfer, the latter being either "reabsorption" of certain substances from the filtered fluid or "secretion" or "excretion" of certain other substances.

1. The Formation of the Glomerular Fluid.—Convincing proof has been furnished that the glomerular fluid is the result of filtration of blood fluid through the walls of the tuft of capillaries into the Bowman capsule. After numerous investigations, inaugurated by Carl Ludwig (1844), it has been conclusively demonstrated that the rate of urine flow varies as the blood pressure in the renal arteries is raised and diminished without changing the amount of fluid passing through the kidneys,[74] provided that the area of the filtering membrane does not change simultaneously, due to changes of the blood distribution inside the kidney through local vasoconstriction or vasodilatation. Furthermore, the rate of urine flow decreases, when the effective gradient of filtration pressure is diminished by raising the intraureteral pressure.

The most direct approach for determining whether the glomerular fluid is a filtrate, has been made possible by withdrawing fluid from the Bowman capsule as fast as it is formed. This procedure of puncturing the pathways of the nephrons, which has been introduced by A. N. Richards,[75] permits the analysis of contents often amounting to not more than 0.5 cmm. Regarding the capsule fluid of the amphibian kidney, the result is that glucose, chloride, urea, creatinine, and hydrogen ions are found to be present in approximately the same concentration as in the plasma, but the fluid is protein-free; in other words, the capsular fluid can be looked upon as an ultrafiltrate. A corresponding condition has been found in the glomerular fluid of rat and guinea-pig.[76]

[74] A. N. Richards and O. H. Plant, Am. J. Physiol., **59**: 144, 1922.

[75] J. T. Wearn and A. N. Richards, Am. J. Physiol., **71**: 209, 1924; J. Biol. Chem., **66**: 247, 1925.

[76] A. M. Walker, J. Oliver, and P. A. Bott, Am. J. Med. Sc., **201**: 625, 1941.

Attempts have been made to obtain a more definite knowledge as to the physical properties of the glomerular membrane as an ultrafilter, by investigating whether colloids other than the plasma proteins, either after being injected into the blood of mammals or after perfusion through the kidneys of amphibia, appear in the urine.[77] The main result is that, grossly, the ability to penetrate the glomerular membrane is a matter of molecular weight. Hemocyanin (5,000,000), casein (190,000), serum globulin (104,000), serum albumin (68,000) are retained. Egg albumin (34,000) and Bence-Jones protein (34,000), gelatin (35,000), insulin (37,000) pass. Still greater is the permeability to P.P.D. tuberculin (13,500). Inulin (5,100) has been found to appear in unchanged concentration in the capsular fluid.[78] Hemoglobin (68,000) shows an ambiguous behavior, possibly due to the intermediary value of its molecular weight. Serum albumin is the first of the plasma proteins to appear in the urine in kidney disease.

2. The Function of the Kidney Tubules.—*a*). *Methods.*—The amphibian kidney (frog, *Necturus*) has been found to be the most satisfactory object for analyzing the formation of urine. Not only does it display the common advantages of the organs of cold-blooded animals, which endure manipulation and isolation much better than mammalian organs, but it has specific anatomical advantages.[79] The dual supply of blood, from the aorta chiefly to the ventral half, where the major part of the glomeruli and of the distal tubules is located, and from the renal portal vein to the main site of the proximal dorsal tubules, has contributed largely to the development of kidney physiology. For, owing to this twofold blood supply, it is more or less possible to provide separately the two halves of the kidney with different solutions, and thus to investigate the functional capacities of the proximal and the distal tubules. A practical procedure for such experiments with the isolated Ringer perfused frog kidney was proposed by Cullis.[80] An important point is to keep the aortic and the portal pressures within the range of about 24:12 cm. of water, corresponding approximately to the normal ratio of the pressures in the supplying vessels.[81] Under these conditions the arterial and the portal fluids, although both are confluent into common roots of the renal veins,[82] usually remain sufficiently separated to allow a differential supply of the proximal and the distal

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[77] L. E. Bayliss, P. M. T. Kerridge, and D. S. Russell, J. Physiol., **77**: 386, 1933; P. A. Bott and A. N. Richards, J. Biol. Chem., **141**: 291, 1941.

[78] J. P. Hendrix, B. B. Westfall and A. N. Richards, J. Biol. Chem., **116**: 735, 1936.

[79] M. Nussbaum, Pflüger's Arch. f. d. ges. Physiol., **16**: 139, 1878; **17**: 580, 1878.

[80] W. C. Cullis, J. Physiol., **34**: 250, 1906; further, F. A. Bainbridge, J. A. Menzies, and S. H. Collins, *ibid.*, **48**: 233, 1914; M. Atkinson, G. A. Clark, and J. A. Menzies, *ibid.*, **55**: 253, 1921.

[81] About some experimental details, see G. Barkan, Ph. Broemser and A. Hahn, Ztschr. f. Biol., **74**: 1, and **37**: 1921.

[82] A. N. Richards and A. M. Walker, Am. J. Physiol., **79**: 419, 1927.

tubular epithelia.[83] This can be shown with dyestuffs, the appearance of which, even in minute concentration, is easily detected in the secretion collected by the ureteral canulas.[84]

a 1. When the kidney is supplied under the normal pressure conditions from the aorta with Ringer, from the portal vein with Ringer plus dyestuff in a low concentration (0.1 mg. %), the dyestuff appears in the urine in a remarkably enhanced concentration (10 times and more), provided that it belongs to a certain group I, which, owing to a special molecular structure (see pp. 563ff) has adequate properties.

a 2. Under the same conditions, another group of dyestuffs, II, fails to appear in the secretion, or it appears at best in an extremely low concentration.

b 1. If the kidney is perfused with Ringer plus a dyestuff I exclusively from the aorta, the perfusion from the portal vein being omitted, the dye appears in about the same increased concentration as in experiment *a* 1.

b 2. If this experiment *b* 1 is repeated, substituting a dyestuff II for a dyestuff I, the dye appears either in the low concentration of the perfusion fluid, or only slightly (2 to 4 times) concentrated. This slight increase, probably, is due to water reabsorption (see pp. 561 and 568).

c. Supplying either a dyestuff I or a dyestuff II from both sides, gives practically the same result as the experiments *a* 1 and *a* 2.

From these facts the following conclusions can be drawn: First, the proximal tubules are able to perform a secretory concentration of dyestuffs I, not of dyestuffs II (experiments *a* 1 and *a* 2). Second, if, as in experiments *b* 1 and *b* 2, the portal pressure fails to balance the aortic pressure, the fluid entering the kidney from the aorta flows down to the capillaries of the portal side, and thus circulates around the proximal tubules. Due to this connection between the aortic and the portal stream-bed, the extent of which varies from individual to individual, eventually, but not necessarily, some fluid may pass over from the portal to the aortic side.

Corresponding results have been gathered with colorless substances. For instance, similar to the group II of the dyestuffs, inorganic ions and organic nonelectrolytes, even some belonging to the regular components of the plasma, may fail entirely to appear in the secretion, when introduced from the portal vein only, the glomeruli at the same time being supplied with Ringer. This has been found with phosphate, chloride, sulfate, xylose,[85] and others.

Thus, this simple method of dual perfusion of the amphibian kidney suffices to permit an extensive study of the correlation between the chemical and physicochemical behavior of solutes and the permeability and secretory

[83] For a scheme illustrating the vascular distribution in the frog kidney, see P. Ellinger, Arch. f. Exper. Path. u. Pharmkol., **145**: 199, 1929.

[84] For the following, see H. Yoshida, Pflüger's Arch. f. d. ges. Physiol., **205**: 274, 1924; F. Scheminsky, *ibid.*, **221**: 641, 1928; T. J. Liang, *ibid.*, **225**: 104, 1930.

[85] H. Yoshida, Pflüger's Arch. f. d. ges. Physiol., **205**: 274, 1924; W. Wohlenberg, *ibid.*, **218**: 448, 1927; T. J. Liang, *ibid.*, **222**: 272, 1929; R. Höber, *ibid.*, **233**: 181, 1933.

capacity of the proximal tubules, of course only semiquantitatively, but exactly enough to justify its often contested value (see also p. 563).

A more direct approach to the problem of tubular function has been made by Richards and Walker.[86] In extending the method of glomerular puncture to the tubules of the amphibian kidney, single tubules are punctured at various levels and the contents collected and analyzed. For this purpose, certain segments can be isolated by blocking the lumen at two points with droplets of mercury or of colored mineral oil. Also, such a segment can be filled or perfused with a certain solution and re-emptied for analysis. Puncturing the Bowman capsule and the proximal and distal tubules at different points can provide information about changes of the composition along the nephrons. The results of this elegant procedure will be referred to later,[87] as well as the use of other more simple methods, such as the study of the excised kidney, of kidney explants, and others.

Beyond these methods, which subject the kidney to more or less unphysiological circumstances, other means have been found to explore tubular function in the normally blood-perfused kidney of the intact animal. The main procedure is based upon the determination of the renal clearance of the substances in question (see p. 574).

b. Passive Penetration.—In analyzing intestinal absorption, it has been found useful to ascertain to what extent the absorbing membrane can be looked upon as being a diffusion membrane, and absorption not more than a passive penetration along concentration gradients, and how far special forces are engaged in bringing about an active transfer. The same procedure may be followed in dealing with the epithelial tubes of the kidney. But here we also are interested to know whether passive penetration across the membrane occurs in one direction as well as in the other.

Penetration toward the lumen of the nephron.—As mentioned before, a number of inorganic ions, phosphate, sulfate, ferrocyanide, chloride, when offered to the proximal tubules from the portal vein, cannot, or can hardly, pass into the lumen of the nephron.[88] The behavior of chloride is of special interest. When, in the arterial perfusion fluid, the normal Cl is replaced by SO_4 and normal Ringer is supplied only from the portal vein, the secretion remains Cl-free.[89] This is an especially striking demonstration of a unidirectional permeability of an epithelial layer, since Cl is moved to completeness in only one direction (see p. 559). But this Cl-shift is a specific reaction of the chlorine ion. Sulfate, phosphate, ferrocyanide, probably are unable to leave the lumen after having entered the nephron through the glomerular membrane. They appear in the frog urine slightly con-

[86] A. N. Richards and A. M. Walker, Am. J. Physiol., **118**: 111, 1936.

[87] About collection and analysis of fluid from single glomeruli and tubules of the mammalian kidney, see: A. M. Walker, J. Oliver, P. A. Bott, and M. C. MacDowell, Am. J. Physiol., **134**: 562, 580, 1941.

[88] H. Yoshida, Pflüger's Arch. f. d. ges. Physiol., **206**: 274, 1924.

[89] T. J. Liang, Pflüger's Arch. f. d. ges. Physiol., **222**: 272, 1929.

centrated, due to water reabsorption (see pp. 561, 568). Many organic anions, especially the anions of sulfonic acid dyestuffs and of related acids, show a corresponding incapacity to pass (see p. 556). About the passive penetration of inorganic cations across the tubular wall, very little is known as yet.

The proper interpretation of this impermeability is that the molecular dimensions of the ions are too great to allow them to penetrate the pores of the sieve-like membrane. Such a viewpoint is suggested by corresponding experiments with organic nonelectrolytes, likewise introduced from the portal side only. With polyhydric alcohols it has been observed[90] that the rate of penetration across the proximal tubular walls seems to increase in the order: mannitol < glycerol < ethylenglycol; with aliphatic acid amides in the order: malonamide < acetamide; with sugars in the order: xylose < dioxyaceton,[91] in other words, in the order of decreasing molecular volume. This is similar to the situation in passive penetration of the intestinal membrane (p. 533). The passivity of the processes is emphasized by the observation that, in contrast to secretion, these penetrations fail to be blocked by adding a toxic (but not disintegrating) substance.

As in intestinal absorption, another factor seems to control the rate of passive penetration, namely, lipoid solubility (see p. 535). There are not many observations which can be mentioned in favor of this correlation. E.g., trimethylcitrate (m/250), pinaconhydrate (m/100), antipyrin (m/400), though having a molecular volume greater than xylose or arabinose, enter the nephron, whereas the pentoses do not.[92]

Penetration from lumen to blood.—So far as passive penetration in the direction lumen to blood is concerned, very little information is available. Regarding the influence of molecular volume, it is interesting to note that, with the technique of Richards and Walker (see p. 557), proximal tubules of *Necturus* have been perfused with Ringer containing urea in concentrations either higher or lower than that of the plasma.[93] The results show that urea passes in either direction along the gradient. From a merely physicochemical viewpoint, this exit toward the blood could be expected, because urea has an exceedingly low molecular volume. In addition, other observations (see p. 572) have suggested that urea leaves the tubules by passive penetration. However, it will be shown that urea, one of the most important compounds concerned in urine formation, exhibits an especially complicated behavior (see p. 573). In the case of phosphate, also, passage in either direction obtains when single proximal tubules of *Necturus* are perfused. This is at variance with what was mentioned before regarding phosphates.

[90] F. E. Schmengler and R. Höber, Pflüger's Arch. f. d. ges. Physiol., **233**: 199, 1933.

[91] T. J. Liang, Pflüger's Arch. f. d. ges. Physiol., **222**: 272, 1929; R. Höber, *ibid.*, **233**: 181, 1933.

[92] R. Höber, unpublished experiments.

[93] A. M. Walker and C. L. Hudson, Am. J. Physiol., **118**: 153, 167, 1936.

c. Reabsorption by Active Transfer.—Reabsorption of inorganic ions.— Assuming that urine formation is begun by filtration in the Malpighian bodies, the fact that normal frog urine is entirely or nearly Cl-free[94] is the strongest evidence of active reabsorption of the filtered Cl against a steep concentration gradient into the Cl-rich plasma, and, since the normal urine of the frog is strongly hypotonic, one cannot evade the conclusion that, simultaneously with Cl, Na likewise is reabsorbed. This reabsorption in frog and in *Necturus* is a function of the distal tubules; in the proximal tubules neither the Cl concentration nor the osmotic pressure of the fluid changes. But in the distal tubules it drops progressively, until it reaches zero.[95] The reabsorption of Cl can also be demonstrated by perfusing the isolated frog kidney from both vessels with Ringer. At the beginning of the experiment, the secretion frequently is found not to contain more than 0.1% NaCl, compared to the 0.65% of the Ringer solution, but during the following hours the concentration slowly rises and finally reaches 0.65%, indicating that the organ under the abnormal conditions is slowly dying.

From a biological viewpoint, it is easy to understand that the heavy burden of doing continuous osmotic work is imposed upon the amphibian kidney to counterbalance the large absorption of water through the skin, and its abundant output through the glomeruli, without loss of the substance that mainly contributes to osmoregulation (see chap. 36). On the other hand, as will be shown later (pp. 561, 568), water, also, is likely to be reabsorbed in the tubules, and this may explain the fact that, after the kidney has been poisoned, e.g., by cyanide, a narcotic, or mercuric chloride, not only the Cl concentration in the urine rises, but also the amount of urine. All the more is it important to point out that active transfer of inorganic anions plus inorganic cations can take place without a concomitant shift of water (see 587).

Previously it was mentioned (p. 557) that sulfate, phosphate, ferrocyanide probably are unable to re-enter the amphibian body after having passed through the glomeruli. This would explain why effects similar to those observed with intestinal absorption are produced by these ions. According to Goldschmidt and Dayton (p. 542), from a mixture of sulfate and chloride introduced into an intestinal loop, the Cl shift into the body increases with rising sulfate concentration. In a similar way, when the isolated frog kidney is supplied alternately with Ringer, and with Ringer in which a part of Cl is replaced by SO_4, again it appears that the presence of SO_4 favors the reabsorption of Cl.[96] Obviously, in either case, sulfate acts by its low diffusibility (p. 543); it is this factor which determines its diarrheic and its diuretic effect, if brought into action from inside the epithelial tubes. It further has been known for a long time that during

[94] F. A. Bainbridge, S. H. Collins and J. A. Menzies, Proc. Roy. Soc., B. **86**: 355, 1913.

[95] A. M. Walker, C. L. Hudson, T. Findlay, and A. N. Richards, Am. J. Physiol., **118**: 121, 1937; see also H. L. White and F. O. Schmitt, *ibid.*, **76**: 483, 1926.

[96] H. Yoshida, Pflüger's Arch. f. d. ges. Physiol., **206**: 274, 1924.

diuresis effected by sulfate the percentage of Cl in the urine may drop to zero.[97]

Another inorganic anion, which is of particular interest because it helps to maintain the acid-base balance in amphibia as well as in mammals, is the bicarbonate ion. In perfusion experiments with frogs, the *p*H of the perfusion fluid, buffered by CO_2 and HCO_3, ordinarily is kept equal to 7.5, corresponding to the normal plasma value. In frog urine the *p*H varies between 7 and 4.5. By omitting bicarbonate in the perfusion fluid, the *p*H is brought down to about 4.8. The HCO_3 content of acid urine frequently has been found to approach zero. For these reasons it was suggestive to assume that the acidification of urine is based upon reabsorption of HCO_3.

The following experimental results seem to be in favor of this interpretation. By testing the reaction of the glomerular fluid with indicator dyestuffs (phenol red, fluorescein) or by direct electrometric determination, evidence is obtained that plasma and the glomerular fluid have the same *p*H.[98] Investigation of the *p*H changes along the tubules with phenol red shows the acidification of the contents to be a function of the distal tubules, and to be restricted to a short segment near the distal end of the distal convolution.[99] This marked localization in contrast to the more extended distribution of the reabsorbing power for Cl in the distal tubules indicates that two independent mechanisms are involved in the transport of these ions.

However, so far no unambiguous evidence is obtained that bicarbonate is reabsorbed. Secretion of acid likewise must be taken into consideration. The following observations[100] appear to be more in favor of the first interpretation, although they, likewise, give no conclusive evidence. By adding sulfanilamide and phenol red to the perfusion fluid, the normal acid reaction of the frog urine, as indicated by a yellow color, is changed to an alkaline reaction (pink color). This process is reversible. Besides sulfanilamide itself, derivatives containing the sulfonamide group unsubstituted have the same effect. Such compounds have been found by Mann and Keilin[101] to be poisonous to carbonic anhydrase. This enzyme is a normal component of the kidney.[102] Therefore, the bicarbonate, in equilibrium with the bicarbonate in the perfusion fluid, in passing the critical tubular segment, is not unlikely normally to enter the wall and take part in the catalyzed

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[97] iR. Magnus, Arch. f Exper. Path. u. Pharmakol., **44**: 396, 1900; T. H. Sollmann, Am. J. Physol., **9**: 425, 454, 1903.

[98] H. Montgomery, J. Biol. Chem., **110**: 749, 1935; J. A. Pierce and H. Montgomery, *ibid.*, **110**: 763, 1935.

[99] H. Montgomery and J. A. Pierce, Am. J. Physiol., **118**: 144, 1937; see, further, P. Ellinger, Quart. J. Exper. Physiol., **30**: 205, 1940.

[100] R. Höber, Proc. Soc. Exper. Biol. & Med., **49**: 87, 1942.

[101] T. Mann and D. Keilin, Nature, **145**: 164, 1940.

[102] H. W. Davenport and A. E. Wilhelmi, Proc. Soc. Exp. Biol. & Med., **48**: 53, 1941.

reaction: $HCO_3 + H \rightleftharpoons H_2CO_3 = CO_2 + H_2O$,[103] with the result of an acid reaction in the urine.

Reabsorption of sugars.—Various observations had led to the assumption that the plasma glucose, entering the pathways of the kidney, disappears from the secretion by tubular reabsorption, before definite evidence was presented by G. A. Clark,[104] showing that from the glucose-Ringer perfused frog kidney glucose is no more retained, after the proximal tubules have been poisoned with mercuric chloride by way of the portal vein; and by Wearn and Richards,[105] that glucose passes from the plasma through the glomerular membrane into the Bowman capsule in unchanged concentration.

The site at which the reabsorption takes place is detected by puncturing the tubules at various places. It appears that, in contrast to the behavior of Cl, it is in the proximal tubules that the percentage of glucose falls off to a value near zero.[105] Moreover, it appears that the reabsorption of glucose can be entirely suppressed by phlorizin. Thereafter, the glucose concentration increases along the nephron until at the end of the proximal tubule it is about 40% above the plasma level, at the end of the distal about 200%. This probably is due to reabsorption of water, occurring particularly in the distal and is comparable to the increase of concentration observed with inorganic and organic ions (pp. 556, 558).

Other sugars, besides glucose, hexoses as well as pentoses, were studied at first by H. J. Hamburger,[107] by perfusing the frog kidney from the aorta only, so that the perfusing fluid reached more or less the entire length of the tubules (see p. 556). Under these conditions the sugars appeared to be retained to various degrees. Clearer results are secured[108] by perfusing alternately with two sugars, either from the aorta alone or from both supply routes, and checking simultaneously the kidney function by Cl analyses of the urine, and by determining the secretory concentration of phenol red continuously injected through the portal vein. In this manner the following order of absorption rates has been found: glucose > galactose > mannose > fructose ≧ xylose > arabinose; in other words, the sugars are arranged in an order which does not differ very much from that encountered in intestinal absorption (p. 546). As a matter of fact, the two absorption phenomena resemble each other in more than one respect. First, in both cases we find the poisoning action of phlorizin, and this effect is found to be stronger, the higher the absorption rate of the sugar. This correlation was detected independently in the kidney experiments and in the study of Lundsgaard and of Wertheimer on intestinal absorption (p. 548). Second,

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[103] N. U. Meldrum and J. F. W. Roughton, J. Physiol., **80**: 113, 143, 1933.

[104] G. A. Clark, J. Physiol., **56**: 201, 1922.

[105] Wearn and A. N. Richards, Am. J. Physiol., **71**: 209, 1924.

[106] H. L. White and F. O. Schmitt, Am. J. Physiol., **75**: 483, 1926; A. M. Walker and P. L. Hudson, *ibid.*, **118**: 130, 1937.

[107] H. J. Hamburger, Biochem. Ztschr., **128**: 185, 1922.

[108] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **233**: 181, 1933.

the amino-acid glycine, undergoing a selective absorption in the intestine as well as in the kidney tubules (see p. 550), is equally well absorbed, no matter whether phlorizin is present or not (p. 563). Also, the absorption of Cl or the secretion of phenol red is not depressed by phlorizin. Therefore, the conclusion may be drawn that, as in intestinal absorption, the sugar absorption is tied up with phosphorylation.

Now, since enzymatic phosphorylation is dependent upon a supply of oxygen (p. 550), and can be suppressed by poisoning with cyanide, sugar absorption also can be conceived of as being an active transfer. The following experiments are in favor of this viewpoint. The Ringer perfused frog kidney is supplied from the aorta with glucose, from the portal vein with phenol red, and in addition, for a short time, with phenylurethane as a metabolic poison (see p. 369). The result is that temporarily, during the narcotic period, the glucose concentration in the secretion increases, the phenol red concentration decreases, since either of these processes depends upon activity of the proximal tubules. For, if the effect of the narcotic were nothing else than an increase of permeability of the tubular wall, the concentration of glucose would not be expected to rise. Therefore, if, in another experiment, the kidney is perfused from both sides with glucose Ringer and brings about by its reabsorbing power a continual shift of the sugar against an increasing concentration gradient, the rise of glucose concentration, reversibly accomplished by phenylurethane, cannot mean anything else than active transfer; in other words, osmotic work (see, further, p. 578).

However, again similar to the conditions in intestinal absorption of glucose (p. 549), the transfer toward the blood by the activity of the tubular epithelia is raised by the administration of thyroxine,[109] and as there are reasons to believe that the increase of absorption is not due to a more abundant blood circulation, to an enlargement of the active mass of cells, to a rise in temperature, etc., and as, further, the increase can be abolished by phlorizin, again the transfer can be assumed to be correlated with phosphorylation. But this interpretation needs more discussion, since it has been discovered that phlorizin is able to retard the active transfer of substances, which are very unlikely to be phosphorylized (see pp. 572 and 617).

The absorption of amino-acids.—So far, there is not much known about the ways and means enabling the amino-acids to pass the amphibian kidney. This is chiefly due to the fact that difficulties were encountered in determining exactly (by the ninhydrin-reaction) the small amounts of these substances, which appear in the secretion. The amino-acids seem to resemble glucose in their behavior,[110] viz., glycine, alanine, tyrosine, tryptophan, supplied from the portal vein, do not enter the tubules, whereas, after they have been supplied by both routes, the secretion does not contain

[109] J. J. Eiler, T. L. A. Althausen, and M. Stockholm, Amer. J. Physiol., **140**: 699, 1944.

[110] S. Robbins and M. L. Wilhelm, Pflüger's Arch. f. d. ges. Physiol., **232**: 66, 1933.

more than 1/5 to 1/10 of the perfusion concentration, the major fraction being absorbed. Further, after inactivating the proximal tubules by a narcotic, the reabsorption is abolished. On the other hand, phlorizin fails to inhibit the absorption of glycine in the kidney,[111] as it fails to do in the intestine (p. 548). Corresponding results have been obtained with dogs.[112]

d. Secretion by Active Transfer.—The word "secretion" will be used in the following paragraph as indicating active transfer in the direction blood to lumen. Secretion is usually associated with increase of concentration, but not necessarily. The analogous term used in the preceding paragraph is reabsorption. It indicated active transfer in the direction lumen to blood, and, as yet this active transfer seems to be confined to "valuable" substances, such as sugar, amino-acid, salt, which serve in the body as foodstuffs or for osmoregulatory or other specific purposes. Whether this biological interpretation of the function, which was suggested by the study of intestinal absorption (see p. 550), may be generalized, can be decided by determining whether "useless" substances, for instance, waste products of metabolism, are also actively reabsorbed in the kidney. It is true, urea appears to re-enter the body in the mammalian kidney, but probably by passive penetration (p. 573). However, in the kidney of Elasmobranchs, where urea has to fulfil a special osmoregulatory function, it undergoes active absorption (p. 573). On the other hand, not only compounds such as those produced as metabolic end products, can be subject to active secretion, possibly owing to a special adaptation of the kidney cells, but numerous foreign substances also have been found to set going the secretory machinery. How this can take place is one of the main problems of the following paragraph.

The secretion of dyestuffs.—Previously (p. 556) it was explained how dyestuffs can be utilized not only to trace the pathways of dissolved substances through the amphibian kidney, but also to provide information about the functional differentiation of the main segments of the nephron by comparing the color intensity of the perfusion fluid and the secretion under various conditions of perfusion; in other words, by measuring the accumulation ratios. However, it must be added that here, as well as in the study of other secretory functions, it is advisable to employ acidic dyestuffs instead of basic ones. Basic dyestuffs are the classical "vital stains" of Paul Ehrlich, which, due partially to their basic character, partially to their lipoid solubility, are admitted to more or less all living cells, whereas certain acidic dyestuffs, especially certain sulfonic acid dyestuffs, are selected by special cells for active transfer or active uptake, and cannot enter after inactivation of these cells (see e. g., pp. 606ff). To this latter category of cells belongs the tubular epithelium of the kidney. There, the appropriate acidic dyestuffs, while being transported, in general display a homogeneous

[111] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **233**: 181, 1933.

[112] J. R. Doty, Proc. Soc. Exper. Biol. & Med., **46**: 129, 1941; and J. R. Doty and E. G. Eaton, Am. J. Physiol., **133**: 262, 1941; R. F. Pitts, *ibid.*, **140**: 156, 1943.

rather faint coloration of the entire protoplasm.[113] In the earlier investigations, the effect of perfusion of the kidney with a dyestuff often was described as it appeared under the microscope in slices of the excised organ. In such preparations the cells contain deeply colored vacuoles, which are mostly artifacts, arising in the dying protoplasm, especially after an *excessive infusion* of dye. On the basis of these observations the long and unproductive discussion referring to the alternative secretion-reabsorption has taken place.

It also has been mentioned previously (p. 556) that the dual dyestuff perfusion does not allow a clear-cut differentiation between proximal and distal functions. Nevertheless, at least *per exclusionem*, it can be shown that secretory activity mainly is due to the proximal tubules. If one of those dyestuffs, which can be concentrated by the kidney, is introduced from the aorta only, the portal vein being supplied with Ringer, the secretion will be slightly colored, not more, or, at best, little more, than the perfusion fluid, although the aortic perfusion not only supplies the glomeruli, but spreads around the distal tubules. But the reverse arrangement (aorta Ringer, portal vein dyestuff-Ringer) conveying the dye to the capillaries around the proximal tubules, is followed by the formation of a markedly colored fluid. However, the color intensity under these conditions is found to vary greatly, among other reasons, because the amount of glomerular fluid, which mixes with the proximal secretion, is a variable factor. The following three experimental objects are free from this complication, and, therefore, can provide somewhat more direct evidence of the tubular activity: the aglomerular kidney (p. 568), the excised surviving frog kidney (p. 567), and the kidney explants of the chicken embryo (p. 569).

The secretion of the sulfonic acid dyestuffs.—The first definite proof of a secretory activity of the kidney tubules was given by E. K. Marshall, Jr.[114] who showed that after a subcutaneous injection of phenol red the amount of dyestuff available in the blood for filtration in the glomeruli at a normal rate of blood circulation is too small to account for the amount of dyestuff accumulated in the urine. The conclusion was drawn that the dyestuff concentration in the urine could not be due to reabsorption of water alone.

More directly, the dyestuff can be proved to be transported through the proximal tubules by the method of the dual perfusion with Ringer. Upon comparison of a great number of readily diffusible sulfonic acid dyestuffs in *low concentration*, e.g., 0.0005 %,[115] it is evident that some

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[113] J. G. Edwards and E. K. Marshall, Jr., Am. J. Physiol., **70**: 489, 1924; A. N. Richards, and J. B. Barnwell, Proc. Roy. Soc. B. **102**: 72, 1927; R. Chambers and G. Cameron, J. Cell. & Comp. Physiol., **2**: 99, 1932; R. Hober, *ibid.*, **6**: 117, 1935.

[114] E. K. Marshall, Jr. and J. L. Vickers, Bull. Johns Hopkins Hosp., **34**: 1, 1923; E. K. Marshall, Jr., and M. M. Crane, Am. J. Physiol., **70**: 465, 1924; see, further, F. Scheminzky, Pflüger's Arch. f. d. ges. Physiol., **221**: 541, 1929.

[115] The technical colors in aqueous solution include all degrees of dispersion from molecular

of the dyes are concentrated by the proximal tubules to a greater or smaller extent, and others are not.[115] This is especially conspicuous, when from a mixture of one specimen of each of these groups of dyestuffs being tested, a more or less complete demixture is brought about by the kidney (see pp. 567, 568). In order to answer the question, as to what factors are involved in such different behavior, among technical acid dyestuffs, those belonging to certain chemical groups have been selected, chiefly azo-, disazo-, triphenylmethane- and sulfonaphthaleine-compounds. Among these the study of the azo-dyestuffs was found to be most enlightening.

The azo-dyestuffs.—The basic structure of the azo-dyestuffs is composed of two ring systems, either benzene or naphthalene, linked together by an azo group. To this skeleton there are attached one or more sulfonate groups as well as other radicals, NH_2, OH, CH_3, and others.[117] The greatest bearing upon the physiological behavior of these dyestuffs appears to be referable to the sulfonate groups,[118] as indicated by the following observations:

1. Ten benzene-azo-naphthalene mono-sulfonates were studied. They appeared accumulated in the secretion, no matter whether the sulfonate was on the benzene or the naphthalene ring.

2. With 8 benzene-azo-naphthalene di-sulfonates the essential point is whether one of the two ring systems carries both sulfonates, or each ring system carries one of them. In the first case, the dyestuffs (5) were accumulated by the kidney, in the second case (3) they were not.

3. A similar behavior is shown by naphthalene-azo-naphthalene disulfonates. With 5 dyes the attachment of both sulfonates to one of the naphthalenes resulted in a marked accumulation, while 4 (out of 5) with one sulfonate on each side were concentrated only very weakly.

4. The result with tri-sulfonates is irregular.

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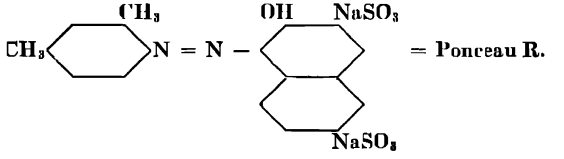
distribution to colloidal solution of the hydrophobic or the hydrophilic type (see Sec. 5, chap. 16). Correspondingly, after an (intravenous) injection in ample concentration, some dyes pass the glomeruli, others do not; some appear more quickly, some more slowly, in the secretion. In general, the semicolloidal and colloidal dyes, in contrast to those in molecular dispersion, are deposited after a time in the form of granules in the proximal epithelia, first in the vicinity of the glomeruli, then farther along the tubules, some for a shorter, others for a longer, period. This is not a reabsorption in the usual sense and is independent of the essential activity of the kidney (R. Höber, Biochem. Ztschr., **20**: 56, 1909; W. von Möllendorff, Anat. Hefte, **53**: Heft 1, 1915; P. Gérard and R. Cordier, Biol. Rev., **9**: 110, 1934. See, further, pp. 615ff.

[115] F. Scheminzky, Pflüger's Arch. f. d. ges. Physiol., **221**: 641, 1929; G. Orzechowski, *ibid.*, **225**: 104, 1930; R. Höber and P. M. Briscoe-Woolley, J. Cell. & Comp. Physiol., **15**: 35, 1940.

[117] For instance:



= Ponceau R.

[118] R. Höber and P. M. Briscoe-Woolley, *loc. cit.*

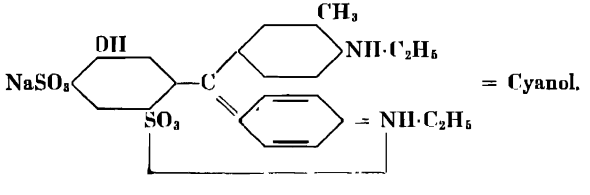
The following interpretation has been proposed: the molecules of the azo-dyestuffs are characterized by a bilateral and possibly elongated structure. Those with the sulfonate groups on one side have the character of a dipole, the non-sulfonated half being nonpolar and organophilic-hydrophobic, the sulfonated half polar and hydrophilic (see chap. 20). According to the concepts developed by Langmuir and Harkins, the dipole molecules can be assumed to be oriented in such a way as to bring about a loose fixation of the organophilic half to the cell surface, whereas the hydrophilic half is turned toward the water. The dyestuffs with hydrophilic groups on either side may be supposed to lack such an orientation and fixation. Eventually, the influence of other radicals besides sulfonates, displaying a lesser affinity toward the organic surface, can invalidate the hydrophilic forces. The attachment to the cell would be conceived as being the first step toward the entrance into, and the transfer across, the cell.

However, another factor favoring the entrance of the dyestuffs has to be discussed, lipoid solubility. Previously it was shown that the term lipoid solubility is somewhat misleading, insofar as it pretends to refer to a simple solution in an organic solvent. However, lipoids are mixtures of organic compounds, which, at least partially, by chemical affinities, affect the distribution of organic substances. Now, many of the azo-dyestuffs have been found to distribute between water and a lipoid, when shaken with both solvents. Employing as the lipoid phase a mixture of oil + oleic acid + diamylamine[119] (see chap. 13), e.g., all benzene azo-naphthalene mono-sulfonates largely enter this mixture from aqueous solution, indicating that the organophilic affinity of the ring system is strong enough to overcome the hydroaffinity of the one sulfonate group. However, among the benzene azo-naphthalene and the naphthalene-azo-naphthalene di-sulfonates there are compounds combining a distinctly nonpolar-polar configuration with a high accumulation ratio, some of them being lipoid soluble, others lipoid insoluble. Therefore, lipoid solubility cannot be conceived to be more than a secondary factor in their secretory transfer.

The tri-phenylmethane dyestuffs.—Similar considerations seem to be serviceable in accounting for the conditions encountered in the secretion of the tri-phenylmethane dyestuffs. The basic structure of these dyestuffs, a central C-atom surrounded by three benzene nuclei,[120] does not lend support to the idea of the necessity of a bilateral configuration. Further, all

[119] E. Nirenstein, Pflüger's Arch. f. d. ges. Physiol., **179**: 233, 1920.

[120] For instance:



sulfonic acid tri-phenylmethanes studied so far (14 specimens) are lipoid insoluble. It may be due to these properties that the Ringer perfused frog kidney either fails to respond to the presence of the tri-phenylmethanes, or does so less than usually observed under the conditions of a perfusion experiment. Whereas the portal perfusion with the solution of an appropriate azo-dyestuff as weak as 0.1 to 0.5 mg. per cent (p. 564) is succeeded by an accumulation in the excretion, in the corresponding experiments with the tri-phenylmethanes the perfusion concentration has to be raised to a level 10 times higher or more, in order to cause the appearance of dyestuffs in even a low concentration, and this only if the perfused dyestuff possesses an aromatic amine in its molecular structure. These observations suggest that chemical affinities are involved in the secretory uptake, in place of the nonpolar-organophilic affinities of the azo-dyestuffs (see, further, p. 570).

Finally, it should be mentioned that there is no better method to differentiate between secretory concentration and concentration by reabsorption of water in kidney function, than to make use of the diverse qualifications of the sulfonic acid dyestuffs for secretory transfer by perfusing a mixture of two dyestuffs, which differ distinctly from each other. For instance, when a frog kidney is perfused from the arterial and from the venous side with Ringer plus 0.3 mg. per cent of the lipoid insoluble triphenylmethane, cyanol, the secretion shows a trace of blue color corresponding to a twofold concentration, due mainly to reabsorption of water. In a second period of the experiment, with perfusion again from both sides with 0.3 mg. per cent cyanol in addition to 0.3 mg. per cent azo-fuchsine I, a lipoid insoluble secretable azo-dyestuff of red color, the secretion shows an intensely pinkish red color corresponding to a twentyfold concentration of the azo-fuchsine. If only reabsorption of water had taken place, both dyestuffs provided at the same concentration to either side of the epithelial wall should have been concentrated to the same level. It is obvious that the separation of the two diffusible dyestuffs is the result of their different secretability.[121]

Kidney function without glomeruli.—The experiments with azo- and tri-phenylmethane-dyestuffs concerning the secretory properties of the proximal tubules are supplemented and partially corroborated by the observation of preparations, in which the function of the glomeruli is more or less excluded.

a. *The excised kidney.* Richards and Barnwell[122] have shown that the excised frog kidney, immersed in well-aerated Ringer, retains its activity for some time. This is indicated by the facts that, first, after 0.01 to 0.03 per cent phenol red has been added to the Ringer solution, dark-red threads appear under the microscope in the lumina of the tubules; second, addition of

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[121] R. Höber, Pflüger's Arch. f. d. ges. Physiol., **224**: 72, 1930; Liang, *ibid.*, **222**: 271, 1929; P. Steffanutti, *ibid.*, **226**: 148, 1930; see, further, p. 568.

[122] A. N. Richards and J. B. Barnwell, Proc. Roy. Soc., London, B. **102**: 72, 1927.

m/100 cyanide prevents the accumulation of the dye. Extending these observations to other dyestuffs, it is easy to show that the dyestuffs, in general, behave just as to be expected from the perfusion experiments, provided that one takes into account that, instead of being distributed by the blood vessels throughout the entire organ, the dyestuffs enter the excised kidney from the surroundings by diffusion across its surface, and that, therefore, whatsoever changes may be brought about in the composition of the tubular contents by the activity of their walls will not be nullified by the fluid, which normally passes into them from the glomeruli.[123] E.g., the microscopic picture ordinarily appearing after exposure of the excised kidney to one of the secretable azo-dyestuffs is that on the dorsal side the lumina appear to be filled with more or less intensely colored fluid, contrasting with the relatively pale epithelial wall, whereas on the ventral side the tubules as well as the glomeruli mostly remain colorless. But this pattern never becomes visible, when, after an ordinary perfusion with dyestuff in low concentration, the kidney is excised and examined under the microscope, even when the kidney had delivered a secretion showing a high accumulation ratio, because the dyestuff solution, even at such a concentration level, is too pale in the thin lumina to become conspicuous under the microscope. Another factor contributing to the distinctness of the microscopic picture in the excised kidney is, that the tubular contents not only fail to be diluted by the glomerular fluid, but, on the contrary, are reduced in volume by water reabsorption (see pp. 558, 561). Thus, it becomes clear that even those triphenylmethane dyes, which were mentioned as appearing in the secretion during a perfusion experiment at best at a very low concentration, can produce an excellent dyestuff pattern in an excised kidney immersed in the 0.03% solution of a suitable tri-phenylmethane (e.g., cyanol).[124] On the basis of other observations, the conformity of the action of the perfused and the excised kidney is immediately obvious. Thus, exposing one kidney to a disulfonated azo-dyestuff with the sulfonate groups attached to one side of the molecule (e.g., azo-fuchsine I), the other kidney to the corresponding dye with the sulfonates opposing each other (azo-fuchsine G), the characteristic pattern appears with the first and does not with the second. Further, the excised kidney, immersed in the violet mixture of blue cyanol and red azofuchsine G, separates the two dyes so that a blue secretion becomes visible in the lumina. But, no pattern appears in the presence of a narcotic, e.g., phenylurethane or phenylurea.

b. *The aglomerular kidney.* Nature has provided the opportunity to study some fish with kidneys lacking or nearly lacking in glomeruli. Attention of physiologists has been turned to this material mainly by Marshall and Grafflin[125] and by Edwards.[126] The chief species investigated have

[123] J. G. Edwards, Am. J. Physiol., **95**: 493, 1930.

[124] R. Höber and P. M. Briscoe-Wooley, J. Cell. & Comp. Physiol., **15**: 63, 1940.

[125] E. K. Marshall, Jr., and A. L. Grafflin, Bull. Johns Hopkins Hosp., **43**: 205, 1928.

[126] J. G. Edwards and L. Condorelli, Am. J. Physiol., **86**: 383, 1928.

been the goose-fish (*Lophius piscatorius*) and the toad-fish (*Opsanus tau*). Their kidneys are composed of blind tubes, the walls of which are formed by epithelial cells, with a brush border resembling the proximal tubules of the glomerular kidney of other vertebrates. Even anticipating the permeability of these tubes toward solutes to be like that of the proximal tubules of the frog or any other animal, there remains the special problem of water transport, since the aortic pressure has been found lower than the secretory pressure in the ureter.[127]

The study of the passage of solutes gave the following results: After intramuscular injection, many substances appeared in the urine of the toad-fish: uric acid, creatinine, iodide, the sulfonic acid dyestuff indigo-carmine, and phenol red, the basic dyestuff neutral red, and others.[128] Even more instructive are quantitative determinations. 1. Ferrocyanide, xylose, glucose do not appear, whereas they are excreted in the urine of fish with glomerular kidneys.[129] Previously (p. 557), it has been mentioned that these substances also failed to pass the proximal wall of frog tubules in the direction blood to lumen. 2. The aglomerular kidney (*Lophius*) differs from the glomerular kidney in being able to concentrate Mg and SO_4 thirty times and even more.[130] 3. Provided the toadfish is handled very carefully, especially avoiding any skin injury, the urine is practically Cl-free[131] (see p. 585). 4. Over a wide range of urea levels in the plasma, the concentration in the urine is found to be the same as in the plasma. This seems to indicate diffusion probably due to the small molecular volume[132] (see, further, p. 572). 5. The toad-fish kidney is able to concentrate phenol red up to 150 times. Further, after the subcutaneous injection of certain diffusible sulfonic acid azo-dyestuffs, the same microscopic picture appears as is seen with excised frog kidney, viz., the lumina of numerous tubes are filled with color, the surrounding epithelial walls contrasting in their paleness.[133]

c. *Explants of the mesonephros of chick embryos.* Chambers[134] has discovered an excellent procedure to study the function of isolated tubules. The mesonephros of an 8 to 10-day chick embryo is cut to pieces, tubules are mechanically separated from their glomeruli and tubular fragments are explanted. In growing, the broken ends often become closed and are converted into cysts by the intake of fluid. Proximal and distal pieces are identified by their characteristic epithelial structure, by their greater distension due to a larger fluid intake, and especially by the fact that only

[127] R. N. Bieter, Am. J. Physiol., **97**: 66, 1931.

[128] E. K. Marshall, Jr., and A. L. Grafflin, J. Cell. & Comp. Physiol., **1**: 161, 1932; also, R. Höber, Pflüger's Arch. f. d. ges. Physiol., **224**: 72, 1930.

[129] E. K. Marshall, Jr., Am. J. Physiol., **94**: 1, 1930.

[130] E. K. Marshall, Jr., and A. L. Grafflin, Bull. Johns Hopkins Hosp., **43**: 205, 1928.

[131] A. L. Grafflin, Am. J. Physiol., **97**: 602, 1931.

[132] E. K. Marshall, Jr., and A. L. Grafflin, J. Cell. & Comp. Physiol., **1**: 161, 1932.

[133] R. Höber, J. Cell. & Comp. Physiol., **6**: 117, 1935.

[134] R. Chambers and R. T. Kempton, J. Cell. & Comp. Physiol., **3**: 131, 1933.

proximal cysts, while embedded in a very dilute solution of phenol red, take up the solution and transmit it into the lumen with a 30 times higher concentration. If in a cyst of a distal segment incidentally a bit of proximal epithelium is included, phenol red will stain only the proximal cells, which are thus sharply differentiated from the colorless distals. The color appears in the cells in homogeneous solution except under abnormal conditions, when cytoplasmic vacuoles are formed (see p. 616). Irrespective of the higher concentration of dyestuff in the cysts, the total molarity of the enclosed fluid (withdrawn from the cysts by a micropipette), in other words, its osmotic pressure has been found to be about 10 per cent lower than that of the surrounding fluid, its hydrostatic pressure, i.e., its secretion pressure higher.[135] This latter fact corresponds to the aforementioned measurements of Bieter, regarding the aglomerular kidney of the toad-fish (p. 569). Further properties of these preparations will be referred to later (see chap. 38).

Secretion of nondyestuffs. Dyestuffs more than any other compounds have proved to be appropriate for utilizing the amphibian kidney to show the functional differentiation of the various segments of a nephron by inspecting the pathways of the color and measuring the color strength. The sulfonic acid azo-dyestuffs, in particular, have provided some inkling as to a correlation between the molecular configuration of a dye and the secretory capacity of the proximal tubules (p. 565).

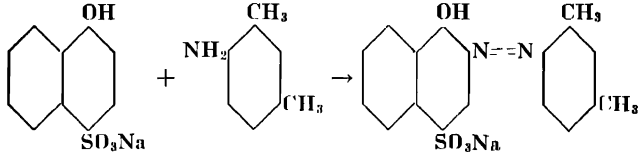
These studies suggested investigation of simpler colorless chemical compounds by applying the following methods:[136] Breaking the azo bond in an azo-dyestuff yields two colorless halves, each of which contains a benzene or a naphthalene ring, and at least one of them a sulfonate radical. The sulfonated half evidently has a nonpolar-polar, organophilic-hydrophilic composition (chap. 20). The question, whether as such it may potentiate a secretory transfer, can be solved by determining whether or not the colorless secretion yields a color, after another suitable half-molecule of an azo-dyestuff has been coupled in a diazoreaction to the substance in question.[137] The intensity of the color eventually appearing in the secretion is indicative of the accumulation ratio.

With a fairly great number of amino- and hydroxy-naphthalene mono- and di-sulfonates, supplied to the kidney from the renal portal vein, the simple result of this procedure has been, that the mono-naphthalene sulfonates undergo secretory concentration by the proximal tubules, the di-sul-

[135] J. Keosian, J. Cell. & Comp. Physiol., **12**: 23, 1938.

[136] R. Höber, P. M. Briscoe-Woolley, J. W. Green, and M. Zimmerman, J. Cell. & Comp. Physiol., **19**: 183, 1942.

[137] For instance:



fonates do not. Further, the amino-benzene mono-sulfonates also fail to be concentrated. Whether these findings may be interpreted as being due to the physicochemical character of a nonpolar-polar configuration is uncertain. Probably, the result depends upon a balance in the molecules between the hydrophilic forces of the sulfonate, on one hand, and the organophilic affinity of a simple benzene or a double naphthalene ring (combined with chemical affinities of the various radicals attached to the ring system), on the other hand.

This concept can be substantiated by the following observations:

1. Certain derivatives of sulfanilamide, $NH_2 \cdot C_6H_4 \cdot SO_2NH_2$, which as such passes the kidney without being accumulated, can be converted into organophilic-hydrophilic substances either by substituting for the sulfonamide group a sulfonate, for instance:

$$NH_2 \cdot C_6H_4 \cdot SO_2NH \cdot C_6H_4SO_2NH_2 \rightarrow NH_2 \cdot C_6H_4 \cdot SO_2NH \cdot C_6H_4 \cdot SO_3Na,$$

or a carboxyl group, or by attaching to the NH_2 a fatty acid radical, for instance:

$$NH_2 \cdot C_6H_4 \cdot SO_2NH_2 \rightarrow NaOOC \cdot CH_2 \cdot NH \cdot C_6H_4 \cdot SO_2NH_2$$[138]

They are then capable of being concentrated.

2. On the background of the experience that an organophilic-hydrophilic structure is one of the essential factors in kidney activity, new light is thrown upon the processes of physiological detoxication. Toxic substances, either arising as metabolic products or somehow entering the body, often are weak electrolytes and are soluble in lipoids, and for these reasons are apt to enter the cells. One customary procedure of the living animal for detoxication of these substances is their conjugation with relatively strong acids, like sulfuric acid, glucuronic acid, and others. This incorporation of the poison into a strong electrolyte, in general, results in incapacity to penetrate the cells. A second effect is the formation of a hydrophobic-hydrophilic compound which can be eliminated by way of the kidney cells (indol → indoxylsulfonate). But, this conjugation also occurs with weaker acids, the strength of which is increased by the conjugation so much that again the product is highly ionized and acquires an organophilic-hydrophilic configuration. E.g., the toxic benzoic acid is conjugated with glycine by coupling the benzoyl radical to the NH_2 group. The conjugation product, hippuric acid, is a fairly strong electrolyte ($pK = 2.3 \times 10^{-4}$) and has an organophilic-hydrophilic structure. Possibly for this reason hippurane, i.e., o-iodohippurate, which is one of the most satisfactory contrast media for roentgenography of the kidney, is found to be eliminated by secretion.[139]

Still more applied in X-ray testing of kidney function is diodrast,

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[138] R. Höber, P. M. Briscoe-Woolley, J. W. Green, and M. Zimmermann, J. Cell. & Comp. Physiol., **19**: 183, 1942; also, R. Höber, Federation Proc., **1**: 240, 1942.

[139] K. A. Elsom, P. A. Bott, and E. H. Shiels, Am. J. Physiol., **115**: 548, 1936.

3.5-diiodo-4-pyridon-N-acetate, whereas skiodan, another organic iodine compound, mono-iodo-methane-sulfonate, fails to be accumulated in the urine.[140] From the structural formulæ one possibly can conclude that this differential behavior is indicative of a correspondingly stronger or weaker polar structure of the molecules.

Diodrast, furthermore, has been utilized for approaching an important physiological problem, viz., in how far the active reabsorptive transfer and the active secretory transfer can be paralleled. By the experimental investigation of this problem, new aspects of the transfer mechanism have been aroused. For reasons previously mentioned, glucose absorption in the intestine and glucose reabsorption in the proximal tubules are assumed to need an intermediary phosphorylation. This concept is especially strongly supported by the inhibitory influence of phlorizin (pp. 548 and 561). Now, as has been shown by White[141] and by Eiler, Althausen, and Stockholm,[142] both the reabsorption of glucose and the secretion of diodrast are diminished by phlorizin. But it is highly improbable that phosphorylation is directly involved in the transfer of diodrast, and another way of interpretation has to be looked for. This will be discussed later (p. 617).

e. Urea and Uric Acid.—Attention is now turned to the output of two metabolic waste products of special physiological significance. In their analysis, great difficulties have been encountered, partly due to divergences in behavior from genus to genus. Again the main material for study was the amphibian kidney. Other investigations will be mentioned later (see p. 578).

Urea is one of the main organic components of the amphibian urine. Its concentration usually surpasses that of the plasma. The percentage in the contents of Bowman's capsule and the plasma have been found practically equal.[143] But, from the capsule downward, the concentration increases along the proximal, and continues to rise along the distal, tubule.[144] Thus, the final concentration reaches in *Necturus* an average value of 2.2, in frog of 7.8 times the plasma concentration. In order to determine whether these differences are attributable to reabsorption of water or to secretion, phlorizinized animals were used, with the result that in *Necturus*, as well as in frogs, the average concentration for glucose was found to be 2.2 times the plasma concentration, i.e., the same values as found for urea in *Necturus*. Therefore, the conclusion is drawn that accumulation of urea in *Necturus* may be referred to absorption of water, but in frogs to both water absorption and secretion. This conforms to earlier experiments of Marshall,[145] comparing urea with xylose, the latter being practically indifferent to the tubular cells (p. 575).

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[140] E. M. Landis, K. A. Elsom, P. A. Bott, and E. H. Shiels, J. Clin. Invest., **15**: 397, 1936.

[141] H. L. White, Amer. J. Physiol., **130**: 582, 1940.

[142] J. J. Eiler, T. L. Althausen, and M. Stockholm, Amer. J. Physiol., **140**: 699, 1944.

[143] A. M. Walker and K. A. Elsom, J. Biol. Chem., **91**: 593, 1931.

[144] A. M. Walker and C. L. Hudson, Am. J. Physiol., **118**: 153, 1937.

[145] E. K. Marshall, Jr., J. Cell. & Comp. Physiol., **2**: 349, 1932.

In another way, evidence is afforded for an active transfer of urea by the frog kidney. Comparing the various urea concentrations in plasma to those in urine, it appears that the accumulation ratio in general is in an inverse relationship to the plasma concentration.[146] The same phenomenon has been observed with dyestuffs, e.g., phenol red and other sulfophthaleins,[147] and in studies upon other kinds of secretory transfer, e.g., plant cells, (see chap. 36), and as such the phenomenon is an argument for assuming active transfer, which is possibly effectuated by a carrier system (see p. 577).

In concluding, it should be made clear that this important metabolite urea is subject to most variable modes of distribution. As just mentioned, among the amphibia, *Necturus* and frog show great differences. In mammals and in some teleosts, absorption—possibly only as a passive penetration in the direction lumen blood—is a preponderant factor[148] (also p. 579), and in elasmobranchs (dogfish) urea is actively reabsorbed by the tubules,[149] apparently in order to participate in the osmotic balance of the blood of these animals, which has been known for a long time to contain 2 to 3 per cent urea, besides a percentage of salt considerably lower than that present in sea water.[150] Finally, in the frog kidney, urea can be stored to such an extent that its concentration at the dorsal side of the kidney, where the proximal tubules are located, by far surpasses the concentration in blood and in urine.[151] The nature of the mechanism accounting for the storage is unknown.

Uric acid.—In order to study the elimination of uric acid by the amphibian kidney, 1 to 3 mg. per cent uric acid (as lithium urate) are supplied to the isolated Ringer perfused frog kidney either from the arterial or from the venous side.[152] In the first case, it appears in the secretion 2 to 2.5 times concentrated; in the second case the average accumulation ratio is 10. In the light of previous considerations (p. 564), this is indicative of a secretory activity of the proximal tubules. This belief is supported, first, by demonstrating that m/1000 cyanide, simultaneously administered through the portal vein, reversibly depresses the concentration and the amount of urate delivered; second, by determining with a suitable

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[146] E. K. Marshall, Jr., and M. M. Crane, Am. J. Physiol., **70**: 465, 1924; A. M. Walker and C. L. Hudson, *ibid.*, **118**: 153, 1937.

[147] E. K. Marshall, Jr., and M. M. Crane, *loc. cit.*: F. Scheminzky, Pflüger's Arch. f. d. ges. Physiol., **221**: 641, 1929.

[148] N. Jolliffe, J. A. Shannon, and H. W. Smith, Am. J. Physiol., **100**: 301, 1932; **101**: 639, 1932.

[149] R. W. Clarke and H. W. Smith, J. Cell. & Comp. Physiol., **1**: 131, 1932; H. W. Smith, Am. J. Physiol., **98**: 279, 296, 1931.

[150] W. von Schroeder, Ztschr. f. Physiol. Chem., **14**: 576, 1890; see also p. 584.

[151] E. K. Marshall, Jr., and M. M. Crane, Am. J. Physiol., **70**: 465, 1924; R. Höber, Pflüger's Arch. f. d. ges. Physiol., **224**: 422, 1930; **234**: 716, 1934; P. B. Rehberg and C. Blem., *ibid.*, **230**: 689, 697, 1932.

[152] B. Lueken, Pflüger's Arch. f. d. ges. Physiol., **229**: 557, 1932.

dyestuff, cyanol (see p. 566), that the supply of the urate is mainly confined to the proximal tubules.[153]

A similar situation has been found in lizards.[154] After poisoning with phlorizin (see p. 561) the percentage of glucose and uric acid in plasma and in urine is compared. The average concentration ratio for glucose is found to be 3.2, for uric acid 51.4. Reabsorption of glucose being stopped by phlorizin, the ratio of 3.2 indicates the glomerular filtrate to be concentrated 3.2 times by tubular reabsorption of water. It follows that by far the most of the uric acid must be eliminated by tubular secretion. The fact that in man uric acid is believed to undergo reabsorption will be referred to later (p. 579).

Again, information is needed as to whether the secretability of uric acid can be assigned to any of its chemical or physicochemical properties As a matter of fact, uric acid and its salts are exceptional in that they have a tendency to form colloidal solutions, which readily turn to unstable ultramicroscopic and microscopic suspensions. As such they may be stored. At least in frog after an intravenous injection, there appear very fine precipitations solely in the cells of the proximal tubules, not in the distal tubules.[155]

f. The Plasma Clearance and Tubular Function.— So far, chiefly, such experiments have been reported as were intended to elucidate the kidney function by subjecting this organ to rather artificial and simplified conditions compatible with the living state of the organs of amphibia, i.e., by isolating the kidney from the rest of the body and utilizing its dual supply with Ringer solution. Now we turn to experiments upon the kidney in its normal functional relationship to all the other organs, especially experiments upon the mammalian kidney. Certainly, this procedure is fraught with essentially greater technical difficulties, and in order to approach the goal of substituting the more qualitative results of the so far described experiments with more quantitative ones, it is necessary to keep the animal under certain basic conditions.

The plasma clearance[156] is the capacity of the kidney to clear during one minute a certain volume of the blood plasma (measured in cc.) of an excretable substance.[157] The amount of this substance is $P \times C$, if P is its concentration in the plasma, C the plasma volume passing through the

[153] For objections to the assumption that the elimination of uric acid is due to secretory activity, see J. Boardley and A. N. Richards, J. Biol. Chem., **101**: 193, 1933.

[154] E. K. Marshall, Jr., Proc. Soc. Exper. Biol. & Med., **29**: 971, 1932.

[155] B. Lueken, *loc. cit.*

[156] See, for the following, H. W. Smith, The Physiology of the Kidney: Oxford University Press, New York, 1937.

[157] The clearance value, in general, is referred to plasma, and not to whole blood, since it is only the plasma which undergoes filtration (or rather ultrafiltration) in the nephrons, and since, due to the great variations in the distribution of the substances in question between the blood corpuscles and the plasma, the concentrations in the whole blood can be far from identical with the concentrations in the plasma.

glomerular membranes into the nephrons. The same amount of the substance reappears within one minute in the urine, provided that in passing the nephrons nothing is withdrawn by reabsorption and nothing is added by secretion. This amount in the urine equals $U \times V$, if U is its concentration and V the volume of the urine excreted. It follows that $UV = PC$, or $C = UV/P$, where, under the conditions mentioned, C, irrespective of the plasma concentration, has been found to be constant, i.e., the circulatory supply of the kidney is constant. Now, if the concentration of an excretable substance, after having passed through the glomeruli, is augmented by the secretory activity of the tubular walls, the constant C, as calculated from the above equation, appears to be increased; if it is diminished by the reabsorptive power of the walls, it appears decreased.

Thus, a way is open for deciding whether a substance passing the kidney, apart from being filtered, undergoes secretion or reabsorption, by comparing the substance with a standard substance, which is neither secreted nor absorbed and which is best supplied simultaneously with the substance in question. Probably, there exist many substances suitable for such standards. They should comply with certain requirements; for instance, they should be chemically, physicochemically, and physiologically rather inert, they should have a medium-sized molecule and thus be filterable; and others. According to A. N. Richards and H. W. Smith, the starch-like polysaccharide inulin has been proved adequate. Suitable properties have been found also in mannitol and sorbitol.[158] Further, in phlorizinized animals, in other words, in animals with kidneys disabled for reabsorbing sugars (p. 561), glucose, xylose, and sucrose behave like inulin. All these substances, when tested with the same animal, have led to fairly identical clearance values, irrespective of variation of the plasma concentration over a fairly wide range. An example using a normal human[159] is given in the following table:

TABLE XLVI.—INULIN CLEARANCE ON MAN

P mg./100 cc.	U mg./100 cc.	V cc./min.	C cc./min.
89	846	13 8	131
92	1058	11.5	132
96	1720	6.7	120
102	2055	6.2	125
106	2695	4.9	125
			Average 127

It appears that, with moderately rising plasma concentrations of inulin,

[158] W. W. Smith, N. Finkelstein, and H. W. Smith, J. Biol. Chem., **135**: 231, 1940.

[159] H. W. Smith, The Physiology of the Kidney: Oxford University Press, New York, 1937.

P, U rises and V falls, evidently due to water absorption, but C, the "clearance value," is fairly constant (average: 127 cc./min.).

However, consistent results cannot be obtained in clearance determinations, unless a certain basal status of the animal is maintained carefully. This means that, during each single experimental period of urine collection, the plasma concentration should be kept constant; further, that changes of the blood pressure and of the rate of blood supplied to the kidney; changes of the water balance and of the composition of the food, should be avoided.[160]

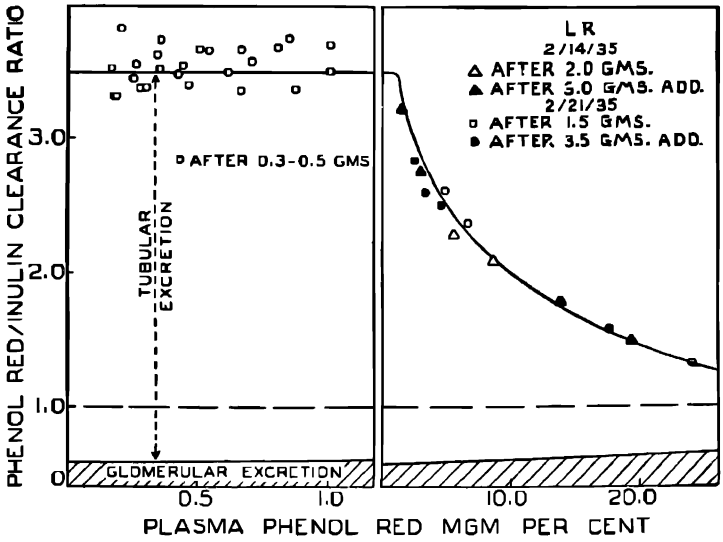

FIG. 61.—The phenol red inulin clearance ratio in man in relation to the concentration of phenol red in the plasma. Left, at plasma concentrations below 1.0 mg. per cent; right, at plasma concentrations above 1.0 mg. per cent.

As to the effect of a secretory transfer upon the clearance values, the results obtained in an experiment with phenol red and inulin simultaneously injected into a man, are outlined in Fig. 61. The phenol red clearance, compared to the inulin clearance (the horizontal broken line in Fig. 61), in terms of phenol red/inulin clearance ratios, surpasses the inulin value (equal to 1) by a maximum of 3.5 times, but it is not constant, as is the inulin clearance (see Table XLVI). Rather, it falls off above $P = 1$ mg. per cent, and, with P-values of 10 to 20 mg. per cent, approaches the inulin clearance figure. This indicates that inulin and phenol red enter the nephrons through the glomeruli at the same ratio as in the plasma, but the *percentage* elimination of phenol red by the kidney decreases when its *absolute* concentration in the blood rises beyond 1 mg. per cent. Apparently,

[160] Consult in this respect: H. W. Smith, *loc. cit.*; further, H. W. Smith, H. Chasis, W. Goldring, and H. Ranges, J. Clin. Investigation, **17**: 263, 1938; **19**: 751, 1940; J. S. Shannon, *et al.*, Am. J. Physiol., **101**: 625, 639, 1932; **102**: 534, 1932; **133**: 752, 1941.

above this concentration limit, the mechanism of the secretory transfer has reached its maximum capacity, and an additional output, after raising the phenol red concentration in the plasma, has no other source than the amounts filtered through the glomeruli, which are in linear relationship to the blood concentration. This behavior is illustrated by Fig. 62, referring to the phenol red concentration by the frog kidney.[161] The total amounts of phenol red excreted per unit of time are plotted against its plasma concentration (curve of dark circles). These amounts are equal to the product UV (p. 575). Their origin is partially glomerular filtration, partially

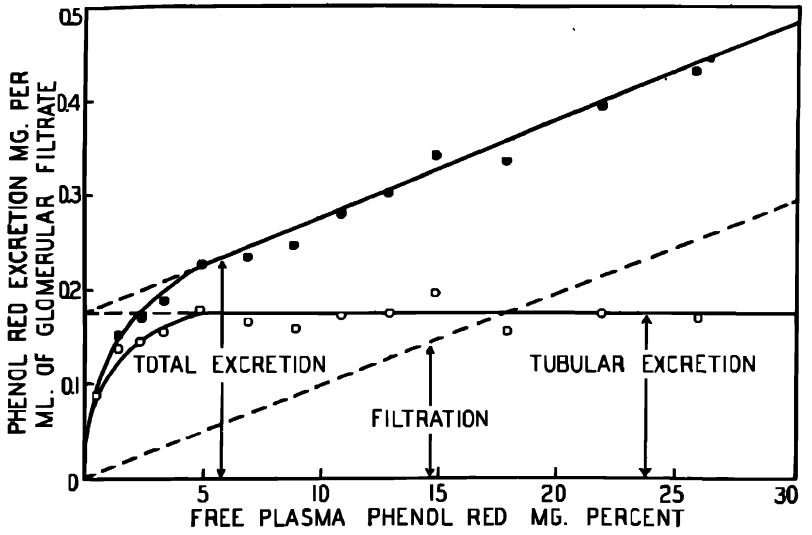

FIG. 62.—The effect of plasma phenol red concentration on the amount of dye excreted by the tubules and the glomeruli of the frog kidney.

tubular secretion. The amounts supplied by filtration at different plasma concentrations (broken line) can be calculated by applying the equation $UV/P = C$. Hence it becomes clear that, with rising P, the part of the total excretion which is due to filtration increases, whereas the part representing the transfer by the tubules (curve of open circles) reaches a maximum at a plasma concentration of about 5 mg. per cent, but is constant beyond that. Obviously, as noted before, there is a maximum capacity of the tubular cells to perform active transport, or, apparently, there are intracellular carriers capable of shifting a limited load. The structural and physicochemical nature of such carrier systems will be discussed later (p. 618).

Some comment is still necessary concerning the ascending part of the curve picturing the tubular transfer. Evidently, this part signifies that, starting with P equal to zero, the efficiency of the transferring system becomes less and less until the maximum uptake has been reached (at 5

[161] R. P. Foster, J. Cell. & Comp. Physiol., 16: 113, 1940.

mg. per cent in the experiment in question). In other words, the accumulation ratio falls off with rising concentration, as has been noted in various earlier observations.[162] It is particularly obvious with active systems, where passive penetration by filtration is negligible, for instance, in the case of the intestinal absorption of glucose (p. 544) or with certain plant tissues (see later, p. 592).

Correlating the results of experiments on various animals, the ratio between active transfer and passive filtration has been found to differ systematically from species to species. In other words, the tubular function can take precedence over, or be subordinate to, the glomerular function. The latter may even be negligible, as in the aglomerular fish.[163] As a matter of fact, when phenol red is injected into the goose-fish or the toad-fish, (p. 568) the effect of tubular excretion causes the dye concentration in the urine to rise with increasing plasma concentration to a maximum, in a manner similar to that shown in Fig. 62 by the curve with open circles. But, there is nothing in the picture of the total excretion by the aglomerular kidney, that can be compared to the ascending curve with dark circles, due to the additional glomerular filtration. Consequently, it is highly significant to the behavior of the aglomerular kidney, that the phenol red concentration in the plasma can exceed considerably the maximum concentration in the urine, indicating that, except through active transfer, the tubular wall is inaccessible to the dye.

So far, the report about the influence of secretion and reabsorption upon the plasma clearance has been chiefly confined to experiments with phenol red, which, from earlier experiments with other methods, was already known to be pushed through the tubular wall in the direction blood → lumen. Clearance determinations with some other substances likewise have led to conclusions, which more or less coincide with the previous results. It has been mentioned before that the differences of the clearance values, before and after phlorizin poisoning, indicate that glucose and xylose are reabsorbed by the normal kidney, in contrast to inulin, the clearance of which is unaffected by the presence of the poison.[164] The same conclusion was drawn from frog perfusion experiments (p. 561). Further, according to both methods applied to frog (p. 562) and dog, amino-acids seem to be reabsorbed by the tubules.[165] With urea a great variety of results was encountered in studies upon frog, *Necturus*, and dogfish, namely, active secretion, indifference, and active reabsorption (p. 572). The clearance method applied to

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[162] E. K. Marshall, Jr., and M. M. Crane, Am. J. Physiol., **70**: 465, 1924; F. Scheminzky, Pflüger's Arch. f. d. ges. Physiol., **221**: 641, 1929; J. S. Shannon, Am. J. Physiol., **113**: 602, 1935.

[163] J. S. Shannon, J. Cell. & Comp. Physiol., **11**: 315, 1938.

[164] J. A. Shannon, Am. J. Physiol., **112**: 405, 1935; J. A. Shannon and H. W. Smith, J. Clin. Investigation, **14**: 393, 1935.

[165] J. R. Doty, Proc. Soc. Exper. Biol. & Med., **46**: 129, 1941; J. R. Doty and A. G. Eaton, Am. J. Physiol., **133**: 262, 1941.

chicken, dog, and man has given evidence of possible reabsorption, but this may be better described as a passive escape through diffusion,[166] because of the low molecular volume of urea. According to the formerly reported results with the frog and the lizard (p. 573), uric acid undergoes active secretion, but clearance determinations in man[167] are interpreted by Smith as indicating active reabsorption.

This section of the book, dealing with passive penetration and active transfer in animal and plant tissues, has opened with the analysis of intestinal absorption and of urine formation, because this analysis brings up a large number of subjects that have to be discussed repeatedly in the following chapters of the section: In the performance of their function, both of these organs, intestinal mucosa and kidney, must dispose of many diverse substances, mixtures of foodstuffs in the one case, mixtures of blood components in the other. Both organs are compelled to admit some substances according to certain general physicochemical properties. But, in addition, in the intestine, special provision is made for the intake of substances, which are necessary for the satisfaction of special metabolic wants; and, in the kidney, there are other arrangements, empowering the output of other substances, either useless or detrimental to the body. Obviously, it is, at least partially, due to these selective activities that the rather definite concentration levels of the blood fluid, so essential to the constant life of the higher animal, are secured. And it is an important problem, as mentioned once before (pp. 554, 571), to test whether these teleologically conceivable selective capacities can be correlated to certain chemically or physicochemically understandable affinities between the dissolved substances and the constituents of the epithelial membranes.

Now, it is the plan to carry on the functional analysis of other transporting organs, which are more specialized in their physiological accomplishments, and correspondingly display an ability to select different substances for an active transfer, which is more restricted than that of the intestine and the kidney in their function of checking the passing substances at one of the main inlets and one of the main outlets of the body. According to this plan, the following organs will be studied: 1. the body surfaces of animals and the root system of plants; the discussion of their properties will raise the question of osmoregulation; 2. the digestive glands, the salivary and the stomach glands, pancreas, and liver; 3. the membranes separating the blood fluid and the aqueous humor and the cerebrospinal fluid.

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[166] P. B. Rehberg, Biochem. J., **20**: 461, 1926; D. D. Van Slyke, A. Hiller, and B. F. Miller, Am. J. Physiol., **113**: 611, 1935; J. A. Shannon, *ibid.*, **117**: 206, 1936; **122**: 782, 1938. About alterations of the clearance values due to water reabsorption in the proximal and in the distal tubules, see V. P. Dole, *ibid.*, **139**: 504, 1943.

[167] H. Berglund and A. R. Frisk, Acta Med. Skandinav., **86**: 233, 1935.

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THE PERMEABILITY OF THE BODY SURFACE OF ANIMALS AND PLANTS

It is obvious that, in many regards, this discussion will be limited to those living objects which normally are in intimate contact with an aqueous environment. The problem of natural permeability of their surface seems to be simplified by the fact that, in general, the permeability to non-electrolytes can be neglected, compared to the permeability to water and to inorganic ions. The main information regarding animals cannot be acquired by another procedure than by studying the properties of the entire animal in relation to its aqueous environment. But, then, it must be remembered that the permeation of water and electrolytes through the surface may be accompanied by the simultaneous intake and output through the intestinal tract, the kidney, the gills, and other organs. The amphibia alone provide the opportunity to investigate the properties of the isolated undamaged surviving surface, since in them the skin is only loosely attached to the body and, therefore, rather uninfluenced by the processes going on in the interior of the body. Concerning the properties of plants, the studies will be restricted here to the roots, including some functionally comparable systems.

1. The Osmotic Properties of the Marine Invertebrates.—For a long time, the more general concepts about the skin permeability of animals living in an aqueous surrounding were derived by Bottazzi, Frédéricq, Duval, and others from comparing the osmotic properties of the internal environment and the external environment (milieu interne and milieu externe of Claude Bernard). It was stated that, under ordinary conditions of life, the two media are approximately isotonic in the case of the marine invertebrates and the marine elasmobranchs. In other words, these animals are in osmotic equilibrium with the sea water; i.e., $\Delta_i \cong \Delta_o \cong 2.0°$, whereas the body fluid of the marine teleosts is hypotonic (Δ_i = about 0.8° to 1.0°) and that of the fresh water invertebrates (Δ_i = about 0.1° to 0.8°), the fresh water teleosts (Δ_i = about 0.55°) and the fresh water amphibia (Δ_i = about 0.4°) hypertonic.

This suggests the belief that maintaining a difference of osmotic pressure of several atmospheres across a thin cell layer, as effected by the marine teleosts and by fresh water invertebrates, teleosts, and amphibia, requires regulatory arrangements, which may be unnecessary in the other groups. In order to test this hypothesis, observations upon the osmotic properties of some marine invertebrates may be quoted first. In the Mediterranean

Sea ($\Delta_o = 2.3°$) Δ_i of the body fluid of *Mytilus edulis* was found to be 2.1°, while in sea water of the Baltic Sea of a certain provenience ($\Delta_o = 0.94°$) it was 0.95°. Similarly, in the North Sea ($\Delta_o = 1.7°$) Δ_i of *Arenicola marina* was 1.7°, in water of the Baltic Sea of another provenience ($\Delta_o = 0.8°$) 0.75°.[168] These osmotic equilibria in animals living in far-distant habitats can be explained as simply being reached either by the passive exchange of solutes or of water, or, possibly, they are due to complicated adjustments over a long period of time. However, when, in an acute experiment, *Aplysia*, a marine snail, suddenly is transferred into 75 per cent sea water, the weight increases within one hour about 25 per cent, and falls during the following 6 hours below the original weight, and the body approaches a lower osmotic equilibrium.[169] As will be shown later, this is probably due to nothing more than—as in a common osmometer experiment—an initial rapid osmotic uptake of water, followed by a slower escape of solutes.

But there are other observations on marine invertebrates suggestive of the existence of special osmoregulatory processes. When *Carcinus maenas* ($\Delta_i = 1.75°$) is transferred from sea water ($\Delta_o = 1.4°$) to diluted sea water ($\Delta_o = 0.63°$), Δ_i drops down to 1.3° within 26 hours. But, thereafter, this difference $\Delta_i - \Delta_o$ (1.3° − 0.63°) remains constant throughout the subsequent 15 days.[170] It cannot be inferred that this is caused by the slowness of diffusion across the exoskeleton of the crustaceans, because a similar reaction is observed with a marine annelid, *Nereis diversicolor*.[171] Presumably, those invertebrates, which, living in brackish water, are subject to frequent and large concentration changes of the external environment, are especially able to compensate in some way for the fluctuations of the osmotic pressure[172] (see also p. 584).

The belief that the aforementioned body weight or volume changes of *Aplysia* are attributable to penetration of both water and solutes is strongly supported by the experiments of Bethe, demonstrating particularly the passive permeability of the body surface to inorganic and organic compounds, which appears to be much more widespread than was earlier assumed. After *Aplysia* has been transferred into 3 parts of sea water +1 part of isotonic sucrose, the weight does not remain constant, as might be expected, but diminishes progressively, e.g., within 20 hours to about 50 per cent of its initial value. The reason is that, after the animal has been immersed in the mixture, the salt concentration is lower outside than inside. Hence, salt leaves by diffusion. As the result of that, the osmotic pressure becomes lower inside than outside, and water escapes. When, in an experi-

[168] C. Schlieper, Biol. Rev., **51**: 309, 1930.

[169] A. Bethe, J. Gen. Physiol., **13**: 437, 1930.

[170] M. Duval, Ann. Inst. Ocean., Monaco, **23**: 233, 1925.

[171] C. Schlieper, Ztschr. vergl. Physiol., **9**: 478, 1929.

[172] C. Schlieper, Biol. Rev., **5**: 309, 1930; A. Bethe, Pflüger's Arch. f. d. ges. Physiol., **234**: 629, 1934.

ment of this kind, sucrose is replaced by glucose or glycerol, the decrease of weight is smaller with glucose and still less with glycerol, as glucose passes more easily into the animal than sucrose, and glycerol still more rapidly.[173] More direct evidence of inorganic ions being able to penetrate in one or the other direction has been afforded by placing *Carcinus* and *Aplysia* alternately in artificial sea water, either containing the main cations and anions in normal concentration, or having a deficiency or a surplus of one of them, e.g. Ca.[174] One of the results is the following:

TABLE XLVII.—CONCENTRATION OF Ca AND Mg (MG./CC.) IN BLOOD OF *Carcinus*. INFLUENCE OF CHANGING THE Ca CONCENTRATION IN THE SURROUNDING MEDIA

External media	After hours	Ca	Mg
Artificial sea water	139	0 58	0.62
Same without Ca	139	0 30	0 59
Artificial sea water	240	0 70	0 50

It is obvious that in this experiment only Ca shows a decrease of concentration in the blood corresponding to the outside deficit. The loss of blood Ca often is accompanied by a loss of tonus and of reflex activity. A similar shift of ion concentration was obtained with Ca, Mg, and Cl.

It may be objected that, in experiments like these, the exchange of ions does not take place across the hard shell, but rather within the intestinal tract. However, the same result is obtained after closing the mouth and the anus. Direct proof of penetration of the shell has been furnished by cementing a metal cylinder upon the dorsal side of the carapace and filling it with dilute NaI. The iodide ion, which appears in the blood, is believed to pass chiefly across the small "holes" of the shells, which are occupied by cutaneous sensory organs.[175]

By these experiments upon crustaceans and echinoderms, not only the penetration of ions across the body surface has been shown to exist, but even seemingly to predominate over the water exchange, as followed from the slight changes of body weight under anisotonic conditions. However, this test is fallacious. In reality, e.g., with the animal in dilute sea water, the water can enter the body by osmosis but only in an amount equal to that of the fluid lost through ejection of jets of gastric juice, urine, and secretion of the antennal glands accompanying the increase of hydrostatic pressure inside the exoskeleton. This is one manner of osmoregulation under the special anatomical conditions of hard-shelled animals.[175]

So far, one might be led to conclude, from the majority of experimental

[173] A. Bethe, J. Gen. Physiol., **13**: 437, 1930; L. Frédéricq, Arch. internat. de Physiol., **19**: 309, 1922.

[174] A. Bethe, Pflüger's Arch. f. d. ges. Physiol., **221**: 444, 1928; **234**: 629, 1934.

[175] E. Berger and A. Bethe, Pflüger's Arch. f. d. ges. Physiol., **228**: 769, 1931.

[176] A. Bethe, E. von Holst, and E. Huf, Pflüger's Arch. f. d. ges. Physiol., **235**: 330; 1935; E. Huf, *ibid.*, **237**: 240, 1936.

data referred to, that the body surface of the marine invertebrates is really accessible to the components of their external media, like a sponge. But this concept is inconsistent with the fact that the percentage of individual inorganic ions, found in the blood serum of these animals under natural conditions, seems to diverge considerably from their percentage in the environment, and to display species-specific differences. Thus, with certain crustaceans, the Mg percentage is remarkably lower, the K percentage higher, than in the environment.[177] This would be incompatible with a merely physicochemical viewpoint of passive diffusion and osmosis, but could be attributable to physiological regulations, which, under the artificial conditions of an experiment, could be overshadowed by unusually gross effects. In any case, this conflict possibly will be abated by observations referred to in the following two paragraphs, which have furnished clear evidence that, under fairly normal conditions, a slow active transfer of very slight amounts of ions across the body surface can be effectuated by teleosts and by amphibia.[178]

2. The Osmotic Properties of Marine and Fresh Water Teleosts.— On the basis of present knowledge, it is believed that a steep and persistent concentration gradient between outside and inside is significant of the majority of teleosts. Approximate values of the gradient for marine and fresh water teleosts are listed in the following table in terms of differences of freezing point depressions, of atmospheres of osmotic pressure, and of molarities.

TABLE XLVIII

Marine teleosts				Fresh water teleosts			
Sea water...	2 3Δ_o	28 atm.	1 25 mol./l	Fresh water	0Δ_o	0 atm.	0 mol./l
Blood fluid..	1 0Δ_i	12 atm.	0 54 mol./l	Blood fluid	0 55Δ_i	6.6 atm.	0.3
Gradient....	1 3	16	0 71	Gradient.	−0 55	−6 6	−0 3

In general, both classes of fish die a short time after the normal concentration gradient has been changed, with the marine animals by diluting the sea water, with the fresh water animals by adding it. But there are some observations showing that, if this change of the surroundings is accomplished slowly enough by the gradual addition of more and more water

[177] A. Bethe and E. Berger, Pflüger's Arch. f. d. ges. Physiol., **227**: 571, 1931; A. B. Macallum, J. Physiol., **29**: 213, 1903.

[178] It was mentioned previously (p. 581) that the marine elasmobranchs resemble the marine invertebrates in that both groups of animals are in approximate osmotic equilibrium with their surroundings. But they differ in that with elasmobranchs no more than 40 to 50 per cent of the osmotic pressure of their blood fluid is due to salts; the rest is chiefly due to urea (p. 573). In this respect, the blood of the elasmobranchs is comparable to that of the marine teleosts, which is hypotonic to sea water (p. 581). Concerning the interesting complex osmoregulatory faculties of this class of animals and the particular role of urea, see C. Schlieper, Biol. Rev., **5**: 309, 1930; H. W. Smith, Am. J. Physiol., **98**: 279, 296, 1931.

or salts respectively, death can be avoided. This is shown, for instance, by the following experiment upon *Cyprinus carpio*.[179]

TABLE XLIX.—ADJUSTMENT OF A FRESH WATER TELEOST (CYPRINUS) TO SALT WATER

Δ_o gradually raised to	Δ_i	Experiment duration, days
0 02	0 50	0
0 48	0 57	21
0 84	0 83	48
1 04	1 06	12

It appears that, with stepwise increase in outside concentration, the inside concentration rises and retains the higher level over a long period. Obviously, the slow change has stimulated some adjustments, though not an osmoregulation.

Possibly light may be thrown upon the problems of such a gradual adjustment by the following observations upon the marine teleosts *Opsanus tau* (toadfish) and *Myoxocephalus*.[180] Under normal conditions, the flow of urine is very slow and the urine is free from chloride, the plasma chloride is not higher than 150 millimol./l. But even a slight injury of the skin, the avoidance of which requires extreme precaution, is followed by an enormous rise of urinary flow and of urine chloride (200 millimol. and more) and by a marked increase of plasma chloride, possibly accompanied by increased swallowing of sea water. It seems not unlikely that the aforementioned adjustment of *Cyprinus* is due to avoiding sudden changes of the osmotic conditions on the body surface, which would be succeeded by damage and disruption of the epithelial layers.

TABLE L.—INFLUENCE OF VARYING CONCENTRATIONS OF SEA WATER UPON THE BODY JUICE
Fundulus

Sea water (in mol. salt/e)	Δ s.w.	Δ_i after 1 day	Δ_i after 8 days
m/2	2 071	0.850	0 790
m/4	1 038	0.810	0.820
m/8	0.540	0.780	0.760
m/16	0.285	0 755	0.735
m/32	. . .	0.770	0.770
m/64	. . . . .	0 765	0.730
m/128	. . .	0 715	0 750

Most distinct osmoregulatory capacities are observed among the teleosts with those species, which, due either to migratory life (eel, salmon) or to their living in brackish water, are accustomed to great changes in the

[179] M. Duval, Ann. Inst. Ocean., Monaco, **23**: 293, 1925.

[180] A. L. Grafflin, Am. J. Physiol., **97**: 502, 1931; further, see W. E. Garrey, Biol., Bull., **8**: 257, 1905.

external media. E.g., the behavior of *Fundulus* is demonstrated in a well-known experiment of Loeb and Wasteneys.[181] Fish were distributed in various dilutions of sea water and kept in each solution for 8 days. The Δ of the body juice was determined after the first and the eighth days of each period with the results shown in Table L.

It appears, first, that after the first day of each period there is only a slight change of the internal osmotic pressure; and, second, irrespective of the hypertonicity or hypotonicity of the external media, the internal media are kept fairly constant.

But this result appears only in case the surface of the body remains intact. It does not occur when the surface is exposed to an unbalanced electrolyte solution (Sec. 4 and 5), instead of sea water. E.g., when sea water is substituted by an isotonic NaCl solution, the fish becomes very weak and Δ of its body juices increases from 0.89° to 1.11° after only one and one-half hours, indicating an abnormal increase of permeability of the body surface.

Information concerning the location of the osmoregulatory mechanism has been provided by experiments upon the eel.[182] For various reasons, the gills were looked upon as being of prime importance in regulatory activity. In an effort to test this hypothesis, the following procedure was applied: The head of the eel was severed from the body and a heart-gill preparation established with the heart, pumping, in a closed system, a constant amount of the internal medium through the gills and returning it through the dorsal aorta to the auricle, while in another closed circuit the external medium, coming from a reservoir, entered the oral cavity and, leaving it by way of the branchial apertures, returned to the reservoir. A Ringer-Locke solution of suitable concentration was used as internal medium, taking into account that for fresh water eels, Δ of the blood was reported to range from 0.56° to 0.63°, for sea water eels from 0.64° to 0.74°.

The result of analyses of the two media, which control each other, leaves no doubt that, in sea water eels, chloride is shifted against the concentration gradient from the perfusion fluid through the gill membrane into the sea water, which contains Cl in about three times higher concentration. This shift seems to increase with increasing Cl concentration in the perfusion fluid. With fresh water as the external medium, the gills seem to be inactive; they allow water to enter the perfusion fluid, and a surplus of water is believed to be eliminated by the activity of the kidneys, which, under these conditions, excrete a large amount of very dilute urine.[183]

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[181] J. Loeb and H. Wasteneys, J. Biol. Chem., **21**: 223, 1915.

[182] A. B. Keys, Ztschr. vergl. Physiol., **15**: 352, 364, 1931; Proc. Roy. Soc., London, B. **112**: 184, 1933.

[183] Reasoning that the function of active transfer of chloride ions might be reserved to specific structures in the gills, which by a histological investigation could be differentiated from the respiratory elements—just as, in the stomach glands, the parietal and the nonparietal cells have been distinguished as HCl- and as enzyme-producing units (see p. 599), Keys and Willmer,

The heart-gill preparation also has served to expand previous observations that a considerable excretion of N-compounds, especially of urea and ammonia, takes place in the gills. H. W. Smith[184] has fixed fresh water teleosts in a two compartment tank in such a way that the water surrounding the head and the body can be analyzed separately. It appears that the branchial output exceeds the urinary excretion by several fold. There is no reason in these experiments not to ascribe the urea output to a simple diffusion, since the concentration of the blood urea was always found to be higher than that in the water around the gills. With the heart-gill preparation, considerable amounts of urea can be detected in the external medium only when the internal medium contains urea.[185]

3. The Osmotic Properties of Amphibia.—The frog, living in fresh water ($\Delta_o \sim 0.1°$), maintains its internal medium at a level of $\Delta_i \sim 0.43$.[186] After having been kept dry for a while, resulting in a loss of 25 to 30 per cent body weight, the frog regains the lost weight in moist surroundings, even when water uptake by mouth is prevented. In water, the weight increases progressively after the cloaca has been closed, because the abundantly excreted urine accumulates in the intestinal tract. If, under such conditions, the frog is placed in salt solution instead of water, the increase of body weight diminishes with increasing salt concentration and changes to a decrease at about 0.8 per cent NaCl. These observations of Overton are indicative of a skin permeability to water in either direction. There is, further, a definite, though low, permeability to dissolved substances, particularly to inorganic ions. This is shown by the fact that after placing the frog in KCl solution, K appears in the urine in considerable amounts, but no symptoms of K-intoxication become evident, even in isotonic KCl, because the K-level in the blood is kept low by the activity of the kidneys.[187] According to Krogh,[188] frogs living in fresh water suffer continuously a small loss of salt through the excretion of large amounts of urine. If no food is taken, and if the surrounding water is renewed frequently, or the frog sprayed for a long period with distilled water, the stores of body salts are depleted and the animal grows weak. In this state, the skin displays a marked absorptive power. From 30 to 50 cc. of $\frac{1}{1000}$ Ringer's (6.5 mg. per cent NaCl) Cl is completely absorbed within a few hours. This absorption is not due to an osmotic inward current of water, for it also takes place

through studies of the microscopic picture of the gill epithelia of teleosts, including the eel, have discovered, on the bases of the gill leaflets, special cells far exceeding in size the predominant "respiratory epithelia," and have discussed the justification for calling them "chloride-secreting cells" (A. B. Keys and E. N. Willmer, J. Physiol., **76**: 368, 1932).

[184] H. W. Smith, J. Biol. Chem., **81**: 727, 1929.

[185] A. B. Keys, Ztschr. vergl. Physiol., **15**: 364, 1931.

[186] E. Overton, Verhandl. d. phys.-med. Ges., Würzburg N. F., **36**: 295, 1904; further, B. Brunacci, Pflüger's Arch. f. d. ges. Physiol., **150**: 87, 1913.

[187] J. K. Parnas, Biochem. Ztschr., **114**: 1, 1921; S. J. Przylecki, Arch. Internat. de. Physiol., **19**: 148, 1922; E. F. Adolph, J. Exper. Zool., **47**: 1, 1927; **49**: 321, 1927.

[188] A. Krogh, Skandinav. Arch., **76**: 60, 1937.

in isotonic sugar solution, from which no water is taken up. Br is absorbed at the same rate as Cl, but I is not absorbed. Cl is absorbed together with Na. This shows the absorption to be an active transfer by the skin. K together with Cl enters only in small amounts compared with Na + Cl, Ca does not enter or does so only slightly.[189]

The frog skin offers a rare opportunity to investigate osmoregulatory power, since, because of its loose attachment to the body, fairly normal pieces can be cut out as diaphragms for separating two fluids. In 1892, W. Reid[190] began this kind of experiment with the intestinal mucosa (p. 552), which repeatedly failed to give definite results, because of the great frailness of the mammalian tissue. But he was successful with fresh frog skin in showing that this membrane, fixed as a diaphragm in a differential osmometer, the two compartments of which were filled with Ringer solution, establishes a hydrostatic pressure difference by shifting the solution from outside to inside. This transport also takes place against a hydrostatic pressure of 2 to 4 cm. water established at the beginning of the experiment. But after the skin has been treated with m/1000 KCN, the transporting power is abolished, and, if a marked pressure difference was already established, the application of KCN results in a reversed movement of the fluid until the difference has been abolished.[191] These various observations provide clear evidence that the experiment of Reid is a demonstration of an active transport of water.

However, beyond this there is an active transport of solutes, as in Krogh's experiments on the whole animal.[192] This has been shown as follows: The skin of the two legs is stripped off and the two cylindric bags are used like vessels of an osmometer.[193] They are filled with measured amounts of Ringer, and are dipped in Ringer. In different experiments the skin sacs are oriented differently. Either they are used as they appear after stripping, inside out, or they are used in their natural orientation. According to the aforementioned observations, these osmometers, turned either way, can be expected to shift fluid from the exterior to the interior surface of the skin, the two sacs controlling each other. However, when one sac of a pair is exposed from both sides to a poison, not only is the shift of fluid diminished or stopped, but—as shown in Table LI—the permeability of Cl in the Ringer solution is changed also.

It appears that in each of the five experiments the concentration of Cl is diminished on the natural outside of the skin; in other words, Cl is shifted

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[189] According to Krogh (Ztschr. vergl. Physiol., **24**: 656, 1937), the osmotic properties of fresh water teleosts are similar to those of the frog. About the fluctuations of the salt content in the fresh water invertebrate *Astacus*, see F. Herrmann, Ztschr. vergl. Physiol., **14**: 479, 1931; E. Huf, Pflüger's Arch. f. d. ges. Physiol., **232**: 559, 1933.

[190] E. W. Reid, J. Physiol., **11**: 312, 1890; **26**: 436, 1901; British Med. J., **1**: 323, 1892.

[191] E. Huf, Pflüger's Arch. f. d. ges. Physiol., **236**: 1, 1935; **238**: 97, 1936.

[192] E. Huf, Pflüger's Arch. f. d. ges. Physiol., **235**: 655, 1935; **237**: 143, 1936.

[193] S. S. Maxwell, Am. J. Physiol., **32**: 286, 1913.

from outside to inside, yielding a higher concentration than that initially present on both sides, and this seems to be dependent upon a supply of metabolic energy, as can be concluded from the effect of poisoning. KCN blocks the oxygen consumption of the skin, bromoacetic acid (which is similar to iodoacetic acid) the production of lactic acid, which normally is subjected to aerobic breakdown.[194] Therefore (see experiment 5), the concentrating ability of the skin, after having been depressed by bromoacetic acid, is improved by the addition of lactate. These results will be discussed more in detail at the end of this section (pp. 597ff). In conclusion, it may be added that the accumulation of Cl at the inside of the skin disappears as the skin dies.

TABLE LI.—ACTIVE TRANSFER OF CL FROM OUTSIDE THE SKIN TO INSIDE
δCl_i (increase or decrease) of Cl_i (= Cl contained inside the sac), BrAA = bromoacetic acid

Exp. no. skin	Duration in hrs.	Skin orientation	Surface sq. cm.	δCl_i in mg. Cl/cc.	δCl_i in per cent
1. No poison	15	Turned	10.7	−1.20	−100.0
KCN 0.001 m.			9.3	−0.01	− 0 8
2. No poison	15	Natural	11.0	+0 44	+100.0
BrAA 0.0036 m.				+0.02	+ 4 5
3. No poison	4	Turned	10.3	−0 36	−100 0
BrAA 0.0014 m.			10.3	−0 15	− 41 7
4. No poison	4	Turned	10.7	−0.37	−100 0
BrAA			10 9	−0 16	− 43.3
5. After BrAA lactate	4	Turned	10 9	−0.38	−100 0
no lact.			10 1	−0.10	− 26 3

The problem concerning the location of the activity will be referred to later (pp. 628ff).

4. Irreciprocal Permeability of the Frog Skin.—Before the active transfer of dissolved substances directed across the frog skin from outside to inside was discovered, a preferential unidirectional passive movement of solutes, as well as of solvents, had been observed and analyzed in many studies. This "irreciprocal permeability" is due to various factors, which together result in some kind of asymmetrical composition of the skin, that, in the first place, is structural in nature; i.e., the skin is built up by a number of strata (see also p. 319). These are 1. different in their chemical composition and, for this reason, can react specifically with the penetrating substances. Especially do they differ in *p*H. 2. They are solvents of greater or lesser dissolving power toward the substances. This will result in the formation, inside the skin, of diffusion gradients of different steepness, and even of different direction. 3. Their mechanical resistance to the entrance of various components of the solution differs and can vary insofar

[194] E. Huf, Biochem. Ztschr., **288**: 116, 1936.

as, e.g., the same electrolyte solution can alter only the inside or the outside of the skin. 4. They are built up by colloids of various nature which, e.g., under the influence of the same ions, will either swell or shrink. All these factors can influence each other in such a way that diffusion or osmosis across the skin is directed either inside-outside or outside-inside.[195]

5. Active Transfer in Some Plant Tissues.—The general picture representing the fundamental properties of the plasma membrane in allowing passive entrance of organic compounds into the cells has been outlined mainly on the basis of experimental studies of plant tissues by two botanists, Overton,[196] and Collander;[197] (see also chap. 10). In this picture, the plasma membrane is shown to act partly as an organic solvent and partly as a molecular sieve. However, it was also recognized, very early, that upon such a stable framework there must be superimposed dynamic devices able to bring about the complex mixture of substances making up the intracellular contents. This was most obvious in studies of inorganic ions, penetration of which cannot be accounted for without assuming that the concentration gradients, varying in steepness as well as in direction (p. 244), are the result of driving forces, regardless of the very common experience that inorganic ions, when studied by osmotic, chemical, or electrical methods, do not display, or hardly display, an ability to pass the surface of the plant cells. But during the last two decades it became more and more clear that this bewildering discrepancy, which is most obvious in plant cells, rests upon the two facts: first, that indeed the rate of diffusion of ions in either direction across the protoplasmic wall is practically zero; and, second, that independent processes, unlike diffusion, establishing an unbalance, also go on with great slowness, and, even at all, only provided a sufficient supply of energy can be furnished by the cell, in order to start the specific machineries in one or the other direction. In the earlier permeability studies, measurements were made mostly by observing rates of plasmolysis (or deplasmolysis) with single cells, or with a small number of cells, by observing conductivities, or applying chemical and spectroscopic methods in experiments frequently too short in duration, and dealing with too limited amounts of material, to allow the detection of small changes in concentration and composition. Great progress in this field is due to F. C. Steward and W. R. Hoagland, who have developed methods for the utilization of large quantities of plant tissue, which, if properly prepared, gives a homogeneous material suitable for observation over a considerable period

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[195] See E. Wertheimer, Pflüger's Arch. f. d. ges. Physiol., **199**: 383, 1923; **200**: 82, 354, 1923, **201**: 488, 591, 1923; **203**: 524, 1924; **205**: 162, 1924; **208**: 669, 1925; V. Bauer, *ibid.*, **209**: 301, 1925; Przylecki, Arch. Internat. de Physiol., **20**: 144, 1922; **23**: 97, 1924; R. Mond, Pflüger's Arch. f. d. ges. Physiol., **205**: 172, 1924; K. Ansom, *ibid.*, **225**: 467, 1930; E. F. Adolph, Am. J. Physiol., **96**: 587, 1930; E. Huf, Protoplasma, **25**: 614, 1936; A. E. Eckstein, Pflüger's Arch. f. d. ges. Physiol., **237**: 125, 1936.

[196] E. Overton, Vierteljahrsschrift Naturforsch. Ges. in Zürich, **40**: 1, 1895; **44**: 88, 1899.

[197] R. Collander and H. Barlund, Acta Bot. Fenn., **11**: 1933.

of time.[198] Young roots or storage organs are placed in weak solutions of certain salts which can serve as an adequate medium, since under normal living conditions in the soil, these organs also are in contact with such solutions in small amounts, serving as a source for their ionic needs. Insofar they may be compared to the fresh water amphibia and teleosts described in this Sec., pp. 584 and 587.

Methods. Storage organs which have been found useful are carrots, artichokes, turnips, dahlias, and especially potatoes.[199] A great number of discs of these organs, 1 to 0.75 mm. in thickness, are placed in water and are well aerated for several days. By this treatment the dormant starch-filled cells become more and more depleted of starch, their slow respiration rises, protoplasma streaming becomes visible, and, accompanied by the revived aerobic metabolism, salts, which in low concentration are added to the water, pass across the thin protoplasmic wall, and are accumulated in the cell sap. Roots have been used in the following way:[200] Seedlings of barley are grown in diluted nutrient solution. Thereafter, in order to avoid complications arising from interrelation between root and shoot, the roots are excised and are kept well aerated for about 10 hours in the experimental salt solution.

The result of these treatments can be determined by freezing and thawing the lots of either discs or roots, pressing them and analyzing the fluid for mineral content. This procedure has been proved to yield fairly uncontaminated vacuolar sap.[201]

Since it is rather generally agreed that from equivalent solutions the cations mostly are absorbed at a rate decreasing in the series $K > Na > Ca$, Mg, the anions in the series $NO_3 > Cl > SO_4$, most experiments regarding absorption have been performed with KNO_3 and KCl. In many experiments it has been found useful to substitute for Cl the near-related Br, eventually also Rb for K, in order to secure conclusions by employing nontoxic ions, not initially present in the cells.

Results. Through experiments of this kind it has been found that, starting with very dilute solutions, cations and anions enter the cell with rising concentration against their gradients, but in such a way that the accumulation ratio is higher, when the tissue is exposed to lower concentrations. This is shown in the following table concerning the absorption of KBr by carrot discs from solutions varying 100 times in strength.[202]

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[198] Concerning studies on single cells, particularly on giant cells like *Valonia, Nitella, Chara* with respect to active transfer and passive penetration of inorganic ions, as described by Osterhout, S. C. Brooks, D. R. Hoagland and Davis, R. Collander, and others, see chap. 11 and 21.

[199] F. C. Stewart, Protoplasm, **15**: 29, 497, 1932; **15**: 576, 1932; **17**: 436, 1932; **18**: 208, 1933; for earlier work on storage organs see A. Nathansohn, Jahrb. Wiss. Bot. **38**: 241, 1903; **39**: 607, 1904; **40**: 403, 1904; W. Stiles and F. Kidd, Proc. Ry. Soc., London, B. **90**: 448, 487, 1919.

[200] D. R. Hoagland and T. C. Broyer, Plant Physiol., **11**: 471, 1936; D. R. Hoagland, Bot. Rev., **3**: 307, 1937.

[201] T. C. Broyer, Bot. Rev., **5**: 531, 1939.

[202] F. C. Steward, Protoplasma, **15**: 29, 1932.

TABLE LII.—CARROT DISCS IN KBr SOLUTIONS. TIME 69.5 HOURS

External conc. $\times 10^3$	Final internal conc. $\times 10^3$	Accumulation ratio
20.0	68 1	3.41
2.0	14.4	7 22
0.2	2 49	12 45

This accumulation cannot be referred to binding forces, since the analytical results and the measurements of electrical conductivity of the sap are in fairly good agreement. The maximum value in this experiment is relatively small. With barley roots exposed to very dilute solutions, accumulation ratios, which are even in excess of 1,000, have been reached. The lower values, observed with discs of storage tissue, are dependent upon the thickness of the slices as well as upon other factors. The thinner the discs, the greater is the accumulation. During the aforementioned long preliminary aeration of the discs, according to microscopic evidence, only the outermost layers of the initially dormant cells are reactivated, i.e., for potato a layer of about three cells, equal to 0.35 mm. thickness, whereas the discs ordinarily employed were about 0.75 mm. and more in thickness.

The vitality of the accumulating power can be proved in many ways: first, by taking advantage of the fact that it is intimately connected with the metabolic activity, or, better, with the aerobic respiration. This is illustrated by the following table:[203]

TABLE LIII.—REVERSIBLE EFFECT ON SALT ACCUMULATION IN ROOTS OF PASSING NITROGEN THROUGH SOLUTION

Condition	Increase of conc. in sap (milliequiv./l)		CO_2 production (mg./g. tissue/ hour)
	K	Br	
6 Hours in air	31 6	26.0	0.422
6 Hours in N_2	−0.8	3.3	0 213
6 Additional hours in air	23 5	17.3	0.320

It appears that the transfer of K and Br, which is high in air, falls off markedly in N_2, but is restored in a following period of air treatment. The table further shows that CO_2-production, which is a regular indicator of aerobic respiration of an active organ, fails to display a deep decline in N_2, proving that the active transfer needs O_2 and that anaerobic CO_2 production apparently is not involved in this function. Correspondingly, m./500 to m./2000 KCN, which depresses O_2 consumption more than CO_2 production, is an effective and reversible inhibitor of the uptake of K and Br.[204] But

[203] D. R. Hoagland and T. C. Broyer, Plant Physiol., 11: 471, 1936.

[204] D. R. Hoagland and T. C. Broyer, J. Gen. Physiol., 25: 865, 1942.

even the retention of salt, which has been already accumulated, is insured only in the presence of O_2.[205] Finally, it is particularly interesting that anaerobically (N_2) very little K and Br enter the roots, even though a marked inward gradient exists for these ions, whereas aerobically both of them are not only absorbed, but even accumulated to a higher concentration than that of the relatively strong external concentration.[206]

Evidence from many experiments has been obtained that the accumulating process usually has a more complicated aspect than so far described. In the plant tissues, Steward[207] has differentiated between "primary"

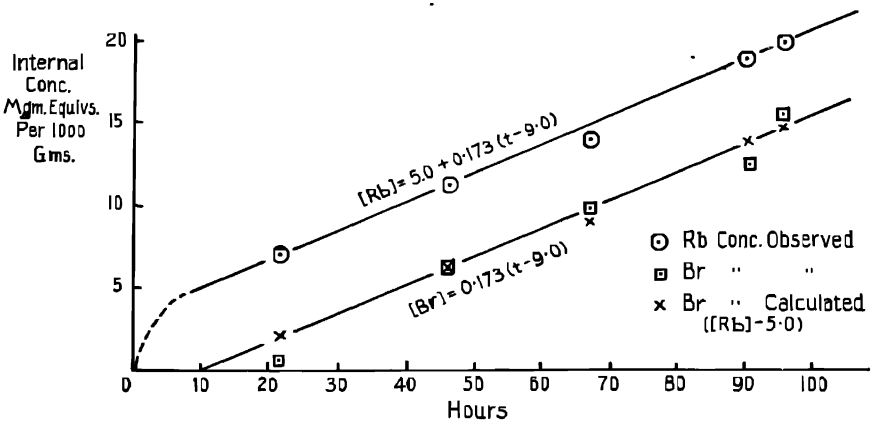

FIG. 63.—The effect of time on the absorption of Rb and Br by potato discs.

absorption and "induced" absorption. The first is defined as an uptake, where cation and anion simultaneously, in equivalent amounts, enter the cell sap; whereas, in induced absorption, external and internal media influence each other by an exchange of their different components, the net effect being an increase of the salt content. This exchange appears in various forms.[208] For example, from a solution of K_2SO_4 a greater amount of K is removed than SO_4, with the result that the external solution becomes acid by an exchange of internal H for K, while, from a solution of $Ca(NO_3)_2$, more NO_3 enters the cell than Ca, and this causes alkalinity by the escape of HCO_3. The amount of such exchange depends on various circumstances, for example, on the metabolic activity determining the magnitude of active transfer, or on the concentration of exchangeable ions accumulated during the period of growth ("high salt" and "low salt" roots), or on the existing

[205] See, also, F. C. Steward, Protoplasma, **18**: 208, 1933.

[206] About the differentiation between accumulation of Rb in the protoplasmic mantle and in the cell sap of *Valonia* and *Nitella* see the studies of S. C. Brooks (Sec. 5, p. 345).

[207] F. C. Steward, Tr. Faraday Soc., **33**: 1006, 1937; F. C. Steward and G. Preston, Plant Physiol., **15**: 23, 1940.

[208] W. Stiles and F. Kidd, Proc. Roy. Soc., London, B. **90**: 448, 487, 1919; D. R. Hoagland, Univ. of California Agr. Exper. Sta., **12**: 1923; S. C. Brooks, Protoplasma, **8**: 389, 1929; D. R. Hoagland and T. C. Broyer, Am. J. Bot., **27**: 173, 1940.

*p*H accounting for the metabolic production of exchangeable organic acids, and other factors.

However, these various factors, which obscure more or less the clear effect of primary absorption, acquire a greater significance mainly when the [illegible] the tissues has subsided or has not yet fully developed. whereas the primary absorption, unmixed with the passive exchange, is most conspicuous in vigorously respiring and growing systems. Examples of such active transfer of cation and anion in equivalent amounts have been obtained with various objects.[209] An example regarding Rb Br, presented to potato discs, is shown in Fig. 63.[210] The uptake of the ions was followed through a period of about 100 hours. It is evident that, in the period 21 hours to 96 hours, the absorption of anions and cations is in approximately equal amounts. During the first 21 hours, before samples for the analysis are collected, by some manner of induced absorption Rb must have entered with greater rapidity, while for Br there is a period of lag of very slow absorption.[211]

In conclusion, it should be emphasized that the essential features of the active transfer in plant tissues strikingly resemble active transfer in animal tissues. As to the transfer of salts, the simultaneous absorption of cation (Na) and anion (Cl) by the kidney epithelium, although not strictly proven, can hardly be doubted, since NaCl, after being filtered through the glomerular membrane into the Bowman capsule, disappears completely during passage down the tubules (p. 559). The same type of active absorption of Na and Cl ions is effective in the frog skin (p. 588), whereas, in the intestine, this kind of shift appears dimmed, due to the greater importance of passive diffusion in the penetration of this membrane. Another point of similarity regards the inverse relationship between the outside concentration and the accumulation ratio (p. 592). This relationship was found very conspicuous in the kidney in the secretion of urea and of dyestuffs (p. 573) and will be found in the secretion of dyestuffs by the liver (p. 607). This has led to the assumption of carriers being involved in the transport into and across the cells, and being differentiated to accept only specific loads, and these only up to a maximum amount. Whether such a hypothesis will suit also the conditions observed in plant tissues awaits further investigation.

[209] See, for instance, F. C. Steward and W. E. Berry, J. Exper. Biol., **11**: 1, 1934; W. E. Berry and F. C. Steward, Ann. of Bot., **48**: 1, 1936.

[210] F. C. Steward and Harrison, Ann. of Bot., N.S., **3**: 427, 1939.

[211] Concerning controversial viewpoints, stressing that the absorption of anions is associated with increase of aerobic metabolism, which releases anion accumulation, and that the accumulation of cations is brought about only indirectly by the anion stimulated respiration, see H. Lundegårdh, Biochem. Ztsch., **261**: 235, 1933; **290**: 104, 1937.

37

THE ELABORATION OF DIGESTIVE JUICES

As has been mentioned before, focussing in this section our chief interest upon active transfer across layers of living cells, we have seen that intestinal and kidney membranes are most versatile in performing this kind of work, in contrast to the body surface of animals and plants, which appears to be much more specialized in transporting ability to inorganic ions. The latter behavior is likewise characteristic of the digestive glands. Defining digestion as a process which performs or supports the chemical breakdown of foodstuffs, the digestive glands comprise mainly salivary glands, stomach mucosa, pancreas, and intestinal mucosa. The chemical breakdown is brought about in the first place by specific intracellular products, the enzymes, which, in general, appear to be included in granules. The discharge of these contents is dependent upon innervation. But very little knowledge about this accomplishment has been made available by physiological study. What is known is mostly morphological in nature, such as details of the microscopic structure of the secretory cells, displayed by the living as well as by the preserved materials. However, the process of the formation of the digestive juices, i.e., mainly saliva, gastric juice, pancreatic juice, and the composition of these fluids as compared to their source, the blood plasma, is accessible to physiological experiment, which is chiefly concerned with studying the "ultrafiltrate" of the juices, i.e., neglecting the colloidal substances, which are mainly the enzymes, but stressing the elucidation of the forces involved in the segregation of the fluids. There is only one more glandular secretion participating in the digestive processes, the bile, which stands apart, first, because of its lack of enzymes; second, because of the content of special characteristic organic compounds in great amounts, namely, the bile salts and bile pigments; third, because its discharge is independent of innervation.

1. The Formation of the Salivary Fluid.—Disregarding the colloidal components of the saliva (enzymes, proteins, mucin), the salivary fluid is mainly a very dilute solution of inorganic salts, to which, in relation to the degree of activity, varying small amounts of lactic acid, phosphate, and other substances are added as split products of glycogen and phosphocreatine.[212]

[212] However, the main part of these metabolites leaves the gland with the venous blood. Concerning the metabolism of the salivary glands, see: M. Bergonzi and V. Bolcato, Arch. Soc.

Ordinarily, saliva is decidedly hypotonic to the blood plasma, and, accordingly, chemical energy is spent in order to perform the necessary osmotic work. The freezing point is found to vary in a wide range and is correlated to the species, to the kind of gland (submaxillary, parotid), to the height of glandular activity, and to the kind of innervation (sympathetic, parasympathetic). Frequently, Δ of submaxillary saliva is found as low as 0.1° to 0.25°.[213]

The ionic composition of the salivary fluid is different from that of the blood serum.[214] Most striking is the surplus of K in the saliva, and the ash of saliva shows an exceedingly high percentage of K. It may be asked whether the ionic composition is the result of some kind of selective permeation. Another possibility would be that the source of the K-surplus is the protoplasma of the cells, which, discharging their granular contents through their inner surfaces into the lumens, could lose some K.

Referring to the first alternative, the permeability of the salivary gland has been studied in different ways, either in experiments on the total animal (rabbits),[215] or on the isolated submaxillary gland (cat) artificially perfused with hemoglobin-Ringer.[216] In either procedure, various substances were added to the circulating fluid and the saliva produced after electric stimulation of the chorda was analyzed. The results obtained bear upon 1. organic nonelectrolytes, selected with regard either to their molecular size or to their lipoid solubility; 2. dyestuffs; 3. inorganic electrolytes.

a). *Organic nonelectrolytes.*—Among these nonelectrolytes, neither disaccharides [MV = 346] nor hexoses (glucose, galactose, fructose) [183] are apt to enter the saliva, but the triose dioxyaceton [89] does.[217] Asparagin [170 eff.], alanin [150 eff.] glycine [110 eff.],[218] and malonamide [104] either do not penetrate, or only slightly, while propionamide [91] and particularly acetamide [69] pass with ease. When malonamide [104] is compared with butyramide [113] and dimethylurea [103], the molecular volumes of these three compounds being about the same, butyramide and dimethylurea are found to enter the saliva in a considerably greater amount than

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Sc. Méd. et Biol., Montpellier, **14**: 573, 1930; **15**: 235, 1931; R. Ferrari and R. Höber, Pflüger's Arch. f. d. ges. Physiol., **232**: 299, 1933; D. Northrup, Am. J. Physiol., **114**: 46, 1935; J. H. Wills, *ibid.*, **135**: 164, 1941.

[213] R. Rosemann, Handb. d. norm. u. pathol. Physiol., vol. **3**: 818: Springer, Berlin, 1927.

[214] R. Rosemann, *loc. cit.*; M. J. Gregersen and E. N. Ingalls, Am. J. Physiol., **98**: 441, 1931; E. J. de Beer and D. W. Wilson, J. Biol. Chem., **95**: 671, 1932.

[215] L. Asher, Biochem. Ztschr., **14**: 1, 1908; J. N. Langley and H. M. Fletcher, Phil. Tr. Roy. Soc., B. **180**: 109, 1889.

[216] R. W. Amberson and R. Höber, J. Cell. & Comp. Physiol., **2**: 201, 1932; F. Schmengler, Pflüger's Arch. f. d. ges. Physiol., **234**: 351, 1934.

[217] Amberson and Höber, *loc. cit.*; J. Jeangros, Biochem. Ztschr., **200**: 367, 1928.

[218] "Eff." means "effective" and indicates the molecular volume of the amino-acids, due to their dipole character, to be larger than the calculated volume. See F. Schmengler, Pflüger's Arch. f. d. ges. Physiol., **232**: 591, 1933; R. Höber and J. Höber, J. Cell. & Comp. Physiol., **10**: 401, 1937.

malonamide. This is very likely to be due to the influence of their lipoid solubility, obliterating the influence of the molecular volume. One irregularity has been observed in the perfusion experiments, which so far cannot be accounted for; urea passes markedly more slowly than acetamide, although they resemble each other in their small molecular volume and in their lipoid insolubility. It may be recalled that also in other regards experiments with urea turned out to give unexpected results (p. 572).

b). *Dyestuffs.*—Three acidic dyestuffs, which have been tested, indigocarmine, fluorescein and erythrosin, do not pass into the saliva.[219]

c). *Inorganic ions.*—The behavior of the inorganic ions is particularly pertinent to this subject. With regard to anions it has been stated that, after intravenous injection, sulfate, phosphate, and ferrocyanide fail to enter the saliva.[220] Further, the low Δ values of 0.1° to 0.25° are enough proof that, at least under certain conditions (see p. 598), Cl does not pass readily. Only iodide was found in the parotid saliva after an intravenous injection of 20 mg. NaI per kg. It appeared 7 times concentrated.[221] Regarding the cations, there are data[222] indicating that "at rest" K and Na are predominant over Ca and Mg. This order is corresponding to what has been observed with the intestine (pp. 536ff), with the frog skin (p. 588), and with plant tissues, and taking into consideration that sugars and amino-acids do not pass, and that saliva, in general, is markedly hypotonic to serum, one may say that the salivary gland often behaves as though very little permeable to solutes.

TABLE LIV.— NA AND K IN SUBMAXILLARY SALIVA (DOG) DURING REST AND ACTION OF THE GLAND

Gland	Saliva		Serum	
	Milliequiv./l	Mg. per cent	Milliequiv./l	Mg. per cent
Resting Na..	10	23	146	336
Resting K	10 to 20	39 to 78	5	20
Active Na....	80	184	146	336
Active K.....	10 to 20	39 to 78	5	20

However, it is important to notice that this picture changes somewhat, when the glands are thrown into greater activity by any kind of stimulation. According to the old observations of Heidenhain,[223] the salt content of the saliva is raised by raising the glandular activity, and even may approach

[219] H. Mathys. Biochem. Ztschr., **234**: 419, 1931.

[220] J. N. Langley and H. M. Fletcher, Philos. Tr. Roy. Soc., **B, 180**: 109, 1889; L. Asher, Biochem. Ztschr., **14**: 1, 1908.

[221] W. Lipschitz, Arch. f. Exper. Path. u. Pharmakol. **147**: 142, 1929.

[222] See R. Rosemann, Handb. Norm. und Pathol. Physiol., vol. **3**: 818; Springer, Berlin, 1926; G. W. Clark and J. S. Shell, Dental Cosmos, **69**: 500, 605, 1927.

[223] R. Heidenhain, Pflüger's Arch. f. d. ges. Physiol., **17**: 1, 1878.

isotonicity. For instance, the NaCl percentage in the chorda-saliva of the submaxillary gland (dog), as calculated from the Cl values, can increase from 0.19 per cent to 0.55 per cent, with 0.7 per cent in the serum,[224] and these results are complemented by figures for Na and K, presented in table LIV.[225]

It appears, first, that during rest there is slightly more K in the saliva than Na, but that during activity Na prevails by far; second, that during activity Na is increased 8 times, whereas K remains unchanged.[226] This suggests that the output of Na salt and the output of K salt are somehow independent processes. Perhaps this can be interpreted as an increase of permeability of the activated gland cells in such a way that NaCl, which is present in the serum in a paramount concentration, and is preponderant in the serum even during great activity (see Table LIV), enterst he cells, together with water, across their outer surface, and passes into the secretion, while K may continuously leave the cells through their inner surface as wear-and-tear product of the protoplasm. In order to test a concept like this, the reappearance in the saliva of Rb, Ca, and Mg, of SO_4 and HPO_4, presented in varying concentrations to the resting and to the active gland from the circulating fluid, should be studied.[227]

It has been mentioned before (p. 595) that during stimulation lactic acid and inorganic phosphate appear (in great amount) in the blood, and eventually in the saliva as split products of glycogen and phosphocreatine. After poisoning the isolated hemoglobin-Ringer perfused submaxillary gland with cyanide, the discharge of lactic acid and phosphate into the perfusion fluid is increased; after iodoacetate, that of lactic acid decreased, of phosphate increased.[228] These effects of poisoning are associated with concentration changes of the ionic output into the saliva; the release of Cl is reversibly raised, and, under these conditions, K likewise is set free reversibly and in a great amount, even so much so that it seems to surpass the concentration in the perfusion fluid. This would be indicative of a heavy, though reversible, loss of intracellular K.

From the above, the following fragmentary picture of the process of the secretion of salivary fluid may be drawn: the essential work to be done is the segregation of water from the blood fluid, serving the purpose of wetting,

[224] M. Werther, Pflüger's Arch. f. d. ges. Physiol., **38**: 293, 1886.

[225] M. I. Gregersen and E. N. Ingalls, Am. J. Physiol., **98**: 441, 1931.

[226] But, see also J. H. Wills, and W. O. Fenn, Am. J. Physiol., **124**: 72, 1938; further, G. O. Langstroth, D. R. McRae, and G. W. Stavraky, Proc. Roy. Soc., London, **B, 125**: 335, 1938.

[227] According to Hebb and Stavraky (Quart. J. Exper. Physiol., **26**: 141, 1936), the submaxillary gland, which during stimulation of the chorda or of the sympathetic nerves remains glucose-tight (p. 596), becomes permeable to glucose following administration of adrenalin, when this sugar is injected intravenously. During hypoglycemia, produced by starvation and phlorizin, a relatively high dose of adrenalin in addition to nerve stimulation throws into the saliva a small amount of sugar, which immediately and markedly increases in amount when glucose is injected.

[228] R. Ferrari and R. Höber, Pflüger's Arch. f. d. ges. Physiol., **232**: 299, 1933.

imbibing, and swelling of the food materials. This segregation is initiated by the rapid rise of osmotic pressure inside the gland cells, following the breakdown of organic substances. The coincident movement of inorganic ions is a mere accessory phenomenon, due to the incomplete semipermeability of the outer surface of the active cells, which allows the Na and Cl ions to take the steep downhill path from the plasma through the cells into the secretion, and perhaps due to the permanent leakiness of their inner surface to K. The organic metabolites, leaving the cell, evidently are diverted more toward the blood fluid than toward the saliva. It is unknown how this is accomplished.

2. The Formation of the Gastric Secretion.—As in the previous section and for the same reason, in the following outline of gastric secretion all those observations will be omitted, which, mainly on the basis of biochemistry as such and from histological evidence, have contributed to the present knowledge concerning the action of the gastric gland in the elaboration and the elimination of enzymes and of mucus. In other words, interest will again be centered around the composition and the forces, which are involved in the production of the abundant aqueous ultrafiltrate-like gastric fluid. The problem concerned is very different from that encountered in the study of the salivary secretion, since the gastric secretion is nearly isotonic with the blood fluid, and therefore needs no energy supply for the shift of water, and since by its composition, undoubtedly, active transfer of solutes is manifested as demonstrated by the fact that, due to its main constituent, HCl, the secretion is about three million times more acid than the blood fluid from which it derives.

For several reasons, it has been assumed for a long time that the parietal cells of the gastric glands are the seat of HCl production. But the parietal cells are not more than a small fraction of all the cells of the gastric glands. "Non-parietal" cells, which include the so-called "chief cells" and the surface epithelia, are supposed to elaborate the pepsin and the mucus. However, aside from these colloidal substances, gastric juice, besides H and Cl, also contains other ions, K, Na, Ca, Mg. Therefore the question may be raised whether the gastric fluid is not the result of the simultaneous activity of several glands.

This question has been attacked mainly by Gray and his co-workers in the following way.[229] In dog, a continuous secretion of gastric juice is maintained by subcutaneous injection of histamine at 10-minute intervals, and the collected fluid analyzed for the concentration of H, K, Na, Ca, and Cl with reference to the volume rates of secretion. The results are listed in the following table:

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[229] J. S. Gray, G. R. Bucher, and H. H. Harman, Am. J. Physiol., **132**: 504, 1941; J. S. Gray and G. R. Bucher, *ibid.*, **133**: 542, 1941; see also F. Hollander, Am. J. Digest. Dis., **1**: 316, 1934; A. C. Liu, J. C. Yuan, and R. K. S. Lim, Chinese J. Physiol., **8**: 1, 1934; further: J. S. Gray, Gastroenterology, **1**: 390, 1943; F. Hollander, *ibid.*, **1**: 401, 1943.

TABLE LV.—CHEMICAL COMPOSITION OF 9 POOLED SAMPLES OF GASTRIC JUICE

Volume rates vol./20 min.	Concentrations in milliequiv./l					
	Cl	HCl	BCl	Na	K	Ca
6.0	155 1	93.5	61 6	52 8	7 4	1 350
9.7	159.0	121 0	38.0	30 0	7.2	0.766
15.2	162.1	130.4	31.7	23 6	7 4	0 654
21.2	164 2	138 0	26.2	18 5	7.2	0 530
29 1	162 6	142 0	20.6	12 8	7 4	0 400
(00)	(166)	(154)	(12)			

It appears that the concentrations of Cl (in milliequiv./liter) surpass those of H, that with rising volume rates (cc./20 min.) H, expressed as HCl, increases much more than does Cl, and that the concentration of H at the highest rates of secretion approximately reaches the concentration of Cl. This is evidence of an independent secretion of HCl and of neutral chloride BCl. The concentrations of neutral chloride are calculated in the table as the differences of the concentration of Cl and H (HCl). This shows that the fraction of neutral chloride secreted falls off steeply with rising concentration of total chloride, so that the output of neutral chloride finally becomes rather insignificant in comparison to the total output of HCl. This is especially evident from the extrapolated results (in brackets) indicating that, at maximum volume rates of secretion, the concentration of the gastric fluid is equal to 154 milliequiv./liter HCl plus 12 milliequiv./liter BCl. Probably the volume of BCl secretion is small and increases very little, compared to the large increase in the output of HCl. From the table, it furthermore appears that the concentration of BCl has been fractionated into those of the chlorides of Na, K, and Ca, and that with rising volume rates the milliequivalents of Na and Ca decrease, those of K remain constant. This suggests that, while Na and Ca move independent of the shift of H, K is secreted by all the cells.

This result has led to the assumption that the parietal cells are the seat of the formation of the acid, that the nonparietal cells secrete Na and Ca, that both of them, parietals as well as nonparietals, equally eliminate K, and that the increase in parietal secretion is accompanied by a relatively small increase of nonparietal secretion. It will be seen that strong support to this assumption is available.

The next question is as to how the amazingly great concentration of hydrochloric acid can be accomplished. This question can be reduced to the somewhat simpler problem of raising the hydrogen ion concentration to a level about 3 million times above the blood level. For, it can be assumed that Cl, the concentration of which in the gastric secretion is about 1.6 times higher than that in the plasma, is passively dragged along into the parietal cells according to the principle of electroneutrality,[230] provided that the parietals are anionpermeable, like the red blood corpuscles.

[230] See H. W. Davenport and R. B. Fisher, Am. J. Physiol., **131**: 165, 1940.

Definite proof of the parietal cells being involved in the production of HCl has been advanced by the discovery of Davenport that the parietal cells are the seat of large quantities of carbonic anhydrase.[231] Discs of the blood-free mucosa are punched out of the stomach walls and cut into thin slices parallel to the surface. The enzyme concentration in these slices is determined by their catalytic activity[232] and compared with the number of parietal cells contained in histological preparations of the corresponding slices. The result is a very high correlation between cell count and enzyme concentration, this concentration in cat cells being even six to seven times greater than in the red corpuscles.

The bearing of carbonic anhydrase upon the formation of HCl can be assumed to be this: CO_2, which is continuously produced by the metabolic activity of the parietal cells, is rapidly hydrated to H_2CO_3 at a rate corresponding to the high content of carbonic anhydrase, and HCO_3, resulting from the immediate ionization, is exchanged with equivalent amounts of plasma-Cl, while the H-moiety of the H_2CO_3 molecule remains in the cells. This Cl-shift from the plasma into the cells would appear to be just opposite to what is happening with other tissue cells which are lacking in the enzyme. In these tissues, likewise, CO_2 is produced, but diffuses as such into the plasma, and enters from there the red blood corpuscles. Due to the large content of enzyme in the erythrocytes, it is again catalyzed to H_2CO_3, immediately ionized to HCO_3 and H, and, while H is fixed to the hemoglobin, HCO_3 leaves the cells in exchange for entering Cl. This is the normal well-known shift of Cl from the plasma into the red cells. The "reversed Cl shift" (Davenport) from the red cells to the plasma in the blood passing through the gastric mucosa could be expected to take place and has actually been found.[233] Its occurrence is further indicated by the fact that blood passing through the secreting stomach becomes more alkaline, due to the copious entrance of HCO_3,[234] whereas in passing other active tissues (muscle) it becomes more acid.

Through these investigations it became clear that the origin of the hydrogen ion moiety of the hydrochloric acid can be assumed to be CO_2, derived from the metabolism of the active parietal cells, the other moiety Cl being supplied from the blood plasma. But this remarkable advance in knowledge leaves unsolved the most essential problem, viz., the mechanism by which the accumulation of H to such tremendous height can be accomplished.

3. The Penetration of Glands by Acidic and Basic Organic Substances.—In view of the enormous accumulation of H in the gastric juice,

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[231] H. W. Davenport, J. Physiol., **97**: 32, 1939; also H. W. Davenport and R. B. Fisher, *ibid.*, **94**: 16, 1938; see, further, H. W. Davenport, Gastroenterology, **1**: 383, 1943.

[232] N. U. Meldrum and F. J. W. Roughton, J. Physiol., **80**: 113, 143, 1933. See also p. 560).

[233] F. D. Mann, J. H. Grindlay, and F. C. Mann, Am. J. Digest. Dis., **8**: 451, 1941.

[234] M. E. Hanke, R. E. Johannesen, and M. M. Hanke, Proc. Soc. Exper. Biol. & Med., **28**: 698, 1931.

there can be no doubt that this achievement is a matter of active transfer. There are many observations concerning organic substances which, after intravenous injection, appear in the gastric juice in so much enhanced concentration as to be easily mistaken for real secretion, until a more careful analysis of the possible implications shows them to be only incidental effects of glandular activity. This refers particularly to dyestuffs, which, even with a relatively low accumulation ratio, give the illusion of a high concentration, due to the intensity of their color. Observations concerning such false effects have been mentioned already in the introduction to this section (pp. 525ff). On the other hand, in the subsection concerning liver, such accumulation ratios for dyestuffs will be cited as to indicate by their large values (sometimes greater than 1,000) an unambiguous active transfer.

Penetration of dyestuffs already several times has played a role in this section. Particularly, kidney experiments have provided evidence that, in general, the proximal tubules cannot be penetrated by acidic dyestuffs unless certain physicochemical properties, based upon a nonpolar-polar configuration of their molecules, probably leading to a characteristic interlinkage with the cell surface, are present. Moreover, as we will see later, the dyestuff transporting activity of the liver cells apparently is bound up with specific physicochemical properties of the dye different from those involved in the transfer by the kidney. On the other hand, from various more sporadic observations, one may conclude that in the intestinal mucosa, in the body surface of animals, and in the salivary glands, conditions favorable to dyestuff accumulation are absent. Somewhat different is the behavior of the gastric mucosa and of the pancreatic gland, the accumulating power of which appears to be connected with the acid and alkaline reactions of their secretion.

Entrance of dyestuffs into the gastric and the pancreatic juice.—Dogs were injected intravenously with numerous dyestuffs[235] belonging to various chemical groups, and varying in diffusibility, in lipoid solubility, in dissociation strength, in oxidizing and reducing power, and in adsorbability to proteins. The gastric juice was collected and the appearance of dye studied. The result shows that the deciding factor is essentially the basic or the acidic character of the dyes. For instance, in the experiments of Ingraham and Visscher, among 27 dyestuffs appearing in the gastric juice, all but one are basic, whereas acidic dyestuffs fail to appear (those dyes being called basic, the staining power of which is carried by the cation, while in the acidic dyes the anion bears the color). The result is correlated to a corresponding series of observations concerning the pancreatic juice. Here, in the same type of experiment, mainly the acidic (anionic), but also the amphoteric, dyestuffs under some conditions, were found to enter the juice. In general, the effect is more evident with gastric than with pancreatic secretion, due

[235] M. B. Visscher, Symposium on the Mechanism of Secretion: Federation Proc. **1**: 246, 1942; see, further, A. B. Dawson and A. C. Ivy, Am. J. Physiol., **73**: 304, 1925; R. C. Ingraham and M. B. Visscher, J. Gen. Physiol., **18**: 695, 1935.

to its more definite acid character compared to the weaker alkalinity of pancreatic juice. This is especially emphasized by the high accumulation ratio observed with basic dyes entering the stomach, the concentration of toluidine blue, for instance, compared to the blood being found to be 36.5, that of thionin 36.8, of neutral red 26.4.

However, this result is by no means a sign of secretory activity. The dyestuffs are electrolytes, basic dyes being, in general, weaker than the acidic, and, therefore, due to hydrolysis, present in the blood more or less as free bases. These free bases, as undissociated molecules and especially as lipoid soluble substances, can diffuse across the cells (see Sec. 4, chap. 13) and enter the gastric juice, where they are converted immediately into ions, which, in general, are unable to pass the living cell. Thus, the basic dyestuffs are captured in the stomach in amounts increasing with the acidity of the juice.[236]

Such simulated secretion is a common widespread phenomenon. Sometimes its real nature is very obvious, sometimes veiled, especially when, as in the case of the entire organism, complications arise from the coincident intervention of several organs. One instructive example is seen in dealing with the excretion by the isolated frog kidney of the sulfonic acid dyestuff phenol red and the basic neutral red.[237] If the kidney is perfused from the aorta with Ringer containing NH_4Cl, the *p*H of the glomerular filtrate running along the tubules can be decreased from 7.4 to 6.3, due to membrane hydrolysis with uptake of the NH_3 by the epithelia. If, under these conditions, neutral red is added to the Ringer solution simultaneously entering the kidney from the portal vein, a very high concentration of neutral red is attained in the urine with an accumulation ratio greater than 40. When phenol red instead of neutral red is applied, nothing happens, no concentration change occurs following the addition of NH_4Cl. In case the kidney is perfused from the aorta with Ringer made alkaline by the addition of $NaHCO_3$, the course of the experiment is the reverse of the former, with the concentration of neutral red falling off, the accumulation ratios decreasing. After the supply of $NaHCO_3$ has been stopped, the ratios rise again. However, when phenol red is used, its concentration is very little affected by this change to an alkaline reaction. The reason is that phenol red is one of those dyestuffs, which, because of their molecular structure, are actively transferred by the kidney rather independent of the existing *p*H, whereas neutral red passes by diffusion here as well as in the stomach. The difference is dramatically shown by the fact that, even though the activity of the kidney tubules has been suppressed by cyanide or by urethane, the concentration ratio of neutral red, which is low during

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[236] Regarding similar distribution effects, see E. Overton, Ztschr. f. physik. Chem., **22**: 189, 1897; W. J. V. Osterhout, J. Gen. Physiol., **8**: 131, 1925.

[237] R. Chambers and R. T. Kempton, J. Cell. & Comp. Physiol., **10**: 199, 1937; R. T. Kempton, *ibid.*, **14**: 73, 1939.

the aortic perfusion with $NaHCO_3$, rises quickly after NH_4Cl has been substituted for the bicarbonate.

The phenomenon of simulated secretion is not restricted to dyestuffs. It is met wherever weak electrolytes are distributed between fluids of different *p*H. Therefore it is of great importance in the field of drug distribution, especially as a main factor controlling the toxicity of alkaloids.[238] For instance, it has been known for a long time that, after intravenous injection, morphine hydrochloride is accumulated in large amounts in the stomach. The salts of other alkaloids, strychnine, cocaine, nicotine, directly introduced into the stomach of the cat or dog, are not absorbed at strongly acid reaction, but are rapidly absorbed, and therefore generally toxic, after the gastric juice has been rendered alkaline.[239]

With regard to the various possible implications obscuring the pattern of distribution of weak organic bases in experiments on the total animal (p. 603), the following study with sulfonamides may have a special informatory value.[240] Gastric juice, under maximal histamine stimulation (p. 599) is collected from Heidenhain gastric pouches of dogs, to which various sulfanilamide drugs are administered. Comparing the concentration of these substances in the gastric juice and in the plasma, the concentration ratios are calculated. It appears from the following table that sulfanilamide and sulfapyridine are accumulated, the others not.

TABLE LVI.—EXPERIMENTS ON DOGS CONCERNING THE DISTRIBUTION OF SULFA DRUGS BETWEEN BLOOD PLASMA AND GASTRIC JUICE

Sulfa drugs	Conc. ratio juice: plasma	Solubility mg. % 37°		Solubility ratio	Rate of "Diffusion"
		0.153*n*. HCl	Plasma		
Sulfanilamide	2.60	3980	1540	2 6	10
Sulfapyridine	3.21	810	62	13	2 7
Sulfathiazole	0.16	1120	348	3	0 175
Sulfadiazine	0.28	188	175	1	0 252
Acetylsulfanilamide	0 30	256	278	0 9	0 47

The following interpretation has been proposed. When sulfanilamide dissolved in plasma is allowed to pass across a membrane into an HCl solution with its concentration corresponding to that in the gastric juice (0.153 m.), until diffusion equilibrium has been reached, the concentration ratio cannot be expected to be 1, because sulfanilamide reacts with HCl. From the saturation concentration in this HCl and in plasma, 3980 mg.

[238] See, among many others, E. Overton, Ztschr. f. Physik. Chem., **22**: 189, 1897; O. Gros., Arch. f. Exper. Path. u. Pharmak., **63**: 89, 1910; E. Wertheimer, Pflüger's Arch. f. d. ges. Physiol., **199**: 383, 1923; W. J. V. Osterhout, J. Gen. Physiol., **8**: 131, 1925.

[239] J. Travel, Jour. Pharmacol. and Exper. Therap., **69**: 21, 1940.

[240] H. W. Davenport, Yale J. Biol. & Med., **14**: 589, 1942; M. Cooke, H. W. Davenport and L. S. Goodman, Yale J. Biol. & Med., **14**: 13, 1941.

per cent and 1540 mg. per cent respectively, the concentration ratio (solubility ratio) is calculated to be 2.6. This is the same value as found in the experiment. Consequently 2.6 is not an accumulation ratio in the usual sense, indicating a glandular active transfer (p. 525ff), but it is like a distribution coefficient. The question arises, why, in the experiments with the other sulfonamides, identity of the two values is lacking, and the solubility ratio always is greater than the concentration ratio juice:plasma. An explanation can be arrived at by following the changes of concentration of the sulfa drugs in the plasma, after known amounts have been injected intravenously. The drugs leave the plasma by diffusion, not only through the stomach wall into the gastric juice, but also into the entire body through a great variety of pathways, and according to their rates of diffusion in water or in organic solvents and other factors. The "rates of diffusion" measured in this way are listed in the last column of the table. It appears that sulfanilamide is the fastest in passing across the tissues, sulfapyridine is next, the others much slower. Thus, one understands that the distribution of sulfanilamide comes to an equilibrium; whereas the other drugs fall short of it to a greater or lesser degree.

4. The Formation of Pancreatic Juice.—Disregarding again the enzymes, the chief function of the pancreatic gland is the elaboration of a secretion which is slightly alkaline (*p*H 8.5), owing to its content of bicarbonate ion, as the character of the gastric secretion is chiefly determined by its high concentration of the hydrogen ion (*p*H 0.9), the character of the salivary gland by its high concentration of water, which makes this glandular product a strongly hypotonic fluid. The problems of these three performances seem to be related inasmuch as they bear upon the distribution of the most common components of the body fluids, H_2O, H, and HCO_3, but the three glands have attacked their problems in utterly different ways.

Concerning the pancreatic secretion, one main question is, whether the bicarbonate ion is derived from the metabolism of the pancreas, as is the hydrogen ion in the gastric gland, or whether its origin is the blood serum. Mainly two kinds of experiments (on dogs) have provided evidence that the source is the serum. First, with highly active glands, the concentration ratio of total juice CO_2 and plasma CO_2 has been found to be 4 to 5, and after an intravenous injection of radioactive $HCO_3(C^{11})$ the radioactivity is distributed between juice and serum in approximately the same ratio,[241] whereas in similar experiments regarding radioactive Na the distribution ratio was 1.[242] Second, if the metabolic CO_2 were the source of the HCO_3 in the juice, one would have expected an influence of sulfanilamide, which by poisoning the carbonic anhydrase present in the gland tissue would inhibit the transformation of CO_2 to HCO_3. But, even in the presence of exceed-

[241] E. G. Ball, H. F. Tucker, A. K. Solomon, and B. Vennesland, J. Biol. Chem., **140**: 119, 1941.

[242] M. L. Montgomery, G. E. Sheline, and I. L. Chaikoff, Am. J. Physiol., **131**: 578, 1941.

ingly large amounts of sulfanilamide, the juice does not undergo an essential change in composition.[243]

The next question is, how to account for the rise of the HCO_3 concentration to a level four to five times higher than that of the plasma. It has been found [244] that the concentrations of Na and K are approximately the same in juice and in serum. This, together with the findings that an experimental change of concentration of these ions in the serum yields a corresponding change in the juice, allows the conclusion that the gland is freely permeable to Na and K. Further, with increasing rates of secretion, the alkalinity of the juice rises, due to a greater concentration of HCO_3, and, simultaneously, the percentage of Cl decreases, the sum of HCO_3 and Cl concentrations remaining nearly constant. The concentrations of HCO_3 and Cl in the gland tissue correspondingly change in the opposite direction. These facts are interpreted as being due to a free permeability of the gland cells to Na, K, and HCO_3, and to a poor permeability to Cl. Therefore, by a certain degree of Donnan effect[245] after a rapid secretion of the gland has been elicited (by secretin), more HCO_3 enters the juice and less Cl, in such a way that electroneutrality is maintained. This may be explanatory of the concentration ratio of juice HCO_3 to serum HCO_3 found during the increased glandular activity.

Recapitulation of the above considerations shows that, according to present knowledge, the properties of these gland cells are rather unique. Disregarding the differences in rates of penetration (not only Cl being slower, but also Ca, Mg and phosphate), the active cells appear to be permeable to the main inorganic cations and anions. Correspondingly, serum and pancreatic juice are in osmotic balance, as are serum and gastric juice (p. 599). It will be particularly useful to extend the investigations to experiments with nonelectrolytes.

Studies concerning the permeability of the pancreas to dyestuffs have been mentioned previously (p. 602).

5. The Permeability of the Liver.—About 80 years ago, when only a small number of colored natural products were available for experiment concerning the pathways of dissolved substances through the living body, there was discovered the neat picture of the bile capillaries, which appears in the liver after an injection of indigo-carmine, and which furnished striking evidence of a secretory activity of the hepatic epithelium. In more recent

[243] H. F. Tucker and E. G. Ball, J. Biol. Chem., **139**: 71, 1941; E. U. Still, A. L. Bennett, and V. B. Scott (Am. J. Physiol., **106**: 509, 1933) have studied the gaseous metabolism of the gland by comparing the rate of blood flow and juice production, and the O_2 and CO_2 content in the pancreatic artery and vein during rest and activity. The most interesting result is that, at the onset of secretion (produced by an intravenous injection of secretin), for several minutes the venous CO_2 falls below the level of the arterial CO_2, whilst HCO_3 appears in the juice.

[244] E. G. Ball, J. Biol. Chem., **86**: 433, 449, 1930.

[245] K. H. Meyer, J. F. Sievers, and H. Hauptmann, Helvet. Chem. Acta, **19**: 1936; **20**: 634, 1937; T. Teorell, Proc. Nat. Acad. Sc., U.S.A., **21**: 152, 1935; W. Wilbrandt, Ergebn. d. Physiol., **40**: 204, 1938.

studies of this phenomenon[246] it was found that the bile capillaries likewise become visible as a dark blue network after the indigo-carmine dissolved in Ringer has been perfused from the abdominal vein through the isolated frog liver, and that, subsequently, in a cannula inserted at the fundus of the gall bladder, a dark blue secretion can be collected, the color concentration of which surpasses by far that of the perfusing solution. Extension of these observations to other technical dyestuffs showed that many of them likewise can be accumulated in the secretion, but, peculiarly enough, the visible network of the stained bile capillaries has so far never been obtained except with indigo-carmine. According to Höber and Titajew,[247] among the dyestuffs which appear in high concentration in the secretion are many well-diffusible acidic (sulfonic and carboxylic) compounds having very diverse chemical structures, partly lipoid soluble, partly lipoid insoluble. It appears that, in its power to accumulate the dyestuffs, the liver differs very much from the kidney, which can differentiate between the nonpolar-polar and the polar-polar sulfonic azo-dyestuffs, and can secrete sulfonic acid tri-phenylmethane dyes which the kidney cannot.[248] It has further been observed that basic dyestuffs likewise are secreted, but apparently to a lower degree, probably for the simple reason that, due to their basic character, these vital stains (see p. 263) are retained and stored in the hepatic cells. Colloidal dyestuffs, significant for their low diffusion rates, either fail to reappear at all or pass only in small concentration.

The great affinity of the liver to dyestuffs may be conceived of as being connected with their great adsorbability, because of the prevalent nonpolar organophilic groups in their molecules. This would make it understandable that no accumulation occurs with dyestuffs which show a predominance of hydrophilic sulfonate radicals. Another viewpoint is found in the correlation between the perfusing concentration and the accumulation ratio, resembling the analogous correlation found in studies of dyestuff transfer by the kidney (see p. 573). In the aforementioned experiment of Plattner, e.g., indigo-carmine was supplied in 0.05% solution, and the accumulation ratio was found not higher than 2 to 6. However, when a 100 times smaller concentration is used in the experiments of Höber and Titajew, the ratio can exceed 1,000, and, with a dilution of 500 times, ratios of even several thousand can be reached. This group of observations can be interpreted as indicating adsorption, as will be discussed later with regard to the concept of "carriers" as transporting devices (see p. 577; also p. 618).

But in view of these amazingly high concentrations found in the liver secretion, the question must be raised whether water reabsorption in the liver tissues might not play an important role. This might be suggested

[246] F. Plattner, Pflüger's Arch. f. d. ges. Physiol., **205**: 91, 1924.

[247] R. Höber and A. Titajew, Pflüger's Arch. f. d. ges. Physiol., **223**: 180, 1929.

[248] R. Höber and P. M. Briscoe-Woolley, J. Cell. & Comp. Physiol., **15**: 35, 1940; **16**: 63, 1940; R. Höber, J. Gen. Physiol., **23**: 185, 1939; R. Höber, Cold Spring Harbor Symp., **8**: 14, 1940.

with regard to the aglomerular kidney (p. 568), and still more to the surviving excised frog kidney (p. 567). As mentioned before, the frog kidney which is perfused from either supply route by Ringer containing a dyestuff adequate for secretion by the proximal tubules, as indicated by a marked accumulation ratio urine: perfusing fluid (e.g., 30 times), does not show any coloration of the tubular lumina under the microscope, because the lumina are too thin to allow a sufficient absorption of light by the inclosed dyestuff solution. The dyestuff concentration is the result of the glomerular influx, the secretory tubular delivery of dye, and the reabsorption of water by the tubules. With an excised kidney bathed in dyestuff-Ringer, the dyestuff concentration resulting from the activity of the proximal tubules alone can be observed easily, since the glomerular influx is absent, and absorption can proceed so that the color filling the lumina increases in distinctness until finally it reaches very high values. But that the secretory activity, also, plays an important role is shown by the fact that this behavior is displayed only in the presence of a secretable dye, and, furthermore, that the picture does not appear, if to the bathing dyestuff-Ringer solution a narcotic or cyanide is added. The situation in the Ringer perfused liver is decidedly different, anatomically, since, obviously, there is no unit comparable to the glomerulus, from which the secretion could be diluted, and physiologically, since inhibition of active reabsorption of water by a narcotic or by cyanide should be followed by an increased output of fluid in the gall bladder cannula, which does not occur. On the other hand, after inactivation of the liver by phenylurethane, the formation of the blue network of the bile capillaries, otherwise following the injection of indigo-carmine, fails to appear (Plattner, *loc. cit.*), and also the entrance of the dye into the cannula is reversibly diminished or stopped.

The idea that adsorption might be involved in the accumulation of dyestuffs is further supported by the finding that, with substances which, in general, are lacking in adsorbability, secretory concentration is not obtained, when supplied to the liver at molarities similar to those effective with dyestuffs.[249] Thus, xylose, glucose, lactose, inulin (with molecular weights ranging from 150 to about 5100) reappear in the secretion of the Ringer perfused frog liver without a significant change in concentration, as though having passed an inert filter, whereas dyestuffs (with a molecular weight of about 500) simultaneously perfused show a considerable accumulation ratio. Only the very large starch molecule fails to pass the liver. In view of these facts, the question arises, whether the carbohydrate molecules smaller than starch pass the filter of the liver across the hepatic cells or across the intercellular pathways. A similar alternative was encountered earlier concerning the transcellular or intercellular passage of salts across the epithelial layer of the intestine (see p. 540). Not only from the viewpoint

[249] C. Haywood and R. Höber, J. Cell. & Comp. Physiol., **10**: 305, 1937; see also C. Haywood Federation Proc., **2**: 20, 1943.

of general physiology, but also from that of liver function, it seems unlikely that the normal hepatic cells should allow the free penetration of the sugars, although a similar conclusion could be suggested by another series of observations.

In order to investigate the chemical conditions of the secretory activity of the isolated liver, a great number of seemingly rather indifferent organic substances was tested for their influence upon the dyestuff secretion.[250] After a deep coloration of the fluid in the gall bladder cannula has been attained, 1/8 to 1/10 of the perfusing dyestuff-Ringer solution is substituted by the isotonic solution of the organic substances in question, with the effect that the dyestuff concentration in the secretion falls off rapidly, eventually to zero, and reappears after resumption of Ringer perfusion. This inhibitory effect is produced by nonelectrolytes and electrolytes. The main nonelectrolytes are the disaccharides, hexoses, pentoses, polyhydric alcohols (C_6 to C_3), isoelectric amino-acids, succinamide; the main electrolytes are the Na salts of rather strong dibasic and monobasic aliphatic oxy- and hydroxy-acids, like gluconate, succinate, pyruvate, and others. All these substances are rich in polar groups (—OH, —COO, —NH_2) and therefore more or less surface inactive, hydrophilic, and as such dehydrating (or antidispersing) with respect to hydrophilic colloids (pp. 296ff, 245ff). In general, the larger molecules are the more active ones; for instance, mannitol and succinate have a stronger dehydrating influence and a more vigorous liver effect than erythritol or glycolate.[251] More light is thrown on the nature of these physiological actions by the additional finding that the so far mentioned organic substances have their physicochemical, as well as their physiological, antagonists in substances which, on the one hand, are surface active, dispersing toward various hydrophilic colloids (starch, gelatin, lecithin) and, on the other hand, are stimulants to the liver secretion (choleretic action), unless they are administered in such high concentration, or for such length of exposure time, that the dispersing action causes disintegration, cytolysis, and irreversible loss of activity (see p. 340). Substances having such action are the sodium salts of the higher fatty acids (higher than C_5), of oleic acid, salicylic acid, bile acid, and others, belonging to the groups of hydrotropic (p. 336), solubilizing nonpolar-polar substances. The antagonistic properties of the two groups of compounds appear most strikingly in experiments in which the inhibitory effect upon the dyestuff secretion of the liver by a substance belonging to the first group is neutralized by the addition of a member of the second group, which restores the concentration of the dyestuff. Thus, the entrance and the accumulation ratio of the dye might be regarded as dependent upon a certain consistency of the superficial, and of the internal, structures of the hepatic cells.

[250] R. Höber and E. Moore, J. Gen. Physiol., **23**: 191, 1939; R. Höber, Pflüger's Arch. f. d. ges. Physiol., **229**: 402, 1932; J. G. Valdecasas, *ibid.*, **228**: 169, 1931; R. Höber, Cold Spring Harbor Symp., **8**: 40, 1940.

[251] See I. R. Katz, Biochem. Ztschr., **262**: 355, 1933; **263**: 421, 1933.

38

SOME REMARKS ABOUT THE ENERGETICS OF THE ACTIVE TRANSFER, THE TRANSFERRING DEVICES, AND THEIR MECHANICS

At the beginning of this section, active transfer by an organ, as contrasted with passive penetration, was defined as the performance of osmotic work, empowered by the expenditure of some fraction of the energy which is liberated in the metabolic reactions. One may supplement this definition by mentioning light as another source of energy. This is mainly significant in plant life, and its biological effects are ordinarily mediated through the products of photosynthesis, the metabolic reactions of which likewise provide the energy for active transfer.

1. Energy Supplied for Active Transfer.—As yet, the information about the origin of energy utilized in active transfer is rather meager. But it may be reasonable to surmise that carbohydrates, as the most common source of energy, wherever physiological work has to be done, appear in this role also here, even though the substances transported up hill are very different in their chemical nature.

Surveying, at first, all those substances which in the discussion of this section have been proved to be subject to active transfer, we encounter 1. hexoses shifted by the proximal tubules of the kidney (p. 561) and by the intestinal mucosa (p. 544); 2. amino-acids, presumably shifted at the same places (pp. 550 and 562); 3. the metabolic end products, urea and uric acid (p. 572), migrating through the wall of the kidney tubules in the opposite direction from that of hexoses and amino-acids; 4. organic sulfonic acids, as dyestuffs, as dyestuff intermediates and related compounds, which are transported by the kidney (p. 563), the liver (p. 606) and certain plant cells (p. 265); 5. special inorganic ions, HCO_3 transported in the distal kidney tubules, H by the parietal cells of the gastric glands (pp. 560 and 599); 6. inorganic salts, or, better, inorganic cations plus anions, by the intestinal wall (p. 536), the gills of fish (p. 586), the frog skin (p. 589, the kidney (p. 559), and various plant cells (pp. 590ff).

Now, among these substances, several seem to be forced through the cellular machinery by means of energy derived from carbohydrate. This appears most conspicuously from microscopic evidence in certain experiments of Steward, which have been mentioned before (p. 591). Normally,

the cells of potatoes live in a state of low metabolic activity, they are dormant storage cells filled with starch. Placed in highly diluted salt solution and strongly aerated, they change into metabolically vigorous cells, oxygen consumption and CO_2 production rise, the starch granules disappear, and, simultaneously, osmotic work begins, salts are transported and accumulated in the enlarging cell sap vacuoles.

But the specifically physiological way of disposing of the potential energy of the carbohydrates is better revealed in the following manner. As is well known, carbohydrate is broken down stepwise in a sequence of intermediary reactions to the metabolic end product. These reactions are mediated by enzymes. The main steps are the formation of several phosphorylation products, of pyruvic and of lactic acid, and others, ending with the final dehydrogenations and decarboxylations (Sec. 6). This chain of reactions can be interrupted at various places by poisoning one or the other enzyme active in the concatenation of single reactions. The effect is a block in the normal carbohydrate breakdown, which must be followed by more or less impairment of the supply of energy indispensable in each kind of activity which relies on the utilization of carbohydrate. But, in case of such an event, the activity should be revived or restored by repairing the chain at the point of interruption; in other words, by providing the intermediary product which, after addition of the poison, ceases to be produced. As a matter of fact, this conjecture has been verified most satisfactorily by employing, for instance, iodoacetate, which blocks the chain of reactions between the intermediates triosephosphoric acid and phosphoglyceric acid, and thus prevents the formation of pyruvic and lactic acids. Then the arrested transfer can be set going again by addition of pyruvic or lactic acid, just as the working activity of muscles and the irritability of nerves is re-established by lactate after poisoning with iodoacetate.[252,253] This has been proved in various experiments upon the kidney, the skin, and the liver.

It has been mentioned earlier (p. 569) that the secretory action of the proximal tubule of the kidney can be easily studied on cystic explants of the mesonephros of the chick embryo, which, when immersed in a dilute solution of phenol red, takes up this acidic dyestuff into the lumina and accumulates it there to a very high concentration. If enough iodoacetate is added to the dyestuff solution to paralyze the uptake of dye, this effect can be counteracted by addition of pyruvate, lactate, or succinate, the latter being one of the breakdown products appearing in the aerobic phase of the carbohydrate metabolism. But glycerophosphate has been found to be indifferent.[254]

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[252] E. Lundsgaard, Biochem. Ztschr., **217**: 162, 1930; **220**: 8, 1930.

[253] T. B. Feng, J. Physiol., **76**: 477, 1932.

[254] L. V. Beck and R. Chambers, J. Cell. & Comp. Physiol., **6**: 441, 1935. For some related observations on the frog kidney, see R. Ferrari and R. Höber, Pflüger's Arch. f. d. ges. Physiol., **232**: 299, 1933.

Further, it has been mentioned before that the frog skin displays an active transfer in that it transports Cl ions against a concentration gradient. This is evidenced in previously described experiments, in which the isolated skin is interposed between samples of the same frog-isotonic Ringer solution (p. 588). It has been known for a long time[255] that the surviving skin is able to drive a current of water from its epithelial to its serosa side. Now the more recent detailed studies of Huf[255] have revealed the facts that, first, during the shift of fluid the Cl concentration diminishes on the epithelial and rises on the serosa side, indicating that the transport of water has been accompanied by an additional transfer of Cl; and, second, as evident from the table (p. 589), the active transfer of Cl can be diminished by bromoacetate (which resembles iodoacetate), and that the poisoning action of bromoacetate can be counteracted by lactate (see experiment 5).

Also the dyestuff concentrating capacity of the isolated Ringer perfused liver of the frog (p. 507), which is decreased by iodoacetate as well as by other poisons, seems to recover after additional provision of lactate.[257]

But, finally, it might be objected that the toxic effect of iodo- or bromoacetate, and the subsequent detoxicating effect of pyruvate or lactate as stated in the aforementioned experiments, cannot be considered a safe proof that the various kinds of active transfer depend directly on energy supplied from carbohydrate breakdown. For, iodoacetate is a rather dangerous substance, the administration of which is readily followed by a universal and irreversible impairment of cell vitality,[258] and, hence, of every kind of cell activity. Even though pyruvate and lactate have been found to restore the inhibited active transfer, this favorable influence might be only an indirect symptom of the revival of the cell energetics, which seem to depend quite generally upon carbohydrate metabolism (see also p. 614).

Inhibition of active transfer can also be produced by chemical means, but without affording clear evidence that the chemicals disrupt the coupling between a specific metabolic process, like the carbohydrate breakdown, and the active action of the transporting mechanism. Such conditions are present during anoxia and during narcosis. Anoxia, produced by cyanide, hydrogen sulfide, or an atmosphere of pure nitrogen, results in cessation of the normal activity, for instance, of the dyestuff secretion by the chick mesonephros[259] or of the uphill shift of Cl by the frog skin,[260] or of the accumulation of sulfonic acid dyestuffs by special cells surrounding the

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[255] E. W. Reid, J. Physiol., **11**: 312, 1890; British Med. J. **1**: 323, 1892; J. Physiol., **25**: 486, 1901; see also p. 552).

[256] E. Huf, Pflüger's Arch. f. d. ges. Physiol., **235**: 655, 1935; **237**: 143, 1936; **238**: 97, 1936.

[257] M. Koll-Schroeder, Pflüger's Arch. f. d. ges. Physiol., **234**: 264, 1934.

[258] K. A. Klinghoffer, J. Biol. Chem., **125**: 201, 1938; R. Öhnell and R. Höber, J. Cell. & Comp. Physiol., **13**: 161, 1939.

[259] R. Chambers, L. V. Beck, and M. Belkin, J. Cell. & Comp. Physiol., **6**: 425, 1935. See also A. N. Richards and J. B. Barnwell, Proc. Roy. Soc., London., **B 102**: 72, 1927.

[260] E. Huf, Pflüger's Arch. f. d. ges. Physiol., **235**: 655, 1935.

vascular bundles of plants.[251] But this inactivation cannot be correlated with the influence upon a specific group of cell constituents serving as the essential source of energy, but rather involves the entire metabolism.[252] Narcosis, as known, e.g., from experiments on the dyestuff perfused isolated kidney,[253] or the liver,[254] even more than anoxia, is a manifestation of the unspecific interruption of numerous cellular reactions, since the narcotics, because of their surface activity, are believed to attach, in general, to the cellular and micellar structures, which are the site of the enzymes, and to interpose in this manner a barrier between the manifold enzymes and their manifold substrates (see pp. 360ff).

No special source of energy of a chemical nature has been suggested as yet as potentiating the active transfer of urea and uric acid in the kidney (see p. 572).

Another procedure for studying the interrelation between the chemical turnover and the osmotic work of living cells is based upon measurements of the influence of temperature. Within a certain range of temperatures, one ordinarily observes an increase of work with an increase of temperature, for instance as shown in Table LVII.[255] This can be interpreted as dependent upon a higher rate of diffusion of the substances entering the energy supplying system or a greater reaction velocity in this system.

TABLE LVII.—INFLUENCE OF TEMPERATURE ON THE ACCUMULATION OF POTASSIUM IN EXCISED BARLEY ROOT SYSTEMS
(Absorption period: 10 hours)

Temp. (°C.)	K mg./liter		Accumulation ratio K sap: K sol.
	Culture solution	Cell sap	
6	9 62	34 7	3 6
12	9 32	47 7	5 1
18	8 45	73.2	8.7
24	7 98	97.8	12.3
30	7 42	112 0	15 1

In general, the temperature coefficients of diffusion referred to a temperature interval of 10°C. (Q_{10}) have been found to be 1.2 to 1.3, the temperature coefficients of chemical reactions 2 to 3 (see Sec. 1, chap. 2). The Q_{10} values calculated for the experiment in the table are about 2, i.e., like

[251] R. Collander and A. Holmström, Acta Soc. pro Fauna et Flora Fenn., **60**: 129, 1937; R. Collander, Jahrb. f. wiss. Bot., **60**: 354, 1921.

[252] In this regard it is worth mentioning that the inhibition of phenol red transport by the tubular cells of the chick mesonephros, following poisoning with hydrogen sulfide, cannot be counteracted either by lactate or by pyruvate; L. V. Beck and R. Chambers, J. Cell. & Comp. Physiol., **5**: 441, 1935.

[253] F. Scheminzky, Pflüger's Arch. f.d. ges. Physiol., **221**: 641, 1929.

[254] R. Höber and A. Titajew, Pflüger's Arch. f.d. ges. Physiol., **223**: 180, 1929.

[255] D. R. Hoagland and T. C. Broyer, Plant Physiol., **11**: 471, 1936.

the temperature coefficients of metabolic reactions (but often they have been found to be as high as 5).

Below a certain temperature limit the work performed by the system is practically zero. This is evidenced by the following experiment[265] upon the mesonephros of the chick embryo (p. 569). The uptake and accumulation of phenol red by the explant can be inhibited completely for many hours by cold (3° to 6° Celsius). After the culture has been returned to body temperature, appreciable amounts of dye appear within a few minutes in the lumina and are accumulated. However, when the culture is exposed to low temperature, after the lumina have been filled with color, the dye requires hours to escape. Obviously, the uptake at high temperature is rapid, active, and unidirectional, the outgo slow and passive. This resembles the situation in the experiments of Collander and Holmström (p. 614, also 265) upon the influence of oxygen on the intake and output of dyestuffs by certain plant cells. For the intake, oxygen is required, but during the subsequent lack of oxygen the captured dye escapes, although very slowly. Another example deals with the absorption of potassium halides by *Nitella* with light as the ultimate source of energy[267] through the metabolic reactions of the products of photosynthesis, and lowering the temperature to 5° causes, e.g., Cl to leak out of the healthy cells, but at room temperature the Cl is recaptured (p. 251).[268]

2. The Cellular Devices for Active Transfer and Their Mechanism. When a chemical process has to be carried out in such a way that a greater or smaller portion of the energy liberated becomes available for work, for instance, for osmotic work, it requires some kind of machinery to bring about an orderly movement of the molecules taking part in the chemical process instead of an otherwise random movement. We are still very far from perceiving any such structural arrangement, even though, as long as cells, either living or fixed, have been investigated by the microscope, emphasis was laid upon the interpretation of their visible constituents in the light of a certain functional significance. More successful in this regard were the combined efforts of histologists and physiologists to design a mechanical model of a muscle on the basis, particularly, of the newly attained knowledge about the physics of fibrillar structures, their physical chemistry, and the linkage of this with an orderly system of chemical

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[265] R. Chambers and R. T. Kempton, J. Cell. & Comp. Physiol., **3**: 131, 1933; R. Chambers, L. V. Beck, and M. Belkin, *ibid.*, **6**: 425, 1935.

[267] D. R. Hoagland, and A. R. Davis, J. Gen. Physiol., **6**: 47, 1923, also **5**: 629, 1923 and D. R. Hoagland, P. L. Hibbard and A. R. Davis, **10**: 121, 1926.

[268] See, further, the observations of Osterhout concerning the uptake of K by *Valonia*, which ceases in the dark and evidently is kept going by the photosynthetic reactions (A. G. Jacques and W. J. V. Osterhout, J. Gen. Physiol., **17**: 727, 1933; W. J. V. Osterhout, Ergebn. d. Physiol., **35**: 967, 1933); further, the experiments of I. E. Harris, (J. Biol. Chem., **141**: 579, 1941) and W. Wilbrandt (Pflüger's Arch. f. d. ges. Physiol., **243**: 519, 1940), upon uptake of K against the concentration gradient by human erythrocytes and its linkage with glycolysis.

reactions (see Sec. 7). In the field of the present chapter, the appearance and the variable location of vacuoles and granules in secretory cells, at rest or in action, at first suggested postulating forces pushing these bodies in one direction toward the lumen, where the enzymes, or hormones, or mucine, or other substances produced within the cells, are extruded. In addition it seemed fruitful to consider whether the more simple substances, like single molecules, produced elsewhere and present in the cell surroundings could be taken up, then accumulated, transferred across the cell body, and thrown out in a similar way. However, this picture looks less promising, since there are observations on living cells, according to which, during the shift of molecularly dispersed substances or small molecular aggregates, transporting vehicles such as granules or vacuoles cannot be detected, even with a high-power microscope. This was emphasized, e.g., by Chambers and Kempton[269] in their observations upon the secretion of phenol red by the mesonephros of the chick embryo (p. 569). As the dye enters from the surrounding highly diluted solution, it appears to be dissolved in the visibly stained protoplasma and is expelled in a markedly increased concentration at the luminal side. Also, in conflict with assigning to the vacuoles an important role as carriers, is the observation that, in the excretion of dyestuffs by the kidney of mouse or frog, the high tide of elimination is already passed before dyestuff vacuoles appear under the microscope.[270] However, there are reasons to believe that the vacuoles containing the dyestuffs are formed at first in submicroscopic size by an intraplasmatic secretion, thus producing the appearance of a monophasic homogeneous distribution inside the cell, and that they grow only after a time to visible bodies. Hence, this discussion as to the role of vacuoles as essential parts of a transporting machinery remains inconclusive.

Another source of information has been furnished by following the absorption of hexoses in the intestine and their reabsorption in the kidney (pp. 544, 561). As was mentioned earlier, Wilbrandt and Laszt,[271] on the basis of their finding that the differential absorption of the hexoses in the intestine disappears after poisoning with iodoacetate, suggested that the preferential absorption of glucose is tied up with reaction between the sugar and inorganic phosphate. Lundsgaard[272] and Wertheimer[273] showed that the preferential absorption of glucose by the intestine, also, is abolished by phlorizin, as has long been known for the reabsorption in the kidney, and Lundsgaard further found that in tissue extracts phlorizin can be a strong inhibitor of phosphorylation as well as of dephosphorylation. Gomori[274]

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[269] R. Chambers and R. T. Kempton, J. Cell. & Comp. Physiol., **3**: 131, 1933.

[270] W. von Moellendorff, Ergebn. d. Physiol., **18**: 141, 1920; Ztschr. f. d. ges. Anat., 3. Abt., **24**: 278, 1922; R. Höber, J. Cell. & Comp. Physiol., **6**: 117, 1935.

[271] W. Wilbrandt and L. Laszt, Biochem. Ztschr., **259**: 398, 1933.

[272] E. Lundsgaard, Biochem. Ztschr., **254**: 209, 221, 1933.

[273] E. Wertheimer, Pflüger's Arch. f. d. ges. Physiol., **233**: 514, 1933.

[274] G. Gomori, Proc. Soc. Exper. Biol. & Med., **42**: 23, 1939.

and Kritzler and Gutman[275] succeeded in demonstrating in microscopic slices that the proximal tubules of the kidney, in which the sugar reabsorption takes place, are extremely rich in phosphatase, which is largely concentrated at or near the luminal border of the epithelium, and which at alkaline reaction can liberate, from glycerophosphate, inorganic phosphate which becomes visible by precipitation in the tissue slices. They further showed that this dephosphorylation fails to be inhibited by phlorizin. These various separate observations can be linked together tentatively into a fairly reasonable picture of sugar absorption, when the influence of *p*H[276] is taken into consideration. First, dephosphorylation of glycerophosphate (and hexosediphosphate), which was mentioned as going on at alkaline reaction (*p*H 7 to 9.2) and also in the presence of phlorizin, is inhibited at *p*H 6 to 7 by phlorizin. Second, the inverse process, the phosphorylation of glucose likewise is inhibited by phlorizin at *p*H 5 to 7.4. Finally, taking into account the earlier mentioned fact (p. 550) that phosphorylation of glucose is an aerobic reaction, viz., that it is a mechanism by which, in the presence of the adenylic acid system, oxidative energy can be utilized in the cell, the absorption can be described as an active transfer, as follows: At the luminal border of the epithelium, the *p*H conditions in the phosphorylizing system of enzymes may be such as to allow the aerobic formation of hexosephosphate, as well as its entrance into the cells. There, at the protoplasmic reaction of about *p*H 7, dephosphorylation takes place (even in the presence of phlorizin), and the freed sugar, which was shifted as hexosephosphate across the boundary separating the exterior and the interior of the body, reappears.[277]

But, this hypothetical description of intestinal and renal transfer of glucose certainly is not more than a fragmentary picture, taking into account the following formerly mentioned findings: First, the secretion of diodrast by the kidney tubules is stopped by phlorizin (p. 572); second, there is no reason to assume that diodrast is phosphorylized; third, the localization of phosphatase at the brush border of the renal epithelia, which is suggestive of being instrumental in absorption, loses its meaning in secretion, that starts on the other side of the cells. Therefore, the conclusion has been drawn[278] that the enzymatic phosphorylation of sugar serves not only as a specific share in the mechanism of the transfer of sugar, but also as a more general source of oxidative energy furnished by the previously (p. 550)

[275] R. A. Kritzler and A. B. Gutman, Am. J. Physiol., **134**: 94, 1941; see, further, M. L. Menten, J. Junge, and M. H. Green, Proc. Soc. Exper. Biol. & Med., **57**: 82, 1944.

[276] H. Kalckar, Enzymologia, **2**: 47, 1937; L. V. Beck, Proc. Soc. Exper. Biol. & Med., **49**: 435, 1942.

[277] This is somewhat similar to the reappearance (by resynthesis) of fat in the intestinal epithelia near their striated borders after it has been subjected to a transitory saponification, which provides the conditions for passage of the short distance between the lumen and the cell body.

[278] T. L. Althausen and M. Stockholm, Amer. J. Physiol., **123**: 577, 1938; J. J. Eiler, T. L. Althausen, and M. Stockholm, *ibid.*, **140**: 699, 1944.

mentioned intracellular linkage between the carbohydrate and the adenylic acid systems, and as such co-operative in the shift of various substances. This energy output also may be thought of as being increased by thyroxin (see p. 562).

The localization of enzymes, as it might serve for the transport of substances across the cells, constitutes a "chemical organization" comparable to the morphological organization, which was discussed before with respect to the cell vacuoles, and may be effective elsewhere as well. Thus, carbonic anhydrase has been mentioned as engaged in the accumulation of HCl in the gastric juice (p. 601) and in the reabsorptive shift of HCO_3 in the distal tubules of the kidney (p. 560). However, so far nothing of importance is known either concerning the structural organization of the parietal cells of the gastric glands as the site of the enzyme, or concerning the spatial relations between the specifically active tubular segment and the place where the enzyme acts.

Other elementary components of a cellular transferring machinery, which have been mentioned previously, are the so-called carriers, hypothetical structures inside the cells, which have been suggested chiefly by the observations of Macleod and Magee (p. 546), Verzár (p. 547), and Donhoffer (p. 547), regarding intestinal absorption, and by those of H. Smith and Shannon (p. 576) regarding kidney function. These and other observations have revealed the fact that the total amount of the substance migrating across the cells is composed of two fractions; one is the result of diffusion, and so increases with rising concentration of the substance; the other increases only in a certain range of low concentrations extending from zero to a limiting value, beyond which it becomes constant (see Fig. 62, p. 577). This has been reasonably conceived of as indicating a system of intracellular carriers or vehicles, which, in the presence of a certain concentration of the available substance, are filled to capacity, and which subsequently discharge their entire burden together with the fraction passed by diffusion. The efficiency of this kind of transport depends, among other things, mainly upon a special affinity between the substance in question and the carrier system, with the result that the total concentration in the output is raised in comparison with that in the entering fluid.

Many questions are suggested by this concept: first, regarding the nature of the affinity between the carrier and the load. The carrier may be looked upon as an adsorbent, or as a solvent, or as a chemical substrate undergoing a stoichiometrical reaction with the transported substance.

There are several properties of the transferring systems particularly favorable to the idea of adsorption. In the case of the kidney, among a great number of lipoid insoluble organic electrolytes, so far mainly those have been found to be picked up and to be accumulated which have a nonpolar-polar molecular configuration (p. 566). This has suggested the interpretation that the nonpolar part of the molecule, due to some residual valence forces, attaches to the organophilic elements in the cell body,

leaving the polar part oriented toward the adjacent aqueous phase. A symmetrical hydrophilic structure, or a predominant influence of the hydrophilic character, is detrimental to the active transfer, and presumably to adsorption. The behavior of the liver presents a somewhat different aspect (p. 607). The marked influence of the location of nonpolar and polar groups in organic electrolytes found in the kidney studies, for instance, with lipoid insoluble dyestuffs, fails to be manifested by the liver. Rather, all kinds of dyestuffs are secreted except those which are highly colloidal, and those which contain in their molecules a great number of the strongly hydrophilic sulfonate groups. On the other hand, lipoid insoluble organic substances like xylose, glucose, and lactose pass the surviving liver like a dead filter, without any change of concentration (p. 608); and inulin (M.W. = 5100), even under conditions as nearly normal as possible, i.e., after intramuscular injection to the aglomerular toad-fish (p. 568), reappears in the secretion of the liver at an average concentration of 0.57, compared to a serum concentration of 1.[279] It is tempting to believe that this different behavior of the two groups of substances toward the secreting system of the liver is accounted for by the great adsorbability of the dyestuffs, in contrast to the various carbohydrates, which are unsuitable for adsorption because of their highly hydrophilic character.

Adsorption, likewise, might be revealed as an essential factor of the active transfer by quantitative studies, such as, for instance, that graphically reproduced in Fig. 62 (p. 577), representing the excretion of phenol red by the frog kidney. In this figure, the tubular action, as related to the concentration of dyestuffs in the perfusing fluid, appears as a curve, the slope of which is initially steep and gradually swings to a plateau, interpreted as representing the maximum load of the carrier. The curve resembles an adsorption isotherm with its increments decreasing with increasing concentration, until an adsorption maximum has been attained, possibly indicating a saturation of the surface by the adsorbent.

The figure can also be explained by thinking of the carrier system as a second solvent dispersed in the aqueous phase of the cell, the substance to be carried being distributed between the two phases, with a distribution factor decreasing with increasing concentration. Such a situation obtains, when the substance is present in the two solvents in different molecular states, for instance, single molecules in one solvent, bimolecular in the other (see chap. 5. subchap. 9, and chap. 23, subchap. 2).

In supplement to the discussion of the hypothetical carrier system, it should be said that the energy required by the system would be utilized for various purposes, for the loading process, for shifting the carrier across the cell, for setting free the substance from its attachment, and for its elimination from the cell. Further, it may be added that the unidirectional shift seems to be predetermined. in some cases, by structural components of

[279] C. Haywood, Federation Proc., 2: 20, 1943.

the protoplasm, like the filamentous mitochondria, providing, through their arrangement, intracellular channels parallel to the long axis of the cell; but, in addition, there is an intracellular polarity,[280] demonstrable in the kidney epithelia by a characteristic arrangement of vitally-stained granules, which is destroyed, when the cells are isolated and when simultaneously they lose their ability to transport and to accumulate the phenol red.

3. Mechanism of the Simultaneous Active Transfer of Cations and Anions.—From this extremely fragmentary description of some separate elementary constituents of the transferring machinery, we finally turn to the most difficult problem, namely, to explain the fact that, by means of an appropriate set-up of these elementary units, the energy liberated by metabolic reactions may ultimately serve to bring about the mechanical effects of active transfer, accumulation, and forwarding of the substances in question to the terminus of the process.

In discussing this fundamental problem, first some simple model experiments will be recalled, which could possibly shed some light upon the transport of the most common components of living organisms, inorganic ions and water. In the introduction to this section (p. 526), studies were mentioned concerning the spontaneous movement of ions against the concentration gradient by employing a selectively ionpermeable membrane, for instance, in the following way.[281] A cationpermeable membrane separates two salt solutions, each of them containing H and K ions in concentrations such that $H_i > H_o$ and $K_i = K_o$, the kind of anions being irrelevant. Then H will tend to move from inside to outside, but for the reason of electroneutrality it cannot do so, unless, for each H leaving, one K enters against an ever-increasing concentration gradient. Equilibrium is reached (eventually after a long time), when $H_i : H_o = K_i : K_o$. For example, in an experiment of Netter employing a dried collodion membrane (p. 526), after 14 days, i.e., before the final state was reached, $K_i/K_o = 10$. In a corresponding experiment, with a selective anionpermeable membrane and the anions HCO_3 and Cl, the equilibrium would be reached if $HCO_{3i} : HCO_{3o} = Cl_i : Cl_o$. It was mentioned previously that the establishment of similar membrane equilibria has been observed under physiological circumstances, for instance, with muscle fibers where—disregarding several complicating factors (chap. 17)—the high percentage of K, compared to the much lower percentage in the serum, can be referred to their specific permeability to K ions.

However, the quotation of these observations seems to be without informatory value for the present question, since in the transport of ions by a number of organs—kidney, intestine, skin, various plant tissues, and others—we are dealing first with the simultaneous or approximately simultaneous accumulation of cations plus anions; and, second, with

[280] R. Chambers, Cold Spring Harbor Symp., **8**: 144, 1940.

[281] H. Netter, Pflüger's Arch. f. d. ges. Physiol., **220**: 107, 1928; S. C. Brooks, Protoplasma, **8**: 389, 1929; S. C. Brooks, Tr. Faraday Soc., **33**: 1002, 1937.

processes demanding the liberation of energy, whereas, in the model experiments, the concentration gradients arise spontaneously, and either for anions or for cations, due to the permeability of the membrane, which is either anion- or cation-permeable. At first sight, then, it may appear promising to adjust the model experiment to the exigencies of the physiological accumulation of both cations and anions, by simply producing artificial membranes, which have a mosaic structure, i.e., which are composed partly of cationpermeable and partly of anionpermeable areas, each of them independently accumulating cations or anions respectively. For instance, with H and HCO_3 on one side, K and Cl on the other side of the mosaic membrane, corresponding to the aforementioned experiments, K and Cl would be expected to be shifted across the membrane against their gradient.

A mosaic membrane can be prepared[282] on a mercury surface, by placing close to each other droplets of alcohol-ether solutions of collodion and of rhodamin-collodion (p. 317), which, flowing together and drying, form membranes with the suitable properties. By measuring the potentials across such a membrane interposed between two different salt solutions, the membrane can easily be shown to exhibit the expected behavior, as is evident from the following table:

TABLE LVIII.—MEMBRANE POTENTIALS OF MOSAIC MEMBRANES
(+ and − refer to the pole in solution 2)

Solution 1	Solution 2	Permeation	mV
0.1 mol. NaCl	0.1 mol. KCl	K > Na	−23.0
0.1 mol. NaCl	0.1 mol. NaSCN	SCN > Cl	+35.5
0.1 mol. NaCl	0.1 mol. KSCN	K = SCN	+ 2.5
0.1 mol. NaCl	0.1 mol. KCl	K > Na	−23.0

However, Sollner[283] demonstrated that this model cannot serve its purpose, i.e., the individual cation- and anion-permeable areas cannot combine their accumulating power to yield an accumulation of salt (cation + anion). This is made clear by the following scheme, Fig. 64.

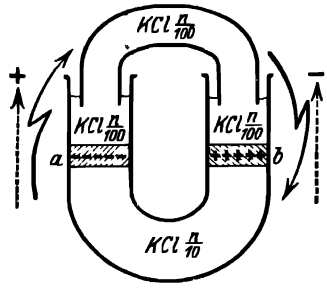

FIG. 64.—Scheme of an electric circuit current passing a porous membrane.

The figure pictures, on the left side, one cationpermeable area (the substance of the membrane being electronegative); on the right side, one anionpermeable area (formed by an electropositive substance), *the two areas electrically influencing each other* by way of the two U-pieces, just as adjacent areas of a mosaic membrane must, when the entire membrane is in contact with the 0.1 mol. KCl solution on one side, with the 0.01 mol.

[282] R. Höber and F. Hoffmann, Pflüger's Arch. f. d. ges. Physiol., **220**: 558, 1928.
[283] K. Sollner, Biochem. Ztschr., **244**: 370, 1931.

KCl solution on the other side. The dotted arrows in the figure indicate the direction of the transport of positive K and of negative Cl ions respectively, the solid arrows the direction of an electric circuit current with the membrane potential on the left side promoting the cations, on the right side the anions in equivalent amounts from the more concentrated to the less concentrated solution, so that cations and anions neutralize each other. It is obvious that the mosaic membrane, at variance to the first assumption, must allow or even accelerate the leveling out of the concentration gradient of both cations and anions, rather than produce an accumulation of salt.

But nevertheless it does not seem quite futile to utilize the idea of a mosaic membrane in an effort to adapt the model better to the physiological circumstances. First, allowance must be made for energy derived in the case of physiological material from metabolism. In addition, it should be considered that natural plasma membranes, instead of being built up by such inflexible material as collodion, actually are composed of many substances of great diversity of physical and chemical nature, and so are not unlikely to possess mosaic properties. These points have been particularly emphasized by S. C. Brooks,[284] and by Wilbrandt.[285]

As to the first point, Brooks has proposed the following way to introduce the factor of energy supply into the exploration of ion accumulation in model experiments. The "inside" of the mosaic membrane is placed in contact with H and HCO_3 ions, the most common products of respiratory metabolism continually arising inside the cell in excess, and therefore tending to pass the membrane, the H ions across the cationpermeable areas of the membrane, HCO_3 correspondingly across the anionpermeable. In addition, K and Cl ions are distributed from the beginning of the experiment "inside" and "outside" in approximately equal concentrations, analogous to the formerly (p. 620) described experiment of Netter. Then, if single selectively anion- and single selectively cation-permeable *areas* were *electrically segregated* from each other, simultaneously with the escape of H and HCO_3, K and Cl would be accumulated inside. But under ordinary conditions, i.e., without such insulation of the cation- and anion-permeable areas, such an accumulation of K + Cl could not persist, because, according to Fig. 64, it would be annihilated by the electric currents. However, the question can be raised whether a continuous and abundant supply of metabolic H and HCO_3 would not overcompensate the return of K and Cl and thus produce *a steady state of accumulation.*

As to the second point, the complexity and the flexibility of the plasma membrane, the evidence presented by Steward and Hoagland should be reconsidered (pp. 592ff). They found, especially with reactivated potato discs and with growing barley roots, that with rise and fall of O_2 consumption and CO_2

[284] S. C. Brooks, Protoplasma, **8**: 389, 1929; Tr. Faraday Soc., **33**: 1002, 1937.

[285] W. Wilbrandt, Ergebn. d. Physiol., **40**: 204, 1938; see also G. E. Briggs, Proc. Roy. Soc., London, **B 107**: 248, 1930; **108**: 317, 1931.

production, in other words, with corresponding changes of H and HCO_3 concentrations, the accumulation ratio for salts increases and decreases, eventually to zero, that the accumulation of Cl, evoked at first in *Nitella* by illumination, vanishes again in the dark, which renders O_2 unavailable (chap. 21). Further, the rate of accumulation is high with Cl, Br, NO_3, low with SO_4, and high with K, lower with Na and still lower with Ca. This would be in agreement with the numerous observations on artificial and natural membranes, showing that the penetrating power of Cl surpasses that of SO_4, and that of K is greater than that of Ca. But, in addition, it has been observed that the rate of metabolism also depends upon the ions present; the metabolism being stimulated by K, depressed by Ca, and also variously modified by anions such as NO_3 or Cl or HCO_3.[285] These findings may be accounted for as owing to the structural flexibility of the natural plasma membrane, mediated by its colloidal components, and at variance with the behavior of the inflexible collodion membrane in the model experiment.[287] Further, such a flexibility would possibly be signified by a deformation of the pore diameters in a sieve structure, also to a change in the thickness of the membrane, both of which would produce an increase or a decrease in the potential drop across the membrane.

4. Model Experiments Concerning the Active Transfer of Water.—The important problem of active transfer of water against its concentration gradient, which has been touched upon on many occasions in the previous discussion, especially in connection with the transport of solution by the intestinal wall, by the kidney tubules, and by the various membranes of the body surface, may seem to have been unduly neglected so far; and also here the discussion will be confined to the description of model experiments dealing with active transfer of water through electrical forces, developed in artificial membranes and manifested as electroosmosis. Physiological transport of water, in general, is a very complex phenomenon, the thorough analysis of which leads to the distinction and evaluation of a series of factors, in part conceivable as a passive penetration along gradients of hydrostatic or osmotic or colloidosmotic pressure (also controlled by the diffusion rates of the solutes), in part controlled in a somewhat undefined manner by the action of endocrines (especially the pituitary gland and the adrenal gland), but in part also definitely a clear-cut active transfer by glands, where the secretion pressure has been found to exceed the arterial pressure. This became especially obvious in studies upon the aglomerular kidney (p. 569; further p. 570), where the ureteral pressure seems to be four to five times higher than the pressure in the kidney capillaries.[288] Possibly the physicochemical conditions for such an accomplishment are comparable,

[285] F. C. Steward and G. Preston, Plant Physiol., **15**: 23, 1940; D. R. Hoagland and T. C. Broyer, *ibid.*, **11**: 471, 1936; W. O. Fenn and D. M. Cobb, J. Gen. Physiol., **17**: 629, 1934; A. H. Hegnauer, W. O. Fenn, and D. M. Cobb, J. Cell. & Comp. Physiol., **4**: 505, 1934; Am. J. Physiol., **112**: 41, 1935; H. Lundegårdh, Biochem. Ztschr., **290**: 104, 1937; H. Lundegårdh and H. Burstrom, Planta, **18**: 683, 1933.

[287] See, however, C. W. Carr and K. Sollner, J. Gen. Physiol., **27**: 77, 1943; K. Sollner and P. W. Beck, *ibid.*, **27**: 451, 1944.

[288] R. N. Bieter, Am. J. Physiol., **97**: 66, 1931.

to some extent, to those established in models arranged to illustrate "anomalous osmosis."

During the last three decades, it has become increasingly obvious that the phenomenon of "anomalous osmosis," discovered about 100 years ago by Dutrochet, is caused by superimposing electrical forces upon the osmotic forces acting in normal osmosis. This could be anticipated already from numerous observations by Thomas Graham and others that anomalous osmosis in general is bound up with the presence of electrolyte solution. In anomalous osmosis, the fluid movement across the pores of a membrane separating an electrolyte solution and water (or two electrolyte solutions of different concentrations) is either unexpectedly great or small with respect to the osmotic strength of the solutions, or it is even reversed in direction resulting in "negative osmosis." More specifically, it was found that, with solutions of equivalent concentrations, the rate, as well as the direction, of osmosis differed from one electrolyte to another. According to the investigations of Girard, Bartell, Loeb, Freundlich, Sollner,[289] and others, two factors are of outstanding importance; first, the liquid junction potential (E-potential) between the two solutions, which are in contact with each other across the pores of the membrane; and, second, the electrokinetic potential (ζ-potential) between the capillary walls of the membrane and the movable solution filling the pores (see chap. 18). The height and the direction of the E-potential vary according to the electrolyte concentration and the nature of the electrolyte, particularly its transfer numbers u and v in free solution, to the sign of the charge of the pore walls and the charge density, and to the size of the pores; these factors, in their combination, allow the ions to penetrate freely the membrane, or restrict their movement toward a selective cation or anion penetration (p. 317). The ζ-potential depends upon the electric charge due to the nature of the membrane material and upon the relative adsorbability of the cations and anions of the electrolyte present, the cations being more adsorbed by a membrane carrying a negative charge, and the anions by one carrying a positive, and polyvalent ions having a stronger influence on the ζ-potential than monovalent ones (see p. 303). The result is the formation of a Helmholtz double layer, whereby the charges of the one layer are seated in the surface of the mobile fluid vein, so that, due to the E-potential across the membrane, and according to its direction, an attraction or a repulsion is exhibited toward the fluid vein. This, however, in itself, is not enough to bring about an electroosmotic liquid movement. Electroosmosis requires the continuous flow of an electric current, that means the existence of a closed circuit. Therefore, if electroosmosis occurs, a closed circuit must

[289] Girard, Compt. rend. Acad. d. sc., **145, 148, 150, 153**: 1908–1911; F. E. Bartell and co-workers, J. Am. Chem. Soc., **35**: 646, 1914; **38**: 1029, 1916; **44**: 289, 1922; J. Physic. Chem., **24**: 444, 593, 1920; **27**: 101, 252, 346, 1923; J. Loeb, J. Gen. Physiol., 1–5, 1919–1922; H. Freundlich, Kolloid-Ztschr., **18**: 11, 1916; K. Sollner, Ztschr. f. Elektrochemie, **38**: 36, 234, 1930; K. Sollner and A. Grollman, *ibid.*, **38**: 274, 1932.

exist. If all pores would be electrically identical, then obviously no current could flow. However, if different pores have different electric characteristics, e.g., due to their different sizes, then they yield different potentials. These potentials, according to Michaelis, may vary from the free diffusion potential to the membrane potential found with selectively anion- or cation-permeable membranes (p. 317). Pores yielding different pore potentials, according to Sollner, must act upon each other, and small electric currents must circulate through neighboring pores, the higher electromotive force of one pore enforcing an electroosmotic fluid shift in the other, and in a direction dictated by the electric charge of the fluid vein. This situation is depicted in the schemes, Figs. 65 to 67, after Sollner.

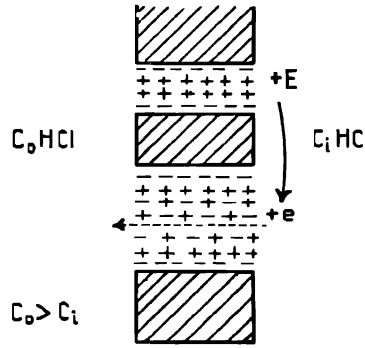

FIG. 65.- Concerning the theory of positive anomalous osmosis.

Figure 65 portrays two pores of a sieve membrane separating two solutions of HCl with the concentrations C_o and C_i ($C_o > C_i$). The narrow upper pore is cationpermeable only; its wall substance (collodion) carries negative charges arranged in opposition to positive charges (H ions) of the

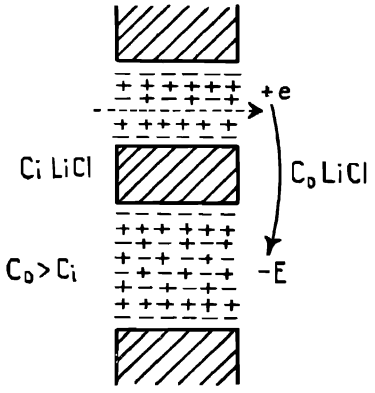

FIG. 66.—Concerning the theory of negative osmosis.

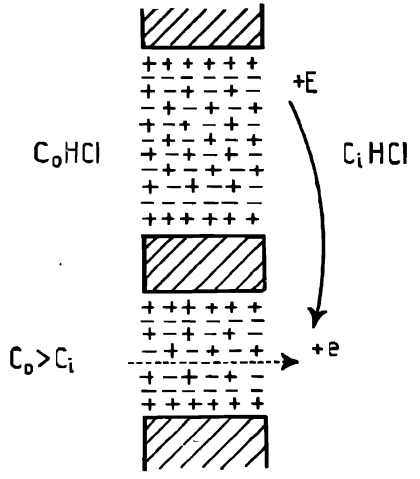

FIG. 67.—Concerning the theory of negative osmosis.

electrical double layer, whose charges are partially fixed by electrostatic forces, partially movable. The potential across this pore is positive and high, due to the mobility of H ions ($+E$). The lower pore permits penetration of anions as well as cations, the potential has the same sign as in the case of the upper pore ($+e$), but is lower, i.e., it approaches that of free diffusion. Therefore, since $+E$ is greater than $+e$, a driving force exists, which compels the liquid vein, with its electropositive surface, to

move in the direction of the positive current (dotted arrow), and thus increases the conventional osmotic flow associated with the concentration difference $C_o - C_i$. This is known as *positive anomalous osmosis.*

Figures 66 and 67 picture *cases of negative osmosis.* According to Fig. 66, this can be brought about by the diffusion of LiCl from the higher concentration C_o to the lower C_i. In this case $v_{Cl} > u_{Li}$, the wider pore (below) becomes the seat of the negative liquid junction potential $-E$, while the upper pore, because of its narrowness and the negative charge of its wall, a condition favoring mobility of cations only, gives a low membrane potential $+e$. Therefore, the higher potential $-E$ enforces electroosmosis in the upper pore, directed from C_o to C_i.

Finally, Fig. 67 shows a scheme for negative osmosis, where HCl diffuses through a positive membrane, the lower pore, compared to the upper one, favoring to some extent the passage of anions, and therefore displaying a somewhat smaller positive potential $(+e)$.

Some further comments are required regarding details, which have been, or will be, found to be important for further experimental progress in this field.

First, anomalous osmosis is associated with the presence of appropriate electrolyte concentrations, as appears clearly from the well-known figures of Loeb,[290] illustrating his experiments with collodion membranes. This effect, approximately equal to $E \times \zeta$ can approach zero, as E with low concentrations, ζ with high, approximate zero. In many experiments, concentrations between m/16 and m/128 have been found to give gratifying results; beyond that range, only normal osmosis occurred.

Second, in securing clear results, very much depends upon suitable membranes. In the case of collodion membranes, enormous differences in electrical properties can be produced by "activation" through certain chemical manipulations.[291] Furthermore, the rigid collodion membranes can be better adapted to physiological purposes by impregnating or coating with colloidampholytes (gelatin, albumin, hemoglobin).[292] In this way they become sensitized to the influence of ions, particularly of ions which, like H, or like certain polyvalent anions, are products of metabolism. Thus, the ζ-potentials can be changed, and indirectly the E-potentials, the latter also eventually being decreased or annihilated at the isoelectric point of the colloidampholytes. For instance, the protamine salmine,[293] with an isoelectric point of *p*H 12.0 to 12.4, is useful for preparing very active electropositive collodion membranes, which maintain a definite positive charge at acid reaction, and even at the alkaline reaction *p*H 8. Colloid-

[290] J. Loeb, J. Gen. Physiol., **2**: 173, 387, 1920.

[291] K. Sollner, J. Abrams, and C. W. Carr, J. Gen. Physiol., **24**: 457, 1941; **25**: 7, 1941; **25**: 17, 1942; further, L. Michaelis and W. A. Perlzweig, *ibid.*, **10**: 575, 1927; W. Wilbrandt, *ibid.*, **18**: 933, 1935.

[292] K. Heesch, Pflüger's Arch. f. d. ges. Physiol., **190**: 198, 1921; J. Loeb, J. Gen. Physiol., **5**: 89, 109, 895, 1922; see, further, p. 318.

[293] J. Abrams and K. Sollner, J. Gen. Physiol., **26**: 369, 1943.

ampholytes, due to their property of shrinking and swelling, may also change the pore diameters, according to the ions present, and thus modify the anomalous osmosis.

Third, of greatest significance for the physiological problem in question, i.e., the transport of water, considered as a positive or a negative electroosmosis, is the time factor. It has been noted by Loeb and others that, with a series of different electrolytes, the optimum time for observing anomalous osmosis with ordinary collodion membranes such as he made use of, is about twenty minutes after the beginning of the experiment; later, the phenomenon becomes less and less conspicuous, due to diffusion of the electrolyte across the membrane. In addition, the electroosmotic current, if it is directed along the diffusion gradient, contributes to equalization of the concentrations on either side of the membrane, since a shift of ions is necessarily associated with the electroosmotic shift of water. As in the previously discussed case (p. 622), there arises again the crucial question of the accumulation of ions, i.e., whether such model experiments are of any value as long as it has not been satisfactorily determined how or whether the electroosmotic shift of water can be made continuous by a metabolic energy supply. This problem will be discussed after reference to one of the few quantitative experiments, which have so far been performed to test the theory.[294]

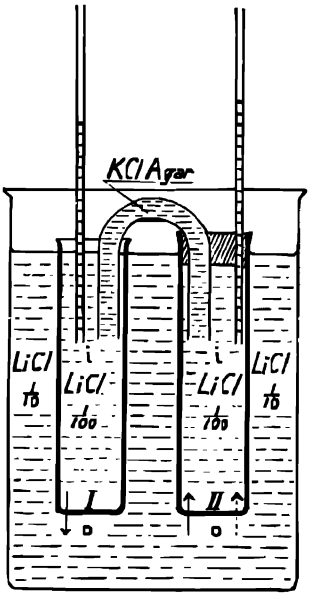

Fig. 68. Device for measuring anomalous osmosis.

The set-up was made as shown in Fig. 68. Stable precipitation membranes of magnesium silicate were formed in parchment test tubes, they were filled with 0.01 m. LiCl and fixed in a beaker containing 0.1 m. LiCl. The electrical charge of the membrane substance is negative. The width of the pores in membrane I is large, compared to those of membrane II. Then, the behavior corresponds to the scheme in Fig. 66. The *E*-potential of I is 16 mV; of II, near to zero. Therefore, a positive electric current circulates from inside II by way of the agar bridge to inside I, and back outside from I to II (solid arrows). Further, potential I being greater than II, an electroosmotic current of positively charged fluid is forced through II from outside to inside, indicating, by a rise of the meniscus in capillary II, a negative osmosis (dotted arrow). The rise in the experiment was not more than 4 mm. due to several unfavorable (though well controlled) conditions, chiefly to that, instead of one membrane, containing closely spaced pores of different widths and thus influencing each other over a very small distance, two membranes had to be employed, one providing the

[294] K. Sollner and A. Grollman, Ztschr. f. Elektrochemie, **38**: 274, 1932.

electromotive force, which drove the fluid by electroosmosis through the other distant one. But the position of the meniscus was found to be constant for more than one hour, and when it finally fell, it quickly regained its level after the beaker and the tubes had been refilled, i.e., after restitution of the concentration gradient, which otherwise vanished quickly at the wide membrane 1. That the maintenance of the position of the meniscus is due to the circulation of the electroosmotic current, was proved by the fact, that the meniscus fell immediately, if the current was interrupted by closing the agar bridge at its left end.

Returning now to physiological studies, it may be recalled that 70 years ago, Engelmann[295] suggested that fluid transfer in absorbing and in secreting organs is of electroosmotic origin. However, about 20 years later this hypothesis was rejected by Reid[296] on the basis of his own well-known experiments upon the shift of water across diaphragms of surviving frog skin and mammalian intestine (p. 552). His main objection was that purely secreting membranes often exhibit electric currents in the same direction as the absorbing membrane. But, in the meantime, much progress has been made, particularly concerning the electrophysiology of the excised frog skin, which has rendered promising an effort to correlate the electrical and the mechanical properties (water transport) of this material, which by its stability provides better conditions for such a task than any other material.

The resting potential of excised frog skin, mounted between two samples of Ringer solution and provided with oxygen, can be maintained fairly constant over several hours, the inside being electropositive, the outside negative. Hypoxia or anoxia decreases the potential, often reversibly. The same effect is brought about indirectly by adequate doses of KCN, Na_2S, or CO.[297] Therefore, the normal frog skin potential can be believed to be the result of the respiratory metabolism of the living organs. This conception has been substantiated by investigations of the influence of bromoacetate, lactate, and cyanide.[298] With bromoacetate (resembling iodoacetate) the normal carbohydrate breakdown is interrupted in such a way that the production of lactate stops. This does not affect the potential so long as the supply of lactate in the skin remains; however, when it is exhausted by tissue oxidation, the potential falls off, but can be restored by adding more lactate. KCN finally depresses the potential, irrespective of the presence of lactate, O_2, and bromoacetate. It may be concluded that oxidation of sugar is somehow connected with the electrical activity.

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[295] W. Engelmann, Pflüger's Arch. f. d. ges. Physiol., **5**: 97, 1872.

[296] E. W. Reid, British Med. J., **1**: 323, 1892; J. Physiol., **25**: 436, 1901; Philos. Tr. Roy. Soc., London, B. **192**: 211, 1900.

[297] E. J. Lund, J. Exper. Zool., **51**: 265, 291, 1928; W. L. Francis and R. J. Pumphrey, J. Exper. Biol., **10**: 379, 1933; W. L. Francis, *ibid.*, **11**: 35, 1934; A. B. Taylor, J. Cell. & Comp. Physiol., **7**: 1, 1935; E. Huf, Pflüger's Arch. f. d. ges. Physiol., **235**: 655, 1935.

[298] E. Huf, *loc. cit.*

Regarding the need of energy supply for keeping up the potential, it should be mentioned that various narcotics, known as depressants of metabolism, have been found to decrease reversibly the skin potential even to zero[299] (see chap. 23, subchap. 2).

But, not only the potential of the skin is dependent upon oxidation of sugar, but also, as followed from the corresponding effects of the aforementioned chemicals, its mechanical activity in pushing water from outside toward the body fluid, even against a hydrostatic pressure of 2 to 4 cm.[300] Simultaneously with the water, a shift of Cl occurs, which, as noted previously (p. 589) is influenced by the same compounds as the shift of water.

Now, in order to analyze this machinery, its components must first be located in the various strata of the skin. In general, it is believed that the layer of surface epithelium is the active part. In particular, the electrical activity has been assigned to this region. According to Amberson and co-workers,[301] the frog skin potential is the sum of three potentials. One located in the outside layer is the skin potential proper. It disappears, when the skin is killed by placing it for a short time in an acid solution (about *p*H 4). The other two are observed with the dead skin, when it is bathed in two salt solutions of different concentrations. One of these is a simple diffusion potential, the other is like the potential of a protein membrane, which above or below its isoelectric point (about *p*H 4.5) is more permeable to cations or to anions (p. 319). Other reasons for assuming that the layer of the living epithelium is the active part are based upon the often-noted observation that, in order to affect the potential with ions or narcotics, smaller doses or a shorter exposure are required to bring about the change from the outer surface than from the inner surface.

Thus, in summary, it appears, first, from the physiological experiments, that a correlation exists between the transport of water, the electrical activity and the metabolism; second, from the physicochemical studies, that a model membrane of a certain porous structure, bathed in two electrolyte solutions, has the power to produce electromotive forces, which can drive an electroosmotic current across the membrane as long as the two electrolyte solutions do not reach diffusion equilibrium. As a matter of fact, the last-described model experiment (p. 627) has shown that the electroosmotic transport can be maintained, if care is taken to re-establish the decreasing osmotic imbalance. Therefore, the essential physiological problem is how, in the surviving skin, the persistency of the osmotic imbalance is guaranteed. A preliminary solution may be sought in the following,

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[299] W. L. Francis and R. J. Pumphrey, *loc. cit.*; E. J. Boell and A. B. Taylor, J. Cell. & Comp. Physiol., **3**: 355, 1933; further, W. Reid, *loc. cit.*

[300] E. Huf, Pflüger's Arch. f. d. ges. Physiol., **235**: 655, 1935; **235**: 1, 1935; **238**: 97, 1936.

[301] W. R. Amberson and H. Klein, J. Gen. Physiol., **11**: 823, 1928; M. Sumwalt, W. R. Amberson, and E. M. Michaelis, J. Cell. & Comp. Physiol., **4**: 49, 1933; M. Sumwalt, Biol. Bull., **56**: 193, 1929.

which is similar to a previously proposed answer (p. 622). The skin epithelia are the site of oxidation of sugar, and, therefore, the site of a permanent production of H and HCO_3. A concentration gradient of these ions, directed from the interior of the cell to the exterior, can be visualized as permanent, if removal of the ions is insured by the tissue fluid in the deeper strata of the skin. The epithelial layer, then, may be pictured, e.g., by a scheme like that of Fig. 67, the wider pore possibly comparable to the plasma membranes of the epithelia, the narrower pore to the intercellular cementing substance, the right side of the scheme representing the deeper subepithelial layers of the skin, the left side the metabolizing stratum of the epithelial bodies. Then, the higher potential $+E$, due to $u_H > v_{HCO_3}$, would indicate the positive potential ordinarily led off from the inner surface of the skin, which causes the negative osmosis to go from the outer surface of the skin toward the inner surface. But, of course, this is not more than a rather arbitrary and fragmentary approach to an understanding, but, perhaps, comparable to the more specified approach of correlating chemical and mechanical work in muscle, which has been mentioned before (see p. 521).

5. Supplementary Remarks Concerning the Action of the Chorioidal Plexuses and the Ciliary Body.—Although a great amount of work has been done in order to clarify the forces involved in the production of the cerebrospinal fluid and the aqueous humor, no final standpoint has been attained. However, it appears highly probable that secretory action is responsible for the formation of these products by the chorioidal plexuses in the brain and the ciliary body in the eye. This assumption is supported by numerous, though still controversial, findings regarding the composition of the blood fluid, compared to its ultrafiltrates and to the fluids in question; the gradients of capillary, colloidosmotic, and Donnan pressures; the penetration of these organs by solutes, particularly by dyestuffs; the histology of their surface-epithelial cells, and the changes in their vacuoles and mitochondria which follow the injection of parasympathetic drugs; the respiratory metabolism and the effect of metabolic poisons.[302] The inconclusive results are due widely to the location, to the small size and irregular form and the complicated vascularization of these organs, and to their extreme sensitivity to insults, especially in the case of the eye. In the experiments, which will chiefly be referred to here as a supplement to our other discussions about active transfer, a simplification of the experimental conditions has been attempted by studying the freshly excised organs.[303] The ciliary body was removed from the enucleated eye of rabbits, guinea-pigs, and cats, the chorioidal plexuses from the brain of pigs. Both prepa-

[302] See the reviews of F. Plaut, Handb. norm. und pathol. Physiol., **10**: 1179, Springer, Berlin, 1927; M. Baurmann, *ibid.*, **12**: 1319, 1931; L. B. Flexner, Physiol. Rev., **14**: 161, 1934; J. Biol. Chem., **121**: 615, 1937.

[303] J. S. Friedenwald and R. S. Stiehler, Arch. Ophth., **20**: 761, 1938; R. D. Stiehler and L. B. Flexner, J. Biol. Chem., **126**: 603, 1938.

rations were laid flat on a glass plate and moistened with Ringer, and the distribution mainly of basic or acidic dyestuffs was studied under the microscope. Their solutions were provided to the stroma directly or indirectly by a preceding intravascular injection, and to the epithelium by placing the edge of a piece of tissue in such a way on the glass that the epithelial cells could be brought in immediate contact with the dye solution. With both preparations, the authors came to the conclusion that the basic dyestuffs move in the surviving organs from the stroma, across the basal membrane of the epithelium, into the epithelial cells, the acidic dyestuffs in the opposite direction, or, in other words, the colored cations pass in one, the colored anions in the other, direction. But, it must be said that the short description, omitting details, leaves some doubt as to an unquestionable demonstration of this shift.[304] In the first place, the satisfactory condition of the material seems open to question in view of the low viability of mammalian secretory epithelium. Moreover, it seems hardly possible not to encounter difficulties in the interpretation, arising from the strikingly different staining power of basic and acidic dyestuffs in general, which are the result of the greater adsorption of basic dyes to proteins, and the lipoid solubility of the basic contrasted with the minor importance of this property in the acidic dyes. In addition, the individual dyestuffs of each group differ with regard to the more diffuse or more differential distribution inside the cells, and they differ with regard to their capacity to permeate the living cells.

However, if the interpretation of the microscopic localization of the dyes as indicating an opposite movement of cations and anions be adopted, it appears a reasonable conclusion of the authors to postulate an electric force not only to distribute the dyestuff ions, but also to establish an electroosmotic stream of fluid into the ventricles of the brain and into the anterior hemisphere of the eye bulb, respectively.

In searching for the source of energy, the respiratory metabolism of the chorioidal plexus and that of the ciliary body were studied by estimating the free energy of oxidation, or, more specifically, by determining the oxidation-reduction potential with a series of redox-indicator dyes, which were introduced into the stroma and the epithelium of the tissues, and the bleaching of which was observed under the microscope. Under aerobic conditions, the redox potential in the epithelium of the ciliary body, for instance, was found to be $+0.1$ volt, in the stroma -0.13 volt (referred to E_o' at pH 7; chap. 4, see p. 74). After the preparation had been asphyxiated in nitrogen, the potential difference between epithelium and stroma disappeared; for instance, the potential of both epithelium and stroma decreased to -0.29 volt each (as indicated, e.g., by the beginning reduction and decoloration of injected safranin).

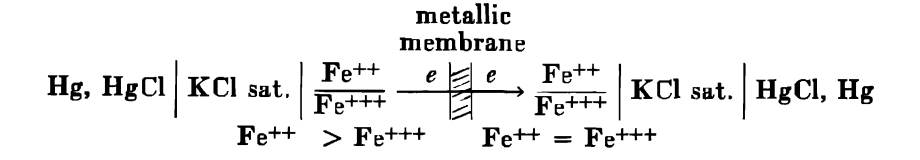
But, after, several years ago, on the basis of a correlation between

[304] See also the illustrations, Fig. 1, in the paper of Friedenwald and Stiehler, *loc. cit.*

the biopotentials of plant tissues and their respiration, E. J. Lund[305] had come to the conclusion that the currents drawn from the surface of plant roots are due to oxidation-reduction potentials, such interpretation often has been rejected[306] for the reason that an oxidation-reduction process is accomplished by a transfer of electrons, and electrons happen to be conducted only along a metallic pathway, but not along a nonmetallic pathway, like a physiological membrane, which, like water, can be a carrier only of ions (see Sec. 6, chap. 26). However, it also has been questioned, whether this objection is valid for all distances, since, obviously, it is not true within molecular distances; and, for this reason, more recent studies regarding the conductance of artificial precipitation membranes, which so frequently have served as models for many kinds of natural membranes, may demand special attention. In 1867, Becquerel discovered the following phenomenon. A test tube, which has received very fine cracks, is filled with a solution of $Cu(NO_3)_2$ and immersed in a solution of Na_2S. After some time, at the inner surface of the tube, there appear precipitations of metallic copper, and at the outside the liquid becomes dark-yellow from the formation of polysulfides. In other words, at the inside reduction, at the outside oxidation, takes place. According to Freundlich,[307] Ostwald has interpreted the phenomenon in the following way: the solutions are separated by a film of CuS, which is formed in the cracks and which is impermeable to Cu^{++} and S^{--}. At the outer surface S^{--} is oxidized: $2S^{--} = S^{-}—S^{-} + 2e$. The liberated electrons (e) pass the film and bring about the reduction:

$$Cu^{++} + 2e = Cu.$$

Electroneutrality is guaranteed by the film being permeable to NO_3^- and Na^+. So far, this hypothesis may be compared to the ordinary conception of the effect of an oxidation-reduction system, which provides an electric current of ions, when two solutions of different oxidation-reduction potentials are separated by a diaphragm (a "membrane") of a noble metal (Pt, Au) and by leading off in the usual way with saturated KCl (see p. 64).[308] For example:



It is obvious that in this scheme Fe^{++} and Fe^{+++} could be substituted by the reduced and the oxidized forms of a redox-indicator dye.

[305] E. J. Lund and W. A. Kenyon, J. Exper. Zool., **48**: 333, 1927; **51**: 265, 1928.

[306] See, e.g., the last articles in Cold Spring Harbor Symp., **1**, 1933.

[307] H. Freundlich, Kolloid-Ztschr., **18**: 11, 1916.

[308] See, e.g., I. M. Korr, Cold Spring Harbor Symp., **7**: 74, 1939.

But, these model experiments fail to be apt to picture the physiological circumstances for two main reasons. First, the CuS membrane of the Becquerel phenomenon, which is supposed to represent the delicate natural membranes, is a metallic or at least a half-metallic conductor, belonging to a larger group of metal sulfides and metal oxides, which likewise are suitable to substitute the CuS membrane in Becquerel's experiment.[309] But, so far, there is no reason to believe that natural membranes composed of organic material behave like metal conductors or can substitute metallic electrodes. Second, it often has been neglected in interpreting the Becquerel phenomenon, that, in order to reduce the Cu ions to metallic Cu and to oxidize S^{--} to S^{-}—S^{-}, energy has to be liberated. According to Freundlich and Sollner, this energy could be made available by a porous structure of the CuS membrane. It would mean that a circuit current is established in the membrane, one part of which, due to metallic (electronic) conduction of CuS, passes across its substance, the other part across the pores, which are accessible to the free ions Na and NO_3.

Therefore, returning to the studies of Friedenwald, Stiehler, and Flexner, it appears that the manner of interpreting their observations cannot be agreed upon. However, instead, another way opens, which is suggested by an additional set of experiments regarding the correlation between redox potentials and tissue activity. Applying the technique of redox-indicators, it has been shown that significant redox-potential differences (or the lack of them) become evident, wherever one compares the respiratory activity inside and outside single cells (amebæ, ova of echinoderms, plant cells, and others),[310] or at different parts of an active organ. The latter behavior is especially marked in studying the developmental onset of function. Thus, according to Flexner and Stiehler,[311] the fetal chorioidal plexus, in its presecretory phase, displays neither the aforementioned shift of dyestuff cations and anions, nor any difference in the intensity of the indophenol blue reaction between the stroma and the epithelium, which would indicate a different distribution of indophenol oxidase (cytochrome-cytochrome oxidase) as a sign of a gradient of respiratory activity (see Sec. 6). In the secretory stage, on the other hand, the oxidase is limited to the epithelium, and is present there in a greater concentration. In addition, in recent studies, Friedenwald, *et al.*,[312] succeeded in separating the two major histological structures of the chorioidal plexus from each other, and showed

[309] H. Freundlich and K. Sollner Ztschr. f. Physik. Chem., **A 138**: 349, 1928; **A 152**: 313, 1931; J. J. Bikerman, *ibid.*, **A 153**: 451, 1931.

[310] J. Needham and D. M. Needham, Proc. Roy. Soc. London, **B 99**: 173, 383, 1926; M. M. Brooks, U. S. Public Health Repts., **38**, 1923; Amer. J. Physiol., **76**: 360, 1926; B. Cohen, R. Chambers and P. Reznikoff, J. Gen. Physiol., **11**: 585, 1928; R. Chambers, Cold Spring Harbor Symp., **1**: 205, 1933; further, several articles in Cold Spring Harbor Symp., **7**: 1939.

[311] L. B. Flexner and R. D. Stiehler, J. Biol. Chem., **126**: 619, 1938; also J. S. Friedenwald and R. D. Stiehler, Arch. Ophth., **20**: 761, 1938.

[312] J. S. Friedenwald, H. Herrmann, and R. Buka, Bull. Johns Hopkins Hosp., **70**: 1, 1942.

that the cytochrome oxidase-[illegible] system (chap. 27) is located almost entirely in the epithelial cells. Thus, these biochemical findings can be correlated with the results of the redox-indicator method. Also it has been shown by Flexner[313] that, using the kidney of fetal pigs of different ages, at the presecretory stage, only a feeble and equally distributed content of cytochrome-cytochrome oxidase can be detected, whereas, in the secretory stage, marked differences appear between the physiologically active portions, the proximal tubules, parts of Henle's loops, the distal tubules on the one hand, the glomeruli and collecting ducts on the other. All these gradations in reactivity disappear during asphyxia, just as functioning is stopped in all living tissues requiring aerobic conditions. According to Friedenwald, *et al.*,[314] the flow of water, normally insuring the turgor of the eye bulb, also falls off during asphyxia, a phenomenon strengthening

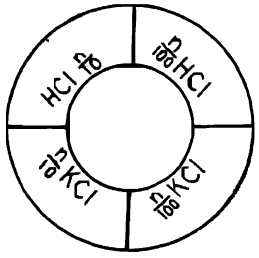


FIG. 69.

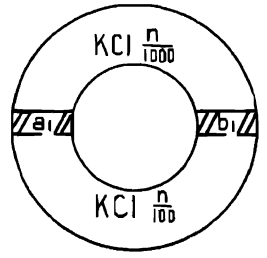


FIG. 70.

the assumption of the authors that the shift of water is due to electroosmosis, somehow connected with the oxido-reduction potential differences.

But all this suggests quite another solution of the main problem of this section. In paying attention to the fact that the energy liberated by metabolic reactions is utilized in different objects for various kinds of work, e.g., in the kidney for the secretion of certain solutes in one segment of the tubules, for the reabsorption in another, the conclusion will be drawn that the result of the energy output is determined by the specific construction pattern of the acting machinery. It seems clarifying that, referring to the previously described model experiments of Sollner (pp. 627ff), and, furthermore, referring to the present theory of the Becquerel phenomenon, which does not involve the assumption of an electron shift, the following picture may be offered. Dolezalek and Krüger[315] have demonstrated that, if a circular canal is filled with electrolyte solutions, as pictured in Fig. 69, i.e., with different sectors filled by solutions containing cations and anions of suitable concentrations and mobilities, and in direct contact with each other, a circular electric current flows, detectable by a magnet suspended within the ring formed by the canal; in other words, cations circulate in one, anions in the other, direction, as long as the concentration differences

[313] L. B. Flexner, J. Biol. Chem., **131**: 709, 1939.

[314] J. S. Friedenwald, W. Buschke, and H. O. Michel, Arch. Ophth., **29**: 535, 1943.

[315] F. Dolezalek and F. Krüger, Ztschr. f. Elektrochem., **12**: 669, 1906.

between the solutions persist. On the basis of this experiment, Sollner[315] has proposed a fluid circle as in Fig. 70; a_1 is a collodion membrane with narrow, b_1 with wide, pores; a_1, through the prevailing permeability to K, is the site of an electromotive force, which drives the fluid through b_1 the liquid junction potential of which is practically zero (p. 317). This may be comparable to the situation in a sieve membrane, which, due to the variety of pore diameters (pp. 233, 318), could be the site of local circular electric currents (pp. 621ff). If, now, a basic dyestuff is added to the solution inside the canal above b_1 ("stroma"), the dye cations will become visible below (in the "epithelium"), migrating with the electroosmotic current of water; if an acidic dyestuff is added below b_1, the dye anions will appear above (in the "stroma"). Dye ions, according to their size, may or may not be allowed to pass a_1. As suggested previously (p. 622), the activity of such a system could possibly be maintained by a supplementary provision of suitable ions below the sieve membrane. In living tissues, such conditions would be secured by the production of metabolic ions in the epithelium, e.g., H and HCO_3, arising from carbohydrate metabolism.

This interpretation, then, differs from that of Stiehler, Friedenwald, and Flexner in that, instead of viewing the oxidation-reduction systems as directly producing a current of electrons, which forces the water and the solutes across the blood-aqueous and blood-cerebrospinal barriers, it considers these systems to be the source of ions, which arise from metabolism, and which, due to the selective ionpermeabilities of the systems, originate an electric current.

[315] K. Sollner, Ztschr. f. Elektrochem., **36**: 36, 1930.

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