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# ADVANCES IN ENZYMOLOGY

## AND RELATED SUBJECTS

Volume I

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# ADVANCES IN ENZYMOLOGY

## AND RELATED SUBJECTS

*Edited by*

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Volume I

*With 56 illustrations*

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# Preface

This collection of independent monographs is initiated at a time when research is subject to the gravest of interruptions and original thinking liable to the greatest distraction.

ADVANCES IN ENZYMOLOGY may be of service to those investigators who are devoting their efforts to extending our knowledge in this field and related subjects; and to all who are interested in the realm of enzyme behavior.

Meantime, the Editors wish to render tribute, although somewhat belatedly, to the concept of SCHWANN of the order of the cell as a symbol of the harmony and organization of interfacial phenomena, and of organic and physical chemistry.

We express our obligation to the contributors for their unreserved cooperation and to the publishers for their support.

New York, N. Y.
Ames, Iowa,
February, 1941

F. F. N.
C. H. W.

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## Errata

On pp. 53 and 61 instead Smith and Rogers, J.Am.Chem.Soc., **52**, 1824 (1
read Smyth and Rogers, J.Am.Chem.Soc., **52**, 1824 (19
On pp. 100 and 157 instead transaminidation read transamidination
On page 157 instead aminidine read amidine
On page 355 instead 5.Mai read 5.Juli

# PROTEIN STRUCTURE

HENRY B. BULL

*Chicago, Illinois*

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The elucidation of protein structure involves the solution of two general problems. The first deals with the quantitative estimation of the amino acids, as well as the non-amino acid portions of proteins. The second has to do with the arrangement of the amino acids in relation to one another and the nature of the forces which maintain this arrangement. We shall present the arguments of this review in three sections. The first is of an introductory nature and contains observations on amino acids and protein prosthetic groups together with a general discussion of the evidence for the peptide linkage being the only important co-valent chemical bond connecting the amino acid residues together. The second part deals with the fibrous protein, while the third part attempts to summarize the knowledge concerning the structure of the globular proteins.

## I. Introduction

It hardly seems necessary to enter into the chemistry of the amino acids in any completeness or detail.

Vickery (1) has listed 25 amino acids as having undoubted occurrence in proteins. In addition to these there are 22 amino acids whose status is doubtful. In any case, however, there is a vast range of possible amino acids which can be used for protein synthesis by living tissue. For many of these amino acids there is no reliable method of estimation. Vickery

states that nine amino acids can be satisfactorily determined in a quantitative manner. These are cystine, tyrosine, tryptophane, methionine, aspartic acid, glutamic acid, arginine, histidine, and lysine. There are six amino acids for which more or less satisfactory methods are available, but which have not been extensively employed. There are eight amino acids for which we have no good method, and our knowledge concerning their amounts in proteins is little better than qualitative.

It can be appreciated from this discussion that protein analyses reported in the literature must be evaluated with a critical eye. There are only a few proteins for which more than 70 per cent of the protein has been accounted for, and of the fraction which has been analyzed perhaps not much over half is to be considered as reasonably accurate. Undoubtedly, accurate amino acid analyses could throw much light on protein structure. How much light the analyses now available throw is a matter of speculation.

In passing, a significant fact for protein structure should be pointed out. It has been found that the groups attached to the alpha carbon atom of all naturally occurring amino acids are arranged in the same stero-configuration as they are in *l*-lactic acid (2). There has been a recent controversy (3) on this point. Some workers believed that they were able to show that, in tumor tissue, glutamic acid occurs partly as the unnatural isomer (related to *d*-lactic acid). The paper by Behrens, *et al.* (4), seems to completely refute this claim. It is pointed out in this paper that *l*-glutamic acid is itself racemized in hot hydrochloric acid and, accordingly, the appearance of a small amount of the unnatural isomer is to be expected in the course of hydrolysis of any protein. They conclude that there is no more of the unnatural isomer of glutamic acid in the hydrolysis of tumor protein than can be accounted for on the basis of racemization of the *l*-form.

The question presents itself as to the type of linkage involved in binding the amino acids together in a protein. The early studies of Hofmeister (5) and of Fischer (6) centered attention on the peptide linkage

$$—CH_2—NH—CO—$$

The objection to this type of linkage as being the only important one connecting the amino acids arose from enzymatic studies. The proteolytic enzymes appeared to fall into two well-defined classes: the proteinases which split substances of high molecular weight and the polypeptidases which attack only degradation products of proteins. It is realized now, however, that this does not constitute a valid argument against the importance of the peptide linkage. For example, Bergmann and his associates

(7) have shown that the occasion for the different types of proteolytic enzymes arises, not from different types of linkages, but from the position of the peptide linkage. All proteolytic enzymes hydrolyze the peptide linkage, but it is attacked by a particular proteolytic enzyme only if it occurs in the proper environment of amino acid residues.

The evidence for the peptide linkage being the only important co-valent bond connecting amino acid residues in proteins has been summarized by Vickery and Osborne (8) along the following lines:

1. Native proteins contain little amino nitrogen, while the end-products of protein hydrolysis contain large amounts.
2. The biuret test is characteristically given by substances having the peptide linkage. Upon complete hydrolysis of the protein, the biuret test can no longer be obtained.
3. The peptide linkage is found in other naturally occurring substances, for example, glutathione and hippuric acid.
4. Synthetic polypeptides can be prepared which are hydrolyzable by the enzymes of the digestive tract.
5. Polypeptides have frequently been found in the products of incomplete protein hydrolysis.
6. Hydrolysis of proteins liberates amino acids and carboxyl groups in equivalent amounts.

It is generally accepted at the present time that the peptide linkage is the only important co-valent bond between amino acid residues in proteins. In this connection, the recent theory of Wrinch postulates the existence of rings in the globular proteins which are closed by a different type of co-valent bond. Astbury also postulates a co-valent bond in $\alpha$-keratin which is different from the peptide bond. These theories will be considered in the course of this review.

While, as we have seen above, a carboxyl group and the $\alpha$-amino group of each amino acid are involved in the peptide linkage and are thus not reactive as such in the native protein, there are various other groups in the amino acids which are active. These are the side chains of the amino acid residues (the so-called R-groups). The nature of these side chains of the amino acid residues has a profound influence on the chemical and physical properties of a protein. If the free groups of these residues are predominantly carboxyl groups, the protein will have an acid character, while if the amino groups predominate, the protein will have a basic nature. Many of the carboxyl groups have their acid character masked by the formation of an amide. Those carboxyl groups which have not formed an amide together with all amino groups can apparently be titrated by acids and bases.

They are, therefore, free and not involved in co-valent bonds. The nature of the side chains also determines to some extent the hydrophilic properties of a protein; many polar side chains tend to make the protein water-soluble. No doubt, the side chains also play an important role in determining the type of folding of the polypeptide chain or chains in the protein molecule.

While in actual amount amino acids constitute the major portion of any protein, not infrequently there is present a group of a non-amino acid nature. Such groups are called prosthetic groups. These groups are of a diverse kind. Some of them perform an obvious biological function. For example, the hemin of hemoglobin has to do with oxygen transport in blood, while those of the intracellular oxidation-reduction enzymes are the active part of the oxidation-reduction system. On the other hand, the role of other prosthetic groups remains obscure. It has been proposed that some prosthetic groups act as cementing substances holding the protein molecules together in the living cell (9). When the tissue protein is treated with salts, etc., incidental to the preparation of a "pure" protein, the tissue protein is simply being cleaved at the connecting prosthetic groups. An extreme view along somewhat the same line is that there is only one blood serum protein which is called "orosin" and, accordingly, the separation of the serum proteins into several albumins and globulin fractions is an artifact. The reviewer has considerable reservations on this point of view.

It is generally the case that a prosthetic group increases the stability of a protein. Thus, hemoglobin is a much more stable protein than is globin obtained from hemoglobin by the removal of the hemin.

Some prosthetic groups are bound very tightly to their protein. For example, the prosthetic group of egg albumin (10) which is composed of four molecules of mannose and two of glucosamine, together with an unidentified nitrogenous constituent, cannot be removed from the protein without hydrolyzing the protein. On the other hand, the prosthetic group of several of the oxidation-reduction enzymes can be removed with relatively gentle treatment.

The nature of the combination of proteins with phospholipids is an important problem which has not received the attention it deserves. It is, however, a messy one for a chemist to undertake. It is well recognized that an ether extraction of minced tissue or of blood proteins is not capable of removing all the phospholipids present. For example, Sorensen (11) found that only one-fifth of the phospholipids of blood serum could be removed by ether alone. In phospholipid analysis of tissue the ether extract is supplemented by the use of alcohol, but it has not as yet been demonstrated that even the addition of alcohol removes absolutely all of the phos-

pholipid. It is true, however, that by far the largest proportion of the phospholipid-protein association is of a very loose character and does not involve chemical bonding. It is the feeling of the reviewer that it is possible that some of the several protein fractions obtained from blood serum may be traceable to the presence or absence of different amounts of phospholipid bound to the serum proteins.

So far, none of the studies on the natural phospholipid-proteins have differentiated between the phospholipids which might be present. In this connection, Chargaff (12) found that only cephalin formed a water-insoluble complex between *p*H 1.9 and *p*H 11 with the basic protein, salmine. Lecithin and sphingomyelin formed no such complex. Cephalin also formed an isoluble complex at *p*H values 2, 3, and 4 with egg albumin. At *p*H values higher than 4 no complex formed. Lecithin and sphingomyelin did not show complex formation with egg albumin at any *p*H. The behavior of cephalin as contrasted with that of lecithin and sphingomyelin is probably due to the fact that both lecithin and sphingomyelin have isoelectric points around *p*H 7, while that of cephalin is very low and hence cephalin is negatively charged over a very wide range of *p*H and, accordingly, combines with a positively charged protein through a salt linkage.

For any one interested in doing work on the phospholipid-proteins, probably the most readily available protein showing this association is vitellin from egg yolk. There are apparently two well-defined egg yolk proteins: vitellin and livetin (13). The vitellin carries with it the phospholipid and behaves as a globulin. It slowly loses its phospholipid in contact with water, and thereby becomes more and more insoluble in dilute salt solutions.

In order to facilitate the discussion of protein structure, the author pro poses to divide proteins into fibrous and globular as Astbury has done. Under fibrous proteins are included such proteins as collagen, elastin, and the various forms of keratin. Under globular proteins are to be included proteins which do not form fibers. Actually, the distinction comes down to the degree of asymmetry. The fibrous proteins are very asymmetrical and frequently show elasticity, while the globular proteins show much less asymmetry. The distinction is, at best, a matter of a degree and, at worst, does not exist.

## II. Fiber Proteins

The animal body makes use of a number of proteins for structural purposes. These are all fibrous proteins and with the exception of muscle

myosin are extracellular. And, again, with the exception of muscle myosin, they are typically water-insoluble. The fiber proteins with the possible exception of the protamines are the simplest proteins which are known. They present, therefore, the best opportunity we have of learning something about protein structure.

In Table I is shown in round numbers the amino acid residues in 100 gm. of dried protein. The accuracy of these analyses is not to be taken too seriously; they are presented simply to show the approximate content of the major component amino acids. The analyses are in no case anywhere near complete.

The first 6 amino acids in Table I give rise to side chain residues which are hydrophobic in nature, *i. e.*, the paraffin chains predominate. The last 5

TABLE I

NUMBER OF AMINO ACID RESIDUES IN 100 GRAMS OF DRY PROTEIN

| Amino acid | Silk fibroin | Elastin | Collagen (gelatin) | Keratin (human hair) |
|---|---|---|---|---|
| Glycine | 0.54 | 0.39 | 0.35 | 0.06 |
| Alanine | 0.28 | ... | 0.10 | 0.02 |
| Valine | ... | 0.12 | ... | ... |
| Leucine fraction | 0.02 | 0.23 | 0.05 | 0.05 |
| Cystine | ... | ... | ... | 0.06 |
| Proline | ... | 0.13 | 0.15 | 0.03 |
| Hydroxyproline | .. | 0.02 | 0.11 | ... |
| Glutamic acid | ... | ... | 0.04 | .. |
| Aspartic acid | ... | ... | 0.03 | 0.05 |
| Arginine | ... | ... | 0.05 | 0.02 |
| Lysine | ... | ... | 0.04 | ... |

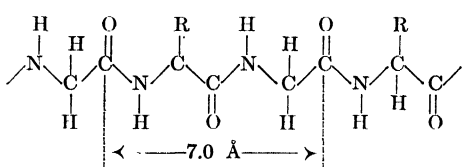
amino acids, on the other hand, have side chain residues which are hydrophilic, *i. e.*, have a polar group in them. It will be seen immediately that, for all the fiber proteins, the hydrophobic groups are more numerous than the hydrophilic ones. In fact, if we sum up the polar residues for a given fiber and divide this into the sum of the non-polar residues, we obtain a kind of a non-polar index of a protein. This has been done using as complete analyses as are available in the literature, and we find the following indices: elastin 29, silk fibroin 19, collagen 3.3, and keratin 2.8. The non-polar character of these fibers is at least part of the reason for the general insolubility in water.

The fact that keratin has the greatest number of polar groups is probably significant in view of the known tendency of keratin to form a well-

defined, folded molecular structure ($\alpha$-keratin). The presence of cystine (disulfide linkage) is also probably of significance in this connection.

Silk fibroin is the simplest of the fibers with an overwhelming predominance of glycine and alanine. Collagen is characterized by the large amounts of proline and hydroxyproline along with glycine. Elastin may, perhaps, be looked upon as a modified type of collagen. It is very much less polar and, accordingly, it might be anticipated that there would be less attraction between elastin molecules than is the case with collagen, with the consequence that the molecules would have a greater tendency toward random orientation. This is, in fact, substantially what x-ray studies indicate.

**Silk Fibroin.**—As we have seen, silk fibroin has the simplest chemical composition of the fiber proteins. It also has the simplest structure as revealed by x-ray diffraction studies. It was first shown by Meyer and Mark (14) that the x-ray diffraction pictures of silk fibroin could be interpreted in terms of a stretched polypeptide chain. Silk fibroin is characterized by a spacing along the fiber axis of 7 Å units. This spacing is accounted for on the basis of a unit consisting of glycine and some heavier amino acid residue; the distance between repeating units being 7 Å units (the heavier residue is, for the most part, alanine). The distance of one amino acid residue would, therefore, be 3.5 Å units.




Silk fibroin
(Meyer and Mark)

The distance of the peptide chains from one another in the direction perpendicular to the paper is 4.6 Å units (the so-called "back bone spacing"), and in the direction of the side chain residues of 5.2 Å units.

The diffraction pattern of silk has only a few spots and these spots are not sharp. The amount of crystalline material is not of a high order as compared, say, with cellulose. Mark estimates it to be from 40 to 60 per cent of the material present. The structural picture given above for silk fibroin seems to meet with everyone's satisfaction; it may indeed be regarded as established beyond a reasonable doubt.

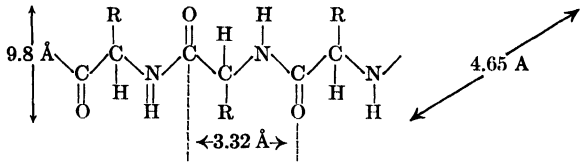
**Keratin.**—In the keratin group are included all types of hair fibers, the protein of bird feathers, nails, claws, and even porcupine quills. Hair (in-

cluding wools) is distinguished from the other types of keratins by its long-range elasticity. In fact, it may, in general, be stretched by about 300 per cent of its original length and will return to its initial length after the load has been removed. This stretching takes place much more easily in water and in other polar solvents than it does in air or liquids of low dielectric constant (this indicates the importance of electrostatic bonds in maintaining the unstretched keratin in its contracted form). If heat and moisture be applied for a sufficient time to the hair in its stretched condition, the fiber will set and will not return to the contracted state upon release of the force producing the stretching.

The changes associated with the stretching of hair have been investigated in some detail. Astbury has been most active in this field of investigation, and along with his collaborators has published a long series of papers dealing with this topic. Actually, the first paper (15) of this series is the most important one and contains most of the primary information. Astbury was the first to show that the stretching of hair is accompanied by changes in the x-ray diffraction pattern. The normal, unstretched hair is called $\alpha$-keratin and is characterized by a repeating distance of 5.15 Å units along the axis of the fiber. The back bone spacing is absent, and the side chain spacing is 9.8 Å units. The structure shows a fair degree of orientation (perhaps somewhat less than silk). As the fiber is stretched, the diffraction picture remains unchanged up to about 2 per cent extension, but beyond this extension the diffraction picture of $\beta$-keratin begins to appear. The $\beta$-diffraction becomes prominent at about 30 per cent extension, and at 60 per cent extension the diffraction picture is entirely of the $\beta$-type. The $\beta$-keratin is characterized by a repeating distance along the fiber of 3.32 Å units, a back bone spacing of 4.65 Å units, and a side chain spacing of 9.8 Å units. Astbury feels that the stretching of hair cannot be entirely explained on the basis of a transformation of a disorganized, randomly arranged structure to that of an organized, oriented one. Indeed, the repeating space of 5.15 Å units along the unstretched fiber forces the conclusion that there must be an intramolecular folding of a very definite kind. So much seems to be reasonably well established.

Astbury and his collaborators have published structures for $\alpha$-keratin and for $\beta$-keratin which they feel account for the observed x-ray diffraction pictures. The structure for $\beta$-keratin is that of a fully stretched peptide chain, the repeating distance of 3.32 Å units being the length of one amino acid residue in direction of the chain.

**The R-groups are the side chains of the amino acid residues and their nature depends upon the type of amino acid involved. Note that the R-groups are shown to alternate**

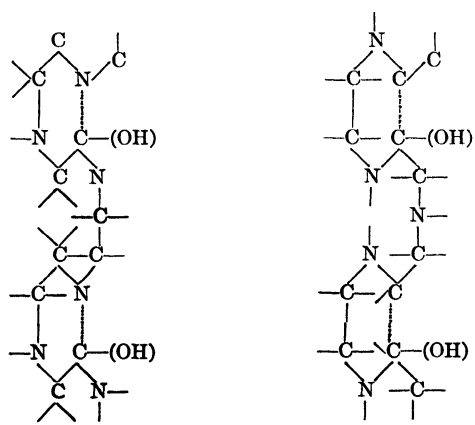



β-Keratin (Astbury)

up and down along the chain; this is a necessary condition in any fully stretched peptide chain and follows from the fact that the amino acids are structurally related to *l*-lactic acid. The distance along the chain of 3.32 Å units per residue is less than that for silk fibroin (3.5 Å units). This discrepancy has not been satisfactorily explained. Corey (91) reports that the distance along a fully stretched chain corresponding to one amino acid residue is 3.67 Å which is greater than that reported for silk fibroin and still greater than that found for β-keratin. Corey states that this fact suggests that in these substances the chain is never fully extended in a truly co-planar configuration but that interactions, steric and otherwise, with its immediate neighbors, cause slight distortions, probably involving rotation about the C—C bond.

The structure proposed by Astbury for α-keratin involves the folding of two amino acids into a six-membered ring. Two types of co-valent linkages were proposed to effect closure of the rings (16). The first type is a lactam-lactim transformation, and the second involves a keto-enol change.

These structures have been criticized by Neurath (17) who has shown rather definitely that they are much too condensed to permit residues other than those of glycine and possibly alanine to occupy the positions called




α-Keratin (Astbury)

for by the carbon atoms of the hexagonal rings to which they are attached.

The source of the embarrassment lies in the valence angle and bond distance requirements. As soon as any peptide chain is folded, all atoms attached directly to atoms in the peptide chain lose their freedom and the R-groups, instead of alternating above and below the chain as they must do in a fully stretched peptide chain ($\beta$-keratin), must now all be on the top or bottom of the chains where there is insufficient room to accommodate them.

Huggins (18) has proposed a spiral form of the peptide chain to account for the $\alpha$-keratin structure. It is claimed that this structure is in accord with all space requirements. The peptide chain is held in its spiral form by means of hydrogen bridges between the oxygen and nitrogen atoms (the hydrogen resonates between the oxygen and nitrogen). This structure has not been published and, accordingly, it seems inappropriate to discuss it at greater length at the present time.

It appears to the reviewer that any structure for $\alpha$-keratin must recognize two important factors. A definite role must be assigned to the side chains in whatever type of bonding assumed. Why, for example, does silk not show a contracted form analogous to that of $\alpha$-keratin? This can only be understood when it is remembered that the side chains of silk are typically non-polar, and hence, non-reactive. And, secondly, the effect of neighboring chains must be considered. We, in fact, never deal with the contractions of a single molecular chain, but only with bundles of chains which must be rather closely knit together. A single molecular chain would, if it contained sufficient number of polar groups, probably contract to such an extent that it would approach a spherical form.

In addition to the unstretched and stretched forms of hair keratin, there is still another form of keratin—the supercontracted form. If $\alpha$-keratin is stretched and then treated with heat and moisture for a short time (not long enough to obtain a set in the $\beta$-form) and allowed to contract, it will contract to a shorter length than that of the original unstretched fiber. This is what Astbury calls the supercontracted keratin. The supercontracted keratin shows long range elasticity. The x-ray shows it to be amorphous with little or no orientation. Astbury (19) has proposed a definite molecular folding of a very condensed type. Neurath (17) has shown that such a structure is incompatible with the space requirements of the various groups which are present. The reviewer feels that supercontracted keratin is a case of randomly folded, randomly arranged peptide chains, and that the stretching of supercontracted keratin is closely analogous to the stretching of rubber.

The influence of various groups in keratin on its elastic properties is not, as yet, very well understood, although it is known that deamination of wool

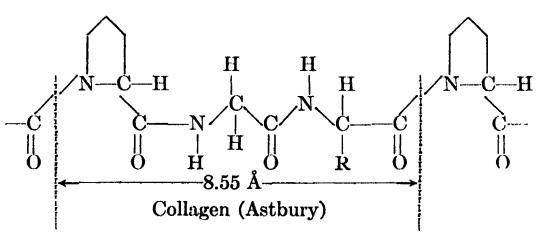
(20) leaves the x-ray diffraction pictures of both $\alpha$- and $\beta$-keratin unchanged which means that the essential framework of the keratin complex remains intact. Deaminated wool will, however, not set in the stretched, $\beta$-keratin form. The amino groups are, therefore, involved in the setting of the $\beta$-keratin structure.

There has been some speculation (21) about the role of the disulfide linkages in keratin (there is little or no sulfhydryl (SH) present), but the situation remains ambiguous. It is the understanding of the reviewer that Dr. Milton Harris and his collaborators will shortly have some interesting observations to report along this line.

**Collagen Group.**—Astbury (22) includes in the collagen family the white fibers of connective tissue (ordinary collagen of the leather chemists) tendons, cartilage, the scales and fins (elastoidin) of fishes, the ichthyocol of swim-bladders, the byssus threads of the bivalves such as *Pinna nobilis*, the so-called ova-keratin of egg capsules of the skate, filaments ejected by the sea cucumber, jelly fish, etc. Gelatin, the water-soluble derivative of collagen, is also included. The protein of the red blood cell membrane may also belong to the collagen group.

The essential feature of the collagens is their inelasticity at body temperature, and an x-ray diffraction pattern showing spacings along the fiber of 2.86 Å units (Wyckoff and Corey (23)) give 2.91 Å units as the repeating spacing) as contrasted with the repeating distance of 3.32 Å units of $\beta$-keratin. We have already noted that the collagens are characterized by a high content of proline and hydroxyproline.

Astbury has recently proposed a structure for collagen which is given below.




Collagen (Astbury)

The rings are either proline or hydroxyproline and constitute one-third of the total number of residues present. Another third of the residues is glycine. Three residues in this structure will occupy 8.55 Å units along the chain which gives an average value per residue of 2.85 Å units. The reviewer is not clear in his mind as to how the average spacing of a residue could produce a diffraction spacing. It would seem to him that, in analogy

with silk fibroin where two amino acids gave rise to a spacing, the spacing in collagen should be 8.55 Å units instead of 2.86 Å as observed. There are strong spacings along the fiber other than the 2.86 Å unit spacing, but none equal to 8.55 Å units. Some of the Wyckoff and Corey (23) spacings along the fiber are 2.91 Å, 4.03 Å, 7.21 Å, 21.6 Å, etc. The longest spacing they report is 103 Å, while Clark and co-workers (24) have reported a spacing as high as 432 Å.

The above structure for collagen helps to explain the inelasticity of this fiber; it is not easy for this structure to fold, because any folding would result in the side chains swinging over to the side of the back bone where the proline rings lie. The chains, therefore, lie in straight lines, and as such are not capable at ordinary temperatures of becoming elastic.

If collagen be heated to a sufficiently high temperature (about 60° C.), it spontaneously contracts to as little as a quarter of its original length. The contracted fiber, when hot, shows long range elasticity. If it is immersed in cold water after contraction, it spontaneously becomes longer again, but it never recovers completely its initial, uncontracted length, and some of the long range elasticity remains.

Astbury reports that the x-ray photograph of thermally contracted collagen is not simply that of a disoriented crystalline collagen; it is a new photograph altogether—an amorphous pattern. For example, if ordinary collagen be made into a pellet so that the fiber has random orientation in the x-ray beam, it is found that the photograph from this material is much different from that of the contracted collagen, although both show disoriented patterns.

Astbury believes that intramolecular folding has taken place in the contracted collagen. He states that disorientation alone of initially parallel chain bundles cannot account for more than 50 per cent of the observed contraction. It is, however, questionable if any definite and single type of molecular folding is involved. The situation appears to be analogous to that of supercontracted keratin which we have already considered.

**Elastin.**—Elastin, in contrast to collagen, shows highly elastic behavior at room temperature; biologically, this is as it should be. The diffraction picture of unstretched elastin (*ligamentum nuchae*) is completely amorphous; stretching to 200 per cent extension does not change the type of x-ray photograph (no new spacings appear), but does result in some orientation. Astbury suggests that it is possible that elastin may be a type of collagen which contracts (becomes elastic) at a lower temperature. The chemical analysis gives some color to this suggestion (high proline content), but

ievertheless there remains a considerable difference between the two types f fibers.

The reviewer has found that the walls of the great aorta of a normal human yielded n x-ray diffraction pattern* closely resembling, if not identical with, that obtained by stbury for the *ligamentum nuchae.* Incidentally, the walls of a highly sclerotic aorta aken from an individual who had died of syphilitic complications gave a diffraction attern which differed from that of the normal in several important respects; the athology of the aorta had induced drastic changes in the fiber protein.

**Muscle Fiber.**—Attempts have been made to study muscles and muscle ontraction by means of x-ray diffraction. Myosin, the principal muscle rotein, has many of the characteristics of a fiber protein, and Weber (25) as shown that the diffraction pictures of muscle are due to the myosin ontained in the muscle. Astbury and Dickinson (26) reported that air-ried myosin fibers could be made to show the $\alpha$-$\beta$ transformation and also upercontraction in analogy to keratin. Then later Astbury and Dickinson 27) reported that the $\alpha$-$\beta$ transformation could be brought about by stretch-ng muscle itself. No details were given. Still later Astbury (28) states:

"The changes that take place in x-ray photographs of living muscle on isotonic or ometric contraction are essentially similar to the changes observed in the x-ray photo-raphs of keratin in corresponding states. In particular, isotonic contraction, like upercontraction of keratin, results in a disorientation of the grids that is dispropor-onately small in relation to the contraction observed. In other words, the small dis-rientation is a secondary effect, the primary cause of contraction being a further olding of the main chains of the grids."

o far as the reviewer is aware, Astbury has published no experimental re-ults on living muscle and, accordingly, it is not possible at the present time o evaluate the role of intramolecular folding in muscle contraction.

## III. Globular Proteins

As indicated previously, the term "globular" as applied to such proteins s those of blood serum, egg albumin, and numerous other water-soluble roteins is not free from objection. Many of these proteins are undoubt-dly asymmetrical. We shall retain this term, however, since it does con-ey the idea that this class of proteins shows less asymmetry than do fiber roteins.

The structure of globular proteins is in a much more uncertain state than that of the fiber proteins; they are much more complicated. Nothing

* This work done through the courtesy of Dr. Jack Wilson of the General Electric -ray Corporation.

is really known as to the arrangement or type of folding of the polypeptide chain or chains in the molecule. We know that within experimental error all of the basic and acidic groups arising from the side chains of the amino acid residues are available for acid-base titration, so that these groups are not involved in any strong co-valent bonds, although they can and, in all probability do, lend stability to the molecule by virtue of their electrostatic charges. Whatever the arrangement of the peptide chain, we have reason to believe that it is a highly specific one and is, in general, unstable; most native proteins in solution, even at room temperatures, are slowly or rapidly undergoing spontaneous denaturation.

It is proposed to discuss the structure of globular proteins under the following four headings: (1) Size (molecular weights); (2) Shape; (3) Possible type of folding and intramolecular arrangement of the peptide chains; (4) Evidence from protein denaturation.

**Size.**—There has been, and continues to be, a discussion of whether or not one should speak of molecular weights or particle weights in reference to proteins. The reviewer takes the position that the use of the term "molecular weight" in this connection is legitimate.

Without a doubt, the most important tool ever devised for the physical study of proteins has been the ultracentrifuge. This work was pioneered by Svedberg and his co-workers and has since been used by several laboratories in this country and abroad. It would take us too far afield to deal with the technique of the ultracentrifuge, and the reader is referred to the recent book by Svedberg and Pedersen (29). The general theory of the ultracentrifuge seems to be sound enough, and the ambiguities which exist would be expected to give rise to second order errors. The ultracentrifuge gives an anhydrous molecular weight. The reviewer is not yet completely satisfied with the treatment accorded hydration, and it is very possible that hydration does cause small errors. The difficulty, as the reviewer sees it, is involved in the uncertain density of the water of hydration; there has been a volume contraction of the water with the result that the density of such water is greater than that in bulk.

It is almost impossible, by the nature of things, to estimate the actual experimental errors involved in ultracentrifugation determinations. Certainly, the early measurements of the molecular weights of proteins were badly in error. For example, the first determination of the molecular weight of egg albumin was given as 34,500 $\pm$ 1000 (30), while the more recent value is 40,500 by the equilibrium ultracentrifugation method and 44,000 by the rate ultracentrifugation method (29). This represents an extreme difference of over 25 per cent between the early and later (rate

sedimentation) determinations. There is still almost 9 per cent difference between the present values of the molecular weight of this protein by the two ultracentrifugation methods. Theoretically, the equilibrium method is on somewhat sounder basis (it is, in part, equivalent to an osmotic pressure method), but the long time required for a determination leaves the equilibrium method open to serious objection. All things considered, the rate sedimentation method seems to be the more reliable. The molecular weight obtained by this method for egg albumin (44,000) and that by the latest osmotic pressure measurements (31) (45,160) are in fairly good agreement and give, if the osmotic pressure method be accepted as the standard, an idea of the errors involved in the more recent ultracentrifuge measurements of the molecular weights of proteins.

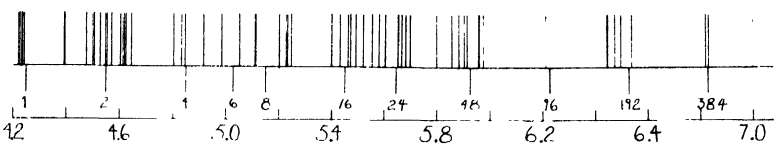



Fig. 1.—A logarithmic "spectrum" of the molecular weights of proteins. The lines of numbers beginning with 1 and ending with 384 are the supposed number of the 17,600 units in the proteins, *i. e.*, the molecular weight classes. The lower line of numbers is an arithmetic scale of logarithms, while the top line indicates the position of the logarithm of the molecular weights taken from "*The Ultracentrifuge*" by T. Svedberg and I. O. Pedersen, Oxford, 1940.

There is one phase of the molecular weight problem which must be critically dealt with. Svedberg and his co-workers (29) have repeatedly claimed that the molecular weights of proteins fall into certain classes. For example, 17,600 is believed to be the unit molecular weight, and the molecular weights of all proteins are supposed to be whole number multiples of this unit. The first class is $1 \times 17{,}600$ or 17,600, the second class is $2 \times 17{,}600$ or 35,200, the third class is $4 \times 17{,}600$ or 70,400 (no proteins are listed which contain 3 units), the fourth class is $6 \times 17{,}600$ or 105,000, etc. The most massive protein according to Svedberg contains 384 of the 17,600 units. The idea of molecular weight classes seems to have been more or less generally accepted and has inspired more than one theory of protein structure. It is exceedingly important to decide whether or not the classification of proteins on this basis is a valid one.

Clearly, if the division of the proteins into molecular weights such as outlined above is in accord with reality, there must be some guiding prin-

ciple back of protein synthesis which determines the molecular size irrespective of the type of animal or plant tissue in which they originated. In short, it throws the burden on the chemical nature of the amino acids. On the face of it, it would seem most unlikely that such a molecular weight classification could be true. For example, what guiding principle could exist which would make zein from corn (molecular weight 40,000) and egg albumin from a hen (molecular weight, 45,160), differing as they do in amino acid content, belong to the same molecular weight class?

The author regards the classification of proteins on a molecular weight basis with extreme reservation. In Fig. 1 is shown a logarithmic "spectrum" of the molecular weights of proteins. The molecular weights have been taken from "the ultracentrifuge" by Svedberg and Pedersen (29). The rate sedimentation values have been used where available.

As far as can be told by inspection of the figure there are no molecular weight classes. There is an apparent tendency for certain molecular weights to cluster around 17,000, but no one knows how many "proteins" have smaller molecular weights than 17,000; the clustering may simply indicate that there are a large number of small molecular weight proteins in nature. The reviewer is not impressed by the finding that the higher molecular weights seem to be approximate multiples of 17,600; all numbers, if they are large enough, are approximate multiples of 17,600. It would be most interesting to analyze the molecular weight distribution statistically and see if the distribution departs significantly from a random one. Until this has been done and a significant difference found, the reviewer regards the classification of proteins on a molecular weight basis as unworthy of serious consideration; the burden of proof is on the people who say that such a classification is possible.

The intriguing question arises as to why proteins have definite molecular weights. Why not a distribution of sizes such as one finds in the case of a gold sol? To the reviewer, the answer to this problem is to be found more in physiology than in chemistry. The synthesis of proteins is brought about by enzymes in tissues under highly specific conditions. The recent work of Tiselius and Eriksson-Quensel (32) on the hydrolyzing action of pepsin on egg albumin appeals to the reviewer as being most suggestive. These workers found that the all-or-none principle was involved; the protein molecules were either not attacked or else were broken into fragments of molecular weights of about 1000. It is not inconceivable that in tissue where uniform conditions prevail certain enzymes could synthesize such units, the size and nature of which would be severely controlled. These

units could then be combined by another enzyme system to form a protein which contained a definite number of units.

Bergmann and Niemann (33) bring up the point that if the synthesizing enzymes are proteins (which they probably are), there must be an enzyme capable of synthesizing the synthesizing enzyme or else they must be capable of synthesizing themselves.

Block (34) believes that the basic amino acids, arginine, lysine, and histidine are the controlling amino acids as far as protein synthesis is concerned, and that the ratios of these basic amino acids to each other determine the type of protein which results. Block cites as evidence the similarity of proteins with similar basic amino acid ratios. The basic amino acid complex is called an "Anlage." Bergmann and Niemann (35) find two objections to Block's hypothesis. They point out that it is strange that if the "Anlage" has so decided an effect on the structure of a protein, it is difficult to understand why wool keratin and silk fibroin, which supposedly have the same "Anlage," have, in fact, so different a general structure. Second, the variations noted in the experimental ratios are greater than would normally be ascribed to an experimental error; in general, the constancy of the ratios that would be expected if the "Anlage" theory were correct is apparently not observed.

We wish to consider briefly the association-dissociation of proteins. For many years it has been realized that the serum proteins are capable of considerable interaction (36). This early work led Sorensen to his investigations on this subject and finally to his concept of proteins as reversibly dissociable component systems. Sorensen (37) summarizes his studies in the following manner:

"Our investigations cover only soluble proteins, *i. e.*, proteins which may be dissolved in water or alcohol with or without the presence of salts, at neutral, acid, or alkaline reaction, without suffering irreversible decompositions. These substances are represented by the ordinary formula $A_x B_y C_z \ldots$, where $A$, $B$, and $C$, etc., indicate entire complexes, namely polypeptides, whereas the subjoined indices $x$, $y$, $z$, etc., indicate the numbers of the said complexes contained in the whole component system. Within each complex all the atoms or atom groups are interlinked by main valences, whereas the various complexes or components are reversibly interlinked by means of residual valences."

Sorensen was led to his conclusions through solubility studies. He found, for example, that by salt fractionation it was possible to obtain from many times recrystallized serum albumin various crystalline serum albumins of differing physical and chemical compositions which, when combined in the proper proportions, again yielded a crystalline protein having the prop-

erties and composition of the original. It is a question, however, whether these experiments mean what Sorensen thought they meant. In the first place, as far as the experiments with serum albumin are concerned, Hewitt's (38) very careful work has shown that serum albumin can be separated into two definite, well-characterized fractions. One of these fractions is a carbohydrate-rich protein with high solubility which does not crystallize, and the other is a protein with lower solubility and no carbohydrate which can be crystallized easily. Thus, there is no need to regard serum albumin as a co-precipitation system which results from the labile dissociation and rearrangement of the components of a single protein complex. Furthermore, Steinhardt (39) has pointed out that the solubility of proteins can be greatly influenced by a relatively small percentage of a low molecular weight impurity. The extraction of several protein "fractions" from what appears to be a pure, crystalline protein is not to be wondered at and is not to be regarded as a fractionation of various molecular species of the protein.

Quite apart, however, from uncertain indications from solubility experiments, there is a definite evidence from ultracentrifugation studies that proteins can, and do, undergo association and dissociation and with a fair degree of ease. Many proteins have *p*H stability regions outside of which the protein decomposes. In the initial phase, at any rate, this is a definite and stoichiometric affair. In general, the protein splits, if it does split, into halves and then into quarters and then into eighths, etc. Sometimes these dissociations are reversible. Other agents besides protons and the hydroxyl ion will bring about dissociation such as salts, urea, etc. Proteins themselves influence the state of division of other proteins in solution. Dilution also has its influence on the state of aggregation. The book (29) by Svedberg and Pedersen should be consulted for a fuller discussion. It is clear that in some proteins the bonds joining two halves or fourths or eighths, etc., of the molecule together are very weak and easily broken.

There are a number of physical measurements in addition to the ultracentrifuge which can yield protein molecular weights. Among these is osmotic pressure. The reviewer has found that it is possible to make osmotic pressure measurements of protein solutions which are accurate (small experimental variations). If the protein can be isolated in a pure state, it is probably the most unambiguous method for determining the molecular weights of proteins. Not all the osmotic pressure measurements reported in the literature have been done with sufficient care. The results reported by Adair and collaborators are to be recommended. The osmotic pressure

method has been generally neglected, but in the future, with the availability of purer proteins, it will, no doubt, find the extensive usage it deserves.

The size of the protein molecule can also be determined by means of x-ray diffraction studies. The dimensions of the unit cell of the protein crystal can be measured with great accuracy, and if the number of molecules in the unit cell are known along with the density, the hydrated molecular weight can be directly calculated. (It is usually more convenient to make measurements on wet crystals than on dry ones.) The amount of water in the crystal can be determined by drying, and the anhydrous molecular weight estimated. Actually, the order of accuracy is apt to be low; the uncertainty arises from the density measurements. It might be thought that this method would give information concerning the very important question of protein hydration and, as a matter of fact, Sponsler (40) has used x-ray diffraction studies in a very able and convincing manner to estimate the hydration of gelatin (35 per cent water of hydration), but with crystals of globular proteins there is considerable water of crystallization which bears no relation to the hydration of dissolved proteins. The molecular weights of these proteins which have been determined by x-ray diffraction studies (41) (insulin, chymotrypsin, pepsin, hemoglobin, and edestin) are in general agreement with the Svedberg values.

Diffusion measurements alone are not sufficient for molecular weight determinations, as they are complicated by hydration and molecular asymmetry.

On various occasions chemical analyses have been used to estimate protein molecular weights. Quite early, for example, the iron content of hemoglobin molecule was rather accurately determined (42). On the assumption that one hemoglobin molecule contained one iron atom, it was possible to calculate by simple proportion the molecular weight of hemoglobin. It was found that the minimal molecular weight of hemoglobin was about 16,000. It has since been shown that each hemoglobin molecule has four iron atoms and, accordingly, the true molecular weight is four times the minimal molecular weight. By the same token an accurate analysis of any one amino acid would yield a minimal molecular weight. The objection to this type of calculation is clear to be seen; there is no way of knowing by what factor the minimal molecular weight must be multiplied in order to give the true molecular weight.

More recently, Bergmann and his collaborators (43) have attacked this problem with considerable ingenuity and have devised a scheme by which they calculate the total number of amino acid residues in a protein molecule. When the total number of residues is multipled by the average

residue weight, the molecular weight is obtained. The Bergmann theory goes considerably deeper than a mere calculation of the molecular weights of proteins; it deals also with the arrangement of the amino acid residues in relation to one another. It seems appropriate, however, to consider the theory at this point.

The principle of Bergmann's calculation depends upon the assumption of a fixed and invariant periodicity of occurrence of amino acid residues in the peptide chain. It seems unnecessary to give details of their calculations; they can be found in easily available journals. For those proteins investigated, they find the total number of amino acid residues to be 288 or a whole number multiple thereof. Bergmann generalizes this finding and concludes that all proteins have 288 residues or a whole number multiple thereof. He further believes that the molecular weight classes reported by Svedberg are a reflection of the 288 unit. That is, the 35,200 class has 288 residues, while the 70,400 class has 576 residues, etc. The variation of the molecular weight within any given class is caused by variation of the average residue weight.

While the number 288 is not a necessity for Bergmann's theory, it is a great convenience. The individual frequency as well as the individual number of residues of any given amino acid must be exactly divisible into the total number of residues, and it so happens that 288 has the largest number of exact divisors of any number from 0 to 576. By assuming a total number of residues of 288 rather than some other number he has to do less violence to the analytical figures in order to make them fit his theory.

The reviewer has the following comments to make concerning Bergmann's theory:

1. As pointed out previously, Bergmann's theory requires a regular and invariant periodicity of occurrence of the amino acids in a single peptide chain (there can be more than one peptide chain in the molecule providing they are identical). While there is some evidence from x-ray studies which indicates a definite periodicity of amino acid residues in fiber proteins, for globular proteins there is no evidence either direct or indirect for such periodicity.

2. The calculation of the average residue weight is attended with considerable uncertainty. The method used obtains the average residue weight of the amino acids for which analyses are available; there is no way of knowing the average residue weight of the amino acids which have not been determined. The uncertainty over the average residue weight is important in two ways: First, if the average residues weight is in error, the calculated frequencies of the individual residues will be incorrect, which

means in turn that the total number of residues will also be in error. Second, the error in the average residue weight will be compounded in the molecular weight because the molecular weight is obtained by multiplying the total number of residues by the average residue weight.

3. It is very doubtful if the present analytical results for proteins are sufficiently accurate to be used in the fashion in which Bergmann uses them. This point has been clearly brought out by Neuberger (44). Incidentally, the recent analysis of gelatin by Bergmann and Stein (45) for proline and glycine leads to a frequency along the peptide chain of 3 for glycine and 7 for proline. Evidently there would be a conflict between these two acids every 21st position along the chain if the two acids are to have invariant periodicities in the chain.

4. If one uses the molecular weights of proteins as obtained from physical measurements and estimates the average residue weight from analytical results, the total number of residues can be directly calculated. Such calculations can be made for only a comparatively few proteins, because the appropriate data are not available for more. The results from such calculations are shown in Table II.

TABLE II

| Protein | Molecular weight | Average residue weight | Total calculated | No. of residues demanded by Bergmann |
|---|---|---|---|---|
| Lactalbumin | 17,400 | 120 | 145 | 144 |
| Egg albumin | 43,960* | 124 | 352 | 288 |
| Insulin | 41,000 | 124 | 330 | 288 |
| Horse hemoglobin | 65,000† | 119 | 552 | 576 |
| Edestin | 310,000 | 119 | 2610 | 2304 |

* The carbohydrate prosthetic group has been subtracted.

† The hemin has been subtracted. Cattle globin cannot be given because the molecular weight of cattle hemoglobin has not been determined. This is to be regretted because it will be recalled that cattle globin was one of the proteins which seemed best in accord with Bergmann's treatment.

Recently, Hotchkiss (92) has developed a method for estimating the average residue weight of the amino acids by titrating the acid and basic groups before and after hydrolysis of a given weight of protein. He applied this technique to lactoglobulin and Linderstrøm-Lang (93) has since applied it to insulin. These workers report an average residue weight of 116.7 for lactoglobulin and 123.2 for insulin. Using the rate sedimentation molecular weight as reported by Svedberg for these proteins, it is found that the lactoglobulin molecule contains 349 and insulin molecule 332 amino acid residues.

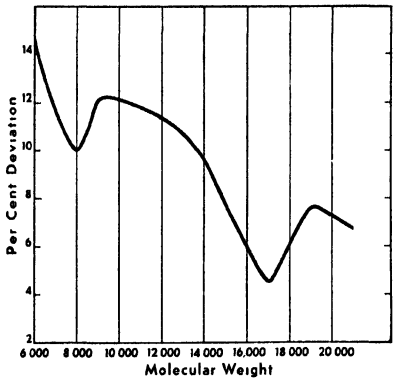



Fig. 2.—The average per cent deviations from whole number residues for lactalbumin plotted against assumed molecular weights of this protein. Calculations made at thousand molecular weight intervals. Data taken from chapter by H. O. Calvery in *"Chemistry of the Amino Acids and Proteins"* by C. L. A. Schmidt, Springfield, Ill., 1938.

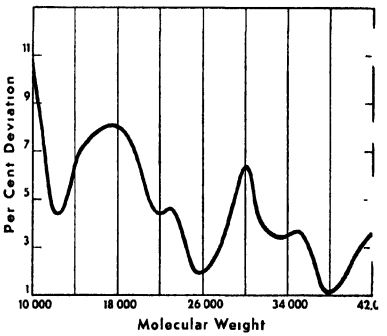



Fig. 3.—The average per cent deviations fro whole number residues for insulin plotte against assumed molecular weights of this pr tein. Calculations made at thousand molecul weight intervals. Data from Vincent du Vi neaud, *Cold Spring Harbor Symposia on Qua titative Biology*, **6**, 275 (1938).

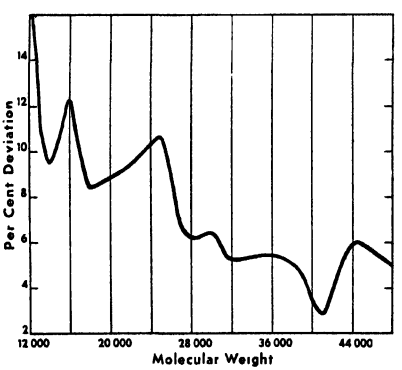



Fig. 4.—The average per cent deviations from whole number residues for hen's egg albumin plotted against assumed molecular weights of this protein. Calculations made at thousand molecular weight intervals. Data from H. B. Vickery, *Symposium on Proteins*, New York Academy of Science, February 2, 1940 (in press).

5. Finally, basic to Bergmann's whole theory is the concept of molecular weight classes and the idea that these classes represent quantized residue numbers. We have already stressed the uncertainty involved in the classification of proteins according to molecular weights.

The reviewer has started out with the simple hypothesis that the only requirement is that the individual amino acid residues must occur as whole numbers (there cannot be fractions of residues). This is equivalent to assuming that there is only one molecular species of protein present. The technique followed is to calculate from chemical analyses the number of residues of the various amino acids present for a series of arbitrary molecular weights. The number of residues for each amino acid for each molecular weight are then changed to the nearest whole number, and the per cent changed is calculated for each amino acid. The per cent deviations from whole number of residues for all the amino acids for each molecular weight are then averaged and these averages plotted against the corresponding assumed molecular weights. Evidently the molecular weight corresponding to the minimum average deviation from whole numbers should be the molecular weight best in accord with the chemical analysis. Such plots are shown in Figs. 2, 3, 4, 5, and 6 for lactalbumin, insulin, egg albumin, cattle globin, and horse hemoglobin.

In all cases the molecular weight can only be obtained if it is already approximately known. It is interesting, however, that there is never any doubt as to which minima and, accordingly, which molecular weight to select. Another curious point is that the minima show a certain symmetry of occurrence. For example, lactalbumin has two minima, one at about 8000 and another at 17,000. Insulin has a symmetry of 3 (interestingly enough, x-ray diffraction studies also show a symmetry of 3). Cattle globin shows 12 minima (one is not shown on graph). Horse hemoglobin also shows 12 minima (two not shown on graph). Egg albumin shows 6 minima (one not shown on graph). The minima of this protein are, however, very unsymmetrically placed. The reviewer does not propose any theory of protein structure based upon these results—the whole thing may be a curious coincidence. This method of calculation aims to present the results of protein analyses in a graphic manner. When better analytical values are available, it may allow valuable conclusions to be drawn from such data.

**Shape.**—We know next to nothing regarding the actual shape of protein molecules. The best that we can do at the present time is to make estimates of the asymmetries of the molecules. In most cases we do not even know whether the asymmetry which we estimate takes the form of rods or

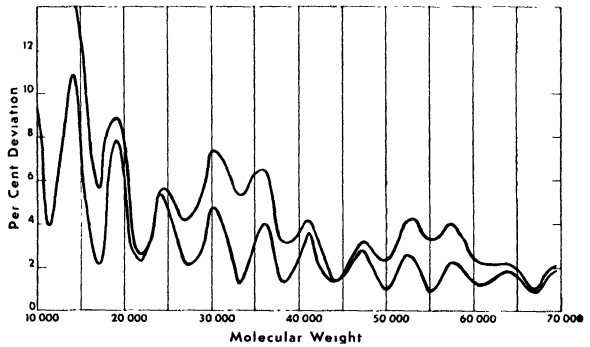



Fig. 5. —The average per cent deviations from whole number residues for cattle globin plotted against assumed molecular weights of this protein. Calculations made at thousand molecular weight intervals. The upper curve includes the cysteine analysis; the lower one does not. Data from Carl Niemann, *Cold Spring Harbor Symposia on Quantitative Biology*, **6**, 58 (1938).

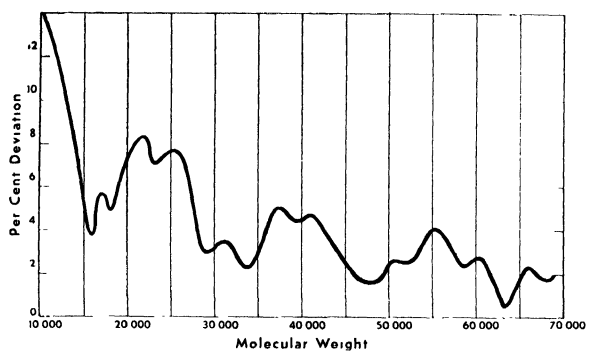



Fig. 6.—The average per cent deviations from whole number residues for horse hemoglobin plotted against assumed molecular weights of this protein. Calculations made at thousand molecular weight intervals. Data from H. B. Vickery, *Symposium on Proteins*, New York Academy of Science, February 2, 1940 (in press).

of disks, although in the case of tobacco mosaic virus protein Lauffer and Stanley (46) have shown rather definitely that this very asymmetric protein is in the form of rods. Asymmetry is expressed in terms of the ratio

of the major and minor axis of a prolate or of an oblate ellipsoid of revolution. The protein molecule may not be ellipsoidal at all but, as a first approximation, this is probably a satisfactory assumption.

The problem of asymmetry is complicated by hydration. It is known, for example, that asymmetry of particles increases viscosity and decreases diffusion from what one would expect from a solution of spheres of the same molecular volume. Hydration also increases viscosity and decreases diffusion of molecules in solution. It is thus difficult to know to what extent hydration and asymmetry enter into any particular cases.

Perrin (47) as well as Herzog, Illig, and Kudar (48) have derived equations for the relation between the asymmetry of particles and the ratio of the experimental diffusion constant to that of spheres of the same volume. These equations express the asymmetry as the ratio of the major and minor axis of a prolate or of an oblate ellipsoid. They blame the entire departure of the observed diffusion constant from that of a spherical molecule of the same volume on asymmetry. There is every reason to believe that this is, in fact, not true, but that part of the departure is due to hydration. The question arises as to how much of the departure is due to hydration and how much to asymmetry. Consider the case of egg albumin.

The anhydrous molecular weight of egg albumin is 45,160 (31). It is hydrated in solution to the extent of about 36 per cent (49); accordingly, the hydrated molecular weight of egg albumin is 61,440. The density of egg albumin containing 36 per cent water is 1.202 (49) and, therefore, the hydrated molecular volume is 51,110 cc. If the egg albumin is assumed to be spherical, the radius of the molecule is calculated to be $2.72 \times 10^{-7}$ cm. Substituting this value in the Sutherland-Einstein diffusion equation for spherical particles, we find the diffusion constant of hydrated, spherical egg albumin molecules in pure water and at 20° C. to be $7.79 \times 10^{-7}$ sq. cm. per second. By the same method of calculation, the diffusion constant of unhydrated spherical egg albumin molecules (molecular weight of 45,160 and density of 1.34) in pure water and at 20° C. is $8.96 \times 10^{-7}$ sq. cm. per second. The experimental value for the diffusion constant at 20° C. and pure water is $7.76 \times 10^{-7}$ sq. cm. (50) per second. The agreement between the diffusion constant calculated for hydrated, spherical molecules and the experimental value is indeed remarkable and indicates that the egg albumin molecule is substantially spherical.

Neurath (51) has used the Perrin equation to calculate the asymmetries of various proteins. He made these calculations with the full realization that they represented first order approximations and are subject to change as knowledge is accumulated. Table III shows the asymmetries calcu-

lated for anhydrous molecules using the technique of Neurath. Also shown are the asymmetries calculated on the assumption of a weight hydration of 35 per cent. In order to carry out these last calculations, it was necessary to make some assumption regarding the density of the water of hydration. This was assumed to be 1.00. This treatment of hydration is admittedly open to serious objection, but it seems more realistic to handle it this way than to neglect it altogether. The assumption of 35 per cent hydration has some basis in fact, thus Sponsler (40) found gelatin to be hydrated to the extent of 35 per cent, and the Neurath-Bull value of 36 per cent hydration for egg albumin has already been discussed. On the other hand, 35 per cent hydration for the higher molecular weight proteins is probably too high unless the molecules actually swell.

TABLE III

ASYMMETRIES CALCULATED FOR ANHYDROUS PROTEINS AND FOR PROTEINS HYDRATED TO THE EXTENT OF 35 PER CENT

(Diffusion data have been used and Perrin's equation for prolate ellipsoids employed.)

| Protein | Molecular weight | Asymmetries | |
|---|---|---|---|
| | | Anhydrous | Hydrated |
| Cytochrome c | 15,600 | 5.4 | 3.1 |
| Lactalbumin | 17,400 | 3.6 | 1.5 |
| Hordein | 27,500 | 11.5 | 8.0 |
| Gliadin | 27,500 | 11.0 | 7.3 |
| Bence Jones | 37,000 | 5.8 | 3.5 |
| Zein | 40,000 | 30.0 | 21.0 |
| Insulin | 41,000 | 3.2 | 1.0 |
| Lactoglobulin | 41,500 | 5.0 | 2.8 |
| Egg albumin | 45,160 | 3.6 | 1.2 |
| Hemoglobin (horse) | 68,000 | 4.2 | 2.6 |
| Serum albumin (horse) | 70,000 | 4.9 | 2.8 |
| Diphtheria toxin | 72,000 | 4.6 | 2.6 |
| Serum globulin (horse) | 167,000 | 7.8 | 4.7 |
| Antipneumococcus serum globulin (man) | 195,000 | 9.5 | 6.4 |
| Antipneumococcus serum globulin (rabbit) | 157,000 | 9.5 | 6.2 |
| Catalase | 250,000 | 4.8 | 3.4 |
| Excelsin | 295,000 | 3.2 | 1.0 |
| Edestin | 310,000 | 4.2 | 2.1 |
| Amandin | 330,000 | 5.2 | 3.1 |
| Urease | 480,000 | 4.0 | 1.8 |
| Thymoglobulin (pig) | 630,000 | 7.7 | 4.8 |
| Antipneumococcus serum globulin (horse) | 920,000 | 15.5 | 11.0 |

Hydration reduces the apparent asymmetries in all cases. The large molecules show as much reduction as the small ones. As has been pointed out, however, 35 per cent hydration for the larger molecules is probably excessive and, accordingly, the asymmetries of the larger molecules, taking hydration into consideration, are probably closer to the anhydrous figures. The correction of asymmetries, due to hydration, is smaller for more asymmetrical molecules than for the less asymmetrical ones, but in no case can the effect of hydration be neglected.

Any theory of protein structure has to take into account the fact that many of the globular proteins are not symmetrical, notably zein, gliadin, hordein, and the various serum globulin fractions. Also to be noted is variation of asymmetries from protein to protein. Even proteins of approximately the same molecular weights can show many different asymmetries. This indicates to the reviewer that there is no structure common to all proteins, but each has its own individual structure. At the present time it is not possible to interpret the apparent asymmetries in terms of any of the other properties of the proteins, although there does seem to be a small positive correlation between asymmetry and the ratio of the nonpolar residues to the polar residues.

The fact that most water-soluble proteins approach a spherical shape is to be understood. Certainly, a long peptide chain containing many polar groups would be expected to fold up into more or less of a globular protein.

Neurath (51) has also considered the asymmetries of protein association and dissociation products. He finds that it is possible to visualize rather clearly the direction in which the protein molecule splits upon dissociation. Some proteins split along the major axis, others along the minor axis. The effect of hydration was not considered, but as can be seen from the results from Table III hydration will decrease all asymmetries, but it will decrease the smaller asymmetries the most, so that taking hydration into account would tend to accentuate Neurath's results and hence make his conclusions more certain.

Viscosity studies have been used to estimate the molecular asymmetry of proteins. Viscosity is, however, inherently a more complicated phenomenon than diffusion. There have been a number of equations derived which relate asymmetry of the particles to the viscosity of a solution of the particles. The latest and, in some respects, the most complete is that of Simha (52). Frankly, the reviewer has considerable reservation in regard to all viscosity equations so far published. It is exceedingly difficult to test these equations experimentally. Polson (53) derived an equation which is empirical to the extent that the equation was formulated by using

the asymmetries obtained from Perrin's diffusion equation (neglecting hydration). He plotted the square of the asymmetries obtained by the use of Perrin's equation against the specific viscosities and found a straight line relation (this indicates protein molecules to be rigid). He formulated an equation to express this straight line relation. Values for asymmetries obtained by the use of Polson's equation are at least consistent with diffusion studies. Polson's equation and Simha's equation give results which are in good agreement for asymmetries greater than about 7 to 1 (80), but for asymmetries less than this the agreement between the two equations becomes progressively worse. A curious fact is that for spherical particles the Polson equation extrapolates to an Einstein coefficient of 4.1 instead of 2.5 as demanded by Einstein's viscosity equation. Such a large discrepancy cannot be explained on the basis of hydration.

There are other ways of estimating asymmetry. If the protein solution shows stream double refraction, the protein molecules are undoubtedly asymmetric. The length of the protein molecule can be estimated from such measurements. Unfortunately, the degree of asymmetry must be considerable before stream double refraction appears, unless one has relatively enormous flow gradients.

A departure of the dispersion curve of the dielectric constant as a function of frequency from the Debye curve can be used to calculate asymmetry, but again we must consider hydration.

X-ray diffraction studies of crystalline proteins give us the dimensions of the unit crystal, although most crystals have more than one molecule per unit cell and, accordingly, it is impossible to determine the molecular asymmetry in such cases without additional data. Fankuchen (41) reports from x-ray studies that horse hemoglobin has the form of a probate ellipsoid with an asymmetry of about 2 to 1. Unfortunately, as Fankuchen points out, proteins with a high degree of asymmetry will not crystallize in a three dimensional crystal, and it is not possible to test the viscosity and diffusion equations involving the appreciable asymmetries by means of x-ray diffraction studies.

**Arrangement of the Peptide Chain.**—One of the central problems which the protein chemist must solve is that of the arrangement of the peptide chain or chains within the protein molecule. This is a problem for which, at the present time, there is no effective approach. We must content ourselves almost entirely with speculation. As long as we realize that what we say is almost pure speculation, no harm will come to us, but we must be careful not to take ourselves too seriously.

It seems evident that in the globular proteins the peptide chains are

folded and, furthermore, this folding must be a highly specific affair. Astbury (19) has suggested that the globular proteins differ in no qualitative way from the fiber proteins; the peptide chain simply undergoes a more extensive folding.

A theory of peptide chain folding which has received wide attention is that of Wrinch. Wrinch has published a long series of papers dealing with her theory (55). Briefly, the theory is about as follows: Instead of the peptide linkage which we have already considered and which might be looked upon as a "two-armed" linkage, she believes that the peptide link can form a "four-armed" building unit. Namely:

$$\rangle N—CHR—C\langle (OH)$$

The essential feature consists in the migration of the hydrogen atom from the nitrogen to the carbon to form a hydroxyl group. From this four-armed building unit she is able to construct the cyclol fabric which consists of six-membered rings of two kinds. These two kinds of rings are the diazine and triazine rings. A section of this cyclol fabric is shown in Fig. 7.

One point about this structure which is worth noting is that half of the R-groups rise vertically upward from the median plane of the fabric, while the other half are attached by bonds that form an angle of 71° with the normal to the plane. Thus, the $\beta$-carbons of all the side chains lie on the same side of the fabric; this is an important point to remember.

A feature of the cyclol fabric is that it can be folded into a cage structure. In the case of the nitrogen atoms only 3 of the tetrahedral valence angles are occupied. The fabric can thus be folded along trigonally arranged lines by shifting the bonds on certain nitrogen atoms to the previously unoccupied positions. This folding takes place through 109° and produces polyhedra with definite size and shape. All the possible polyhedra are truncated tetrahedra, whose eight faces are all parallel to the corresponding faces of a regular octahedra. Their sizes are such that they can be comprised of only 72, 288, or, in general, 72 $n^2$ residues, where $n$ is any integer. The cages containing 72 residues are called $C_1$, those containing 288 residues $C_2$, etc. The cage structure is essentially an octahedron with slits on some of its edges. It has a hollow interior with sides of the lace-like cyclol fabric. Wrinch and Langmuir (56) have assigned definite dimensions to the $C_2$ structure (288 residues). It has a surface area of 2970 sq. Å units and a volume of 11,710 cu. Å units.

The advantages claimed for the cyclol-cage structure may be summarized along somewhat the following lines:

1. It provides for definite molecular sizes and the molecular sizes which

it allows are limited. In fact, as noted above, it must contain 72 $n^2$ residues. This is in accord with Bergmann's theory of amino acid residue periodicity and conclusions drawn from this assumed periodicity. It is also in agreement with Svedberg's idea of molecular weight classes.

2. It provides for protein molecules which are globular in nature; as we have seen, many of the proteins show a low order of asymmetry.

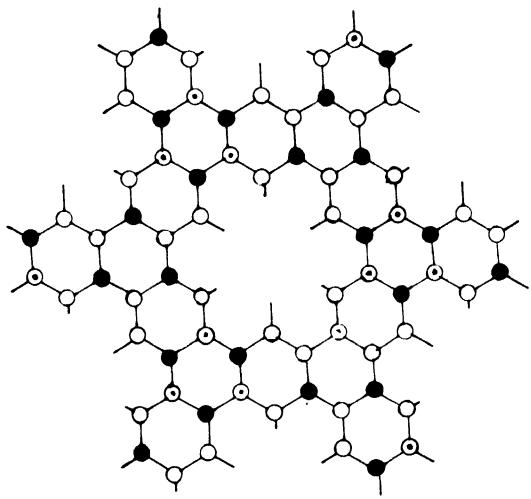


Fig. 7.– A fragment of the cyclol fabric. The median plane of the lamina is in the plane of the paper. The lamina has its "front" surface above and its "back" surface below the paper. ● are nitrogen atoms, O are C(OH) with hydroxyls upwards. ⊙ are C(OH) with hydroxyls downwards, O— are C H R with direction of chain initially outwards, O are C H R with direction of side chains initially upwards.

3. It is said to be compatible with the x-ray studies of Crowfoot on crystalline insulin (56).

4. It provides a structure which would be expected to be disrupted or torn apart with comparative ease (57). Globular proteins, in general, denature with ease, and, accordingly, it is believed that the cage structure fulfills this requirement.

5. Finally, it provides a unified theory of protein structure.

Wrinch has a powerful proponent (58) of her theory; she also has some severe critics. Possibly the most effective of her critics are Pauling and Niemann (59). Their paper must be read in detail to appreciate their arguments. The reviewer does not propose to review their paper or even summarize it, but much of which is to follow has been inspired by them.

We have already noted the uncertain nature of the Svedberg classification of proteins on a molecular weight basis. We have also expressed our lack of faith in Bergmann's frequency hypothesis with his belief that a protein molecule must contain 288 residues (the $C_2$ cage of Wrinch) or a multiple thereof. Since we have covered this ground in some detail, it seems pointless to enter into these arguments again. It appears very possible, however, that Wrinch has been trying to explain something which does not exist, *i. e.*, the occasion for quantized residue numbers.

The reviewer has to confess his inability to judge the validity of the conclusions drawn from x-ray diffraction studies on insulin by Langmuir and Wrinch. People who are eminently equipped to pass judgment in the matter seem almost unanimous in their opinion that these conclusions were extremely premature (60).

The cyclol-cage structure is by no means unique in its explanation of protein denaturation. In fact, it is not entirely satisfactory in this regard. The denaturation of some proteins can, apparently, be reversed or, at any rate, regain their solubility and crystallizability after having been denatured (61). It would seem that once the cyclol fabric were torn open it could never be reconstructed into anything approaching its original structure.

While it is true, as we have seen, that several proteins do show a low order of asymmetry and are in this respect in accord with the cage structure, there are several proteins which show marked asymmetry, notably zein, gliadin, hordein, and the various serum globulin fractions. It appears quite impossible on this score for zein (asymmetry of 20 to 1) to have a cage structure.

The fact that the Wrinch theory provides a unified theory of protein structure has some disadvantages. Proteins differ greatly in their properties. A common structure for all should lead to more properties in common.

A point which appeals to the reviewer is that there simply is not enough space allowed for the R-groups. As we have noted, the surface area of the $C_2$ cage is 2970 sq. Å units. Since all residues must be on the same side of the fabric, this allows only 10.6 sq. Å units per residue. This is far too small

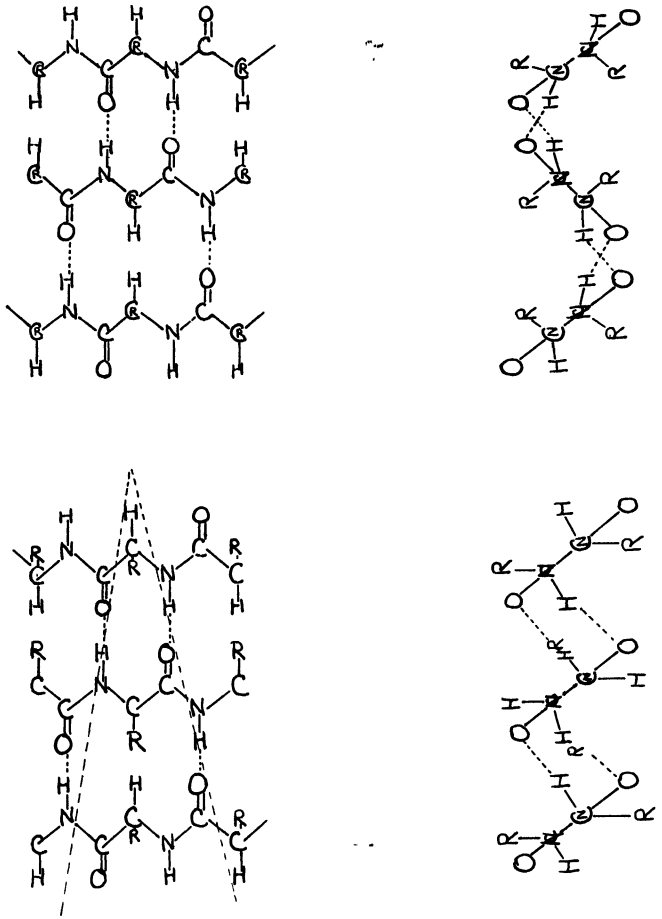


Fig. 8.—Two basic patterns containing zig-zag peptide chains. In the *A*-pattern (top), the sequence —C H R—N H—C O— runs in opposite directions in adjacent chains; in the *B*-pattern (bottom) the sequence is the same in all the chains.

an area as the area of the average residue must be in the neighborhood of 20 sq. Å units.

The assumption of the cyclol structure pains the instincts of a chemist since no such structure has ever been synthesized or described. It has

never been shown to have natural occurrence outside of the minds of its proponents.

The Wrinch theory has served a worthy purpose in forcing examination of protein structure. It has aroused the interest of many able chemists who had previously been indifferent to these matters. At the present time, however, the evidence against it is of such a nature that it seems profitless to continue its consideration.

It is the understanding of the reviewer that Wrinch has devised a new type of cyclol folding which is said to accommodate the R-groups. This communication was to have appeared in the June, 1940, issue of the *Philosophical Magazine*. Unfortunately, due to delays caused by the present war, this issue of the magazine was not available to the reviewer at the time this discussion was being written.

Recently, Huggins (62, 63) has suggested two hypothetical types of structures derived by folding from basic patterns containing extended zig-zag chains. These chains are joined together by O—H—N hydrogen bridges. The two basic patterns of the extended zig-zag peptide chains are shown in Fig. 8.

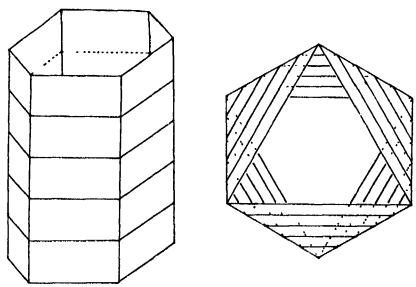


Fig. 9.—Two hypothetical types of folding of zig-zag peptide chains. The chains are held together by hydrogen bridges between the nitrogen and oxygen atoms. Structure *A* (left) corresponds to the folding of pattern *A* shown in Fig. 8 and structure *B* (right) corresponds to the folding of pattern *B*.

The first type of pattern (*A*) can be folded, without breaking any bonds or doing violence to any of the accepted ideas concerning bond lengths, about lines at right angles to the chain axes. The angle between adjacent "planes" of the fabric at each fold is 120°, if the bond angles are the usual tetrahedral angles. By repeated folding an open-ended hexagonal prism structure can be produced in which the peptide chains are now large rings. Huggins believes that 24 amino acid residues would be the smallest possible number in each ring. For a 288 residue protein the model would be 14.5 Å units wide and 44 Å units high. This structure would not impose any limit on the number of residues which can be accommodated, except that they must be multiples of 24.

The second type of pattern (*B*) folds without strain to give open-ended octahedra. Making the requirement that all corners are alike, Huggins

concludes that the residues per ring must be 12 $n$ and the number of rings 6 $n$. The total number of residues in such a structure is then 72 $n^2$. The smallest possible structure would be 2 $n$ or 288 residues (there is too much crowding in a 72 residue structure). The top and bottom of the triangular rings would be about 29 Å units on a side. The vertical height would be roughly 43 Å units. These two structures are shown in Fig. 9.

Apparently, Huggins proposed these structures largely as possible alternates to the Wrinch structure. He wished to show that it was possible to have structures which would perform all the functions of the Wrinch structure without some of its defects. For example, Huggins was careful to allow sufficient space for the R-groups. His structure also has the advantage that it involves types of bonding which has been shown to occur in various compounds and, in fact, the reviewer understands that there is experimental evidence that N—H—O hydrogen bridges actually occur in proteins (90).

The reviewer must confess that he is still not satisfied with the role accorded the R-groups. It is true that Huggins did do them the courtesy of providing space for them in his model, but he assigns them no bonding function or considers their influence on the structure in any way. As a matter of fact, the R-groups, starting with the $\beta$-carbon atoms, make up over 50 per cent by weight of a protein such as egg albumin. Many of these R-groups are comparatively massive and about half of them contain strongly polar groups. The reviewer does not see how the conclusion can be escaped, that if the R-groups contribute to protein stability, and evidence indicates that they do, they must influence the nature of the folding of the peptide chain. This conclusion implies a different type of folding for each protein, since the R-groups differ from protein to protein. This conclusion is in keeping with the specificity of proteins as they are known to the protein chemist. Thus, if it is true as Wrinch (64) has claimed and Huggins implied, that insulin and pepsin have the same peptide chain folds, it limits the basis for explaining the known, and tremendous, difference between these two proteins.

Whatever the type of folding of the peptide chain in the protein, it must give rise to a comparatively dense structure. The density of dry, native egg albumin is 1.2655 using xylene as the displacing liquid (49) and 1.350 using dry hydrogen as the displacing gas (65). The density of egg albumin in hydrogen gas is well in the range of the densities of the contained amino acids. The difference between these two density determinations indicates that about 7 per cent of the volume of the protein molecule, which is penetrable by hydrogen, is not penetrable by xylene.

The important question arises as to how much, if any, the protein molecules swell in solution. That is, does any appreciable quantity of water enter the protein molecule when the dry protein is dissolved in water? This amounts to asking whether or not the peptide chain arrangement can be displaced by water. Density measurements cannot solve this problem. There is, however, some indirect evidence from viscosity and x-ray diffraction studies which indicate that, in some cases at least, the amount of water entering into the molecule and causing swelling must be very small. The viscosity of an egg albumin solution from *p*H 2.5 to *p*H 10.5 is, after allowance has been made for the electroviscous effect, practically constant (66). This indicates that there has been no change in shape or size of the egg albumin molecule over this range of *p*H. Since the protein molecule cannot swell under the influence of wide changes in proton and hydroxyl ion concentration, it is natural to conclude that the structure is a very fixed and rigid one, and it is not to be anticipated that appreciable swelling will take place when the dry protein is dissolved in water.

X-ray diffraction studies (67) on tobacco mosiac virus protein indicate that, while the micelle or tactoid can, and does, swell enormously, the molecule itself maintains its exact dimensions irrespective of the water content. On the other hand, there is some x-ray evidence (68) which suggests that possibly the very large molecules of bushy stunt virus swell in solution.

It is the instinctive feeling of the reviewer that native protein molecules which are capable of denaturation, cannot swell appreciably when the dry protein is dissolved in water.

**Evidence from Protein Denaturation.**—It is characteristic of many globular proteins to undergo what is known as denaturation. A number of agents will induce this reaction, such as heat, ultraviolet light, x-rays, strong acids or bases, urea, guanidine hydrochloride, alcohol, sulfonated alcohols, surface forces, etc. In fact, it is much easier to find chemicals which will cause protein denaturation than it is to find chemicals which will not. In general, the denaturation reaction involves four changes in the native protein. These changes are: (1) decreased solubility, (2) increased viscosity of solution, (3) exposure of oxidizing and reducing groups (sulfhydryl and disulfide linkages among others), and (4) large loss of specific biological properties.

While the first serious physical measurements of the denaturation reaction go back to the early work of Chick and Martin (69), the first reasonable interpretation of denaturation was made by Wu (70). More recently Mirsky and Pauling (71) have elaborated on the ideas of Wu. The general thesis of this theory is that denaturation can be looked upon as a change

from the unique, highly specific structure of the nature protein to a more random arrangement of the denatured molecule. An analogy is the change from a crystalline to an amorphous state. There is every reason to believe that, in broad outline, this theory is true.

It appears that much can be learned about protein structure through a study of protein denaturation. In fact, the two questions are so intimately connected that a complete solution of one or of the other would probably provide a solution for both.

The loss of solubility is probably the reflection of at least two changes in the native molecule. Polar groups which were available for water binding in the native protein are now, due to the shift from the rigid and unique configuration of the native molecule, linked with other polar groups in the protein molecule itself. This results in decreased solubility (less attraction for water). That this is indeed true is shown by the fact that the water of hydration of both heat denatured and of surface denatured egg albumin is less than for native (49). Secondly, there is an increase in asymmetry of the molecule. This results in a greater area of cohesion between the protein molecules and, accordingly, less solubility. There is also a third possible factor at work. Proteins contain a large number of non-polar groups and it might be that upon denaturation these hydrophobic groups are exposed.

The increase in asymmetry upon denaturation is clearly indicated by viscosity studies. It was shown by a number of early workers that there is an increase in the viscosity of a solution of protein upon denaturation. The reviewer has determined the viscosities of solution of native, of heat denatured, and of urea denatured egg albumin (72). Using the equation of Polson (53), it was estimated that the asymmetry of the native molecule was 3.9 to 1, of heat denatured 7.4 to 1, and of urea denatured 9.2 to 1. However, if the effect of the hydration of the native and of the heat denatured is considered and the hydration of the urea denatured egg albumin assumed to be that of the heat denatured (16 per cent), the asymmetries come out to be: native 1.2:1, heat denatured 5.8:1, and urea denatured 7.3:1. With or without considering hydration, it is evident from these results that even urea denatured egg albumin still has considerable structure and is not simply a polypeptide chain in the $\beta$-keratin form since the egg albumin molecule, if it existed as a $\beta$-keratin chain, should be over 1000 Å units long and about 10 Å units wide, which would yield an asymmetry greater than 100 to 1. This shows, even in a denatured globular protein, that the peptide chain is greatly folded and collapsed. We would, as a matter of fact, anticipate this state of affairs from the large number of polar

R-groups in egg albumin. Neurath (73) has shown a similar increase in asymmetry of serum albumin upon urea denaturation.

The thiol groups of proteins have been subjected to investigation by Hopkins (74), Mirsky and Anson (75), Greenstein (76), and others. Some native proteins such as egg albumin, edestin, globin, and excelsin do not give a nitroprusside test, showing that the sulfhydryl (SH) groups are nonreactive in these native proteins. It is only upon denaturation that a positive nitroprusside test is obtained. Other proteins such as hemoglobin, myosin, and crystalline lens protein have detectable thiol groups even in their native state, but their number is increased upon denaturation. The reagents used to detect the sulfhydryl groups such as nitroprusside, ferricyanide, or porphyrindin dye are believed to oxidize the sulfhydryl to the corresponding disulfide. Iodoacetate and iodoacetamide also react with sulfhydryl groups. This reaction may yield a disulfide compound or it may involve the substitution of the hydrogen of the sulfhydryl by a carboxymethyl group. In the first case 2 sulfhydryl groups would react with two molecules of the iodine compound, while in the second case only one molecule of each is necessary.

$$2RSH + 2CH_2ICOOH \longrightarrow RS{-}SR + 2HI + C_2H_4(COOH)_2$$

or

$$RSH + CH_2ICOOH \longrightarrow RSCH_2COOH + HI$$

It has been shown that in the case of amino acids containing SH the reaction with iodoacetate proceeds according to the second of the above reactions.

There are two general ways of looking at the sulfhydryl problem. The first is that SH groups appear upon the rupture of the disulfide bond incidental to denaturation. It is known, for example, that the disulfide linkage can be hydrolyzed to yield (77, 78)

$$RS{-}SR + H_2O \longrightarrow RSOH + HSR$$

This reaction is favored by a high *p*H. It is possible, but doubtful. There is no reason to believe that urea, alcohol, and other reagents can effect such a cleavage. The reagents which cause denaturation are known to be without effect on the synthetic disulfide peptides (79).

The second point of view regards the SH groups as present in the native protein, but for some reasons, yet unknown, are unreactive. This second way of looking at the problem gives rise to two possibilities. One is that the SH groups are buried in the interior of the native molecule and hence cannot be attacked by the sulfhydryl reagents, and it is only upon denaturation that they are brought to the suface of the molecule.

The second possibility has been suggested by Neurath (80). He points out that since the estimation of sulfhydryl groups by nitroprusside, porphyrindin dye, or ferricyanide involves the oxidation of sulfhydryl to disulfide, two cysteine residues are required for the reaction to proceed. In the native protein it may so happen that individual cysteine residues are not close enough to form a disulfide bond. In this case the reagents will be ineffective in testing for SH groups. Upon denaturation, however, the molecule assumes a much looser and random arrangement and, accordingly, even distant, cysteine residues may come, at least temporarily, into close contact. Oxidation of the SH groups may then occur. The action of iodoacetate or iodoacetamide may, as indicated above, involve only one SH group. The work of Balls and Lineweaver (81) on papain indicates that this may be the case. Iodoacetate causes inactivation of papain, whereas the porphyrindin does not affect it. Since it is known that the presence of SH groups is essential for the action of this enzyme, it looks as though the dye were incapable of oxidizing the SH groups while the iodoacetate could and did react with them. Also, Anson (82) reported that about 60 per cent of the SH groups could not be detected in denatured egg albumin following treatment of the native protein at *p*H 9 iodoacetamide. Rosner (83), however, could detect no reaction between native egg albumin and iodoacetate at *p*H 7.3. The problem must be looked upon as unsolved. Unsolved also is the role of the thiol groups in protein denaturation. Is their appearance only incidental to the reaction, or do they play a significant part? This is largely equivalent to asking if the thiol groups are important in protein structure. It must be remembered that with many proteins they make up only a very small part of the total number of residues present.

Proteins lose their biological specificity upon denaturation. Thus, most native proteins which manifest a high degree of immunological specificity, to a large extent, lose it upon denaturation (84). Enzymes also lose their enzymatic activity upon denaturation (85). Evidently, these biological properties are dependent upon a unique configuration of the peptide chain in the protein molecule.

The question of molecular dissociation upon denaturation has not received the attention it deserves. Burk (86) has conducted a series of studies on the osmotic pressure of proteins denatured by urea. He finds that the molecular sizes of a number of proteins are unchanged upon treatment with urea. These proteins are egg albumin, serum albumin, serum globulin, gliadin, zein, and pepsin. On the other hand, there are several proteins which are split by urea. These are hemoglobin into halves,

myogen into halves, edestin into fourths, excelsin into sixths, and amendin into sixths. Perhaps the factor which determines whether or not a protein will split upon denaturation is the number of peptide chains in the molecule. If there is only one chain there will be no splitting, while if there are two the molecule will be split into halves, etc. In this connection Steinhardt (87) studied the action of urea on horse hemoglobin. He found, in keeping with the results of Burk, that hemoglobin is indeed split into halves and, furthermore, as far as he could judge, these two halves were identical.

Beginning with the work of Chick and Martin (69) there have been many studies on the kinetics of protein denaturation. One of the important conclusions which has emerged from this kinetic work is that heat denaturation is characterized by a very large temperature coefficient.

Energies of activation have been calculated for heat denaturation and extraordinarily large values were found. Steinhardt (88) made a very careful study of the heat denaturation of pepsin. He found that of the total energy of activation of 63,500 calories a large fraction, namely 45,200 calories, were needed to ionize 5 protons from the pepsin molecule. In other words, the only form of pepsin which would heat denature had a net charge on it. The rest of the energy of activation (18,300 calories) was involved in the breaking of bonds of some kind in the pepsin molecule.

While no other protein has received the detailed attention that pepsin has, the effect of *p*H on the energy of activation of other proteins indicates quite clearly that before denaturation can take place, the molecule has to have a net charge on it.

Mirsky and Pauling (71) had attempted to explain the entire energy of activation on the necessity of having to disrupt many hydrogen bridges—some thirty for egg albumin. Evidently their picture was incomplete. Eyring and Stearn (89) came to the conclusion from the examination of the energies of activation that denaturation involved the breaking of two kinds of bonds: (1) salt linkages which are sensitive to *p*H changes, and (2) homopolar bonds which are insensitive to *p*H changes. These last they considered to be disulfide linkages. The salt linkages occur between the charged amino and charged carboxyl groups and give rise to the zwitter ionic structure. These are the bonds which are disrupted by the ionization of protons and which Steinhardt found accounted for the largest fraction of the total activation energy.

The energy of activation studies on heat denaturation of proteins simmers down to this: There are two types of linkages broken upon denaturation: (1) linkages which are sensitive to *p*H changes, and (2) linkages which are largely insensitive to *p*H changes. The first type of linkage is undoubt-

edly electrostatic in nature (salt linkage) and arises from the carboxyl and amino groups on the R-groups. The reviewer suggests that the second type of bonds are N—H—O hydrogen bridges between the neighboring peptide chains in the molecule or neighboring loops of the same chain. As pointed out before, there is experimental evidence (90) for such linkages in proteins; Huggins (62) has also suggested that they play a major role in determining the nature of the folding of the peptide chain. While such bonds would be sensitive to protons and hydroxyl ions, their sensitivity might be expected to be reduced because of the following two reasons: (1) Neither the N nor the O belong to charged centers, and hence their ionization is not involved and, accordingly, protons would not be so potent in breaking such a bond, and (2) the N—H—O bonds between the peptide chains are buried in the molecule, and hence protected by the protruding R-groups.

We have evidence, then, from denaturation studies, that the important bonds which hold the peptide chain in position in the native protein and give to it its specific and unique structure are of two types, namely, electrostatic bonds between the charged amino, and the charged carboxyl groups of the R-groups and the N—H—O bridges between the peptide chains and arise, in the sense of Huggins, directly from the peptide chains and do not involve the R-groups.

The reviewer regards it as worth emphasizing that the R-groups apparently contribute the burden of the energy of activation of heat denaturation. In short, from an energy standpoint they are the most important factor in maintaining the peptide chain in its unique configuration. It is unthinkable that they do not have a profound influence on the type of peptide chain folding. Since the number, nature, and distribution of these polar R-groups are different for each protein, it seems logical to conclude that each protein has its own individual and highly specific pattern of peptide chain folding.

### Bibliography

1. H. B. Vickery, "Symposium on Proteins," N. Y. Acad. of Sci., February 2, 1940 (in press).
2. M. Bergmann and C. Niemann, *Ann. Rev. Biochem.*, **7**, 99 (1938).
3. F. Kögl and H. Erxleben, *Z. physiol. Chem.*, **258**, 57 (1939).
4. O. K. Behrens, F. Lipmann, M. Cohn, and D. Burk, *Science*, **92**, 32 (1940).
5. F. Hofmeister, *Ergeb. Physiol.*, **1**, 759 (1902).
6. E. Fischer, *Ber.*, **35**, 1095 (1902).
7. M. Bergmann and J. S. Fruton, *Compt. rend. trav. lab. Carlsberg*, **22**, 62 (1938).
8. H. B. Vickery and T. B. Osborne, *Physiol. Rev.*, **8**, 393 (1928).
9. K. O. Pedersen, *Proc. Roy. Soc.* (Lond.) **B127**, 20 (1939).
10. A. Neuberger, *Biochem. J.*, **32**, 1435 (1938).

11. S. P. L. Sorensen, *Kolloid-Z.*, **53**, 306 (1930).
12. E. Chargaff, *J. Biol. Chem.*, **125**, 661 (1938).
13. T. H. Jukes and H. D. Day, *J. Nutrition*, **5**, 81 (1932).
14. K. H. Meyer and H. Mark, *Ber.*, **61**, 1932 (1928).
15. W. T. Astbury and A. Street, *Phil. Trans. Roy. Soc.* (London), **A230**, 75 (1931).
16. W. T. Astbury and D. M. Wrinch, *Nature*, **139**, 798 (1937).
17. H. Neurath, *J. Phys. Chem.*, **44**, 296 (1940).
18. M. L. Huggins, Gibson Island Conference, July, 1940.
19. W. T. Astbury, *Trans. Faraday Soc.*, **34**, 378 (1938).
20. W. T. Astbury and J. A. T. Dawson, *J. Soc. Dyers and Colourists*, **54**, 6 (1938).
21. J. B. Speakman, *Nature*, **132**, 930 (1933); **138**, 327 (1936); F. F. Elsworth and H. Phillips, *Biochem. J.*, **32**, 837 (1938).
22. W. T. Astbury, First Proctor Memorial Lecture, published by the International Society of Leather Trades' Chemists, London, S. E. 1.
23. R. W. G. Wyckoff and R. B. Corey, *J. Biol. Chem.*, **114**, 407 (1936).
24. G. L. Clark, E. A. Parker, J. A. Schaad, and W. J. Warren, *J. Am. Chem. Soc.*, **57**, 1509 (1935).
25. G. Boehm and H. H. Weber, *Kolloid-Z.*, **61**, 269 (1932).
26. W. T. Astbury and S. Dickinson, *Nature*, **135**, 95 (1935).
27. W. T. Astbury and S. Dickinson, *Ibid.*, **135**, 765 (1935).
28. W. T. Astbury, *Compt. rend. trav. lab. Carlsberg*, **22**, 45 (1938).
29. T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford at the Clarendon Press, New York, 1940.
30. T. Svedberg and J. B. Nichols, *J. Am. Chem. Soc.*, **48**, 3081 (1926).
31. H. B. Bull (unpublished results).
32. A. Tiselius and I. Eriksson-Quensel, *Biochem. J.*, **33**, 1752 (1939).
33. M. Bergmann and C. Niemann, *Science*, **86**, 187 (1937).
34. R. J. Block, *Cold Spring Harbor Symposia on Quantitative Biology*, **6**, 79 (1938).
35. M. Bergmann and C. Niemann, *Ann. Rev. Biochem.*, **7**, 99 (1938).
36. W. B. Hardy, *J. Physiol.*, **33**, 251 (1905); H. Chick, *Biochem. J.*, **8**, 404 (1914).
37. S. P. L. Sorensen, *Compt. rend. trav. lab. Carlsberg*, **5**, 1 (1930).
38. L. F. Hewitt, *Biochem. J.*, **30**, 2229 (1936).
39. J. Steinhardt, *Cold Spring Harbor Symposia on Quantitative Biol.*, **6**, 301 (1938).
40. O. L. Sponsler, J. D. Bath, and J.W. Ellis, "*Water Boundto Gelatin as Shown by Molecular Structure Studies*," *National Colloid Symposium*, Stanford Univ., July, 1939.
41. I. Fankuchen, "*Symposium on Proteins*," N. Y. Acad. of Sci., February 2, 1940 (in press).
42. O. Zinoffsky, *Z. physiol. Chem.*, **10**, 16 (1886).
43. M. Bergmann and C. Niemann, *J. Biol. Chem.*, **118**, 301 (1937); **122**, 577 (1938). M. Bergmann, *Harvey Lectures*, **31**, 37 (1936).
44. A. Neuberger, *Proc. Roy. Soc.* (Lond.), **B127**, 25 (1939).
45. M. Bergmann and W. H. Stein, *J. Biol. Chem.*, **128**, 217 (1939).
46. M. A. Lauffer and W. M. Stanley, *Ibid.*, **123**, 507 (1938).
47. F. Perrin, *J. phys. radium*, **7**, 1 (1936).
48. R. O. Herzog, R. Illig, and H. Kudar, *Z. physiol. Chem.*, **A167**, 329 (1934).
49. H. Neurath and H. B. Bull, *J. Biol. Chem.*, **115**, 519 (1936).
50. A. Polson, *Kolloid-Z.* **87**, 149 (1939).
51. H. Neurath, *J. Am. Chem. Soc.*, **61**, 1841 (1939).

52. R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).
53. A. Polson, *Kolloid-Z.*, **88**, 51 (1939).
54. J. W. Williams and C. C. Watson, *Cold Spring Harbor Symposia on Quantitative Biol.*, **6**, 208 (1938).
55. D. M. Wrinch. See list of references in *Ibid.*, **6**, 122 (1938).
56. D. M. Wrinch and I. Langmuir, *J. Am. Chem. Soc.*, **60**, 2247 (1938).
57. D. M. Wrinch, *Phil. Mag.*, **25**, 705 (1938).
58. I. Langmuir, *Proc. Phys. Soc.*, **51**, 592 (1939).
59. L. Pauling and C. Niemann, *J. Am. Chem. Soc.*, **61**, 1860 (1939).
60. D. Crowfoot, *Proc. Roy. Soc.* (London), **A164**, 580 (1938); J. D. Bernal, *Nature*, **143**, 74 (1939); D. P. Riley and I. Fankuchen, *Ibid.*, **143**, 648 (1939).
61. M. L. Anson and A. E. Mirsky, *J. Phys. Chem.*, **35**, 185 (1931).
62. M. L. Huggins, *J. Chem. Phys.*, **8**, 598 (1940).
63. M. L. Huggins, Gibson Island Conference, July, 1940.
64. D. M. Wrinch, *Phil. Mag.*, Ser. **7**, **24**, 940 (1937).
65. H. B. Bull, *Cold Spring Harbor Somposia on Quantitative Biol.*, **6**, 140 (1938).
66. H. B. Bull, *Trans. Faraday Soc.*, **36**, 80 (1940).
67. J. D. Bernal and I. Fankuchen, *Nature*, **139**, 923 (1937).
68. J. D. Bernal, I. Fankuchen, and D. P. Riley, *Ibid.*, **142**, 1075 (1938).
69. H. Chick and C. J. Martin, *J. Physiol.*, **40**, 404 (1910); **43**, 1 (1911–12); **45**, 61, 261 (1912–13).
70. H. Wu, *Chinese J. Physiol.*, **5**, 321 (1931).
71. A. E. Mirsky and L. Pauling, *Proc. Nat. Acad. Sci.*, **22**, 439 (1936).
72. H. B. Bull, *J. Biol. Chem.*, **133**, 39 (1940).
73. H. Neurath, *Cold Spring Symposia on Quantitative Biol.*, **6**, 196 (1938).
74. F. G. Hopkins, *Nature*, **126**, 328, 383 (1930).
75. A. E. Mirsky and M. L. Anson, *J. Gen. Physiol.*, **18**, 307 (1935); **19**, 427 (1935–36).
76. J. P. Greenstein, *J. Biol. Chem.*, **125**, 501 (1938); **128**, 233 (1939); **130**, 519 (1939).
77. F. Dickens, *Biochem. J.*, **27**, 141 (1933).
78. J. B. Speakman, *Nature*, **132**, 930 (1933).
79. J. P. Greenstein, *J. Biol. Chem.*, **125**, 501 (1938).
80. H. Neurath, *Cold Spring Symposia on Quantitative Biology*, 1940 (in press).
81. A. K. Balls and H. Lineweaver, *J. Biol. Chem.*, **130**, 669 (1939).
82. M. L. Anson, *J. Gen. Physiol.*, **23**, 321 (1940).
83. L. Rosner, *J. Biol. Chem.*, **132**, 657 (1940).
84. H. Zinsser and Z. Ostenberg, *Proc. New York Path. Soc.*, **14**, 78 (1914).
85. J. H. Northrop, *J. Gen. Physiol.*, **13**, 756 (1930); **16**, 33, 323 (1932).
86. N. F. Burk, *J. Biol. Chem.*, **120**, 63 (1937).
87. J. Steinhardt, *Ibid.*, **123**, 543 (1938).
88. J. Steinhardt, *Kgl. Danske Videnskab. Selskab, Math.-fys. Medd.*, **14**, 11 (1937).
89. H. Eyring and A. E. Stearn, *Chem. Rev.*, **24**, 253 (1939).
90. A. M. Buswell, K. F. Krebs, and W. H. Rodebush, *National Colloid Symposium Univ. of Michigan*, June, 1940.
91. B. Corey, *Chemical Reviews*, **26**, 227 (1940).
92. R. D. Hotchkiss, *J. Biol. Chem.*, **131**, 387 (1939).
93. K. Linderstrøm-Lang and C. F. Jacobsen, *Enzymologia*, **8**, 64 (1940).

# PHYSIKALISCH-CHEMISCHE GESICHTSPUNKTE ZUM PROBLEM DER VIRUSAKTIVITÄT

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INHALT

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## Einleitung

Es gibt wohl kaum eine physikalische Untersuchungsmethode, die zur Charakterisierung von Virusproteinen nicht herangezogen worden wäre. Schlussfolgerungen aus diesen, zum Teil mit grösster Exaktheit und Präzision durchgeführten experimentellen Ergebnissen sind jedoch nur dann einwandfrei, wenn bei einer Auswertung alle, bei solchen Messergebnissen gemachten "*Annahmen*" und Vernachlässigungen berücksichtigt werden.

Eine Fehlerquelle, die oftmals nicht genügend berücksichtigt wird, betrifft die zur Aggregation führenden Anziehungskräfte der gelösten Partikelchen untereinander. Die Nichtberücksichtigung dieser Tatsache hat besonders bei der Auswertung von Messungen, in denen die Gültigkeit des Stokes'schen Gesetzes angenommen wurde, zu Irrtümern geführt, z.B. bei Viskositätsmessungen und Diffusionsmessungen bei der Ermittlung von Sedimentationskonstanten und Teilchengrössenbestimmungen. Eine grosse Anzahl Forscher hat immer wieder auf diese Umstände aufmerksam gemacht. Frampton (1) erörterte diese Frage z.B. für sedimentierende T.M.*-Virusteilchen, Waldmann (2) für M.K.S. und Pockenvirus. Für Lösungen von Eiweisskörpern ist von F. F. Nord (3) und Mitarbeitern

* T.M. = Tabakmosaik.
M.K.S. = Maul- und Klauenseuche.

gleichfalls verschiedentlich nachdrücklichst auf Fehlschlüsse aus Sedimentations- und Viskositätsmessungen bei Nichtberücksichtigung dieser Tatsachen hingewiesen worden (vgl. auch neuerdings G. A. Kausche, E. Pfankuch, H. Ruska (41)).

Besonders zu beachten ist ferner bei der Beurteilung der physikalischen Messergebnisse der Einfluss der Wasserstoffioninkonzentration. So kann man hier bei je nach dem eingestellten *p*H der Lösung verschiedene Teilchengrössen erhalten, wie aus Untersuchungen an Eiweisskörpern, z.B. bei Insulin, Hämocyanin und anderen, hervorgeht. Ferner kann die Sedimentation eines Eiweisskörpers oder Virusproteins gestört werden, wenn die Partikelchen eine elektrische Ladung tragen, was bei Eiweisskörpern meist der Fall ist, oder in dissoziierter Form vorliegen.

In diesem Fall kann durch das Zentrifugieren eine Trennung der positiv und negativ geladenen Teilchen erfolgen, sodass ein dem Zentrifugenpotential entgegengerichtetes Potential entsteht. Auf diese Erscheinung wurde unter anderem von J. W. Beams (4) hingewiesen. Man verhindert praktisch diesen Umstand durch Hinzufügen von niedermolekularen Elektrolyten und Puffersubstanzen, was jedoch auch nicht völlig einwandfrei ist, da eine selektive Bindung von Pufferionen stattfindet, die wiederum das physikalische Verhalten der Eiweisslösungen beeinflussen können, sodass es keineswegs gleichgültig ist, ob die Einstellung des jeweils gewünschten *p*H-Wertes mit $Na^{\cdot}$ oder $NH_4^{\cdot}$ bezw. $Cl'$ oder Phosphationen erfolgt. Erstmalig von Wöhlisch (5), später von Farkas und Grośk (6) wurden diese interessanten Tatsachen bei der Bestimmung des isoelektrischen bezw. isoionischen Punktes an Fibrinogen nachgewiesen. Auf die Bedeutung des isoelektrischen Punktes wird im Zusammenhang mit dem Aggregationsproblem weiter unten eingegangen. G. S. Adair und M. E. Adair (7) haben dann die gleichen Erscheinungen bei Haemoglobin durch Messung von Membranpotentialen festgestellt, wobei je nach den angewandten Puffersubstanzen eine Verschiebung des isoelektrischen Punktes erfolgt. Auch von Tiselius (8) ist auf eine spezifische Pufferionenwirkung bei Elektrophoreseversuchen hingewiesen worden, sodass eine Ermittlung z.B. der Wertigkeit von Eiweissionen in Gegenwart von Puffersubstanzen nicht ohne weiteres möglich ist.

Trotz der angeführten Fehlerquellen lassen sich aber durch die vergleichsweise Anwendung zahlreicher verschiedenartiger Untersuchungsmethoden ziemlich sichere Aussagen über den Charakter und die Wirkungsweise z.B. eines Eiweisskörpers oder Virusproteins machen. Bei der Untersuchung der verschiedenen Lösungen von Viruseiweissen sind oftmals hemmende oder infektionssteigernde Umstände ermittelt worden, es bestehen jedoch bisher keine einheitlichen Ansichten über die Ursachen der Viruswirksamkeit, und es seien im folgenden darum einige dieser zu infektionshemmender bezw.-steigernder Wirkung führenden Umstände besonders berücksichtigt.

Von G. Pyl (9) war aufgrund seiner Untersuchungen über die Temperaturabhängigkeit der pathogenen Aktivität beim M.K.S.-Virus der jeweilige Dispersitätsgrad des Virus für die Aktivität verantwortlich gemacht worden, während E. Pfankuch und G. A. Kausche (10) die spezifische Aktivität von pflanzlichen Virusproteinen aufgrund von Untersuchungen am T.M.- und Kartoffel-X-Virus auf das Vorhandensein besonders wirksamer Gruppen am Molekül zurückführen. Dass beide Ansichten zu Recht bestehen, soll in den nachstehenden Ausführungen an Hand des experimentellen Materials erläutert werden.

## I. Einfluss des Ladungscharakters auf die spezifische Viruswirksamkeit

Stanley konnte in seinen Untersuchungen am T.M.-Virus feststellen, dass die Acidität des Milieus die Viruswirksamkeit beeinflusst, sodass oberhalb bezw. unterhalb *p*H 9.5 und *p*H 2.6 die biologische Aktivität auf ein Minimum absinkt. Bei *p*H 9.8 (11) wurde gefunden, dass das Protein 2 Komponenten gebildet hatte, die eine Sedimentationskonstante

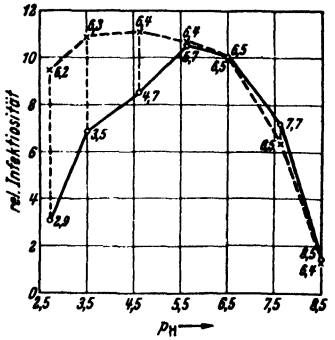



Fig. 1.—Reaktivierung von T. M.-Virus. (Nach G. A. Kausche.)
——— Inaktivierungsversuch.
– – – – Reaktivierungsversuch.

von 185 bezw. 125 hatten, während es bei *p*H 11.7 in noch kleinere Bruchstücke zerfiel mit den Sedimentationskonstanten 8.1 und 3.8. G. A. Kausche (12) konnte diese Versuche dann ergänzen durch die Feststellung, dass im sauren Bereich inaktivierte T.M.-Lösungen reaktiviert werden konnten durch Erhöhung des *p*H-Wertes (Fig. 1), während im alkalischen Bereich inaktivierte Lösungen irreversibel geschädigt, nicht wieder re-

aktivierbar waren. Ferner konnte er feststellen, dass durch Variation der Viruskonzentration zwei Aktivitätsmaxima auftreten, von denen das eine in der Gegend von *p*H 3.5, das zweite bei *p*H 7.5 liegt (Fig. 2).

Diese Untersuchungen waren in gepufferten Lösungen durchgeführt worden und konnten somit auf Grund der eingangs angeführten Bedenken gegen derartige Messungen im wesentlichen nur Anhaltspunkte über die Haltbarkeit der Viren geben, nicht jedoch über die spez. H'-Ionenwirk-

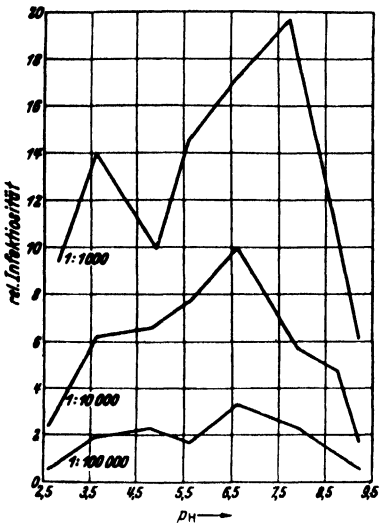



Fig. 2.—T.M.-Virus: Aktivitätsmaxima in Abhängigkeit von der Konzentration. (Nach G. A. Kausche.)

samkeit, den Einfluss des Ladungscharakters bezw. die Wertigkeit der Virusproteine selbst. In eigenen gemeinsamen Versuchen mit G. A. Kausche (13) konnte aber festgestellt werden, dass das T.M.-Virus in ungepufferten Lösungen sich durch Zentrifugieren in eine weniger saure und eine stärker saure Komponente zerlegen lässt (Fig. 3). Hierbei war die Komponente mit dem niederen *p*H-Wert weniger infektiös als die alkalischere. Es wurde hier also der eingangs erwähnte Fehler beim Zentrifugieren von ungepufferten Lösungen von Eiweisskörpern zur Ermittlung der Abhängigkeit der pathogenen Aktivität vom Ladungscharakter der Virusproteine herangezogen.

Bei den in Fig. 3 dargestellten Versuchen ergab sich nun ausserdem die interessante Tatsache, dass die zeitliche Änderung dieser Lösungen hinsichtlich des *p*H-Wertes entgegengesetzt verlief. Die gestrichelten Kurven in Fig. 3 zeigen den *p*H-Verlauf der Verdünnungsreihe von Sediment bezw. überstehender Flüssigkeit in Abhängigkeit von der Konzentration. Die ausgezogene Kurve zeigt die gleichen Lösungen nach einer gewissen Zeit, es scheint sich hier um die Einstellung eines Gleichgewichtszustandes zu handeln, wie man es von organischen Kolloiden (Seifenlösungen, Modellsubstanzen von Eiweisskörpern, etc.) her kennt, der lediglich durch das Zentrifugieren gestört war, eine Erscheinung, auf die noch später eingegangen werden soll.

Es konnte dann festgestellt werden, dass die Komponente mit dem niederen *p*H-Wert tatsächlich weniger infektiös war, durch Versuche, in denen

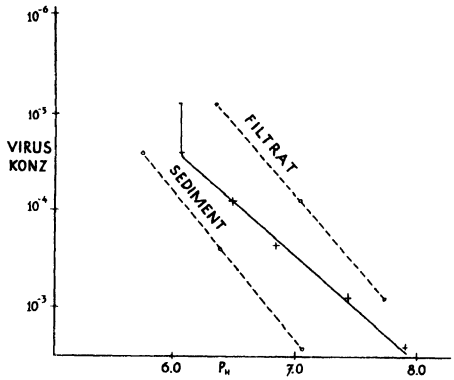



Fig. 3.—T.M.-Virus: Ladungstrennung durch Zentrifugieren. (Nach Holzapfel-Kausche.)

lediglich durch physikalische Eingriffe, z.B. Frieren der ungepufferten salzfreien Lösungen, eine Änderung des *p*H-Wertes herbeigeführt wurde und im Impftest die Lösungen mit den höheren Wasserstoffionenkonzentrationen weniger aktiv waren (13). Die elektrischen Ladungsverhältnisse sind ganz allgemein für verschiedene Virusarten noch umstritten. So wurden ausser den oben erwähnten Erscheinungen beim T.M.-Virus stark von einander abweichende Ergebnisse ermittelt. Die meisten Autoren haben für die untersuchten Virusarten (Pocken, Herpes, Poliomyelitis, M.K.S., Tollwut und Mosaikkrankheit) negative elektrische Ladung festgestellt. Guardabassi (14) stellte für T.M.-Virus und M.K.S.-Virus dagegen positive Ladung fest, während Olitzky und Boetz (15), Poppe und Busch (16) einen isoelektrischen Punkt für M.K.S.-Virus nachweisen konnten.

Der Existenzbereich für M.K.S.-Virus wird mit *p*H 7.0 bis 9.5 und einem Optimum bei *p*H 7.6 angegeben (17). Köbe (18) stellte bei Elektrophoreseversuchen fest, dass nur ein Bruchteil des vorhandenen Virus zur Anode wanderte unabhängig davon, wie lange der Versuch ausgedehnt wurde. Die Kathodenflüssigkeit selbst war nicht infektiös, steigerte aber die Aktivität des Anodenvirus um das Vierzigfache. Obzwar hier keine Parallelversuche zu den oben mitgeteilten von Holzapfel und Kausche am T.M.-Virus vorliegen, scheint die Analogie doch sehr ins Auge fallend, sodass man wohl für M.K.S.-Virus die Trennung in zwei Komponenten verschiedener Aktivität annehmen kann, die allerdings nicht notwendig, wie weiter unten ausgeführt werden wird, in jedem Fall zwei verschiedenen organischen Körpern angehören muss im Sinne eines Enzymsystems, das aus Trägersubstanz und aktiver Gruppe besteht, wobei beide sich wesentlich von einander unterscheiden. Trotzdem muss diese Möglichkeit natürlich im Auge behalten werden, obwohl es bisher noch nicht eindeutig gelungen ist, das infektiöse Agens mit chemischen oder physikalischen Mitteln von dem Trägerprotein zu trennen, und die Ansicht noch nicht widerlegt ist, dass der bisher in den einzelnen Fällen als Virusprotein isolierte Körper der Träger der biologischen Wirksamkeit ist.

## II. Einfluss von Bestrahlungen

Eine grössere Anzahl von Autoren hat die Wirkung von Röntgen -U.V.- und $\gamma$-Strahlen auf T.M.-Virus untersucht.

Bawden und Pirie (19) erhielten mit Röntgen- bezw. U.V.-Bestrahlung eine Inaktivierung von T.M.-Virus, G. A. Kausche und H. Stubbe (20, 21) fanden dagegen innerhalb eines bestimmten Dosierungsbereiches für Röntgen- bezw. $\gamma$-Strahlen Aktivierungseffekte. In eigenen Versuchen wurde ferner gemeinsam mit G. A. Kausche bei der Ermittlung der gleichzeitig damit auftretenden *p*H-Änderung U.V.-bestrahlter T.M.-Lösungen festgestellt, dass eine *p*H-Verschiebung ins alkalische Gebiet stattfand, wobei in kurzzeitig bestrahlten Lösungen (z.B. bei einem ungepufferten Ausgangs-*p*H von 6.97 und einer Konzentration von 3.15 mg. Virusprotein/ccm.) eine schwache Aktivitätserhöhung nachweisbar war, länger bestrahlte Lösungen ergaben die von den oben genannten Autoren nachgewiesenen Inaktivierungen.

Sichere Aussagen über die spez. Wirkung von Strahlung auf derartige Lösungen von Eiweisskörpern zu machen, ist nicht ohne weiteres möglich, weil hierbei die verschiedensten Effekte nebeneinander auftreten können und es von Fall zu Fall verschieden sein wird, welcher Effekt als Primäreffekt zu betrachten ist. So entsteht bei der Bestrahlung von Wasser und wässrigen Lösungen neben $H_2O_2$ freier Wasserstoff (22), sodass in wässrigen Lösungen als Sekundäreffekt von Bestrahlungen z.B. Hydrolyse und

Oxydationsreaktionen auftreten können neben einem Primäreffekt, der die gelösten Moleküle selbst betrifft; (so rechnet Löbering (23) z.B. mit der Möglichkeit, dass durch Röntgenstrahlen die Dipolmomente der Eiweisskörper verschoben werden).

Svedberg und Brohult (24) erhielten bei U.V.-Bestrahlung von Hämocyanin-Lösungen eine Aufspaltung in kleinere Bruchstücke, und zwar variabel je nach dem Ausgangs-*p*H-Wert. Cluzet und Ponthus (25) schliessen aus ihren Beobachtungen bei der Einwirkung radioaktiver Substanzen auf eiweisshaltige Medien auf eine chemische Desaminierung einerseits und eine Änderung der Teilchenladung sowie der Teilchengrösse anderseits. Die gleichen Effekte können natürlich auch bei der Bestrahlung von Virusproteinen angenommen werden. Auf die zahlreichen in diesem Zusammenhang durchgeführten Untersuchungen an Virusproteinen und infiziertem Gewebe sei daher nur kurz hingewiesen.

Auffallend ist weiterhin die Tatsache, dass Trockenpräparate durch Bestrahlung ebenso wie Viruslösungen inaktiviert werden (19), und zwar in der Sonne getrocknete Viruspräparate im Gegensatz zu solchen, die im Schatten getrocknet waren, wobei die letzteren ihre Infektiosität beibehielten. Dieser Umstand legt die Vermutung nahe, dass durch Strahlung aktivierter Sauerstoff eine Rolle spielt.

Eine Strahlenwirkung wird im wesentlichen eine Vermehrung oder Verminderung der biologisch aktiven Gruppen oder einen Teilchenzerfall hervorrufen. Der Nachweis im einzelnen wird jedoch oftmals leichter auf chemischem als auf physikalischem Wege zu erreichen sein. So konnte z.B. Stanley (26) mit Formaldehyd, $HNO_2$, und $H_2O_2$ eine Inaktivierung beim T.M.-Virus erhalten und beim näheren Studium des Einflusses von Formaldehyd konnten Ross und Stanley (27) eine Abnahme der freien Amino- und reduzierenden Gruppen nachweisen. Die Inaktivierung konnte durch Dialyse bei *p*H 3 wieder rückgängig gemacht werden unter gleichzeitiger Zunahme der Amino- und reduzierenden Gruppen.

In Übereinstimmung mit den bei Bestrahlungsversuchen auftretenden *p*H-Änderungen sowie den oben erwähnten Versuchen über den Ladungscharakter der Virusproteine scheinen freie Aminogruppen bei der Virusinfektion von besonderer Bedeutung, eine Annahme, die weiterhin bestätigt wird durch die in zahlreichen Untersuchungen nachgewiesene Zunahme des *p*H-Wertes in Tumorgeweben.

Bei einer Reaktion der Aminogruppen des Virusproteins mit Formamid kann die folgende Reaktion

$$RNH_2 + OHCR \rightarrow RN = CHR + H_2O$$

die Inaktivierung bewirken.* Diese Reaktion ist deswegen besonders interessant, weil sie vielleicht eine Brücke zu den Untersuchungen von Kögl und Erxleben (28) bildet. Werden z.B. racemische Amine mit optisch aktiven Aldehyden in Aldimine übergeführt, so können diese fraktioniert kristallisiert und im Anschluss daran in die optisch aktiven Amine aufgespalten werden (Erlenmeyer).

Bei der Einwirkung salpetriger Säure auf primäre Amine entstehen Alkohole neben freiem $N_2$. Hierbei wäre demnach auch keine Reaktivierung der mit $HNO_2$ behandelten Virusproteine zu erwarten, wenn dabei Gruppen primärer Amine angegriffen werden.

Der Einfluss von $H_2O_2$ ist unter der Annahme eines Angriffs auf freie Aminogruppen voraussichtlich als dehydrierende Autoxydation zu verstehen, da nach Wieland die Anwesenheit bezw. Bildung von $H_2O_2$ für den Verlauf dehydrierender Autoxydationen unerlässlich ist.

Im Gegensatz zu diesen Inaktivierenden Methoden könnte eine durch Wasserstoff bezw. $H^{\cdot}$-Ionen bewirkte Hydrolyse z.B. der RCONHR-Gruppen von Eiweisskörpern zu einer Aktivierung führen, wenn die Möglichkeit einer Anreicherung von Molekülen mit freien $NH_2$-Gruppen vorliegt. Über die möglichen Voraussetzungen hierzu wird weiter unten berichtet.

## III. Das Aggregationsproblem

Besondere Unklarheit besteht hinsichtlich der Aggregationstendenz der Eiweisskörper und Virusproteine, wie sie insbesondere bei den letzteren für T.M.- und M.K.S.-Virus diskutiert worden ist. Als erste hatten Bawden und Pirie (19) darauf hingewiesen, dass die Unterschiede von gereinigtem und ungereinigtem T.M.-Virus auf eine Aggregation der Virusteilchen zurückzuführen ist, gleichgültig ob die Reinigung auf chemischem Wege oder durch Ultrazentrifuge erfolgte, wobei die Autoren annahmen, dass die stäbchenförmigen Moleküle sich mit den Enden zusammenlagern. Diese Ansicht ist aber für T.M.-Virus erst in neuerer Zeit allgemein anerkannt worden.

Das Gleiche gilt für die Aggregatbildung bei M.K.S.-Virus, wie von G. Pyl (9) auf Grund der Temperatur- und Konzentrationsabhängigkeit der Lösungen angenommen wurde und zu einer lebhaften Diskussion Anlass gab, was umso erstaunlicher ist, da ausgezeichnete Untersuchungen über die Bildung von Aggregaten bei den verschiedensten anorganischen und

* Über Reaktionen von Formaldehyd mit Aminosäuren siehe auch, A. Wadsworth und M. C. Pangborn, *J. Biol. Chem.*, **116**, 423 (1936).

organischen Substanzen vorlagen, sodass auf die grosse Zahl von diesbezüglichen Untersuchungen nur kurz hingewiesen sei. In diesem Zusammenhang seien jedoch die Versuchsergebnisse von F. F. Nord und Mitarbeitern (3) mit sogenannten Eiweissmodellkörpern erwähnt, die in Analogie zu den Virusproteinen die gleichen Effekte ergaben und darüber hinaus Schlüsse über die Ursachen der Aggregatbildung gestatten (29, 30), auf die weiter unten eingegangen werden soll.

Über die theoretischen Voraussetzungen des Aggregationsmechanismus bestehen verschiedene Anschauungen, die im einzelnen bei Briegleb (31) bezw. Meyer-Mark (32) ausführlich zusammengestellt sind und auf die hier nur kurz Bezug genommen werden kann. So wird einerseits die starke Assoziationsneigung der Alkohole, Carbonsäuren, des $H_2O$ und $NH_3$ auf wellenmechanische Austauschresonanzeffekte zurückgeführt, andererseits als Folge einer Coulomb'schen Wechselwirkung bezw. van der Waals'scher

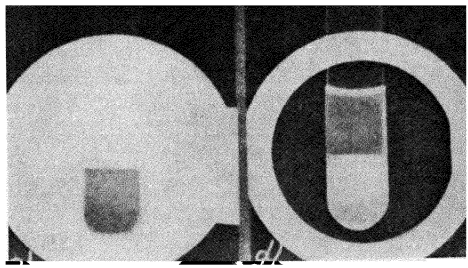


Fig. 4.—T.M.-Viruslösung, die sich beim Stehen in zwei Schichten getrennt hat. Die Aufnahme zwischen gekreuzten polarisierten Platten zeigt, dass die Bodenschicht spontan doppelbrechend ist, während die Oberschicht dieses Phänomen nicht besitzt. Die rechte Aufnahme bringt dasselbe System zwischen parallelen pol. Platten. (Nach M. A. Laufer, W. M. Stanley.)

Kräfte betrachtet. Die für den ersteren Fall postulierte "hydrogen bond" ist dabei nach Sidgwick so vorzustellen, als ob Wasserstoffatome jeweils mit einer bestimmten Frequenz zwischen zwei Molekülen den Platz tauschten. Darüber hinaus steht einer Anschauung nichts im Wege, die unter Annahme einer "Gruppenresonanz" nicht nur Elektronen (wie beim $H_2$-Molekül) oder z.B. H-Atome zwischen Molekülen für scheinbar austauschbar hält, sondern diese Ansicht auf ganze Molekülgruppen über-

trägt, wobei eine bestimmte Molekülgruppe bei genügender Annäherung und der entsprechenden sterischen Anordnung mit der gleichartigen eines Nachbarmoleküls in Resonanz geht, sodass diese Molekülgruppe mit einer bestimmten Frequenz wechselseitig in den Anziehungsbereich des Nachbarmoleküls gelangt.

Takahashi und Rawlins (40) (vgl. auch 40*a*) konnten feststellen, dass T.M.-Viruslösungen starke Strömungsdoppelbrechung zeigen. Im Zusammenhang damit konnten Bawden und Pirie (19) beobachten, dass konzentrierte Lösungen von T.M.-Virus sich beim Stehen in zwei Schichten trennen, von denen die untere doppelbrechend ist, während die obere erst beim Fliessen Strömungsdoppelbrechung zeigt (Fig. 4). Von Langmuir (33) wurde eine Erklärung hierfür zu geben versucht. Diese Erscheinung, die nach Bungenberg de Jong und Kruyt (34) mit Koazervation bezeichnet wird und bei organischen Solen ebenso aber auch bei anorganischen Substanzen, z.B. Bentonit und $V_2O_5$ beobachtet wurde, war von den beiden Autoren als durch die Entladung und Dehydratation bezw. Desolvatation der Kolloidpartikelchen verursacht angenommen worden. Die Koazervation ist nach diesen Autoren als Vorstufe der Koagulation zu betrachten. Langmuir (33) setzt sich mit der Anschauung verschiedener Forscher auseinander, die die Bildung der flüssig kristallinen Bodenphase auf van der Waals'sche Kräfte zurückführen, wobei er durch Einführung des osmotischen Druckes $p$ die in früheren Überlegungen vernachlässigte Berücksichtigung der Temperaturbewegung und der Anziehungskraft der Gegenionen mit in Rechnung stellt.

Betrachtet man nun einmal eine einfache Aminosäure vom Typus

$$NH_2RCOOH$$

vom Aggregationsstandpunkt, so ergeben sich folgende Gesichtspunkte: neben der Tendenz der Moleküle unter Bildung von Kondensaten miteinander zu reagieren, wobei die bekannten Eiweissketten entstehen können

$$\begin{matrix} R & NH & CO & R & NH & CO \\ NH & CO & R & NH & CO & R \end{matrix} + NH_2O$$

besteht die Neigung einzelner Molekülgruppen zu einer Zusammenlagerung unter Bildung von Doppel- und Mehrfach-Molekülen. Diese Zusammenlagerung kann naturgemäss nur bei nicht dissoziierten und nicht hydratisierten Molekülen zu Aggregation führen, da im ersten Fall die Abstossung der gleichsinnig geladenen Ionen, im zweiten die Blockierung durch die hydratisierenden Moleküle einer festeren Zusammenlagerung hinderlich wäre. Diese Vorstellung würde mit dem Zustand der Moleküle am Koagu-

lationspunkt und demnach der Auffassung von Bungenberg de Jong und Kruyt in Übereinstimmung sein.

Für das vorstehend genannte Beispiel einer einfachen Aminosäure würde bei einer Zusammenlagerung zweier Moleküle die Möglichkeit zur Bildung von drei verschiedenen Aggregationstypen bestehen:

(I*) $NH_2—R—C(O\cdot H\cdot O)(O\cdot H\cdot O)C—R—NH_2$

(II) $NH_2R\ COOH$
$NH_2R\ COOH$

(III) $COOH\ R\ NH_2$
$NH_2R\ COOH$

die, wenn man jedes Aggregat für sich betrachtet, charakteristische Unterschiede zeigen. Die Aggregate I bezw. III würden basische bezw. saure Endgruppen tragen, was z.B. bei Leitfähigkeit und Elektrophoreseversuchen in Erscheinung treten müsste ebenso wie bei *p*H-Messungen. Ist also die Wirksamkeit eines Eiweisskörpers oder Virusproteins an eine bestimmte Gruppierung der aktiven Gruppen am Molekül zurückzuführen, so können Abweichungen vom normalen Verhalten auf diese Weise zwangsläufig erklärt werden, und ebenso würden die oben angeführten Beispiele über den verschiedenartigen Ladungscharakter der Virusproteine verständlich. Die Änderung des Dispersitätsgrades kann von Bedeutung werden, wenn man sich vorstellt, dass bei einer Aggregation zwei aktive Gruppen von kleineren Partikeln, wenn dieselben sich zu einem grösseren Teilchen zusammenlagern, gegenseitig in ihrer freien Wirksamkeit hindern.

Immerhin wird diese Anschauung sowohl den eingangs erwähnten Annahmen von G. Pyl (9) wie der Ansicht von E. Pfankuch und G. A. Kausche (10) gerecht. Experimentell bewiesen ist sie u.a. z.B. für assoziierte Moleküle von Essigsäure und Buttersäure in Benzin, für die Smith und Rogers (35) ein elektrisches Moment 0 ermittelten, da im Benzin die Carboxylgruppen assoziiert sind. Untersuchungen von K. L. Wolf und Mitarbeitern (36) kann man als Modellversuche für Assoziationserscheinungen in diesem Zusammenhang betrachten.

Für die bei der Zusammenlagerung zweier Molekülgruppen in Frage stehenden Bindungsenergien, können die aus den Verdampfungswärmen sich errechnenden Molkohäsionen als Grundlage dienen, wie sie von M. Dunkel (37) für die wichtigsten Gruppen organische Moleküle abgeschätzt

* "Bindungsmechanismus zwischen Carboxylgruppen durch wellenmechanische Austausch-Wechselwirkung nach Gillette und Sherman," *J. Am. Chem. Soc.*, **58**, 1135 (1936).

worden sind, und zwar wären für das oben angeführte Beispiel einer einfachen Aminosäure folgende Werte zu berücksichtigen:

| Gruppe | Molkohäsion |
|---|---|
| $NH_2$ | 3530 cal. |
| COOH | 8970 " |
| R* = —$CH_2$— | 990 " |

Besonders hoch ist die Molkohäsion der $CONH_2$-Gruppe mit ca. 13,200 cal. und der CONH-Gruppe mit ca. 16,200 cal., was im Hinblick auf die grossen Eiweissmoleküle bedeutsam erscheint. Enthält also beispielsweise ein Eiweisskörper oder Virusprotein eine sterisch nicht behinderte $CONH_2$-Gruppe, so kann diese unter Umständen leicht zu einem Aggregat mit einer gleichartigen Gruppe eines anderen Moleküls führen; sind ferner auf Grund der Versuchsbedingungen die Mehrzahl der Moleküle mit diesen Gruppen aggregiert, so muss für die Lösung jeder Einzelbindung die Energie von 13.2 Kcal. aufgewandt werden, im Gegensatz zu einer $NH_2$-Gruppe mit 3.53 Kcal. bezw. COOH-Gruppe mit 8.97 Kcal.

Die Häufigkeit, mit der die verschiedenen Komponenten positiv neutral oder negativ geladene Partikel vom monodispersen Einzelteilchen bis zum Mizellaggregat† in einem gegebenen System vorhanden sind, wird einem Gleichgewichtszustand zustrebend ausser durch die Konzentration der verschiedenen Verbindungen des Gesamtsystems noch durch die Temperatur und vielfach die Vorgeschichte bestimmt. (Praktisch kann man die Aggregation verhindern durch Zusatz von Dispergierungsmitteln und Arbeiten in grosser Verdünnung, sodass die dispergierten Teilchen nicht miteinander in Wechselwirkung treten können, man kann sie begünstigen durch Konzentrationserhöhung sowie Herabsetzung des Dissoziationsgrades.)

Dass tatsächlich auch die Virusproteine in Lösungen einen Gleichgewichtszustand zustreben, kann aus den Untersuchungen von Kausche und Holzapfel (p. 46) geschlossen werden, besonders aber auch aus den Versuchen von G. Pyl durch den Nachweis, dass die Infektionsfähigkeit einer Lösung von M.K.S.-Virus von der Temperatur reversibel abhängig ist (9), wobei die Gleichgewichtseinstellung jedoch keinesfalls momentan erfolgen

* R kann hierbei eine beliebige Grösse sein, je nachdem welcher Körper in Frage kommt. Der jeweilige Aggregationswert für R ergibt sich näherungsweise aus der Tabelle von Dunkel.

† Die Möglichkeit zur Bildung der verschiedenen Lösungssysteme molekulardispers, kolloidal bezw. mizellar besteht prinzipiell für jede organische oder anorganische Substanz und ist von den oben genannten Bedingungen abhängig; (vgl. hierzu Meyer-Mark, "Hochpolymere Chemie," I, 298, Leipzig, 1940). Mark, "Physical Chemistry of High Polymeric Systems," New York, 1940, p. 292.

muss, sondern sich über grössere Zeiträume erstrecken kann (vgl. hierzu 30).

Es besteht also die Möglichkeit, durch Störung des jeweiligen Gleichgewichtes, sei es durch physikalische Eingriffe (Sedimentieren, Temperaturerhöhung bezw. -erniedrigung, Bestrahlung) oder durch chemische Zusätze die Viruswirksamkeit zu erhöhen oder zu vermindern.

Gegenüber chemischen Einflüssen ist das Aggregationsproblem naturgemäss von grösster Bedeutung, wenn es sich z.B. um oxydativen Abbau einzelner Gruppen, um Umaminierung, Methylierung oder dergl. handelt. Sind die fraglichen Molekülteile aggregiert oder infolge aggregierter Nachbargruppen blockiert, so kann eine entsprechende chemische Reaktion weitgehend verzögert oder verhindert werden. Um bei unserem oben angeführten Modell zu bleiben, würde z.B. eine Decarboxylierung der Komponente I gegenüber der Komponente III stark erschwert sein. Ähnliche Beispiele lassen sich beliebig viele anführen. So könnten sie möglicherweise auch bei den von M. Samec (38) durchgeführten Untersuchungen an Stärkesubstanzen die charakteristischen Abweichungen bei Erythroamylosen und Amyloamylosen durch Besonderheit der Mizellarstruktur infolge verschiedenartiger Aggregation erklären.

Zur Bildung von Aggregaten ist aber bei gegebener Temperatur naturgemäss immer eine Mindestkonzentration notwendig, sodass es durchaus möglich erscheint, dass gewisse Wirkstoffe im Organismus, solange sie in kleinsten Mengen anwesend sind, günstig wirken können, sobald jedoch eine zu hohe Konzentration vorhanden ist, oder eine Erhöhung der Temperatur die Stosszahl der fraglichen Moleküle untereinander so heraufsetzt, sodass gleichartige Moleküle miteinander aggregieren, kann die günstige Wirkung der betreffenden Substanz durch eine Blockierung der wichtigen Molekülgruppen (z.B. Aggregation von OH- bezw. COOH-Gruppen) in eine gegenteilige Wirkung umschlagen und möglicherweise schädigende Einflüsse (z.B. Schädigung des Redoxsystems) auslösen. Es ist möglich, dass die Viruswirksamkeit in manchen Fällen auf ähnlichen Ursachen beruht.

## IV. Gestalt und Teilchengrösse

Die Grösse und Gestalt der Viren ist oftmals verantwortlich gemacht worden für die Aktivität dieser Proteine und infolgedessen Gegenstand zahlreicher Untersuchungen, doch besteht über die letzte infektiöse Einheit noch keine eindeutig gesicherte Bestimmung.

Die bisherigen Untersuchungen phytopathogener Virusarten haben die

Ansicht von W. M. Stanley (39) bestätigt, dass es sich hierbei um einen Eiweisskörper von Nucleoproteincharakter handelt. Zahlreiche verschiedene Messmethoden gaben fernerhin sichere Anhaltspunkte über die Gestalt. So konnte in den Versuchen von Takahashi und Rawlins (40)




Fig. 5.—T.M.-Virus elektronenoptische Aufnahme. (Nach G. A. Kausche und H. Ruska (41*a*).)

die Ansicht von Bawden und Pirie (19), Bernal und Fankuchen (47), bestätigt werden, dass T.M.-Virus stäbchenförmige Gestalt besitzt, eine Annahme, die dann besonders eindrucksvoll in den elektronenoptischen Aufnahmen von G. A. Kausche und H. Ruska (41*a*) zum Ausdruck kommt (Fig. 5). Im Gegensatz hierzu zeigen die Menschen- und Tier-pathogenen Viren in den elektronenoptischen Aufnahmen neben sphärischen Teilchen z.T. längliche und zerflossene Gebilde. Die ersten elektronenoptischen Virusaufnahmen wurden von F. Krause (42) mit Vakzinevirus gemacht, doch leiden diese ebenso wie neuere elektronenoptische Aufnahmen am M.K.S.-Virus von G. Pyl und von M. Ardenne (43) unter Abbildungsschwierigkeiten infolge des geringen Kontrastes, es ergaben sich aber in beiden Fällendeutlich etwas zerflossene längliche oder runde Partikelchen.

Bei den Grössenbestimmungen der verschiedenen Viren hatte Thornberry (44) mit Filtrationsversuchen nach Elford (45) einen Teilchendurchmesser für verschiedene Pflanzenviren und T.M.-Virusarten von 15 bis 22 mμ bezw. für gereinigtes Virus von 11–17 mμ ermittelt. Wyckoff

(46) berechnete einen Teilchendurchmesser von 12.3 mμ für T.M.-Virus und aus den röntgenographischen Aufnahmen (47) ergab sich ein Wert von etwa 15 mμ. Dieser Wert steht auch mit den Grössen in Übereinstimmung, die aus weiteren Untersuchungen ermittelt wurden (48). In Ergänzung hierzu ergaben die elektronenoptischen Aufnahmen (41) einen Teilchendurchmesser von etwa 15 mμ. Über die Bedenken hinsichtlich der Genauigkeit der verschiedenen Methoden berichten Kausche, Pfankuch, und Ruska (41).

Der Durchmesser von M.K.S.-Virus wurde bei Ultrafiltrationsversuchen mit 8 bis 12 mμ gefunden. Für die länglichen Gebilde der elektronenoptischen Aufnahmen des Vakzinevirus gibt Krause (42) eine Breite von 0.15 bis 0.2μ und eine Länge von 0.4 bis 0.6μ an.

Besonders aufschlussreich waren die elektronenoptischen Aufnahmen hinsichtlich der Länge der T.M.-Virusproteine. Wyckoff (46) hatte durch Ultrazentrifugenversuche nachgewiesen, dass Teilchen der Grösse 15 × 150 mμ infolge Aggregation leicht in ein Aggregat mit höherer Sedimentationskonstante übergehen, Kausche, Pfankuch, und Ruska (41, 41*a*) konnten nun zeigen, dass Teilchen der verschiedensten Länge nebeneinander existieren und z.T. zu faserigen Gebilden verknüpft sind. Diese Mizellen waren auf Grund des physikalischen Verhaltens der Proteinlösungen gleichfalls zu erwarten. Das bevorzugte Auftreten einzelner Stäbchenlängen bestimmte diese Autoren zu der Ansicht, dass Viren mit der Länge von 150 mμ und 300 mμ Träger der kleinsten infektiösen Einheit sind. Neben diesen Teilchen zeigten die elektronenoptischen Aufnahmen jedoch hin und wieder kleinere kugel- oder kurzstäbchenförmige Gebilde, die die Verfasser als Bruchstücke der Virusmoleküle betrachten und annehmen, dass sie nicht als Träger der pathogenen Aktivität in Frage kommen. Sie begründen diese Annahme mit der Tatsache, dass es gelingt, mit chemischen Agenzien die Teilchen der oben angeführten Grössen in kleinere Bruchstücke aufzuspalten, die dann pathogen inaktiv sind.

Diese Schlussfolgerung wäre nur dann zutreffend, wenn nachgewiesenermassen in einem solchen Fall keine chemische Veränderung am Molekül stattfinden könnte. Dass eine solche chemische Veränderung leicht stattfindet, ist weiter oben erörtert worden.

G. Pyl (9) konnte, wie bereits mehrfach erwähnt, lediglich durch thermische Änderungen des Dispersitätsgrades eine Infektionserhöhung nachweisen, allerdings liegen zu diesen Versuchen noch keine genauen Teilchengrössenbestimmungen vor, sodass hier der Einwand berechtigt ist, dass die kleineren entstehenden Aggregate lediglich Bruchstücke der grossen Mizellaggregate sind und eine untere Grenze der Teilchengrösse vorhanden

ist, die mit den angegebenen Grössen übereinstimmt, während kleinste Teilchen inaktiv sind. Derselbe Einwand trifft bei den Kryolyseversuchen zu (49, vgl. auch (29) und (30)), bei denen gleichfalls lediglich durch einen physikalischen Eingriff (Frieren und Wiederauftauen) eine auf eine Teil-

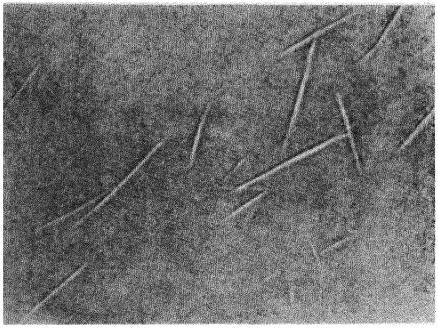
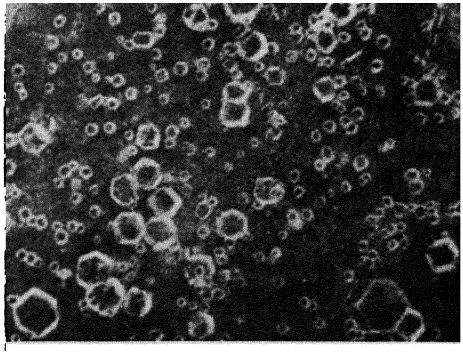


Fig. 6.—(*a*) T.M.-Virus krist. Proteinnadeln mit zweidimensionaler Regelmässigkeit. (Nach W. M. Stanley.)



(*b*) Bushy Stunt—Viruskristalle mit dreidimensionaler Regelmässigkeit. (Nach F. C. Bawden und N. W. Pirie.)

chenverkleinerung zurückzuführende Aktivitätserhöhung stattfand. Völlige Klarheit könnten hier erst neuere Untersuchungen bringen, bei denen unter jeweiliger Teilchengrössenbestimmung mit physikalischen Methoden

ohne chemische Änderungen Virusproteine in Lösung dispergiert bezw. aggregiert und hinsichtlich der pathogenen Aktivität kleinster Teilchen untersucht werden.

Die Tatsache, dass bei einigen Viren Stäbchenform, bei anderen z.T. sphärische Formen auftreten, machen es bei oberflächlicher Betrachtung nicht wahrscheinlich, dass die Gestalt der Viren die Ursache der Infektionsfähigkeit ist. Dagegen ist die aussergewöhnliche Grösse der Virusproteine möglicherweise eine Folge ihrer Reaktionsfähigkeit und damit Infektionsfähigkeit, die die einzelnen Teilchen zu erhöhter Aggregation und somit zur Bildung grosser Mizellaggregate befähigt. Immerhin bleibt aber zu bedenken, dass die Denaturierung von Eiweisskörpern sich dahingehend

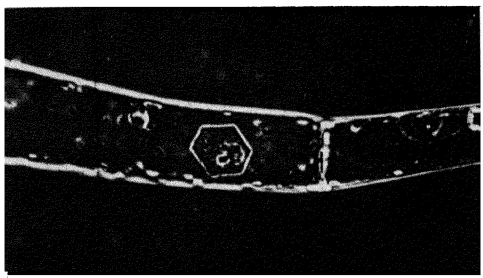
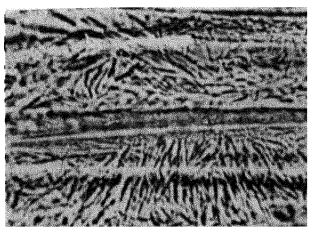
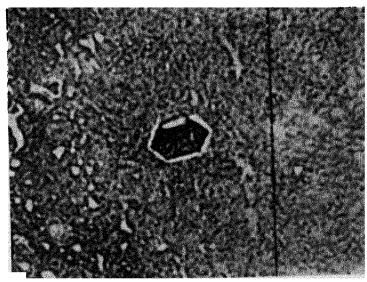


Fig. 7.– (*a*) T.M.-Virus: hexagonale Kristalle in Epidermis und Haarzellen von Nikotiana tab. Samsun. (Nach G. A. Kausche.)



(*b*) Epidermiszelle von türk. Tabak, bei der die hexagonalen Kristalle durch Zusatz von HCl in Nadeln übergeführt wurden. (Nach H. Purdy-Beale.)



(*c*) Hexagonale Kristalle aus T.M.-Viruslösung in vitro. (Nach G. A. Kausche.)

deuten liess, dass die sphärischen Teilchen in eine weniger lösliche gestreckte Form übergingen.

Neben den angeführten Proteinnadeln und sphärischen Teilchen sind die Viren befähigt dreidimensionale wohlausgebildete Kristalle zu bilden (Fig. 6). Die Entstehung dieser Kristalle scheint jedoch an gewisse Voraussetzungen geknüpft zu sein. H. Purdy-Beale (50) glaubte das Auftreten der hexagonalen Kristalle auf einen Übersättigungszustand des Gewebes mit molekular gelöstem Virus in Verbindung zu bringen. Es gelang ihr auch im Gewebe selbst durch Einführung von HCl die hexagonalen Kristalle des T.M.-Virus in die bekannten Stäbchen zu überführen. Kausche (51) ist es dann erstmalig gelungen, die Nadelaggregate in Vitro in die hexagonale Kristallform überzuführen und somit die Annahme von H. Purdy-Beale über den Zusammenhang dieser beiden Zustandsformen eindeutig zu beweisen (Fig. 7). Die Tatsache, dass der H-Ionenkonzentration bei den Virusproteinen eine besondere Rolle zukommt und nach den Versuchen von H. Purdy-Beale auch die Kristallisation derselben beeinflussen kann, spricht dafür, dass ein spezifischer Einfluss der Bindungsart der Moleküle untereinander besteht. Es soll deshalb noch kurz in diesem Zusammenhang darauf hingewiesen werden, dass z.B. Nahrungsmittel und Heilmittel nach H. G. Grimm (52) dreidimensionale van der Waals'sche bezw. zwischenmolekulare Bindungen aufweisen. Da der Bindungstyp für Eigenschaften und Verhalten der verschiedenen Körper von grösster Bedeutung ist, so kann hierin möglicherweise eine der Ursachen der Viruswirksamkeit liegen. Eine Möglichkeit zur Beeinflussung der verschiedenen Bindungsarten ergibt sich aus den vorstehend gemachten Ausführungen.

Es war nicht die Aufgabe, der vorliegenden Arbeit, einen Überblick über das gesamte Virusgebiet zu geben, da hierüber bereits z.T. ausgezeichnete Zusammenfassungen vorliegen. Es sollte im wesentlichen die Blickrichtung auf gewisse spezielle Probleme der Virusforschung im Zusammenhang mit neueren Gesichtspunkten aus anderen Forschungsgebieten gelenkt werden. Hierbei konnten aus der Fülle des auf dem Virusgebiet vorliegenden Untersuchungsmaterials einige Tatsachen zusammengestellt werden, die auf eine besondere Bedeutung für die Frage der Virusaktivität schliessen lassen, so die *p*H-Abhängigkeit, der Ladungscharakter der Proteine, der Dispersitätsgrad, und die Bedeutung chemisch aktiver Gruppen, sowie die generelle Bedeutung des Aggregationsproblems.

### Literaturverzeichnis

1. V. L. Frampton, *J. Biol. Chem.*, **129**, 233 (1939); *Science*, **90**, 305 (1939).
2. O. Waldmann, *Naturwissenschaften*, **20**, 129 (1932).

3. F. F. Nord, *Ibid.*, **24**, 781 (1936); F. F. Nord und F. E. M. Lange, *Ibid.*, **23**, 722 (1935); H. Leichter, G. Umbach, und F. F. Nord, *Biochem. Z.*, **291**, 191 (1937); vgl. auch K. Freudenberg, *Naturwissenschaften*, **27**, 22 (1939).

4. J. W. Beams, *Rev. Modern Phys.*, **10**, 257 (1938).

5. E. von Wöhlisch, *Zentr. exp. Med.*, **40**, 137 (1924).

6. G. von Farkas und B. Groák, *Zeitschr. exp. Med.*, **66**, 596 (1929).

7. G. S. Adair und M. E. Adair, *Biochem. J.*, **28**, 1230 (1934); vgl. auch H. Freundlich, "Kapillarchemie II," Leipzig, 1932, p. 344; A. S. MacFarlane, *Trans. Faraday Soc.*, **1940**, 258; Künzel, *Biochem. Z.*, **209**, 419 (1929).

8. A. Tiselius, *Nova Acta Regiae Soc. Sci. Upsaliensis*, **7**, 4 (1930); A. Tiselius und H. Svenson, *Trans. Faraday Soc.*, **36**, 19 (1940).

9. G. Pyl, *Z. Hygiene*, **114**, 501 (1932).

10. E. Pfankuch und G. A. Kausche, *Biochem. Z.*, **299**, 344 (1938).

11. W. M. Stanley, *J. Phys. Chem.*, **42**, 62 (1938).

12. G. A. Kausche, *Naturwissenschaften*, **28**, 61 (1940).

13. G. A. Kausche und L. Holzapfel, *Ibid.*, **28**, 61 (1940).

14. M. Guardabassi, *Riv. Biol.*, **11**, 615 (1929).

15. P. K. Olitzky und L. Boetz, *J. Exp. Med.*, **45**, 685 (1927).

16. K. Poppe und G. Busch, *Z. Immun. Forschung*, **68**, 510 (1930).

17. G. Pyl, *Naturwissenschaften*, **20**, 132 (1932).

18. K. Köbe, *Zbl. Bakter. I Org.*, **123**, 285 (1932).

19. F. C. Bawden und N. W. Pirie, *Proc. Roy. Soc.*, **(B)123**, 274 (1937).

20. G. A. Kausche und H. Stubbe, *Naturwissenschaften*, **26**, 740 (1938).

21. G. A. Kausche, *Ibid.*, **26**, 741 (1938).

22. Vgl. u.a. O. Risse, *Z. physik. Chem.*, **(A)140**, 133 (1929); W. Duane und O. Scheuer, *Le Radium*, **10**, 33 (1913); H. Fricke und E. I. Hart, *J. Chem. Phys.*, **3**, 56 (1935); P. Günther und L. Holzapfel, *Z. physik. Chem.*, **(B)42**, 346 (1939).

23. I. Löbering, *Ber.*, **70**, 1963 (1937).

24. The Svedberg und Sv. Brohult, *Nature*, **142**, 830 (1938).

25. I. Cluzet und P. Ponthus, *Arch. phys. biol.*, **14**, 5 (1937).

26. W. M. Stanley, *Ergeb. Physiol.*, **39**, 301 (1937) (vgl. auch 11).

27. A. F. Ross und W. M. Stanley, *Proc. Soc. Exp. Biol. Med.*, **38**, 260 (1938).

28. F. Kögl und H. Erxleben, *Z. physiol. Chemie*, **258**, 57 (1939); *Klin Wochschr.*, **18**, 801 (1939).

29. L. Holzapfel und F. F. Nord, *Ber.*, **71**, 1217 (1938); L. Holzapfel und F. F. Nord, *Biodynamica*, **3**, No. 57 (1940).

30. L. Holzapfel, *Kolloid-Z.*, **85**, 272 (1938).

31. G. Briegleb, "Zwischenmolekulare Kräfte und Molekülstruktur," Stuttgart, 1937.

32. Meyer-Mark, "Hochpolymere Chemie I," Leipzig, 1940. Mark, "Physical Chemistry of High Polymeric Systems," New York, 1940, p. 292.

33. I. Langmuir, *J. Chem. Phys.*, **6**, 873 (1938).

34. Bungenberg de Jong und Kruyt, *Kolloid-Z.*, **50**, 39 (1930); vgl. auch Bungenberg de Jong und Bekker, *Biochem. Z.*, **212**, 318 (1929); **221**, 403 (1930).

35. Smith und Rogers, *J. Am. Chem. Soc.*, **52**, 1824 (1938).

36. Z.B. K. L. Wolf, H. Fram, und H. Harms, *Z. physik. Chem.*, **(B)36**, 237 (1937).

37. M. Dunkel, *Ibid.*, **(A)138**, 42 (1928); M. Dunkel und K. L. Wolf, *Müller-Pouillet Lehrbuch der Physik*, IV, 3, 578.

38. M. Samec, *Ber.*, **73**, 89 (1940).
39. W. M. Stanley, *Phytopathology*, **26**, 305 (1936).
40. Takahashi und Rawlins, *Proc. Soc. Exp. Biol. Med.*, **30**, 155 (1932); *Science*, **77**, 26 und 284 (1933); **85**, 103 (1937).
40*a*. M. A. Laufer und W. M. Stanley, *J. Biol. Chem.*, **123**, 50 (1938).
41. G. A. Kausche, E. Pfankuch, und H. Ruska, *Naturwissenschaften*, **27**, 292 (1939).
41*a*. G. A. Kausche und H. Ruska, *Biochem. Z.*, **303**, 221 (1939).
42. F. Krause, *Naturwissenschaften*, **26**, 122 (1938).
43. G. Pyl und M. v. Ardenne, *Ibid.*, **28**, 531 (1940).
44. Thornberry, *Phytopathology*, **25**, 36, 601, 938 (1935).
45. Elford, *Proc. Roy. Soc.* (London), **(B)112**, 384 (1933).
46. R. W. G. Wyckoff, *J. Biol. Chem.*, **121**, 122, 219, 239 (1937).
47. J. S. Bernal und Fankuchen, *Nature*, **139**, 923 (1937).
48. Neurath und Saum, *J. Biol. Chem.*, **126**, 435 (1938); V. L. Frampton und I. Neurath, *Science*, **87**, 468 (1938); M. Laufer, *Ibid.*, **87**, 469 (1938); M. Laufer, *J. Biol. Chem.*, **126**, 443 (1938).
49. I. Remesov, *Bull. Biol. Med. Exper. U.R.S.S.*, **3**, 606 (1937).
50. H. Purdy-Beale, *Contrib. Boyce Thompson Inst.*, **8**, 413 (1937).
51. G. A. Kausche, *Naturwissenschaften*, **27**, 77 (1939).
52. H. G. Grimm, *Ibid.*, **27**, 1 (1939).

# THE SPECIFICITY OF PROTEINASES

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Proteolytic enzymes are classified into proteinases and peptidases. The proteinases are supposed to split genuine proteins, as well as proteoses and peptones, into polypeptides. The polypeptides, in turn, are assumed to be hydrolyzed by the peptidases, finally yielding a mixture of amino acids. This concept is based on the assumption that the specificity of proteolytic enzymes is adapted to the molecular size of their substrates, thus enabling the enzymes to differentiate between substrates of high and low molecular weight. The proteinases have been supposed to act exclusively on substrates of high molecular weight but not to act on lower polypeptides and dipeptides. The opposite is assumed for the peptidases. However, no chemical basis for the restriction of the action of proteinases on substrates of high molecular weight has been provided (1–3).

The proteinases are further classified into three groups in the well-known hypothesis that pepsinases attack protein cations, tryptases digest protein anions, while papainases act upon protein zwitterions. In a certain analogy, peptidases have been separated into three groups which attach themselves to the amino or carboxyl groups of their substrates or to both kinds of groups simultaneously.

The concept that the specificity of the proteolytic enzymes is adapted to a few physical properties of the substrates, such as their molecular size and ionic nature, has been generally accepted because of its simplicity. It should be remembered, however, that this concept is only a working hypothesis which is insufficiently supported by convincing experimental evidence. For example, the conclusion that pepsin does not act on low molecular weight substrates was based on a few experiments with synthetic substrates containing only glycine, alanine, and leucine (1). Furthermore, several investigators have observed that during the action of proteinases on proteins, free amino acids are liberated in significant amounts (4–6). The theory is particularly unsatisfactory in the case of proteinases since it does not reveal whether the attack of the enzymes on proteins is a haphazard one or whether it is directed toward particular points in the substrate, and what the specific nature of these points of attack may be. This ambiguity makes it impossible to differentiate the specific action of the proteinases belonging to one of the three groups; thus the members of the group of tryptases (*e. g.*, beef trypsin and beef chymotrypsin) cannot be considered identical in their specific action, but no hint as to the differences between the action of the individual tryptases is given by the ionic theory.

A protein molecule may contain several hundred amino acid residues representing about 20 different kinds of amino acids. Therefore, each kind of amino acid is repeated several times throughout the protein molecule to form a rather intricate pattern. The frequency with which each kind of amino acid is repeated and the resulting pattern determine the biological properties of the protein. This influence of the general pattern also finds expression in the enzymatic behavior of each peptide bond. The sensitivity of an individual peptide bond is determined largely by the nature of the adjacent amino acid residues. Until recently, theories of the specificity of proteinases did not take into account such differences in the specific nature of the amino acid residues and peptide bonds. It has long been known that various proteins differ in their digestibility by certain enzymes; however, the specific influence of the various amino acid residues was unknown and therefore it was impossible to advance an unambiguous explana-

tion for such differences and still less possible to test such explanations experimentally.

Serious difficulties are encountered if one attempts to use proteins for a

TABLE I

SYNTHETIC SUBSTRATES FOR PROTEINASES

| Enzyme | Substrate | Remarks |
|---|---|---|
| Pepsin | Glycyl-*l*-glutamyl\|*l*-tyrosine | |
| | Carbobenzoxy-*l*-glutamyl\|*l*-tyrosine | |
| Trypsin | Benzoylglycyl-*l*-lysine\|amide | |
| | Benzoyl-*l*-arginine\|amide | |
| Chymotrypsin | Benzoyl-*l*-tyrosyl\|glycineamide | |
| Cathepsin (Beef Spleen) | Carbobenzoxy-*l*-glutamyl\|*l*-tyrosine | |
| | Benzoyl-*l*-arginine\|amide | |
| | Carbobenzoxy-*l*-leucylglycyl\|glycine | |
| Papain | Benzoylglycine\|amide | Presence of activator (cysteine, HCN, etc.) required for hydrolysis |
| | Benzoyl-*l*-arginine\|amide | |
| Bromelin | Carbobenzoxyglycyl\|*l*-glutamylglycineamide | |
| Ficin | Benzoyl-*l*-arginine\|amide | |

study of the interdependence of the pattern of the amino acids in the substrate and the specific action of a proteinase. In order to determine the precise nature of the action of an enzyme on a protein molecule, one needs to know the exact structure of the protein, and it would also be necessary to identify the split-products produced through the action of the enzyme on the protein. It will readily be seen that such a task is at present completely outside the range of our experimental possibilities. Let us suppose, for example, that an enzyme such as trypsin hydrolyzes a protein consisting

TABLE II

COMPARISON OF RATES OF HYDROLYSIS OF SYNTHETIC SUBSTRATES AND PROTEINS

| Enzyme | Substrate | Substrate per cc., mg. | *p*H | Time, hrs. | Hydrolysis | | $NH_2$-N per mg. of substrate, mg. |
|---|---|---|---|---|---|---|---|
| | | | | | Per cent | Increase in $NH_2$-N per cc., mg. | |
| Pepsin | Edestin | 20 | 2.0 | 24 | .. | 0.25 | 0.012 |
| 1.0 mg. of protein N per cc. | Carbobenzoxy-glutamyltyrosine | 22 | 4.1 | 24 | 38 | 0.27 | 0.012 |
| Trypsin* | Casein | 40 | 7.3 | 1 | .. | 0.19 | 0.005 |
| 0.007 mg. of protein N per cc. | α-Hippurylly-sineamide | 17 | 7.2 | 1 | 35 | 0.25 | 0.015 |
| HCN-Papain | Gelatin | 40 | 5.0 | 2 | .. | 0.55 | 0.014 |
| 0.05 mg. of protein N per cc. | Benzoylarginine-amide | 18.5 | 5.0 | 2 | 80 | 0.57 | 0.03 |

* These values were determined by Dr. K. Hofmann.

of 144 amino acid residues at 12 peptide bonds; then, even if the structure of the protein were known, there is no method available which enables one to identify the 13 products of hydrolysis and thus to establish the points of scission within the protein molecule.

In order to overcome this difficulty, methods for the synthesis of peptides and substituted peptides derived from many kinds of amino acids have been developed. A great number of these peptides have been tested with regard to their sensitivity toward the various proteolytic enzymes.

It was found that many of these synthetic substances were hydrolyzed by proteinases such as pepsin, the trypsins (trypsin and chymotrypsin), the cathepsins (liver, spleen, kidney, tumor), papain, ficin, and bromelin. A selection of these substrates is given in Table I.

## I. Role of Molecular Weight of the Substrate

The experimental findings summarized in Table I establish the fact that the specificity of proteinases is by no means adapted only to substrates of high molecular weight. Crystalline pepsin, crystalline trypsin, and crystalline chymotrypsin split substrates of molecular weights not higher than 300–400. Papain hydrolyzes hippurylamide (molecular weight 178) and leucineamide* (molecular weight 130). Furthermore, it will be noted from Table II that the speed with which the low molecular weight substrates are hydrolyzed by proteinases is in some cases of the same order of magnitude as the splitting of genuine proteins.

It is clear, therefore, that the differentiation of proteinases and peptidases on the basis of the molecular weight of their substrates has now lost its validity.

## II. The Nature of the Linkages Split by Proteinases†

The structure of most of the hitherto synthesized substrates for proteinases is rather simple, so that they are split by the proteinase at only one peptide bond. Under these conditions, the products of the hydrolysis may easily be isolated and identified. All the well-established proteinases have been found in these experiments to be adapted to the hydrolysis of genuine peptide bonds. This is a strong indication that in the action of proteinases upon proteins peptide bonds are hydrolyzed.

About 15 years ago it had been observed by Waldschmidt-Leitz and Künstner (7) that during the action of proteinases upon proteins, basic and acidic groups were liberated in approximately equivalent amounts. This result was interpreted as indicating the hydrolysis of peptide bonds. While the conclusions of these authors agree with the results obtained with substrates of low molecular weight, these experiments with proteins cannot be regarded as being decisive. During the hydrolysis of a protein

* Investigations are in progress to determine whether leucineamide and hippurylamide are split by the same enzymatic constituent of papain.

† In this paper the term "proteinase" denotes those proteolytic enzymes that attack proteins.

by a proteinase, a multitude of linkages are hydrolyzed in each protein molecule and the analytical determinations applied by Waldschmidt-Leitz and his collaborators merely compare the sum of the many basic

TABLE III

BEHAVIOR OF SYNTHETIC SUBSTRATES TOWARD CRYSTALLINE SWINE PEPSIN

| Substrate | Hydrolysis, per cent | |
|---|---|---|
| | 24 hrs. | 48 hrs. |
| Carbobenzoxy-*l*-glutamyl\|*l*-tyrosine | 53 | .. |
| *l*-Glutamyl-*l*-tyrosine | .. | 3 |
| Glycyl-*l*-glutamyl\|*l*-tyrosine | 32 | .. |
| Carbobenzoxyglycyl-*l*-glutamyl\|*l*-tyrosine | 48 | .. |
| Carbobenzoxy-*l*-glutamyl\|*l*-tyrosylglycine | 39 | .. |
| Carbobenzoxy-*l*-glutamyl-*l*-tyrosineamide | .. | 15 |
| Carbobenzoxy-*l*-glutaminyl-*l*-tyrosineamide | .. | 3 |
| Carbobenzoxy-*l*-glutamyl\|*l*-phenylalanine | 26 | .. |
| Carbobenzoxy-*l*-glutamyl-*d*-phenylalanine | .. | 2 |
| Carbobenzoxy-*d*-glutamyl-*l*-tyrosine | .. | 0 |
| Carbobenzoxy-*l*-glutamyl-*l*-diiodotyrosine | .. | −1 |

*p*H 4.0; enzyme concentration, 1.4 mg. of protein N per cc.

groups produced with the sum of the many acidic groups liberated. Neither homogeneous proteins nor homogeneous proteinases had been employed in this method. Moreover, the ratio of amino to carboxyl groups found in the first stages of enzymatic hydrolysis deviated appreciably from equivalency. Therefore, a more precise proof, such as that obtained by means of the low molecular weight substrates, was highly desirable.

Linderstrøm-Lang, Hotchkiss, and Johansen (8) have advanced the interesting hypothesis that native proteins do not contain peptide linkages and are not attacked directly by proteinases. The denatured form of the protein, on the other hand, is assumed to contain peptide bonds and therefore to be susceptible to proteinase action. When a proteinase digests the small amounts of denatured protein present in each sample of the native protein, more denatured protein is formed and subsequently digested by the enzyme, the native and denatured form of the protein being assumed to be in equilibrium.

It is well known that peptide bonds may exist in a number of desmotropic forms which may be converted into one another under the proper conditions.* On the other hand, no structural combination of amino acids, which is different from a peptide bond but can easily be converted into a peptide linkage by an equilibrium reaction, is known. It seems justifiable, therefore, to interpret the theory of Linderstrøm-Lang, Hotchkiss, and Johansen as postulating the possible presence in proteins of desmotropic forms of peptide linkages that are resistant to proteinases but can easily be converted into forms which are hydrolyzable by proteinases. Our conclusion that proteins must contain linkages similar to those present in the synthetic substrates is not rendered invalid by the considerations of Linderstrøm-Lang, Hotchkiss, and Johansen.

## III. Specificity of Gastro-Intestinal Proteinases

### *1. Pepsin*

Crystalline swine pepsin splits a group of peptides containing *l*-tyrosine or *l*-phenylalanine, *e. g.*, carbobenzoxyglutamyltyrosine, glycyl-*l*-glutamyl-

* Such isomerization of peptide bonds has already been considered in earlier theories on the action of proteolytic enzymes (9). The possible isomerization of peptide bonds may give rise to a large number of desmotropic forms of a protein. There seems to be no valid reason, from the standpoint of structural chemistry, to suppose that each individual protein should exist in only two desmotropic forms which could be unambiguously designated as the native and the denatured forms.

*l*-tyrosine, and carbobenzoxy-*l*-glutamyl-*l*-phenylalanine (10). A list of synthetic peptides illustrating the specificity of crystalline swine pepsin is presented in Table III. The hydrolysis of the synthetic substrates by swine pepsin occurs exclusively at the peptide linkage that involves the amino group of the aromatic amino acids. It is apparent that the character of these substrates contradicts the generally accepted view that the specificity of pepsin is adapted to the splitting of cationic substrates. Many of the substrates for pepsin do not contain any group that could form cations. On the contrary, when in the pepsin substrate carbobenzoxyglutamyltyrosine the carbobenzoxy group is removed and thus a basic group established next to the peptide bond, as in glutamyltyrosine, pepsin no longer attacks the glutamyltyrosine linkage.

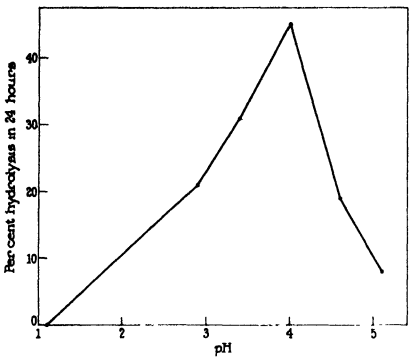



Fig. 1.—*p*H dependence of hydrolysis of carbobenzoxy-*l*-glutamyl-*l*-tyrosine by beef pepsin. Enzyme concentration, 1.6 mg. of protein N per cc.

If the basic group is located at a greater distance from the glutamyltyrosine peptide bond, pepsin is capable of hydrolyzing this peptide bond. It must therefore be concluded that a basic group within the substrate is not necessary for pepsin action and, under certain conditions, is even inhibitory. It should be noted, furthermore, that the typical substrates for pepsin, such as carbobenzoxyglutamyltyrosine and glycylglutamyltyrosine, contain two carboxyl groups. It has been found that the presence of such carboxyl groups is essential for pepsin action, and if one of them is masked by amide formation the action of the enzyme is slowed down; and if both carboxyls are masked, no enzyme action occurs at all. The presence in the substrate of a tyrosine or phenylalanine residue is not sufficient for pepsin action and must be supported by the presence of one or several carboxyl groups. This need for carboxyl groups explains the fact that the combination of glutamic acid and tyrosine residues gives the more sensitive substrates.

The splitting of carbobenzoxy-*l*-glutamyl-*l*-tyrosine by swine pepsin is most rapid around *p*H 4. A similar *p*H optimum was observed for beef

pepsin (Fig. 1). The generally accepted optimum for pepsin action on proteins is around *p*H 2, although many deviations from this value have been observed. It seems justifiable to suppose that the *p*H-optimum is partly dependent upon the structure of the substrate. In a protein numerous peptide bonds are hydrolyzed by pepsin and the observed optimum is the average of the optima of the various linkages.

In a recent paper Tazawa (11) has claimed that the hydrolysis of substrates such as carbobenzoxyglutamyltyrosine by pepsin is due to the presence in crystalline pepsin of a "specific polypeptidase" and not to the "real peptic proteinase." Tazawa reports that *l*-histidine anhydride is a suitable synthetic substrate for the "peptic proteinase." We have repeated Tazawa's experiments, using a twice-crystallized preparation of swine pepsin (Philpot (12)). As will be seen from Table IV, histidine anhydride* is not attacked at all by this enzyme preparation in 48 hours,

TABLE IV

BEHAVIOR OF *l*-HISTIDINE ANHYDRIDE IN THE PRESENCE OF CRYSTALLINE SWINE PEPSIN

| Substrate | Pepsin per cc., mg. | Normality HCl | *p*H* | Increase in $N_2$ per 0.5 cc. aliquot, cc. | | Hydrolysis of substrate, per cent | |
|---|---|---|---|---|---|---|---|
| | | | | 24 hrs. | 48 hrs. | 24 hrs. | 48 hrs. |
| Histidine anhydride | None | 0.165 | 1.24 | 0.02 | 0.03 | 3 | 5 |
| Histidine anhydride | None | 0.110 | 1.94 | 0.03 | 0.00 | 5 | 0 |
| None | 20 | 0.04 | 1.87 | 0.12 | 0.14 | .. | .. |
| None | 20 | 0.02 | 2.36 | 0.07 | 0.09 | .. | .. |
| Histidine anhydride | 20 | 0.165 | 1.58 | 0.13 | 0.16 | 2 | 3 |
| Histidine anhydride | 20 | 0.120 | 2.46 | 0.09 | 0.08 | 3 | −1 |
| Carbobenzoxy-*l*-glutamyl-*l*-tyrosine | 20 | ... | 4.1† | 0.48 | .. | 80 | .. |

* Determined by means of the glass electrode (Young).

† Acetate buffer.

Concentration of substrate, 0.05 millimol per cc. of test solution.

* The histidine anhydride, prepared according to the method of Abderhalden (13), gave the following analysis:

$C_{12}H_{14}O_2N_6$, (274.3) Calculated: C 52.54, H 5.15, N 30.64.
Found: C 52.49, H 5.14, N 30.66 (Dumas).

Micro-Kjeldahl analysis of this substance gave a low value.

whereas carbobenzoxyglutamyltyrosine is split to 80 per cent in 24 hours.

The synthetic substrates hydrolyzed by swine pepsin were also found to be split by the pepsins of beef, sheep, and chickens. The experimental data are reported in Table V.

TABLE V

ACTION OF VARIOUS PEPSINS ON SYNTHETIC SUBSTRATES*

| Substrate | Crystalline swine pepsin (1.4 mg. of protein N per cc.) | Beef pepsin | | Sheep pepsin (1.6 mg. of protein N per cc.) | Chicken pepsin (1.6 mg. of protein N per cc.) |
|---|---|---|---|---|---|
| | | Wilson (1.6 mg. of protein N per cc.) | Armour (1.7 mg. of protein N per cc.) | | |
| | *p*H 4.0 | *p*H 4.2 | *p*H 4.1 | *p*H 4.0 | *p*H 4.2 |
| | Hydrolysis in 24 hrs., per cent | Hydrolysis in 24 hrs., per cent | Hydrolysis in 24 hrs., per cent | Hydrolysis in 24 hrs., per cent | Hydrolysis in 24 hrs., per cent |
| Carbobenzoxy-*l*-glutamyl-*l*-tyrosine | 53 | 44 | 53 | 43 | 34 |
| Carbobenzoxy-*l*-glutamyl-*l*-phenylalanine | 28 | 32 | 42 | 23 | 22 |
| Carbobenzoxy-*l*-glutaminyl-*l*-phenylalanine | 17 | 20 | 23 | 16 | 10 |
| Carbobenzoxyglycyl-*l*-tyrosine | 10 | 5 | 7 | 4 | 5 |
| Carbobenzoxy-*l*-glutamyl-*l*-tyrosylglycine | 39 | 30 | 36 | 25 | 20 |
| Carbobenzoxyglycyl-*l*-glutamyl-*l*-tyrosine | 48 | 44 | 52 | 50 | 50 |
| Glycyl-*l*-glutamyl-*l*-tyrosine | 32 | 15 | 21 | 36 | 10 |
| *l*-Glutamyl-*l*-tyrosine | 1 | 0 | 0 | −1 | 0 |

* Since the various pepsin preparations probably contained varying amounts of non-enzymatic protein material, quantitative comparison of the activity per milligram protein nitrogen cannot be made.

**Beef Pepsin.**—In the purification of this enzyme the commercial products obtained from Wilson and Co. and Armour and Co. were employed. Five hundred grams of the crude material were stirred with 1000 cc. of 0.5 *N* sulfuric acid and the insoluble material was centrifuged off. To the supernatant solution an equal volume of saturated magnesium sulfate solution was added with stirring. The resulting precipitate was centrifuged and then dissolved in 500 cc. of 0.002 *N* hydrochloric acid. The enzyme

was alternately precipitated by means of magnesium sulfate and redissolved in 0.002 *N* hydrochloric acid three more times. It was then precipitated again by means of magnesium sulfate, filtered, and dissolved in normal acetate buffer (*p*H 4.5). Attempts to obtain crystals from this purified material were unsuccessful.

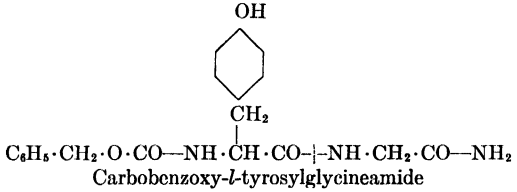
**Sheep Pepsin.**—Four hundred grams of the crude material obtained from Wilson and Co. were worked up in a manner similar to that described for beef pepsin. Highly active preparations, which could not as yet be crystallized, were obtained.

**Chicken Pepsin.**—We are indebted to Dr. Roger M. Herriott for a sample of purified chicken pepsin.

The pepsins have similar specificities in that they have a number of synthetic substrates in common. However, a closer comparison of the reaction kinetics of these pepsins is desirable.

### 2. *Chymotrypsin*

At *p*H 7.6 chymotrypsin splits synthetic substrates of the type carbobenzoxy-*l*-tyrosylglycineamide and carbobenzoxy-*l*-phenylalanylglycineamide (14). The splitting occurs at the peptide linkage that involves the carboxyl of the aromatic amino acid:

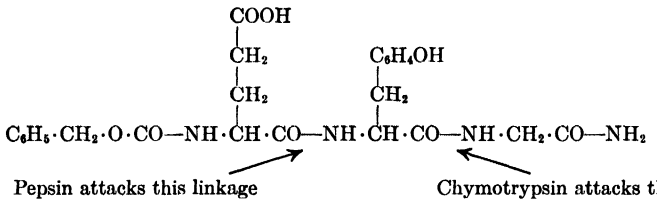



Carbobenzoxy-*l*-tyrosylglycineamide

It is of interest that the phenolic hydroxyl of tyrosine in the substrate is not indispensable for chymotrypsin action. Moreover, the typical substrates for chymotrypsin do not contain free amino or carboxyl groups. Therefore, it may be concluded that chymotrypsin does not require an ionized substrate for its action.

Chymotrypsin and pepsin resemble each other in that they are adapted to substrates containing a tyrosine residue. They differ, however, in the manner in which they attack the tyrosine-containing substrate. This may best be illustrated by comparing their action upon the same substrate, *i. e.*, carbobenzoxy-*l*-glutamyl-*l*-tyrosylglycineamide.

Carbobenzoxy-*l*-glutamyl-*l*-tyrosylglycineamide




Pepsin, as well as chymotrypsin, splits only one of the several peptide bonds present in this substrate, but pepsin splits a peptide bond other than that split by chymotrypsin. Pepsin action occurs at the peptide linkage between the glutamyl and the tyrosyl residues, while chymotrypsin action takes place between the tyrosyl and the glycyl residues. This difference between pepsin and chymotrypsin becomes even more pronounced when the electrochemical character of the substrate carbobenzoxyglutamyltyrosylglycineamide is modified (Table VI). For example, the carbo-

TABLE VI

ENZYMATIC HYDROLYSIS OF SYNTHETIC SUBSTRATES BY PEPSIN AND CHYMOTRYPSIN

| Substrate | Pepsin | | Chymotrypsin | |
|---|---|---|---|---|
| | Time, hrs. | Hydrolysis, per cent | Time, hrs. | Hydrolysis, per cent |
| Carbobenzoxy-*l*-glutamyl-*l*-tyrosylglycineamide | 24 | 57 | 4 | 79 |
| | 48 | 87* | 24 | 85† |
| Carbobenzoxy-*l*-glutamyl-*l*-tyrosylglycine | 24 | 54 | 4 | 1 |
| | 48 | 88* | 24 | 1 |
| *l*-Glutamyl-*l*-tyrosylglycineamide | 24 | 0 | 4 | 22 |
| | 48 | −1 | 24 | 30 |

* Carbobenzoxy-*l*-glutamic acid isolated.

† Carbobenzoxy-*l*-glutamyl-*l*-tyrosine isolated.

Enzyme concentration: Pepsin, 1.6 mg. of protein N per cc.; Chymotrypsin, 0.3 mg. of protein N per cc.

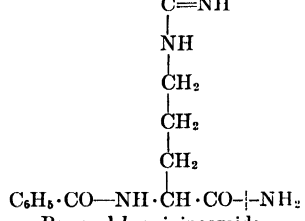
benzoxy group may be removed and thus the basic amino group be regenerated. The resulting glutamyltyrosylglycineamide is no longer split by pepsin because the presence of the basic amino group affects the specific properties of the neighboring peptide bond, making it resistant to pepsin action. In contrast to pepsin, chymotrypsin is not inhibited by the pres-

ence of the amino group, which is not in direct proximity to the chymotrypsin-attacked peptide bond. Therefore, glutamyltyrosylglycineamide is hydrolyzed by chymotrypsin. On the other hand, the compound carbobenzoxyglutamyltyrosylglycine, in which the acidic carboxyl is uncovered, was tested. Pepsin hydrolyzes the compound, while chymotrypsin is no longer effective.

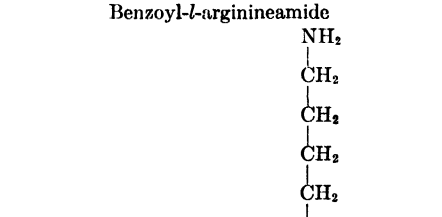
The stereochemical selectivity of chymotrypsin is shown by the fact that carbobenzoxy-*d*-tyrosylglycineamide is resistant to the enzyme. A curious influence of the *d*-isomer on the behavior of the *l*-isomer toward chymotrypsin was observed when an equimolecular mixture of the *l*- and *d*-forms of carbobenzoxytyrosylglycineamide was subjected to chymotrypsin action. Under these conditions the presence of the *d*-form completely inhibited the hydrolysis of the *l*-form. This has been explained by assuming the formation of a racemic compound the components of which have a greater affinity for one another than that of the enzyme for the *l*-form (15).

### *3. Trypsin*

At *p*H 7.2 to 7.6 crystalline beef trypsin hydrolyzes benzoyl-*l*-arginineamide and benzoylglycyl-*l*-lysineamide* at the peptide linkage involving the carboxyl of the basic amino acid (16, 17).

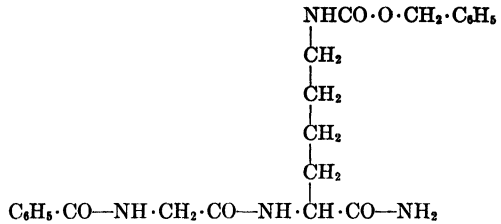


Benzoyl-*l*-arginineamide



Benzoylglycyl-*l*-lysineamide

* Both substrates were employed in the form of their hydrochlorides.



Benzoylglycyl-(ε-carbobenzoxy)-*l*-lysineamide

(Not split by trypsin)

Replacement of arginine or lysine in the above substrates by other amino acids yields compounds that are resistant to trypsin action. It is interesting that these resistant compounds include benzoyl-*l*-histidineamide and benzoylglycyl-*l*-histidineamide. All the hitherto found synthetic substrates for crystalline trypsin contain the basic side chain of arginine or lysine. If in benzoylglycyllysineamide the ε-amino group is covered by substitution with the carbobenzoxy group, the sensitivity toward trypsin disappears.

It had been thought previously that the hydrolysis of benzoylglycyllysineamide was due to an enzyme different from trypsin, namely heterotrypsin. Later experiments clearly showed that this substrate is hydrolyzed by the same enzyme that splits benzoylarginineamide (17). The assumption of a separate enzyme hydrolyzing the lysine-containing substrate has therefore been abandoned.

## IV. Some General Remarks Concerning the Specificity of Gastro-Intestinal Proteinases

Pepsin, trypsin, and chymotrypsin represent the three best recognized proteinases. A knowledge of the general character of their specificity is necessary, therefore, for the study of other less well-defined proteinases. It will have been seen from the foregoing that the specificity of each of the gastro-intestinal proteinases is sharply defined with respect to the structural details required in its substrate. The structural details that have thus far been found to influence the specificity of these proteinases are the following:

*a.* From the data available, it appears that the susceptibility of a substrate toward a given proteinase is determined by the presence of certain amino acid residues. Thus, the typical substrates for pepsin contain tyrosine or phenylalanine residues. The same holds true for the substrates of

chymotrypsin. On the other hand, the typical substrates for trypsin contain lysine or arginine residues.

A structural modification of the typical amino acid residue usually deprives it of its special character, thus rendering the substrate resistant toward the enzyme. Examples are the resistance of α-hippuryl-ε-carbobenzoxylysineamide toward trypsin, and of carbobenzoxy-*l*-glutamyl-*l*-diiodotyrosine toward pepsin.

*b.* The proteinase always attacks the substrate at a peptide bond that involves the typical amino acid. Theoretically, the typical amino acid may participate in peptide bonds through its α-amino or its α-carboxyl group. The specificity of a given gastro-intestinal proteinase is such that it is adapted to only one of these possible sites of action. Thus, pepsin acts at the peptide linkage that involves the amino group of the typical amino acid (tyrosine, phenylalanine), while chymotrypsin acts at the peptide linkages involving the carboxyl end of these typical amino acids. Trypsin, however, acts at the carboxyl end of lysine or arginine residues.

*c.* When conditions (*a*) and (*b*) are fulfilled, a peptide may nevertheless become insensitive to the action of a proteinase if a free amino group or a free carboxyl appears in the peptide chain and in close proximity to the peptide bond.

In the case of pepsin, glutamyltyrosine is not attacked in contrast to carbobenzoxyglutamyltyrosine, thus showing the inhibitory effect of the free amino group. Similarly, chymotrypsin is inhibited by the presence of a free carboxyl as in carbobenzoxytyrosylglycine, while the corresponding amide is split rapidly.

On the other hand, pepsin is not inhibited by the presence of a free carboxyl and can therefore act at the peptide bond adjacent to the terminal carboxyl as well as at more central peptide bonds, if the previously mentioned other prerequisites for pepsin action are met.

*d.* In addition to the factors mentioned in sections (*a*) to (*c*) the action of a proteinase may also be modified to a lesser degree by other structural factors such as the variation of a non-typical amino acid residue; thus carbobenzoxyglutamyltyrosine is much more sensitive to pepsin action than is carbobenzoxyglycyltyrosine. Another example is the slower hydrolysis of carbobenzoxytyrosylglycylglycineamide by chymotrypsin, as compared with the hydrolysis of carbobenzoxytyrosylglycineamide. It cannot be decided at present whether the effect in the latter case is due to the lengthening of the peptide chain or to the specific influence of the added glycine residue.

It is not intended to discuss the *p*H optima of the gastro-intestinal

proteinases since the basis for the pronounced differences of these optima is at present not clear. In an earlier part of this paper it was pointed out that the explanation of the *p*H optima as due to the ionization of the substrate is no longer adequate.

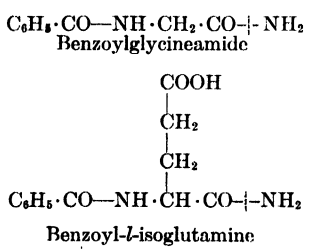
## V. Proteinases and Peptidases

Since typical proteinases such as pepsin, trypsin, and chymotrypsin digest substrates of very low molecular weight, the most important criterion for the differentiation of peptidases and proteinases has become invalid. One may ask, therefore, whether there is an essential difference between proteinases and peptidases. Is there a fundamental difference between the splitting of carbobenzoxyglutamyltyrosine by pepsin and by carboxypeptidase (18)? Several years ago (19) the suggestion was made to differentiate proteolytic enzymes that are restricted to terminal peptide linkages (exopeptidases) from those enzymes that can act at terminal peptide linkages, as well as at more centrally located peptide bonds (endopeptidases). All the facts obtained thus far by means of well-defined substrates support this classification of the proteolytic enzymes.

## VI. Specificity of Intracellular Proteinases

### *1. Papain*

As early as 1924, Willstätter and Grassmann (20) observed that HCN-activated papain hydrolyzed leucylglycylleucine. However, this line of investigation was not pursued, probably because of the prevailing view that proteinases could not hydrolyze substrates of low molecular weight. Subsequent experiments showed that benzoylglycineamide and benzoyl-*l*-isoglutamine were attacked by HCN-Papain (21). Benzoylglycineamide




Benzoylglycineamide

Benzoyl-*l*-isoglutamine

contains neither amino nor carboxyl groups, and therefore the hydrolytic action of papain could not be ascribed to any of the then known peptidases.

Further studies yielded a considerable number of other simple substrates for activated papain (16, 22, 23). A representative selection of these is given in Table VII. It will be noted that papain has a much broader range of specificity than any of the gastro-intestinal proteinases. It

TABLE VII

SYNTHETIC SUBSTRATES FOR HCN-PAPAIN

| Substrate | Time, hrs. | Hydrolysis, per cent |
|---|---|---|
| Benzoylglycine\|amide | 4 | 59 |
| Benzoyl-*l*-isoglutamine | 2.5 | 64 |
| Benzoyl-*l*-arginine\|amide | 2 | 76 |
| Benzoylglycyl\|*l*-leucylglycine | 4 | 90 |
| Benzoyl-*l*-lysine\|amide | 4.5 | 55 |
| Benzoyl-*l*-tyrosylglycine\|amide | 3 | 67 |
| Glycylglycyl-*l*-leucylglycine | 24 | 21 |
| *l*-Leucine\|amide | 24 | 40 |
| Benzoylglycyl\|*l*-histidineamide | 24 | 75 |

*p*H 5.0 (citrate buffer).
Enzyme concentration, 0.1 mg. of protein N per cc.

ıydrolyzes peptides derived from many types of amino acids. Furthermore, papain splits substrates with or without free $\alpha$-amino or $\alpha$-carboxyl groups.

It should be emphasized, however, that it is probable that the splitting of the above substrates is not due to a single enzyme but to a number of different enzymes. Tentative evidence for the existence of more than one enzymatic component in papain was obtained by comparing the activity of several papain preparations toward *l*-leucineamide and carbobenzoxyglutamine (24).

The complex nature of papain has already been suggested (26) and has also been used as the basis for a theory of papain activation (27). However, the experimental evidence for these views was later found to be inadequate (28).

Balls and Lineweaver (25) have reported the isolation, from papain latex, of a crystalline enzyme preparation capable of clotting milk and of splitting benzoylglycineamide and hemoglobin. The crystals represented 2 per cent of the original enzyme activity. These authors found that the ratio of milk clotting to hemoglobin digestion varied with different papain fractions and concluded that papain may contain more than one enzyme. It appears necessary at the present time to investigate the enzymatic homogeneity of papain by performing a systematic fractionation of the crude enzyme and by a quantitative determination of the activity of each fraction toward a number of simple substrates. This fractionation may finally yield enzyme preparations of constant specificity. Experiments along these lines are now in progress and have given added evidence for the enzymatic inhomogeneity of papain.

Preliminary experiments with bromelin (29), the proteolytic enzyme of the pineapple, and with ficin (30) indicate specificities of the activated enzymes similar to that of activated papain.

### 2. *Intracellular Proteolytic Enzymes of Animal Tissues*

The specificity of this group of physiologically important enzymes has been studied extensively by numerous authors.* Hedin (31) differentiated two enzymes in spleen, one acting on fibrin at *p*H 4, and the other at *p*H 7–8. Later Waldschmidt-Leitz and collaborators (32) concluded from the action upon gelatin, benzoylglycylglycine, leucylglycylglycine, and leucylglycine that liver and spleen extracts contain four types of proteolytic

* A bibliography may be found in Oppenheimer (1).

enzymes: a proteinase acting at a weakly acid *p*H (named "cathepsin" by Willstätter), a catheptic carboxypeptidase, an aminopeptidase, and a dipeptidase. The proteinase and carboxypeptidase could be activated by the addition of $H_2S$. In setting up this classification it was assumed that a proteinase was incapable of acting on simple peptides and that any such hydrolysis (*i. e.*, benzoylglycylglycine) must be due to the action of specific peptidases. The changes in the concept of the specificity of pro-

TABLE VIII

SEVERAL SYNTHETIC SUBSTRATES FOR BEEF SPLEEN CATHEPSIN

| Substrate | Time, hrs. | Hydrolysis, per cent | | | |
|---|---|---|---|---|---|
| | | No activator | Cysteine | Ascorbic acid | Iodoacetic acid |
| Carbobenzoxy-*l*-glutamyl-*l*-tyrosine | 2 | 53 | 61 | .. | 53 |
| Benzoyl-*l*-argininеamide | 4 | 0 | 54 | 0 | 0 |
| Benzoylglycineamide | 24 | 8 | 43 | .. | .. |
| Carbobenzoxy-*l*-isoglutamine | 20 | 3 | 53 | .. | .. |
| Carbobenzoxy-*l*-leucylglycylglycine | 2 | 0 | 79 | 3 | .. |
| Carbobenzoxy-*l*-glutamyl-*l*-glutamic acid | 2 | 0 | 55 | 1 | .. |
| *l*-Leucylglycine | 4 | 5 | 34 | 18 | .. |
| *l*-Leucylglycylglycine | 4 | 4 | 89 | 32 | .. |
| *l*-Glutamyl-*l*-tyrosine | 4 | 4 | 37 | 15 | .. |
| *l*-Leucineamide | 2 | 3 | 53 | 27 | .. |

*p*H 4.5–5.2; enzyme concentration, 0.25 mg. of protein N per cc.

teinases necessitated by the previously mentioned results with crystalline pepsin, trypsin, and chymotrypsin make it apparent that the hydrolysis of the four substrates used by Waldschmidt-Leitz can no longer be regarded as definite evidence for the presence of as many specific enzymes. In view of this, it seemed desirable to reinvestigate the specificity of tissue proteolytic enzymes and to characterize the component activities by means of an extensive series of simple substrates. This approach, when coupled with suitable fractionation procedures, should result in the isolation of several separate enzymes of constant specificity.

In experiments along these lines the activation behavior of beef spleen "cathepsin" was studied by means of simple substrates. It was found (33) that an enzyme preparation from beef spleen rapidly hydrolyzes a number

of peptides which contain tyrosine (or phenylalanine) and glutamic acid—such as carbobenzoxy-*l*-glutamyl-*l*-tyrosine (Table VIII). The splitting of carbobenzoxy-*l*-glutamyl-*l*-tyrosine occurs optimally at about *p*H 5 at the linkage between the glutamyl and tyrosine residues. The rate of the enzyme action on this substrate is not significantly increased by cysteine. It is not inhibited, on the other hand, by iodoacetic acid. This enzyme was designated "beef spleen cathepsin I." Carbobenzoxyglutamyltyrosine was also found to be hydrolyzed at the same linkage by crystalline swine pepsin at *p*H 4.0. It seems, therefore, that beef spleen cathespin I bears resemblance in specificity to pepsin.

There is a group of synthetic substrates that are split by beef spleen cathepsin only after addition of an activator such as cysteine or glutathione. This group of substrates includes acylated amino acid amides such as benzoyl-*l*-argininamide, benzoylglycineamide, and carbobenzoxy-*l*-isoglutamine, and acylated peptides such as carbobenzoxy-*l*-leucylglycylglycine and carbobenzoxy-*l*-glutamyl-*l*-glutamic acid (Table VIII). The enzyme that hydrolyzes benzoyl-*l*-argininеamide has been designated "beef spleen cathepsin II." It has not been decided whether the other substrates of this group are split by cathepsin II or another enzymatic component of beef spleen. Cysteine-activated cathepsin II is inhibited by iodoacetic acid.

The aforementioned substrates are not attacked by beef spleen cathepsin when ascorbic acid is added in place of cysteine. However, it was found that peptides, such as *l*-leucylglycine, *l*-leucylglycylglycine, and *l*-glutamyl-*l*-tyrosine, and amino acid amides, such as *l*-leucineamide, are split by beef spleen cathepsin in the presence of either ascorbic acid or cysteine (Table VIII). The enzyme that splits *l*-leucineamide has been designated "beef spleen cathepsin III."

Ascorbic acid may be replaced in these experiments by *d*-glucoascorbic acid or araboascorbic acid. In both cases the observed activation was of the same order of magnitude as in experiments in which ascorbic acid was used (Table IX).

The occurrence of these two types of proteolytic activity is not restricted to extracts of beef spleen. Enzyme preparations from beef kidney also showed activity toward carbobenzoxyglutamyltyrosine in the absence of added activator and, when activated by cysteine, hydrolyzed benzoyl-argininеamide and other substrates (Table X).

In Table XI data are reported on the stereochemical specificity of the beef spleen enzyme preparation both with and without added activator. It will be seen that replacement of an *l*-amino acid that participates in a

peptide bond by the *d*-isomer renders the bond resistant toward the enzymatic action of the beef spleen preparation.

An attempt has been made by means of simple substrates to characterize the specificity of proteolytic enzymes obtained from tumor tissues (34). The action of several tumor extracts on various substrates is summarized in Table XII. It is of some interest that in several respects the tumor extracts exhibit specificities similar to those previously described for beef spleen

TABLE IX

ACTIVATION OF BEEF SPLEEN CATHEPSIN BY ASCORBIC ACID AND RELATED SUBSTANCES

| Substrate | Activator | Hydrolysis, per cent | |
|---|---|---|---|
| | | 4 hrs. | 8 hrs. |
| *l*-Leucineamide | None | 2 | 5 |
| | Ascorbic acid | 29 | 53 |
| | *d*-Glucoascorbic acid | 29 | 51 |
| | Araboascorbic acid | 31 | 55 |
| | Reductone | 12 | 18 |
| | Cysteine | 72 | 95 |
| Benzoyl-*l*-arginineamide | None | 0 | .. |
| | Ascorbic acid | 2 | .. |
| | Cysteine | 59 | .. |

Enzyme concentration, 0.23 mg. of protein N per cc.; concentration of activator, 0.008 mM per cc.; *p*H 5.1.

TABLE X

SYNTHETIC SUBSTRATES FOR BEEF KIDNEY ENZYME PREPARATION

| Substrate | Hydrolysis in 4 hours, per cent | |
|---|---|---|
| | No activator | Cysteine |
| Carbobenzoxy-*l*-glutamyl-*l*-tyrosine | 53 | 64 |
| Benzoyl-*l*-arginineamide | 0 | 44 |
| Carbobenzoxyglycylglycylglycine | 3 | 56 |
| *l*-Leucineamide | 0 | 8 |

*p*H 4.6–5.2; enzyme concentration, 0.22 mg. of protein N per cc.

and kidney. The tumor extracts tested thus far hydrolyzed carbobenzoxy-*l*-glutamyl-*l*-tyrosine both in the absence and in the presence of cysteine. The hydrolysis of benzoylarginineamide was observed only in the presence of cysteine. *l*-Leucineamide was hydrolyzed by extracts of several tumors rather slowly or not at all.

TABLE XI

STEREOCHEMICAL SPECIFICITY OF BEEF SPLEEN ENZYME PREPARATIONS

| Substrate | Time, hrs. | Hydrolysis, per cent | |
|---|---|---|---|
| | | No activator | Cysteine |
| Carbobenzoxy-*l*-glutamyl-*l*-tyrosine | 2 | 53 | 61 |
| Carbobenzoxy-*d*-glutamyl-*l*-tyrosine | 24 | 1 | 0 |
| Carbobenzoxy-*l*-glutamyl-*l*-phenylalanine | 4 | 15 | 67 |
| Carbobenzoxy-*l*-glutamyl-*d*-phenylalanine | 12 | 1 | 3 |
| *l*-Leucineamide | 2 | 3 | 53 |
| *d*-Leucineamide | 24 | 2 | 9 |
| Carbobenzoxy-*l*-leucylglycine | 2 | 5 | 58 |
| Carbobenzoxy-*d*-leucylglycine | 2 | 0 | 3 |

*p*H 4.6–5.3; 0.25 mg. protein N per cc.

It will be recalled that the enzyme preparations from normal tissues described above hydrolyze only the *l*-forms of carbobenzoxyglutamyltyrosine and leucineamide. It was of considerable interest, therefore, to find that an extract of Bashford mouse carcinoma split *d*-leucineamide at approximately the same rate as *l*-leucineamide. Furthermore, the Bashford carcinoma extract and an extract from a carcinoma of the human breast exhibited enzymatic activity toward carbobenzoxy-*d*-glutamyl-*l*-tyrosine.* In spite of their preliminary character, these experiments indicate the need for a closer study of the specificity of the proteolytic enzymes of normal

* Waldschmidt-Leitz and Mayer (35) have recently reported that the serum of normal human beings hydrolyzes peptides containing *dl*-amino acids only to 50 per cent, while the serum of cancer patients splits these peptides to nearly 100 per cent. Properly calculated, the data of these authors would indicate that normal sera as well as sera of cancer patients hydrolyze peptides containing *dl*-amino acids to an extent far exceeding 50 per cent hydrolysis. Furthermore, Bayerle and Podloucky (35*a*) have reported their inability to reproduce the experimental results of the above authors.

and pathological tissues as a means of approaching the problem of intracellular protein metabolism.

## VII. The Activation of Intracellular Proteolytic Enzymes

Since the discovery by Vines (36) of the fact that papain could be activated by HCN, there have been numerous experimental studies of the mechanism of this phenomenon. Until recently, quantitative studies have

TABLE XII

ACTION OF TUMOR EXTRACTS ON SYNTHETIC SUBSTRATES

| Substrate | Activator | Mouse sarcoma | Bone sarcoma | Brown-Pearce carcinoma | Bashford carcinoma | Breast carcinoma |
|---|---|---|---|---|---|---|
| | | Hydrolysis in 4 hrs., per cent | Hydrolysis in 6 hrs., per cent | Hydrolysis in 24 hrs., per cent | | |
| Benzoyl-*l*-arginine-amide | None | 3 | 3 | 2 | 1 | 1 |
| | Cysteine | 65 | 51 | 59 | 58 | 68 |
| Carbobenzoxy-*l*-glutamyl-*l*-tyrosine | None | 55 | 53 | 35 | .. | .. |
| | Cysteine | 67 | 52 | 40 | 44 | 34 |
| *l*-Leucineamide | Cysteine | 10 | 8 | 9 | 20 | 0 |
| *l*-Leucylglycylglycine | Cysteine | 61 | 3 | 14 | 87 | 26 |
| Carbobenzoxy-*d*-glutamyl-*l*-tyrosine | Cysteine | 0 | 0 | 0 | 18 | 36 |
| *d*-Leucineamide | Cysteine | 3 | 0 | 0 | 25 | 0 |

*p*H 4.7–5.2.

Enzyme concentration: Mouse sarcoma, 0.22 mg. protein N per cc.
Bone sarcoma, 0.66 mg. protein N per cc.
Brown-Pearce carcinoma, 0.97 mg. protein N per cc.
Bashford carcinoma, 0.70 mg. protein N per cc.
Breast carcinoma, 0.59 mg. protein N per cc.

involved the use of only proteins (gelatin, fibrin, denatured egg albumin, etc.) and peptones as substrates. Mendel and Blood (37), in an important paper published in 1910, concluded that HCN and other activators acted in a manner analogous to that of coenzymes. This conclusion was ac-

cepted by Willstätter and Grassmann (20) who regarded the activation as resulting in a broadening of the specificity range of papain. Several facts were responsible for the subsequent abandonment of the theory of Mendel and Blood. Most of the known activators—cysteine, glutathione, $H_2S$, thioglycolic acid, sulfite—are reducing agents. Moreover, the intracellular enzymes are accompanied in plant and animal tissues by natural activators that have been found to be sulfhydryl compounds (38) and therefore also reducing agents. Finally, Bersin (39) has reported that natural papain is inactivated by treatment with oxidizing agents such as iodine or $H_2O_2$ and may be reactivated by the addition of one of the previously mentioned activators. An examination of Bersin's data shows that only a partial reactivation was achieved.

From these and similar experiments Bersin has concluded that inactive papain represents the disulfide form of the enzyme and that the process of activation consists in a reduction of disulfide groups to sulfhydryl groups. In his view, the number of SH groups is a measure of the enzymatic activity (40). This view was widely accepted (1, 41).

In the course of a purification of papain it was observed that the precipitation of HCN-activated preparations by isopropyl alcohol resulted in a nearly complete loss of activity (42). The quantitative study of this behavior by means of synthetic substrates gave results which could not be reconciled with the oxidation-reduction theory.

A preparation of purified papain, to be designated in this discussion as "Papain A," was activated by HCN and subsequently precipitated by means of isopropyl alcohol, resulting in a preparation designated as "Papain B." The activity of the enzyme preparation toward carbobenzoxyisoglutamine and benzoylarginineamide was determined before and after this precipitation. If the activation of papain by HCN consisted simply in the reduction of disulfide groups of the enzyme to sulfhydryl groups, then the precipitated Papain B without added activator should have exhibited approximately the same activity as did the HCN-Papain before precipitation. However, the precipitate Papain B was found to behave like an unactivated papain toward the above substrates. Furthermore, the precipitated Papain B, on addition of HCN, regained nearly all the activity of the original HCN-Papain A. This activated Papain B was precipitated a second time by means of isopropyl alcohol and the resulting inactive enzyme preparation C, on activation with HCN, regained the activity of the original HCN-Papain A (Table XIII).

In order to explain these results, it seemed necessary to return to the

TABLE XIII

HYDROLYSIS OF SYNTHETIC SUBSTRATES BY PAPAIN FOLLOWING ISOPROPYL ALCOHOL PRECIPITATION

| Papain solution | Enzyme solution per 2.5 cc., cc. | Added activator | Hydrolysis, per cent | | | |
|---|---|---|---|---|---|---|
| | | | Carbobenzoxyisoglutamine | | Benzoylarginineamide | |
| | | | 2 hrs. | 4 hrs. | 2 hrs. | 4 hrs. |
| A | 0.3 | None | 1 | 0 | 6 | 7 |
| B | 0.3 | | 1 | 1 | 9 | 13 |
| C | 0.3 | | −1 | 0 | 4 | 9 |
| A | 0.3 | HCN | 35 | 54 | .. | .. |
| B | 0.3 | | 29 | 49 | .. | .. |
| C | 0.3 | | 27 | 48 | .. | .. |
| A | 0.15 | | 22 | 29 | 46 | 61 |
| B | 0.15 | | 22 | 28 | 40 | 55 |
| C | 0.15 | | 19 | 27 | 36 | 52 |
| A | 0.3 | Cysteine | 42 | 58 | .. | .. |
| B | 0.3 | | 43 | 57 | .. | .. |
| C | 0.3 | | 41 | 58 | .. | .. |
| A | 0.15 | | 22 | 28 | 65 | 79 |
| B | 0.15 | | 25 | 30 | 68 | 86 |
| C | 0.15 | | 22 | 29 | 66 | 82 |

*p*H 5.0.

TABLE XIV

STEREOCHEMICAL SPECIFICITY OF HCN-PAPAIN

| Substrate | Time, hrs. | Hydrolysis, per cent |
|---|---|---|
| Carbobenzoxy-*l*-leucylglycylglycine | 5 | 50 |
| Carbobenzoxy-*d*-leucylglycylglycine | 5 | 0 |
| Benzoyl-*l*-leucineamide | 24 | 79 |
| Benzoyl-*d*-leucineamide | 24 | 0 |
| Benzoylglycyl-*l*-leucylglycine | 4 | 90 |
| Benzoylglycyl-*d*-leucylglycine | 19 | 12 |

*p*H 5.0; enzyme concentration, 0.1 mg. of protein N per cc.

coenzyme theory of Mendel and Blood and to assume that HCN combines with the "inactive" papain to form a dissociable HCN-Papain compound which represents the HCN-activated enzyme. On precipitation of the enzyme by means of isopropyl alcohol, the HCN enzyme compound dissociates and the precipitate contains the HCN-free enzyme that is inactive toward synthetic substrates.

Since the SS-SH theory of papain activation postulates that the function of the activator is to reduce disulfide groups, one would be forced to conclude that, regardless of the activator applied, the same activated enzyme would result.

On the other hand, the coenzyme theory provides, for each enzyme, a number of different enzyme-activator compounds, since each enzyme of this group can be activated by a number of different activators. It may be expected that the various enzyme-activator compounds derived from the same enzyme would differ more or less in their specificities. The application of simple synthetic substrates offers a convenient method for the quantitative study of such specificity differences. Experiments designed to test this theory are in progress, and preliminary results obtained by Dr. George W. Irving, Jr., indicate that there is an appreciable difference in the action of HCN-Papain and cysteine-papain on carbobenzoxyleucylglycylglycine. Similar observations have been made with the substrate benzoylarginineamide.

The fact that the specificity of an intracellular proteinase is not rigidly determined but may be altered in various ways by different activators is of obvious significance for the general problem of protein metabolism. While glutathione has been accepted as a possible naturally occurring activator, it is clear that other substances (cysteine, cysteine peptides, ascorbic acid, etc.) shown to occur in living tissues are also potential activators and by combining with an intracellular proteinase may modify its specificity. In order to test this theory further, it seems desirable to extend the systematic study of the influence of activators on the specificities of the intracellular proteinases of various animal and plant tissues.* In order to make such investigations possible, two conditions must be fulfilled. First, it is necessary to secure homogeneous enzyme preparations; and second, methods must be made available for the quantitative study of enzyme specificity in terms of reaction velocities.

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* In this connection it should be of interest to examine the question whether the specificities of the gastro-intestinal proteinases may be modified by combination with other substances.

## VIII. Kinetics and Specificity

The specificity of a proteinase is characterized by the rates at which the peptide bonds of a number of substrates are hydrolyzed in the presence of the enzyme. The ultimate goal is, of course, a precise quantitative description of the specificity in terms of the reaction rates at which the pure enzyme hydrolyzes the peptide bonds of suitable, well-defined substrates. The criteria of enzymatic homogeneity and purity usually cannot be met and it is at present not possible, for most of the proteinases, to determine the specific reaction rates of the pure enzymes. It is therefore necessary to characterize the specificity of a proteinase by comparing the rates at which the impure proteinase hydrolyzes the peptide bonds of a number of substrates. Such a comparison is usually performed on a semi-quantitative basis in order to establish what types of peptide linkages are selectively attacked by the proteinase. The information obtained by comparing the rates at which one proteinase hydrolyzes a number of substrates can also be employed to compare the specificities of several proteinase preparations. In this way the comparative specificities of closely related proteinase preparations may be studied, thus enabling one to follow the enzymatic homogeneity and purity attained in fractionation and purification procedures. Furthermore, by means of these data, it is possible to determine the specificity differences between various proteinases by comparing the relative rates of hydrolysis of a number of substrates by each enzyme. Frequently these differences are so sharp that they are revealed by merely qualitative results (*cf.* comparative specificities of pepsin, trypsin, and chymotrypsin). On the other hand, in other cases (intracellular proteinases such as papain, bromelin, ficin, etc.) the various enzymes will all hydrolyze several substrates but at somewhat different relative rates. In these cases it is therefore necessary to perform a quantitative study of the reaction velocities as the basis for the comparison of specificities.

In the past, the quantitative study of the reaction velocity of proteinase action has involved the use of proteins as substrates. In a previous section it was pointed out that in the enzymatic hydrolysis of a protein numerous different peptide linkages are split. Furthermore, the study of proteinase action by means of synthetic substrates has established the fact that the rate of enzymatic hydrolysis varies greatly depending upon the nature of the amino acids participating in the sensitive peptide bond as well as upon other structural details of the substrate. Consequently, when a protein is digested by a proteinase, the various sensitive peptide linkages of the protein are split at very different rates. Thus the measured rate of libera-

tion of carboxyl or amino groups represents a resultant of the rates of numerous simultaneous and consecutive individual reactions.

Knowledge of the kinetics of proteinase action is usually needed for studies of the reaction mechanism or, still more frequently, for the quantitative estimation of the enzyme. It seems clear that such a knowledge can be obtained only through a study of the kinetics of a reaction in which only one peptide linkage per substrate molecule is hydrolyzed. This criterion is met by the application of simple substrates that provide only one sensitive peptide bond for the action of the proteinase.

When simple substrates are hydrolyzed under experimental conditions where the substrate is in great excess, where no enzyme destruction occurs and the split-products do not inhibit the reaction, it is to be expected that a first order kinetics should be observed. This has actually been shown for crystalline trypsin, using benzoyl-*l*-arginineamide and hippuryl-*l*-lysineamide as substrates (42*a*). The numerical values for the first order reaction constants were found to be strictly proportional to the amount of enzyme employed. Furthermore, experiments with papain, ficin, and beef spleen "cathepsin" showed that the hydrolysis of benzoyl-*l*-arginineamide and several other substrates follows a first order kinetics (30).

## IX. Enzymatic Synthesis

Experiments designed to reverse the hydrolytic action of proteinases into synthetic action have been performed frequently during the past decades. Wasteneys and Borsook (43) concluded from their experiments with commercial pepsin that the equilibrium between hydrolysis and synthesis may be shifted in favor of synthesis when rather concentrated solutions of protein split-products are used at *p*H 4.

Several authors have attempted to correlate protein metabolism with the redox potential of the proteolytic system. Voegtlin, Maver, and Johnson (44) have reported that, on oxygenation and in the presence of a proteinase, protein digests and tissue extracts exhibit a reversal of proteolysis. These authors have suggested that proteolysis may be favored by the presence of sulfhydryl groups which on oxygenation may be converted into disulfide groups; the presence of the latter is assumed to favor synthesis. Strain and Linderstrøm-Lang (45) have reported their inability to reproduce the above results.

Previous investigators of enzymatic protein synthesis *in vitro* have in general operated as follows: A protein was hydrolyzed enzymatically and the resulting mixture of split-products, the nature, number, and relative

amount of which were not known, was incubated under various conditions with a crude enzyme preparation. A decrease of amino nitrogen or, more frequently, an increase of insoluble or precipitable substances was taken as indication for an enzymatic synthesis of protein. Clearly, such a procedure gives only the resultant of a great number of single component steps. However, the exact nature of any of these component steps cannot be ascertained in such complex systems. Here, again, the method employing simple, well-defined synthetic substrates has served as a valuable tool. Through this technique it has become possible to effect enzymatic syntheses of single CO—NH linkages by means of each of the following enzymes: papain, bromelin, liver and spleen cathepsin, and chymotrypsin (46, 47, 15).

The action of a proteolytic enzyme upon a peptide bond is generally regarded as consisting in a catalytic acceleration of the establishment of the equilibrium between hydrolysis and regeneration of the peptide bond. Thus, the hydrolysis of benzoylglycineamide (hippurylamide) into hippuric acid and ammonia in dilute aqueous solution under the influence of papain may be represented as follows:

$$C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot CO—NH_2 + H_2O \rightleftarrows C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot COOH + NH_3$$

The equilibrium is in this case, as in the vast majority of other cases involving the hydrolysis and synthesis of peptide bonds, overwhelmingly in favor of hydrolysis. Therefore, when equilibrium is established, there is present only a very small amount of hippurylamide and a much larger proportion of hippuric acid and ammonia. This equilibrium amount of hippurylamide is smaller than the amount that would have been present in a saturated aqueous solution. Therefore, if solid hippurylamide is brought into contact with an aqueous solution of papain, hippurylamide will be dissolved until a concentration is reached which exceeds the equilibrium concentration. Most of the dissolved amide will be hydrolyzed under the influence of the enzyme, leaving an amide concentration that is much below the concentration of the saturated solution. Consequently more solid amide will go into solution, again most of the dissolved amide will be hydrolyzed, and the process will continue until the solid amide has disappeared and nearly all of the amide is hydrolyzed.

A reversion of the enzymatic hydrolysis of hippurylamide, *i. e.*, its enzymatic synthesis from hippuric acid and ammonia, would only be possible if one could work under conditions at which the solubility of the synthetic product is smaller than its concentration at equilibrium. Such experimental conditions have not as yet been found for hippurylamide. How-

ever, in the case of the phenyl derivative of hippurylamide, *i. e.*, hippuryl anilide, the concentration in a saturated aqueous solution is smaller than the concentration present when the following equilibrium has been established.

$$\text{Hippuric acid} + \text{aniline} \rightleftarrows \text{Hippuryl anilide} + H_2O$$

Therefore, when in a dilute solution containing equivalent amounts of hippuric acid and aniline the above equilibrium reaction is accelerated by the addition of papain, the solution becomes supersaturated with respect to the anilide. The anilide will partly crystallize out, thus disturbing the equilibrium. More hippuric acid and aniline combine with one another with the formation of anilide, more anilide crystallizes, until eventually the greater part of the hippuric acid and aniline are combined into hippuryl anilide.

The following examples illustrate the synthesis of tripeptides by the enzymatic formation of genuine peptide bonds. The enzyme was in all cases cysteine-papain (24).

Benzoyl-*l*-phenylalanylglycine + glycine anilide →
Benzoyl phenylalanylglycylglycine anilide

Carbobenzoxy-*l*-phenylalanylglycine + glutamic acid anilide →
Carbobenzoxy-*l*-phenylalanylglycyl glutamic acid anilide

Carbobenzoxy-*l*-phenylalanylglycine + tyrosineamide →
Carbobenzoxy-*l*-phenylalanylglycyltyrosineamide

Since this type of enzymatic synthesis produces insoluble compounds, its progress can easily be demonstrated visually. For example, when cysteine-papain is added, at 40°, to a solution containing equivalent amounts of acetyl-*dl*-phenylalanylglycine and aniline, after several minutes a copious crystallization of acetyl-*l*-phenylalanylglycine anilide is observed (48). The same synthetic reaction has been performed with beef spleen "cathepsin" as the catalyst.

The enzymatic syntheses discussed above involve only a single reaction step resulting in a product which upon formation separates out because of its low solubility. Such a simple one-step synthesis differs from the synthesis of a natural protein in that protein synthesis consists of a great number of steps most of which seem to proceed in homogeneous solution. In such a system the product of one reaction step is not removed from the equilibrium through crystallization, but by participating in another equilibrium reaction. Recently it has become possible to copy *in vitro* this natural type of reaction, *i. e.*, the enzymatic synthesis of a peptide bond in homogeneous solution by means of a coupled reaction sequence

(24). Equimolecular amounts of the two peptides acetyl-*dl*-phenylalanylglycine and glycyl-*l*-leucine, when in contact with papain in aqueous solution, combine to a very small extent by an equilibrium reaction to form acetyl phenylalanylglycylglycylleucine. This small amount of synthetic acetylated tetrapeptide is at once further transformed by the same enzyme through a second and a third reaction step, in which first the leucine residue and then one of the glycine residues are split off. This splitting of the acetylated tetrapeptide, which had been formed by the first step, upsets the equilibrium of this step. In an effort to restore equilibrium, more of the acetyl phenylalanylglycine and glycylleucine combine to form the acetylated tetrapeptide which, in turn, is hydrolyzed, etc., until all of the glycylleucine is used up by the synthetic reaction. The second and third steps of this reaction sequence provide the driving forces for the completion of the first step which consists in a synthesis.

Acetyl-*dl*-phenylalanylglycine + glycyl-*l*-leucine
↑ ↑ ↓
Acetyl-*l*-phenylalanylglycylglycyl-*l*-leucine
↓ ↓ ↑
Acetyl-*l*-phenylalanylglycylglycine + *l*-leucine
↓ ↓ ↑
Acetyl-*l*-phenylalanylglycine + glycine

The hope has frequently been expressed that the highly specific action of proteolytic enzymes may be applied to a gentle breakdown of proteins and the identification of the split-products. It was believed that the arrangement of the amino acids in these split-products should be the same as in the original protein. Unfortunately, the possible occurrence of synthetic reactions during enzyme action may result in the formation of peptides containing amino acid sequences not present in the original protein.

## X. Stereochemical Specificity of Proteinases

The investigation of the stereochemical specificity of proteinases by means of synthetic substrates has shown that in general only those peptide linkages are split in which either the *l*-form of an asymmetric amino acid or glycine is involved. Examples to illustrate this behavior for pepsin, chymotrypsin, and beef spleen "cathepsin" were presented in earlier sections of this paper. For papain a similar restriction to the *l*-form of the substrates was observed in the case of carbobenzoxyleucylglycylglycine and benzoylleucineamide. However, in the case of benzoylglycylleucyl-

glycine the *d*-form was also hydrolyzed by papain although at a much slower rate than the *l*-form (Table XIV). Since these experiments were performed with a crude enzyme preparation, there is no assurance that the hydrolyses of the *d*- and *l*-forms were due to the same enzymatic component of papain. It is clear that the limited information available at present does not permit the generalization that proteinases hydrolyzing *d*-peptides are absent in normal plants and animals. In fact, the possible occurrence of such enzymes is suggested by the reported finding of *d*-proline in ergot (49), *d*-glutamic acid in the capsular substance of bacteria of the *mesentericus* group (50), and of *d*-amino acids in gramicidin (51), as well as the enzymatic experiments of Berger and Johnson (52).

In a previous section it was pointed out that the enzymatic synthesis of peptide bonds is performed by the same proteinases that hydrolyze peptide bonds. Since synthesis and hydrolysis represent component processes of equilibrium reactions, it is to be expected that similar stereochemical specificities should be found for enzymatic synthesis as were observed for hydrolysis. In fact, HCN-Papain will catalyze the reaction:

Benzoylleucine + aniline → Benzoylleucine anilide

only when the *l*-form of the benzoylleucine is employed. Similarly, under the influence of cysteine-papain, carbobenzoxy-*dl*-glutamic acid and aniline will form only the carbobenzoxy-*l*-glutamic acid anilide, leaving the carbobenzoxy-*d*-glutamic acid in solution. This antipodal selectivity serves as a useful general method for the resolution of racemic amino acids (53).

In the Pasteur method for the biochemical resolution of racemates one of the stereoisomers is selectively destroyed by a microorganism. The enzymatic formation of insoluble anilides is similar to the Pasteur method in that it depends on the antipodal selectivity of a biological agent; however, in the synthetic action of a proteinase neither of the optically active components is destroyed and may consequently be recovered.

It is clear that this method may also be applied to the preparation of peptides containing *d*-amino acids. For example, in the presence of cysteine-papain, acetyl-*dl*-phenylalanylglycine and aniline yield the insoluble acetyl-*l*-phenylalanylglycine anilide. The acetyl-*d*-phenylalanylglycine remains unaltered if the enzymatic action is not prolonged. However, if the reaction time is extended, acetyl-*d*-phenylalanylglycine anilide also will be formed (54). In this case the antipodal specificity is expressed in the difference in the reaction rates for the *l*- and *d*-forms. It will be noted that in this synthesis the asymmetric amino acid does not participate in the newly formed CO—NH linkage.

## Conclusion

Finally, it may be permissible to discuss several aspects of the general biological significance of the specificity of proteinases. It is clear that an aqueous solution containing amino acids, peptides, and/or proteins represents a system in which there is an interplay of innumerable equilibrium reactions. In the absence of catalysts, these reactions proceed at an extremely slow rate. The role of the proteolytic enzymes is to accelerate selectively the rate of only a few of the many possible equilibrium reactions. The acceleration of a few selected equilibrium reactions directs the sequence of metabolic reactions into channels determined by the specificity of the proteinase. This specificity finds its expression in the rate of each of the favored reactions. Clearly, any change in the relative rates of the favored reactions will result in a different sequence of metabolic reactions. Thus the specificity of proteinases may be considered as the directing agency in determining the precise course of hydrolysis and synthesis of proteins in living systems. It was pointed out earlier that the specificity of a number of intracellular proteinases may be modified by the presence of accessory substances that can function as coenzymes. The possibility must therefore be envisaged that living cells are capable of altering the course of their protein metabolism depending upon the nature of the available coenzymes. Such flexibility in the specificity of intracellular proteinases should be of some importance in the adjustment of the organism to changes in environmental factors.

There is another group of phenomena indicating the influence of environmental factors. It has been observed that the enzymatic fate of a peptide is frequently influenced by the presence of other peptides. For example, glycyl-*l*-leucine is not hydrolyzed by cysteine-papain; neither is acetyl phenylalanylglycine. However, in a previous section it was shown that these two compounds are combined by cysteine-papain to form acetyl phenylalanylglycylglycine. This acetyl tetrapeptide is in turn hydrolyzed by the enzyme to yield acetyl phenylalanylglycine, glycine, and leucine. Thus, the final result of this reaction sequence is the hydrolysis of glycyl-leucine.* Acetyl phenylalanylglycine functions as a "cosubstrate" which

* When a tissue extract exhibits the capability of splitting dipeptides, this behavior is usually taken as an indication of the presence of a dipeptidase. It is clear that such a conclusion is justifiable only when the presence of cosubstrates has been ruled out. Furthermore, in cases in which an enzyme splits a dipeptide only in the presence of a thermostable auxiliary substance, it must be determined whether the auxiliary substance acts as a coenzyme or a cosubstrate.

by its presence alters the enzymatic fate of the peptide glycyl-*l*-leucine. A similar observation was made in the action of cysteine-papain on glycine anilide where the addition of acetyl phenylalanylglycine, horse serum, or one of several proteins resulted in a hydrolysis of the previously resistant glycine anilide. It should be pointed out that the cosubstrate effects no change in the specificity of the enzyme.

The occurrence of such cosubstrate reactions appears inevitable in the physiological transformations of proteins and offers an additional mechanism for the adjustment of the proteolytic system of tissues to changes in the chemical environment. Thus the biological process of peptide synthesis and peptide hydrolysis is not only determined by the specificity of the enzyme and the coenzymes that modify this specificity but is also influenced by the presence of other peptides that may act as cosubstrates.

The specificity of a proteinase enables the enzyme to select among many equilibria but does not shift the point of equilibrium. Therefore, in a given system the shift of the reaction in the direction of hydrolysis or synthesis is not a direct consequence of the specificity of the proteinase; rather, in isolated single equilibrium systems, proteinases generally perform hydrolyses. In order to influence such an equilibrium system in the direction of synthesis it is necessary to couple it with another equilibrium system. The function of this additional system is continuously to remove the synthetic product of the first equilibrium reaction. The nature of the additional equilibrium reaction may vary widely. It appears likely that in living organisms the synthetic reactions necessary for protein formation are made possible through the coupling of proteolytic systems with other equilibrium systems.

## Bibliography

1. C. Oppenheimer, "Die Fermente und ihre Wirkungen," The Hague, Supplement, 1936, p. 618 ff.
2. W. Grassmann and F. Schneider, *Ergeb. Enzymforsch.*, **5**, 79 (1936).
3. E. Waldschmidt-Leitz, *Angew. Chem.*, **47**, 475 (1934).
4. H. O. Calvery and E. D. Schock, *J. Biol. Chem.*, **113**, 15 (1936).
5. M. Bergmann and C. Niemann, *Ibid.*, **118**, 781 (1937).
6. M. Damodaran and P. S. Krishnan, *Biochem. J.*, **32**, 1919 (1938).
7. E. Waldschmidt-Leitz and G. Künstner, *Z. physiol. Chem.*, **171**, 70 (1927); E. Waldschmidt-Leitz, "Enzyme Actions and Properties," New York, 1929, p. 131 ff.
8. K. Linderstrøm-Lang, R. D. Hotchkiss, and G. Johansen, *Nature*, **142**, 966 (1938).
9. M. Bergmann and L. Zervas, *Z. physiol. Chem.*, **224**, 11 (1934).
10. J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, **127**, 627 (1939).
11. Y. Tazawa, *Enzymologia*, **7**, 321 (1940).

12. J. Philpot, *Biochem. J.*, **29**, 2458 (1935).
13. E. Abderhalden and F. Leinert, *Fermentforschung*, **15**, 324 (1937).
14. M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **118**, 405 (1937).
15. M. Bergmann and J. S. Fruton, *Ibid.*, **124**, 321 (1938).
16. M. Bergmann, J. S. Fruton, and H. Pollok, *Ibid.*, **127**, 643 (1939).
17. K. Hofmann and M. Bergmann, *Ibid.*, **130**, 81 (1939).
18. K. Hofmann and M. Bergmann, *Ibid.*, **134**, 225 (1940).
19. M. Bergmann and J. S. Fruton, *Ibid.*, **117**, 189 (1937).
20. R. Willstätter and W. Grassmann, *Z. physiol. Chem.*, **138**, 184 (1924).
21. M. Bergmann, L. Zervas, and J. S. Fruton, *J. Biol. Chem.*, **111**, 225 (1935).
22. M. Bergmann, L. Zervas, and W. F. Ross, *Ibid.*, **111**, 245 (1935).
23. M. Bergmann, L. Zervas, and J. S. Fruton, *Ibid.*, **115**, 593 (1936).
24. O. K. Behrens and M. Bergmann, *Ibid.*, **129**, 587 (1939).
25. A. K. Balls and H. Lineweaver, *Ibid.*, **130**, 669 (1939).
26. M. Bergmann and W. F. Ross, *Ibid.*, **111**, 659 (1935).
27. M. Bergmann and W. F. Ross, *Ibid.*, **114**, 717 (1936).
28. M. Bergmann and J. S. Fruton, *Science*, **86**, 496 (1937).
29. M. Bergmann, J. S. Fruton, and H. Fraenkel-Conrat, *J. Biol. Chem.*, **119**, 35 (1937).
30. J. S. Fruton, G. W. Irving, Jr., and M. Bergmann, *Ibid.*, in press.
31. S. G. Hedin, *J. Physiol.*, **30**, 155 (1904).
32. E. Waldschmidt-Leitz, E. Schäffner, J. J. Bek, and E. Blum, *Z. physiol. Chem.*, **188**, 17 (1930).
33. J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, **130**, 19 (1939); J. S. Fruton, G. W. Irving, Jr., and M. Bergmann, *Ibid.*, in press.
34. J. S. Fruton, G. W. Irving, Jr., and M. Bergmann, *Ibid.*, **132**, 465 (1940).
35. E. Waldschmidt-Leitz and K. Mayer, *Z. physiol. Chem.*, **262**, 15 (1939).
35*a*. H. Bayerle and F. H. Podloucky, *Ibid.*, **264**, 189 (1940).
36. S. H. Vines, *Ann. Bot.*, **17**, 602 (1903).
37. L. B. Mendel and A. Blood, *J. Biol. Chem.*, **8**, 177 (1910–11).
38. W. Grassmann, H. Dyckerhoff, and O. Schoenbeck, *Z. physiol. Chem.*, **186**, 183 (1929).
39. T. Bersin, *Ibid.*, **220**, 209 (1933); **222**, 177 (1934).
40. T. Bersin, *Ergeb. Enzymforsch.*, **4**, 82 (1935).
41. L. Hellerman, *Physiol. Rev.*, **17**, 454 (1937).
42. J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, **133**, 153 (1940).
42*a*. K. Hofmann and M. Bergmann, *Ibid.*, in press.
43. H. Wasteneys and H. Borsook, *Physiol. Rev.*, **10**, 110 (1930).
44. C. Voegtlin, M. E. Maver, and J. M. Johnson, *J. Pharmacol.*, **48**, 241 (1932).
45. H. Strain and K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg*, **23**, 11 (1938).
46. M. Bergmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **119**, 707 (1937).
47. M. Bergmann and H. Fraenkel-Conrat, *Ibid.*, **124**, 1 (1938).
48. M. Bergmann and O. K. Behrens, *Ibid.*, **124**, 7 (1938).
49. W. A. Jacobs and L. Craig, *Ibid.*, **110**, 522 (1935).
50. C. Ivanovics and V. Bruckner, *Z. physiol. Chem.*, **247**, 281 (1937).
51. R. D. Hotchkiss and R. J. Dubos, *J. Biol. Chem.*, **132**, 793 (1940).

52. J. Berger and M. J. Johnson, ***J. Biol. Chem.***, **130,** 655 (1939).
53. J. S. Fruton, G. W. Irving, Jr., and M. Bergmann, *Ibid.*, **133,** 703 (1940).
54. O. K. Behrens, D. G. Doherty, and M. Bergmann, *Ibid.*, **136,** 61 (1940).

# METABOLIC GENERATION AND UTILIZATION OF PHOSPHATE BOND ENERGY

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## I. Historical Introduction

For a long time after its discovery by Harden and Young, phosphorylation of hexose in alcoholic fermentation was thought to be significant only as a means of modeling the hexose molecule to fit it for fermentative breakdown. However, as the outcome of intensive study of the intermediate reactions in fermentation and the relation between muscular action and metabolism, it later became evident that the primary phosphate ester bond of hexose changes metabolically into a new type of energy-rich phosphate bond (1). In this bond large amounts of energy made available by the metabolic process accumulate. The recent recognition that in nature there occurs a widespread utilization of such phosphate bonds (2, 3) as energy carriers, necessitates a still further revision of the earlier view concerning the biological significance of phosphate turn-over. During various metabolic processes phosphate is introduced into compounds not merely, or at least not solely, to facilitate their breakdown, but as a prospective carrier of energy. To outline the metabolic generation and the circulation of this peculiar type of chemical energy is the primary purpose of this paper.

* * *

Through the discovery of creatine phosphate (Fiske and Subbarow (4), Eggleton and Eggleton (5)) a compound of unusual properties was recognized as component of the chemical make-up of cells. The more or less pronounced breakdown of creatine phosphate during muscular contraction early suggested its connection with energy supply. Interest in the compound became stronger after Meyerhof and Suranyi (6) found that unexpectedly large amounts of heat were released by enzymatic decomposition. The biochemistry of the energy-rich phosphate bond was, in fact, herewith opened. Progress, however, was slowed by the conception then current as to the mechanism of muscle action, connecting contraction rigidly with glycogen breakdown to lactic acid. A profound revision of this conception became unavoidable when Lundsgaard (7) showed that anaerobic contraction proceeded qualitatively, although not quantitatively, undisturbed after complete blocking of glycolysis by iodoacetic acid. He found "*a*-lactacid" contraction accompanied by a quite pronounced breakdown of creatine phosphate, exhaustion of the

muscle being coincident with exhaustion of combined creatine. During the contraction period proportionality between creatine phosphate breakdown and action—measured as tension—was found. Using the heat data of Meyerhof, Lundsgaard (8) calculated the tension-heat quotient of Hill ($Tl/H$) (9). He found practical agreement with the quotient calculated earlier for normal muscle where heat of glycogen breakdown was compared with tension (10). In other words, equal amounts of ultimate heat energy, irrespective of its origin from either creatine phosphate or glycogen, did the same amount of mechanical work. By this finding of Lundsgaard the applicability of phosphate bond energy for the driving of the muscle machine was established.

With normal muscle, the Eggletons (5) and Fiske and Subbarow (4) had already found that creatine phosphate, when largely decomposed during a long series of contractions, was reconstituted quite rapidly during recovery in oxygen. Anaerobically likewise creatine phosphate was resynthesized very effectively at the expense of glycolysis (Nachmansohn (11)). Studying anaerobic resynthesis under most favorable conditions, Lundsgaard (12) found a remarkable efficiency of glycolysis. By breakdown of one-half mole of glucose to lactic acid approximately two moles of creatine phosphate were reformed. Comparing the heats of both reactions each about 24,000 cal but of opposite sign it could be concluded that the total heat energy of glycolysis was utilized for conversion into phosphate bond energy. The free energy of glycolysis might be in fact somewhat greater than 24,000 cal (Burk (13)).

The availability of the energy-rich phosphate bond (~ph) in absence of glycolytic or combustion energy and the ease and effectiveness with which glycolysis and combustion energy could be converted into ~ph, suggested (12) that the energy utilized in the mechanical set-up of muscle under all circumstances was derived from energy-rich phosphate bonds, supplied constantly by glycolytic or oxidative foodstuff disintegration. The manner in which this supply took place remained, however, entirely obscure.

The study of intermediate reactions in glycolysis and fermentation with tissue and yeast extracts furnished the first explanation of the chemistry of such energy transfers.

The understanding of the transfer mechanism in anaerobic glycolysis still left much unexplained as to how creatine-phosphate could be synthesized in purely aerobic metabolism, especially in the presence of iodoacetic acid (8), (14). The creatine in muscle must be considered as a natural trap or storehouse for ~ph. Every metabolic process utilizable for the rebuilding of creatine phosphate must generate energy-rich phosphate bonds.

A partial explanation developed when it was found that keto acid oxidation, undoubtedly occurring to some extent in aerobic carbohydrate breakdown, can furnish energy-rich phosphate, which, when brought over to creatine, would reform creatine ~ph (Lipmann (15)). A more general study of purely oxidative phosphorylation, found to occur abundantly in extracts of kidney and liver, was initiated by the work of Kalckar (16) and is being continued in Cori's laboratory (17). Here indications are found that present knowledge of the chemistry of generation and transfer of phosphate bonds is far from complete. More and more clearly it appears that in all cells a tendency exists to convert the major part of available oxidation-reduction energy into phosphate bond energy.

The metabolism of muscle is an almost unique case in nature of a straightforward utilization of chemical energy. Here the need of organization into a uniform type is understandable. In all other cells the energy problem is much more complex. If, as in growth, foodstuff is transformed into protoplasm, the comparison of the free energies of starting material and final product frequently does not show appreciable difference, *i. e.*, storage of energy may be insignificant. The extra "energy of synthesis" needed here is used only in such a manner as to force chemical processes to go in desired directions. Ways and means by which phosphate bond energy is utilizable for such general cell purposes are recognizable and partly understood, and shall be discussed in due course.

## II. Definition of the Term "Group Potential"

As pointed out, the energy derived from metabolic processes is utilized to force desirable synthetic processes. Groups, such as phospho-, acetyl-, and amino-, are brought by metabolic mechanisms intermediately into positions from where they easily can be carried into desired places. More or less energy is lost or better used up because of special paths adopted in forming these groups. These biologically interesting linkages designed to transfer groups with loss of energy will be called "weak" linkages based on the usual chemical nomenclature with respect to cleavage processes. If, with cleavage, large amounts of energy can be made free (negative change in free energy: $-\Delta F$), the tendency to burst the linkage is relatively great: thus a weak linkage (small affinity). If little energy is freed with cleavage, or energy has even to be furnished, the linkage is called strong (large affinity). Now, very often the biochemist and likewise the synthetic organic chemist is not interested to talk so much about the weakness of the linkage by which a group is bound as about the energy accumulated in the link-

age. Instead of emphasizing the negative, the escape of energy through cleavage, he wants to emphasize the positive, the largeness of the energy present in the linkage before cleavage, which determines the *group potential*, the escaping tendency of the group. If in organic chemistry a group is to be brought over into a desired position, compounds with this group in energy-rich linkage (high group potential) are commonly used for the purpose. Acetyl chloride or acetic anhydride is used for acetylation, dimethyl sulfate or diazomethane for methylation, and so forth. Here, it is not the weakness of the linkage but the push, the tension of the group, whereon attention is focussed, although both refer to equivalent attributes. Such clarification seems desirable since useful terms like energy-rich linkage and group potential will be unfamiliar to workers used to the common nomenclature. Attention must be called to the fact that the free energy change does not necessarily measure the group potential but empirically parallelism between both magnitudes may be common.

In the case of the paired hydrogen "group" the familiar term O/R potential designates a group potential. The reaction

$$\text{compound-H}_2 \rightarrow \text{compound} + (2\epsilon + 2\text{H}^+)$$

is defined by the O/R potential of the compound which expresses the group pressure of the paired hydrogen. Michaelis (18) even showed the possibility of subdividing the over-all potential of the pair into the single potentials of the individual hydrogens.

## III. Group Potential of Phospho-organic Compounds

It appears already from the preceding discussion that phospho-organic compounds with widely different group potentials are found in nature. Evidence shall be given here that two rough groups can be distinguished: one larger group with low potentials and a second group with high potentials.

### *1. Ester Phosphate*

To the first group belong all compounds where the phosphate residue is linked to an alcoholic hydroxyl: phospho-hexoses, -pentoses, and -trioses, phospho-glycerols and -3- or -2-glyceric acids, phospho-choline, phospho-serine, etc. In contrast with the energy-rich phosphate bond, designated with ~ph, the energy-poor phosphate ester bond will be designated with -ph in this paper. In many respects phosphate esters behave very much

like the esters of alcohols and organic acids, for which, from equilibrium measurements with or without enzyme, the change in free energy with reaction:

$$\text{ester} + H_2O \rightarrow \text{alcohol} + \text{acid}$$

was calculated to be around $-1000$ cal ($K = 4$–$5$) (19). The relation between $K$, the hydrolysis constant, and $\Delta F°$, the change of standard free energy, is (20):

$$\Delta F° = -RT' \ln K = -4.58\, T \log K.$$

As pointed out the same numerical value but with reversed sign gives some measure of the group potential of the linkage.

Only in the case of phospho-glycerol are equilibrium measurements for an actual member of this group available. The equilibrium point for two concentrations of glycerol (50 and 75 vol. %) was determined by Kay (21) with intestinal phosphatase as catalyst. From both sides nearly identical end-points were reached indicating true equilibrium. A value of 40 was found for $K$, at 38° and $p$H 8.5:

$$\Delta F° = -4.58 \times 311 \times \log 40 = -2280 \text{ cal.}$$

for:

$$\text{glycerophosphate} + H_2O \rightarrow \text{glycerol} + \text{phosphate.}$$

For an entirely different type of phosphate ester, the Cori ester phospho-1-glucose, the following considerations lead to an approximate value. Phospho-1-glucose is in equilibrium with glycogen and inorganic phosphate (Cori (22)):

$$\text{phospho-1-glucose} \rightleftharpoons \text{glycogen} + \text{phosphate, } (K = 5 \text{ (23)}).$$

Therefore the "glucosidic" ester linkage must be approximately equivalent to the glucosidic glucose linkages in glycogen.

Emil Fischer showed that with high concentrations of glucose and galactose the disaccharide isolactose was synthesized with kefir lactase as catalyst. With appreciable synthesis the hydrolysis constant for the disaccharide cannot be much larger than 100, and $\Delta F°$, therefore ca. $-3000$ cal. Similar or smaller values were indicated for various glucosides (cf. Veibel (24)). That indicates that a value not far different from that for phospho-glycerol should be true for phospho-1-glucose. However, this ester must be on the upper

side of the group level at the 3000 cal range (see Fig. 2), since the enzymatic change of the phospho-group from 1- to 6-position was found to be irreversible (Cori (22)). This irreversibility may be due, however, to an irreversible change in the rest of the glucose structure following phosphate transfer rather than to the higher potential of the phosphate group in 1-position.

From these considerations the range of 2–4000 cal is to be assumed for the group potential of the phosphate group, when esterified with an alcoholic hydroxyl.

### 2. *Energy-rich Phosphate Bonds*

The result obtained in the preceding paragraph allows us to consider the large and otherwise quite inhomogeneous group of ester-phosphates as uniform, as far as the phosphate bond is concerned. We may now contrast this group with the group of compounds containing ~ph. Here we meet at least four different linkage types, P-O-P, N-P, carboxyl-P, enol-P, the chemistry of each of which will be discussed in the following chapter. The fact, however, that a reversible interchange of ~ph takes place between the members of this group simplifies the discussion of the bond energies, which are on a higher but again uniform level. Only phospho~ pyruvate might be treated with a certain reserve since phosphate transfer from this compound to the other members seems to date to be irreversible. This linkage may be at a definitely higher level than the average (1). Uniformity is further indicated by the fairly uniform heat content of the enumerated linkages, 8–12,000 cal (Meyerhof and Schulz (25)). However, abundant evidence shows how hazardous it is to take the heat values indiscriminately as a true indication of the free energies which alone determine the direction and extent of a reaction. In the equation relating heat and free energy:

$$\Delta F = \Delta H - T\Delta S,$$

the additional entropy factor, $T\Delta S$, influences unpredictably the actual value of $\Delta F$, to the extent of up to 10,000 cal. In the present case the following discussion will show that corroborative evidence can be brought forward for an approximate equivalence of heat and free energy:

(*a*) The mechanical efficiency, when measured by the tension-length-heat quotient, is the same in iodoacetic acid muscle, *i. e.*, with N~P in

creatine ~ph as the only source of energy, as it is in normal muscle (8, 10). The mechanical effect compared with the heat in normal anaerobic muscle is very high, up to 50%, cf. *e. g.* (26). Since mechanical work measures the free energy, at least 50% of the known heat calories represent convertible energy.

(*b*) In the sequence of intermediate reactions in glycolysis and fermentation the energy-rich phosphate bond in phospho~pyruvate is formed by dehydration of phospho-2-glycerate:

$$\begin{array}{l} CH_2OH \\ \cdot \\ CHO{-}ph \\ \cdot \\ COOH \end{array} \quad \begin{array}{c} -H_2O \\ \rightleftharpoons \\ +H_2O \end{array} \quad \begin{array}{l} CH_2 \\ \| \\ C\cdot O{\sim}ph \\ \cdot \\ COOH \end{array}$$

This reaction is freely reversible, *i. e.*, no energy is required to bring about this peculiar transformation. The peculiar difference between the two compounds is that on the glycerate side the phosphate linkage is an ordinary ester linkage and as such is low in energy, but on the pyruvate side becomes an energy-rich enol~ph linkage. This illustrates a mechanism by which such a transformation takes place: Although the total of energy over the whole compound is equal on both sides of the equilibrium, the intramolecular energy distribution is changed by dehydration in such a way that a much larger part is concentrated now in the organo-phosphate linkage. Besides illuminating the nature of the process these considerations lead to a hitherto unnoticed possibility of calculating the energy present in such a bond. The total change in free energy ($\Delta F$) with the reactions (*a*) and (*b*):

$$\begin{array}{lll} \text{ph-glycerate} & \longrightarrow & \text{phosphate} + \text{pyruvate} \qquad (a) \\ \downarrow \uparrow & & \\ \text{ph}{\sim}\text{pyruvate} + H_2O & \rightarrow & \text{phosphate} + \text{pyruvate} \qquad (b) \end{array}$$

must be the same, since ph-glycerate and ph~pyruvate are energy-equivalent. As we know from phosphoglycerol, to which phosphoglycerate is entirely comparable with regard to ph-linkage the removal of ester phosphate by hydrolysis alone, *i. e.*, by formation of glycerate, means a loss of ca. 3000 cal ($\Delta F_1 = -3000$ cal) only. But dephosphorylation of ph~pyruvate presumably occurs with a much larger loss. This difference must be made up by loss of energy with dehydration of glycerate to pyruvate ($\Delta F_2$). Starting with ph-glycerate (reaction (*a*)) the total reaction can be divided into two partial reactions:

| | |
|---|---|
| I: | ph-glycerate + $H_2O$ → glycerate + phosphate; $\Delta F_1$ |
| II: | glycerate → $H_2O$ + pyruvate; $\Delta F_2$ |
| I + II: | ph-glycerate → phosphate + pyruvate; $\Delta F_1 + \Delta F_2 = \Delta F_{total}$ |

The dehydration of free glycerate to pyruvate must be a largely exergonic* reaction (occurring with loss of free energy) in contrast to the dehydration of ph-glycerate to ph∼pyruvate, *where through attachment of the phosphate residue this energy, dissipated otherwise, is retained in the enol∼ph bond.* If we add to it the $\Delta F_1$ with hydrolysis of the ester-phosphate linkage (reaction I), the sum, $\Delta F_1 + \Delta F_2$, represents $\Delta F$ with hydrolysis of the energy-rich enol∼ph linkage because, as shown by equation (*a*) and (*b*), it must be the same as with cleavage of phosphoglycerate to pyruvate and phosphate. With $\Delta F_1$ now estimated, $\Delta F_{total}$ is calculable upon $\Delta F_2$ being determined. Unfortunately this determination can only be carried out in a rough way. But since at present this procedure represents the only means to determine $\Delta F$ for the splitting of a phosphate linkage of this type, which is highly desirable, the calculation was carried out, using equation:

$$\Delta F_2 = \Delta H_2 - T\Delta S_2. \tag{1}$$

Here $\Delta H_2$ is given by the difference of the heats of combustion of pyruvic and glyceric acid, and $\Delta S_2$ can be calculated from the entropy data given by Parks and Huffman (27) and by Kelley (in (13)).

The heat of combustion of liquid pyruvic acid, as determined by Blaschko (28), is 279,000 cal. Unfortunately, no experimental data are available for glyceric acid. An apparently very reliable value, however, was obtained by the use of the calculation method of Kharasch (29). The reliability of this method is shown by the excellent agreement between calculated and determined values for pyruvic acid and for glycerol (30).

| | | | | |
|---|---|---|---|---|
| Pyruvic acid: | determined | 279,000 cal, | calculated | 280,000 cal. |
| Glycerol: | " | 397,000 cal, | " | 397,200 cal. |

The method of calculation is based on the assumption that for every electron between C and C, and between C and H 26,050 cal are generated with combustion. To the basic electron value "structural correction factors" are added. Both pyruvic and glyceric acids contain 10 of the described electrons. To the basic value of 10 × 26,050 cal are to be added 19,500

* Coryell (169) introduced recently the terms "exergonic" and "endergonic" specifically for reactions occuring with negative and positive change in free energy, to contrast with exo- and endothermic designating now exclusively heat change.

cal for C:O next to COOH in *pyruvic* acid. In *glyceric* acid, however, the structural factors are 13,000 cal for $CH_2OH$ and 13,000 cal for CHOH next to COOH, a total of 26,000 cal to be added to 260,500 cal.

| Pyruvic acid | | Glyceric acid | |
|---|---|---|---|
| 10 $\epsilon$ | 260,500 cal | 10 $\epsilon$ | 260,500 cal |
| C:O | 19,500 cal | CHOH | 13,000 cal |
| | | $CH_2OH$ | 13,000 cal |
| | 280,000 cal | | 286,500 cal |

The method shows clearly that the replacement of two separate hydroxyls by one carbonyl, the structural difference caused by dehydration, involves an appreciable decrease of the heat of combustion. We think it probable that $\Delta H_2$ (equation (1)) of 6500 cal, as calculated for the structural difference between the compounds, represents the most reliable value to be obtained at present. We prefer this to the slightly higher value of 7500 cal, obtained by subtraction of the value for glyceric acid as calculated (286,500 cal) from pyruvic acid determined (279,000 cal).

To obtain the entropies, $S$, of the compounds the atomic entropies of the atoms in their respective positions were added together. The numerical values were taken from Burk's paper (13), who obtained them from Dr. K. K. Kelley by personal communication: C, −13.4, O in terminal OH of COOH or $CH_2OH$, 0.9; O in secondary OH, −4.6; :O in carbonyl and carboxyl, 24.4, H, 11.3.

$$S(CH_3CO\cdot COOH) = (3 \times -13.4) + (4 \times 11.3) + 0.9 + (2 \times 24.4) = 54.7$$
$$S(CH_2OH\cdot CHOH\cdot COOH) = (3 \times -13.4) + (6 \times 11.3) + (2 \times 0.9) + 24.4 - 4.6 = 48.9$$
$$\Delta S_2 = 54.7 - 48.9 = 5.8$$

Now, $\Delta F_2$, the change of free energy with dehydration of glyceric to pyruvic acid, can be found (for $T$ 298°) from $\Delta F_2 = \Delta H_2 - T\Delta S_2 = -6500 - 298 \times 5.8 = -8250$ cal. This represents the change for the pure liquid acids (undissolved). It is assumed that no appreciable differences in dilution and neutralization occur between the two compounds. Such assumption seems justifiable, because both are infinitely soluble in water and their acidic strength is approximately the same.

The final result, the change of free energy with decomposition of phosphoglycerate($\rightleftharpoons$phosphopyruvate), $\Delta F_{total}$, is then obtained by addition of the changes with dephosphorylation of phosphoglycerate, $\Delta F_1$, and with dehydration of glycerate to pyruvate, $\Delta F_2$.

$$\Delta F_{total} = \Delta F_1 + \Delta F_2 = -3000 - 8250 = -11{,}250 \text{ cal.}$$

TABLE I

THERMODYNAMIC DATA FOR HYDROLYSIS OF PHOSPHO-ORGANIC COMPOUNDS

| No. | Compound | $\Delta H$ cal | Reference | $\Delta F$ cal | Reference |
|---|---|---|---|---|---|
| 1 | Phosphoglycerol | .... | ..... | − 2,350 | Equilibrium constant, Kay (21) |
| 2 | Phosphopyruvate | − 8,450 | Meyerhof and Schulz (25) | −11,250 | Calculation, this paper |
| 3 | Phosphoglycerate (hydrolysis to phosphate any pyruvate) | − 8,250 | " " " | | |
| 4 | Phosphocreatine | −10,700 | Meyerhof and Schulz (25) | | |
| 5 | Phosphoarginine | − 7,700 | " " " | | |
| 6 | Adenosine polyphosphate (per easily hydrolyzable P) | −12,000 | Meyerhof and Lohmann (32) | (−10,000) | Approximation from phosphopyruvate; mutual equilibrium |
| 7 | Phosphoglyceryl phosphate | .... | ..... | | |
| 8 | Acetyl phosphate | (− 8,000) | Difference of heat of combustion for acetic acid and acetic anhydride (29) | | |
| 9 | Amido phosphate, $(OH)_2OP{\sim}NH_2$ | −14,000 | Meyerhof and Lohmann (31) | | |

It is to be noticed that an appreciable part of $\Delta F$ is made up by the increase in entropy from glyceric to pyruvic acid, due to the formation of a carbonylic group. A partially but not wholly similar situation was some years ago discussed by Burk (13), who pointed out that in compounds of the same molecular formula the formation of the carbonyl group corresponded to an entropy ($T\Delta S$) of some $-7500$ cal per mol. In the present case the value is, or may be regarded as less (ca. $-$ 2000 cal), because elimination of water is also involved.

The heat change determined by Meyerhof and Schulz (25) for the reaction phosphoglycerate = phosphate + pyruvate was $\Delta H$: $-$ 8250 cal and for phosphopyruvate + $H_2O$ → phosphate + pyruvate, $\Delta H$: $-$ 8450 also practically identical. It appears that $\Delta F$ is about $-3000$ cal larger than $\Delta H$, as is to be expected from the positive value of $\Delta S$. This makes us confident that the calculated value is very nearly right. Therefore, it becomes more easily understandable that the heat change with decomposition of enol-phosphate is appreciably lower than with creatine phosphate and adenosine polyphosphate (see Table I), although accounting for irreversibility of phosphate transfer the free energy change with the latter compounds should even be lower than with enol-phosphate.

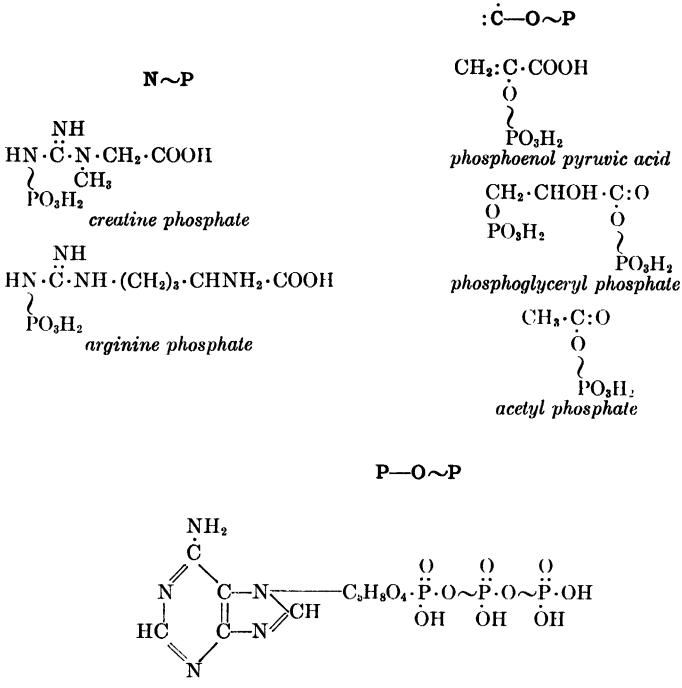
It is thus concluded that the average energy present in an energy-rich phosphate linkage amounts to 9000–11,000 cal, and of an ester linkage to around 3000 cal. The metabolic relation between the two groups of organo-phosphates in the cell laboratory is to be compared with the relation of acid anhydride to acid ester in the organic chemical laboratory. Even the numerical difference of the group potential is approximately the same in both cases, *e. g.*, ca. 8000 cal for the acetyl group (see Table I). The differences in energy levels of the energy-rich anhydride and the energy-poor ester drives the group into the ester bond.

## IV. Chemistry and Distribution in Nature of Energy-rich Phosphate Bonds

### *1. General Survey*

In three of the four bond types to be discussed, the high group potential is caused by the anhydric nature of the bond. Anhydrization takes place either between two phosphates (P-O∼P) or between a phosphate and a carboxyl (O:C·O∼P) or acidic enol (C:C·O∼P). In guanidine phosphates (N∼P), however, the direct connection between phosphorus and nitrogen is held responsible for the high group potential by Meyerhof and Lohmann (31) (see No. 9 in Table I).

## FORMULAE FOR COMPOUNDS




In the formulae represented, the energy-rich linkage expressed by ∼ is placed between oxygen and phosphorus. This decision was made because by reversible shifting of ∼ph from adenosine polyphosphate to creatine (partial Parnas-reaction (33, 34)) the break can only occur between O and the terminal -$PO_3H_2$, which changes place with an hydrogen in the $NH_2$ of the guanidine group of creatine to form HN∼$PO_3H_2$ (compare formulae).

In Table II the commonly used quantitative procedures for determination of the compounds with energy-rich phosphate bond are listed. These methods not only reflect the behavior of the substance but, in most cases, also the manner by which the substance was discovered.

TABLE II

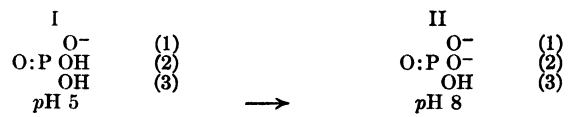
CUSTOMARY METHODS OF DETERMINATION

| No. | Compound | Analytical Denotation | Determined as: |
|---|---|---|---|
| 1 | Creatine phosphate | Acid-unstable P | Difference between colorimetric P and the P found by alkaline Mg (35) or Ca (4) precipitation (= true inorganic P)* |
| 2 | Arginine phosphate | Acid-unstable P | Same procedure adapted to slightly greater acid stability (31)* |
| 3 | (ad-ph)~ph~ph | Easily hydrolyzable P | Difference between colorimetric P immediately and after 7 min. hydrolysis with *n*-HCl at 100° (36) |
| 4 | Phosphopyruvate | ..... | P, mineralized by hypoiodite treatment (1) |
| 5 | Acetyl phosphate | Acid-, alkali-unstable P | Difference between colorimetric P and neutral Ca precipitate (3) (= true inorganic P)* |
| 6 | Phosphoglyceryl phosphate | Acid-, alkali-unstable P | See (2) |

*The test solution for colorimetric determination contains 0.5 *n*-$H_2SO_4$ and 2.5% ammonium molybdate (Fiske and Subbarow (37)). The rate of decomposition of Nos. 1, 5, and 6 is greatly accelerated in presence of molybdate. Decomposition in plain acid is much slower. In dilute trichloroacetic acid at 0° practically no decomposition occurs in short periods.

**Acid-base Changes with Phosphorylation.**—Generally speaking, the attachment of phosphate to an organic molecule is accompanied by increase in acidity since the organo-phosphoric acid is always a stronger acid than ortho phosphoric acid. The complicated change of the acid base equilibrium needs careful consideration because of manifold physiological and methodological implications.

In the physiological range of *p*H ortho phosphate is present in solution in two forms, as primary and as secondary salt:



In both forms, between *p*H 5 and 8, the third hydrogen is entirely undissociated, the first being completely eliminated. It is only the dissociation of the second H which changes within this range of *p*H. On phosphorylation, always the undissociated third OH group enters the organic linkage. Solely from the replacement of a "homoiopolar" bonded H by an organic radical, no change of the acid-base equilibrium would be expected. However, as pointed out, a more or less pronounced change occurs because in the organo-phosphate the second OH always becomes more acidic. Or, with transition from inorganic to organic phosphate, part of form I is changing to form II. The paradoxical situation results that with the disappearence of a potentially acid group, the reaction becomes more acidic; and in the reverse direction becomes more alkaline. The rise with phosphorylation of the dissociation constant (decrease of *p*K) of the second OH is shown in Table III.

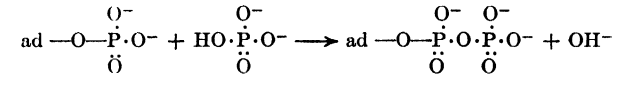
TABLE III

SECOND ACID DISSOCIATION CONSTANTS OF ORGANO-PHOSPHATES

| Compound | $pK_2$ | Reference |
|---|---|---|
| Ortho phosphate | 6.8 | |
| Hexose-di-phosphate | 6.29 | Meyerhof and Lohmann (38) |
| Fructose-6-phosphate (Neuberg ester) | 6.11 | " " " " |
| Glucose-6-phosphate (Robison ester) | 6.10 | " " " " |
| Creatine phosphate | 4.5 | Fiske and Subbarow (4) |

At *p*H 7 with one mole of phosphate combining with hexose or creatine, respectively, 0.25 or 0.4 equivalents of "acid" appear. With the reverse reactions corresponding amounts of base are liberated. The pronounced alkalization taking place with creatine phosphate breakdown was measured in living muscle by Lipmann and Meyerhof (39) and proved the actual occurrence of this reaction in the living organ.

Only with adenosine polyphosphate the change is not paradoxical. Synthesis is accompanied by disappearance and decomposition by appearance of "acid" in solution. At *p*H 7.1 per mol of "pyro" phosphate formed ca. 0.5 equivalents of acid disappear (Lohmann (40)). Because in adenylic acid the third OH group has already undergone esterification anhydrization occurs between the partly dissociated acidic second OH of adenylic acid and the third OH in ortho-phosphate. A net decrease of "acid" therefore results with the reaction:

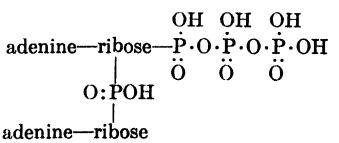


## 2. *Adenosine Polyphosphate*

The P-O∼P linkage, although attached to an organic residue, is in most respects equivalent to the P-O∼P link in inorganic pyrophosphate. The rate of hydrolysis in hot acid and the heat of decomposition are the same for free and bound pyrophosphate. The pyrophosphate first isolated from muscle was the inorganic form (36), then assumed by Lohmann to be a cell constituent. Subsequently, however, Davenport and Sacks (41) showed that in untreated muscle filtrate no inorganic pyrophosphate could be demonstrated with a specific colorimetric method. Reinvestigating the problem, Lohmann (42) eventually isolated adenylpyrophosphate, of which the inorganic pyrophosphate had been a breakdown product. He found that the Ba salt of adenylpyrophosphate splits off the pyrophosphate on standing, especially in alkaline solution. After demonstration of stepwise dephosphorylation (40) the preferable designation adenosine polyphosphate instead of adenylpyrophosphate came into use for the whole group and, specifically, the designations: adenosine-mono-, -di-, and -tri-phosphate. As abbreviations, ad-ph, ad-ph∼ph, ad-ph∼ph∼ph shall be used in this paper. The maximum of two P-O∼P linkages is formed per mole of adenosine. The first phosphate is linked to ribose in an ordinary ester linkage which is, in contrast to the other two, an energy poor linkage. The P-O∼P linkages are readily hydrolyzed with hot acid and frequently are spoken of as easily hydrolyzable phosphate (Table II).

The wide distribution of adenosine polyphosphate in nature is shown in Table IV.

Frequently compounds have been isolated with the properties of a di-adenosine polyphosphate (Deuticke (51), Ostern (52), Warburg and Christian (53)). More recently Kiessling and Meyerhof (54) isolated and carefully studied a polymer obtained from yeast to which they assign the composition of a di-adenosine-tetraphosphate:



Only two of the phosphates are easily hydrolizable. Evidence is offered

TABLE IV

ADENOSINE POLYPHOSPHATE IN TISSUES

| Tissue | Mg. % P | | Reference |
|---|---|---|---|
| | Polyphosphate P | Inorganic P | |
| Yeast | 80 | 160 | Lohmann (43) |
| Muscle, frog | 24 | ... | " " |
| Muscle, rabbit, white | 32 | ... | " " |
| Muscle, rabbit, red | 21 | ... | " " |
| Muscle, crab | 32 | ... | " " |
| Heart, rabbit | 9 | 50 | " " |
| Uterus, rabbit | 8 | .. | " " |
| Liver, rabbit | 7 | 35 | " " |
| Kidney, rabbit | 6; 10 | 45; 18 | Lohmann (43); Eggleton (44) |
| Spleen, rabbit | 17 | 36 | Lohmann (43) |
| Brain, rabbit | 5 | 7 | " " |
| Nerve, rabbit | 6 | .. | Gerard and Tupikova (45) |
| Red blood cells, pig | 9 | 9 | Lohmann (43) |
| Embryo, rat | 9 | 17 | " " |
| C 180 tumor, mouse | 8 | .. | Franks (46) |
| L R 10 tumor, rat | 11.5 | 25 | Boyland (47) |
| Jensen sarcoma | 14 | 29 | Lohmann (43) |

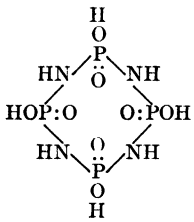
The listed figures represent easily hydrolyzable phosphate corrected for interfering substances. Actual isolation of the adenosine polyphosphate has been carried out with muscle (42), heart (48), and blood (49, 50). Yeast and muscle contain the largest amounts. The content in spleen is high. Malignant tumors contain fairly large quantities in comparison to other parenchymatous tissues. The compound has been demonstrated in bacteria, plant seeds, and arbacia eggs (43). It must be considered a universally present cell constituent and thus phosphate transfer should be considered a universal metabolic reaction.

for the existence of a phosphate bridge between the two riboses. The compound is of interest because the linkage between the adenylic acids is very alkali-sensitive, much like the inter-nucleotidic linkages in nucleic acid. No physiological difference could be detected between the mono- and dinucleotide.

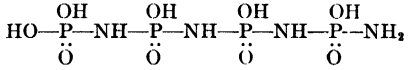
The presence of polyphosphate chains in compounds of biological importance should stimulate interest in the numerous and peculiar products formed by anhydric polymerization of orthophosphates partly in combination with ammonia. Stokes (55) made a very interesting study of poly-

merization products of amido phosphoric acid which he called meta-phosphimino acids and which had the general formula $(HPO_2(:NH))_x$. They correspond to the meta phosphoric acids $(HPO_3)_x$. According to Stokes the phosphimino acids exist in two forms, (*1*) as ring structures of great stability, (*2*) as open chains of small stability. By hydrolysis (*1*) is converted into (*2*). The especially stable ring structure $(HPO_2(:NH))_4$, (I) and the corresponding unstable open chain (II) are shown below.

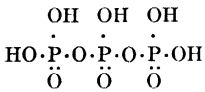
(I)



(II)



An analogous relation should exist between nitrogen-free compounds like tri-metaphosphoric acid, $(HPO_3)_3$, and tri-phosphoric acid (Neuberg and Fischer (56), Huber (57)), the inorganic homologue of ad-ph∼ph∼ph.



By the increase of stability with ring closure it is suggested that the opening and closing of the ring has a large effect on the intramolecular energy distribution. This might have some bearing on the problem of utilization of P∼N and P-O∼P linkages in muscular contraction. Furthermore, the similarity between the P-NH-P and the P-O-P bond in these structures is remarkable.

### *3. Phosphoguanidine Linkages (Phosphagens)*

The ontogenetically newer creatine phosphate in vertebrates and the "older" arginine phosphate in invertebrates are not, as is adenosine polyphosphate, substances universally occurring in cells. Their almost exclusive presence in muscular and nervous organs (44, 45), including the electric organ of fishes (Kisch (63)), suggests connection with the speed of action required by these organs. Their function as reservoirs of energy easily to be released is readily confirmed by experimental facts.

**Creatine Phosphate.**—In Table V the distribution in tissues of creatine P is shown. The substance was isolated only from muscular tissue (Fiske and Subbarow (4)). The figures represented in the table were obtained by determination of "acid unstable" P (see Table II). Therefore it must be emphasized that the significance of all figures below 3 mg. % as found in some parenchymatous organs remains doubtful. Likewise, the demonstration of labile phosphate in tumor tissue, interesting in itself, does not, as pointed out by Franks (46), give conclusive proof for the presence of phospho~creatine. Boyland (47) confirmed Franks's result and furthermore showed a content of 25 mg. % of creatine in rat tumor. He used, however, the colorimetric method for creatine determination which gives not quite convincing results for such small amounts (see Miller and Dubos (58)).

TABLE V

CREATINE PHOSPHATE IN TISSUES

| Tissue | Mg. % P | | Reference |
|---|---|---|---|
| | Creatine P | Inorganic P | |
| Muscle, frog | 54 | 22 | Gerard and Tupikova (45) |
| Muscle, cat | 60 | 26 | Fiske and Subbarow (4) |
| Muscle, amphioxus | 37 | 72 | Meyerhof (61) |
| Brain, dog | 12 | .. | Gerard and Tupikova (45) |
| Nerve, frog | 9 | 7 | " " " " |
| Heart, normal rat | 4–7 | .. | Bodansky (62) |
| Heart, hyperthyroid rat | 1–3 | .. | " " |
| Stomach, rabbit | 2–5 | 25–32 | Eggleton and Eggleton (44) |
| Testicle | 0.6–2.6 | 8–12 | " " " " |
| Uterus, rabbit | 1.4 | 11.6 | " " " " |
| C 180 tumor, mouse | 1.5–2.7 | ... | Franks (46) |
| L R 10 tumor, rat | 2.5 | 25 | Boyland (47) |
| Jensen sarcoma | 1.2 | 22 | " " |

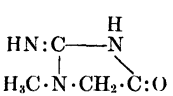
In Table VI the values for free and combined creatine are compared in tissues high in phosphocreatine. The table is taken from a paper by Gerard and Tupikova (59).

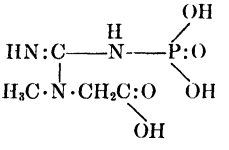
TABLE VI

FREE AND PHOSPHORYLATED CREATINE IN TISSUES

| Tissue | Total creatine, mg. % | % Phosphorylated |
|---|---|---|
| Muscle, frog | 495 | 52 |
| Nerve, frog | 146 | 40 |
| Brain, rat | 230 | 20 |

In muscle over half of the total creatine is combined with P, and about 70% of the available P is found combined with creatine. That makes only a slight surplus of creatine over phosphate. In nervous tissue likewise total creatine is fairly equivalent to available + combined phosphorus. As is noteworthy, testis is an organ known to contain large amounts of creatine [up to 200 mg. % (Hunter (60))] but none or only doubtful amounts of phosphocreatine. It seems to me a question of great interest if the constantly occurring decay of creatine to creatinine might be related to the linking of creatine to phosphate in the body. Comparison between the formulae of creatinine and phospho~creatine makes such a theory quite tempting:





In both compounds the free amino group of the guanidino part is combined with an acidic group. With the tension present in the P-N linkage a change to the more stable and similar C-N linkage, offered by the presence of carboxyl in the molecule, seems suggested.

**Arginine Phosphate.**—In search for a compound similar to phosphocreatine in invertebrate muscle, known not to contain creatine, Meyerhof and Lohmann (31) found phosphoarginine. In chemical behavior phospho arginine is very similar to phosphocreatine (31). Physiologically the compounds are perfectly analogous (Baldwin and Needham (65), Ochoa (64)). Data for phosphoarginine content of invertebrate muscles are represented in Table VII.

TABLE VII

ARGININE PHOSPHATE IN INVERTEBRATE MUSCLE

| Animal | Mg. % P | | Free Arginine | Reference |
|---|---|---|---|---|
| | Inorganic P | Arginine P | | |
| Crab | 35 | 61 | ... | Meyerhof and Lohmann (31) |
| Pecten | 42 | 84 | 0 | Meyerhof (61) |
| Sipunculus | 57 | 171 | Trace | |
| Holothuria tuberculata | 16 | 46 | 0 | |

Practically no free arginine was demonstrable in the fresh oxygenated muscle. Free and total arginine were estimated with arginase. Phosphoarginine is not attacked by arginase.

### 4. *Phosphoenol Pyruvic Acid*

Embden (66) discovered that phosphoglyceric acid, earlier isolated by Nilsson (67), was fermented to pyruvic acid and phosphate:

$$CH_2OPh \cdot CHOH \cdot COOH \longrightarrow CH_3 \cdot CO \cdot COOH + H_3PO_4.$$

Subsequently this complex reaction was analyzed by Lohmann, Meyerhof and Kiessling. Embden's reaction was found to occur in three steps:

$$CH_2OPh \cdot CHOH \cdot COOH \rightleftharpoons CH_2OH \cdot CHOPh \cdot COOH \rightleftharpoons CH_2{:}COPh \cdot COOH + H_2O \longrightarrow CH_3 \cdot CO \cdot COOH + H_3PO_4.$$

First, Parnas, Ostern, and Mann (33) observed that a transfer of the phosphate present in phosphoglyceric acid to adenylic acid occurs in muscle whereby the energy-rich phosphate bond in ad-ph~ph~ph is formed. Lohmann and Meyerhof (1) then found that the removal of phosphate from phosphoglyceric acid occurs only when adenylic acid is present to accept the phosphate. A partial change took place, however, also in absence of adenylic acid, yielding an ester which was less stable against acid hydrolysis. Eventually the transformation product was isolated and identified as phosphopyruvic acid containing phosphate in an energy-rich bond (1). Later on, with Kiessling (68), the first transformation product, phospho-2-glyceric acid, was isolated. In the absence of adenylic acid the following equilibrium mixture was obtained in muscle extract.

| | % | Enzyme | |
|---|---|---|---|
| d(-)phospho-3-glyceric acid | 58.5 | phosphoglycero-mutase | (68) |
| d(+)phospho-2-glyceric acid | 12.5 | | |
| phospho~enolpyruvic acid | 29 | enolase | (1) |

SPECIFIC REACTIONS OF PHOSPHOENOL PYRUVIC ACID (1)

| Reagent | Products of Reaction |
|---|---|
| $Br_2$ | $CH_2Br \cdot CO \cdot COOH + HBr + H_3PO_4$ |
| I | $CH_3CI_3 + CO_2 + 3HI + H_3PO_4$ |
| $HgCl_2$ | $CH_3CO \cdot COOH + H_3PO_4$ (catalytic) |
| *n*-HCl, 100° | Complete hydrolysis after 60 minutes |

With the analysis of the stepwise degradation of phosphoglyceric acid Lohmann and Meyerhof were able to define specifically the effect of fluoride long known to be a powerful poison of fermentation and glycolysis. They found that the enolase reaction is very sensitive to fluoride. This explained the accumulation of phosphoglycerate which had been shown to occur in Nilsson's reaction (67).

2 acetaldehyde + glucose + $2H_3PO_4$ ⟶ fluo | ride
2 ethylalcohol + 2 phosphoglyceric acid ↓ . . . . . .

Fluoride inhibition, thus presenting a means to interrupt the chain of transformations of phosphorylated intermediates at a definite stage, but without interfering with the central O/R reaction, became a powerful tool for the analysis of intermediate processes in fermentation. Fluoride inhibition which is shown by various fermentations and by glycolysis in various tissues (Dickens (69)) indicated early the general occurrence of phosphorylation in fermentation processes. More recently Werkman (70) showed in a variety of fermentations with various organisms that there, like in alcoholic fermentation, phosphoglyceric acid accumulation occurs under similar conditions. Yeast (Effront (71)), however, and propionic acid bacteria (Wiggert and Werkman (73)) can by subculturing with rising concentrations of fluoride become adapted to the poison. Wiggert and Werkman (73) showed, furthermore, that fluoride-fast propionic bacteria, in contrast to normal, did not attack phosphoglyceric acid. This was interpreted to show that a non-phosphorylating fermentation mechanism operates in the fluoride-fast organisms. It seems, however, not unlikely that the fluoride-fast organism has become impermeable to fluoride during the period of adaptation, and that a change of permeability had likewise caused the non-fermentability of phosphoglyceric acid. Effront (72) reports for fluoride treated yeast that it had changed greatly in appearance and in composition, *e. g.*, Ca content was raised from 1.65 to 4.1 in arbitrary units. Runnström's (74) experiments are also sug-

gestive in this direction. He showed that the poisoning effect of fluoride in normal yeast depends greatly on the state of the cell. Aerobically and with addition of glucose, inhibition was invariably less or absent. Anaerobically or without substrate, however, the poisoning effect was greatly exaggerated. Runnstöm interprets this effect as a change of permeability for fluoride with active metabolism.

### 5. *Phosphoglyceryl Phosphate*

A diphosphoglyceric acid was isolated by Negelein and Brömel (2) as the product of enzymatic oxidation of phosphoglyceraldehyde (Fischer ester (75)) + phosphate. This compound was not identical with Greenwald's 2-3-diphosphoglyceric acid (76). The anhydric nature of one phosphate linkage was assumed because of its instability even at neutral reaction. This assumption was corroborated by the showing of an ultraviolet absorption band at 240 Å, analogous to acetanhydride and disappearing after decomposition of the labile ester linkage. The compound was called by Negelein and Brömel R-diphosphoglyceric acid or 1-3-diphosphoglyceric acid. We have suggested the more descriptive name of phosphoglyceryl phosphate (77).

### 6. *Acetyl Phosphate*

A phospho organic compound of stability conditions analogous to synthetic acetyl phosphate was shown to be formed by enzymatic oxidation of pyruvate + phosphate (Lipmann (3)). It was synthesized by a modification of the method of Kämmerer and Carius (78) for the preparation of triacetyl phosphate (unpublished). Instead of using $Ag_3PO_4$, a mixture of $Ag_3PO_4$ and $2H_3PO_4$, and acetyl chloride was used. Lynen (79) described recently a more reliable synthesis by passing through the dibenzyl phosphate. As an acid anhydride, the compound is quite stable in water.

Analogously, succinyl phosphate would be formed by oxidation of ketoglutarate + phosphate. We synthesized this compound from succinyl chloride + $Ag_3PO_4$ and found it very similar to acetyl phosphate (unpublished).

## V. The Phosphate Cycle

Several reaction phases can be distinguished in the constantly occurring metabolic turn-over of phosphate: (*1*) introduction of inorganic phosphate into ester linkage, (*2*) generation of energy-rich phosphate bonds (~ph) by oxidation-reduction, (*3*) the taking over and distribution of ~ph by cell catalysts (*e. g.*, adenylic acid), (*4*) utilization of ~ph and the regeneration

of inorganic phosphate. The machine-like functioning of the revolving sequence of reactions appears from the schematic representation shown in Fig. 1.

For the undisturbed maintenance of this complicated series of reactions a well-balanced equilibrium is needed between the great number of enzymatic reactions involved. Oxido-reductive formation of ~ph and its removal by adenylic acid must follow each other in due course, in order to

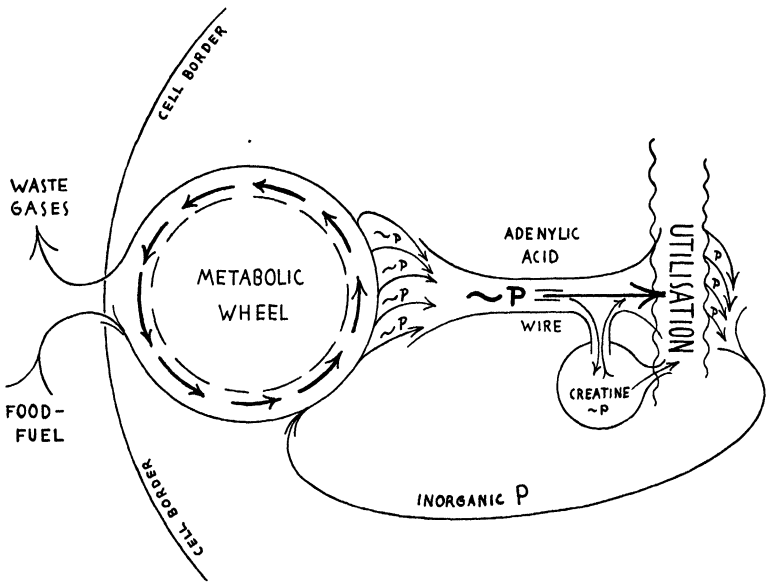



Fig. 1.—The metabolic dynamo generates ~P-current. This is brushed off by adenylic acid, which likewise functions as the wiring system, distributing the current. Creatine~P, when present, serves as P-accumulator.

avoid obstruction of the smooth flow of reactions. A finely coordinated interplay between oxidation-reduction and phosphorylation-*de*-phosphorylation results. This explains why very often adenylic acid functions as apparent coenzyme of O/R reactions. In many if not all such cases the part played by the adenylic acid seems not related to O/R catalysis proper but to removal of obstructing phosphate groups. This was shown to be the case in the O/R reaction in the fermentation sequence (Warburg and Christian (80)):

$$\longrightarrow \text{pyridine} + \text{di-ph-glyceraldehyde} \rightleftharpoons \text{reduced pyridine} + \text{ph-glyceryl}\sim\text{ph} + \text{ad-ph}\sim\text{ph} \longrightarrow \text{ph-glycerate} \longrightarrow + \text{ad-ph}\sim\text{ph}\sim\text{ph}$$

Ph-glyceryl∼ph, if not dephosphorylated, would be accumulated which soon would cause the back reaction to take place to such an extent that a state of equilibrium would be established. Thus the forward reaction would come to a standstill and thereby the whole reaction chain would be broken. A similar case seems to be the oxidation of pyruvic acid where a phosphorylated oxidation product primarily is formed (Lipmann (77,3)) and adenylic acid is required for further oxidation (Banga, Ochoa, and Peters (81)). Disturbance of this finely balanced interplay is prone to lead to "pathological" reactions in cell extracts which in many respects are certainly out of balance. Also in the body, such disturbances quite probably are the cause of pathological conditions. Recent work by Shorr, Barker, *et al.* (82), is suggestive of the existence of a deficiency in phosphate turnover in diabetic tissues.

As an example of a reaction which is "pathological" in certain respects, the Harden-Young fermentation in brewer's yeast extract might be cited.

$$2 \text{ hexose} + 2 \text{ phosphate} \longrightarrow \text{alcohol} + CO_2 + \text{hexosediphosphate}.$$

Here, following and slightly expanding the interpretation of Harden (83), it is assumed that the returning of inorganic phosphate into the cycle is out of function (see Fig. 1). An "over"-phosphorylation of hexose occurs possibly because of interruption of the normal utilization of ∼ph, by way of which the inorganic phosphate is normally recovered. Through the erroneous course the ∼ph takes, hexosediphosphate accumulates, and inorganic phosphate, needed in the cycle, eventually disappears. The result is an almost complete stop of fermentation when the inorganic phosphate, if present only in limited amounts, is irreversibly fixed.

In fact, the phosphate can only fulfill its function as a true catalyst in fermentation by entering and leaving the reaction cycle at the same rate. Nevertheless, in the preliminary phases, very often a more or less pronounced accumulation of phosphate ester does occur. Thus, in extracts of baker's yeast (Lipmann (84)) only in the beginning occurs extensive ester formation. The system never becomes entirely depleted of inorganic phosphate and fermentation continues with almost constant rate. Preliminary ester formation was likewise demonstrated at the onset of fermentation in living cells (Macfarlane (85) Wiggert and Werkman (85a). With slow fermenting *fusarium lini*, however, phosphorylation was not found in the beginning (Nord (86, 87)). First in the later phases of this

fermentation phosphorylation was observed. This was considered as indicating a non-phosphorylating fermentation mechanism.

### *1. Primary Phosphorylation*

Various reactions leading to fixation of phosphate have been known for a long time. However, an exact analysis showed that in many cases there occurs a shift of already fixed phosphate from one compound to another. Only more recently reactions were found which explain the primary introduction of inorganic phosphate into organic linkage. Primary phosphorylation occurs (*a*) by phosphorolysis of glycogen (Cori (22), Parnas (88)), or (*b*) by phosphate addition to carbonyl double bonds (Negelein and Brömel (2), Lipmann (3)). It is quite probable that still other pathways exist (Kalckar (16), Colowick, *et al.* (17)).

(*a*) The details of his work on phosphorolysis were discussed fully by Cori in a recent review (22) to which the reader is referred. With reference to the reversibility of the reaction,

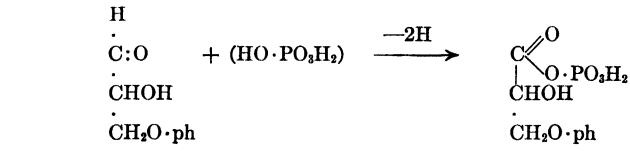
$$\text{glycogen} + \text{phosphate} \rightleftharpoons \text{phospho-1-glucose},$$

it is assumed by Cori that phosphorylation of glucose must occur prior to glycogen synthesis. Thus, taking the body as a whole, phosphorylation of glucose by glycogen phosphorolysis might be called a rephosphorylation because every glucose present in the body as glycogen had earlier to pass through a stage of phosphorylation. It is then preserved in glycogen in a position to take up inorganic phosphate at will, in a spontaneous reaction. By this potentiality, glycogen is made such a preferable substrate for muscle, enlarging the yield of utilizable ~ph by 50 or maybe even 100% (Needham and Pillai (89)) (as will appear later by comparison of glycogen and glucose glycolysis).

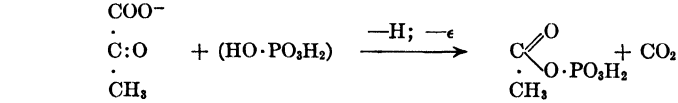
The phosphorolytic equilibrium reaction is catalyzed by an enzyme with adenylic acid as the prosthetic group (22). It is very noteworthy that here the adenylic acid functions as a phosphorylating catalyst apparently without being intermediately phosphorylated. This was shown experimentally to be the case, as would be indeed almost impossible also for energetic reasons. It might be, however, that the phosphate group potential of the ad-ph/ad-ph~ph~ph system can be changed by combination with a protein, as, *e. g.*, the O/R potential of flavin is changed in the flavoproteins (90).

(*b*) The entry of phosphate into compounds with carbonyl groups is a reaction for which no analytic data are available. It might be considered an addition reaction, similar to that shown by other acids like acid-sulfite

and HCN (Lipmann (77)). So far, such addition products have not been isolated. Their occurrence, however, must be assumed because of the necessity of the presence of phosphate in certain enzymatic carbonyl dehydrogenations and because of the appearance of the phosphate there in the products of dehydrogenation (Negelein and Brömel (2), Lipmann (3)). Without any definite assumption as to the nature of the intermediate, it is a fact that phosphate is fixed into organic linkage by the following reactions:



and



The experimental concentrations of the reacting substrate and of phosphate in these reactions are shown in Table VIII.

TABLE VIII

| Compound | Substrate $10^{-2}$ mol/liter | Phosphate $10^{-2}$ mol/liter | Enzyme |
|---|---|---|---|
| Phospho-glyceraldehyde (Fischer-ester) (75) | 0.5 | 3.3 | 2 γ/ml crystalline "oxidation enzyme of fermentation" (yeast) (80), diphosphopyridine nucleotide |
| Glyceraldehyde | 3.2 | 0.33 | 1000 γ/ml of same enzyme |
| Pyruvate | 2 | 1.3 | Extract of dried bact. Delbruecki (118) |

It is to be noted that *non*-phosphorylated glyceraldehyde likewise reacted appreciably, however, only when the enzyme concentration was a thousand times higher.

Before concluding the discussion of phosphate introduction into organic linkage, it should be mentioned that synthesis of an organo-phosphate by the reverse catalysis of phosphatase was shown by Kay (21) in the case of

phosphoglycerol. However, to obtain a measurable synthesis, extreme concentrations of glycerol (50–75%) had to be employed. It can be calculated from the equilibrium constant (see page 104) that under physiological conditions, *i. e.*, with about $10^{-2}$ mol per l. of phosphate and of glycerol less than 0.1% ($10^{-5}$ mole per l.) would be present as phosphoglycerol at equilibrium. Therefore appreciable synthesis cannot occur unless, through subsequent reactions, the ester were not kept at a very low concentration. There is no evidence at present to show that the reverse catalysis by phosphatase is utilized in cells in any of the numerous cases of phosphate ester synthesis.

### 2. *Transphosphorylation*

It appears from the preceding paragraph that phosphate enters into primary organic bonds over the pathway of oxidation-reduction reactions. By these procedures acyl-phosphates and possibly other phosphate bonds with high group potential are generated. Or, in a respiring or fermenting cell the phosphate potential is maintained at a fairly constant and high level. By means of the high phosphate group-pressure a great variety of transphosphorylations occurs in the cell, whereby the high potential of the bond is either maintained, or a fall of potential takes place with formation of an ester bond. From ester bonds secondary transphosphorylations take place by reversible exchange. In Fig. 2 the relationship between the bonds at different potential levels is shown schematically.

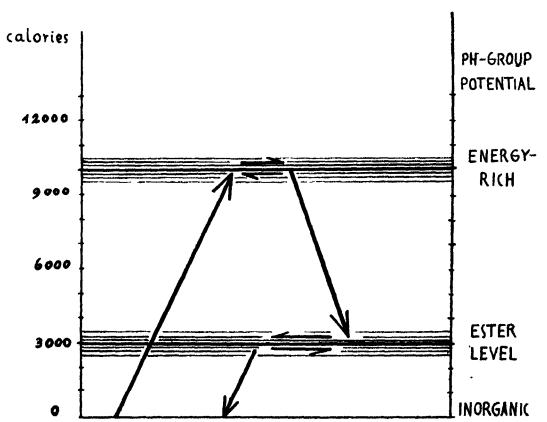



Fig. 2.

Beginning at the left, the potential of inorganic phosphate which represents the base level is lifted to the upper, the 10.000 cal level, *e. g.*, by oxidation of carbonyl-phosphate adducts to acyl~phosphate. Energy-rich phosphate groups may remain at first on the high level, by migrating to adenylic acid, but fall eventually to the lower, the 3000 cal level by assuming ester linkages. They may be retained there but most fall down finally to the inorganic phosphate "base line" through phosphatase action or otherwise. Thus, a more explicit picture of the phosphate cycle is obtained.

The enzymatic mechanism of transphosphorylation appears remarkably

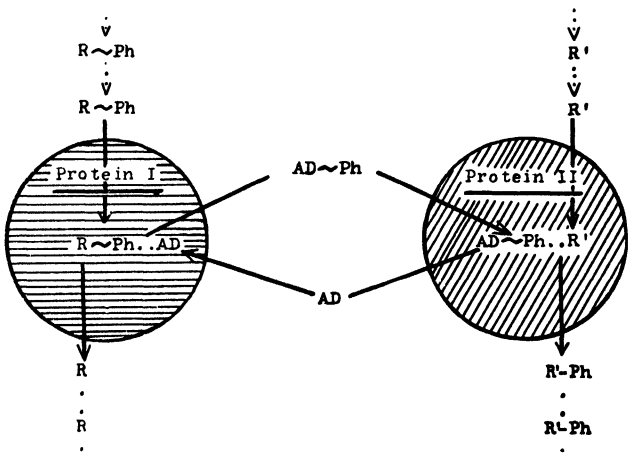



Fig. 3.

similar to enzymatic hydrogen transfer, although definite knowledge about the protein parts of phosphorylating enzymes is still lacking (53, 91). Adenylic acid, much like pyridine nucleotides, acts as a largely dissociated coenzyme, especially with transfer on the upper potential level and from the upper to the ester level (see Table IX).

The *step*-wise de- and rephosphorylation of ad-ph~ph~ph is due to different apo-enzymes (protein parts) (Lohmann (40), see also (91, 80)). On the upper level, adenosine polyphosphate/adenylic acid reacts reversibly, *e. g.*, with the pairs creatine/creatine phosphate (34) and phosphoglycerate/phosphoglyceryl phosphate (80). The reaction from ad-ph~ph~ph to the ester level occurring with a loss of 6000 cal (referring to

TABLE IX

TRANSPHOSPHORYLATIONS

| *On upper potential level* | | *From upper to lower level* |
|---|---|---|
| (64) Arginine∼ph⇌ arginine | | + glucose → ph-6-glucose (88) |
| | | + ph-6-fructose → di-ph-1-6-fructose (88) |
| (33) Creatine∼ph⇌ creatine | | + adenosine → ad-ph (98) |
| (80) ph-Glyceryl∼ph ← ph-glycerate → | ad-ph ⇄ ad-ph∼ph → ad-ph∼ph∼ph | + di-ph-pyridine di-nucleotide → tri-ph-pyridine dinucleotide (99) |
| (1) Pyruvic-enol∼ph → (97) Acetyl∼ph → | ↑ | + thiamine → thiamine-di-ph (100) |
| (16) Enol∼ph *via* ← di-carboxylid acid | "oxydative" ∼ ph→ | + glycerol → ph-glycerol (16) |
| | | + galactose → ph-galactose (16) |
| | | + arabinose → ph-arabinose (16) |

*On lower potential level*

*Intra*molecular

ph-1-glucose → ph-6-glucose (22)
ph-3-glycerate ⇄ ph-2-glycerate (68)

*Inter*molecular

adenosine-ph + glucose ⇄ ph-6-glucose + adenosine (98)
*di*-ph-fructose + *di*-ph-pyridine dinucleotide ⇄ *tri*-ph-pyridine dinucleotide + hexose-*mono*-phosphate (99)

standard conditions) was never observed to be reversible. According to recent experiments of Kalckar (16) and of Colowick, *et al.* (17), the adenylic acid system does not seem to be the only pathway for distribution of ∼ph, as had been thought before.

An essential function of metabolic group transfer is the maintenance of the group potential at a certain level. It is therefore evident that in the transfer of phosphate groups between organic molecules the group never passes through the inorganic phosphate stage. This was proved experimentally by the use of radioactive phosphate (92, 93). Therefore it must

TABLE X

SPECIFIC ACTIVITIES OF PHOSPHATE FRACTIONS TAKEN THREE TO FOUR HOURS AFTER INTRAVENOUS INJECTION OF RADIOACTIVE SODIUM PHOSPHATE

Related to Plasma Phosphate Activity 100

| No. | Animal | Organ | Inorganic | ad-ph~ ph~ph | Ester-ph | Creatine ~ph | Fixed-ph | Reference |
|---|---|---|---|---|---|---|---|---|
| 1 | Rabbit, narcotized* | Kidney | 100 | 36 | 38 | ... | .... | (173) |
| 2 | | Liver | 55 | 38 | 18 | ... | .... | (173) |
| 3 | | Blood corpuscles | 11 | 7.7 | .. | ... | .... | (176) |
| 4 | | Muscle | 7.3 | 0.8 | .. | 1.0 | 0.07 | (173) |

* No definite statement is found about narcosis. However, radioactive phosphate was injected dropwise intravenously over a period of 4 hours. This seems only possible with an animal in narcosis.

TABLE XI

SPECIFIC ACTIVITIES OF MUSCLE PHOSPHATE FRACTIONS

Related to Muscle Inorganic Phosphate = 100

| No. | Animal | Time of exposure, hours | Inorganic | ad-ph~*ph*~*ph* | Ester-ph | Creatine-ph | ad-ph | Reference |
|---|---|---|---|---|---|---|---|---|
| 1 | Frog | 3 (2°) | 100 | 50 | 47 | 49 | .... | (176) |
| | | 3 (21°) | ... | 78 | .... | 78 | .... | |
| 2 | Rabbit | 0.5 | 100 | 80 | .... | .... | 0 | (174) |
| | | 2 | ... | 81 | .... | .... | 0.03 | |
| 3 | Rabbit (narcotized)* | 4 | 100 | 11 | .... | 13.5 | .... | (173) |
| 4 | Cat (amyntal anaesthesia) | 2 (resting) | 100 | 8.5 | 2.9 | 10.3 | .... | (175) |
| | | (15″ tetanus) | 77 | 8.9 | 5.8 | 10.7 | .... | Average of 7 experiments |
| | | mg % P (resting) | 14 | 33 | 7 | 60 | .... | Single experiment |
| | | (15″ tetanus) | 33 | 31 | 25 | 29 | .... | |

* See footnote on p. 129.

be postulated that an intermediary addition compound forms between the molecules exchanging the group. On this basis the above scheme (Fig. 3) of the enzyme reaction is probable (91, 80).

For the study of phosphate transfer in the whole animal the use of radioactive phosphate was introduced by Hevesy. The experiments represented in Tables X and XI were taken from Hevesy's reviews (172, 173) and from papers by Korzybski and Parnas (174) and by Sacks (175). As was expected, a fairly rapid turn-over was found in blood corpuscles, kidney, and liver (Table X). The results obtained with muscle were somewhat ambiguous. As shown in Table X, there appears to be a striking difference in turn-over between the experiments of Nos. 1 and 2 and those of Nos. 3 and 4. With rabbit, in experiment 2 a complete equilibration had taken place after only 30 minutes; in experiment 3, however, with a narcotized rabbit* very little exchange was found after 4 hours' exposure. Similar results were obtained with a narcotized cat by Sacks (Table XI, No. 4). He found that even when assayed immediately after short tetanization, practically no increase of phosphate exchange could be found. From these, however, interesting results, he feels justified to deny the existence of a direct communication between creatine phosphate and adenosine polyphosphate metabolism with the glycolytic reaction. This, we feel certain, is contradicted by the occurrence in the living organ of a rapid and effective anaerobic resynthesis of creatine phosphate at the expense of glycolytic energy (11, 12), and, furthermore, by the exchange found after short periods in experiments with non-narcotized animals (Table XI, Nos. 1 and 2). An explanation of the behavior of muscle in the narcotized animal might be found in the sluggishness of exchange between the muscle cell containing the metabolites and the intercellular fluid, where in the state of resting, the inorganic phosphate is to be found according to experiments of Eggleton (177) and Fenn (178). Then, the reference to inorganic muscle phosphate would be misleading since *inter*cellular inorganic phosphate and *intra*cellular metabolites would be present in separated tissue units. An additional systematic error seems introduced in all these experiments through contamination with plasma inorganic phosphate which, although relatively low in concentration (3–4 mg. %), is up to 14–15 times higher in activity (see Table X, No. 4).

## VI. Metabolic Generation of Energy-rich Phosphate Bonds

Reactions of the type of fermentations occur in the cell with the purpose of making available the energy liberated by degradation of the initial

* See footnote on p. 129.

substrates to the end-products. These are waste products. Therefore, the intermediate stages in a fermentation should not be considered as stages in a somewhat complicated preparatory procedure but rather as the chemical "wheels" which drive a machine. Knowledge of intermediary processes, then, becomes the first step toward a definite comprehension of procedures used to effect maintenance and propagation of the living state.

### *1. Anaerobic Metabolism*

Fermentation and glycolysis represent reactions where the energy, derivable from the partial catabolism of glucose, is converted almost quantitatively into phosphate bond energy. Two ~ph, phosphoglyceryl ~ph and pyruvic enol~ph, are obtained per $^1/_2$ glucose. The enol~ph

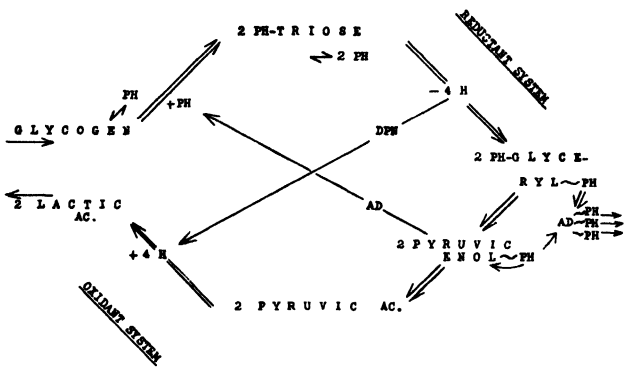



Fig. 4.

arises from primary introduced ester-ph, the acyl~ph by dehydrogenation of the addition product between inorganic phosphate and carbonyl compound. When free glucose is the substrate one of the two ~ph is used to effect a primary ester bond. Thereby the efficiency of generation of *utilizable* phosphate is depressed by 50%. With glycogen the efficiency is higher because the glucosidic linkages between the glucoses in glycogen are on the phosphate ester level and give free entrance to the phosphate by exchanging phosphate for glucose.

A survey of the whole reaction cycle is given in Fig. 4. The basic structure represented is taken from a paper which appeared in 1934 (Lipmann (94)).

With the oxidation-reduction in central position we distinguish three

phases, (*1*) pre O/R transformation period, (*2*) O/R reaction proper, (*3*) post O/R transformation period. The two transformation periods shall be considered first.

**Pre O/R Transformation Period.**—In Fig. 5 the sequence of transformation is shown schematically. Starting with glycogen as in Fig. 4 the introduction of the first phosphate goes by phosphorolysis. In muscle and yeast extracts (95, 96) the second phosphorylation leading to the remarkable symmetric fructose-di-phosphate requires forced introduction from one of the later generated ~ph with adenylic acid as intermediate (Ostern, *et al.* (98)). However, Lundsgaard's (12) results with living muscle suggest a more economic process occurring in the living organ. He found

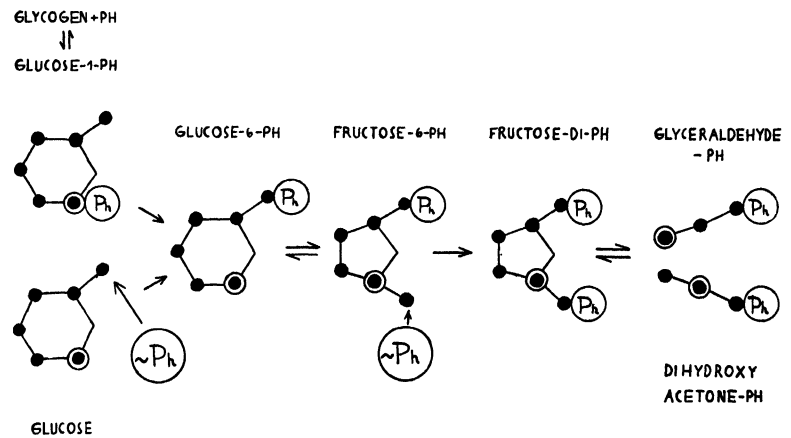



Fig. 5.

up to 1.9 mols. ~ph, determined as creatine ~ph, to correspond with one mole lactic acid formed from $^1/_2$ glucose. It may be, and in fact seems rather plausible, that here a reaction of the following type takes place:

2 hexose-mono-phosphate ⟶ hexose-di-phosphate + hexose.

The interchange of ester phosphate is a known reaction (see Table IX). Glucose-1-phosphate might well transfer its phosphate into 1-position of fructose-6-phosphate by means of a hitherto unknown enzyme. In such a case all ~ph would remain utilizable because glycogen would be in a sequence of spontaneous reactions yielding hexose diphosphate thus explaining fully Lundsgaard's experimental results:

2 glucose (-glycogen) + 2 phosphate ⟶ hexose-di-phosphate + glucose.

The observation of Hegnauer, Fisher, Cori, and Cori (101) that free glucose appears in the recovery of muscle lends support to this equation which suggests a mechanism for the appearance of glucose.

In contrast, when free glucose is the starting material, both phosphate groups have to be introduced forcibly and two out of four ~ph are always used up in this preparatory phase. A reaction type results where the occurrence of an earlier stage depends on the occurrence of a later stage of the cycle. This interrelation between the two subsequent periods makes the reaction autocatalytic at the start.

As the result of this chain the twice phosphorylated hexose is finally split into two parts. The splitting reaction, the aldolase effect of Meyerhof (102), was recently carefully studied by Herbert, Gordon, Subrahmanyan, and Green (103). The reaction is energetically interesting because spontaneous dissociation occurs, in spite of the fact that the reaction is largely endothermic.

It must be considered as the intention with this series of reactions to break the hexose molecule into two parts. This preliminary procedure is carried out even at the expense of energy derived by later phases of the cycle. It seems, then, that the twofold phosphorylation is needed to effect such fission under conditions prevailing in the cell. The final product is phosphoglyceraldehyde which in the presence of inorganic phosphate reacts as the reductant in the O/R reaction.

**Post O/R Transformation Period.**—Table XII shows the sequence of

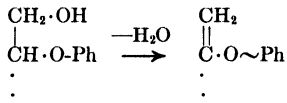
TABLE XII

POST O/R TRANSFORMATION PERIOD

ph-3-glyceryl~ph ⟶
↓
ph-3-glycerate
⇅
ph-2-glycerate
⇅
pyruvic enol~ph ⟶
↓
pyruvate

reactions. The ~ph generated in the oxidation-reduction is removed from the carboxyl of phosphoglyceric acid with conservation of the bond energy through adenylic acid (80, 96). Eventually it appears in muscle as creatine~ph. A second ~ph is yielded by removal of water from phosphoglyceric acid. This reaction was discussed extensively earlier in

this paper. The energy which also is taken over with the phosphate by adenylic acid is mobilized by a dehydration of the following type:



It seems possible that in a similar manner ~ph is generated with other phosphate esters where the carbon, bearing the esterified hydroxyl, lies next to another hydroxylated carbon. Phosphoglycerol, especially β-phosphoglycerol, might be degraded in such manner and utilized as source of ~ph. A reaction of this type might occur intermediately in the bacterial reduction of glycerol to dihydroxypropane as described by Mickelson and Werkman (104).

In lactic acid fermentation (glycolysis in animal tissues) the post O/R transformations are closed by formation of pyruvic acid which in the O/R reaction is reduced to lactic acid. It might be concluded from the very general formation and fermentability of phosphoglyceric acid, precursor of phosphopyruvic acid (see Werkman's review (70)), that in many of the more complicated bacterial fermentations the same sequence of pre O/R and post O/R reactions occurs until the pyruvic acid stage is reached. Subsequently, however, pyruvic acid undergoes further transformations either with or without dehydrogenation.

If the subsequent reactions do not involve dehydrogenation, the structure of the fermentation cycle might remain essentially the same except that the hydrogen acceptor appears somewhat later in the reaction chain. This is the case in alcoholic fermentation which occurs with decarboxylation of pyruvic acid and consequently with acetaldehyde as hydrogen acceptor. Another important non-O/R transformation of pyruvic acid is the so-called hydroclastic reaction,

$$CH_3CO\cdot COOH + H_2O \longrightarrow CH_3COOH + HCOOH.$$

This reaction might be, in fact, a "phosphoro-clastic" reaction since, according to experiments of Silverman and Werkman (105) with *Aerogenes* preparations, the presence of inorganic phosphate seems necessary for this reaction to occur. In analogy to pyruvic acid dehydrogenation in *Bact. Delbrueckii* (3) the "phosphoro-clastic" reaction might be formulated as follows:

$$CH_3CO\cdot COOH + H_3PO_4 \longrightarrow CH_3COOPO_3H_2 + HCOOH.$$

Subsequent condensation to $C_4$-compounds would then occur with an

"active" acetate, *i. e.*, with acetyl phosphate, containing the acetyl residue with a high group potential.

When pyruvic acid is dehydrogenated in the course of a fermentation, it will compete with ph-glyceraldehyde to yield fermentation hydrogen. Then, instead of a single reaction cycle, two or more cycles occur around different O/R reactions. Since pyruvic acid degradation can furnish additional ~ph, the yield of ~ph per mole of glucose should be increased in such fermentations.

While the pre O/R period is preparatory and mostly energy-consuming the post O/R period together with the O/R period proper yield practically

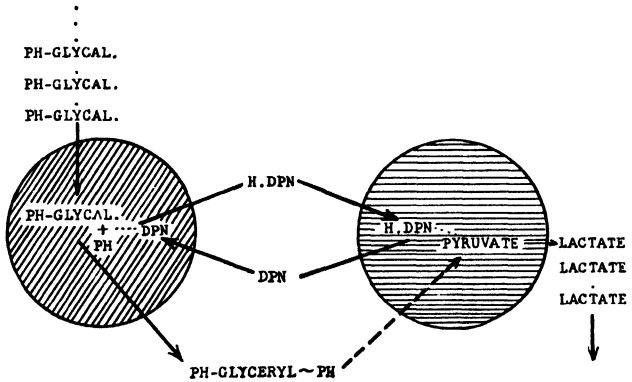



Fig. 6.

all the energy; this is drawn off from the anaerobic reaction cycle in the form of phosphate bond energy.

**The Oxidation-Reduction Reaction.**—The two transformation periods produce the reactants in the O/R reaction. The hydrogen in lactic acid and alcoholic fermentations is carried from reductant to oxidant by diphosphopyridine dinucleotide, DPN(105a), the cozymase. The pyridine combines with two independent enzyme systems for the partial reactions of dehydrogenation and hydrogenation (Warburg and Christian (80), Negelein and Wulff (106)). This is shown in Fig. 6 which is a schematized reproduction of the O/R part of fermentation.

Comparison between hydrogen transfer shown in the figure and of phosphate group transfer (see Fig. 3) shows the analogy of the enzymatic processes. The independent operation of donator and acceptor systems

in almost all biochemical transfer reactions is a fact of great metabolic significance. Thereby the cell is enabled to interchange under different conditions donator as well as acceptor systems. For example, a profound reorganization of hydrogen and of ~ph interchange must take place when oxygen as hydrogen acceptor enters a fermenting cell. The result of this reorganization is usually called the Pasteur effect.

An interesting example of the change of hydrogen acceptor under anaerobic conditions is the shift from butyric to lactic acid fermentation with *Clostridium butylicum*, reported by Kubowitz (107) to take place in the presence of $H_2$, CO, or HCN. From the fact that pyruvic acid fermentation is inhibited or abolished by these substances, it was concluded that

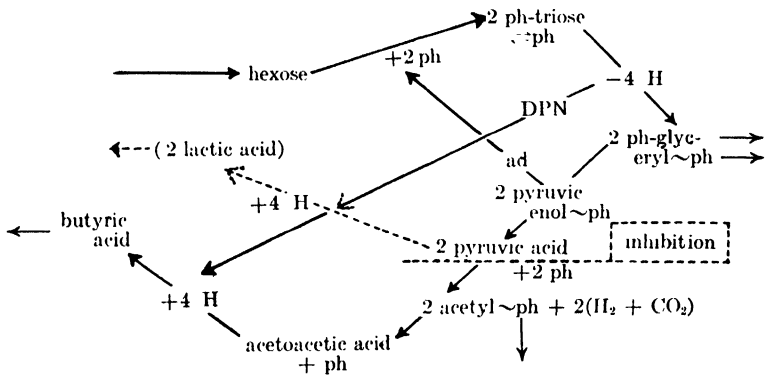



Fig 7

pyruvic acid normally is the precursor of the end-products of butyric acid fermentation which are butyric and acetic acids, $H_2$, and $CO_2$. If the normal pathway is blocked pyruvic acid itself accepts the hydrogen and the result is lactic acid fermentation. The experiments of Kubowitz suggest that in both butyric and lactic acid fermentation, the reductant is the same. A tentative scheme explaining by an acceptor change the transition from butyric to lactic acid production is given in Fig. 7.

According to the experiments of Silverman and Werkman (105) mentioned above, the two pyruvic acids arising from glucose might furnish with subsequent degradation two additional ~ph in butyric acid fermentation. Thereby, as indicated in the scheme, the yield of ~ph would be increased from four to six. On subtraction of the two ~ph returned to

glucose, the external yield would then be increased from lactic to butyric acid fermentation from two to four ~ph, or by 100%.

**Energy Balance.**—In the reaction sequence leading from glucose to two lactic acids, four ~ph are made available, by formation (*1*) of two ~ph in the O/R reaction (Dische (108), Meyerhof, *et al.* (96), Needham and Pillai (89)) due to formation of two acyl-phosphate bonds (Negelein and Brömel (2)), and (*2*) of two enol-phosphates by dehydration of phosphoglyceric acid. In the first reaction ~ph arises from spontaneously introduced inorganic phosphate while in the second reaction it arises from primarily introduced ester phosphate. The manner by which the primary ester bond is formed determines whether the second pair of ~ph is allowed to leave the reaction cycle. The first pair, however, is always in surplus. If glycogen is the substrate, at least one, or in the living muscle probably both, of the ester phosphates can be spontaneously introduced by phosphorolysis. If free glucose is the substrate no spontaneous introduction is possible. Both ester phosphates are to be forced into glucose from energy-rich phosphate bonds produced in the glycolytic cycle itself. With this transfer the energy previously accumulated in the bond is practically lost.

With glycogen as substrate, then, three out of four, or even all the four ~ph remain available. This explains, as first was pointed out by Needham and Pillai (89), why almost four moles of creatine phosphate could be reformed per mole of glycogen-glucose metabolized in the recovery of muscle (12). With glucose, however, where two ~ph are used up for primary phosphorylation, only two out of four ~ph remain available.

The calculation carried out in the earlier part of this paper leads to an average value of $-10{,}000$ cal for the change in free energy with decomposition of an energy-rich phosphate bond. The 40,000 cal, thus represented by the four ~ph, might be compared to the total change in free energy with the decomposition of one mole of glucose (glycogen) to two moles of lactic acid. For this reaction, Burk (13) calculated $\Delta F$ = ca. $-58{,}000$ cal. Therefore, up to 70% of the total energy is converted into phosphate bond energy.

The difference of 30% can grossly be accounted for by the change of free energy with the O/R reaction. The two partial reactions, reduction of quaternary diphosphopyridine dinucleotide ($DPN^+$) and oxidation of tertiary $H \cdot DPN^+$, occur. The reaction between $DPN^+$ and phospho-glyceraldehyde-phosphate is reversible (80). Therefore, no appreciable change of free energy should occur here. The change of free energy with oxidation of $H \cdot DPN^+$ by pyruvate can be estimated approximately from the difference between

the known O/R potentials of the reacting systems. $E_0'$ (*p*H 7) for $\overset{+}{\text{D}}\text{PN}/\text{H}\cdot\text{DPN}$ was calculated by Clark (109) from equilibrium data by Euler, Adler, Günther, and Hellström (110) to be −0.33 volt.* $E_0'$(*p*H 7) for pyruvate/lactate, as determined by Barron and Hastings (111) (cf. 111a), is −0.18 volt. With a difference of 0.15 volt between the $E_0'$ values, the change in free energy with the reaction pyruvate + $\text{H}\cdot\overset{+}{\text{D}}\text{PN}$ → lactate + $\overset{+}{\text{D}}\text{PN}$, is −6400 cal, or per mole of glucose, where two moles of lactic acid are formed, −12,800 cal. Then, four ~ph with 40,000 cal and the O/R reaction with ca. −13,000 cal account for −53,000 cal of the calculated −58,000 cal.

It seems not impossible that, besides the fermentation sequence starting with phosphorylation of hexose, another sequence might exist without primary phosphorylation, as was discussed recently by Nord (171). It would, however, be desirable to offer additional evidence. A fruitful line of approach might be found, if possible intermediate reactions could be checked. If, *e. g.*, the cleavage of non-phosphorylated hexose could occur under conditions prevailing in the cell, which seems not very likely, then triose could be dehydrogenated even with generation of an energy-rich phosphate bond (80). The glyceric acid (171a) eventually formed could be dehydrated to pyruvic acid with a suitable enzyme, since this reaction earlier in this paper was shown to be largely exergonic (−8250 cal). The hydrogen from triose would thus be taken up by the same acceptor as in phosphorylating fermentation. Such a reaction cycle would indeed yield the same amount of utilizable phosphate bond energy as phosphorylating hexose (not glycogen) fermentation, since there, as discussed above, 50% of the generated total energy is always used up for primary phosphorylation.

### *2. Aerobic Metabolism*

**General.**—In the earlier work on coupling between oxidation and phosphorylation (Runnström and Michaelis (112), Lennerstrand and Runnström (113)), reactions were mainly studied where the specific hydrogen acceptor of fermentation was merely replaced by molecular oxygen. Runnström's (113) experiments with yeast maceration juice poisoned with fluoride gave an example of the coupling of the dehydrogenation of fermentation reductant by molecular oxygen with the phosphorylation of free hexose. Oxygen served there as the only available hydrogen acceptor because fluoride inhibited the further transformation of phosphoglyceric

* According to a recent paper by Borsook (109a), this value might be slightly too low. He calculates −0.28 volt.

acid and thereby the formation of the specific hydrogen acceptor of fermentation. The following reaction then occurs:

2 ph-glyceraldehyde + $O_2$ (*via* flavo-protein) + 2 phosphate + hexose =
2 phosphoglycerate + $2H_2O$ + hexosediphosphate (→ 2 ph-glyceraldehyde . . .).

Correspondingly, the amounts of oxygen consumed and of phosphoglycerate formed were found to be equivalent. These experiments show plainly the independence of this coupling mechanism from the nature of the hydrogen acceptor. In similar experiments with kidney extracts, Kalckar (16, 114) found, likewise in presence of fluoride, *aerobic* but not anaerobic phosphorylation of glucose. In contrast to the yeast experiments little or no phosphoglycerate was formed with the kidney extracts. Thereby a fermentation-like coupling with phospho-glyceraldehyde oxidation seemed to be excluded. Hexosediphosphate was the main product of phosphorylation with glucose as a substrate. Other compounds, like glycerol, could likewise be phosphorylated in air. From these experiments the strong suggestion was given that aerobically other reactions than those known from fermentation and glycolysis could yield transferable phosphate. As the first defined non-fermentative reaction the dehydrogenation of pyruvate by *Bacterium Delbrueckii* was shown to be coupled with phosphorylation (Lipmann (15)). Through this finding the independence of the generation of ~ph on fermentative reactions and the broad applicability of the phosphorylation principle in cell metabolism became more easily understandable.

It was already pointed out in the introduction that muscular contraction quite probably is driven by energy derived exclusively from energy-rich phosphate bonds—aerobically as well as anaerobically. In oxygen, the glycolytic breakdown of six moles of glucose might be replaced by the complete oxidation of one mole of glucose by six moles of oxygen. This deduction can be made because the upper limit of the Meyerhof quotient (lactic acid formed anaerobically) : (carbohydrate oxidized aerobically) is six. Then, one-sixth of a mole of glucose oxidized by one $O_2$ is metabolically equivalent to the breakdown of a whole glucose molecule to two moles of lactic acid. Expressed somewhat differently, the transfer of two pairs of hydrogen from "$^1/_6$ glucose" to $O_2$ is metabolically equivalent to the transfer of two pairs of hydrogen from one glucose-equivalent (two triosephosphate) to two moles of pyruvate. In the glycolytic reaction, per mole of glucose, the transfer of four hydrogens furnishes 2–4 energy-rich phosphate bonds. Then, the reduction of $O_2$ by the sixth of glucose must likewise furnish 2–4 ~ph in order to be metabolically equivalent. Since, therefore, one mole of glucose, aerobically, must

generate six times more than the anaerobically accounted 2–4 ~ph, 10–20 must originate with reactions occurring only in aerobiosis. Such an efficiency first makes the extensive utilization of phosphorylation energy in metabolism plausible. Then, only one glucose has to be sacrificed to synthesize 12–24 glucoses into glycogen (22) or to transport the same amount through the intestinal mucosa into the blood (Verzár (116), Lundsgaard (117)).

**Pyruvate Oxidation.**—The connection between pyruvate oxidation and phosphate turn-over was first found and studied with the relatively simple oxidation of *Bacterium Delbrueckii* (Lipmann (77, 118)). Contrary to oxidation in animal tissues this bacterial oxidation is incomplete. It stops after removal of one pair of hydrogens from pyruvic acid. The primary dehydrogenation could more easily be studied here without disturbance, due to subsequent reactions. The primary dehydrogenation product could be defined. It was found that: (*1*) pyruvate is only dehydrogenated in presence of inorganic phosphate, (*2*) pyruvate dehydrogenation generates ~ph (*i. e.*, causes adenylic acid phosphorylation), (*3*) the primary oxidation product contains phosphate and behaves like acetyl phosphate and synthetic acetyl phosphate does transfer ~ph to adenylic acid. From these findings the dehydrogenation could be formulated:

$$CH_3COCOOH + H_3PO_4 + O_2 = CH_3COOPO_3H_2 + CO_2 + H_2O_2.$$

The formation of acetyl phosphate was overlooked earlier because the substance is easily decomposed by acid and alkali (3).

After the demonstration of the coupling reaction in *Bacterium Delbrueckii*, Banga, Ochoa, and Peters (81) found likewise in brain that (*1*) pyruvate is oxidized only in the presence of inorganic phosphate, (*2*) pyruvate furnishes ~ph over adenylic acid for phosphorylation of hexose (119). Since in brain (153), (120), pyruvate is oxidized over the acetyl stage to water and $CO_2$, Ochoa and Peters (120a) concluded that acetyl phosphate, if an intermediate, should be oxidized. They found, however, no oxidation of synthetic acetyl phosphate with dialyzed brain dispersions which oxidized pyruvate. Being aware of the perfect analogy of pyruvate dehydrogenation in the bacteria and in brain, they concluded that acetyl phosphate was formed neither in brain nor in bacteria. For a formation of acetyl phosphate in the latter case, however, we have given in the meantime almost inescapable proof (3). We assume that the failure of brain to oxidize acetyl phosphate, when added alone, does not exclude its intermediate formation. Interconnection between oxidation, phosphorylation, and dephosphorylation, as known from fermentation experience, is finely balanced. It has been shown with

hexose-di-phosphate in the fermentation system that a phosphorylated intermediate might not react if the right conditions for phosphate transfer are not there (96). We interpret the non-oxidation of acetyl phosphate to show that the smooth metabolism of the intermediate requires metabolic coordination of previous and later stages of the reaction chain. This is not possible when the intermediate alone is added to the reaction system. The indication for such interplay is found in the observation (81) that in the absence of adenylic acid pyruvate oxidation is incomplete but not entirely blocked.

Recently, Colowick, Welch, and Cori (121) have found in kidney extracts a very effective glucose phosphorylation promoted by pyruvate and by ketoglutarate oxidation (17). Thereby it is indicated that $\alpha$-keto acid oxidation in general furnishes energy-rich phosphate bonds.

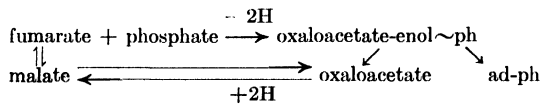
**Dicarboxylic Acid Oxidation.**—It was shown by Kalckar (16) in his previously mentioned experiments with kidney extracts that oxidation of dicarboxylic acids such as succinic, fumaric, and malic acids, gives rise to vigorous phosphorylation of various substrates. From more recent experiments with similar kidney extracts Cori (17) draws the conclusion that the generation of energy-rich phosphate originates with the dehydrogenation step from succinic to fumaric acid. The manner of the coupling of dicarboxylic acid oxidation to phosphorylation is all the more interesting since oxidation of fumarate and malate in absence of glucose yielded in Kalckar's kidney experiments a phosphorylation product which was either phospho-pyruvate or another enol-phosphate. As an explanation he discussed the possibility that pyruvate might be phosphorylated to phospho-pyruvate when formed by breakdown of the intermediary oxaloacetate. Pyruvate itself was not phosphorylated under similar conditions. The phenomenon is of great metabolic importance and invites more discussion. Pyruvate is not phosphorylated by ad-ph~ph~ph, although the reverse reaction from enol~ph to ad-ph is very easy. As has been mentioned, the group potential of the enol phosphate must therefore be appreciably higher than that of ad-ph~ph~ph. It seems doubtful if any of the known energy-rich phosphate bonds, which are all in equilibrium with ad-ph, could transfer their phosphates to pyruvate. An independent generation of enol~ph, however, would be offered by the following series of reactions:

$$\begin{matrix}\mathrm{CH\cdot COOH}\\ \|\\ \mathrm{CH\cdot COOH}\end{matrix} + \mathrm{H_3PO_4} \longrightarrow \left[\begin{matrix}\mathrm{CH_2\cdot COOH}\\ |\\ \mathrm{CHO\cdot (PO_3H_2)\cdot COOH}\end{matrix}\right] \xrightarrow{-2H} \begin{matrix}\mathrm{CH\cdot COOH}\\ \|\\ \mathrm{CO(PO_3H_2)\cdot COOH}\end{matrix}$$

This sequence would explain Kalckar's finding where then primarily phospho-enol-oxaloacetate would be formed by dehydrogenation of an

adduct of fumarate and phosphate. An earlier, subsequently abandoned (123), view of Szent-Györgyi (122) that fumarate can be oxidized without intermediate formation of malate, was based on peculiar differences between fumarate and malate oxidation. We believe that Szent-Györgyi with his earlier view was partly right and that the differences between malate and fumarate oxidation will find an explanation through the coexistence of two different routes, one leading over malate, the other over a fumarate-phosphate adduct.

The coupling of dicarboxylic acid oxidation with phosphorylation and their hydrogen carrier function should not be mere coincidences. The carrier function is ascribed by Szent-Györgyi to the pair malate-oxaloacetate (122). Krebs (124) showed recently that the transport of at least 6 of the 12 pairs of hydrogens given off during oxidation of one mole of glucose transferred through the oxaloacetate-fumarate-malate cycle. The assumed coupling of fumarate-phosphate oxidation with phosphorylation would suggest the following Szent-Györgyi-phosphorylation cycle:




Together with 2 ~ph from pyruvate and ketoglutarate, respectively [citric acid cycle of Krebs (124)], these 6 ~ph would account for 10 of the 12–24 ~ph postulated to be formed by the oxidation of one mole of glucose.

**Resynthesis of Carbohydrate.**—The whole chain of the glycolytic reaction from hexose-di-phosphate to lactic acid is made up by units which, with the exception of phosphopyruvate breakdown, are all reversible reactions. The two transformation periods (*1*) between hexose-di-phosphate and phosphoglyceraldehyde and (*2*) between phospho-3-glycerate and phosphopyruvate are to be considered as unconditionally reversible. Reversibility of the other, the energy yielding reactions, however, is conditioned by ~ph turn-over, *i. e.*, by the eventual removal of energy, accumulated in energy-rich phosphate bonds. The rate and even the direction of such a reaction will be partially determined by the rate and direction of the flow of ~ph between what might be called different reaction orbits. Since energy flows out of the glycolytic reaction almost solely in the form of ~ph, refunding of ~ph, when generated in other, *e. g.*, aerobic reactions, is at least one of the factors needed to reverse the glycolytic reaction. Such considerations are borne out by and partly originated with the demonstration by Meyerhof, *et al.* (96) of the reversible coupling of phospho-glyceraldehyde $\rightleftarrows$ phospho-glycerate oxidation-reduction to

adenylic acid-adenosine polyphosphate phosphorylation and de-phosphorylation for which the chemical explanation was given by Warburg and Christian (80).

In the previous paragraph it was deduced from the value of six for the Meyerhof quotient in various tissues (115) that the oxidation of $^1/_6$ of one mole of glucose is able to furnish as much ~ph as the glycolysis of a whole molecule of glucose. The ~ph, thus originating in oxidation, will compete for adenylic acid with ~ph originating in glycolysis and should, by severing the outflow of ~ph from glycolysis, decrease the rate of glycolysis. It seems doubtful, however, that the reduction of glycolysis to the extent found in many oxidizing cells can be explained by such competition for adenylic acid, alone or together with aerobic competition for DPN$^+$ suggested by Ball (125). Most probably there is still a considerable "braking" effect of oxygen needed as was first suggested by Lipmann (126). Such an effect seems to become more definitely explained by the work of Rapkine (127) on oxidative inactivation of triose phosphate dehydrogenase, by the work of Stern and Melnick (128) on the glycolysis-inhibiting, cytochrome-like "Pasteur enzyme," and by the work of Chaix and Fromageot (129) with propionic acid bacteria.

If we accept a brake-like effect of oxygen on glycolysis which is mostly needed in cells high in anaerobic glycolysis, it then becomes possible that the reaction course of carbohydrate resynthesis is, in long stretches, the reverse course of the glycolytic reaction. This is brought out in the reaction scheme which is represented in Fig. 8.

*The reversal of the O/R reaction* in fermentation was first demonstrated by Green, D. M. Needham, and Dewan (132). Lactate and phosphoglycerate were brought into reaction through addition of cyanide which binds the carbonylic products of the reaction, pyruvate, and phosphoglyceraldehyde. These were thus prevented from reacting back. The mechanism of the rapid reduction of phosphoglycerate observed by Green, *et al.*, became partly explained when by the work of Meyerhof, *et al.*, the rigid coupling of this reduction with the delivery of ~ph was shown. In the Green-Needham-Dewan reaction pyruvic enol~ph, originating by equilibrium reaction from phosphoglycerate, functioned as the source of ~ph. The concomitant liberation of inorganic phosphate from phosphoglycerate observed by Green, *et al.*, was likewise explained by Meyerhof who formulated the over-all reaction as follows,

$$\text{lactate} + \text{phosphoglycerate} + \sim\text{ph} \xrightleftharpoons[\text{adenylic acid}]{\text{DPN}} \text{pyruvate} + \text{phosphoglyceraldehyde} + \text{inorganic ph}$$

Cyanide was needed for fixation in the non-oxidizing extracts while in the cell the removal of the products of reaction occurs by subsequent aerobic degradation. In presence of oxygen, pyruvate is very rapidly removed by dehydrogenation most probably to acetyl~ph and $CO_2$. The

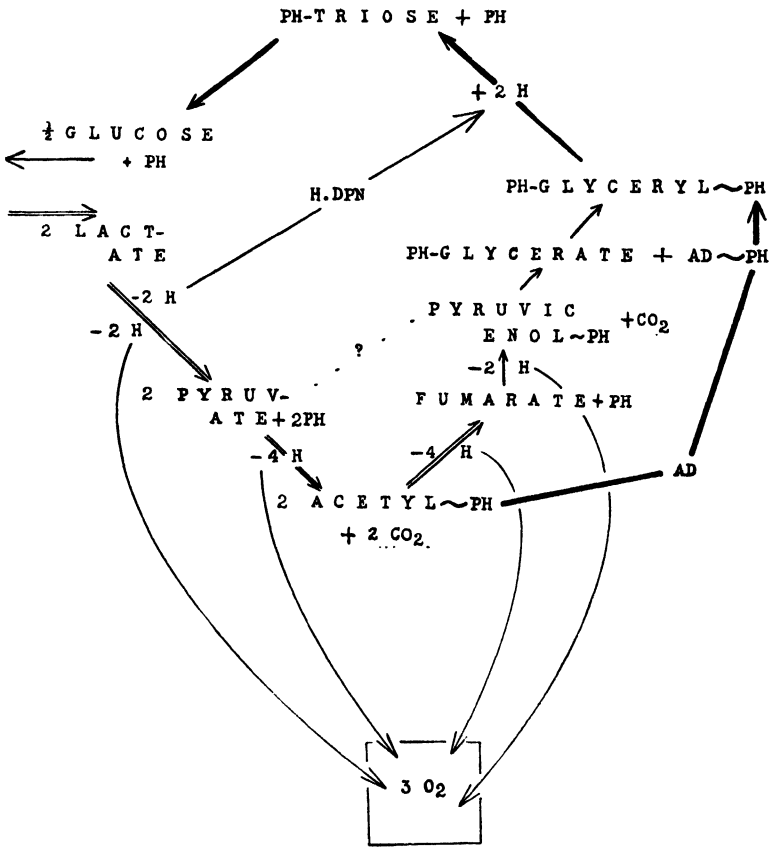



Fig. 8.

~ph generated in the course of this oxidation is utilized in a later stage for phosphoglyceryl~ph formation (see Fig. 8). The removal of phosphoglyceraldehyde, the other carbonyl compound formed in the reverse O/R reaction, leads eventually through subsequent reactions to the forma-

tion of carbohydrate. Phosphoglyceraldehyde presumably condenses to hexose-di-phosphate. The exact mechanism of the reformation of glucose and of glycogen from hexosediphosphate is not understood in detail at the present time (see Cori (22) and Ostern, *et al.* (130)).

*Indirect formation of phosphopyruvate* by way of fumarate oxidation is assumed in our scheme because it is strongly supported by experimental work. Carbohydrate resynthesis from dicarboxylic acids was first demonstrated by Elliott, Benoy, and Baker (133). As mentioned, phosphopyruvate was found a product of fumarate oxidation (16). Almost the strongest support for indirect formation of phosphopyruvate is given by the work of Hastings, *et al.* (134). They showed that the glycogen which is formed from lactic acid with radioactive carbon in the carboxylic group, contains little or no radioactive carbon. Through this finding it is postulated that lactic acid on the way back to carbohydrate loses its carboxylic group. This, however, is to be expected when pyruvic acid formation is only possible by way of dicarboxylic acid oxidation. In this reaction, as indicated in the scheme, two moles of lactate are used for the formation of one mole of phosphorylated pyruvate which then returns eventually to carbohydrate. As maximum, therefore, only one carbohydrate eq. can be yielded through partial oxidation of two lactates. This corresponds to a maximum of 2 for the oxidation "resynthesis-" quotient,

$$\frac{\text{lactate eq. oxidized} + \text{carbohydyate eq. formed}}{\text{lactate eq. oxidized}} \leqq 2.$$

This would seem to be in disagreement with the original figures of 4–5 as calculated by Meyerhof (135) for this quotient from data obtained with muscle in recovery. In this calculation, however, only the surplus of recovery-respiration over rest-respiration was taken into consideration, and recalculation, by taking account of the whole respiration, furnished figures closely averaging two (Burk (131)). This value is in good agreement with the theoretical maximum for indirect resynthesis as given above. The probable maximum of two for the resynthesis-oxidation quotient must not be confounded with the well-established experimental maximum of six for the Meyerhof (replacement-) quotient (in lactate units):

$$\frac{\text{anaerobic fermentation} - \text{aerobic fermentation}}{\text{respiration}} \leqq 6.$$

The large difference between the two maximum values shows in an independent way that most probably the resynthesis cycle theory cannot be applied as an exclusive explanation of the Pasteur effect. Therefore, we propose to distinguish clearly between the above defined "resynthesis"

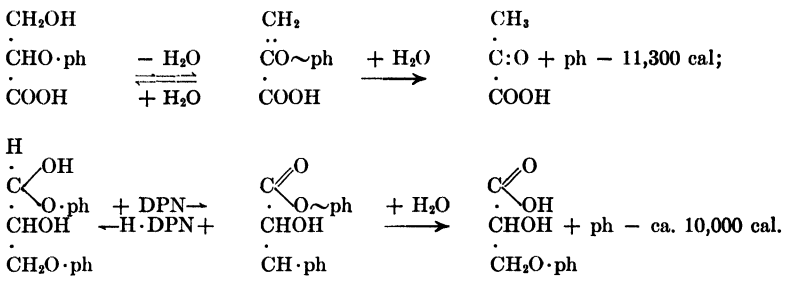
and "replacement" quotients, as they are largely independent experimental values.

Quite recently, Barron and Lyman (136) demonstrated that the resynthesis of carbohydrate from a mixture of pyruvate and fumarate depended upon the presence of thiamin. They concluded that thiamin enzymes catalyze condensation reaction presumably occurring during resynthesis. We prefer the view that condensation reactions occur rather with the reaction products of pyruvic acid oxidation, *e. g.*, acetyl phosphate. They might, therefore, not directly but indirectly be connected with thiamin catalysis. We are, however, aware of the possibility that condensations might occur as reaction units with no distinct formation of an intermediate and might be catalyzed by a composite thiamin-enzyme. Even the pyruvic acid oxidase of *Bacterium Delbrueckii* (Lipmann (137)) might be called a composite thiamin enzyme as it contains flavin adenine nucleotide besides thiamin pyrophosphate, both of which are possibly bound to the same protein.

*The conversion of phosphate group potential energy* into O/R potential energy is shown with remarkable clarity by the reaction leading to recovery of triose from the glyceric acid level. The phosphorylation of the carboxylic group in phosphoglyceric acid increases its electron affinity to such an extent that it becomes an acceptor for the electrons offered by reduced diphosphopyridine dinucleotide (H·DPN). The 10,000 calories brought into the molecule raise its electron affinity without any loss of energy which means a rise in O/R potential of 0.22 volt.

$$\Delta E = \frac{-\Delta F}{n \times \text{Faraday}} = \frac{10{,}000}{2 \times 23{,}000} = 0.22 \text{ volt.}$$

The complete fixation of ~ph energy in the reduction process is shown by the fact that the formation of the acyl phosphate bond requires 10,000 cal, but the formation of the carbonyl-phosphate addition product (*i. e.*, phosphorylation of the product of reduction) occurs spontaneously. In the reverse reaction, by the removal of $2\epsilon + 2H^+$ from the aldehyde-phosphate, the same amount of energy is fixed in the energy-rich phosphate bond. This energy would be dissipated if dehydrogenation had occurred with aldehyde hydrate to non-phosphorylated acid. Although here through a different mechanism, the effect obtained is analogous to that of dehydration of phosphopyruvate as the comparison of the two reactions generating energy-rich phosphate bonds will show:




An important difference, however, is that pyruvate apparently cannot, but phosphoglycerate can, be rephosphorylated by ad-ph∼ph∼ph.

In general, phosphorylation of the carboxylic group will raise the electron affinity of this group, or, expressed differently, the O/R potential of the phosphorylated pair becomes elevated. Thus in the case of pyruvate which on dehydrogenation yields acetylphosphate and $CO_2$ (3) a less potent reductant will be needed to effect fixation of $CO_2$ through reduction of acetyl phosphate + $CO_2$ to pyruvate, as would be needed to effect reductive combination with free acetate. The pyruvate/acetate + $CO_2$ system has a very low O/R potential, according to Franke's (138) calculation about 0.3 volt lower than the hydrogen potential. Phosphorylation of acetate would elevate the O/R potential of this system by ca. 0.22 volt, and bring it up to a level where reductive $CO_2$ fixation becomes quite possible. Mechanisms of this type might play a role in photosynthesis or in bacterial $CO_2$-fixation (Wood and Werkman (139)).

## VII. Utilization of Phosphate Bond Energy

On several occasions during this analysis we have referred to the utilization of potential energy present in energy-rich phosphate bonds although, except in the case of carbohydrate resynthesis, only in a general manner. Undoubtedly, a large part of available metabolic energy passes through such bonds. Not very definite answers can be given to the question as to how the high phosphate group potential operates as the promoter of various processes although more or less loosely defined interconnection with phosphate turn-over is recognizable. Nevertheless, interpretation of some of the phenomena shall be tried. In this discussion we have decided to take wholeheartedly the "inside viewpoint" of the cell. Chemical reactions occurring in living cells are part of cell procedures. A procedure implies purpose and design, and to approach the understanding of cell procedures their purposefulness has to be taken into consideration.

In Fig. 1 the metabolic phosphate cycle was compared with a machine which generates electric current. It seems, in fact, that in cell organization the phosphate "current" plays a similar part as does electrical current in the life of human beings. It is a form of energy utilized for all-round purposes. Indications are found that the phosphate current can be used to carry out mechanical work, to do osmotic work in resorption (Verzár (116), Lundsgaard (117)) to build bones (Robinson (140)), to synthesize protoplasmic material as lecithin, nucleic acid, and so forth. As shall be pointed out, it should be utilizable for organic chemical synthesis in the cell. In all these procedures, we must conclude, use is made of the fall of phosphate group potential from higher to lower levels as was explained earlier (cf. Fig. 2). It seems difficult at present to explain how such a variety of mechanisms can be driven by a uniform source of energy. The task, however, should be simplified to a certain extent by clear recognition of the type of driving power behind all these mechanisms.

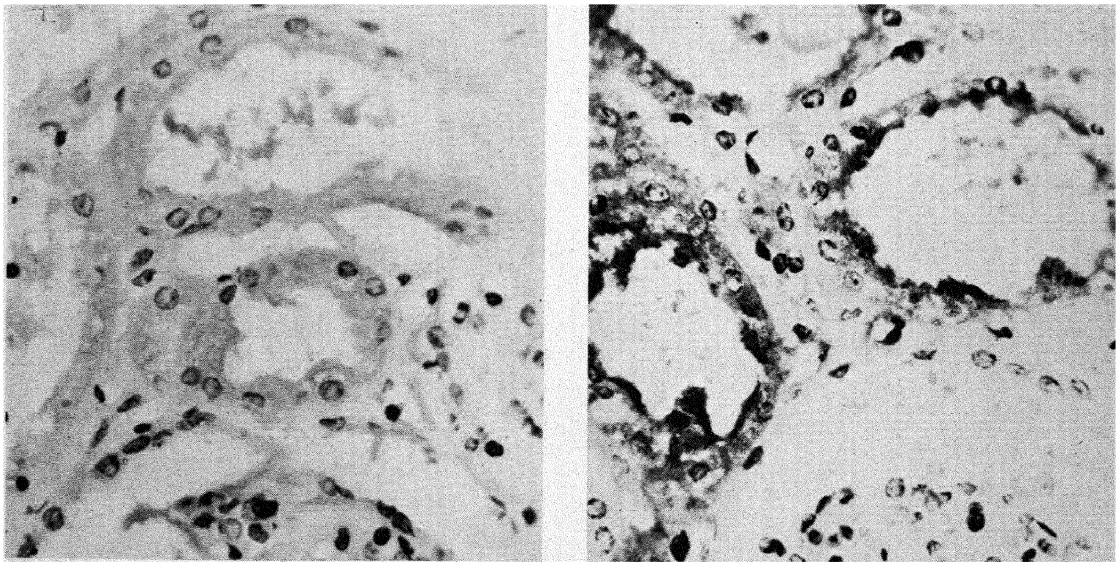
### *1. Muscular Contraction*

The breakdown of creatine phosphate, or, more recently, of ad-ph∼ph∼ph (40) are considered as the chemical reactions nearest to muscular contraction proper. The change from creatine∼ph to ad-ph∼ph∼ph was proposed by Lohmann after it was found that hydrolytic breakdown of creatine∼ph occurred only by way of intermediate formation of ad-ph∼ph∼ph and subsequent hydrolysis of the latter through adenyl pyrophosphatase. Such indirect hydrolysis was likewise found with the other energy-rich phosphate bonds. Lohmann's argument, however, seems not justified, because hydrolytic destruction and utilization need not take the same routes. No decision can be taken from where ∼ph is taken off to operate contraction. Hydrolysis through pyrophosphatase, in fact, completely dissipates the potential energy accumulated in the energy-rich bond. Pyrophosphatase operates rather like an outlet for the adenylic acid system to adjust the flow of ∼ph in case of overproduction, much in the manner of a valve. In cyclic processes of utilization, we think that to know how inorganic phosphate is recovered would frequently imply knowing the mechanism because complete utilization of the potential energy in ∼ph converts it necessarily into inorganic phosphate.

It is an obvious deduction that the energy-rich phosphate should link up to the contracting protein. There is, however, no experimental indication even to encourage such a view. An opening for further experimental approach might be found in Mirsky's (141) work on myosin coagulation in muscle rigor.

### 2. *Absorption*

Connection between active glucose transfer through a cell and its phosphorylation is indicated by the work of Verzár (116) and Laszt (142) on intestine and that of Lundsgaard (117) and Kalckar (16) on kidney. It is not intended to discuss their evidence here. This seems as strong as one can expect for an analysis of a process which necessarily depends on the intactness of cell structure, cf. (114a). Intestinal resorption and the reabsorption of glucose in kidney are analogous metabolic problems. In both, the transflux of a compound through a sheet of cells is effected or its rate increased by a pump-like action of cell metabolism. The compound enters the cell on one side from a slowly moving fluid column and leaves it on the opposite side to be carried away by the rapidly moving blood stream. On the exit side the constant mechanical removal through the blood stream seems to create a sufficiently steep osmotic gradient to guarantee an even outflow. The metabolic pump function must then be concentrated on that side of the cell where the substance is to enter. A phosphate group is here introduced into the entering molecule of glucose from metabolically generated energy-rich phosphate bonds. It might be that the pumping consists merely of a removal, by phosphorylation, of free glucose, thereby creating an increase of its osmotic gradient. We doubt, however, that such a mechanism would be efficient enough. Also, it would require a very efficacious spacial separation of phosphorylation and dephosphorylation which is not indicated (see Figs. 9 and 10). It seems likely that a better utilization takes place of the 10–11,000 cal at disposal in the energy-rich bond. By mere phosphorylation only 3000 out of ca. 11,000 cal are made use of. We are more inclined to assume that the phosphorylated glucose represents the final stage of the transport reaction wherein, one way or another, the potential difference between energy-rich and ester-phosphate bond is utilized. In any case, the once attached phosphate must rapidly be removed again so as to deliver free glucose on the opposite side of the cell into the blood. To do this, significantly large amounts of phosphatase are present in kidney and in intestine. It was, in fact, on account of the high phosphatase content that a connection between phosphorylation and absorption was sought. A histochemical method developed by Gomori (143) allows the specification of the site of phosphatase within the cell and organ. I am greatly indebted to Drs. Kabat and Furth who have used the method for pathological analysis of organs (144), and who supplied the photographs reproduced in Figs. 9 and 10. It seems significant that, as shown by the microphotographs of the kidney, phosphatase is con-



Figs. 9 and 10.—The left-hand photograph shows a kidney preparation without, the right-hand the same with treatment according to Gomori to show the site of phosphatase activity. The active parts are black stained. Only the proximal tubuli contain phosphatase. There, phosphatase is concentrated at the inner cell border.

centrated on the side of the tubulous cells facing the lumen from where glucose is absorbed. In confirmation of Lundsgaard's theory, the phosphatase is only present in the proximal tubuli where glucose reabsorption from the primary urin filtrate takes place (Gomori (143)).

It should be mentioned that fat absorption in intestine is likewise thought to be connected with phosphorylation (see Bloor (145)).

### 3. *Transformation of Fructose into Glucose*

The analysis of this reaction in tissue slice experiments with liver and kidney (146)(146a) has shown that fructose, here, is intermediately phosphorylated to fructose phosphate (Neuberg ester). Then, the ketose ester is partially transformed into aldose ester by the catalysis of Lohmann's phosphohexose mutase (147). From the resulting ester mixture the aldose ester is continuously removed by selective decomposition (see also Kalckar (114a)), and thus glucose appears eventually at the end of the reaction chain,

*fructose* + phosphate ⟶ fructose−phosphate ⇄
glucose−phosphate ⟶ *glucose* + phosphate.

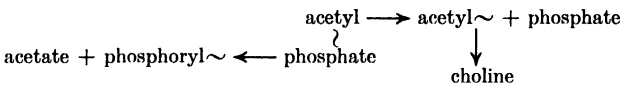
### 4. *Utilizability of Acetyl Phosphate and of Acyl Phosphates for Biosynthesis*

For a long time it has been suspected that by oxidation of pyruvic acid an "active" acetic acid is formed because acetylation of various substances (Knoop and Oesterlein (148), duVigneaud and Irish (149)) and condensation of $C_2$-intermediates to $C_4$-compounds (*e. g.*, Krebs and Johnson (150), Weil-Malherbe (151)) occurs quite easily subsequent to pyruvic acid oxidation. Such reactions are, however, mostly absent when acetate is used as a substrate instead of pyruvate. It might be concluded from general experience that acetate, if added as such, is fairly slowly oxidized. Pyruvate, however, is rapidly metabolized although its degradation goes surely through an acetic acid stage, as shown by the experiments of Mickelson, Reynolds, and Werkman (152), of Lipmann (153), and of Banga, Ochoa, and Peters (81). It was shown recently that the "active" acetate, which forms intermediately in pyruvic acid oxidation, most probably is acetyl phosphate (3),

$$CH_3CO\cdot COOH + H_3PO_4 \xrightarrow{-2H} CH_3COOPO_3H_2 + CO_2.$$

By finding an intermediate of this type the way seems opened for an understanding of the various and peculiar reactions occurring subsequent to pyruvic acid oxidation. Experiments to prove this point are now in progress (Lipmann).

A metabolically quite important utilization of the "active" acetate is acetylation of choline which was shown to be dependent on pyruvic acid oxidation (Mann and Quastel (154)). Alternating decomposition and rebuilding of acetyl choline occurs in nervous tissue (Dale and Gasser (155), Nachmansohn (156)) and is dependent on the oxidative metabolism of the tissue, as was shown by the work of Mann, Tennenbaum, and Quastel (157). For such purpose a constantly high acetyl group potential might be maintained in the cell, as would be utilized for the purpose of acetylation in organic chemistry in form of acetyl chloride. Apparently the analogous acetyl phosphate is utilized in cell metabolism in an identical manner. In this case, then, the purpose with the generation of an acyl phosphate is not to utilize the potential imposed upon the phosphate, but that likewise imposed upon the acyl part, the acetyl group potential generated metabolically by anhydrization with phosphoric acid.

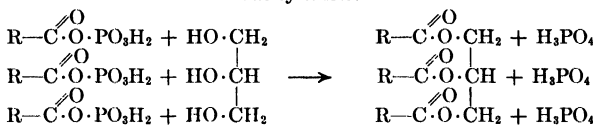



In such a case, anhydrization with phosphoric acid would be utilized to force the organic partner into a desired linkage. Considering the potential energy accumulated in a mixed anhydride linkage the direction of the above reaction would be determined by the nature of the compound offered and the catalytic system present. This will decide which of the partners is to enter into a new linkage, carrying away with it part of the energy accumulated thus utilized more or less efficiently. The esterification of the acetyl in acetyl phosphate with choline-hydroxyl would be perfectly analogous to esterification of hexose-hydroxyl with phosphoryl in adenyl pyrophosphate.

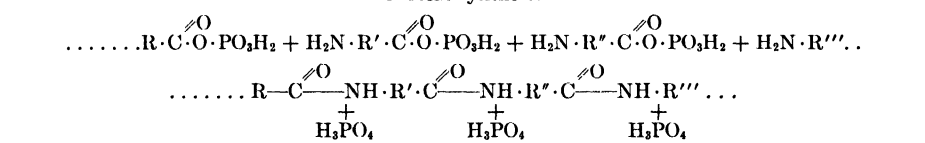
A broader application of this metabolic principle can be visualized if it is remembered that the formation of a mixed acyl phosphate anhydride can be effected through phosphorylation from adenyl pyrophosphate (Warburg and Christian (80)). The major part of the constituents of protoplasma are compounds which contain the ester or the peptide linkage. In the routine procedure of organic chemistry for synthesis of compounds of this type the acyl chloride of the acid part is first prepared and then brought into reaction with the hydroxyl or amino group of the other part. In an analogous procedure the cell might first prepare the acyl phosphate with adenyl pyrophosphate as the source of energy-rich phosphate groups. The acyl phosphate of fatty acids might then condense with glycerol to

form the fats. The acyl phosphate of amino acids likewise might condense with the amino groups of other amino acids to form the proteins.

Fat synthesis



Protein synthesis



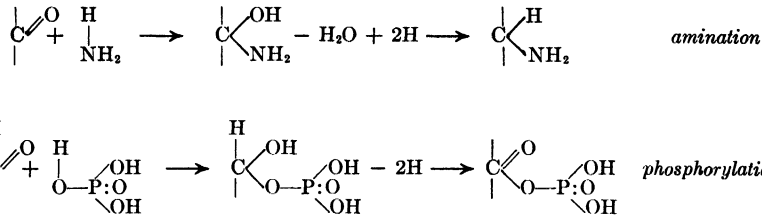
The phosphoryl groups, continuously generated in metabolizing cells, might thus find application for protoplasmic synthesis.

## VIII. Group Transfer as General Metabolic Reaction

The general application of the method of group transfer in cell metabolism becomes more and more evident with the increasing number of transfer reactions being found. The idea that reactions of this type have metabolic importance could already be recognized in the early work of Knoop on amination and acetylation (148). It seems, however, that through the detailed study of phosphorylation mechanisms, appreciation and understanding of this type of cell reactions were greatly furthered. There are undoubtedly rather common traits to be found with the cell procedures of introduction and distribution of various groups. These similarities will be brought out by brief comparison.

### *1. Amination and Transamination*

Like phosphate on its way to acyl-phosphate, ammonia is introduced through addition on a carbonyl double bond. Solidification of the linkage occurs likewise by a subsequent oxidation-reduction reaction. With ammonia, however, *hydrogenation* of the dehydrated addition product takes place (Knoop (148), Euler, Adler, *et al.* (158)), while the phosphate is fixed by *dehydrogenation.* Comparison of the reactions leading to acyl-phosphate (Negelein and Brömel (2), Lipmann (3)) and to glutamate, respectively, show their similarity:




Another method of introduction of ammonia is the addition to —C:C— double bond, exemplified by the aspartase (170) catalysis, which leads from fumarate to aspartate. It seems rather likely that analogous addition of phosphate on —C:C—, presumably in fumarate takes place which would, indeed, offer an explanation for some of the phosphorylation reactions reported by Kalckar (16), as was discussed earlier.

From glutamate, the amino group is propagated by the transamination of Braunstein and Kritzmann (159):

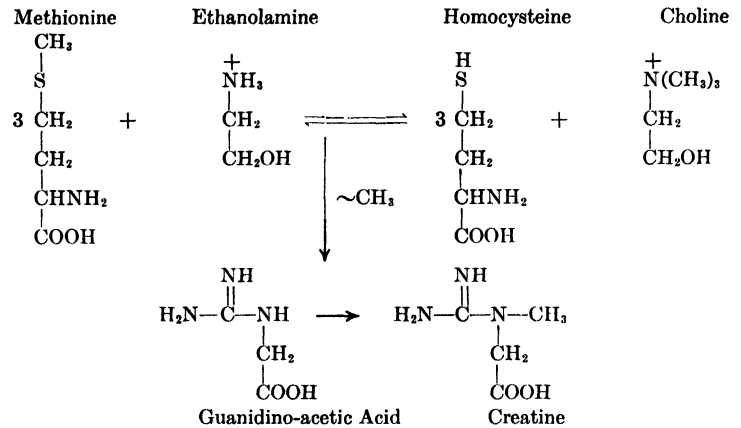
glutamic acid + α-keto acid ⟶ α-keto glutaric acid + amino acid.

It is assumed by Braunstein and by duVigneaud (160) that likewise an addition product by way of amino- and carbonyl-group is formed intermediately. The ease with which acidic groups as well as basic groups are added to the carbonylic double bond seems to be utilized to a great extent in biosynthesis.

The amazing ease with which there occurs an exchange of amino groups not only with free amino acids but even with those fixed in the body proteins has been demonstrated by the well-known isotope work of Schoenheimer, Rittenberg, and their associates (161).

### *2. Transmethylation*

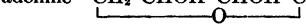
The occurrence of methyl group transfer in animals was discovered by duVigneaud and his colleagues (162). They found that animals are not able to generate metabolically methyl groups in the body but need supply from outside in the form of methionine or choline. Choline- and methionine-methyl were interconvertible. However, creatine-methyl (163), although derivable from choline or methionine, cannot be used as a source of $—CH_3$ by the body. The relation between these compounds is shown in the following formulation:




Lately, these results, obtained by feeding experiments and isotope analysis, were complemented by tissue slice experiments with liver (Borsook and Dubnoff (164)). They showed transfer of $-CH_3$ from methionine to guanidino acetic acid with formation of creatine in the isolated tissue.

Apparently, choline and methionine contain the methyl group with a high group potential, designated here with $\sim CH_3$. From these compounds distribution takes place probably with a variety of transfer enzymes to reversible or irreversible acceptors. A large part of the introduced $\sim CH_3$ is needed for creatine synthesis. By feeding large amounts of guanidino acetic acid together with amounts of methionine, sufficient otherwise to maintain slow growth, duVigneaud, *et al.* (163), created a very interesting metabolic situation. The irreversible loss of $\sim CH_3$, caused by excessive creatine formation, resulted in a large decline of weight with such animals. Parallelism might be seen to the disturbance of fermentation in extracts of yeast (Harden (83)) where the continuous irreversible loss of ~ph to glucose with excessive formation of hexosediphosphate leads to a tremendous decrease in fermentation rate as soon as inorganic phosphate, needed for regeneration of ~ph, is used up. In both cases a group needed for maintenance of growth or fermentation rate, respectively, is lost into an irreversible linkage.

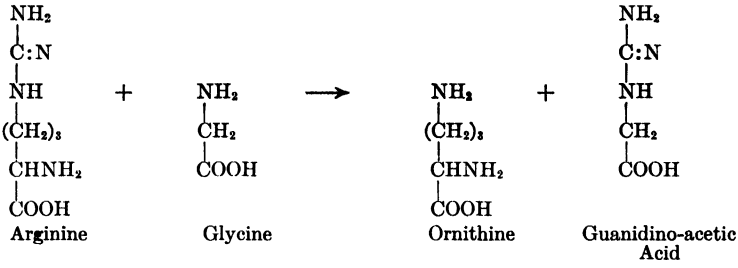
It is a curious incident that an adenine-methio-pentose most probably of the following constitution (Levene and Sobotka (165) was isolated from yeast:



With adenosine present in the prosthetic group of various transfer enzymes this compound might very neatly fit into the place of a methyl-carrier.

### *3. Transaminidation*

Independently, Borsook and Dubnoff (166) with isolated tissue (kidney), and Bloch and Schoenheimer (167) with the whole animal by isotope analysis, demonstrated the transfer of the aminidine group ($-C(=NH)-NH_2$) from arginine to glycine, resulting in formation of guanidino acetic acid, the precursor of creatine:




Thus, creatine synthesis occurs in the body by a succession of two or even three group transfers:

(*a*) Glycine, probably formed by transamination from carbohydrate degradation products,

(*b*) guanidino acetic acid by transfer of the aminidine group from arginine to glycine,

(*c*) finally creatine by transmethylation from methionine to guanidino acetic acid.

The aminidine group is generated in the body by the Krebs (168) synthesis from ammonia, $CO_2$, and ornithine; the methyl has to be introduced into the body as such.

Into this category of reactions belongs likewise the transfer of acetyl groups which seems to be quite closely connected with phosphorylation and was discussed in the paragraph on utilization of energy accumulated in the phosphate bonds.

Conclusion.—It is common with all these reactions that there are group donators and group acceptors. Here as in phosphate transfer distinction has to be made between reversible and irreversible group acceptors. Likewise reversibility and irreversibility must be due to a leveling of the respec-

tive group potentials. The force which drives the group into the irreversible acceptor can be described grossly by the difference of potential levels. It seems that the group potential concept can find manifold applications and will be helpful for the general understanding of transfer reactions.

### Bibliography


1. K. Lohmann and O. Meyerhof, *Biochem. Z.*, **273**, 60 (1934).
2. E. Negelein and H. Brömel, *Ibid.*, **303**, 132 (1939).
3. F. Lipmann, *J. Biol. Chem.*, **134**, 463 (1940).
4. C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **81**, 629 (1929).
5. P. Eggleton and G. P. Eggleton, *Biochem. J.*, **21**, 190 (1927).
6. O. Meyerhof and J. Suranyi, *Biochem. Z.*, **191**, 106 (1927).
7. E. Lundsgaard, *Ibid.*, **217**, 162 (1930).
8. E. Lundsgaard, *Ibid.*, **227**, 51 (1930).
9. A. V. Hill, *Physiol. Revs.*, **2**, 339 (1922).
10. O. Meyerhof, E. Lundsgaard, and H. Blaschko, *Naturwissenschaften*, **18**, 787 (1930).
11. D. Nachmansohn, *Biochem. Z.*, **196**, 73 (1928).
12. E. Lundsgaard, *Ibid.*, **233**, 322 (1931).
13. D. Burk, *Proc. Roy. Soc.*, **B104**, 153 (1929).
14. A. V. Hill and J. L. Parkinson, *Ibid.*, **B108**, 148 (1931).
    O. Meyerhof and E. Boyland, *Biochem. Z.*, **237**, 406 (1931).
    O. Meyerhof, Ch. L. Gemmill, and G. Benetato, *Ibid.*, **258**, 371 (1933).
15. F. Lipmann, *Nature*, **143**, 281 (1939).
16. H. Kalckar, *Biochem. J.*, **33**, 631 (1939).
17. S. P. Colowick, M. S. Welch, and C. F. Cori, *J. Biol. Chem.*, **133**, 359 (1940).
18. L. Michaelis and M. P. Schubert, *Chem. Revs.*, **22**, 437 (1938).
19. M. Berthelot and St. Gilles, *Ann. chim. phys.*, **66**, 225 (1863).
20. G. N. Lewis and M. Randall, "Thermodynamics and the Free Energy of Chemical Substances," New York, 1923.
21. H. D. Kay, *Biochem. J.*, **22**, 855 (1928).
22. C. F. Cori, *Cold Spring Harbor Symposia*, **7**, 260 (1939).
23. G. T. Cori, C. F. Cori, and G. Schmidt, *J. Biol. Chem.*, **129**, 629 (1939).
24. S. Veibel, *Enzymologia*, **2**, 124 (1936).
25. O. Meyerhof and W. Schulz, *Biochem. Z.*, **281**, 292 (1935).
26. D. Burk, *J. Phys. Chem.*, **35**, 432 (1931).
27. G. S. Parks and H. M. Huffman, "The Free Energies of Some Organic Compounds," New York, 1932.
28. H. Blaschko, *Biochem. Z.*, **158**, 428 (1925).
29. M. S. Kharasch, "Heats of Combustion of Organic Compounds," *Bureau of Standards J. of Research*, 2 (1929).
30. Stohmann and Langbein, *J. prakt. Chem.*, (2) **45**, 305 (1892).
31. O. Meyerhof and K. Lohmann, *Biochem. Z.*, **196**, 49 (1928).
32. O. Meyerhof and K. Lohmann, *Ibid.*, **273**, 73 (1934).
33. J. K. Parnas, P. Ostern, and T. Mann, *Ibid.*, **272**, 64 (1934).
34. H. Lehmann, *Ibid.*, **281**, 271 (1935).

35. K. Lohmann. *Biochem Z.*, **194,** 306 (1928).
36. K. Lohmann, *Ibid.*, **202,** 466 (1928).
37. C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66,** 375 (1925); see also K. Lohmann and L. Jendrassik, *Biochem. Z.*, **178,** 419 (1926).
38. O. Meyerhof and K. Lohmann, *Ibid.*, **185,** 113 (1927).
39. F. Lipmann and O. Meyerhof, *Ibid.*, **227,** 84 (1930).
40. K. Lohmann, *Ibid.*, **282,** 120 (1935).
41. H. A. Davenport and J. Sacks, *J. Biol. Chem.*, **81,** 469 (1929).
42. K. Lohmann, *Biochem. Z.*, **233,** 460 (1931).
43. K. Lohmann, *Ibid.*, **203,** 164 (1928).
44. G. P. Eggleton and P. Eggleton, *J. Physiol.*, **68,** 198 (1929).
45. R. W. Gerard and N. Tupikova, *J. Cellular Comp. Physiol.*, **13,** 1 (1939).
46. W. R. Franks, *J. Physiol.*, **74,** 195 (1932).
47. E. Boyland, *Ibid.*, **75,** 136 (1932).
48. K. Lohmann and Ph. Schuster, *Biochem. Z.*, **282,** 104 (1935).
49. H. K. Barrenscheen and W. Filz, *Ibid.*, **250,** 281 (1931).
50. K. Lohmann and Ph. Schuster, *Ibid.*, **294,** 183 (1937).
51. H. J. Deuticke, *Pflügers Arch. ges. Physiol.*, **230,** 537 (1932).
52. P. Ostern, *Biochem. Z.*, **270,** 1 (1934).
53. O. Warburg and W. Christian, *Ibid.*, **287,** 291 (1936).
54. W. Kiessling and O. Meyerhof, *Ibid.*, **296,** 410 (1938).
55. Stokes, *Z. anorg. Chem.*, **19,** 36 (1899).
56. C. Neuberg and H. A. Fischer, *C. R. Carlsberg*, **22** (Volume jubilaire Sörensen), 366 (1938).
57. H. Huber, *Z. angew. Chem.*, **50,** 323 (1937).
58. B. F. Miller and R. Dubos, *J. Biol. Chem.*, **121,** 447 (1937).
59. R. W. Gerard and N. Tupikova, *J. Cellular Comp. Physiol.*, **12,** 325 (1938).
60. A. Hunter, "Creatine and Creatinine," London, 1928.
61. O. Meyerhof, *Arch. sci. biol.* (Italy), **12** (Botazzi-Festschrift), 526 (1928).
62. M. Bodansky, *J. Biol. Chem.*, **109,** 615 (1935).
63. B. Kisch, *Biochem. Z.*, **225,** 183 (1930).
64. S. Ochoa, *Biochem. J.*, **32,** 237 (1938).
65. E. Baldwin and T. Needham, *Proc. Roy. Soc.*, **B122,** 197 (1937).
66. G. Embden, *Klin. Wochschr.*, **12,** 213 (1933).
67. R. Nilsson, *Arkiv Kemi Mineral. Geol.*, **A10,** No. 7 (1930).
68. O. Meyerhof and W. Kiessling, *Biochem. Z.*, **280,** 99 (1935).
69. F. Dickens and F. Simer, *Biochem. J.*, **24,** 1301 (1930).
70. C. H. Werkman, *Bacteriol. Rev.*, **3,** 187 (1939).
71. T. Effront, *Compt. rend.*, **117,** 559 (1893); **118,** 1420 (1894); **119,** 169 (1894).
72. T. Effront, "Moniteur Scientifique," 1905, p. 19.
73. W. P. Wiggert and C. H. Werkman, *Biochem. J.*, **33,** 1061 (1939).
74. J. Runnström and E. Sperber, *Biochem. Z.*, **298,** 340 (1938).
75. H. O. L. Fischer and H. Baer, *Ber.*, **65,** 337 (1932).
76. J. Greenwald, *J. Biol. Chem.*, **63,** 339 (1925).
77. F. Lipmann, *Cold Spring Harbor Symposia*, **7,** 248 (1939).
78. Kämmerer and Carius, *Ann.*, **131,** 165 (1864).
79. F. Lynen, *Ber.*, **73,** 367 (1940).
80. O. Warburg and W. Christian, *Biochem. Z.*, **303,** 40 (1939).

81. I. Banga, S. Ochoa, and R. A. Peters, *Biochem. J.*, **33**, 1980 (1939).
82. E. Shorr, S. B. Barker, E. Cohen, and M. Malam, *Am. J. Physiol.*, **129**, 463 (1940).
83. A. Harden, "Alcoholic Fermentation," London, 1932.
84. F. Lipmann, *C. R. Carlsberg Lab.*, **22** (Volume jubilaire Sörensen), 317 (1937).
85. M. G. Macfarlane, *Biochem. J.*, **33**, 574 (1939).
85a. W. P. Wiggert and C. H. Werkman, *Biochem. J.*, **32**, 101 (1938).
86. F. F. Nord, *Ergeb. der Enzymforsch*, **8**, 149 (1939).
87. J. C. Wirth and F. F. Nord, *Science*, **92**, 15 (1940).
88. J. K. Parnas, *Ergeb. Enzymforsch.*, **6**, 57 (1937).
89. D. M. Needham and R. Pillai, *Biochem. J.*, **31**, 1837 (1937).
90. R. Kuhn and P. Boulanger, *Ber.*, **69**, 1557 (1936).
91. O. Meyerhof, W. Kiessling, and W. Schulz, *Biochem. Z.*, **292**, 25 (1937).
92. O. Meyerhof, P. Ohlmeyer, W. Gentner, and H. Maier-Leibnitz, *Ibid.*, **298**, 398 (1938).
93. T. Korzybski and J. K. Parnas, *Bull. Chim. Biol.*, **21**, 713 (1939).
94. F. Lipmann, *Biochem. Z.*, **274**, 413 (1934).
95. O. Meyerhof, W. Schulz, and Ph. Schuster, *Ibid.*, **293**, 309 (1937).
96. O. Meyerhof, P. Ohlmeyer, and W. Möhle, *Ibid.*, **297**, 113 (1938).
97. F. Lipmann, *Nature*, **144**, 381 (1939).
98. P. Ostern, T. Baranowski, and J. Terszakovec, *Z. physiol. Chem.*, **251**, 258 (1938).
99. H. v. Euler and E. Adler, *Ibid.*, **252**, 42 (1938).
100. M. A. Lipton and C. A. Elvehjem, *Cold Spring Harbor Symposia*, **7**, 184 (1939).
101. A. H. Hegnauer, R. E. Fisher, G. T. Cori, and C. F. Cori, *Proc. Soc. Exptl. Biol. Med.*, **32**, 1075 (1935), see footnote on p. 1077.
102. O. Meyerhof, *Bull. soc. chim. biol.*, **20**, 1033, 1345 (1938).
103. D. Herbert, H. Gordon, V. Subrahmanyan, and D. E. Green, *Biochem. J.*, **34**, 1108 (1940).
104. M. N. Mickelson and C. H. Werkman, *Enzymologia*, **8**, 252 (1940).
105. M. Silverman and C. H. Werkman, *Proc. Soc. Exptl. Biol. Med.*, **43**, 777 (1940).
105a. Cf. K. Linderstrøm-Lang, *Ann. Rev. Biochem.*, **6**, 48 (1937).
106. E. Negelein and H. J. Wulff, *Biochem. Z.*, **293**, 351 (1937).
107. F. Kubowitz, *Ibid.*, **274**, 285 (1934).
108. Z. Dische, *Enzymologia*, **5**, 288 (1936).
109. M. W. Clark, *Cold Spring Harbor Symposia*, **7**, 1 (1939).
109a. H. Borsook, *J. Biol. Chem.*, **133**, 629 (1940).
110. H. v. Euler, E. Adler, G. Günther, and H. Hellström, *Z. physiol. Chem.*, **245**, 217 (1937).
111. E. S. G. Barron and A. B. Hastings, *J. Biol. Chem.*, **107**, 567 (1934).
111a. R. Wurmser and N. Mayer-Reich, *Ann. physiol. physicochim. biol.*, **9**, 923 (1933).
112. J. Runnström and L. Michaelis, *J. Gen. Physiol.*, **18**, 717 (1935).
113. A. Lennerstrand and J. Runnström, *Biochem. Z.*, **283**, 12 (1935).
114. H. Kalckar, *Enzymologia*, **2**, 47 (1937).
114a. H. Kalckar, "Fosforylerings processer i dyrisk Väv," Dissert. (with English Review), Copenhagen, 1938.

115. O. Warburg, "Ueber die katalytischen Wirkungen der lebenden Substanz," Berlin, 1928, p. 510 (*Biochem. Z.*, **184,** 484 (1927)).
116. F. Verzár, *Biochem. Z.*, **276,** 17 (1935).
117. E. Lundsgaard, *Ibid.*, **264,** 209, 221 (1933).
118. F. Lipmann, *Enzymologia*, **4,** 65 (1937).
119. S. Ochoa, *Nature*, **145,** 747 (1940).
120. C. Long and R. A. Peters, *Biochem. J.*, **33,** 759 (1939).
120a. S. Ochoa, R. A. Peters, and L. A. Stocken, *Nature*, **144,** 750 (1939).
121. S. P. Colowick, M. S. Welch, and C. F. Cori, *J. Biol. Chem.*, **133,** 641 (1940).
122. A. Szent-Györgyi and collaborators, *Z. physiol. Chem.*, **236,** 1 (1935).
123. K. Laki, *Ibid.*, **244,** 149 (1936).
124. H. A. Krebs, *Biochem. J.*, **34,** 775 (1940).
125. E. G. Ball, *Bull. Johns Hopkins Hospital*, **65,** 253 (1939).
126. F. Lipmann, *Biochem. Z.*, **265,** 133 (1933); **268,** 205 (1934); **274,** 329 (1934).
127. L. Rapkine, *Biochem. J.*, **32,** 1729 (1938).
128. K. G. Stern, J. L. Melnick, and D. DuBois, *Science*, **91,** 436 (1940).
129. P. Chaix and C. Fromageot, *Enzymologia*, **3,** 288 (1937).
130. P. Ostern, D. Herbert, and E. Holmes, *Biochem. J.*, **33,** 1858 (1939).
131. D. Burk, *Occasional Publications Am. Assoc. Adv. Sci.*, **4,** 121 (1937).
132. D. E. Green, D. M. Needham, and J. G. Dewan, *Biochem. J.*, **31,** 2327 (1937)
133. K. A. C. Elliott, M. P. Benoy, and Z. Baker, *Ibid.*, **29,** 1937 (1935).
134. A. B. Hastings, G. B. Kistiakowsky, R. D. Cramer, F. W. Klemperer, A. K. Solomon, B. Vennesland, Report of Washington Meeting of Nat. Acad. Sci., April 22–23, 1940, in *Science*, **91,** 421 (1940).
135. O. Meyerhof, *Pflügers Arch. ges. Physiol.*, **175,** 20 (1919); **185,** 11 (1920).
136. E. S. G. Barron and C. M. Lyman, *Science*, **92,** 337 (1940).
137. F. Lipmann, *Nature*, **143,** 436 (1939).
138. W. Franke, *Biochem. Z.*, **258,** 280 (1933).
139. H. G. Wood and C. H. Werkman, *Biochem. J.*, **34,** 129 (1940).
140. R. Robinson, *Ergeb. Enzymforsch.*, **1,** 280 (1932).
141. A. E. Mirsky, *J. Gen. Physiol.*, **20,** 455, 461 (1937).
142. L. Laszt and H. Süllmann, *Biochem. Z.*, **278,** 401 (1935).
143. G. Gomori, *Proc. Soc. Exptl. Biol. Med.*, **42,** 23 (1939).
144. E. A. Kabat and J. Furth, *Am. J. Path.* (in press).
145. W. R. Bloor, *Physiol. Revs.*, **19,** 557 (1939).
146. T. Goda, *Biochem. Z.*, **294,** 259 (1937); **297,** 134 (1938).
146a. C. F. Cori and W. M. Shine, *J. Biol. Chem.*, **114,** XXI (1936).
147. K. Lohmann, *Ibid.*, **262,** 137 (1933).
148. F. Knoop and H. Oesterlein, *Z. physiol. Chem.*, **148,** 294 (1925).
149. V. duVigneaud and O. J. Irish, *J. Biol. Chem.*, **122,** 349 (1937–38).
150. H. A. Krebs and W. A. Johnson, *Biochem. J.*, **31,** 645 (1937).
151. H. Weil-Malherbe, *Biochem. J.*, **31,** 2202 (1937).
152. M. N. Mickelson, H. Reynolds, and C. H. Werkman, *Proc. Soc. Exptl. Biol. Med.*, **34,** 748 (1936).
153. F. Lipmann, *Skand. Arch. Physiol.*, **76,** 255 (1937).
154. P. J. G. Mann and J. H. Quastel, *Nature*, **145,** 856 (1940).
155. H. H. Dale and H. S. Gasser, *J. Pharmacol.*, **29,** 53 (1929).
156. D. Nachmansohn, *Yale J. Biol. Med.*, **12,** 565 (1940).

157. P. J. G. Mann, M. Tennenbaum, and J. H. Quastel, *Biochem. J.*, **32**, 243 (1938).
158. H. v. Euler, E. Adler, G. Günther, and N. B. Das, *Z. Physiol. Chem.*, **254**, 61 (1938).
159. A. E. Braunstein and M. G. Kritzmann, *Enzymologia*, **2**, 129 (1937).
160. V. duVigneaud, M. Cohn, G. B. Brown, O. J. Irish, R. Schoenheimer, D. Rittenberg, *J. Biol. Chem.*, **131**, 273 (1939).
161. R. Schoenheimer and D. Rittenberg, *Physiol. Revs.*, **20**, 218 (1940).
162. V. duVigneaud, J. P. Chandler, A. W. Moyer, and D. M. Keppel, *J. Biol. Chem.*, **131**, 57 (1939).
163. V. duVigneaud, J. P. Chandler, M. Cohn, and G. B. Brown, *Ibid.*, **134**, 787 (1940).
164. H. Borsook and J. W. Dubnoff, *Ibid.*, **132**, 539 (1940).
165. P. A. Levene and H. Sobotka, *Ibid.*, **65**, 551 (1925).
166. H. Borsook and J. W. Dubnoff, *Science*, **91**, 551 (1940).
167. K. Bloch and R. Schoenheimer, *J. Biol. Chem.*, **133**, 633 (1940).
168. H. A. Krebs, *Ergeb. Enzymforsch.*, **3**, 247 (1934).
169. C. D. Coryell, *Science*, **92**, 380 (1940).
170. E. F. Gale, *Biochem. J.*, **32**, 1583 (1938).
171. F. F. Nord, *Chem. Revs.*, **26**, 423 (1940).
171a. Cf., however, O. v. Schoenebeck, *Biochem. Z.*, **276**, 421 (1935).
172. G. Hevesy, *Enzymologia*, **5**, 138 (1938).
173. G. Hevesy, *J. Chem. Soc.*, **1939**, 1213.
174. T. Korzybski and J. K. Parnas, *Bull. soc. chim. biol.*, **21**, 713 (1939).
175. J. Sacks, *Am. J. Physiol.*, **129**, 227 (1940).
176. G. Hevesy and O. Rebbe, *Nature*, **141**, 1097 (1938).
177. M. G. Eggleton, *J. Physiol.*, **79**, 31 (1933).
178. W. O. Fenn, *Physiol. Revs.*, **16**, 450 (1936).

# THE CHEMICAL NATURE OF CATALASE

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## 1. Introduction

Hundreds of research papers have been written about catalase and many methods have been devised for purifying it. Only those articles which deal especially with the chemical nature of catalase will be considered in this review.

Thénard noted in 1811 that the tissues of plants and animals as well as certain finely divided metals were able to decompose hydrogen peroxide with the evolution of oxygen. Schönbein (1) observed this action in 1863 and believed it to be a property of all enzymes. But this view was disproved in 1892 by Jacobson (2) and in 1901 by Löw (3).

In 1911 Wolff and de Stoecklin (4) worked out a method for purifying catalase from erythrocytes and obtained a yellow catalase solution free

from hemoglobin. In 1914 Waentig and Gierisch (5) concentrated catalase by repeated alcoholic precipitation, dialysis, and adsorption. They considered catalase to be a protein. Tsuchihashi (6) in 1923 devised an excellent method for denaturing the hemoglobin in his catalase preparations. This was accomplished by adding both alcohol and chloroform to aqueous catalase and shaking. The treatment did not inactivate any catalase.

Hennichs (7, 8) used adsorption upon kaolin and fractional precipitation with alcohol. He obtained a 300–400-fold concentration and his purest catalase had a "Kat. f." of 20,000 to 30,000. He found 3.5 to 4.1 per cent of iron in his preparations and could establish no relationship between iron content and enzyme activity. This, undoubtedly, was due to the presence of ferritin (9) which has an iron content of 20 per cent.

Von Euler and Josephson (10, 11) obtained one sample of horse liver catalase of "Kat. f." 43,000. This contained 0.63 per cent of iron and was preserved in dry form. Another preparation of "Kat. f." 40,000 contained 0.15 per cent of iron. Three years later, von Euler, Zeile, and Hellström (12) found the dried catalase of von Euler and Josephson to have a hemin content of 0.6 per cent. This would be the equivalent of 0.062 per cent iron.

Zeile and Hellström (13) were able to obtain catalase free from hemoglobin, using the method of Tsuchihashi. They showed that their purified material was an iron porphyrin. Zeile (14) demonstrated that the catalase of sprouted pumpkin seeds contains hematin. Stern (15) confirmed the presence of hematin in catalase and proved it to be protohematin.

In 1937 Sumner and Dounce (16, 17) crystallized beef liver catalase. Somewhat later, they obtained crystalline lamb liver catalase (unpublished) and in 1939 Dounce and O. Frampton (18) were able to obtain crystalline horse liver catalase. Sumner, Dounce, and V. L. Frampton (19) have worked out an improved method for the preparation of this substance.

## 2. Occurrence of Catalase

Although catalase is generally distributed in nearly all living tissues, its concentration varies enormously from tissue to tissue. In mammals the greatest quantity occurs in the liver and in the erythrocytes. Zeile (14) has found high concentrations in sprouted seeds. Hennichs (7) states that the "Kat. f." for fresh horse liver is 20. Neglecting any inhibitors present in horse liver, one can calculate that if purest catalase has a "Kat. f." of 40,000, then the concentration of catalase in moist horse liver is:

$$\frac{20 \times 100}{40{,}000} = 0.05 \text{ per cent.}$$

Similarly, for whole human blood, which has a "Kat. f." of about 27, the percentage of catalase is 0.07.

### 3. Physiological Role of Catalase

The only role assigned to catalase is that of protecting living organisms against injury by the hydrogen peroxide produced in certain oxidations. Whether this hydrogen peroxide is the result of normal metabolic reactions, or whether it arises from accidental side reactions, does not seem to be known. Injury to protoplasm would not require a large quantity of peroxide.

Mellon (20) and his collaborators have proposed that the beneficial action of sulfanilamide is due to the ability of this drug to destroy or inactivate the catalase of the blood and tissues, thereby allowing hydrogen peroxide to accumulate in amount sufficient to destroy bacteria. Sumner and Dounce (unpublished) consider that adequate support for this theory is lacking.

### 4. The Stability of Catalase

The catalase from mammalian liver is a rather rugged enzyme, otherwise it would not have been possible for the earlier workers to purify it so successfully by repeated alcohol precipitation and by adsorption. Hennichs (7) observed that his catalase preparations decreased in activity during dialysis and ascribed this decrease to adsorption on the collodion membranes employed. No losses, as a rule, were noted by von Euler and Josephson (10) when they dialyzed catalase at low temperature and von Euler (21) states that parchment does not adsorb catalase. We have observed that enough horse liver catalase is adsorbed upon collodion to color it considerably and also that catalase is strongly adsorbed on glass. Sumner and Dounce (unpublished) have found that crystalline beef liver catalase either in concentrated solution or diluted 1–300,000 will keep without decrease in activity for one month in an ice chest. For some unknown reason catalase which has been diluted somewhat more than 1–300,000 decreases rather rapidly in activity even in the cold and in the absence of light.

### 5. The Inactivation of Catalase by Trypsin

In 1913 Waentig and Steche (22) found catalase to be inactivated by trypsin and by the gastric juice of crabs, but not by weakly acid pepsin. Tauber and Kleiner (23) showed that catalase is inactivated by trypsin

at *p*H 6.4. Sumner and Dounce (24) found crystalline catalase to be inactivated by trypsin.

### 6. The Determination of Catalase Activity and Purity

The method of von Euler and Josephson (10) is very satisfactory for the determination of catalase concentration (K value), or for the determination of catalase purity ("Kat. f." value). However, for this method pure or comparatively pure catalase must be employed. We find (unpublished) that one should employ redistilled hydrogen peroxide and that the catalase should be of such dilution as to give a K value, or velocity constant, of 0.025 to 0.040. While we have always titrated our aliquots of digest with permanganate, it is doubtless as satisfactory to measure the oxygen evolved,

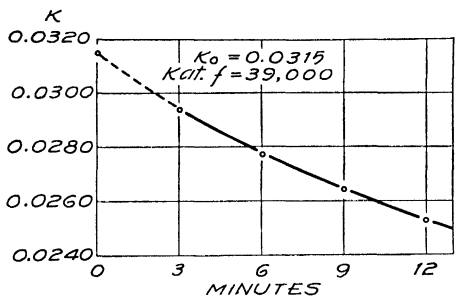



Fig. 1.—Curve for "K" of horse catalase.

provided one possesses the necessary apparatus. Figure 1 shows one of our best curves obtained by plotting K values against time and extrapolating to obtain $K_0$.

When one estimates the catalase in grossly impure material it is advisable to employ some such method as that of Jolles (25). We have modified this method as follows:

Add 1 cc. of sufficiently diluted catalase solution to 50 cc. of 0.01 *N* hydrogen peroxide in 0.015 *M* phosphate buffer of *p*H 6.8, kept at 0° C. in a bath. Mix at once and withdraw 5 cc. and pipette this into 10 cc. of 10 per cent sulfuric acid. Now add 10 cc. of 10 per cent potassium iodide and 1 cc. of 1 per cent ammonium molybdate and mix. Titrate with 0.005 *N* thiosulfate, using soluble starch near the end-point. Titrate other samples after 3, 6, 9, and 12 minutes. Calculate the monomolecular reaction constants, or K values. This method is considerably less accurate than the method of von Euler and Josephson, largely on account of the returning end-point. For this reason it is advisable to titrate each sample after a definite time.

### 7. The Immunochemistry of Catalase

Tria (26) obtained anticatalase by injecting solutions of crystalline beef liver catalase repeatedly into rabbits. The immune rabbit serum was shown to produce a heavy precipitate with crystalline beef liver catalase, a fairly heavy precipitate with crystalline lamb liver catalase and a faint precipitate with crystalline horse liver catalase. Campbell and Fourt (27) also obtained anticatalase; their paper was published at the same time as Tria's. They state that their rabbit anticatalase produced precipitates with dog liver and horse liver catalases, but not with hemoglobin or hematin.

Harkins, Fourt, and Fourt (28) have employed the Blodgett-Langmuir-Porter technique and have adsorbed catalase and anticatalase alternately upon conditioned chromium plates. Normal serum did not form a heavy deposit upon catalase, while anticatalase serum did. The undissociated catalase-anticatalase was observed to be one-fifth to one-tenth as active as dissolved catalase, which the authors have attributed to a decrease in the catalase dispersion. The total activity of two layers of catalase was no greater than the activity of one layer, since the hydrogen peroxide was decomposed before reaching the inner layer. When catalase composed the outer layer some of it was lost to the solution. None was lost when anticatalase formed the outer layer.

### 8. Can Catalase Be Resynthesized after Dissociation?

Theorell (29, 30) was able to split the yellow enzyme of Warburg and Christian into its protein part and its riboflavin phosphate part by dialyzing the salt-free enzyme against 0.02 *N* hydrochloric acid. Upon neutralizing the denatured protein, it became largely renatured and upon adding the neutralized riboflavin phosphate to this the yellow enzyme was reformed. Agner (31) claims to have succeeded in dissociating horse liver catalase into protein and hemin and to have reformed the catalase again, using a similar procedure. However, Tauber and Kleiner (23) could not repeat this, using beef, rabbit, and rat liver catalase. Neither could Sumner and Dounce (24), using crystalline beef liver catalase. Lemberg, Norrie, and Legge (32) suggest that the inability to reassociate catalase is due to the lability of the "blue substance," or biliverdin precursor, about which reference will be made later. Still another possibility is that denatured catalase protein does not readily renature upon neutralization.

## 9. The Absorption Bands of Catalase

The purification of catalase has been aided greatly by the pronounced color of this enzyme and by its characteristic absorption spectrum. There is general agreement that catalase is a ferric compound with three absorption bands at approximately:

627 536 502 mμ

while for peroxidase Keilin and Hartree (33) place the bands at:

645 550 500 mμ

The absorption bands for alkaline methemoglobin are situated at:

597 574 536 mμ

Stern and Lavin (34, 35) have studied the ultraviolet absorption of catalase. A band at 275 mμ indicates the presence of tryptophan, tyrosine, and phenylalanine.

## 10. Theories of the Mechanism of Catalase Action

Haber and Willstätter (36) proposed that catalase decomposes hydrogen peroxide by means of reaction chains. Designating catalase as $Fe^{+++}$, reduced catalase as $Fe^{++}$, and free radicals with arrows, the reactions are:

$$\begin{aligned} H_2O_2 + Fe^{+++} &= \nearrow O\cdot OH + Fe^{++} + H^+ \\ \nearrow O\cdot OH + H_2O_2 &= O_2 + \nearrow OH + H_2O \\ \nearrow OH + H_2O_2 &= H_2O + \nearrow O\cdot OH \end{aligned}$$

This hypothesis assumes that catalase is reduced by hydrogen peroxide. Stern (37) has proposed a modification of the process in which the catalase remains trivalent:

$$\begin{aligned} H_2O_2 + Fe^{+++} &= Fe^{+++} + 2\nearrow OH \\ \nearrow OH + H_2O_2 &= H_2O + \nearrow O\cdot OH \\ \nearrow O\cdot OH + H_2O_2 &= O_2 + \nearrow OH + H_2O \end{aligned}$$

Keilin and Hartree (40) postulate that two reactions occur when catalase decomposes hydrogen peroxide. In the first reaction the catalase, represented as $Fe^{+++}$, is reduced, while in the second reaction molecular oxygen reoxidizes the ferrous catalase back to ferric catalase again:

$$\begin{aligned} (1)\quad & 4Fe^{+++} + 2H_2O = 4Fe^{++} + 4H^+ + 2O_2 \\ (2)\quad & 4Fe^{++} + 4H^+ + O_2 = 4Fe^{+++} + H_2O \end{aligned}$$

It is noteworthy that although hydrogen peroxide is supposed to be a specific reductant for catalase, such powerful reducing agents as sodium hyposulfite or catalytically activated hydrogen have no reducing action

(41). It is strange, indeed, that the oxidizing agent in reaction 2 should be gaseous oxygen, rather than hydrogen peroxide.

Keilin and Hartree have attempted to support their hypothesis by two types of experiment. The first type is said to demonstrate that in pure nitrogen, hydrogen, or carbon dioxide, under conditions where hydrogen peroxide does not decompose spontaneously and where no molecular oxygen is present, the action of catalase on peroxide may be inhibited as much as 99 per cent. The second type of experiment consists in adding sodium azide to catalase solutions. This produces no change in the catalase spectrum, but upon adding hydrogen peroxide the three-banded spectrum disappears and one with two bands takes its place. Soon afterwards there is an evolution of oxygen and the original spectrum reappears. Keilin and Hartree have concluded that these changes demonstrate, first, a reduction of catalase by hydrogen peroxide and, second, a reoxidation of the reduced catalase by the molecular oxygen produced.

Johnson and van Schouwenburg (42) could not repeat the experimental work of Keilin and Hartree demonstrating that catalase is inactive in the absence of oxygen. They passed hydrogen over palladianized asbestos and then through hydrogen peroxide and through catalase until a suspension of luminous bacteria (*Ph. Fischeri*) lost its luminescence. Upon mixing the catalase and the peroxide, the catalase was found to be very active. These authors argue that such a strong oxidant as hydrogen peroxide would oxidize the reduced catalase.

Weiss and Weil-Malherbe (43) also have attacked the hypothesis of Keilin and Hartree. They gassed their apparatus with nitrogen until no oxygen could be detected by the ferrous pyrophosphate method. After this the catalase from erythrocytes or from cucumber seeds was tested for its action on peroxide, but the catalase was no less active than it was in air. Weiss and Weil-Malherbe point out that if the theory of Keilin and Hartree is correct the reaction curve of oxygen production should be autocatalytic, which was not found to be the case. They state also that the reaction postulated:

$$4Fe^{++} + 4H^{+} + O_2 = 4Fe^{+++} + 2H_2O$$

is very improbable, since the second product would not be water, but hydrogen peroxide. Finally, they state that conclusions based upon spectroscopic examination of hydroxylamine and azide catalase cannot be accepted as strong evidence.

Sumner and Dounce (unpublished) have been unable to repeat the demonstration of Keilin and Hartree showing catalase to have a diminished

action upon peroxide in the absence of molecular oxygen. They prefer the two following chemical reactions as a mechanism of catalase action:

$$(1)\quad \text{Fe-OH} + H_2O_2 = \text{Fe-OOH} + H_2O.$$
$$(2)\quad \text{Fe-OOH} + H_2O_2 = \text{Fe-OH} + H_2O + O_2.$$

Here, catalase containing ferric iron and linkages generally similar to those in methemoglobin, is represented as "Fe-OH." It is assumed that catalase peroxide, formed in reaction 1, is no more active an oxidant than peroxide itself. This hypothesis is a modification of the theory of Haber (44), of von Euler, and of Liebermann (45).

## 11. The Blue Substance Produced from Catalase

Stern (46) found that a blue substance was formed when impure catalase was treated with acetone and hydrochloric acid. He sent some of this to Lemberg, who pronounced it biliverdin. Stern reported biliverdin to be present in catalase as an impurity. Lemberg and Wyndham (47) mention this and state:

"The way in which biliverdin had here been obtained by Stern from horse liver suggested that, previous to the treatment with acetone-HCl, biliverdin had been present combined with a protein as hemin is combined with a protein in catalase. There was, however, no evidence that iron had been a part of the molecule, as it is in verdohaemochromogen. Stern mentions his catalase solutions as showing absorption in the far red, which can be ascribed either to biliverdin or to oxidized verdohaemochromogen. Reduced verdohaemochromogen, however, has a distinct absorption band between 660 and 650 mμ, for which we have now searched. We have, indeed, found this band in strong solutions of catalase, after addition of alkali, pyridine, and hyposulfite."

In 1937 Sumner and Dounce (17) reported that crystalline beef liver catalase contains a prosthetic group in addition to hematin and that this is split off to form a blue solution upon adding acetone-HCl to catalase. It was possible to separate the blue substance from the hemin by filtering off the denatured catalase protein and carefully evaporating the acetone of the filtrate in a vacuum. This caused the hemin to crystallize out, leaving the blue compound in solution. As far as the author can tell, Sumner and Dounce were the first to state that the blue substance is a decomposition product of catalase.* The substance was believed by them to be closely related to bile pigment, but not identical with it.

Lemberg, Norrie, and Legge (32) have prepared crystalline beef liver and horse liver catalase and have isolated crystalline dehydrobilirubin

* Stern, "Biological Oxidation," Oppenheimer and Stern, Nordeman Pub. Co., New York, 1939, credits Lemberg with this discovery.

(biliverdin) from these enzymes. In addition to the Gmelin test, their product gave the biliviolin test. It must be understood that biliverdin does not occur in catalase as such but is produced by hydrochloric acid acting upon the opened-up hematin prosthetic groups. These still contain iron and apparently possess a structure akin to that of the "green hemin" of Warburg and Negelein (48), which Lemberg (49) showed to be verdohemochromogen and which he converted into biliverdin. The alpha pseudohaemoglobin and alpha pseudomethemoglobin of Barkan and Schales (50, 51) and of Haurowitz (52, 53) are similar. Fig. 2 shows the

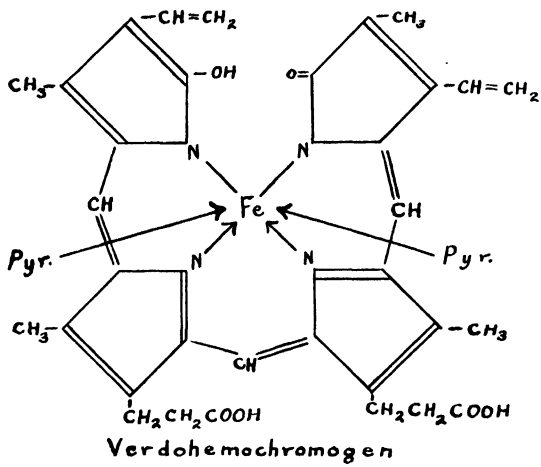



Fig. 2.—Structural formula for pyridine verdohemochromogen.

structural formula for pyridine verdohemochromogen and illustrates the opened chain of pyrrole rings.

Sumner and Dounce (24) observed that upon decomposing catalase with acetone-HCl about one-half of the iron appeared as hemin iron and about one-half as the iron accompanying the blue substance. A small amount of iron remained in the denatured catalase protein and this was regarded as due to incomplete washing out of the decomposition products. Lemberg, Norrie, and Legge (32) have found crystalline catalase to produce about three parts of hemin iron to one of blue substance iron. On the other hand, Agner (54) found only one-tenth of the total iron to occur together with the blue substance when he treated his catalase of 60,000 to 63,000

"Kat. f." with acetone-HCl. Both Agner and Theorell (55) appear to think that the iron associated with the blue substance is derived from some impurity, such as ferritin. But cow liver catalase as prepared by Sumner and Dounce has always been free from ferritin. This is shown by the low content of iron.

## 12. Crystalline Beef Liver Catalase

In a preliminary paper, Sumner and Dounce (16) reported twice-crystallized beef liver catalase to have a "Kat. f." of 43,000. Later, they obtained values of about 26,000 for 8 crystallizations of catalase (17). Still later, their values were occasionally as high as 35,000 (24).

The sedimentation constant for crystalline beef liver catalase slightly contaminated with impurity was found by Sumner and Gralén (56) to be: $s_{20} = 11.3 \times 10^{13}$ and the diffusion constant $D_{20} = 4.1 \times 10^{-7}$. The molecular weight for beef liver catalase was calculated to be 248,000. This agrees with the value of 250,000 to 300,000 found by Stern and Wyckoff (57) for horse liver catalase.

## 13. Agner's Catalase Preparations

Agner (58) has obtained highly purified horse liver catalase, using a combination of the procedures employed by the earlier workers. His yield was 0.8 gm. of catalase from 2 kilograms of liver. The enzyme had a "Kat. f." of 40,000 to 43,000 up to the point where he adsorbed it upon tricalcium phosphate. After adsorption and elution the dialyzed material had a "Kat. f." of 55,000 to 60,000. Cataphoresis in the Theorell apparatus showed no impurity, but ultracentrifugation showed 15 to 20 per cent impurity. This impurity had a lower sedimentation constant than the enzyme. The molecular weight of the enzyme was found to be 225,000.

Agner treated the preparation with picric acid and 0.1 *N* acetic acid until, at *p*H 5.2, a precipitate appeared. This was centrifuged down and more acetic acid added. At *p*H 4.6 a second precipitate formed. Both fractions were neutralized and dialyzed free from picric acid. The "Kat. f." values were low. The first fraction contained about 0.1 per cent iron and almost no copper, while the second fraction contained 0.16 per cent copper and very little iron. The fractions had different sedimentation constants. Agner observed that when both fractions were precipitated together at *p*H 4.6 the preparation retained most of its activity after removal of the picric acid. In this paper Agner appears to suspect that the copper protein may be an integral part of the catalase system.

Later, by means of the Tiselius procedure, Agner (54) was able to remove an inactive acid substance from his catalase preparation. The enzyme was then found by the ultra-

centrifuge to be unitary. The "Kat. f." was now 62,000 to 63,000, while the iron content was 0.092 per cent and the copper 0.035 per cent. By direct determination the hemin was 1 per cent (corresponding to 0.086 per cent iron). When fractionated by the procedure of Sumner and Dounce the hemin iron amounted to 83 per cent of the whole, the protein iron to 7 per cent, and the mother liquor iron (blue substance) to 10 per cent.

At *p*H 4.07, 5.31, 6.00, and 6.86 the copper migrated with the catalase. By shaking catalase solutions with amyl alcohol and sodium diethyl dithiocarbamate the copper could be removed, while the catalase activity decreased only to "Kat. f." 45,000. At this point it should be remarked that Sumner and Dounce have never found significant quantities of copper in either beef liver catalase or in horse liver catalase.

## 14. Is Horse Liver Catalase More Active than Beef Liver Catalase?

Agner's best values for horse liver catalase are approximately twice as high as most of the values of Sumner and Dounce for crystalline beef liver catalase. Theorell (55) appears to accept these figures as proof of a difference in species. Stern (37), on the other hand, has taken the differences as proof of the futility of purifying an enzyme by crystallization. He comments thus upon Agner's value of "Kat. f." 55,000:

"This is far in excess of the activity of Sumner's crystalline beef liver fractions, another illustration of the fact that crystalline form, even after repeated 'recrystallizations' is not a guarantee for the purity of a protein."

In order to clear up the conflicting differences, Sumner, Dounce, and Frampton (19) have investigated horse liver catalse and have worked out an improved method for its crystallization. Their crystalline preparations have varied in activity from "Kat. f." 22,000 to 40,000, although the first sample of crystalline horse liver catalase obtained by Dounce and O. Frampton (18) did possess a "Kat. f." of 50,000.

Sumner, Dounce, and Frampton have also prepared horse liver catalase by the method of Agner, but to their great surprise were not able to obtain "Kat. f." values greater than 32,000. They had no difficulty in crystallizing the catalase prepared according to Agner's method and noted that the crystallization brought about an increase in activity approximately as great as was occasioned by adsorbing upon tricalcium phosphate in a Tswett column, eluting, and dialyzing.

## 15. The Homogeneity of Crystalline Catalase

Sumner, Dounce, and Frampton (19) have employed the diffusion cell

of Lamm in order to test the homogeneity of various preparations of catalase and have normalized the curves. Two preparations of crystalline horse liver catalase obtained by their method and of nearly 40,000 "Kat. f." were found to be completely homogeneous. A third preparation of "Kat. f." 30,000 proved to be homogeneous also, while a preparation of beef liver catalase of "Kat. f." 35,000 was nearly homogeneous.

The diffusion constants for both beef liver catalase and horse liver catalase were found to be $D_{20} = 4.5 \times 10^{-7}$. Using the sedimentation constant of Sumner and Gralén (56), together with this new diffusion constant, the molecular weight for beef liver catalase comes out 225,000.

## 16. Are There Several Catalases, Depending upon the Number of Intact Hemin Residues?

Sumner, Dounce, and Frampton (19) have found that those preparations of catalase which are the most active per unit of weight, or, in other

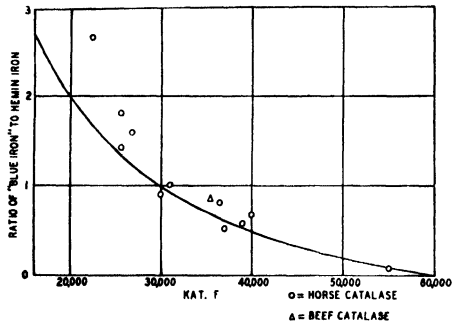



Fig. 3.—Curve, showing ratio of "blue iron" to hemin iron.

words, have the greatest "Kat. f.," produce the least blue substance upon treatment with acetone-HCl and the greatest quantity of hemin. They advance the hypothesis, therefore, that several different catalases exist. The catalase of highest "Kat. f." would contain four hematin groups and no blue substance precursor. This is an idea originally suggested by Lemberg to the author.† The curve in Fig. 3 agrees with the hypothesis

† More recently K. G. Stern suggested in a private communication to A. L. Dounce that catalase may be a mixture of one compound of high activity, containing 4 hematin groups and another substance of no activity containing 4 opened-up hematin groups.

fairly well and indicates that the catalase of great activity is rich in hematin and poor in blue substance precursor. The authors have suggested that animal tissues may contain some enzyme which is able to open up the hematin residues in catalase and which is similar or identical with the enzymes which are claimed to convert hemoglobin hematin to bile pigments (59, 60).

### Bibliography


1. C. F. Schönbein, *J. prakt. Chem.*, **89**, 334 (1863).
2. W. Jacobson, *Z. physiol. Chem.*, **16**, 340 (1892).
3. O. Löw, *U. S. Dept. Agr. Report No.* **68** (1901).
4. J. Wolff and E. de Stoecklin, *Compt. rend.*, **152**, 729 (1911).
5. P. Waentig and W. Gierisch, *Fermentforschung*, **1**, 165 (1914–16).
6. M. Tsuchihashi, *Biochem. Z.*, **171**, 314 (1926).
7. S. Hennichs, *Ibid.*, **171**, 314 (1926).
8. S. Hennichs, *Ber.*, **59**, 218 (1926).
9. V. Laufberger, *Bull. soc. chim. biol.*, **19**, 1575 (1937).
10. H. von Euler and K. Josephson, *Ann.*, **452**, 158 (1927).
11. H. von Euler and K. Josephson, *Ibid.*, **455**, 1 (1927).
12. H. von Euler, K. Zeile, and H. Hellström, *Svensk Kem. Tid.*, **42**, 74 (1930).
13. K. Zeile and H. Hellström, *Z. physiol. Chem.*, **192**, 171 (1930).
14. K. Zeile, *Ibid.*, **195**, 39 (1931).
15. K. G. Stern, *J. Biol. Chem.*, **112**, 661 (1936).
16. J. B. Sumner and A. L. Dounce, *Science*, **85**, 366 (1937).
17. J. B. Sumner and A. L. Dounce, *J. Biol. Chem.*, **121**, 417 (1937).
18. A. L. Dounce and O. Frampton, *Science*, **89**, 300 (1930).
19. J. B. Sumner, A. L. Dounce, and V. L. Frampton, *J. Biol. Chem.*, **136**, 343 (1940).
20. L. E. Shinn, E. R. Main, and R. R. Mellon, *Proc. Soc. Exptl. Biol. Med.*, **44**, 596 (1940).
21. H. von Euler, "Chemie der Enzyme," Leipzig, 1934, p. 28.
22. P. Waentig and O. Steche, *Z. physiol. Chem.*, **83**, 315 (1913).
23. H. Tauber and I. S. Kleiner, *Proc. Soc. Exptl. Biol. Med.*, **33**, 391 (1935).
24. J. B. Sumner and A. L. Dounce, *J. Biol. Chem.*, **127**, 439 (1939).
25. A. Jolles, *Münch. med. Wochschr.*, **51**, 2083 (1904), quoted from von Euler, "Chemie der Enzyme," Teil 2, p. 73; see also K. G. Stern, *Z. physiol. Chem.*, **204**, 259 (1932).
26. E. Tria, *J. Biol. Chem.*, **129**, 377 (1939).
27. D. H. Campbell and L. Fourt, *Ibid.*, **129**, 385 (1939).
28. W. D. Harkins, L. Fourt, and P. C. Fourt, *Ibid.*, **132**, 111 (1940).
29. H. Theorell, *Biochem. Z.*, **272**, 155 (1934).
30. H. Theorell, *Ibid.*, **278**, 263 (1935).
31. K. Agner, *Z. physiol. Chem.*, **235**, 2 (1935).
32. R. Lemberg, M. Norrie, and J. W. Legge, *Nature*, **144**, 151 (1939).
33. D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (London), **B122**, 119 (1937).
34. K. G. Stern, *Z. physiol. Chem.*, **212**, 207 (1932).
35. K. G. Stern and G. I. Lavin, *Science*, **88**, 263 (1938).

36. F. Haber and R. Willstätter, *Ber.*, **64**, 2844 (1931).
37. K. Oppenheimer and K. G. Stern, "Biological Oxidation," New York, 1939, p. 46
38. D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (London), **(B)121**, 173 (1936).
39. D. Keilin and E. F. Hartree, *Ibid.*, **124**, 397 (1938).
40. D. Keilin and E. F. Hartree, *Ibid.*, **144**, 787 (1939).
41. K. G. Stern, *J. Gen. Physiol.*, **20**, 631 (1937).
42. F. H. Johnson and K. L. van Schouwenburg, *Nature*, **144**, 634 (1939).
43. J. Weiss and H. Weil-Malherbe, *Ibid.*, **144**, 866 (1939).
44. F. Haber, *Z. anorg. Chem.*, **18**, 40 (1898); H. von Euler, Ref. Wiedmann, *Beiblätter*, **24**, 949 (1900), quoted from G. Bredig, and K. Ikeda, *Z. physik. Chem.*, **37**, 1 (1901).
45. L. Liebermann, *Arch. ges. Physiol.*, **104**, 119 (1904).
46. K. G. Stern, *J. Biol. Chem.*, **112**, 661 (1935–36).
47. R. Lemberg and R. A. Wyndham, *J. and Proc. Roy. Soc. New South Wales*, **70**, 343 (1937).
48. O. Warburg and E. Negelein, *Ber.*, **63**, 1816 (1930).
49. R. Lemberg, *Biochem. J.*, **29**, 1322 (1935).
50. G. Barkan and O. Schales, *Z. physiol. Chem.*, **248**, 96 (1937).
51. G. Barkan and O. Schales, *Science*, **90**, 616 (1939).
52. F. Haurowitz, *Enzymologia*, **4**, 139 (1937).
53. F. Haurowitz, *Z. physiol. Chem.*, **253**, 83 (1938).
54. K. Agner, *Naturwiss.*, **23–24**, 418 (1939).
55. H. Theorell, *Ann. Rev. Biochem.*, **9**, 666 (1940).
56. J. B. Sumner and N. Gralén, *J. Biol. Chem.*, **125**, 33 (1938).
57. K. G. Stern and R. W. G. Wyckoff, *Science*, **87**, 18 (1938).
58. K. Agner, *Biochem. J.*, **32**, 1702 (1938).
59. H. T. Schreus and Corrie, *Klin. Wochenschr.*, **2**, 1675 (1934).
60. S. Edlbacher and A. Segesser, *Naturwissenschaften*, **34**, 557 (1937).

# ENZYMES AND TRACE SUBSTANCES

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Oxidation enzymes which have been analyzed thus far have been found to conform to a common architectural plan. The enzymes consist of (*1*) a protein in loose or rigid combination with (*2*) a substance of comparatively low molecular weight known as the prosthetic group. Each component of the enzyme is highly specific. That is to say, both the protein portion and prosthetic group are specialized for the particular function of the enzyme.

A considerable number of oxidation enzymes can now be classified on the basis of the chemical constitution of the prosthetic group. Thus flavoproteins, pyridinoproteins, Cu proteins, Fe porphyrin proteins, and diphosphothiamine proteins represent families of oxidation enzymes containing, respectively, flavinadenine dinucleotide, pyridine nucleotide, Cu, Fe porphyrin, and diphosphothiamine as prosthetic group. The members of a family of enzymes have in common the same or very similar prosthetic group but differ in respect to both the protein moiety and the reaction catalyzed. It follows that the protein moiety is more specific and, in fact, determines the nature of the catalysis.

Catalysis by oxidation enzymes invariably resolves itself into some cyclical process. Consider, for example, the aerobic oxidation of *d*-alanine catalyzed by a flavoprotein enzyme from liver or kidney. This reaction takes place in two distinct stages (1):

(a) Alanine + flavoprotein $\longrightarrow$ pyruvic acid + $NH_3$ + reduced flavoprotein

(b) Reduced flavoprotein + $O_2$ $\longrightarrow$ flavoprotein + $H_2O_2$

The flavoprotein undergoes a cycle of reduction by alanine and oxidation by molecular oxygen. The net result is the oxidation of alanine by molecular oxygen with production of $H_2O_2$. At 38° each molecule of the en-

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zyme undergoes this cycle about 2000 times per minute (2). The nature of the cyclical process varies from one family of oxidation enzymes to another, and is determined primarily by the chemical properties of the prosthetic group.

When *d*-alanine is oxidized by the enzyme the sole chemical change is the transfer of 2 H atoms from the substrate to the flavin group of the enzyme. The prosthetic group registers the actual chemical change. What role, therefore, does the protein moiety play in the catalytic process? A mixture of *d*-alanine and flavinadenine dinucleotide does not react. In the presence of the specific protein reaction takes place. Clearly the protein modifies the substrate in some way as to facilitate the transfer of hydrogen. This process of modification of the substrate is referred to by terms such as activation, labilization, etc. It matters little which term is used since the nature of the process is obscure. But there are a few facts which point the way to an explanation of what takes place when the substrate is brought into contact with the enzyme. There is good evidence that a special group of the protein combines with the substrate. This enzyme-substrate compound is in a sense equivalent to a modified substrate molecule, and it is assumed that the substrate modified by chemical combination with the enzyme becomes more susceptible to chemical change. The obscure problem of the activation of the substrate by the enzyme lies beyond the province of this article. For the discussion that follows it is important to recognize that the protein moiety of the enzyme is not mere ballast for the prosthetic group. There are hundreds of enzymes known and each one is represented by a protein uniquely fashioned for its particular purpose. In what this uniqueness consists there are no clues as yet to guide us. It would be idle to deny that enzyme chemists are unable to explain the extraordinary catalytic properties of special proteins in terms either of the properties of the amino acids or of the properties of large molecules formed by peptide linkage of amino acids.

The conception of the turn-over number has proved useful for quantitative evaluation of the catalytic efficiency of enzymes. For each molecule of substrate transformed the enzyme undergoes one complete cycle. The turn-over number is defined as the number of cycles per minute which the enzyme undergoes. Catalase (3), for example, has a turn-over number of 2,640,000 at 0°, whereas the amino acid oxidase (2) has a turn-over number of ca. 2000 at 38.°

The family of Fe porphyrin protein enzymes includes some of the most active catalysts known. A summary of the characteristics of this family is given in Table I. The prosthetic group in each case is protoporphyrin

TABLE I

FE-PORPHYRIN PROTEIN ENZYMES

| Enzyme | Prosthetic group | Function | Turn-over no. |
|---|---|---|---|
| Catalase[1] | Fe-protoporphyrin + bile pigment hemochromogen | Catalyzes reaction: $2H_2O_2 \longrightarrow 2H_2O + O_2$ | ca. $2.4 \times 10^6$ (0°) |
| Peroxidase[2] | Fe-protoporphyrin | Catalyzes oxidation of phenols, diamines, etc., by $H_2O_2$ | ca. $1.5 \times 10^5$ (20°) |
| Cytochrome $c$[3] | Slightly modified Fe-protoporphyrin | Undergoes cyclical reduction and oxidation | $1.4 \times 10^3$ (38°) |
| Hemoglobin | Fe-protoporphyrin | Undergoes cyclical combination with and dissociation from molecular oxygen | ..... |

[1] K. G. Stern, *J. Biol. Chem.*, **112**, 661 (1936).
J. B. Sumner and A. L. Dounce, *Ibid.*, **127**, 439 (1939).
D. Keilin and E. F. Hartree, *Proc. Roy. Soc.*, **121**, 173 (1936).
[2] D. Keilin and T. Mann, *Ibid.*, **122**, 119 (1937).
[3] D. Keilin and E. F. Hartree, *Ibid.*, **122**, 298 (1937).

or a slight modification thereof. It is interesting to note that the cyclical changes which the prosthetic group undergoes are not the same for each member of the family. In catalase the Fe atom of the prosthetic group

TABLE II

DIPHOSPHOPYRIDINOPROTEIN ENZYMES

The prosthetic group of this family of enzymes is nicotinicamide-ribose-(phosphate)$_2$-ribose-adenine [diphosphopyridine nucleotide or coenzyme I].

| Substrate of enzyme | Product of oxidation | Reference |
|---|---|---|
| Alcohol | Aldehyde | Negelein and Wulff[1] |
| Aldehyde | Acetate | Dixon and Lutwak-Mann[2] |
| Lactate | Pyruvate | Straub,[3] Green and Brosteaux[4] |
| Malate | Oxaloacetate | Green[5] |
| Triose | Glycerate | Green, *et al.*[6] |
| 1:3-Diphosphoglyceraldehyde | 1:3-Diphosphoglycerate | Warburg and Christian[7] |
| Glucose | Gluconate | Harrison,[8] Adler and Euler[9] |
| $\alpha$-Glycerophosphate | Triosephosphate | Euler, *et al.*[10] |
| $\beta$-Hydroxybutyrate | Acetoacetate | Green, *et al.*[11] |
| Glutamate | $\alpha$-Ketoglutarate + $NH_3$ | Adler, *et al.*,[12] Dewan[13] |
| Formate | Bicarbonate | Adler and Sreenivasaya[14] |

[1] E. Negelein and H. J. Wulff, *Biochem. Z.*, **289**, 436 (1937); **293**, 351 (1937).
[2] M. Dixon and C. Lutwak-Mann, *Biochem. J.*, **31**, 1347 (1937).
[3] F. B. Straub, *Ibid.*, **34**, 483 (1940).
[4] D. E. Green and J. Brosteaux, *Ibid.*, **30**, 1489 (1936).
[5] D. E. Green, *Ibid.*, **30**, 2095 (1936).
[6] D. E. Green, D. M. Needham, and J. G. Dewan, *Ibid.*, **31**, 2327 (1937).
[7] O. Warburg and W. Christian, *Biochem. Z.*, **303**, 40 (1939).
[8] D. C. Harrison, *Biochem. J.*, **26**, 1295 (1932).
[9] E. Adler and H. v. Euler, *Hoppe-Seyler's Z. physiol. Chemie*, **232**, 6 (1935).
[10] H. v. Euler, E. Adler, and G. Günther, *Ibid.*, **249**, 1 (1937).
[11] D. E. Green, L. F. Leloir, and J. G. Dewan, *Biochem. J.*, **31**, 934 (1937).
[12] H. v. Euler, E. Adler, G. Günther, and N. B. Das, *Hoppe-Seyler's Z. physiol. Chemie*, **254**, 61 (1938).
[13] J. G. Dewan, *Biochem. J.*, **32**, 1378 (1938).
[14] E. Adler and M. Sreenivasaya, *Hoppe-Seyler's Z. physiol. Chemie*, **249**, 24 (1937).

undergoes a cycle of reduction by $H_2O_2$ and oxidation by molecular oxygen (4). Similarly the Fe atom of the prosthetic group of cytochrome *c* undergoes a ferro-ferric valency cycle. But the atom of Fe in both peroxidase (5) and hemoglobin does not change in valency, being ferric in the

one and ferrous in the other. The cyclical changes of the prosthetic group in peroxidase are still somewhat obscure. In hemoglobin the ferrous prosthetic group undergoes a cycle of combination with and dissociation from molecular oxygen.

Diphosphopyridine and triphosphopyridine nucleotides are the prosthetic groups, respectively, of two important families of oxidation enzymes (cf. Tables II and III). Only two specific proteins are known which can utilize both pyridine nucleotides as prosthetic group, *viz.*, the proteins for the glutamic (6) and glucose (7) enzymes of liver. All the others are specific for either the one or the other nucleotide although the chemical differ-

TABLE III

TRIPHOSPHOPYRIDINOPROTEIN ENZYMES

The prosthetic group of this family of enzymes is nicotinicamide-ribose-(phosphate)$_3$-ribose-adenine [triphosphopyridine nucleotide or coenzyme II].

| Substrate of enzyme | Product of oxidation | Reference |
|---|---|---|
| Hexosemonophosphate | Phosphohexonate | Warburg and Christian[1] |
| Isocitrate | $\alpha$-Ketoglutarate + $CO_2$ | Adler, *et al.*[2] |
| Glutamate | $\alpha$-Ketoglutarate + $NH_3$ | Adler, *et al.*[3] |
| Glucose | Gluconate | Das[4] |

[1] O. Warburg and W. Christian, *Biochem. Z.*, **254**, 438 (1932).

[2] E. Adler, H. v. Euler, G. Günther, and M. Ploss, *Biochem. J.*, **33**, 1028 (1939).

[3] H. v. Euler, E. Adler, G. Günther, and N. B. Das, *Hoppe-Seyler's Z. physiol. Chemie*, **254**, 61 (1938).

[4] N. B. Das, *Ibid.*, **238**, 269 (1936).

ence between the two nucleotides consists merely in one phosphoric acid group.

The pyridinoprotein enzymes catalyze the oxidation of their respective substrates by undergoing cyclical reduction and oxidation. It is only the pyridine ring of the prosthetic group which is concerned in the cyclical process. The catalytic efficiencies of the pyridinoprotein enzymes are high, allowing for the chemistry of the oxidations involved. The alcohol and triosephosphoric enzymes, for example, have a turn-over number of about 20,000 at 20° (8, 9).

The flavoproteins have much in common with the pyridinoproteins (cf. Table IV). The iso-alloxazine ring of the flavin prosthetic group is specialized for cyclical reduction and oxidation in the same way as the py-

TABLE IV

FLAVOPROTEIN ENZYMES

| Source | Reaction catalyzed | Prosthetic group | Turn-over no. | Reference |
|---|---|---|---|---|
| Bottom yeast | Oxidation of dihydrocoenzyme I and II | Flavinphosphate | 50 | Warburg and Christian,[1] Theorell[2] |
| Top yeast | Oxidation of dihydrocoenzyme II by cytochrome *c* | Flavinphosphate | 300-500 | Haas, *et al.*[13] |
| Bottom yeast | Oxidation of dihydrocoenzyme II | Flavinadenine dinucleotide | 220 | Haas[3] |
| Kidney | Oxidation of *d*-amino acids | Flavinadenine dinucleotide | ca. 2000 | Warburg and Christian,[4] Straub[5] |
| Milk | Oxidation of purines, aldehydes, dihydrocoenzyme I | Flavinadenine dinucleotide + unknown group | 306—hypoxanthine<br>570—acetaldehyde<br>260—dihydrocoenzyme I | Corran, *et al.*,[6] Ball[7] |
| Liver | Oxidation of aldehydes | Flavinadenine dinucleotide + unknown group | 550—acetaldehyde | Gordon, *et al.*[8] |
| Bottom yeast | Reduction of fumarate | Flavinadenine dinucleotide | 2750 | Fischer and Eysenbach[9] |
| Top yeast | Uncharacterized as yet | Flavinadenine dinucleotide + unknown group | ..... | Green, *et al.*[10] |
| Heart | Oxidation of dihydrocoenzyme I | Flavinadenine dinucleotide | ca. 8000 | Straub,[11] Corran, *et al.*[12] |

[1] O. Warburg and W. Christian, *Biochem. Z.*, **254**, 438 (1932).
[2] H. Theorell, *Ibid.*, **275**, 344 (1935); **278**, 263 (1935).
[3] E. Haas, *Ibid.*, **289**, 378 (1938).
[4] O. Warburg and W. Christian, *Ibid.*, **295**, 261 (1938); **298**, 150 (1938).
[5] F. B. Straub, *Nature*, **141**, 603 (1938).
[6] H. S. Corran, J. G. Dewan, A. H. Gordon, and D. E. Green, *Biochem. J.*, **33**, 1694 (1939).
[7] E. Ball, *J. Biol. Chem.*, **128**, 51 (1933).
[8] A. H. Gordon, D. E. Green, and V. Subrahmanyan, *Biochem. J.*, **34**, 764 (1940).
[9] F. G. Fischer and H. Eysenbach, *Ann.*, **30**, 99 (1937).
[10] D. E. Green, P. Stumpf, and E. Knox (unpublished results).
[11] F. B. Straub, *Biochem. J.*, **33**, 787 (1939).
[12] H. S. Corran, D. E. Green, and F. B. Straub, *Ibid.*, **33**, 793 (1939).
[13] E. Haas, B. L. Horecker and T. R. Hogness, *J. Biol. Chem.*, **136**, 747 (1940).

TABLE V

CU PROTEIN ENZYMES

| Enzyme | Source | Reaction catalyzed | Turn-over no. | Reference |
|---|---|---|---|---|
| Hemocyanin | Arthropods, molluscs | Combination with and dissociation from molecular oxygen | ..... | ..... |
| Polyphenol oxidase | Potato | Oxidation of *o*-diphenols and monophenols by $O_2$ | $3 \times 10^3$ (20°) | Kubowitz[1] |
| Polyphenol oxidase | *Agaricus campestris* (cultivated mushroom) | Oxidation of *o*-diphenols by $O_2$ | $7 \times 10^4$ (20°) | Keilin and Mann[2] |
| Laccase | *Rhus succedanea* (lacquer tree of Indo-China) | Oxidation of *p*-phenylene diamine and diphenols by $O_2$ | $2.5 \times 10^3$ (20°) | Keilin and Mann[3] |
| Monophenol oxidase | *Lactarius piperatus* (wild mushroom) | Oxidation of monophenols by $O_2$ | $4 \times 10^4$ (20°) | Dalton and Nelson[4] |
| Ascorbic oxidase | Summer squash (*C. pepo condensa*) | Oxidation of ascorbic acid by $O_2$ | $5 \times 10^4$ (20°) | Lovett - Janison and Nelson[5] |

[1] F. Kubowitz, *Biochem. Z.*, **299**, 32 (1938).
[2] D. Keilin and T. Mann, *Proc. Roy. Soc.*, **125**, 187 (1938).
[3] D. Keilin and T. Mann, *Nature*, **143**, 23 (1939); **144**, 442 (1939).
[4] A. R. Dalton and J. M. Nelson, *J. Am. Chem. Soc.*, **61**, 2946 (1939).
[5] P. L. Lovett-Janison and J. M. Nelson, *Ibid.*, **62**, 1409 (1940).

ridine ring. Yet the pyridine nucleotides cannot substitute for flavin in the flavoproteins and, conversely, flavin dinucleotide cannot substitute for pyridine nucleotide in the pyridinoproteins. There can be no question, therefore, that the prosthetic group stands in a highly specific relation to the protein moiety of the enzyme.

The protein of the Warburg-Christian flavoprotein can combine with flavinadenine dinucleotide (10), flavinphosphate, or flavin (11) to form the catalytically active enzyme. This lack of specificity for the form in which the prosthetic group is presented is true only of the Warburg-Christian flavoprotein. All the other flavoproteins are specific for flavinadenine

TABLE VI

DIPHOSPHOTHIAMINOPROTEIN ENZYMES

| Source | Reaction catalyzed | Reference |
|---|---|---|
| Yeast | Decarboxylation of α-ketonic acids | Lohmann and Schuster,[1] Green, *et al.*[2] |
| *Bact. Delbrueckii* | Oxidative decarboxylation of pyruvic acid | Lipmann[3] |
| *Gonococcus* | Oxidative decarboxylation of pyruvic acid | Barron[4] |
| Brain | Oxidation of pyruvic acid | Peters, *et al.*[5] |

[1] K. Lohmann and G. Schuster, *Biochem. Z.*, **294**, 188 (1937).
[2] D. E. Green, D. Herbert, and V. Subrahmanyan, *J. Biol. Chem.*, **135**, 795 (1940).
[3] F. Lipmann, *Enzymologia*, **4**, 65 (1937); *Nature*, **143**, 436 (1939).
[4] E. S. G. Barron, *J. Biol. Chem.*, **113**, 695 (1936).
[5] I. Banga, S. Ochoa, and R. A. Peters, *Nature*, **143**, 764 (1939); **144**, 74 (1939).

dinucleotide except for cytochrome *c* reductase which is specific for flavinphosphate (49). Some evidence is now accumulating that the adenine-ribose moiety of the prosthetic group is merely a vehicle for an additional phosphoric acid group, and that phosphoric acid groups are necessary for the combination of protein and prosthetic group. It is conceivable, therefore, that flavin could replace flavinadenine dinucleotide as prosthetic group if an alternative method of attachment to the protein could be devised, say, by way of some metal. Presumably in the case of the Warburg-Christian flavoprotein the protein has the possibility of combination with the three forms of riboflavin. It is significant that massive concentrations of free flavin are necessary to give the same effect as traces of flavinphosphate.

In other words, the phosphoric group increases enormously the ease of combination with the protein.

The simplest known prosthetic group is the metal, copper, which forms a family of enzymes known as copper proteins (cf. Table V). In the respiratory pigment hemocyanin the prosthetic group remains in the cuprous condition and undergoes a cycle of combination with and dissociation from molecular oxygen. In the monophenol and polyphenol oxidases the prosthetic group undergoes a cycle of valency (12) change from the cupric to the cuprous condition and *vice versa*. Thus the cupric metal is reduced by the substrate and the cuprous metal in turn is oxidized by

TABLE VII

AVERAGE SATURATION NUTRITIONAL REQUIREMENTS OF VITAMINS

| | γ Vitamin per kg. body weight per day (approximate) |
|---|---|
| Vitamin A[1] | 60 |
| Vitamin $B_1$[1] | 13 |
| Riboflavin ($B_2$ complex)[1] | 4 |
| Vitamin C[1] | 500 |
| Vitamin D[1] | 1 |
| Vitamin $B_6$ (pyridoxin)[2] | 50 |
| Vitamin K[3] | 25 |
| Nicotinic acid[4] | 500 |

[1] "The Vitamins" (1939). A Symposium, American Medical Association.

[2] R. Kuhn and G. Wendt, *Hoppe-Seyler's Z. physiol. Chemie*, **256**, 127 (1938).

[3] H. J. Almquist and A. A. Klose, *J. Biol. Chem.*, **130**, 787 (1940).

[4] C. A. Elvehjem, R. J. Madden, F. M. Strong, and D. W. Wooley, *J. Am. Chem. Soc.*, **59**, 1767 (1937).

molecular oxygen. The prosthetic group of the polyphenol oxidase of *Agaricus campestris* has a turn-over number (13) of ca. 70,000 at 20° with catechol as substrate. Copper protein compounds have been isolated from liver, blood corpuscles, and serum but as yet there is no information available of their catalytic role, if any.

Decarboxylation of α-ketonic acids is catalyzed by a family of enzymes known as diphosphothiamine proteins (cf. Table VI). The cyclical changes which the prosthetic group undergoes during the catalytic process are still obscure. The turn-over number of diphosphothiamine in yeast carboxylase is ca. 940 at 30°. Thiamine and monophosphothiamine are inactive as prosthetic group.

Yeast carboxylase has been shown to be a diphosphothiamine-magnesium-protein compound (14). Although the enzyme is isolated as a magnesium complex it is possible to replace magnesium by a variety of divalent metals such as Co, Ni, Mn, Ca, etc. This non-specificity does not argue a role of the metal in the actual catalytic process. The most plausible explanation is that the metal acts as a chemical link between the protein and the prosthetic group and has purely structural significance like the phosphoric groups of flavin dinucleotide and other nucleotides.

Our survey of six well-defined families of enzymes would not be complete without mention of isolated examples of new types of prosthetic groups. Carbonic anhydrase, the enzyme which catalyzes the formation of $H_2CO_3$ from $CO_2$ and $H_2O$, has been shown to be a zinc protein compound (15). Glyoxalase, the enzyme which catalyzes the transformation of methyl glyoxal into lactic acid, is a glutathione (16) protein compound. Phosphorylase, the enzyme in animal tissues which catalyzes the synthesis of starch from glucose-1-phosphate, appears to be an adenylic acid protein (48). Both milk (17, 18) and liver (19) flavoproteins have been shown to contain some prosthetic group other than flavinadenine dinucleotide, and there is some evidence, in the case of the milk enzyme at least, that this group may be of greater functional significance than flavinadenine dinucleotide. Finally the aminophorases (20), the enzymes which catalyze the transfer of amino groups from amino acids to $\alpha$-ketonic acid, have been shown to contain a prosthetic group not identical with any yet described.

An important discovery in recent years has been the identification of some of the vitamins with prosthetic groups of enzymes. The identity of the antineuritic, anti-black tongue, and $B_2$-growth factor vitamins with thiamine (21, 22), nicotinic acid (23), and flavin (24), respectively, is now established beyond dispute. An interesting inference can be drawn. The animal cell is self-sufficient as far as the protein moieties of the diphosphothiaminoproteins, pyridinoproteins, and flavoproteins are concerned. These must be obtained, therefore, directly by synthesis. The synthesis of the prosthetic groups apparently does not lie within the capacity of the animal cell.* To a great extent this is also true of the bacterial cell. They must be supplied to the cell from external sources. It is not the prosthetic group as a whole which the cells usually require—only the functional part such as the thiazol ring, the pyridine ring, and the isoalloxazine ring.

* There is variability in the synthesizing powers of the cells of different animals. *E. g.*, ascorbic acid is essential in the diet of guinea pigs and humans but not in that of rats. Although all animals require certain vitamins, yet the number and nature of the vitamins vary from one animal to another.

Given flavin, for example, the animal or bacterial cell can prepare the phosphoric ester, and then by condensing the ester with adenine nucleotide can form the catalytically active flavinadenine dinucleotide. Similarly, nicotinic acid can be aminated, condensed with ribose phosphoric ester, and the nicotinicamide nucleotide then condensed with adenine nucleotide to form the catalytically active diphosphopyridine dinucleotide.

The identification of three vitamins with three prosthetic groups has helped to dovetail the field of animal and bacterial nutrition with that of

TABLE VIII

CONCENTRATIONS AT WHICH HORMONES EXERT THEIR ACTIVITY

| Hormone | Dose | Effect |
|---|---|---|
| Insulin[1] | 10$\gamma$ | Lowers the blood sugar of 2 kg. rabbit |
| *l*-Thyroxine[2] | 100$\gamma$ | Raises metabolic rate of mouse |
| Adrenaline[2] | 0.002$\gamma$ | Arrests contraction of frog's stomach |
| Oestrone[3] | 0.04–0.1$\gamma$ | Induces oestrus in castrated mouse |
| Progesterone[3] | 500–1000$\gamma$ | Produces after fifth day a progestational proliferation of uterine mucosa |
| Androsterone[3] | 60$\gamma$ | Produces 20% daily increase in area of capon's comb (one capon unit) |
| Testosterone[3] | 10$\gamma$ | As above |

[1] J. J. R. Macleod and W. R. Campbell, "Insulin," Williams & Wilkins Co., Baltimore, 1925.

[2] A. J. Clark, "The Mode of Action of Drugs on Cells," Williams & Wilkins Co., Baltimore, 1933.

[3] L. Ficser, "The Chemistry of Natural Products Related to Phenanthrene," Rheinhold Publishing Corp., N. Y., 1936.

physiological function. These vitamins are necessary in the diet as a source of prosthetic groups for essential enzymes. The life period of enzymes is apparently not long and a steady supply of the essential building components must be available. Since enzymes occur in very low concentrations the amount of vitamin necessary is correspondingly of a low order of magnitude (cf. Table VII). The question arises whether there are any criteria by which we can predict vitamin-enzyme relationships from the information now available. Or to put the question in a somewhat more general form, can the data of nutritional requirements provide any clue as to enzyme function? Before attempting an answer to these questions it

will be necessary, first, to establish criteria by which we can recognize enzymic phenomena.

The thesis which we shall develop in this article is that any substance which occurs in traces in the cell and which is necessary in traces in the diet or medium must either be an essential part of some enzyme or the enzyme itself. We shall define a trace concentration as one whose uppermost limit is less than 5γ per gm. dry weight of cell. There are, of course, many trace substances which are chance contaminants of cells but these are excluded from our consideration since they are not essential in the nutritive milieu. It also does not follow that concentrations higher than

TABLE IX

ACTIVITY DILUTIONS OF PLANT AND ANIMAL HORMONES

| Hormone | Concentration in medium γ per liter | Effect |
|---|---|---|
| Biotin[1] | 0.04 | Stimulates growth of yeast |
| Auxin A[1] | 0.1 | Induces curvature of oat seedlings |
| Acetylcholine[2] | 0.25 | Produces fall in blood pressure of cat |
| Thyroxin[1] | 0.5 | Induces metamorphosis of tadpoles |
| Adrenaline[2] | 0.02 | Induces expansion of frog's eye |
| Posterior pituitary extract (crude)[2] | 0.001 | Induces dilation of melanophores in frog's skin |
| | 0.5 | Induces contraction of guinea pig uterus |
| Histamine[2] | 0.11 | Induces vasoconstriction of rabbit's ear |

[1] F. Kogl, *Naturwissenschaften*, 29, 465 (1937).

[2] A. J. Clark, "The Mode of Action of Drugs on Cells," Williams & Wilkins Co., Baltimore, 1933.

the arbitrarily defined trace concentration rule out the possibility of enzymic function. But the degree of probability that a connection exists between a particular substance and some enzyme function becomes very high indeed when the criteria of concentration and nutritional requirements are both satisfied.

The trace substance-enzyme thesis boils down to the view that enzyme catalysis is the only rational explanation of how a trace of some substance can produce profound biological effects. Merely to defend this view and quote evidence in its favor would on the whole be unprofitable. The assessment of the value of a new thesis in a growing experimental science should be purely pragmatic. The discussion will therefore be restricted

to the new lines of experimentation suggested by the trace substance-enzyme thesis and to some of its logical implications.

There are two main lines of attack in enzyme isolation work. The first is to isolate an enzyme in highly purified form and then to make appropriate tests for the presence or absence of known prosthetic groups. The second is the reverse process. The prosthetic group is used as a clue for running down an enzyme.

The isolation of a catalytically active flavoprotein from heart muscle will serve as an example of how the second method is used in practice. Straub (25) and Warburg (26) demonstrated that the prosthetic group of the *d*-amino acid oxidase of liver and kidney was flavinadenine dinucleotide. A study of the distribution of flavinadenine dinucleotide in different tissues revealed that heart muscle is one of the richest sources of the prosthetic group although the *d*-amino acid oxidase is not present in heart muscle. Straub (27) reasoned that the flavinadenine dinucleotide present in heart muscle was the prosthetic group of some enzyme other than the *d*-amino acid oxidase. He made the significant discovery that the dinucleotide occurred in combination with a protein. Purification of this bound flavinadenine dinucleotide led to the isolation of a flavoprotein which was then identified by Corran, *et al.* (28), as an enzyme which catalyzes the oxidation of reduced diphosphopyridine nucleotide. The enzyme had been described in detail before the isolation of the flavoprotein but no success attended the earlier efforts to determine the nature of the prosthetic group.

There are three variations of the tracer method of isolating enzymes. One can track down enzymes by following the bound forms of prosthetic groups, vitamins, or metals. Of course, in some cases vitamins are known to be prosthetic groups and it is immaterial whether a tracer is considered as the one or the other. But some of the vitamins have not as yet been identified with any enzyme function and their bound forms should serve as valuable tracers. Keilin and Mann (15) were the first to use a metal as a tracer. They observed that the Zn present in red blood corpuscles was not dialyzable but associated with protein. They undertook the purification of this compound and isolated a Zn protein which was then identified as the enzyme, carbonic anhydrase. Keilin and Mann similarly tracked down the bound form of Cu in blood and isolated a blue Cu protein known as hemocuprein (29). As yet no enzymic activity has been attributed to hemocuprein.

Copper, cobalt, manganese and zinc (30) are the trace metals which appear to be essential for the normal life of animal cells. The Cu and Zn

proteins thus far isolated do not account for the total bound concentrations of these metals in the body and we must anticipate further isolation of catalytically active Cu and Zn proteins. As for manganese and cobalt there are no published data available on the form in which they occur though Professor D. Keilin has informed the author in a private communication that manganese occurs in a bound form in mammalian blood. The indispensability of manganese and cobalt coupled with their very low concentration in animal tissues argue some important catalytic role.

An interesting confirmation of the trace substance-enzyme thesis has come from the recent work of Wald (31) on the photosensitive pigments of the retina. He has presented evidence that visual purple is a conjugated protein with vitamin A as prosthetic group. In presence of light, visual purple is resolved into its component parts; in the dark the original conjugated protein is resynthesized. The vitamin A prosthetic group undergoes a photochemical cycle of combination with and dissociation from its specific protein. No doubt there will be some who will protest against the inclusion of visual protein as an enzyme. In recent years the conception of what constitutes catalysis has undergone considerable revision and extension. Any protein which *uniquely* performs some specialized physiological function should earn the title of an enzyme. Visual purple is as specific for photoreception as hemoglobin for oxygen transfer or the amino acid oxidase for oxidation of *d*-amino acids. It would be erecting artificial barriers to distinguish between these different physiological functions on the basis of some arbitrary definition of catalysis.

The antidermatitis vitamin has been shown to be a substituted pyridine compound (32). Its chemical similarity with the pyridine nucleotides is sufficiently close to suggest a role as prosthetic group of some oxidation or allied enzyme. Kuhn made the significant observation that in yeast (33) the vitamin occurs in combination with protein and that the free vitamin is liberated only after heat denaturation of the protein complex.

Substances which cannot be synthesized by an organism and which are essential in the diet are called vitamins. Substances which are synthesized by one organ and which are essential for the maintenance of other organs are called hormones. The analogy runs even deeper. The concentrations at which hormones exert their activity are of the same trace order of magnitude as those of vitamins or enzymes (cf. Tables VIII and IX). We may, therefore, in accordance with the trace substance-enzyme thesis consider hormones either as potential enzymes or as prosthetic groups. There is, in fact, a body of circumstantial evidence in favor of this view, at least in the case of two of the hormones.

Insulin, the active principle of the pancreas, plays a pivotal role in the regulation of carbohydrate metabolism in the animal body. As yet it is not known precisely with which step insulin is concerned, though there are strong indications that insulin regulates the conversion of glucose into glycogen (34, 35). Unless we assume "action at a distance" it follows that insulin is acting catalytically at some stage in the synthesis or breakdown of glycogen. Insulin, being a protein, can hardly be compared to a prosthetic group. However, the possibility that insulin is the essential protein part of some enzyme cannot as yet be excluded. In the cases thus far considered the synthesizing powers of the animal cell have been found deficient with respect to the essential heterocyclic rings of the prosthetic groups but not with respect to the corresponding specific proteins. Insulin may therefore be in the unique position of an enzyme or the essential protein part of an enzyme which can be synthesized only by certain cells of the pancreas. It remains to be determined whether insulin as such is the effective agent in the tissues of the body or whether insulin is only the essential protein part of a larger complex.

Thyroxine, the active principle of the thyroid gland, is necessary for the maintenance of the normal metabolic level of animal cells. In thyroidectomized animals the rates both of heat production and oxidation are depressed (36), whereas these rates in the normal animals are increased when the active principle is fed. Beyond these elementary facts little is known either of the mode of action of thyroxine or of the particular path of metabolism which is controlled by thyroxine. It is significant that thyroxine normally occurs in the thyroid gland in combination with a protein—the so-called thyroglobulin. Insufficient consideration has been given thus far to the possible catalytic properties of thyroglobulin although it can be easily prepared in a high state of purity (37). The iodine content of normal tissues apart from the thyroid is at the trace level of concentration (38). There is evidence that iodine occurs in these tissues principally in combination with protein (36). It would be of considerable interest to know whether the iodine-protein compound of normal tissues is identical with thyroglobulin. At the moment one cannot exclude the possibility that thyroglobulin is merely the storage and not the catalytic bound form of thyroxine.

The specific way the animal body responds to the presence of thyroxine bears close comparison with the relation between the protein moiety of an enzyme and its prosthetic group. For example, substituted thyroxine compounds and degradation products of thyroxine show little or no physiological activity. Furthermore the *d*-isomer of thyroxine shows little

if any activity (39, 40). This chemical and stereochemical specificity is the hallmark of enzymic phenomena. One can therefore with some reason propose the view that thyroxine is the specific prosthetic group of some enzyme or family of enzymes concerned in the normal economy of the animal cell.

No attempt will be made in this article to analyze the long list of known hormones from the standpoint of the trace substance-enzyme thesis. Quite apart from the fact that the author is not competent to discuss hormones authoritatively the available scanty data on the detailed biochemical effects of the great majority of the known hormones will not permit of more than guesswork. It is of interest, however, to note that the hormones occur either as proteins such as the parathyroid and anterior pituitary principles or as molecules of the molecular dimensions of prosthetic groups such as adrenalin, corticosterone, and the sex hormones. The concentrations at which they are effective are uniformly of the trace order of magnitude.

To digress somewhat from the main thesis let us consider some of the difficulties in the way of identifying enzymes and trace substances. Only a minute fraction of the total number of enzymes described has been isolated and characterized chemically. Furthermore, the total number of enzymes described is very likely only a small proportion of the total present in the cell. There is no question, therefore, that the limited repertoire of the enzyme chemist is the principal obstacle to more rapid and extensive correlation. Given, for example, a protein hormone in a high state of purity, two courses would be open for deciding whether it possessed catalytic activity. The first would involve an exhaustive series of tests for proteolytic, hydrolytic, oxidase, carbohydrase, etc., activity. Apart from the vastness of the effort required there would be no assurance that consistently negative results would rule out the possibility of some catalytic function since the tests do not cover the entire gamut of enzyme activity. The second and simpler course would involve comparison of the physical and chemical properties of the protein hormone with those of enzymes which have been isolated and characterized. But the success of this course will depend on the number and variety of enzymes whose isolation has been effected. We may anticipate, therefore, that as more and more enzymes are isolated, so the process of identification will be simplified. Identification has been remarkably successful in the field of enzymic oxidation to a great extent because isolation work has been intensively prosecuted. It would not be surprising, therefore, if a similar degree of success attended isolation work on other types of enzymes.

The two-component structure of oxidation enzymes cannot as yet be considered as characteristic of enzymes in general. Prosthetic groups have been demonstrated in isolated cases of non-oxidative enzymes such as hemoglobin, hemocyanin, glyoxalase, carbonic anhydrase, phosphorylase, carboxylase, and visual purple. But it would be unwise in the present state of our knowledge to venture a decision as to whether the other main groups of enzymes such as lipases, carbohydrases, phosphatases, proteolytic enzymes, etc., contain prosthetic groups. Some of the proteolytic enzymes such as papain are inactive in absence of some "activator" such as glutathione (41, 42). Others of this group are independent of any external additional factor, though this may mean simply that the "activator" is rigidly bound to the protein. However, to equate these "activators" of proteolytic en-

TABLE X

PHARMACOLOGICAL ACTIVITY OF SOME DRUGS

| Drug | Amount in $\gamma$ | Effect |
|---|---|---|
| Eserine[1] | 0.1 | Potentiates action of acetylcholine in frog's heart |
| Atropine[1] | 0.01 | Restores activity of frog's sinus that had been arrested with muscarine |
| Strophantin[1] | 0.01 | Inhibits beat of frog heart |
| Hyoscine[1] | 0.01 | Partially constricts cat's eye |
| Digitoxin[1] | 100 | Arrests systolic beat of frog's heart |

[1] A. J. Clark, "The Mode of Action of Drugs on Cells," Williams & Wilkins Co., Baltimore, 1933.

zymes with prosthetic groups would be premature until more is known of the mode of action of "activators." The necessity for a prosthetic group would appear to depend on the nature of the catalysis. Where some special function such as hydrogen transfer, combination with oxygen photoreception, etc., lies beyond the capacity of the protein, an auxiliary substance in the form of the prosthetic group becomes necessary. But where the function can be completely taken over by the protein, a prosthetic group would be superfluous. The thesis which we have been developing thus far applies whether dealing with a two- or one-component enzyme. The same general considerations and rules apply to both types of enzymes.

To return from the above digression consider how synthetic drugs fit in

TABLE XI

EFFECT OF DRUGS ON ENZYME REACTIONS

| Drug | Concentration ($M$) | Enzyme affected | % Inhibition | Reference |
|---|---|---|---|---|
| Phenyl urethane | $4 \times 10^{-7}$ | Liver esterase | 100 | Stedman and Stedman[1] |
| Eserine | $4 \times 10^{-7}$ | Choline esterase | 100 | Easson and Stedman[2] |
| Phlorizin | $7 \times 10^{-3}$ | Adenosine phosphorylase | 100 | Ostern, *et al.*[3] |
| Benzedrine | $2.2 \times 10^{-3}$ | Amine oxidase | 100 | Mann and Quastel[4] |
| Ephedrine | $2 \times 10^{-2}$ | Amine oxidase | 50 | Blaschko, *et al.*[5] |
| *p*-Aminophenol | $5 \times 10^{-5}$ | Xanthine oxidase | 100 | Bernheim and Bernheim[6] |
| Iodoacetic acid | $5 \times 10^{-4}$ | Triose and triosephosphoric dehydrogenases | 100 | Green, *et al.*[7] |
| HCN | $1 \times 10^{-4}$ | Urico-oxidase, cytochrome oxidase, catalase, peroxidase, polyphenol oxidases | 100 | Keilin[8] |
| *l*-Glyceraldehyde | $2.5 \times 10^{-3}$ | Anaerobic glycolysis of embryonic and tumor tissue | 100 | Needham and Lehman[9] Mendel, *et al.*[10] |
| Sulfanilamide | $2 \times 10^{-6}$ | Carbonic anhydrase | 50 | Mann and Keilin[11] |

[1] E. Stedman and E. Stedman, *Biochem. J.*, **26**, 1214 (1932).
[2] L. H. Easson and E. Stedman, *Ibid.*, **31**, 1723 (1937).
[3] P. Ostern, T. Baranowski, and J. Terszakowéc, *Hoppe-Seyler's Z. physiol. Chemie*, **251**, 258 (1938).
[4] P. J. G. Mann and J. H. Quastel, *Nature*, **144**, 943 (1939).
[5] H. Blaschko, D. Richter, and H. Schlossman, *Biochem. J.*, **31**, 2187 (1937).
[6] F. Bernheim and M. L. C. Bernheim, *J. Biol. Chem.*, **123**, 307 (1938).
[7] D. E. Green, D. M. Needham, and J. G. Dewan, *Biochem. J.*, **31**, 2327 (1937).
[8] D. Keilin and E. F. Hartree, *Proc. Roy. Soc.*, **119**, 114 (1936).
D. Keilin and E. F. Hartree, *Ibid.*, **121**, 173 (1936).
D. Keilin and T. Mann, *Ibid.*, **122**, 119 (1937).
D. Keilin and E. F. Hartree, *Ibid.*, **125**, 171 (1938).
D. Keilin, *Ergeb. Enzymforsch.*, **2**, 239 (1933).
[9] J. Needham and H. Lehmann, *Biochem. J.*, **31**, 1210, 1913 (1937).
[10] B. Mendel, M. Bauch, and F. Strelitz, *Klin. Wochschr.*, **10**, 118 (1931).
[11] T. Mann and D. Keilin, *Nature*, **146**, 164 (1940).

with the trace substance-enzyme thesis. Many of them exert profound pharmacological activity at the trace order of magnitude (cf. Table X), although they are not normal constituents of cells. It would be manifestly absurd to assume that these synthetic substances are potential prosthetic groups of enzymes. However, the effects of many of these drugs can be rationally explained in terms of enzymic phenomena, particularly in terms of specific inhibition of enzymes.

There are three important types of enzyme inhibitors. As an illustration of the first type consider the catalytic oxidation of succinate ($COOH-CH_2CH_2COOH$) to fumarate. The enzyme concerned does not attack any other dicarboxylic acid. In presence of malonate ($COOHCH_2COOH$) the rate of oxidation of succinate is depressed (43, 44). The degree of inhabition for a given concentration of malonate is far and above that of any other dicarboxylic or polycarboxylic acid at the same concentration. This specific inhibition of the succinic enzyme by malonate is explained as follows. The process of oxidation of succinate takes place in two separate phases. First the enzyme combines with the substrate, and then the actual transfer of hydrogen takes place within the enzyme-substrate complex. Substances configurationally related to the substrate can combine with the enzyme but do not undergo further change. They inhibit the oxidation of the substrate by competing with the substrate for the combining or active group of the enzyme. This type of inhibition, known as competitive inhibition, is met with in all types of enzymes.

The second type of inhibition is best illustrated in the effect of cyanide on the catechol or polyphenol oxidase. The prosthetic group of this enzyme is the atom of copper. The catalytic process involves a valency cycle from the cupro to the cupric condition, and *vice versa*. HCN forms a non-dissociable compound with the prosthetic group and thereby immobilizes the enzyme. In this type of inhibition the inhibitor locks the functional part of the prosthetic group, whereas in competitive inhibition the inhibitor locks the functional part of the protein moiety.

The third type of enzyme inhibitor is not as well defined as the other two. There are organic molecules, simple or complex, which specifically inhibit a particular enzyme although they are not configurationally related to the substrate. Apparently this type of inhibitor can at very low dilutions lock the enzyme in the form of a catalytically inactive compound. To explain how these organic molecules are able to immobilize specifically a given enzyme would involve information on the chemical nature of the active groups of enzymes—information which is not always available.

Iodoacetic acid in $5 \times 10^{-4}$ $M$ concentration completely inhibits the activity of the triosephosphoric and triose pyridinoprotein enzymes (45) although without any comparable effect on a large number of other enzymes. The alkaloid, eserine (46), at a concentration as low as $4 \times 10^{-7}$ $M$, completely inhibits the working of choline esterase and this effect is highly specific for choline esterase.

Comparatively little work has been done on the whole in the way of correlating the pharmacological effects of drugs with the effects on enzymes. But in a few outstanding cases such as that of eserine, ephedrine, benzedrine, sulphanilamide and phlorizin (cf. Table XI) some successful results have been obtained. Quastel and Jowett (47) have correlated the inhibitory effects of evipan, luminal, and chloretone on oxidation processes in brain cortex slices with the narcosis induced by these substances. The concentrations at which the enzymic and pharmacological effects are obtained are of the same order of magnitude.

It is conceivable that some of the hormones may exert their effects by specific inhibition of key enzymes. The physiological literature abounds with references to antagonism between hormones as, for example, between adrenaline and insulin in their effects on glycogen storage. Either one or the other hormone can enter into the constitution of the particular enzyme concerned but not both. Thus unless we are dealing with alternative metabolic paths the conception of specific enzymic inhibition must be invoked to explain hormone antagonism.

The fundamental assumption of the trace substance-enzyme thesis is that there is no rational explanation available of how traces of some substance can exert profound biological activity except in terms of enzymic phenomena. Although it is true that no alternative explanation can be put forward, it does not in consequence follow that the assumption is correct. Our aim, however, has not been to dogmatize but to point out the possibilities for experimentation and correlation which are implicit in the trace substance-enzyme thesis.

## Bibliography

1. E. Negelein and H. Brömel, *Biochem. Z.*, **300**, 255 (1939).
2. O. Warburg and W. Christian, *Ibid.*, **298**, 150 (1938).
3. D. Keilin and E. F. Hartree, *Proc. Roy. Soc.*, **121**, 173 (1936).
4. D. Keilin and E. F. Hartree, *Ibid.*, **119**, 114 (1936).
5. D. Keilin and T. Mann, *Ibid.*, **122**, 119 (1937).

6. H. v. Euler, E. Adler, G. Günther, and N. B. Das, *Hoppe-Seyler's Z. physiol. Chemie*, **254**, 61 (1938).
7. N. B. Das, *Ibid.*, **238**, 269 (1936).
8. E. Negelein and H. J. Wulff, *Biochem. Z.*, **289**, 436 (1937); **293**, 351 (1937).
9. O. Warburg and W. Christian, *Ibid.*, **303**, 40 (1939).
10. O. Warburg and W. Christian, *Ibid.*, **298**, 368 (1938).
11. R. Kuhn and H. Rudy, *Ber.*, **69**, 2557 (1936).
12. F. Kubowitz, *Ibid.*, **299**, 32 (1938).
13. D. Keilin and T. Mann, *Proc. Roy. Soc.*, **125**, 187 (1938).
14. D. E. Green, D. Herbert, and V. Subrahmanyan, *J. Biol. Chem.* **135**, 795 (1940).
15. D. Keilin and T. Mann, *Nature*, **144**, 442 (1939).
16. K. Lohmann, *Biochem. Z.*, **254**, 452 (1932).
17. E. Ball, *J. Biol. Chem.*, **128**, 51 (1938).
18. H. S. Corran, J. G. Dewan, A. H. Gordon, and D. E. Green, *Biochem. J.*, **33**, 1694 (1939).
19. A. H. Gordon, D. E. Green, and V. Subrahmanyan, *Ibid.*, **34**, 764 (1940).
20. M. G. Kritzmann, *Biochimia*, **3**, 614 (1938); *Nature*, **143**, 603 (1939).
21. R. R. Williams and J. K. Cline, *J. Am. Chem. Soc.*, **58**, 1504 (1936).
22. J. K. Cline, R. R. Williams, and J. Finkelstein, *Ibid.*, **59**, 1052 (1937).
23. C. A. Elvehjem, R. J. Madden, F. M. Strong, and D. W. Wooley, *Ibid.*, **59**, 1767 (1937).
24. R. Kuhn, P. György, and T. Wagner-Jauregg, *Ber.*, **66**, 317 (1933); **66**, 1034 (1933).
25. F. B. Straub, *Nature*, **141**, 603 (1938).
26. O. Warburg and W. Christian, *Biochem. Z.*, **295**, 261 (1938); **298**, 150 (1938).
27. F. B. Straub, *Biochem. J.*, **33**, 787 (1939).
28. H. S. Corran, D. E. Green, and F. B. Straub, *Ibid.*, **33**, 793 (1939).
29. T. Mann and D. Keilin, *Proc. Roy. Soc.*, **126**, 303 (1938).
30. A. T. Shohl, "Mineral Metabolism," New York, 1939, pp. 234–247.
31. G. Wald, *J. Gen. Physiol.*, **18**, 905 (1935).
32. R. Kuhn, G. Wendt, and O. Westphal, *Ber.*, **72**, 310 (1939).
33. R. Kuhn and G. Wendt, *Ber.*, **71**, 780 (1938); **71**, 1118 (1938); **71**, 1534 (1938).
34. J. J. R. Campbell and W. R. Macleod, "Insulin," Williams and Wilkins Co., Baltimore, 1925.
35. H. F. Jensen, "Insulin," London, 1938.
36. A. T. Cameron, "Recent Advances in Endocrinology," Philadelphia, 1934.
37. M. Heidelberger and K. O. Pedersen, *J. Gen. Physiol.*, **19**, 95 (1936).
38. A. T. Shohl, "Mineral Metabolism," Rheinhold Publishing Corp., N. Y., 1939, pp. 225–232.
39. G. L. Foster, W. W. Palmer, and J. P. Leland, *J. Biol. Chem.*, **115**, 467 (1936).
40. C. R. Harington, *Biochem. J.*, **22**, 1429 (1928).
41. W. Grassmann, O. v. Schoenebeck, and H. Eibeler, *Hoppe-Seyler's Z. physiol. Chemie*, **194**, 260 (1930).
42. E. Waldschmidt-Leitz, A. Purr, and A. K. Balls, *Ibid.*, **198**, 260 (1931).
43. J. H. Quastel and W. R. Wooldridge, *Biochem. J.*, **22**, 689 (1928).

44. J. H. Quastel and A. H. M. Wheatley, *Ibid.*, **25,** 117 (1931).
45. D. E. Green, D. M. Needham, and J. G. Dewan, *Ibid.*, **31,** 2327 (1937).
46. L. H. Easson and E. Stedman, *Ibid.*, **31,** 1723 (1937).
47. M. Jowett and J. H. Quastel, *Ibid.*, **31,** 565 (1937).
48. G. T. Cori, S. P. Colowick, and C. F. Cori, *J. Biol. Chem.* **123,** 381 (1938).
49. E. Haas, B. L. Horecker, and T. R. Hogness, *Ibid.*, **136,** 747 (1940).

# PHOTOSYNTHESIS, FACTS AND INTERPRETATIONS

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*Chicago, Ill.*

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For decades research in the field of photosynthesis has been revealing

many important facts, but it is astonishing how uncertain the results, and particularly the interpretations of some of the basic experiments, still are.

In this report on the last few years' progress we are restricting ourselves only to a discussion of such problems and their theoretical correlations which, according to our judgment, are basic. We are well aware that our choice of topics cannot be other than subjective.

## I. Quantum Efficiency

One of the most interesting questions in the whole problem of the photochemical reduction of carbon dioxide concerns the efficiency with which the absorbed light energy is used in the plants. This question seemed to have been definitely settled since Warburg and Negelein (1) found, about 19 years ago, that 4.4 quanta of red light were sufficient to liberate one molecule of oxygen with the simultaneous reduction of one molecule of carbon dioxide.

The value of four quanta per molecule of carbon dioxide, in other words, the quantum yield of 0.25 mol. $CO_2$ per quantum, was considered as highly significant. The number four appeared to be in striking agreement with the number of four hydrogen atoms which have to change their places during the conversion of $CO_2$ or $CO(OH)_2$ into $(HCHO)_n$ with the simultaneous decomposition of water. On the other hand, the yield was so close to the theoretical limit given by the laws of thermodynamics, that it was very hard to devise a mechanism of carbon dioxide reduction which would not contradict one or the other experimental fact, and at the same time explain the high efficiency.

We know now that the high quantum efficiency mentioned is only apparent, and that the true efficiency is probably only a third of it, namely, 12 quanta per $CO_2$ molecule reduced. The foundations on which the hypotheses concerning the amazing efficiency and the four-step mechanism rested have disappeared.

With a method different from that of Warburg and Negelein (1), Manning, Stauffer, Duggar, and Daniels (2) (1938) attempted to find out the average efficiency of photosynthesis under conditions approximating those of a natural environment, and soon realized that even the most favorable conditions failed to induce the plants to use less than 12 quanta for the reduction of one molecule of carbon dioxide. These observations have not been readily accepted. There was, for instance, doubt whether the algae had been grown under optimal conditions. At the same time one of the authors of this article (G.) had the opportunity of watching experiments which W. Arnold was carrying out in van Niel's laboratory at Pacific

Grove. Arnold tried to measure the efficiency of photosynthesis by a calorimetric method. Though the experimental device appeared to work well, the quantum yields found always remained smaller than half of that reported by Warburg and Negelein. Because of this discrepancy the method was held inadequate, the work discontinued, and the result never published. This attitude appeared to be the more justified, as it became known that Rieke (3) using the original manometric technique, had reproduced the results of Warburg and Negelein. In Rieke's paper, however, there is a remark that the values for the computed quantum yields varied consistently with the method of computation. One has to conclude, according to Rieke, "either that there is an obscure systematic error in one method of measurement, or that under the conditions of these experiments, photosynthesis and respiration do not follow a simple course." The second explanation has been finally confirmed by Emerson and Lewis (4) who succeeded in definitely showing that the quantum yield measured in the classical way has no well-defined meaning.

It was known that in order to obtain the "best" quantum yields with Chlorella, the rapidly growing algae had to be adapted to low light intensities. They had to be "trained" to use the light energy economically. Warburg and Negelein had discovered that without the special treatment, the efficiency of their plants was much smaller. Because, for obvious reasons, only the smallest number of quanta which suffice to decompose carbon dioxide in a plant is of theoretical importance, they, as well as later students, used the "trained" algae exclusively. Using the same culture methods, Emerson and Lewis duplicated the measurements in question. But they succeeded in improving the method of "training," and brought the number of quanta necessary to reduce carbon dioxide down to three. This was hard to believe as such an efficiency would go beyond the thermodynamical limits mentioned above. A closer examination of the gas exchange of the algal suspension in the manometers revealed a sudden outburst of considerable amounts of gas, later identified as carbon dioxide, during the first minutes of illumination (cf. Fig. 1). Now a basic assumption in computing such manometric measurements of photosynthesis of algae suspended in acid phosphate solutions has been the constancy of the assimilatory quotient. It was believed that the ratio of $CO_2$ absorbed/$O_2$ liberated, never deviated much from the value of unity. The quick changes in the nature and the rate of the gas exchange revealed by the curve of Fig. 1 make it clear that it is not possible to compute true quantum yields from measurements of this type. This surprising initial evolution of carbon dioxide immediately after the start of illumination depends on the com-

position of the culture medium in which the algae had grown, and mainly on its concentration of micro-elements. Hence the possibility of "training" the plants for apparently still higher quantum yields.

In recent years more cases of an abnormal assimilatory quotient have been found, particularly during so-called induction periods (see chapter: "Long Induction Periods"). It is clear that any attempt to measure the quantum efficiency of photosynthesis must either avoid such complications or include precise analyses of the gas exchange of the plants.

As soon as it was established that the quantum number of four in photosynthesis was not real, the question arose as to what the efficiency in the re-

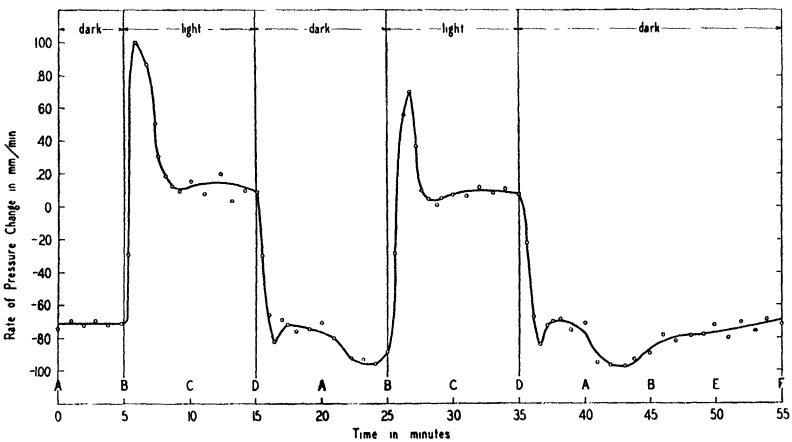



Fig. 1.—Photosynthesis in *Chlorella pyrenoidosa*. Deviations from steady rate of pressure change in successive periods of light and darkness. (According to Emerson and Lewis.)

duction of carbon dioxide truly was. Emerson and Lewis (5) and independently Rieke (6) have made further measurements, avoiding the newly discovered sources of error by either suspending the algae in carbonate buffer mixtures, or by using longer light periods and thinner suspensions. There is no need to go into the details of the method at present. With *Chlorella pyrenoidosa*, Emerson and Lewis found an average of 10 to 11 quanta per $CO_2$ molecule. Rieke arrived consistently at an average of 12 quanta for Chlorella as well as for Scenedesmus, the single measurements scattering from 11 to 14 quanta. A survey, made by Emerson and Lewis, of the efficiency in about twelve different algae and a higher plant, revealed

no significant deviation from the behavior of Chlorella. All authors agree that as long as young, healthy cultures are used, no great importance is to be attached to the composition of the medium nor to the intensity at which the algae have been grown. Therefore, we may for the moment say that the natural efficiency of plants using visible light for the reduction of carbon dioxide is about 12 quanta per molecule of $CO_2$, in agreement with the lowest numbers obtained by Manning, *et al.*, in the paper mentioned above, and the average number obtained with a calorimetric method in a more recent publication by Magee, DeWitt, Smith, and Daniels (7).

Yet there is still one discordant note. Just when the students in this country, who had been working for years on the efficiency of photosynthesis, had agreed on a value of about 12 quanta, Eichhoff in Noddack's laboratory using Warburg's method, again found (8) four quanta per $CO_2$ reduced. He did not use acid suspension media, but carbonate buffer mixtures, and illuminated the algae 15 minutes before measuring. Hence the phenomena discovered by Emerson and Lewis can scarcely have made pressure changes big enough to account for his figures. Eichhoff cultivated the algae in a different and more primitive way. Repeating his culture method, Emerson as well as Rieke found either no difference from their earlier results, or yields which were only smaller.

The chapter on quantum yields is by no means closed, however, if we consider photosynthesis under anaerobic conditions without the evolution of oxygen in purple bacteria and in green algae (see Chapters V and VI). French (9), measuring the efficiency of carbon dioxide reduction with hydrogen in Athiorhodacea found a maximum of five to eight quanta, depending on the method of computation. These organisms are particularly suitable for such measurements as their dark metabolism appears to be negligibly small.

Thiorhodaceae, red sulfur bacteria, have been studied by Eymers and Wassink (10) (1938). Using young cultures of a purple sulfur bacterium, grown under optimal conditions, they found for each molecule of carbon dioxide taken up by the bacteria, 12 to 18 quanta of infrared light absorbed. At the same time French (11) had casually reported that *Spirillum rubrum*, a purple bacteria, absorbed 15 quanta per molecule $CO_2$ reduced. These two strains of purple bacteria have a very strong dark metabolism which leads to the formation of carbon dioxide and of substances serving again as hydrogen donors in the light (Gaffron (12) (1934, 1935), Roelefson (13) (1934)). It is unknown to what degree this will cause a continuous internal cycle of $CO_2$ reduction and formation while the bacteria are illuminated. The numbers of quanta measured by Eymers and

Wassink indicate, therefore, the apparent yield only, the true yield possibly being much higher.

This is an interesting situation because lately it has been found that the assimilatory system of the green plants is sometimes capable of reactions which have been thought to be restricted to the purple bacteria—namely, carbon dioxide reduction with hydrogen donors (see Chapter VI). What has just been said about the metabolism of the Thiorhodaceae can be applied here: the internal carbon dioxide cycle which uses up part of the absorbed light energy may also lower the measurable quantum yield in green algae. This is certainly true in the "reduced state" of the assimilatory mechanism, if the hydrogen donors are carbohydrates. To what an extent the internal carbon dioxide cycle exists under aerobic conditions is an open question.

## II. Saturation Phenomena

### *1. Saturation in Continuous Light*

It has long been known that the rate of photosynthesis is directly proportional to the intensity of incident radiation, as long as the latter is not very strong. But if we increase the intensity beyond a value of 10 to 20 thousand meter candles, the linearity of the curve representing the relation between rate of carbon dioxide reduction and light intensity disappears. The curve begins to bend toward the abscissa and becomes finally parallel to it. The rate of photosynthesis has become independent of changes in the light intensity. The plant is saturated with light, though of course the chlorophyll itself will continue to absorb more and more light quanta if we continue to increase the incident radiation above the amount necessary to "saturate" photosynthesis.

This "light saturation" in continuous illumination caused by high intensities is a general feature of photosynthesis irrespective of the plant material. The absolute value of the light intensity expressed in meter candles, for instance, necessary to produce saturation varies, however, very much among different plants. The favorite objects of modern studies in photosynthesis, the unicellular green algae (Chlorella, Stichcoccus, Scenedesmus, etc.), are saturated with light at intensities between 20,000 and 40,000 lux, whereas wheat and other higher plants sometimes need 100,000 lux to approach the point of light saturation. An interesting fact is that under different conditions the same plants may require different intensities to reach light saturation, a point which we shall discuss later.

Since the work of Willstätter and Stoll it has been generally recognized

that not only chlorophyll (14) which has absorbed a certain amount of light energy but also enzymatic factors play a part in the reduction of carbon dioxide. The investigations of Blackman (15) and of Warburg (16) have led to the distinction between light and dark reactions in photosynthesis. From photochemistry we know that the rate of reactions proceeding under the influence of light quanta absorbed by the reacting molecules is practically insensitive to changes in temperature, because these changes will not alter the high excitation energy of the molecules to any appreciable amount. On the other hand, the reactions in living cells have more or less the same temperature coefficient as similar chemical reactions *in vitro*. With rising temperature the metabolism is accelerated. In photosynthesis as long as its rate is determined by the light intensity alone, we find practically no influence of temperature. Approaching light saturation, however, the effect of temperature becomes more pronounced and under the condition of light saturation as defined above the rate of carbon dioxide reduction is influenced by the temperature to the same extent as the general metabolism of the plant. Consequently Warburg assumed that at low light intensities it is a purely photochemical reaction which limits the rate of process as a whole, while at light saturation an ordinary chemical (enzymatic) or dark reaction determines its rate. In other words, light saturation has been interpreted as being caused by the limited capacities of the dark or "Blackman" reaction to respond to the conditions brought about by the absorption of light energy.

It is clear that in studying the influence of various factors on the rate of photosynthesis at very low as well as very high light intensities one can decide whether these factors influence the photochemical or the "Blackman" reaction or both. By this method it was established that narcotics would interfere principally with the photochemical reaction and only to a lesser degree with the "Blackman" reaction. The effect of narcotics seems to be due mainly to their being adsorbed to interfaces in the cell. Consequently their action on the photochemical part of photosynthesis has been interpreted as an interruption of the energy transfer (either as excitation energy or in the form of a hydrogen atom) from the light-absorbing constituent to another part of the assimilatory mechanism. This explanation appears to be generally accepted. No other one has been proposed nor have facts been discovered which would call for a different one.

The narcotics have been termed unspecific poisons to distinguish them from those the action of which consists in a chemical attack on a specific component of a metabolic system. The best known poison of this latter kind is hydrogen cyanide. It has an affinity for heavy metals which in

combination with organic compounds serve as metabolic catalysts in living cells. An example is the action of cyanide on cell respiration by combining with the iron atom of one of the iron-porphyrin catalysts involved in the uptake and transfer of molecular oxygen.

Warburg (16) found that the Blackman reaction is extremely sensitive to cyanide; so it is $H_2S$, hydroxylamine, and azide, all specific poisons known to have an affinity toward iron-porphyrin catalysts.

Pratt and Trelease (17) have shown that heavy water retards the photosynthesis of algae suspended in a salt medium made up with $D_2O$. Only the dark reaction is affected. We cannot say as yet whether this influence is a specific one due to replacement of the normal substrate $H_2O$ by $D_2O$, or a non-specific one, similar to the inhibitions by heavy water observed in other catalytic reactions.

Higher concentrations of carbon dioxide have long been known to poison the plant metabolism and to inhibit photosynthesis. A recent study by Livingston and Franck (18) indicates that the depressing influence of large excesses of carbon dioxide is mainly connected with the dark reactions. Lack of $CO_2$ diminishes the yield of photosynthesis at low as well as at high light intensities.

### *2. The Blackman Period*

Another experiment supporting the assumption that the process of photosynthesis consists of light and dark reactions has been Warburg's (16) observation on the effect of intermittent light. A certain amount of light of high intensity can reduce more carbon dioxide when given in a succession of short flashes than in continuous illumination. The interpretation is that during the pauses in the illumination the dark reaction will find time to act on the photoproducts piled up during the short illumination periods. The utilization of light is the better the more completed the dark reaction is during the pauses. Warburg used a rotating disk in which windows are cut out of the same width as the solid remaining parts. The total amount of light transmitted by the disk is then reduced to half, regardless of the frequency of rotation. On increasing the frequency of the rotation Warburg found that the yield of photosynthesis finally increased from one-half of that found in continuous light to the full amount; in other words, the effect of the light absorbed increased a hundred per cent. It is obvious that with equal light and dark periods no greater gain is possible (Wohl (19)). Emerson and Arnold (20) later used unequal light and dark periods. They made the light flashes extremely short, $10^{-5}$ sec., the dark periods of variable length. Thus it was possible to separate light and

dark reactions completely. If in such an experimental set-up the time spaces between flashes are made sufficiently long, all dark reactions may have ample time to run to completion. The light quanta of the next flash will be used with optimal efficiency so far as the general conditions of the plant permit. Using light flashes of such short duration in combination with the sufficiently long dark intervals the yield of carbon dioxide reduced for a given amount of light can be improved several hundred per cent as compared with light of the same intensity applied uninterruptedly. It is useful, however, to keep in mind that the mean intensity for the unit of time is, of course, much lower with intermittent than with continuous light. In first approximation we may expect the same results with strong flashing light as with continuous light of low intensity.

By gradually shortening the long dark intervals between flashes, until the yield per flash begins to decrease, it is possible to obtain an approximate value for the length of time required for the completion of the dark reaction. This "Blackman" period was found by Emerson and Arnold to be 0.02 sec. at 25°. The fact that the period turned out to be so short was very surprising as will be discussed in the chapter, "Chemical Kinetics of Photosynthesis." One expected a period of about 2000 times longer (Gaffron and Wohl 24).

Another result, important for any theoretical interpretation, is the kind of influence, observed by Emerson and Arnold (20), that poisoning with cyanide and low temperature had on the "Blackman" period. The period becomes longer, but the yield of photosynthesis per flash remains unaltered provided the dark time between flashes is enlarged enough to allow the dark reaction to run to completion. The lengthening of the period again shows that the velocity of the dark reaction determines the saturation rate, since, as was discussed above, cyanide and changes in temperature greatly influence the saturation rates, but not the rates at low intensities.

The last result can be interpreted in two different ways. The first is as follows: During the light flash a certain amount of a substrate is made which has to be transformed *in toto* by the catalyst before the photosynthetic apparatus is ready to repeat quantitatively the same performance. The material produced is so stable that the molecules remain unaltered until they make their encounter with an active catalyst-molecule. The second interpretation assumes that the catalytic reaction, sensitive to the inhibitors, precedes the flash. In that case a preparatory dark reaction has to be completed to produce the total efficiency of the photosynthetic apparatus. Since the flashes and dark pauses follow one another peri-

odically both assumptions are indiscernible by the method described. It is possible, on the other hand, to learn more about the catalytic processes by studying the shape of the curve in which the yield per flash is plotted as a function of the time of the dark pauses. The shape of the curves varies in a characteristic manner with the order of the catalytic reaction. Arnold (21) found that under normal conditions a reaction of the first order fits the result best. On the other hand, the experiments published by Emerson and Arnold are not sufficient to tell whether that reaction order is still maintained if, when using low temperature or adding cyanide, the "Blackman" period is lengthened. Weller and Franck (22) have therefore recently extended Emerson and Arnold's experiments. The results are in good agreement with the earlier ones, but it was found that the shape of the curves is entirely altered in the case of the inhibited reactions. The curves measured under conditions in which cyanide is used as an inhibitor strongly support the view that, in the presence of cyanide and with small time intervals between flashes, the reaction is of zero order but goes over to first order when the dark pauses become great and the yield per flash approaches that without cyanide. Curves measured at low temperature look different. They seem to correspond to a transition stage between first and zero order reactions. The implication of these results will be more fully discussed in the chapter, "Chemical Kinetics of Photosynthesis."

Finally it may be mentioned that the yield per light flash depends upon the partial pressure of carbon dioxide just as the saturation values in continuous light do. A prolongation of the time distance between flashes does not improve the yield if $CO_2$ is limiting. The most plausible interpretation is the assumption that $CO_2$ is bound to an acceptor molecule by a reversible reaction so that the equilibrium concentration of the bound $CO_2$ depends upon its partial pressure.

Since the saturation rate of photosynthesis is determined by the limitations which the dark reactions impose, it is of interest to see whether there are also limitations on the efficiency of the photochemical part of photosynthesis. The best method would be to study the photosynthetic output produced by a single flash as a function of its light intensity. But since Emerson and Arnold proved that the yield per flash becomes independent of preceding or following light flashes, provided the time lapse is made long enough, it is of course possible to use a long series of flashes. Then by measuring the $O_2$ production as a function of the intensity of the flashes one can calculate the relation between yield per flash and its intensity. This was done by Emerson and Arnold (20) and Arnold and Kohn (23) with the result that an upper limit was found which was astonishingly low.

The maximum number of $CO_2$ molecules which could be reduced by a single light flash was about 2000 times smaller than the number of chlorophyll molecules present. This number varied with the plant material, but in general this so-called flash saturation value does not show greater differences than the saturation rate of different plants in continuous light.

### 3. *Chemical Kinetics of Photosynthesis*

The results of the measurements of saturation curves and the data obtained in the flashing light experiments have shown beyond any doubt that the light saturation is produced by one or another catalytic dark reaction which limits the rate of photosynthesis. But it has already been indicated above that difficulties arise as soon as one wishes to correlate quantitatively the saturation rate of photosynthesis with the fact that the limiting dark reaction is completed in 0.02 second (24). Knowing that 10–12 quanta are used to reduce one carbon dioxide molecule, one can calculate from the total number of quanta absorbed how quickly the photochemical reaction would proceed without limitations by dark reactions, and one can compare that reaction velocity with that of the dark reaction. Using the general principle that a whole reaction cannot proceed faster than its slowest partial reaction, one has to expect that both velocities would be equal at half the maximum rate. The photochemical reaction calculated that way is indeed ~6000 times slower than the dark reaction. The two ways to avoid the difficulties are obvious. One has to suppose that there is a special mechanism in a plant by which the photochemical reaction can run 6000 times faster than calculated above, or one has to assume that by back reactions the maximum efficiency of the limiting dark reaction is reduced to 1/6000 of the amount calculated from the time used for its completion. The first assumption is known under the name, "Hypothesis of the Photosynthetic Unit."

**Theories Using the Hypothesis of the Photosynthetic Unit.**—The calculation used in the previous paragraph to evaluate the velocity of the photochemical part of photosynthesis (unlimited by dark reactions) tacitly assumed that each chlorophyll molecule absorbs its quanta independently from the others and transfers its excitation energy individually to a carbon dioxide molecule or to a partially reduced derivative of $CO_2$. The theories based on the hypothesis of a photosynthetic unit abandon the last-mentioned supposition, replacing it by the assumption that several thousands of chlorophyll molecules form a cooperative unit (20, 24) in delivering the

light energy absorbed by any of them to a single $CO_2$ molecule or to a partially reduced derivative.

Such cooperation makes the photochemical reduction of a $CO_2$ molecule proceed several thousand times faster, since the energy needed to reduce a single $CO_2$ molecule is absorbed by a unit of thousands of chlorophyll molecules instead of by a single one. At the same time it eliminates the difficulty which the interpretation of the low value of the flash saturation offered. If the total concentration of $CO_2$ connected at any given moment with the photosynthetic apparatus can never be greater than 1/6000 of the chlorophyll concentration, it is understandable that no more molecules than just that amount can be reduced by a single light flash (20). The picture of the unit gives also a plausible interpretation of the lack of longer induction periods (24). A detailed discussion can be found in the chapter devoted to the induction periods.

The theories falling under the heading of the unit differ in their assumptions as to how the cooperation between the chlorophyll molecules is accomplished. Gaffron and Wohl (24) discussed the possibility that several thousand chlorophyll molecules are bound together in a form of a one-dimensional crystal to which carbon dioxide molecules are attached, one to each end. The energy absorbed at any place in that row of chlorophyll molecules will migrate to the carbon dioxide throughout the crystal. Such an energy migration known in the literature of physics as exciton migration (25) has recently been observed by Scheibe and co-workers (26). If an exciton travels fast enough through a row of several thousand molecules so that fluorescence losses during the journey are negligible, then the molecules must show a strong resonance coupling. The resonance coupling influences the spectrum considerably as compared with the spectrum of the molecules in a non-coupled state existing in a molecularly dispersed solution. The changes demanded by the theory are actually found in Scheibe's polymerized dyes, but they are absent in the chlorophyll in plants. Furthermore, since the fluorescence in plants is only very weak, Franck and Teller (27) conclude that the crystal picture of the unit is very improbable.*

Wohl (29) proposed as an alternative another model for the unit. The chlorophyll molecules are not coupled together, but each excited chlorophyll molecule transfers its energy to other kinds of molecules which act as energy carriers. The carriers transport the energy to the reduction centers which contain a $CO_2$ molecule. Each impact of an energy carrier with a re-

* The fluorescence yield observed in plants is only of the order of one thousandth. (Vermeulen, Wassink, and Reman (28)).

duction center results in a transfer of energy.* The number of these centers is several thousand times smaller than the number of chlorophyll molecules. While this picture is certainly not an impossible one it attributes to the carrier molecules unusual properties. Wohl's special picture in which the energy-carrying molecules are regarded as molecules in a metastable state of the electronic system is difficult to reconcile with experiences in physics about impacts of the second kind and metastable states. It would therefore be better to replace it by the assumption that the carriers transport the energy as chemical energy. For instance, one could assume that the carrier molecules are transformed in a tautomer by taking over the excitation energy. An impact with the reduction center may then catalyse the back reaction into the original compound and in this way liberate the energy stored up as potential chemical energy in the tautomer.

Ornstein, Wassink, Reman, and Vermeulen (30) recently changed and improved the picture of the unit. They combined Wohl's hypothesis of the small number of reduction centers connected with $CO_2$ with ideas recently emphasized by van Niel.† The main features of Ornstein and coworkers' theory are these. Chlorophyll molecules absorb light quanta and use the excitation energy to sensitize chemical processes in molecules of a substance present in abundance. These molecules are dissociated into one part which acts as a hydrogen donor and another one which has the character of a radical of a peroxide. The hydrogen donors migrate to the reduction centers and reduce $CO_2$, while pairs of peroxide radicals combine and produce the molecular oxygen. If the hydrogen donors find no reduction centers connected with matter to be reduced but only such centers choked by already reduced matter, they recombine with peroxide radicals and are in that way eliminated. The saturation rate of photosynthesis is given by the rate with which the reduced matter is catalytically removed from the centers. From the point of view of kinetics this theory is unobjectionable. But there are experimental reasons which seem to show that theories making use of the basic hypothesis of the unit are not in agreement with reality. For these experiments indicate that the number of $CO_2$ molecules

* The energy will be used for the reduction processes if the center is connected with $CO_2$ or intermediates, but the energy goes over into heat if the centers are connected with the photochemical end-product of photosynthesis.

† Photosynthesis is, according to van Niel, not a direct photochemical reduction of $CO_2$, but rather the photochemical production of hydrogen donors, which in their turn reduce $CO_2$ in dark reactions. This assumption would make photosynthesis a special case not differing in principle from other kinds of reduction syntheses observed in living matter. See the chapter, "Metabolism of Purple Bacteria and van Niel's Theory."

connected with the photosynthetic apparatus is comparable to the number of chlorophyll molecules. In this respect we mention the following groups of observations: Experiments in which the fluorescence of green plants is studied as a function of the light intensity and as a function of $CO_2$ concentration, which will be discussed in the chapter on steady fluorescence; the phenomenon of the so-called pick-up of $CO_2$ in the dark which is described in the chapter on induction periods; to this group finally belong experiments on plant metabolism and photosynthesis with carbon dioxide containing radioactive carbon, Ruben, Hassid, and Kamen (31). Since these basic experiments furnish the most direct evidence, they may be briefly discussed here. The molecules of the labeled $CO_2$ react reversibly with acceptor molecules in the plant, apparently a carboxylation reaction. The acceptor molecules have the molecular weight of about 1000, but are not chlorophyll molecules. The carboxylation process is assumed to be a catalytic one since the reaction is sensitive to cyanide.

It becomes necessary, therefore, to see whether theories making no use of the unit are able to give a satisfactory account of the facts.

**Theories Explaining the Saturation Phenomena by Back Reactions.**—Franck and Herzfeld (32) in a paper called "An Attempted Theory of Photosynthesis" introduced the idea that losses produced by way of a reversal of the photochemical reactions were responsible for the saturation phenomena. While they succeeded in getting correct values for saturation in continuous light and for the observations made in flashing light, they had to introduce several assumptions made entirely *ad hoc* to get these results. Wohl (29) criticized this theory, and a part of his criticism is justified. It seems, however, superfluous to discuss the merits of, and the objections to, that theory, since the authors themselves abandoned the special picture, replacing it by a new one (33) in which the idea of the back reactions is retained and in which use is made of new observations.

The main idea of the theory is the following. Saturation in continuous light under normal conditions is caused by the limited effectiveness of a catalytic reaction. The substrate on which the catalyst works are freshly formed photochemical products. These products are unstable; they have a natural lifetime, small compared to the working period of a catalyst-molecule and long compared to the time needed for the freshly formed products and the catalyst-molecules to diffuse together.

The term "working period" indicates that there is an average time lapse during which a catalytic molecule that reacted once with its substrate is unable to repeat that performance. At low intensities the amount of catalyst present is sufficient to handle the total production of freshly

formed photoproducts; they will be stabilized without loss, and can wait until the next reaction, if such is needed, transforms them further. At saturation intensities most of the freshly formed products will suffer a back reaction due to the fact that the catalyst is limiting. The Blackman period observed by Emerson and Arnold under normal conditions of temperature, etc., is supposed to be the working period of the limiting catalyst. Since no freshly formed photoproduct survives a whole working period, the maximum number of $CO_2$ molecules reduced by a single flash is equal to the number of catalyst-molecules present divided by the number of photochemical steps necessary to reduce a molecule. In the following we will designate this catalyst by the letter "B." To explain the saturation in continuous and in flashing light, it is enough to introduce the assumption that the catalyst B limits only one of the photochemical steps. But one must also take into account that no induction period of appreciable length is observed when an illumination with weak light immediately follows an exposure to saturation intensities. The conclusion is obvious—the distribution of intermediates is identical in weak and in strong light. That can only be accomplished if the back reactions occurring at high light intensities will not change the distribution of intermediates. The back reactions, therefore, have to take place with the same probability at each step. Hence one is forced to assume that the same catalyst is used to stabilize all the different photoproducts. That again makes it probable that the type of photochemical reactions is the same in each step. Fortunately it is quite plausible to assume that the steps are similar to one another. One can describe them as sensitized reactions taking place in a molecular complex which contains a chlorophyll molecule and consisting in a shift of a hydrogen atom from one bond to another. It is a very great help for every theory of this kind that one is no longer restricted to the small number of four photochemical steps which previously was believed to suffice for the reduction of a carbon dioxide molecule. Franck and Herzfeld's theory makes use of this fact in assuming an eight step reaction leaving it as an open question whether the quantum yield of $^1/_{10}$ to $^1/_{12}$, recently found, will make it necessary to change this particular picture.

Four reactions are supposed to belong to the type in which a molecule contained in the complex at the chlorophyll acts as a hydrogen donor and four others are of the type wherein the dehydrogenated donor gets the hydrogen atom back indirectly from the water. The four last-mentioned reactions produce peroxide radicals which act as the source of the oxygen. One immediately sees a close connection to Willstätter's ideas, to van Niel's, and others. In Franck and Herzfeld's theory, the often-discussed

possibility is also studied—that the molecules acting as hydrogen donors are the chlorophyll molecules themselves. Some facts concerning the general behavior of chlorophyll in photochemical reactions *in vitro* collected in a paper of Franck and Livingston (34) speak in favor of that hypothesis. Firstly, it was possible to describe a great number of photo-oxidation processes sensitized by chlorophyll, and the related phenomena of fluorescence, phosphorescence, and reversible bleaching by the hypothesis that an excited chlorophyll molecule can act as a hydrogen donor*, and, secondly, there are strong indications that the dehydrogenated chlorophyll (monodehydrochlorophyll) has (at least in the visible region) the same absorption spectrum as the normal chlorophyll. The bond affected will not then belong to the main system of conjugated double bonds responsible for the color of chlorophyll. To show the sequence of the photochemical steps and catalytic dark reactions postulated by this theory, one can write down some model equations in which the chlorophyll molecules may just as well be denoted as hydrogen donors. It must be emphasized, though, that the identity of the specific compounds mentioned below is not essential for the general theory.

Four hydrogen atoms have to be given to the $CO_2$-acceptor-complex to reduce it and, therefore, four reactions of the following type occur.

1.
$$\text{HChph} - CO_2 \text{ complex} + h\nu. \rightarrow \text{Chph} - \text{Intermed.}^{i_1}$$
quickly followed by:
$$\text{Chph} - \text{Intermed.}^{i_1} + \text{Cat. B} \rightarrow \text{Chph Intermed.}^{s_1} + \text{Cat. B}$$
The intermediates not stabilized by the catalyst give back reactions
$$\text{Chph} - \text{Intermed.}^{i_1} \rightarrow \text{HChph} + CO_2 + \text{acceptor}$$

and correspondingly reactions 2, 3, and 4 take place according to:

2, 3, 4
$$\text{HChph} - \text{Intermed.}^{s_n} + h\nu. \rightarrow \text{Chph Intermed.}^{i_{(n+1)}}$$
followed by:
$$\text{Chph Intermed.}^{i_{(n+1)}} + \text{Cat. B} \rightarrow \text{Chph Intermed.}^{s_{(n+1)}} + \text{Cat. B}$$
or back reactions:
$$\text{Chph Intermed.}^{i_{(n+1)}} \rightarrow \text{HChph} + \text{Intermed.}^{s_n}$$

The symbol HChph represents the normal chlorophyll, while Chph is the symbol for monodehydrochlorophyll. Intermed.$^{i_n}$ means an unstable intermediate and intermed.$^{s_n}$ a stable one.

Four quanta are necessary to restore the hydrogen to four monodehydrochlorophylls, and one gets reactions of the type

* The ability to act as a hydrogen donor is exhibited by other dyestuffs as well as by chlorophyll; if it turns out, as Manning's (35) experiments seem to indicate, that other plant pigments also can sensitize photosynthesis, this fact may be of importance.

$$\text{Chph} + \text{ROH} + h\nu. \rightarrow \text{HChph} - \text{RO}$$
$$\text{HChph} - \text{RO} + \text{Cat. B} \rightarrow \text{HChph} + \text{RO} + \text{Cat. B}$$
$$2\text{RO} + \text{H}_2\text{O} \rightarrow \text{ROOH} + \text{ROH}$$
$$\text{ROOH} + \text{Cat. C} \rightarrow \text{ROH} + {}^1/_2\text{O}_2 + \text{Cat. C}$$

We now have to discuss how this theory accounts for the prolongation of the Blackman period by inhibitors, and the corresponding influence on the saturation rate of photosynthesis. In doing so we will use the influence of cyanide as an example. An inhibitor like cyanide influences a catalytic reaction by diminishing the number of active catalyst molecules available. But the catalyst B cannot be the one which is sensitive to cyanide, since the maximum amount of $CO_2$ molecules reduced by a single light flash is not changed by the addition of this poison. On the other hand, as stated above, the maximum amount of $CO_2$ reduced per flash is a measure of the number of catalyst B molecules present. The conclusion becomes evident that a sufficient dose of cyanide reduces the number of another catalyst (which may be called catalyst A) to such an extent that now the dark reaction into which that catalyst A enters becomes limiting. In continuous light, therefore, the saturation value will be reduced as soon as the limitation of the action of catalyst A by cyanide becomes noticeable. The sensitiveness of photosynthesis in a given plant to cyanide will depend entirely upon the relative abundance of the molecules of catalyst A and catalyst B. If catalyst A is very far from limiting at the normal saturation rate, the number of its active molecules can be reduced considerably by cyanide without affecting the rate. On the other hand, the sensitivity to cyanide will become great if the surplus of catalyst A is small under normal conditions. In this way it is easy to interpret the strange facts that photosynthesis in closely related plants like the algae Chlorella and Scenedesmus show quite different sensitivity to cyanide.

In the experiments with flashing light in the presence of cyanide, the limitation imposed by catalyst B remains responsible for the occurrence of an upper limit of $CO_2$ reduced per light flash. Lack of a sufficient amount of catalyst A will, on the other hand, be responsible for the prolongation of the Blackman period and for the changes in the shape of the curve representing yield per flash *versus* dark time. The shape of the curve as observed by Weller and Franck indicates a reaction of the zero order at small time intervals between flashes and a transition to a first order reaction when the time intervals are large and the difference between yields per flash in the poisoned and non-poisoned cases gradually vanishes. We are to expect just these reaction orders if catalyst B determines the total amount which can be reduced while catalyst A, since it operates on a stable substrate, can make use of many consecutive working periods in the large dark pause

until the whole amount of substrate put at its disposal by the action of the light flash is worked up.

The results of the experiments in which the radioactive carbon dioxide was used as an indicator make it very probable that catalyst A is the one that has to perform the carboxylation reaction between the acceptor molecule and $CO_2$. That conclusion is supported by the fact that the pick-up—*i. e.*, the continuation of the uptake of $CO_2$ for a short time after the light is shut off—takes a longer time (Aufdemgarten (36), McAlister (37)) if photosynthesis is inhibited by cyanide. Finally, some observations on fluorescence, which are in accordance with that assumption, will be discussed in the next chapter.

### *4. Steady Fluorescence of Green Plants*

In the last few years most of the papers discussing fluorescence of chlorophyll in living leaves and algae are devoted to the anomalies of the fluorescence in the beginning of an illumination period. These phenomena will be discussed later in connection with the chapter, "Induction Periods." Measurements of the steady value of the fluorescence, which is reached after an illumination of several minutes, however, are apt to indicate whether any relation exists between the strength of the fluorescence and the steady rate of photosynthesis. If experience about the relation between photochemistry and fluorescence made with very simple molecules could be applied directly to complicated molecules, one would expect that a molecular complex would not fluoresce at all if it contains the light-absorbing group as well as that which accepts the energy for chemical purposes. But as Franck and Herzfeld (73) have shown, there is always a time lapse between the moment of absorption and the use of the energy if the molecules involved are complicated. During that short time part of the excited molecules lose energy, emitting it as fluorescent light. The chlorophyll occupied with photosynthesis in plants under optimal conditions re-emits only about one quantum in a thousand as fluorescent light (28). That amount is negligible in the energy budget, but yet the fluorescence is of importance since changes in the fluorescence can be used as an indicator of changes in the kind or the concentration of substances connected with the chlorophyll. As an example we mention that Kautsky (38) and Wassink, Vermeulen, and Katz (28) found that an addition of a narcotic substance like urethane which reduces the rate of photosynthesis raises the fluorescent yield. The narcotic is supposed to cover the surface of the chlorophyll and to hinder its contact with the substances which normally act as the energy acceptors. But only a small part

of the energy lost for photosynthesis appears as fluorescent light. Most of the excitation energy of the chlorophyll covered with the narcotic is transferred in heat. The question then becomes important as to whether the fluorescent yield changes in the range of intensities where the quantum yield of photosynthesis ceases to be constant. Wassink, Vermeulen, and Katz (28) answered that question in the negative. They found that under normal conditions the fluorescence of the alga, Chlorella, was a linear function of light intensity well into the region of photosynthesis saturation. They concluded therefrom that all theories have to be discarded in which each chlorophyll molecule is supposed to transfer its energy (or a hydrogen atom) directly to $CO_2$, and to its derivatives in different degrees of reduction. Their argument is as follows (30): In the region where the rate of photosynthesis is proportional to light intensity, according to these theories each chlorophyll molecule is connected with substances which are able to take over the excitation energy. If, however, light saturation is reached most of the chlorophyll should be in contact with insensitive photochemical products since a catalytic reaction failed to transform them or replace them quickly enough by sensitive ones. But the last argument is not generally applicable to the class of theories mentioned. It eliminates theories in which not only a direct contact of chlorophyll with the different substances to be reduced is required, but also in which the added hypothesis is contained that the photochemical products which have to be transformed by the catalyst will accumulate if the catalytic reaction becomes limiting. Such an accumulation is, for instance, impossible if, as in Franck and Herzfeld's theory, the insensitive photoproducts are supposed to fall back immediately into a sensitive state. The molecules transformed by the catalyst are, of course, also photosensitive.

By using the values for the maximum possible lifetime of these insensitive substances calculated by this theory, one can demonstrate that the amount of these substances connected with chlorophyll remains too small to have an observable influence on the fluorescent yield even if the catalyst B would be entirely inefficient. So far, therefore, the result of Katz, Wassink, etc., will neither contradict nor support a theory like that of Franck and Herzfeld. On the other hand, one must expect that an easily detectable influence on the strength of fluorescence will occur if the dark reaction in which catalyst A enters becomes limiting, since catalyst A acts on a stable substrate which will accumulate (see page 215). For reasons mentioned above, it is assumed that A catalyzes the reaction between $CO_2$ and the acceptor molecule. If a limitation in that reaction occurs, chlorophyll connected with $CO_2$-free acceptor will result. Since the chlorophyll ac-

ceptor complex without $CO_2$ is a photo-insensitive substance, one must expect that the yield of fluorescence will be greater than normal. Indeed Katz and Wassink (28) already observed that Chlorella do not show the linear relation between fluorescence and the exciting light intensity if photosynthesis was strongly inhibited by cyanide. The fluorescence rises rather more quickly than linear or, in other words, the fluorescence yield grows. That is just what is to be expected since the reaction poisoned is that promoted by A.

One must also expect, in the absence of cyanide, a rise in the yield of fluorescence if one uses very strong light far beyond the saturation value. The first group of the model equations on page 214 discloses the reason. The first unstable intermediate will, by back reactions, break up into free $CO_2$ and a free acceptor molecule. These two must be brought together again with the help of catalyst A. The higher the intensity of the light becomes, the more back reactions occur and the more the claims on catalyst A mount. Finally it cannot satisfy the demand, with the result that part of the chlorophyll will be connected with free acceptor molecules and that amount will rise with the light intensity. At what light intensities the corresponding rise of the fluorescent yield will start depends upon the surplus of catalyst A available at the saturation intensity. Franck, French, and Puck (39), in the case of the leaves of Hydrangea, found that this plant material has apparently such a small amount of catalyst A that at normal conditions catalyst A and catalyst B are both responsible for saturation. The rise of the fluorescent yield starts before saturation is reached and is continuous to intensities about two times higher than the saturation value. At still higher values of irradiation the fluorescent yield becomes constant again. It is apparently at these highest values that practically all the Chph is free from $CO_2$ and intermediates. Addition of cyanide, and low temperature, shift the region of the rise toward lower intensities in accordance with expectations. At both the lowest and the highest light intensities, steady fluorescence is not influenced by either cyanide or by lowering of the temperature, but in the region of medium intensities, steady fluorescence in the inhibited leaves is much stronger than in the normal ones.

McAllister and Myers (37) observed curves of the fluorescence of wheat in which its intensity rises more quickly than linearly if $CO_2$ becomes limiting. The deviation occurs at intensities which are far from sufficient to give saturation in the presence of enough $CO_2$, but great enough to produce saturation under $CO_2$ limitation. This observation is again in accordance with expectations, since the fluorescent yield has to become

greater the moment the acceptor-$CO_2$-complexes are not formed in a sufficient amount. Regardless of what is the cause of that deficiency—lack of $CO_2$ or of catalyst A—a rise in the fluorescent yield will be produced. McAllister and Myers were unable to reach the very high intensities which are necessary for real saturation in young wheat. The high intensities with which they have to work produce a slow gradual fall of the fluorescence which distorts the curves. That effect is most probably caused by photo-oxidation (see chapter, "Photo-oxidation").

Franck, French, and Puck (39) encountered the same difficulty, but they avoided distortions by special procedures. The reason for mentioning that source of error at all lies in the desire to emphasize that the influence of photo-oxidation becomes strong just at those intensities where the limitation of catalyst B occurs. That seems quite natural since in the range of these intensities an increasing amount of chlorophyll will become empty. Finally the deduction has to be mentioned that a plant will not have its normal distribution of intermediates after an illumination which is strong enough to produce much empty chlorophyll. One must expect that a plant illuminated with weak light might, after such strong exposure, show a long induction period. That is indeed the case, but it is doubtful whether lack of intermediates or damage by photo-oxidation is responsible for the occurrence of the prolonged induction periods.

It might be of interest to mention that a sudden addition of $CO_2$ to a plant irradiated under $CO_2$ limitation always produces a brief lowering of the fluorescent intensity. The minimum produced is followed by a fluorescent outburst, a phenomenon which has nothing to do with catalyst A as will be explained in the chapter, "Induction Periods." We interpret the minimum as produced by the transition from the insensitive free acceptor to the photo-sensitive $CO_2$ complex.

### 5. *Liberation of Oxygen by Illuminated Chloroplasts*

Generally photosynthesis stops completely when the structure of the living plant cell is hurt. It has been known for quite some time, however, that isolated chloroplasts may deliver detectable amounts of oxygen upon illumination. In a short communication which came to our notice, while this article was being written, Hill and Scarisbrick report that illuminated chloroplasts can be induced to liberate oxygen continuously at an appreciable rate. To this end it is necessary to suspend the chloroplasts in a solution containing $M/2$ sucrose, $M/15$ phosphate buffer, $M/500$ ferric oxalate, and $M/10$ potassium ferric cyanide. Such a suspension of chloroplasts will reduce ferric oxalate photochemically with the simultaneous liberation

of molecular oxygen. To avoid a reverse reaction between the free oxygen and the newly formed ferrous oxalate, the other iron salt, potassium ferric cyanide, will re-oxidize the oxalate before the latter has a chance to react with oxygen. The over-all reaction thus becomes a reduction of potassium ferric cyanide with the simultaneous liberation of oxygen. This experiment is important because it shows that in the isolated chloroplasts the photochemical mechanism, as well as the oxygen-liberating system, is still intact. Only the connection with the carbon dioxide-acceptor complex has been lost. Replacing the missing natural hydrogen acceptor by ferric oxalate allows the photochemical cycle to continue.

Though in this short communication the authors do not discuss any reaction mechanism, it is quite apparent that their findings are in the best agreement with the experimental results and theoretical considerations presented at various instances in the course of this article.

## III. Induction Periods

### *1. Long Induction Periods*

If one assumes that after a long dark period a plant is free from intermediates of photosynthesis it follows that at the beginning of an illumination period all the intermediates have to be produced step by step. A steady rate of photosynthesis will only be reached after an adaptation time during which such concentrations of all intermediates have been built up that they can now compete with $CO_2$ for a place at the chlorophyll. Since we know that about 12 quanta are necessary to reduce a $CO_2$ molecule we can calculate the time which would be used to reach the steady state for a given strength of illumination. We introduce the assumption (well supported by experiments with radioactive carbon discussed on page 231) that $CO_2$ has to combine with acceptor molecules present in a concentration comparable to that of chlorophyll before it can be reduced photochemically. Once the first intermediate has been formed, it and all following intermediates will stay bound to its own acceptor molecule while the reduction progresses. Since we are interested only in the order of magnitude of the adaptation time, we ascribe to the acceptor the same concentration as that of chlorophyll. In that case a leaf would use about 2 hours to reach the equilibrium when irradiated with so much light that the steady rate of photosynthesis just over-compensates the respiration. During the first hour it should take up practically no carbon dioxide at all. If one irradiates a leaf with a light intensity which produces in the steady

state half of the maximum rate the adaptation time still would last several minutes. But such induction periods are not observed in plants after dark pauses of about 24 hours and they are apparently also absent at the beginning of the very first illumination of algae grown in organic media in the dark. We have the choice, therefore, between assuming that after a long dark period a plant contains intermediates of photosynthesis even when cultivated entirely in the dark, or rejecting the idea that the intermediates have concentrations comparable to that of the chlorophyll.

The hypothesis of the photosynthetic unit takes the point of view last mentioned (24). It stipulates a concentration of $CO_2$ complex and of intermediates which is 6000 times smaller than that of chlorophyll, and correspondingly the time to reach the steady state will be 6000 times shorter than the values given above. Induction periods which are so short are unobservable by the methods used in manometric experiments. Indeed the lack of a long induction period has been one of the fundamental reasons for the introduction of the hypothesis of the unit. Since as explained elsewhere in this article there are theoretical and experimental reasons for rejecting the picture of the unit one has to conclude that intermediates of photosynthesis are always present in a plant. This statement does not imply an unusually great stability of all the intermediates since it is quite probable that a slow back reaction takes place at the photosynthetic apparatus which replaces the losses of intermediates produced by their instability. In the case of plants cultivated in the dark that conclusion seems inevitable. It is, on the other hand, improbable that the dark equilibrium of the intermediates formed entirely by back reactions would produce the same even distribution in concentrations, which we have to stipulate after a steady rate of photosynthesis is reached. One has to expect therefore that after very long dark periods or in algae cultivated entirely in the dark illumination will be accompanied by irregularities in the rate which last longer than those obtained after short dark periods. The initial rate may be higher or lower than the final one, but the gas exchange would not remain negligibly small for a long time as would be the case if there were no intermediates at all present initially. Indeed all kinds of long lived irregularities occur after long dark periods. They may be produced to some extent by the effect under discussion, but also by quite other reasons. It is, therefore, not possible to decide at present whether the last assumption is in agreement with the facts or not. More experiments are desired. Dark pauses up to several hours may not alter the light distribution of intermediates considerably. The quantum yield $^1/_{10}$ to $^1/_{12}$ places such a surplus of energy at the plant's disposal that a

great part of it may be used as heat of activation of the intermediates, and in that case the intermediates will be very stable.

### 2. *Short Induction Periods*

Such long induction periods as one would expect, if the concentrations of intermediates had to be equalized, do not occur after dark pauses shorter than an hour. However there is the well-known short induction period which is already fully developed after a dark pause lasting about a minute (provided that one has the normal conditions with respect to temperature, food supply in the plant, etc.). This induction period has been studied by Osterhout and Haas (40), Warburg (16), van der Paauw (41), Smith (42), and others. New progress in its study was made only recently by the introduction of observation methods which allow recording of quick changes in the rate of photosynthesis.

Blinks and Skow (43) used potential measurements of a mercury electrode to register the $O_2$ development during the first seconds or minutes of photosynthesis and they followed the course of $CO_2$ uptake by measurements with a glass electrode. Their method gives the best resolving power, but it is difficult to get quantitative results. McAlister (37, 44) spectroscopically measures the change of the $CO_2$ content in a gas passing over an illuminated plant, making use of the strong absorption band of carbon dioxide gas at $4\mu$ in the infrared. Aufdemgarten (36) uses the thermal conductivity of a gas for analysis of its $CO_2$ content. After passing over a photosynthesizing plant the thermal conductivity of the gas is measured with the well-known hot wire method wherein the resistance is measured as a function of temperature. Plotting the rate of photosynthesis as a function of time at the beginning of an illumination period one gets complicated curves which change with the type of the plant material and its previous treatment.

Before analyzing the details we may discuss in general the changes of rate of photosynthesis during the induction period in a higher plant. The most extensive studies of that kind have been made by McAllister (44) who measured the induction losses in wheat after dark pauses of several minutes.

During the first two minutes the rate of photosynthesis rises irregularly to its final value. The time course of the induction period is independent of the light intensity, the curves are similar to one another so that the induction loss expressed as a fraction of the final rate is always constant. The induction loss is roughly equal to the photosynthetic production during one minute at the final rate. But that last statement of McAlister is only true if one excludes the region of the very low light intensities. We know from manometric experiments and from the study of the fluorescence (see later) that the relative induction loss will become smaller if one lowers the intensity below a certain point. It will eventually converge to zero at light intensities in the neighborhood of the compensation point. It is also possible to produce the induction periods stepwise. One observes, for instance, an induction loss in going from darkness to an

intensity of light which produces half of the saturation value of photosynthesis, and a second induction loss in going from that light intensity to full saturation (44). The sum of the losses is equal to the induction loss occurring when one goes directly from darkness to the saturation intensity.

The size of induction loss depends upon the length of the preceding dark pause. It increases in linear proportion with that time interval until the dark time becomes a minute. From there on it grows only very slowly with time. The finer details of the rate curves during the induction period we shall discuss together with the corresponding anomalies of the fluorescence of the chlorophyll.

### 3. *Fluorescence Outburst During the Induction Period*

Kautsky (45) was the first to observe a quick rise of the fluorescence followed by a much slower decay in leaves which are suddenly irradiated after a dark period. He suspected also a relation of that outburst to the induction period. In this connection we have to mention the papers of Kautsky, *et al.* (45), Franck and Wood (46), Katz, Wassink, and Vermeulen (28), of McAlister and Myers (37), and Franck, French, and Puck (39) (the last one still unpublished). We shall refer, however, mostly to the papers of McAlister and Myers and Franck, French, and Puck. Their results have been obtained with the leaves of higher plants under conditions similar to those under which the induction losses described above have been studied. We shall make use also of the curves of the rate of photosynthesis and of fluorescence measured simultaneously by McAlister and Myers. The last-mentioned paper shows that in general each depression in the rate of photosynthesis corresponds to an outburst of fluorescence and *vice versa*. In many instances the time rate curves are exact mirror images.

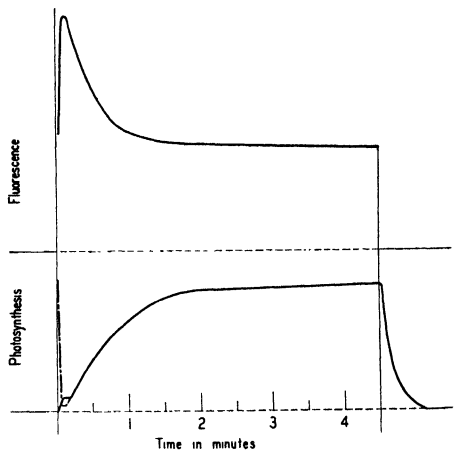



Fig. 2.—The course of fluorescence and photosynthesis in wheat during the induction period. (According to McAlister and Myers.)

Figure 2 gives an example of two curves measured in $N_2$ containing about 1 per cent oxygen and .03 per cent $CO_2$.

McAlister and Myers' method (37) is not quick enough to show that the rapid rise of the fluorescence in the first second of irradiation has its counterpart in a quick decay of photosynthesis from a high value to a low one.

We therefore present a curve measured by Blinks and Skow (43) observed on a higher plant, which shows clearly that in the very first moment a strong oxygen production occurs (Fig. 3).

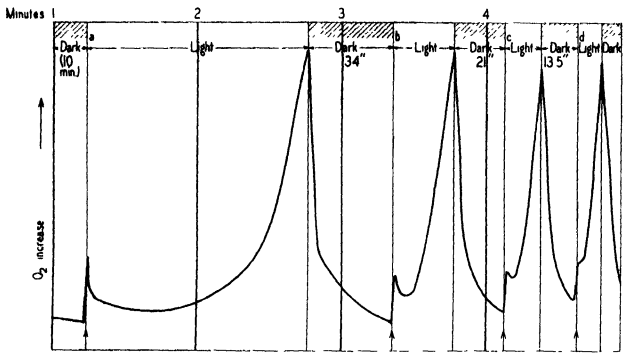



Fig. 3.—Oxygen liberation during the induction period of photosynthesis in a leaf of castor bean (*Ricinus*) as measured by the mercury electrode. (According to Blinks and Skow.)

Figure 4 shows a more complicated type of curves. They were observed by McAlister and Myers with wheat plants in a higher $CO_2$ concentration. Franck, French, and Puck found this type often in different leaves especially if the concentration of $CO_2$ was 1 per cent or higher. But the sensitiveness of the plant material toward a surplus of $CO_2$ was sometimes so great that the second maximum became visible already in relatively small $CO_2$ concentrations.

We may conclude from these examples that in general a maximum of fluorescence corresponds to a minimum of photosynthesis.*

* In algae cultivated in a gas atmosphere containing only a small amount of carbon dioxide, McAlister and Myers find exceptions to this rule. Just as in the experiments of Katz and Wassink the total changes of the strength of fluorescence observed under these conditions are so much smaller (about 10 per cent against 200–300 per cent in the cases mentioned above) that they seem to be produced by another mechanism. We will postpone the discussion of that point. (See page 232.)

With such a relation between the rate of photosynthesis and the intensity of fluorescence now established it is possible to study with the fluorescence method alone those problems which involve extremely rapid changes of metabolic rates. That was done by Franck, French, and Puck mostly with Hydrangea leaves and under conditions which produced phenomena of the type in Fig. 2. The main results are the following: The fluorescence outburst occurs always if a sudden and not too small a rise of photosynthesis is produced. The shape of the decay of the outburst is independent of the light intensities as long as the latter remains relatively high. At low intensities the outburst becomes smaller and eventually vanishes.

It is important that the outburst of fluorescence is not only produced by the rapidly increasing rate of the photochemical reaction following a rise in light intensity, but also by an increase of photosynthesis due to a greater supply of carbon dioxide. In such an experiment the steady photosynthesis in a leaf is kept below the normal value for the given intensity by a lack of carbon dioxide. It is remarkable how quickly the fluorescence responds to the addition of more carbon dioxide to the surrounding atmosphere. The shape of the curve is influenced, of course, by the fact that the gas exchange cannot be made infinitely rapid but takes a few seconds to be completed. The time necessary to reach the maximum of fluorescence is a function of the incident intensity and is shorter the higher the intensity. But the velocity of increase rises strictly proportional to the intensity only at low intensities, at higher intensities less rapidly, and finally reaches a constant value. Because the rate of photosynthesis shows the same relation to light we conclude that the time to reach the maximum is (under normal conditions) indirectly proportional to the final value of photosynthesis. If, on the other hand, the saturation value of photosynthesis is greatly reduced

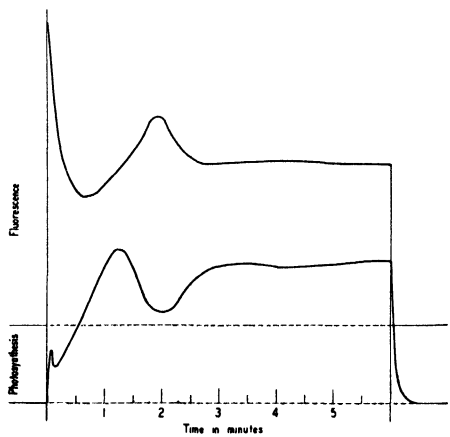



Fig. 4.—The course of fluorescence and photosynthesis in wheat during the induction period. (According to McAlister and Myers.)

under the influence of the low temperature of cyanide or a surplus of $CO_2$ one does not observe corresponding influences on the rising part of the fluorescence curve; its shape and its time course remain practically unaltered.

After an illumination period equal to or longer than the duration of the outburst a dark pause is necessary to repeat the phenomenon. Franck and Wood (46) had already found the same dependence of the outburst upon the duration of the dark pause as that described by McAllister (44) for the induction loss. Low temperature prolongs the dark time necessary for a recovery of the outburst.

The slow decrease of the fluorescence indicating the corresponding rise in the rate of photosynthesis is of another nature than the first rapid reaction. Instead of being completed in a second or less it requires about one minute under normal conditions and this period is considerably protracted under the influence of low temperature, excessive concentration of carbon dioxide, or poisoning with cyanide (to which it is less sensitive than the Blackman reaction).

The effect of carbon dioxide is demonstrated in Fig. 5. The bright spot is a place where the illuminated leaf is exposed to 20 per cent $CO_2$ in air. This part is brighter because here the fluorescence has not yet faded away as in the other regions of the leaf. It shows a picture of the intensity distribution of the fluorescence in a Hydrangea leaf. The picture was taken after the uniform illumination of the leaf had lasted one minute. From a group of experiments which furnish the key to an understanding of the induction phenomena in higher plants we have chosen the following example. A plant is illuminated continuously with light of such a low intensity that only a steady fluorescence, without any outburst, is observed after the light has been turned on. Then a strong illumination is added for a period of only two or three seconds. This produces the usual increase in fluorescence. But when the weak illumination is resumed the fluorescence which results at first is much higher than it was before the short exposure to strong light and thereafter falls gradually to the former steady value which is reached in several seconds. The time necessary for the fading of this outburst depends on temperature just as does the outburst during normal induction periods.

Such experiments show that a rapid photochemical reaction at the beginning of a sufficiently strong illumination results in the production of chemical substances responsible for the higher fluorescence yield, and that these substances afterwards are removed by way of a dark reaction similar to the respiratory metabolism of the plant.

**Explanation of the induction period.**—All these recent experiments described in the preceding chapter make it clear that the induction period is a much more complex phenomenon than has been assumed on the basis of the earlier measurements of Osterhout, Warburg, van der Paauw, and others.

the production of new peroxide is lowered so much that it can be handled by the active fraction of catalyst C. This state of conditions corresponds to the minimum of oxygen production and the maximum of fluorescence. From now on two reactions determine the course of events. The reactivation of the peroxide-splitting enzyme under the influence of the reducing substances formed in photosynthesis and the disappearance of the inhibitor of catalyst B. As shown by the experiments the latter reaction appears to be related to respiration.

Not only are the facts described in the preceding chapter in agreement with this theory but also the influence of intermittent light in decreasing the induction loss is explained (44). If the surplus of peroxide molecules formed in the first flash is small it will be taken care of by the catalyst in the following dark interval. An accumulation of peroxides is avoided, no secondary inhibitor is formed, and the rate of photosynthesis rises to its final value. The induction period of photosynthesis which occurs after a period of darkness has been attributed to the limited action of the oxygen liberating enzyme not only because the considerations presented are plausible but because none of the other catalysts of which we know could possibly be responsible for this induction period.

A partial inactivation of catalyst B would never produce an outburst of fluorescence. Its specific substrate is so unstable that it will not accumulate. Catalyst A, even if inactive to a large extent, could not inhibit the rate of photosynthesis quickly enough in the beginning of an illumination period. It would limit the rate only after the normal supply of intermediates and of $CO_2$ acceptor complex have been depleted by photosynthesis. That would take about $^1/_2$ minute at saturation intensity instead of the observed value of $^1/_2$ second. In addition the yield of fluorescence is much lower in complete absence of carbon dioxide than it is at the maximum of the outburst.

Algae which have been starved (McAlister and Myers (37)) may contain only little of the oxidizable substance yielding inhibitors in the reaction with the intermediate peroxides. In this case the form of the fluorescence curve has been found different; the outburst and the induction loss are much smaller.

The stationary concentration of the oxidizable substance depends upon the rate of the oxidative metabolism in the plant. This would agree with the observations of Katz, Wassink, and Vermeulen who found the most conspicuous changes of the fluorescence induced by oxygen mainly at very low partial pressures of oxygen between zero and a few millimeters Hg. This is the region of oxygen pressures wherein the transition from fermenta-

tion to respiration occurs. We may expect that plants kept in nitrogen will show stronger fluorescence outbursts with a protracted second phase and this also has been observed. Wassink and Katz consider the curve shown in Fig. 6 as the general type of fluorescence curves they obtain in Chlorella.

The percentage change in the intensity of fluorescence occurring during the induction period is very small under the conditions of their experiments. But if these conditions are changed by a different previous treatment of the plants it is possible to obtain from Chlorella a much larger outburst like that of higher plants.

The algae used by Katz, Wassink, etc., apparently contained less oxidizable material than the leaves, so that the inhibiting substances would proceed at a slower rate and attain a much smaller concentration. Actually one can explain each detail of these fluorescence curves. We assume that the first sharp maximum which occurs during the first few seconds of illumination is due to the same mechanism which produces the fluorescence outburst and induction phenomena in higher plants; but is much smaller in magnitude

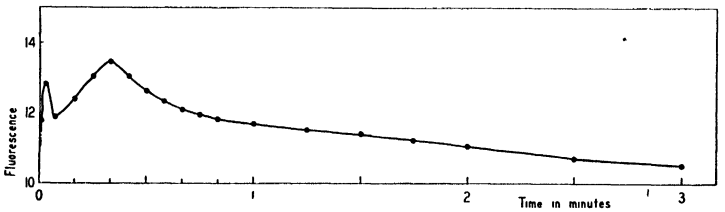



Fig. 6.—The course of fluorescence during the induction period of photosynthesis in Chlorella. (According to Wassink and Katz.)

in algae because of the decreased concentration of the oxidizable material here. The second weak maximum of the fluorescence rise and the slow decrease which occurs in these algae can be explained by an accumulation of the peroxides themselves without any interaction of oxidized metabolic substances. As pointed out in the paper of Franck, French, and Puck, one has to expect in that case that the induction losses are smaller than in the usual case and, too, that here the photosynthetic quotient will deviate strongly from unity for some time during the induction period.

We know that in the "reduced state" of the assimilatory system, see Chapter VI, brought about by a prolonged anaerobic period no oxygen is formed. The intermediate peroxides are reduced by hydrogen donors before they can react with catalyst C. It is not surprising, therefore, that an induction period like that occurring under normal aerobic conditions has not been observed. In the "reduced state" the reduction of carbon dioxide appears to begin and to continue at a rather high rate, unless the "reduced state" itself is reversed. The observations referred to have been made, however, only with the somewhat sluggish manometric

method. Measurements of the changes in fluorescence, under these conditions, are obviously important.

### *4. Uptake of Carbon Dioxide in the Dark; Experiments with Radioactive Carbon*

It was mentioned in Chapter II that the uptake of carbon dioxide by the plant is a reaction by which the $CO_2$ is chemically bound to an acceptor molecule. The most direct information about this process we owe to the work of Ruben, Kamen, and Hassid, *et al.* (31). They used $CO_2$ labeled by a content of radioactive carbon. The authors gained results from which they conclude that the reaction is a carboxylation of the type $RH + CO_2 \rightarrow RCOOH$ where R has a molecular weight of about 1000. The question whether it is really a carboxylation process which binds the $CO_2$ is still not quite settled. On the one side one can state that the binding energy of the $CO_2$ cannot be much smaller than $\sim$10 K. cal; on the other hand, there are no simple carboxylation reactions known which give such strength of the binding. But whatever the outcome of this question may be, it is certain that there are acceptor molecules in a concentration comparable to that of chlorophyll which combine with $CO_2$ in a catalytic dark reaction.

The same conclusion can be gained out of the so-called pick-up phenomenon also mentioned in Chapter II. The amount of $CO_2$ taken up by a plant in the dark immediately after an illumination period is not measured quantitatively since the methods to observe it are not developed far enough, but the methods of McAlister and Myers (37) and of Aufdemgarten are at least a proof that the amount taken up is not much smaller than that of chlorophyll. Aufdemgarten (36) found furthermore that the time necessary for the pick-up is considerably prolonged by the application of cyanide. The same is true (McAlister and Myers) if the irradiation takes place under conditions of a strong $CO_2$ limitation. Both results are to be expected because if the carboxylation reaction is hindered either by the insufficiency of $CO_2$ or by poisoning of catalyst A with cyanide (cf. Ruben, Hassid, and Kamen) the effect of the light will be to deplete the photosynthetic apparatus of R—COOH and increase the concentration of RH. Consequently, the carboxylation reaction will continue in the dark until equilibrium is reached. Under these conditions (little $CO_2$ or presence of cyanide) the prolonged pick-up has its counterpart in anomalies in both the fluorescence and photosynthesis at the beginning of the next

irradiation period, which will be superimposed on the usual induction anomalies. If the dark pause is not too long there is an abnormal distribution of intermediates at the beginning of the new irradiation since a surplus of RCOOH is present. Such an abnormal distribution is responsible for the second maximum of the fluorescence and the corresponding minimum in $CO_2$-uptake of plants which are irradiated in an atmosphere containing a surplus of $CO_2$ after a previous irradiation under $CO_2$ limitation. It is also responsible for the anomalies in fluorescence observed by McAlister and Myers (mentioned above) when algae are irradiated under severe $CO_2$ limitation.

## IV. Photo-Oxidation Processes in Plants

Chlorophyll sensitizes photo-oxidation reactions of easily oxidizable substances if dissolved in organic solvents which do not quench its fluorescence (49). The quantum yield is high. Chlorophyll derivatives also act as sensitizers for photo-oxidations in aqueous solutions if the dye is adsorbed at the surface of proteins and the like, but with a much lower quantum yield (50). Since the chlorophyll in leaves is supposed to be adsorbed on proteins, the occurrence of photo-oxidation processes in plants was foreseen. Noack (51) has shown that artificially added substances whose oxidation products can be recognized by their color are actually photo-oxidized in plants, and there have been many attempts to find out whether photo-oxidation processes of the normal constituents of the plant take place under natural conditions. We shall discuss here a few new papers related to this problem.

Indirect conclusions on the occurrence of photo-oxidation are often based on observations of the rate of photosynthesis. Very long exposures to light of relatively high intensity and shorter ones to excessively strong light reduce the rate of photosynthesis. The damage is reversible as long as it is slight, but becomes irreversible if the process advances too far. M. G. Stålfelt (52) observed such effects recently in some lichens (*Usnea dasypoga* and *Ramalina farinacea*). His results show that an illumination lasting for ten hours produces a decline of photosynthesis even at intensities well below the light saturation values. Air without an extra addition of $CO_2$ was used, so one must conclude that the saturation rate was determined by a $CO_2$ limitation. During night darkness the photosynthetic apparatus recovered. Whether the effect was actually produced by photo-oxidation remained an open question.

Burr and Myers (53) studied the decay of photosynthesis produced in

Chlorella by shorter but excessively strong illumination. In this case there was no doubt that photo-oxidation was the cause of the obstruction of photosynthesis, since a gradual transition was found by the authors, from a state where the photosynthetic rate was slightly lowered to destructive processes resulting in killing the cells and bleaching them out entirely.

The effect occurs even in the presence of a surplus of $CO_2$ but begins to take place at smaller light intensities if $CO_2$ is lacking. Since Burr and Myers used much stronger light, their experiments do not contradict Emerson's (54) earlier results with Chlorella, to the effect that these algae are insensitive to strong irradiation provided enough $CO_2$ is present.

It is tempting to try to make a connection between these experiments and the theoretical discussions of page 214. It is a conclusion of that theory that chlorophyll can be depleted of $CO_2$ and intermediates if, even in the presence of enough $CO_2$, the plant is illuminated with sufficiently strong light. Whether the depletion will occur at intensities not much higher than saturation intensities or only by excessively strong illumination depends, according to that theory, upon the relative abundance of catalysts A and B. Lack of $CO_2$ shifts the critical illumination to smaller values. The depletion of $CO_2$ is supposed to appear just in the range of intensities which are able to damage the plant. It therefore seems that the observations on photo-oxidation support the afore-mentioned theory, since it is obvious that the less of the chlorophyll which is combined with $CO_2$ or intermediates of photosynthesis, the more of it will be free to use its excitation energy to sensitize other photochemical reactions.

A method of observing photo-oxidation in plants directly was introduced by van der Paauw (55). Franck and French (56) have recently adapted the same method to their research. Part of a leaf was mounted just above the surface of a KOH solution in the vessel of a Warburg manometer. With the help of a rotating magnetic field the leaf was spun around an axis perpendicular to its surface. The irradiating light passed through the KOH solution. In that way it was possible to reduce the $CO_2$ content of the air to a minimum but, nevertheless, the leaf photosynthesized part of its respiration carbon dioxide, caught before it was able to leave the leaf. By making the necessary corrections for photosynthesis produced in that way and for the respiration, it was possible to study photo-oxidation as a function of the light intensity and of the partial pressure of $O_2$. Furthermore, comparisons were made between photo-oxidation in living leaves and in leaves which were freshly killed by submerging them in boiling water.

Some of the main results are the following: Photo-oxidation occurs in dead and live leaves with about the same yield depending, however, upon internal factors (amount of food present, previous treatment, etc.). The effect is not constant over long periods of time but becomes less the longer

the leaf is irradiated. It is therefore not easy to find out whether the effect is strictly linear with light intensity or proportional to the square root of the intensity. Further experiments are needed to decide that question. The quantum yield is small at intensities great enough to produce saturation under normal conditions. The rate of oxygen consumption by photo-oxidation is of the same order of magnitude as the rate consumed by respiration. The dependence of photo-oxidation upon oxygen pressure shows the same sort of curve as that observed by Gaffron (50) for photo-oxidation processes *in vitro* using porphyrin adsorbed at the surface of proteins in an aqueous solution. The curve is a kind of saturation curve. An oxygen content of 21 per cent produces about $^3/_4$ of the maximum effect, and in pure oxygen of atmospheric pressure, photo-oxidation is practically saturated. One also gets the same type of curve for the diminishing of the saturation values of photosynthesis by a rising partial pressure of oxygen as observed by Warburg (16) with Chlorella. But, just as in the experiments of Stålfelt and Burr and Myers, the indirect influence of photo-oxidation on photosynthesis was much greater than the direct one. In Warburg's observations the difference of the photosynthetic rate in $N_2$ and in $O_2$ was at least ten times greater than the strongest photo-oxidation, observed by French and Franck, in the absence of $CO_2$. It has been found that the effect of high oxygen concentration varies much with the internal factors in the plant, just as does the directly observed photo-oxidation. The dependence of the oxygen influence upon light intensity is, according to Warburg, similar to that of a catalytic poison (no influence at weak light and strong influence at saturation). On the other hand, Katz, Wassink, and Vermeulen (28) observed that the losses caused by high oxygen concentration are the same percentage at all light intensities. More experiments are necessary with plant material treated in different ways and in atmospheres containing different amounts of $CO_2$. Simultaneous measurements of the fluorescent intensity are desirable, to find out whether photo-oxidation again produces surface-active substances which may, by forming a layer, reduce the rate of photosynthesis and may at the same time protect the plant against too strong and destructive a photo-oxidation.

## V. The Metabolism of the Purple Bacteria and van Niel's Theory of Photosynthesis

Since 1883 the purple bacteria have been suspected of having a photochemical metabolism similar to that of the green plants. Engelmann (57) observed that the bacteria, when irradiated with the spectrum of visible

light, accumulated at those wave lengths which were most strongly absorbed by the pigments in the living bacteria. He also detected an accumulation of bacteria in the infrared light. Though quite a number of observations concerning the purple bacteria have in the meantime been published, a definite knowledge of the metabolism of the organisms has been gathered only during the past ten years.

It has been established, first by the work of van Niel (58), and independently by Gaffron (59), that the purple bacteria have a truly photosynthetic metabolism. Our present knowledge can be summarized as follows.

1. Under anaerobic conditions the purple bacteria reduce carbon dioxide with the aid of light energy absorbed by one of their pigments. This pigment, bacterio-chlorophyll, is very closely related to the chlorophyll of plants. The radiation which can be used for the photochemical reaction includes the infrared light up to 900 mμ.

2. The photochemical reduction of carbon dioxide proceeds only in the presence of specific organic or inorganic substances. A stoichiometric relation prevails between the amount of carbon dioxide reduced and the quantities of hydrogen donors which disappear.

3. The formation of free oxygen by purple bacteria has never been demonstrated.

Comparing this brief summary of the photochemical metabolism of the purple bacteria with photosynthesis in green plants, there appears to be no similarity beyond the fact that both the green plants and the bacteria make use of absorbed light energy to assimilate carbon dioxide. The differences become even more conspicuous if one compares the great number and variability of the reactions found in purple bacteria with the unique, uniform, and apparently rigid mechanism of photosynthesis present in all chlorophyllous plants without exception.

In the entire domain of the green plants, from unicellular algae, Chlorella, and Scenedesmus, to all species of higher plants, carbon dioxide reduction has hitherto been known either to proceed according to the equation

$$CO_2 + H_2O \xrightarrow{\text{light}} CH_2O + O_2 \qquad (1)$$

or not to proceed at all. For each molecule of $CO_2$ reduced, exactly one molecule of oxygen appeared and it was only the rate, not the nature or the products of the process, which could be changed at will by changing the experimental conditions. (For references see the monographs of Spoehr (60) and Stiles (61).) Because, moreover, chlorophyll was found to be identical in all plants, this substance was considered the key to the whole

problem, and there was apparently no other choice left but to follow the relationship between light, chlorophyll, and carbon dioxide more closely than before.

Contrary to what is the case in green plants, it is possible to distinguish the great number of species of photosensitive, colored bacteria by the different nature of their photosynthetic reactions. They can be divided roughly into three main groups: (1) green bacteria, which apparently use hydrogen sulfide exclusively as a hydrogen donor; (2) red sulfur bacteria (*Thiorhodaceae*), distinguished by their ability to grow autotrophically in the presence of a number of inorganic sulfur compounds, *viz.*, S, $H_2S$, $Na_2SO_3$, $Na_2S_2O_3$; (3) purple bacteria (*Athiorhodaceae*), which do not grow with inorganic sulfur compounds but require organic material, the favorite substrates being organic aliphatic acids.

Very little is known about the green bacteria. The occurrence of various metabolic processes among the individual strains of the other two groups often overlaps the group divisions. For this reason it is not possible to draw a clear dividing line between the metabolism of the red sulfur bacteria and the purple bacteria by studying the characteristics of only one specimen in each group as Gaffron (12) (1937) had thought possible in the beginning. Differences in the specificity toward a certain substrate, for instance organic acids, or molecular hydrogen, may be as great between two closely related strains of the same group as the differences in metabolic reactions seemingly characteristic for each entire group. Rhodovibrio, a purple bacteria, reduces carbon dioxide in the presence of any one of the whole range of the soluble fatty acids, from acetic to lauric acid. Thiocystis and Chromatium, two typical red sulfur bacteria, do not assimilate butyric and the higher acids under any circumstances, but can be induced to take up acetic and propionic acids, if the salt content and the *p*H of the medium are properly adjusted. In this way van Niel (62) (1936) was able to confirm his and Muller's (58) (1933) earlier findings that typical red sulfur bacteria will thrive with purely organic material. Thiocystis and Rhodovibrio, though belonging to different groups, can both reduce carbon dioxide with molecular hydrogen. In Spirillum rubrum, a purple bacteria, this faculty is absent. These instances will be multiplied in the future.

The metabolism of purple bacteria is of negligible importance compared with that of green plants, in its effect on both the total carbon dioxide turn-over in the organic world and on the amount of light energy transformed into chemical energy. The cited studies on purple bacteria were undertaken, not because of a desire to collect examples of variations in

bacterial metabolism, but in the hope of throwing light on the problem of photosynthesis in plants by investigating processes which might be similar, but chemically more accessible.

Once these very different types of photochemical carbon dioxide reduction had been established, the main problem became to find a point of view from which all these metabolic types could be looked upon as variants of the same sequence of chemical steps.

The simplest and most conservative assumption has been expressed by Nakamura (63). According to him the purple bacteria produce free oxygen which oxidizes the hydrogen donors present in the process of respiration. This is an old hypothesis which, if true, would mean that investigating the photochemical metabolism of the bacteria is of little service for the analysis of photosynthesis in plants since both reactions are absolutely identical. Nakamura arrived at this conclusion, because in contact with air his bacteria, "Rhodobacillus," absorbed less oxygen when irradiated than in the dark. This effect was studied and observed, however, in the absence of carbon dioxide, the substrate for the photochemical reaction, and in the absence of suitable organic material, the substrate for respiration. If, in these experiments, neutralized fatty acids were added to the bacterial suspension, the rate of oxygen consumption rose 500 per cent and the light reaction, still devoid of its substrate $CO_2$, was apparently powerless to compete with the accelerated oxidation. Since, as we shall discuss later, the purple bacteria do not produce free oxygen, Nakamura's observations have been interpreted by Gaffron (47) (1937) and by Wassink, *et al.* (10, 28) (1938), as an internal competition for the same hydrogen donors by oxidation and carbon dioxide reduction. Such a description stipulates, of course, a certain kind of mechanism, and it is important, therefore, that van Niel was able to demonstrate a competition between respiration and photoreduction even in the presence of sufficient carbon dioxide and of added substrates. (Personal communication.)

The most common observation with respect to the influence of oxygen on photosynthesis in red sulfur or purple bacteria is simply an inhibition of the photochemical reaction. The degree of sensitivity of different organisms to oxygen varies, however. Typical red sulfur bacteria will not grow at all unless the last traces of oxygen have been removed from the culture medium (58). Many of the purple bacteria, on the other hand, have been cultivated aerobically on suitable organic media. "Thus in the dark these bacteria substitute an oxidative metabolism for the photosynthetic mode of life which they carry out in the light" (van Niel (58), 1931, p. 106). Oxygen takes over the part played by the radiant energy under anaerobic

conditions. But this part is never connected with the formation of free molecular oxygen. This is one of the traits common to all these organisms which sets them apart from green plants.

Fig. 7, for instance, demonstrates the photochemical assimilation of carbon dioxide as a function of a certain amount of normal- and iso-butyrate by Rhodovibrio. The absorption of $CO_2$ by the bacterial suspension in an atmosphere of 95 per cent $N_2$ and 5 per cent $CO_2$ stops abruptly when the organic substrate is exhausted. And van Niel writes:

"If one determines, by purely chemical methods, the amounts of carbon dioxide and hydrogen sulfide which disappear during the development of Thiorhodaceae, one finds that in no case is there any demonstrable disappearance of $CO_2$ in excess to that which can be accounted for on the basis of the equation

$$2CO_2 + 2H_2O + H_2S \longrightarrow 2(CH_2O) + H_2SO_4.$$"

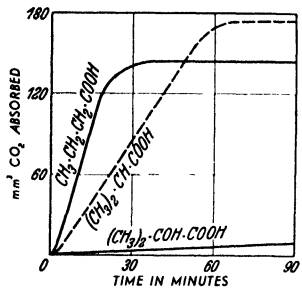



Fig. 7.—Absorption of carbon dioxide by an illuminated suspension of the purple bacterium *Rhodovibrio* in presence of 0.1 cc. *M*/20 of either butyrate, isobutyrate or oxy-butyrate. (According to Gaffron.)

Results like these are incompatible with the intermediate formation of free oxygen. All tests for oxygen, in particular that with luminous bacteria, in the way described by Harvey (64) have given negative results. For more detailed discussion the reader is referred to the papers of van Niel (62, 65) (1936).

We can definitely state, therefore, that in all these anaerobic photochemical reactions, carbon dioxide is reduced only in the presence of a certain amount of a specific substrate.

It is difficult to see, under these circumstances, how the reaction between a fatty acid, for instance, octylic, $C_8H_{16}O_2$, and carbon dioxide could have any similarity with the decomposition of carbonic acid into oxygen and carbohydrate, the more so as the *Athiorhodaceae* excrete no detectable reaction products into the culture medium when assimilating the aliphatic acids together with carbonic acid. The substrates are quantitatively transformed into cell material (Gaffron (59), Muller (58)).

Fortunately there exist simpler reactions in purple bacteria. The red sulfur bacteria and the green bacteria which reduce carbon dioxide with hydrogen sulfide form either sulfuric acid or sulfur which can be determined in the cultures at the end of the experiments together with the amounts

of carbon dioxide and of sulfide which have disappeared. In this way van Niel (58) (1931) obtained results which, for instance, could be represented by the equation

$$CO_2 + 2H_2S \xrightarrow{\text{light}} (CH_2O) + H_2O + 2S. \quad (2)$$

Also the assimilation with molecular hydrogen in Rhodovibrio (Gaffron (66), 1935) mostly gave results fitting the equation

$$CO_2 + 2H_2 \xrightarrow{\text{light}} (CH_2O) + H_2O \quad (3)$$

though in other bacteria sometimes more hydrogen is absorbed (Wessler and French (67)).

Adopting the view of Kluyver and Donker (68) (1926) that one should consider water as the specific hydrogen donor for the reduction of carbon dioxide in plants and the oxygen as the oxidation product of water, van Niel formulated the over-all reaction of photosynthesis in green plants in strict analogy

$$CO_2 + 2H_2O \xrightarrow{\text{light}} (CH_2O) + H_2O + O_2 \quad (4)$$

to his results with green bacteria.

The insertion of the second molecule of water apparently introduced *ad hoc* in equation (4) marks the turning point between the old conceptions of photosynthesis as a unique process of carbon dioxide decomposition by light and the modern idea that photosynthesis should be looked upon as one of a group of similar oxido-reduction processes, all following the general formula

$$CO_2 + 2H_2A \xrightarrow{\text{light}} (CH_2O) + 2A + H_2O. \quad (5)$$

Van Niel (69) (1935), therefore, insisted that the carbon assimilation with organic substances should not be regarded as an exception to this reaction type. This interpretation was, at least, the most likely one for Gaffron's (66) earlier data on the photoreduction of pyruvate and lactate by Rhodovibrio as van Niel showed. In other words, the organic material is dehydrogenated while carbon dioxide is reduced. The photochemical dehydrogenation of the fatty acids may proceed similarly to the oxidative assimilation of these substances in other living tissues. Parallelisms are found in the influences of length and configuration of the carbon chain and the behavior of phenyl substituted acids. Phenylpropionic acid, for instance, will be attacked by Rhodovibrio in the light and the remaining

benzoic acid can be recovered quantitatively from the medium (70). This use of only partly digestible substrates for analyzing the course of a reaction has lately been successfully applied to the metabolism of the purple bacteria by substituting alcohols in place of fatty acids. Alcohols are not a normal substrate for those strains of purple bacteria which have been studied up to recently. Only such organic substances which contain an aliphatic carboxyl group are assimilated at a high rate.

Foster (72), however, using alcoholic culture media, isolated directly from soil new strains of purple bacteria, which grow abundantly with alcohols as substrates for the photochemical reaction.

Foster (72) in van Niel's laboratory succeeded in restricting the photoreduction with organic material to the first step, thus arriving at simple stoichiometric relations of the reactants. He used iso-propanol as a substrate. In the course of the reaction the iso-propanol is oxidized to acetone. The latter apparently is not a suitable substrate; it accumulates and can be recovered. This experiment verified the equation

$$CO_2 + 2(CH_3)_2CHOH \xrightarrow{\text{light}} (CH_2O) + 2(CH_3)_2CO + H_2O.$$

On account of the collective evidence provided by the metabolism of the photosynthetic bacteria, we accept van Niel's theory that all photosynthetic reactions with carbon dioxide can be summarized in the general form of oxido-reductions as given in equation (5).

The next question is how much such an equation will reveal about the mechanism of carbon dioxide reduction, in particular about the nature of the photochemical steps. The answer is that such an over-all equation, though excluding the classical theory of carbon dioxide being decomposed inside a $CO_2$-chlorophyll complex, is still compatible with a great number of detailed mechanisms. The chemical explanations and schemes based on the principle of oxido reduction which have been discussed by various authors during the last decade are very much alike, and for want of real knowledge in the chemistry of carbon dioxide reduction, have moved around in narrow circles. We shall not discuss their lesser or greater merits. But it is important to note the general trend in the theoretical development of the problem of photosynthesis. It has been determined mainly by two facts: On the one hand, by the firm belief that one molecule of carbon dioxide is reduced with only four quanta of visible light, in both plants and bacteria; on the other, by the growing knowledge about the chemical reduction of carbon dioxide in colorless bacteria in the dark.

These two facts led van Niel (69) to suggest that each quantum was

responsible for the activation of one hydrogen atom and that the stepwise reduction of the carbon dioxide should be considered as a subsequent dark process. But such a scheme would be difficult to assume for thermodynamical reasons if the quantum yield in photosynthesis was really four. In Chapter I we have seen that the actual number of quanta used in photosynthesis per molecule of carbon dioxide is certainly twice, probably three times as large. With this new knowledge a serious handicap of the van Niel scheme has disappeared.

Van Niel's hypotheses were summarized in a paper written in French in 1936 (65). Translated, his essential paragraphs read:

I. The pigment system absorbs radiant energy. It is activated in such a manner that it may now react as a hydrogen donor. This is a typical photochemical reaction.

II. Carbonic acid is reduced by the activated pigment system. This reaction, which goes on in the dark, transforms the pigment system into an oxidized complex. The system cannot react successfully as a hydrogen donor, unless it has been brought back to a reduced state.

III. In an oxido reduction, the oxidized pigment complex is reduced with the aid of the specific hydrogen donor, $H_2A$. This reaction re-establishes the initial state of the pigment system with the simultaneous formation of the oxidation product A.

Because, as we shall see below, carbon dioxide under certain circumstances is readily reduced in the dark also in green plants, we have adopted van Niel's point of view that in photosynthesis hydrogen donors are produced which reduce the carbon dioxide. The only difference between the scheme presented on page 214 and van Niel's is the following.

In our scheme both the activation by light absorption and the transfer of the hydrogen to a new position are assumed to be one elementary act, which is followed by a catalytic dark reaction which serves to stabilize the products. According to van Niel the formation of a hydrogen donor and the transfer of the hydrogen are supposed to be two acts. From the point of view of chemistry, the difference is a negligible one. It becomes important only if one enters into discussions in the field of chemical kinetics. Reactions 1 and 3 in the model equation given on page 214, however, differ from the corresponding reactions in van Niel's scheme in that we have to assume reaction 3 to be a photochemical one also. It has to be written, if possible, as the same type of reaction, *i.e.*, an intermolecular hydrogen transfer. Such a variant of the van Niel scheme is called for in the case of the green plants.

Because of the new observation that the assimilatory system of some green plants can be brought into a condition in which it reacts similarly to the system present in purple bacteria, we can assume the mechanism to be

essentially the same in plants as well as in bacteria, even in those cases where energetically there is no need for such a system. With the divergent and partly doubtful determinations of quantum yields of photosynthesis in bacteria described in Chapter I, the interesting possibility is not yet excluded that in purple bacteria one of the sequence of light reactions mentioned above is replaced by dark reactions. The quantum yields then should be different from those found in plants by a factor of $^1/_2$ or $^1/_3$.

## VI. Carbon Dioxide Reduction in the Absence of Oxygen and the "Reduced State" of the Assimilating System in Plants

Willstätter and Stoll (14) devoted a part of their investigations on photosynthesis to studies of this reaction in the absence of oxygen. They observed that replacing air by nitrogen had no effect on photosynthesis in plants which had been left under anaerobic conditions for only a short period. A complete and long extended inhibition of photosynthesis was found, however, after the plants (picked leaves) had been kept in nitrogen for 2, 15, or 24 hours. The most important new fact was that the assimilating cells though injured by secondary influences recovered part of their capacity to reduce carbon dioxide under the influence of continued strong illumination. The authors concluded that very small amounts of oxygen were indispensable for the assimilatory mechanism, and that this oxygen need not be present as free gas but as part of an enzyme from which it would separate by dissociation at extremely low partial pressures of oxygen.

Ten years later the investigations on the metabolism of the purple bacteria revealed a reduction of carbon dioxide with the aid of light energy, similar in many respects to photosynthesis in plants (see the preceding chapter). In contrast to the observations of Willstätter and Stoll, oxygen did not have a favorable but a decidedly inhibiting influence on the photoreduction in purple bacteria.

Because this reversed sensitivity toward oxygen might be directly related to the difference in the mechanism of carbon dioxide reduction in plants and in bacteria, a series of investigations on photosynthesis under anaerobic conditions were resumed (Gaffron 74, 47, 75, 76).

The experiments of Willstätter and Stoll, and similar ones, have since been repeated with unicellular algae. The main results, which we have collected from several papers, are as follows. Many algae, Scenedesmus, Ankistrodesmus, Rhaphidium, etc., may survive unharmed not only hours but days of anaerobic conditions. Chlorella, which has been used so frequently in modern investigations on photosynthesis, appears to be more

sensitive to lack of oxygen and shows permanent injuries if the anaerobic dark periods are too long. Yet several hours without oxygen do not damage the cells of Chlorella in a measurable way. Green algae are therefore more suitable material for anaerobic experiments than the detached leaves of higher plants. With these algae it has been found that the assimilatory system suffers a very definite, though completely reversible alteration. The presence in the cell of reducing substances like carbohydrates or molecular hydrogen produce a "reduced state" of the photosynthetic mechanism provided the anaerobic incubation lasts for a considerable time. The shortest effective period observed was an hour. Generally the dark period has to last for several hours. In the "reduced state" the absorption of light energy brings about the reduction of carbon dioxide, but the photochemical reaction is no longer coupled with the liberation of molecular oxygen. Instead various substances serving as hydrogen donors are simultaneously dehydrogenated. The oxidizable substances normally present in a plant cell are likely to form carbon dioxide when broken down in the course of an oxido reduction. It is difficult, therefore, to prove that a production of carbon dioxide in the light may be due to carbon dioxide being reduced, particularly since Emerson and Lewis (4) (1940) observed a liberation of carbon dioxide under aerobic conditions during the induction period which is probably due to quite different causes. Reversing therefore the historic development, we shall describe the properties of the "reduced state" of the assimilatory system in green plants beginning with the reduction of carbon dioxide by molecular hydrogen.

### *1. Photoreduction with Hydrogen in Algae*

**Adaptation to the Use of Molecular Hydrogen.**—Molecular hydrogen has been considered as an inert gas for all plant cells. In the course of investigations on photosynthesis under anaerobic conditions, it was found that among the unicellular green algae some families can be adapted to a metabolism which includes the use of molecular hydrogen (76). This ability has been found so far in Scenedesmus (several strains), Ankistrodesmus, and Rhaphidium (one strain each). The capacity to react with hydrogen reveals itself in these algae after an anaerobic adaptation period running from at least one hour to twelve hours. It seems to be more firmly "rooted" after many hours of incubation. Whether the adaptation to hydrogen consists merely in an activation of an inert enzyme ("hydrogenase") already present because of the reducing forces of fermentation processes and products, or whether the enzyme is formed in a sequence of more than one reaction is not yet known. During the time of adaptation the

algae begin to absorb hydrogen slowly. The amount absorbed is due to oxygen impurities in the gas phase and to internal conditions of the algae varying with the preceding method of cultivation. Algae grown autotrophically and devoid of much organic reserve material appear to absorb the least—scarcely enough to produce a measurable rate of hydrogen uptake.

When illuminated, the algae begin to consume large quantities of hydrogen, provided carbon dioxide is present in the suspension medium. In the absence of carbon dioxide the rate of hydrogen absorption in the light becomes very small but remains measurable. This reaction may be due to the formation of $CO_2$ by fermentation. By introducing a known amount of carbon dioxide into a vessel containing adapted algae and determining the amount of hydrogen which disappears together with the carbon dioxide, the assimilatory quotient of the photochemical reaction has been found to be equal to 2. We have, therefore, a reaction which appears to be identical with that found in one strain of purple bacteria and represented by equation (3) in the foregoing chapter.

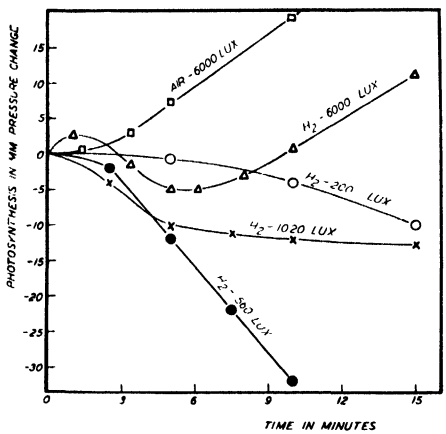



Fig. 8.—Beginning of photosynthesis in the alga Scenedesmus adapted to hydrogen. Influence of the light intensity on rate and type of the photochemical process. (According to Gaffron.)

**Transition from Photoreduction to Photosynthesis under the Influence of Light.**—Like normal photosynthesis at lower light intensities, the rate of the photoreduction with hydrogen increases proportionally to the incident radiation. But before any kind of saturation is attained a new process interferes which replaces the particular anaerobic metabolism by normal photosynthesis. But this transition is not a sudden one. There is a threshold of intensity which, if passed, causes a decrease in the rate of the hydrogen reaction. If the illumination at this critical intensity is continued, the reaction with hydrogen will disappear completely, giving way to normal photosynthesis with the production of oxygen. This

threshold changes with internal conditions, but the critical intensity always remains lower than that necessary to reach saturation of photosynthesis in air.

The transition from photoreduction with hydrogen to normal photosynthesis will be faster the higher the intensity. If the algae adapted to the assimilation with hydrogen are suddenly irradiated with "saturation" intensity, normal photosynthesis will appear after an abnormal induction period. Below the critical intensity, however, the reaction with hydrogen may continue indefinitely if both substrates are present in excess.

The phenomena just described are demonstrated in Figs. 8 and 9. After a return to normal photosynthesis under the influence of strong light, the reaction with hydrogen

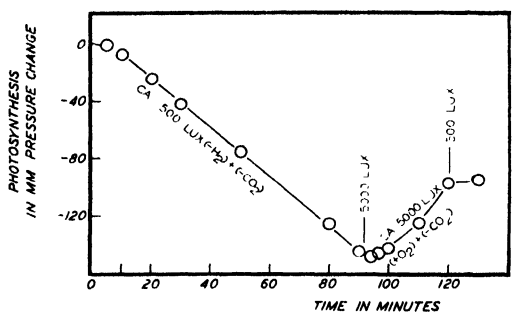



Fig. 9.—Change of photoreduction with hydrogen to photosynthesis and liberation of oxygen under the influence of light of high intensity in Scenedesmus. (According to Gaffron.)

will not reappear by merely lowering the intensity. This is due partly to the aerobic conditions which now prevail, but partly also to the fact that it takes time to establish the "reduced state." In the absence of carbon dioxide the "reduced state" of the assimilatory system is much more resistant to the action of high light intensity. A period of illumination with high light intensity, sufficient to stop the reaction with hydrogen simultaneously reviving normal photosynthesis, will produce no change if, during its duration, all carbon dioxide is removed. After dimming the light and restoring the carbon dioxide supply, photoreduction with hydrogen will continue (77).

**The Effect of Oxygen in the Dark and the Oxy-Hydrogen Reaction.**—In a closed vessel small amounts of oxygen will soon be absorbed by the algal suspension. The respiration of the algae eventually produces the strict

anaerobic conditions apparently necessary for the development of the "reduced state." If constantly replaced, an impurity of half a per cent of oxygen in the hydrogen gas, however, seems to prevent the transition from the normal to the "reduced state." We do not know whether there are individual variations with respect to the inhibitory concentration of oxygen between different species of algae or different conditions of the same algae.

A few per cent of oxygen added to the atmosphere in contact with the adapted algae will enforce the return to normal conditions in the course of a few minutes. But it depends very much upon the internal conditions of the algae whether $^1/_2$, 1, or 3 per cent of oxygen in the gas phase are sufficient to destroy the "reduced state," for this reason. The oxygen is rapidly taken up by the algae and reduced to water with the aid of the hydrogen. In other words, they are able to perform the "Knallgas" reaction (see the last chapter). Only when the rate of the Knallgas or oxy-hydrogen reaction, which increases proportional to the concentration of oxygen, cannot keep up with the absorption of oxygen by the cell, the "reduced state" disappears. The partial pressure of molecular oxygen necessary to destroy the "reduced state" is much greater than that which is produced by the plant during the transition period under the influence of an excessive illumination. But it is important that the rate of the oxy-hydrogen reaction, just the moment before return to normal conditions, is very nearly that of the maximum photoreduction of which the cell is capable without turning to aerobic photosynthesis.

**Induction Phenomena.**—Describing the normal induction period in terms of a loss of carbon dioxide that could have been reduced at the respective intensity if no inhibition existed after a dark period, we have said in Chapter III that the induction loss is proportional to the final rate of photosynthesis and that the length of the period is more or less independent of the intensity. In Fig. 8 we see that the reaction with hydrogen does not begin immediately at its final rate. We have clearly an induction phenomenon.

But this induction is very different from, if not to say the reverse of, the aerobic induction. The length of the period depends on the incident radiation and is shorter the higher the intensity is, whereas the induction loss appears to be more or less the same. Such behavior is best explained by having a certain amount of substance which reacts photochemically with carbon dioxide, so as to allow the reduction with hydrogen to assume its pace only after the inhibitory substance has been decomposed. Such an assumption would also explain why cells which contain larger amounts of organic compounds have longer induction periods than those

which have been grown autotrophically and allowed to destroy their reserve material by respiration.

A study of the induction loss which might occur after a short dark interval of one or two minutes between periods of continued illumination meets with difficulties because the absorption of hydrogen is apt to continue for several minutes after the light has been turned off, probably because some substances have been dehydrogenated during illumination and now take up hydrogen in the dark.

The theory discussed in Chapter III predicts that the aerobic induction should be absent as long as the light intensity remains below the conversion threshold which separates photoreduction from photosynthesis in these plants.

TABLE I

CYANIDE INHIBITION OF THE ADAPTATION TO HYDROGEN IN THE ALGA RHAPHIDIUM POLYMORPHIUM

Temperature, 25°. Light intensity, 1200 lux.

| Type of reaction | Rate of gas exchange in mm./20 min. | | Inhibition (per cent) |
|---|---|---|---|
| | Unpoisoned | Poisoned with $10^{-4}$ *M* HCN | |
| Respiration | − 2.8 | −1.3 | 52 |
| Photosynthesis | + 8.0 | +7.0 | 13 |
| Adaptation to $H_2$ in the dark | − 1.3 | −0.16 | 92 |
| Photoreduction in $H_2$ | −60.0 | +3.0 | 100 |
| Photosynthesis | + 7.0 | +5.0 | 29 |

**The Effect of Organic Substances and of Specific Poisons.**—If the suspension medium contains organic substances like glucose or yeast autolysate the induction becomes prolonged, and the rate of hydrogen absorption very much depressed (76). This inhibition is perfectly reversible in respect to normal aerobic photosynthesis. Organic substances which will prohibit any visible gas exchange of the adapted algae in hydrogen will scarcely affect the photochemical production of oxygen in air. The threshold of the light intensity at which photoreduction changes into photosynthesis appears to be moved up to higher intensities in the presence of some organic substances. The "reduced state" becomes more stable.

But there are only a few preliminary experiments on this interesting phenomenon.

Cyanide concentrations, which do not decrease the rate of photosynthesis considerably, are nevertheless sufficient to prevent the adaptation to the use of hydrogen (Table I), while they have no effect when added after the adaptation has been completed.

Hydroxylamine, a poison which in a concentration of 0.0005 *M* inhibits normal photosynthesis completely, reduces the rate of the photoreduction only to about one-half in a concentration of 0.03 *M*, that is, a concentration which is sixty times greater. (Table II.) Any experimental procedure which normally would lead to the conversion of photoreduction to photosynthesis ends in total inhibition if the medium contains hydroxylamine.

TABLE II

EFFECT OF HYDROXYLAMINE ON THE RATES OF NORMAL PHOTOSYNTHESIS IN AIR AND OF PHOTOREDUCTION WITH HYDROGEN IN HYDROGEN

Temperature, 25°. 0.018 cc. of Scenedesmus D 1 in 3 cc. of *M*/10 carbonate buffer (85 p. pr. + 15 p. sec.). $NH_2OH$ added 30 minutes before illumination.

| Time (min.) | Light intensity (lux) | Rate of gas exchange in cmm./10 min. | | | |
|---|---|---|---|---|---|
| | | Oxygen produced | | Hydrogen absorbed | |
| | | Control | *M*/300 $NH_2OH$ | Control | *M*/300 $NH_2OH$ |
| 20 | 1200 | +8.1 | −3.5 | −14.3 | −11.3 |
| 285 | 0 (Dark) | −2.2 | −1.3 | − 0.5 | − 0.8 |
| 40 | 1200 | +9.2 | −1 3 | −20 0 | −18.0 |

This insensitiveness to hydroxylamine of the anaerobic photosynthesis in plants is shared by the photoreduction in bacteria. It is noteworthy, however, that Nakamura (77) observed a difference in the anaerobic light metabolism of his bacteria in the presence of hydroxylamine, after the amount of substrate ($H_2S$) introduced had been assimilated.

### 2. *The "Reduced State" in the Absence of Hydrogen*

The adaptation to hydrogen is speeded up considerably if the algae have been kept in nitrogen under anaerobic conditions for some time. The success of this preparation depends to some extent upon the amount of

fermentable material present in the cells. The "reduced state" can apparently be brought about not only with molecular hydrogen, but with other hydrogen donors as well. In fact, studies of this kind preceded the experiments with hydrogen. Planned as a continuation of the experiments of Willstätter and Stoll, cited above, they rendered a great number of seemingly divergent results (74, 75). We shall mention only a few.

Upon illumination after a dark period, less than an hour, in pure nitrogen, photosynthesis appears not much changed in comparison to aerobic

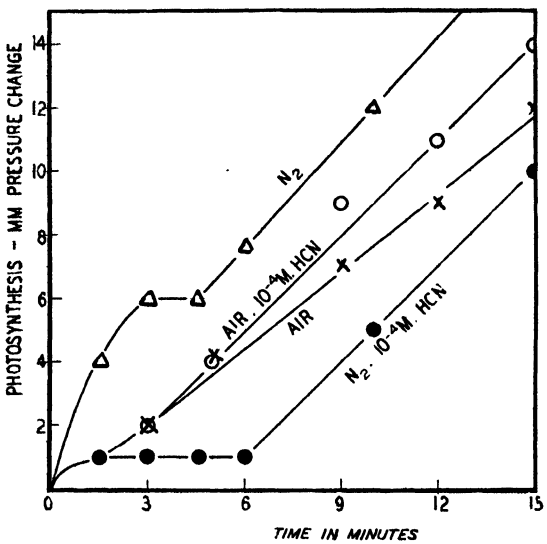



Fig. 10.—The influence of $10^{-4}$ M. cyanide on the course of the aerobic and anaerobic induction period of photosynthesis in Scenedesmus. (According to Gaffron.)

conditions. But after anaerobic periods of several hours up to twenty-four hours, photosynthesis will start quite differently. The common induction period, the initial inhibition of carbon dioxide reduction occurring after long aerobic dark periods, is absent (see Chapter III). Upon irradiation a photochemical reaction begins immediately, but in most cases the rate and the nature of the gas exchange are not those of the control in air.

At low light intensities only a slow and rather stationary evolution of carbon dioxide is observed which may continue for a long time. No oxygen

is liberated. A change to normal photosynthesis can be induced at any moment by irradiation with strong light. The transition from this "inhibited" condition to normal photosynthesis takes one to several minutes, depending on the intensity of the light and the preceding culture conditions of the algae.

Immediate irradiation with strong light after the long anaerobic period produces this transition right away (cf. Fig. 8). Considerable amounts of

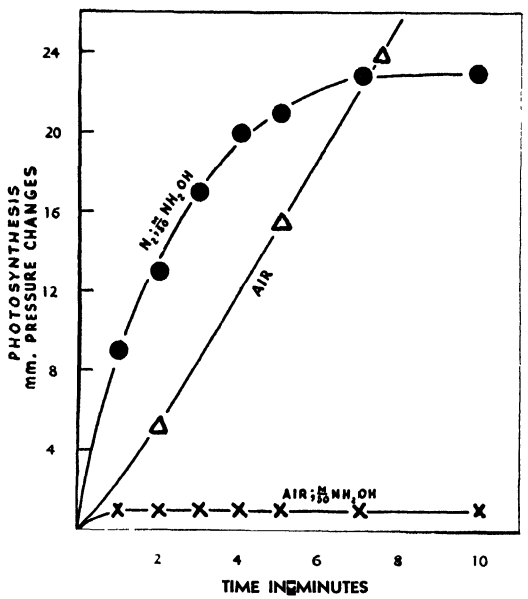



Fig. 11.—The effect of $2 \cdot 10^{-2}$ M. hydroxylamine on photosynthesis in Scenedesmus after 12 hours in the dark in air and in nitrogen. Hydroxylamine added 50 minutes before exposure. (According to Gaffron.)

carbon dioxide are formed. The rate of liberation of oxygen, initially small or absent, increases rapidly. Meanwhile the production of carbon dioxide stops and this gas begins to be consumed. The exchange of oxygen and carbon dioxide gas gradually attains its normal relation and final rate in the course of several minutes. Algae grown in inorganic media give very brief effects, while those cultivated and incubated with glucose solutions require several minutes' time to return to normal conditions. If the

algae are brought into contact with air before illumination, they show an accelerated oxygen uptake for some time. But a few minutes of aerobic conditions are sufficient to obliterate the phenomena described.

The similarity to the conditions existent during the assimilation with hydrogen becomes even more apparent if we consider the effect of such poisons as hydrogen cyanide and hydroxylamine. Figures 10 and 11 contain examples (70). Cyanide, added to the algal suspension before the anaerobic period in a concentration which inhibits normal photosynthesis

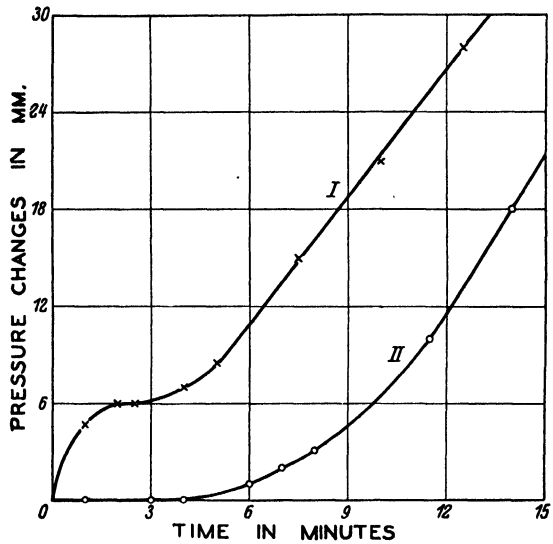



Fig. 12.—Beginning of photosynthesis in Scenedesmus after an anaerobic period of 16 hours. (I) In nitrogen, (II) in carbon monoxide. (According to Gaffron.)

only slightly or not at all, suppresses or changes the anomalous reactions. Added in excessive amounts to the algal suspension after the conversion of the photochemical system, but thirty minutes before exposure, hydroxylamine allows the abnormal reaction to proceed until the transition to normal photosynthesis should have been completed. At this moment it stops the reduction of carbon dioxide. The algae kept in air as a control are, of course, completely poisoned from the very beginning. The peculiar conditions into which the assimilatory system has been brought during the anaerobic period is even more emphasized by the fact that carbon monoxide

also prohibits the development of the anomalous photochemical reaction. If in the anaerobic period CO is used in place of $N_2$, the result is a total inhibition of any gas exchange on subsequent illumination (Fig. 12). This inhibition may last for minutes or up to an hour in spite of strong irradiation and it disappears presumably because somewhere in the cell the first molecule of oxygen has had a chance to make its appearance. There are several possible explanations of the carbon monoxide effect, though none is wholly satisfactory (74). An effect of carbon monoxide on normal photosynthesis has never been observed. But it is perhaps useful to remember that the very complicated metabolism of Thiocystis, a red sulfur bacterium, is decidedly influenced by this gas. (Gaffron (12) 1935.)

With Chlorella or with other algae in the presence of large amounts of glucose in the suspension medium, a Willstätter-Stoll effect has sometimes been found after anaerobic periods, *viz.*, a complete inhibition similar to that observed in the presence of carbon monoxide or cyanide. It remains to be investigated whether or not in these cases we have a true inhibition, the absence of carbon dioxide reduction, or only an apparent one, due to the fact that the hydrogen donors reacting in the "reduced state" have the composition of carbohydrates.

### 3. *Theoretical Conclusions*

We have said in Chapter V that the oxidation-reduction formula of photosynthesis can be developed further in many ways. But as yet there has been little experimental support for the preference of one scheme to another. With the knowledge that both types of photosynthesis may occur in the same cell and that they are interchangeable at will, we are now in a better position to make a choice between the various hypotheses. Considerations as to the special mechanism involved in the photochemical reduction of carbon dioxide can start from one of two assumptions. Firstly, that the process which involves the liberation of oxygen is an integral part of every kind of photosynthesis, but it is followed in bacteria and in the "reduced state" of plants by a secondary reduction of this free oxygen. Or secondly, that the anaerobic type of photosynthesis is the same in all cells but that it is supplemented in green plants by the capacity of liberating gaseous oxygen. Of these two possibilities we have to discard the first and accept the second for the following reason. Only one form of carbon dioxide reduction is known which is coupled with the liberation of oxygen. The great number of similar reactions in bacteria proceed without the formation of a detectable amount of free oxygen. Photosynthesis in plants,

therefore, is the exception to the general rule. One has to admit, however, that with the green algae in the "reduced state," it is not so easy to prove experimentally that an intermediate formation of oxygen does not occur.

Using luminous bacteria to test for the production of oxygen in algae adapted to hydrogen, M. Doudoroff (cf. 76) observed very clear differences in comparison to control suspensions in nitrogen, which luminesce much stronger. Nevertheless at light intensities below the intensity of the conversion threshold, a faint luminescence could sometimes be observed in the mixed suspension of algae and bacteria. On the other hand, we know from Harvey's original experiment (64) with luminous bacteria, and from the experiments of Blinks and Skow (see Chapter III) that the oxygen of photosynthesis is not retained in the plant cell, but appears outside in less than one-fiftieth of a second. Therefore the pressure of gaseous oxygen inside the cell is almost the same as that outside. Now we have seen that in order to produce the reduction of oxygen with algae adapted to hydrogen, it is necessary to use a measurable partial pressure of oxygen. This partial pressure would be sufficient to produce brilliant luminescence in the test with the luminous bacteria. And if the algae would have to produce all the oxygen corresponding to this partial pressure first in order to get the reaction going at the observed rates, long and differently shaped induction curves should have been found.

We conclude, therefore, that in the "reduced state" of the plant system carbon dioxide reduction proceeds by way of an oxido reduction just as in the purple bacteria without any intermediate formation of oxygen.

But does this mean a direct replacement of the hydrogen donor, water, by other substances like hydrogen or carbohydrate derivatives? Both van Niel and Nakamura (for different reasons) have previously favored the idea that it is always water which is decomposed in the photochemical reaction, in plants as well as in bacteria, and that the dehydrogenation of the specific hydrogen donors, other than water, takes place in a later phase. In Chapter II the photochemical reactions are formulated in a somewhat different way. Water is not decomposed in the first photochemical step but in the very next, and this leads to the intermediate peroxides. For the present discussion, it makes little difference which formulation is used. The assumption that molecular hydrogen and other reducing substances will reduce the intermediate peroxides, and not deliver their hydrogen directly to the pigment system, is all that is important. Considering the way in which photosynthesis changes to photoreduction and back again in green algae, it is logical to prefer a mechanism in which the part played by the photochemical system remains the same. The difference between

photoreduction and photosynthesis results, then, from the two ways by which the oxidized part of the photochemical oxido-reduction system is regenerated. It can be reduced either by hydrogen donors or by the removal of oxygen which finally appears as free gas.

We shall assume that the system of hydrogen carriers transferring the hydrogen from the donors to the photochemical mechanism is inactivated in air. Hence the absence of photoreduction under aerobic conditions in plants and in *Thiorhodaceae.* We have seen that in the presence of hydrogen donors only a sufficiently high concentration of oxygen is dangerous to the "reduced state" because of the rapid reduction of the oxygen to water. The question arises, as to how an excess of free oxygen, or of light, or both can bring about the inactivation of the "reduced state."

The inactivation of the reducing system can occur in two ways. Either by molecular oxygen directly or by an oxidized intermediate in both the dark or the light reaction. The experiments point in favor of the inactivating action of an excess of oxidized intermediates, or photoperoxides respectively. The mere presence of molecular oxygen in partial pressures up to 1 vol. per cent does not interfere with the course of the oxy-hydrogen reaction, if the latter proceeds fast enough, but the presence of very small amounts of cyanide which inhibit this reaction leads progressively to total inactivation. On the other hand hydroxylamine inhibits the liberation of oxygen under circumstances where photoreduction still can proceed, yet it does not prevent the inactivation of the reducing system at high light intensities.

The transition from the normal to the "reduced state" of the photochemical system and back can thus far be explained by the activation and inactivation of the hydrogen transferring system. We need not assume that the oxygen liberating catalyst is changed by anaerobic conditions. If the reduction of the peroxides is the much faster reaction, even an active "photocatalase" would have little chance to produce oxygen. One indication that it is still active is the fact that in Doudoroff's experiments with luminous bacteria a sub-critical intensity could, in the long run, produce luminescence. And we like to recall that Harvey (1928) found an instantaneous response of the luminous bacteria when he momentarily irradiated the alga, Fucus, previously kept for two hours under anaerobic conditions in hydrogen.

The main difference between the assimilatory system in plants and in photoactive bacteria would thus be that the latter have not the proper enzyme for decomposing the intermediate peroxides. Consequently in the absence of hydrogen donors or at very high light intensity, the photo-

peroxide in the purple bacteria will accumulate, and saturation will eventually be reached either because of back reactions or because of secondary inhibitions similar to those discussed in Chapter III.

This theoretical picture of photosynthesis in the presence and in the absence of oxygen appears to be consistent with the known facts, and is supported by many of them, but it is far from being complete. We need only mention that, as shown above, carbon monoxide and small concentrations of cyanide, neither of which inhibits aerobic photosynthesis, prevent not only the appearance of a photochemical reduction after the anaerobic incubation period, but also the normal resumption of oxygen evolution, an observation which suggests a closer coupling between the formation of the intermediate peroxides and the reduction of the carbon dioxide acceptor complex.

## VII. The Reduction of Carbon Dioxide in the Dark

For many decades microbes have been known which grow in surroundings completely devoid of organic material. In other words, they are capable of providing the necessary carbon by reducing carbon dioxide. These bacteria do not contain any photoactive dyes and can grow in the dark. The necessary energy for the reduction of carbon dioxide is provided by an oxidation reaction. It was found that several groups of autotrophic bacteria exist, which can be classified according to the inorganic material which is used as substrate for the oxidation reaction. (For reference see M. Stephenson (78).)

Some oxidize ammonia to nitrite and nitrate; others, hydrogen sulfide, sulfur, and thiosulfate, to a more oxidized sulfur compound. Again, others convert ferrous carbonate into ferric carbonate. The organisms which are capable of reducing the greatest amount of carbon dioxide per quantity of substrate oxidized seem to be the bacteria which use molecular hydrogen. As these autotrophic microbes, though ubiquitous, can be considered as rare organisms, they have been described as such in the textbooks of bacteriology and botany.

A decade ago nobody considered a possible similarity between carbon dioxide reduction in the dark by these microbes and photosynthesis in green plants. In the classical papers of Willstätter and Stoll, and of Warburg and co-workers, reduction of carbon dioxide in the dark is not mentioned. But since 1936 a number of papers have appeared in rapid succession showing that heterotrophic bacteria, supposed to assimilate the necessary carbon by respiring or fermenting organic material, are able to reduce

free carbon dioxide. Woods (79) found that the decomposition of formic acid into carbon dioxide and hydrogen in *B. coli* is reversible. Barker (80) described the reduction of $CO_2$ to methane in connection with the fermentation of aliphatic alcohols. Wood and Werkman (82) proved that propionic acid bacteria synthesize succinic acid from glycerol and carbon dioxide. These reduction processes are possible because the reduction of carbon dioxide is coupled with dissimilating processes which provide the chemical energy or, in other words, the "active" hydrogen. Therefore, Gaffron (83) wrote in 1937:

"These reactions prove that a chemical reduction of carbon dioxide is possible in principle in any living cell. The reduction does not depend on chlorophyll or radiant energy or on unique conditions like those prevailing with the iron and sulfur bacteria. Viewed in connection with our results this means that photosynthesis in green plants is perhaps the special case in which a reduction process requiring energy ends with a gain in energy only because that energy has not been furnished by the cell metabolism, but by the activated chlorophyll."

Since then it has been firmly established that carbon dioxide is not simply an end-product of any cell metabolism, but a metabolite, a substrate as well. A decisive step forward has been made with the introduction of radioactive carbon, as a new tool, in biological research.

In all those cases where the metabolism of a plant or animal cell produces carbon dioxide in amounts greatly surpassing the quantity that can be reduced, only the tracer method with a carbon isotope can detect the existence of a synthetic reaction involving carbonic acid.

With this method Ruben and Kamen (84) found a fixation of carbon dioxide in yeast, *B. coli*, barley root, ground rat liver, etc. Evans (85) demonstrates the formation of $\alpha$-keto glutarate from pyruvate and carbonic acid in pigeon liver and Hastings and co-workers (personal communication) observed that glycogen formed in liver slices from lactate in the presence of radioactive bicarbonate contained some of the active carbon.

These examples may suffice to demonstrate the point. The question arises whether the assimilatory system in green plants can produce the reduction of carbon dioxide in the dark as in the instances given above if the energy is provided by some respiratory or fermentation process in the cell. By reduction we do not mean, in this case, the reversible transformation of carbon dioxide into a carboxyl group in the acceptor molecule. This enzymatic dark reaction, discovered by Ruben, Kamen, and Hassid (31), does not proceed further once all possible carboxyl groups have been formed, unless the plant absorbs radiant energy. It is, of course, correct to say that carbon dioxide becomes reduced when a C—C bond is estab-

lished. But the energy change necessary to produce this important preliminary step must be rather small because it is so easily reversible. No coupling with another energy-yielding process is needed, contrary to what would be the case for a total reduction of carbon dioxide to the level of a carbohydrate which would require at least 110 Cal., not counting activation energies. These same experiments demonstrate that the normal respiration evidently is not used to provide the energy for a total reduction.

But the situation is different if we consider reduction reactions which take place in the dark under anaerobic conditions. Here a total reduction of carbon dioxide in the dark has been found. In the preceding chapters we have seen that in the "reduced state" of the photosynthetic mechanism the reduction of carbon dioxide causes oxidation of various hydrogen donors, particularly of molecular hydrogen. Under the same conditions the reverse is also true. The oxidation of hydrogen donors causes carbon dioxide to be reduced. It has been mentioned that at low partial pressures oxygen does not destroy the "reduced state." In the algae adapted to the use of molecular hydrogen the addition of oxygen will start the oxy-hydrogen reaction. A volume of hydrogen is taken up which at best reaches twice the volume of oxygen added, if other competing hydrogen donors do not interfere.

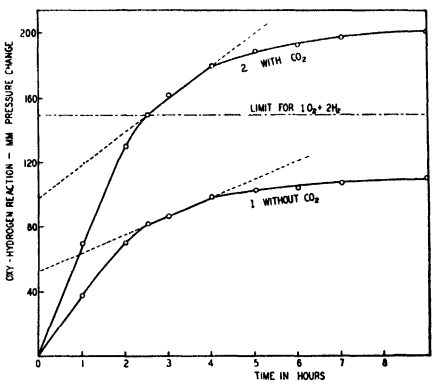



Fig. 13.—The course of the oxyhydrogen reaction with 50 mm. of oxygen in Scenedesmus. (1) In absence, (2) in presence of carbon dioxide. (According to Gaffron.)

But this simple relation exists only if carbon dioxide is absent. In the presence of this gas the reaction is accelerated and much more hydrogen disappears than can be accounted for by the formation of water.

An instance may be given: In a manometer vessel with a suspension of Scenedesmus adapted to hydrogen one might expect 50 mm. of oxygen to be consumed together with 100 mm. of hydrogen, leaving a total negative pressure of 150 mm. The actual course of the ensuing reaction is shown in Fig. 13.

Gas analyses have proved that simultaneously with the excess of hydrogen, carbon dioxide is also absorbed by the algae (86). The yield of the chemical reduction of carbon dioxide varies with the concentration of that

gas, with the hydrogen ion concentration in the suspension liquid, and also with other factors. The maximum increase in the uptake of hydrogen, due to the presence of $CO_2$, appears to be one "extra" molecule of hydrogen for each molecule of oxygen transformed to water. Not only does the total amount of hydrogen absorbed increase in the presence of carbonic acid, but also the rate is remarkably accelerated. In spite of the fact that the oxidative dark reduction of carbon dioxide competes with the photoreduction for the same hydrogen donor, it differs from the light reaction not only in that it begins with an uptake of molecular oxygen, but also in being

TABLE III

COMBINED INFLUENCE OF OXYGEN AND LIGHT ON THE HYDROGEN METABOLISM IN SCENEDESMUS D 3. THE MAXIMUM OF THE STEADY RATE OF HYDROGEN ABSORPTION IS THE SAME IN THE DARK AND IN THE LIGHT

Temperature, 25°. Algae suspended in $M/100$ $NaHCO_3$. Gas phase: 4% $CO_2$ in $H_2$. Preceding dark time, 12 hours. Rate of gas exchange in mm. pressure change/5 min.

| Initial rate of dark reaction | Mm. of $O_2$ added | Rate afterwards First 5′ | 30′ later | Final rate in the dark | Remarks |
|---|---|---|---|---|---|
| (*a*) Rate of oxyhydrogen reaction in the dark. | | | | | |
| ± 0 | 48 | − 8 | − 6 | ... | Normal |
| − 3 | 61 | −13 | −11 | ... | Normal |
| − 1.5 | 300 | −60 | − 3 | ... | Inactivated |
| (*b*) Rate of photoreduction in the light. Intensity 2000 lux. | | | | | |
| 0 | 0 | −10 | −11 | 0 | Normal |
| (*c*) Influence of reaction (*b*) on reaction (*a*). Intensity 2000 lux. | | | | | |
| − 5 | 0 | −13 | −12 | − 3 | Normal |
| −11 | 0 | −12 | .. | −14 | Only 10′ light, normal |
| −14 | 0 | −15 | + 1 | − 3 | Inactivated |

much more sensitive to cyanide. An attempt to describe the dark reaction in more detail would read as follows: The acceptor-carbon-dioxide-complex is oxidized by a molecule of oxygen in a reaction which occurs in two steps corresponding to the two oxygen atoms. The energy released by each of these steps will be used to activate a group in the complex so that it acts momentarily as a hydrogen donor. The restitution of the initial state, therefore, requires four hydrogen atoms to reduce the oxidized acceptor complex and two more to replace those which have been transferred from the intermediate hydrogen donors to carbon dioxide. These six hydrogen

atoms are ultimately derived from the specific donor in question—in our case, molecular hydrogen. Hence the possible yield cannot be more than 1 $CO_2$ for 2 molecules of oxygen absorbed.

It is not yet known whether the intermediate oxidation products of the oxyhydrogen reaction are the same as those formed photochemically. But it can be shown that both compete for the same hydrogen donors.

The maximum rate of hydrogen uptake before conversion occurs is the same for the photochemical and for the purely chemical reduction of carbon dioxide. Table III demonstrates that a combination of light plus oxygen is tolerated by the "reduced state" as long as the sum total of the rates of both light and dark reactions does not surpass the maximum mentioned.

As far as can be judged from these first results, the reduction of carbon dioxide in the dark with the aid of the oxyhydrogen reaction takes the same course in green algae as in Bacterium pycnoticum investigated by Ruhland (87). Also in this bacterium the oxidation of hydrogen continues in the absence of carbon dioxide and the ratio $-H_2/-O_2$ falls below 2. A difference lies in the sensitivity of the "reduced state" of the algae and hence of the reaction itself toward higher oxygen concentrations. This inactivation of the oxyhydrogen reaction by oxygen is not a specific property of the algae, however. Other bacteria show the same phenomenon (88, 89).

## Bibliography


1. O. Warburg and E. Negelein, *Z. physik. Chem.*, **102**, 235 (1922).
   O. Warburg and E. Negelein, *Ibid.*, **106**, 191 (1923).
2. W. M. Manning, J. I. Stauffer, B. M. Duggar, and F. Daniels, *J. Am. Chem. Soc.*, **60**, 266 (1938).
3. F. F. Rieke, *J. Chem. Phys.*, **7**, 238 (1939).
4. R. Emerson and C. M. Lewis, *Am. J. Botany*, **26**, 808 (1939).
5. R. Emerson and C. M. Lewis, AAAS Meeting, Seattle, June 20, 1940.
6. F. F. Rieke (still unpublished).
7. J. L. Magee, T. W. deWitt, E. Coolidge Smith, and F. Daniels, *J. Am. Chem. Soc.*, **6**, 3529 (1939).
8. H. T. Eichhoff, *Biochem. Z.*, **303**, 112 (1939).
   H. T. Eichhoff, W. Noddack, *Atti X° congr. intern. chem.*, **4**, 427 (1939).
9. C. S. French, *J. Gen. Physiol.*, **20**, 711 (1937).
10. J. G. Eymers and E. C. Wassink, *Enzymologia*, **2**, 258 (1938).
11. C. S. French, *J. Gen. Physiol.*, **21**, 71 (1937).
12. H. Gaffron, *Biochem. Z.*, **269**, 447 (1934).
    H. Gaffron, *Ibid.*, **279**, 1 (1935).
13. P. A. Roelofsen, *Proc. Acad. Sci. Amsterdam*, **37**, 660 (1934).
14. R. Willstätter and A. Stoll, "Untersuchungen über die Assimilation der Kohlensäure," Berlin, 1918.
15. F. F. Blackman, *Ann. Botany*, **19**, 28 (1905).

16. O. Warburg, "Über die katalytischen Wirkungen der lebendigen Substanz," Julius Springer, Berlin, 1928.
17. R. Pratt and J. F. Trelease, *Am. J. Botany*, **25**, 133 (1938).
R. Pratt, F. N. Craig, and S. F. Trelease, *Science*, **85**, 271 (1937).
18. R. Livingston and J. Franck, *Am. J. Botany*, **27**, 449 (1940).
19. K. Wohl, *Z. physik. Chem.*, **B37**, 169 (1937).
20. R. Emerson and W. Arnold, *J. Gen. Physiol.*, **15**, 391 (1932); **16**, 191 (1932).
21. W. Arnold, *Ibid.*, **17**, 145 (1933).
22. S. Weller and J. Franck (still unpublished).
23. W. Arnold and H. J. Kohn, *J. Gen. Physiol.*, **18**, 109 (1934).
24. H. Gaffron and K. Wohl, *Naturwissenschaften*, **24**, 81, 103 (1936).
25. R. Peierls, *Ann. Physik*, **13**, 905 (1932).
26. G. Scheibe and co-workers, *Z. angew. Chem.*, **49**, 563 (1936); **50**, 51, 212 (1937); *Naturwissenschaften*, **25**, 75, 474, 795 (1937); **26**, 412 (1938); *Kolloid-Z.*, **82**, 1 (1938).
Cf. also E. Jelley, *Nature*, **138**, 1009 (1936); **139**, 378, 631 (1937).
27. J. Franck and E. Teller, *J. Chem. Phys.*, **6**, 861 (1938).
28. D. Vermeulen, E. C. Wassink, and G. H. Reman, *Enzymologia*, **4**, 254 (1937).
Cf. also E. C. Wassink, D. Vermeulen, G. H. Reman, and E. Katz, *Ibid.*, **5**, 100 (1938).
E. C. Wassink and E. Katz, *Ibid.*, **6**, 144 (1939).
29. K. Wohl, *Z. physik. Chem.*, **B37**, 105 (1937); **B37**, 122 (1937); **B37**, 169 (1937); **B37**, 186 (1937); **B37**, 209 (1937).
K. Wohl, *New Physiologist*, **39**, 33 (1940).
30. L. S. Ornstein, E. C. Wassink, G. H. Reman, and D. Vermeulen, *Enzymologia*, **5**, 110 (1938).
31. S. Ruben, M. D. Kamen, W. Z. Hassid, and D. C. Devault, *Science*, **90**, 570 (1939); *J. Am. Chem. Soc.* **62**, 3443; 3450; 3451 (1940).
32. J. Franck and K. F. Herzfeld, *J. Chem. Phys.*, **5**, 237 (1937).
33. J. Franck and K. F. Herzfeld (still unpublished).
34. J. Franck and R. Livingston (still unpublished).
35. W. M. Manning, AAAS Meeting, Columbus, December, 1939.
Cf. also K. Mothes, I. Baatz, and H. Sagromsky, *Planta*, **30**, 289 (1939).
36. H. Aufdemgarten, *Planta*, **29**, 643 (1939); **30**, 343 (1939).
37. E. D. McAlister and J. Myers, *Smithsonian Misc. Coll.*, **99**, No. 6 (1940).
38. H. Kautsky, *Biochem. Z.*, **284**, 412 (1936).
39. J. Franck, C. S. French, and T. Puck (still unpublished).
40. W. J. V. Osterhout and A. R. C. Haas, *J. Gen. Physiol.*, **1**, 1 (1918).
41. F. van der Paauw, *Rec. trav. botan. Néerland.*, **29**, 497 (1932).
42. E. L. Smith, *J. Gen. Physiol.*, **21**, 151 (1937).
43. L. R. Blinks and R. K. Skow, *Proc. Nat. Acad. Sci.*, **24**, 420 (1938).
44. E. D. McAlister, *Smithsonian Misc. Collections*, **95**, No. 24 (1937); *J. Gen. Phys.*, **22**, 613 (1939).
45. H. Kautsky and co-workers, *Naturwissenschaften*, **19**, 964 (1931); **19**, 1043 (1931); **24**, 317 (1936); **26**, 576–577 (1938); *Ber.*, **65**, 1762 (1932); **66**, 1588 (1933); **68**, 152 (1935); *Biochem. Z.*, **274**, 423 (1934); **274**, 435 (1934); **277**, 250 (1935); **278**, 373 (1935); **302**, 1137–1166 (1939).
46. J. Franck and R. W. Wood, *J. Chem. Phys.*, **4**, 551 (1936).

47. H. Gaffron, *Naturwissenschaften*, **25**, 460, 715 (1937).
48. L. R. Blinks and R. K. Skow, *Proc. Nat. Acad.*, **24**, 413 (1938).
49. H. Gaffron, *Be .*, **60**, 755 (1927).
50. H. Gaffron, *Biochem. Z.*, **179**, 157 (1926).
51. K. Noack, *Z. Botan.*, **12**, 273 (1923); *Naturwissenschaften*, **14**, 385 (1926).
52. M. G. Stålfelt, *Planta*, **30**, 384 (1939).
53. G. O. Burr and J. Myers, *J. Gen. Physiol.*, **24**, 45 (1940).
54. R. Emerson, *Cold Spring Harbor Symposia*, **3**, 128 (1935).
55. F. van der Paauw, *Rec. trav. Botan. Néerland.*, **29**, 497 (1932).
56. J. Franck and C. S. French (still unpublished).
57. T. W. Engelmann, *Arch. ges. Physiol.*, **30**, 90 (1883).
58. C. B. van Niel, "Contributions to Marine Biology," Stanford Univ. Press, 1930, p. 161.
C. B. van Niel, *Arch. Mikrobiol.*, **3**, 1 (1931).
C. B. van Niel and F. M. Muller, *Rec. trav. Botan. Néerland*, **28**, 245 (1931).
F. M. Muller, *Arch. Mikrobiol.*, **4**, 131 (1933).
59. H. Gaffron, *Biochem. Z.*, **260**, 1 (1933); **269**, 447 (1934).
60. H. A. Spoehr, "Photosynthesis," Chem. Catalog Co., New York, 1926.
61. W. Stiles, "Photosynthesis," London, 1925.
62. C. B. van Niel, *Arch. Mikrobiol.*, **7**, 323 (1936).
63. H. Nakamura, *Acta Phytochim.*, **9**, 189 (1937).
64. E. N. Harvey, *Plant Physiol.*, **3**, 85 (1928).
65. C. B. van Niel, *Bull. de l'Association de Microbiol. Faculté de Nancy*, Number **13**, 3 (1936).
66. H. Gaffron, *Biochem. Z.*, **275**, 301 (1935).
67. S. Wessler and C. S. French, *J. Cellular Comp. Physiol.*, **13**, 327 (1939).
68. A. J. Kluyver and H. J. L. Donker, *Chem. Zelle Gewebe*, **13**, 134 (1926).
69. C. B. van Niel, *Cold Spring Harbor Symp., Quant. Biol.*, **3**, 138 (1935).
70. H. Gaffron (unpublished).
71. C. B. van Niel (unpublished).
72. T. Foster, *J. Gen. Physiol.* **24**, 123 (1940).
73. J. Franck and K. F. Herzfeld, *J. Phys. Chem.*, **41**, 97 (1937).
74. H. Gaffron, *Biochem. Z.*, **280**, 337 (1935).
75. H. Gaffron, *Amer. Journ. Bot.*, **27**, 204, 1940.
76. H. Gaffron, *Nature*, **143**, 204 (1939); *Am. J. Botany*, **27**, 273 (1940).
77. H. Nakamura, *Acta Phytochim.*, **9**, 220 (1937).
78. M. Stephenson, "Bacterial Metabolism," Longmans, Green and Co., London, 1939.
79. D. D. Woods, *Biochem. J.*, **30**, 315 (1936).
80. H. A. Barker, *Arch. Mikrobiol.*, **7**, 420 (1936); **7**, 464 (1936).
81. H. A. Barker, S. Ruben, and M. D. Kamen, *Proc. Nat. Acad. Sci.*, **26**, 426 (1940).
82. H. G. Wood and C. H. Werkman, *Biochem. J.*, **30**, 618 (1936); **32**, 1262 (1938); H. G. Wood and C. H. Werkman, *Biochem. J.*, **34**, 7 (1940).
83. H. Gaffron, *Biochem. Z.*, **292**, 269 (1937).
84. S. Ruben and M. D. Kamen, *Proc. Nat. Acad. Sci.*, **26**, 418 (1940).
85. E. A. Evans, and L. Slotin, *J. Biol. Chem.*, **136**, 805, (1940).
86. H. Gaffron. *Science*, **91**, 529 (1940).

87. W. Ruhland, *Jahrb. wiss. Botan.*, **63,** 321 (1924).
88. H. Wieland and H. T. Pistor, *Liebigs Ann.*, **522,** 116 (1936); **535,** 205 (1938).
89. O. B. Claren, *Ibid.*, **535,** 122 (1938).
90. R. Hill and R. Scarisbrick, *Nature*, **146,** 61 (1940).

# THE BACTERIAL PHOTOSYNTHESES AND THEIR IMPORTANCE FOR THE GENERAL PROBLEM OF PHOTOSYNTHESIS

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## I. Introduction

The following review presents a discussion of the experimental foundations and development of a generalized concept of photosynthesis. The original working hypothesis was the result of an attempt to reinterpret the metabolism of the green and purple sulfur bacteria on the basis of extensive qualitative and quantitative data. During the past decade various objections have been raised against its validity. A careful evaluation of the experimental evidence shows, however, that there is as yet no reason for abandoning this concept.

Kinetic studies on the metabolism of the purple bacteria have meanwhile been conducted. Also investigations on the chemical nature of the

pigment systems in purple and green bacteria have been published. An application of this knowledge to the problem of photosynthesis has resulted in a certain clarification of the fundamental reactions in this process. Although the final picture thus obtained is only a crude approximation, it serves the function of integrating a vast body of information.

## II. Early Studies on the Metabolism of the Purple Bacteria

In the beginning of the twentieth century three conflicting theories on the physiology of the purple bacteria existed side by side. Each one represented an attempt to explain the results of experiments which were carried out by different investigators, and with different objectives in mind.

Engelmann (1), in 1883, discovered a marked phototactic response of certain red-colored bacteria, also known as "purple" bacteria. A careful study of this phenomenon revealed characteristic similarities with the phototactic behavior of motile green algae which he had previously studied. So fundamental, indeed, was the resemblance that Engelmann was led to the conclusion that the physiology of the purple bacteria should be much the same as that of the algae. For the latter he had demonstrated a close correlation between phototaxis and photosynthetic activity. Hence the inference was drawn that the purple bacteria (*Bacterium photometricum*) were also able to photosynthesize. Inasmuch as this would imply the liberation of oxygen by the organisms upon illumination, experiments were carried out with a view to effectively demonstrating its evolution. But even Engelmann's sensitive "motile bacteria method"—so successfully used with algae—did not yield the expected results. A reinvestigation in 1888 (2) convinced Engelmann, however, of the photosynthetic nature of the organisms. Their phototactic responses, the fact that rapid growth was restricted to conditions including proper illumination, and the chemotactic accumulation of small, colorless spirilla around clumps of illuminated purple bacteria were considered satisfactory evidence in its favor.

The marked absorption of the bacteria in the infrared region of the spectrum, together with the phototactic accumulation in accordance with the absorption bands and with the effect of infrared irradiation on the "indicator spirilla" convinced Engelmann that

> "the idea, heretofore considered strictly valid, *that the evolution of oxygen by all plants is limited to the action of visual radiation, is wrong.* . . . The experiments with the purple bacteria have actually demonstrated that not only the visible but *also the dark rays can be active in producing oxygen, in causing assimilatory processes.*"

Meanwhile Winogradsky (3, 4) had embarked upon a study of the sulfur bacteria, in the course of which he hit upon an explanation of the role of the sulfur globules that at times fill up the cells to an astonishing extent. Culture experiments showed that the accumulation of sulfur droplets depends upon the presence of hydrogen sulfide in the external environment, that after the disappearance of this compound the sulfur inclusions also gradually vanish, that during this phase sulfate is excreted into the medium, and that the sulfur-free cells soon die off unless provided with a fresh supply of sulfide. On the basis of these observations Winogradsky postulated that the large supply of sulfur in the healthy organism served the function of a reserve of oxidizable material, and that the oxidation of hydrogen sulfide and of sulfur represented a substitute for the respiration of organic substances, characteristic for the normal functioning of most organisms. The oxidation of the sulfur compounds was thus interpreted as a means of providing the organisms with energy; and this concept also furnished an explanation for the abundant growth of the sulfur bacteria in a medium in which organic matter was practically lacking, such as hot-spring effluents. For it is clear that an organism which can supply its energetic requirements by the oxidation of an inorganic constituent of the environment can thereby eliminate the respiration of organic matter and thus may be in a position to use the latter exclusively for the building up of new cell material.

In Winogradsky's subsequent studies on similar microorganisms capable of oxidizing other inorganic compounds, the above concept was carried to its ultimate consequence; it provided for a mode of life in which organic matter could be entirely dispensed with, because the energy obtained from the oxidation of inorganic substances would enable the organism to reduce carbon dioxide and live autotrophically in darkness. Pfeffer later coined the name chemosynthesis for this group of processes for which Pringsheim, more recently, has proposed the term chemo-autotrophic.

The original experiments which led Winogradsky to his brilliant theory were carried out with non-pigmented sulfur bacteria, such as members of the genera *Beggiatoa* and *Thiothrix*. The communities of sulfur bacteria, found in nature, ordinarily comprise both colorless and purple or red organisms. At that time the chief interest in the latter group centered around the problem of their morphology, and Winogradsky conducted only a few essential experiments concerning their physiology. Yet, these again showed that hydrogen sulfide is required for the accumulation of sulfur globules in the bacteria; that the exhaustion of sulfide is followed by the gradual disappearance of the sulfur reserve, which, in turn, goes hand

in hand with the increase of sulfate in the environment; and that a gradual disintegration and death of the purple bacteria follow the prolonged exposure of the sulfur-free organisms to a medium not containing any sulfide. Consequently, Winogradsky did not hesitate to proclaim that fundamentally the physiology and metabolism of both colorless and red sulfur bacteria were identical.

Almost simultaneously, then, the first two theories, namely, those of Engelmann and Winogradsky, concerning the activities of the purple bacteria were developed, each on the basis of what seemed satisfactory and adequate evidence. During the following two decades it was primarily Winogradsky's concept that was further developed, mainly through studies with other types of chemo-autotrophic bacteria. Engelmann's work seemed all but forgotten.

Then, in 1907, Molisch published a monograph on the purple bacteria (5), in which Engelmann's and Winogradsky's experiments were criticized on the basis of many new findings.

The claim for demonstration of oxygen production was examined, and, most important, found wanting. Even with the luminous bacteria method, developed by Beijerinck as the—even today—most sensitive and most specific test for molecular oxygen, Molisch could obtain no evidence for the liberation of this gas by illuminated purple bacteria.

But also Winogradsky's concept of the metabolism of the purple bacteria was considered erroneous. This as a result of the successful isolation in pure culture of a number of purple bacteria which did not store sulfur and could not develop except in the presence of organic substances. The same behavior was claimed for such typical sulfur bacteria as the red *Chromatium*, although in this respect Molisch's experiments are clearly inconclusive, because the studies on (crude) cultures of a *Chromatium* species were made only in media with and without organic substrates and, in the latter case, never with added hydrogen sulfide.

Considering Engelmann's and Winogradsky's hypotheses thus disposed of, Molisch felt obliged to advance a third. This seemed necessary because the purple bacteria appeared to present features which did not permit of considering them as ordinary saprophytes. The unusual behavior of the organisms again showed itself in connection with light; Molisch expressed it as follows:

"It has thus been shown that light may influence the development of the purple bacteria more or less favorably. As far as my experiments go, light does not seem necessary for the development of pure cultures; many of these Rhodobacteria can grow quite well in darkness, especially in solid media. But in liquid cultures the favorable

influence is readily ascertainable. Particularly striking is the effect of light on cultures in water with decaying organic matter (hay, marine plants, animals) in which an abundant development of the purple bacteria appears conditioned by the presence of light."

Hence there exists a certain similarity in behavior between purple bacteria and algae; the latter can, in pure cultures, also be grown in darkness, provided organic substances are supplied. Nevertheless, in one point they differ fundamentally:

"the alga, developing in the light, does not require organic nutrients because it can assimilate carbon dioxide; the purple bacterium, as far as one can tell at the present moment, is always in need of an organic substrate, in darkness as well as in the light."

Thus the new thesis was developed: the purple bacteria cannot assimilate carbon dioxide, but they assimilate organic compounds in the light.

In order to understand the reasons for the controversial nature of the results obtained by the three investigators whose work has here been reviewed, it is necessary to realize clearly that they used different biological material for their investigations. Engelmann's studies were carried out with colored organisms; Winogradsky established the fundamentals of the metabolism of the sulfur bacteria by experiments on the colorless forms, and confirmed the main features for the purple representatives; while Molisch, as was first stressed by Buder (6, 7), used primarily a group of pigmented organisms which does not belong to the sulfur bacteria and is in part characterized by its inability to metabolize hydrogen sulfide, testing some of his conclusions by a few cursory experiments on sulfur-containing representatives. The following diagram may help to visualize this confused state of affairs.

| Group designation | Types of organisms contained in the group | Studied by |
|---|---|---|
| "Sulfur bacteria" | Colorless representatives | Winogradsky |
| | Purple (red) | Engelmann (Winogradsky; Molisch) |
| "Purple bacteria" | Purple (red), non-sulfur bacteria | Molisch |

It is also well to keep in mind that Winogradsky was already quite aware of the necessity of light for the development of his red bacteria. But the remarkable role and fate of hydrogen sulfide, so well established and understood for the colorless forms, led him to dismiss the possibility of a direct effect of illumination on the metabolism of the pigmented ones. Its function was interpreted as secondary; the purple bacteria, having anaerobic

tendencies, cannot be grown in the presence of air. Yet, for the oxidation of hydrogen sulfide and sulfur to sulfuric acid oxygen is needed, and this is supplied by small, photosynthetic, green bacteria during illumination, in small enough amounts so as not to harm the purple organisms. This explanation was completely adopted by Skene (8) who, in 1914, confirmed Winogradsky's experimental results.

On the other hand, Engelmann realized the importance of Winogradsky's studies on the fate of the sulfur globules, and used this in part to account for the negative outcome of many of his experiments on oxygen evolution. If the penetration of oxygen from outside were slow enough, the rate of sulfur oxidation might well exceed the rate of oxygen supply. The bacteria would then be using the oxygen produced intracellularly by photosynthesis, so that the evolution could not become apparent.

During the following twelve years each one of the three major hypotheses received some additional support, mostly in the form of confirmation of the earlier experimental results. Not until 1919 was an earnest attempt made to straighten out the existing confusion and to develop a more or less unified concept. This was done by Buder (6, 7) who, after having repeated much of the older work, integrated the controversial ideas of Engelmann, Winogradsky, and Molisch.

Buder's working hypothesis was founded on the following considerations:

1. The fundamental experiments of Engelmann leave no doubt that light fulfills an important function in the metabolism of the purple bacteria. Such a function can be most easily interpreted as permitting a photosynthetic mode of life. The occasional reports claiming the demonstration of oxygen evolution fit in well with this hypothesis.

2. The necessity of hydrogen sulfide or sulfur for the normal metabolism of the purple sulfur bacteria also cannot be denied. But, because the organisms generally are found living under conditions where free access of oxygen is excluded, they are unable to function chemosynthetically, unless they can supply themselves with oxygen. Thus is explained the necessity of both light (for photosynthetic oxygen production) and hydrogen sulfide (for the normal chemosynthetic process).

3. The non-sulfur purple bacteria, chiefly studied by Molisch, are not chemosynthetic, but respire organic compounds like most saprophytic microorganisms. By virtue of their ability to photosynthesize they can, however, carry out such respiratory activity under conditions where oxygen is not available; the oxygen produced photosynthetically is used by these organisms for the oxidation of organic substances rather than hydrogen sulfide.

Particularly interesting is the fact that the purple bacteria thus appear to have two sources of energy at their disposal, radiant as well as chemical energy. The ability to use the former enables them to exist under anaerobic conditions where similar non-pigmented, and hence non-photosynthetic organisms, cannot function.

Five years later Bavendamm (9), the first investigator to claim having obtained pure cultures of purple sulfur bacteria, confirmed the necessity of both hydrogen sulfide and light for their development, and subscribed to Buder's theoretical deductions.

However attractive this concept may seem, it contains an element which makes it impossible to accept the hypothesis in its original form. As long as both light and hydrogen sulfide (or organic substances) are considered essential to the development of the purple bacteria, one must admit the necessity for also assuming an intimate link between the photosynthetic and oxidizing reactions. Without such a link it should be possible to raise these organisms, either in the light and in the absence of oxidizable materials, or in the dark by providing a proper substrate and oxygen.

The existence of an intimate connection between photosynthetic activity and respiratory phenomena was first clearly expressed by Kluyver and Donker (10) in a discussion of the possibility of considering the metabolism of the purple sulfur bacteria as a process of hydrogen transference in which oxygen does not function as the ultimate hydrogen acceptor.

This concept has been the starting point for qualitative and quantitative studies on the metabolism of purple bacteria in the course of which new facts have been established which gradually have led to a more or less coherent picture.

## III. The Metabolism of Green and Purple Sulfur Bacteria as an "Abnormal" Photosynthetic Process

The development of simple and adequate methods for obtaining crude and pure cultures of purple sulfur bacteria (12, 13) has rendered these organisms ideal objects for some fundamental qualitative and quantitative experiments which clarify their metabolic peculiarities. They can be grown in strictly mineral media, containing hydrogen sulfide and bicarbonate, and such cultures prove that *development takes place under strictly anaerobic conditions but only when properly illuminated.* This result demonstrates conclusively the photosynthetic nature of the metabolism; the only carbon source from which the cell constituents can be manufactured is carbon dioxide, and these synthetic reactions are dependent upon light.

By using media with increasing sulfide concentration it can further be shown that the amount of growth is proportional to the quantity of sulfide (within the physiologically permissible range). In the absence of sulfide no growth can be observed. The precipitation of barium sulfate upon the addition of barium chloride and acid to culture media in which purple sulfur bacteria have developed proves the formation of sulfate during growth.

From these experiments it seems justified to draw the conclusion that the bacteria do not develop by means of ordinary photosynthesis in which carbon dioxide and water are converted into carbohydrate and oxygen. It is true that the oxygen might not appear because it is consumed during the conversion of sulfide into sulfate. But if this were so, the photosynthetic reaction should continue after the complete oxidation of the sulfur compounds, now with the liberation of molecular oxygen, and growth would not be proportional to and dependent upon the presence of sulfide.

A major obstacle to the assumption of an ordinary photosynthetic activity with secondary use of oxygen in respiration is, finally, the lack of oxygen production under all conditions. With dense cultures of purple bacteria, bottled up together with suspensions of luminous bacteria, not the least indication of oxygen evolution has ever been observed, regardless of whether or not the suspension liquid contains sulfide, or the bacteria sulfur inclusions.

Even more convincing are the results of quantitative experiments. The following Table I represents a summary (11, 12):

TABLE I

QUANTITATIVE RELATIONS BETWEEN $H_2S$, $H_2SO_4$, AND $CO_2$ IN ANAEROBIC, ILLUMINATED CULTURES OF SULFUR PURPLE BACTERIA

| Series | Analysis of culture after days | $H_2S$ oxidized, mg. | $H_2SO_4$ produced | | $CO_2$ used | |
|---|---|---|---|---|---|---|
| | | | Calculated, mg. | Found, mg. | Calculated, mg. | Found, mg. |
| I | 15 | 9.14 | 26.32 | 25.86 | 23.63 | 22.0 |
| | 22 | 9.14 | 26.32 | 25.90 | 23.63 | 22.8 |
| | 29 | 9.14 | 26.32 | 25.83 | 23.63 | 22.4 |
| | 36 | 9.14 | 26.32 | 25.87 | 23.63 | 21.6 |
| II | 27 | 8.8 | 25.5 | 24.7 | 22.9 | 20.7 |
| | 34 | 17.2 | 49.6 | 43.3 | 44.5 | 39.2 |
| | 42 | 18.7 | 53.9 | 51.4 | 48.4 | 46.8 |
| | 48 | 18.7 | 53.9 | 53.4 | 48.4 | 46.9 |
| III | 14 | 12.7 | 36.7 | 35.8 | 33.0 | 32.1 |
| | 24 | 24.9 | 71.5 | 70.9 | 64.2 | 63.8 |
| | 25 | 24.8 | 71.5 | 70.2 | 64.2 | 63.1 |
| | 47 | 24.8 | 71.5 | 71.4 | 64.2 | 63.9 |

It will be observed that the disappearance of carbon dioxide from the culture medium stops completely as soon as the oxidation of the sulfide has become complete. A further incubation, even for 21 days, does not lead to an increase in the amount of carbon dioxide used.

This dependence of carbon dioxide utilization on oxidation of hydrogen sulfide and sulfur can be approximately expressed by the equation

$$2CO_2 + 2H_2O + H_2S = \underset{2(CH_2O)^*}{\text{bacteria}} + H_2SO_4$$

and on the basis of this equation the calculated values for "$H_2SO_4$ produced" and "$CO_2$ used" have been computed.

From one other point of view the quantitative dependence of the photosynthetic carbon dioxide utilization on the oxidation of sulfide and sulfur deserves comment. The amount of synthesized cell material, expressed in moles of ($CH_2O$), is nearly twice as large as the quantity of oxidized sulfide. If the process were analogous to the chemosynthetic sulfide oxidation, one could at best expect one-eightieth this yield. This shows that, even if the photosynthetic reaction were to yield the oxygen for the oxidation of sulfide, and thus make chemosynthesis possible, still all of the photosynthetically converted carbonic acid is also used for cell syntheses. And this leads to the consequence that a consideration of the "chemosynthetic" reaction as of vital importance to the cell would then have to imply that it be essential for the synthesis of only one-eightieth of the material. Since both photo- and chemosynthetic reactions yielding cell material start with carbon dioxide it seems irrational to consider such a concept seriously.

Commonly accompanying the purple sulfur bacteria in nature, particularly in localities with high hydrogen sulfide concentration, are the green bacteria. Crude and pure cultures can readily be obtained by methods analogous to those used for the former group (12). Such cultures behave essentially in the same way as do the purple bacteria; development is restricted to anaerobic conditions in which both light and sulfide are available, and its extent is again proportional to the amount of sulfide. However, these organisms appear incapable of oxidizing the sulfide to sulfate; the oxidation seems to proceed only as far as sulfur. Also here a quantitative relationship between sulfide oxidation and the disappearance of carbon dioxide was disclosed, which can be expressed approximately by the equation

$$CO_2 + 2H_2S \longrightarrow (CH_2O) + H_2O + 2S.$$

Taking all these facts and deductions together one cannot escape the

* The symbol ($CH_2O$) is used in this paper to denote the conversion products of the carbon dioxide. It is, admittedly, a simplification. But, because in many of the experiments the end-product of the reaction consists of bacterial cells, and in others probably of some as yet unknown organic reserve product stored in the organisms, this first approximation has been decided upon mainly to serve as a guide.

conclusion that in the purple and green sulfur bacteria *the photosynthetic reaction depends entirely upon the presence of oxidizable sulfur compounds.*

How can such a dependence be understood? If one compares the equations for the photosynthetic process of green plants (1*a*, 1*b*) and green sulfur bacteria (2) a striking similarity is revealed.

$$CO_2 + H_2O \longrightarrow (CH_2O) + O_2, \text{ or} \tag{1a}$$

$$CO_2 + 2H_2O \longrightarrow (CH_2O) + H_2O + O_2 \tag{1b}$$

$$CO_2 + 2H_2S \longrightarrow (CH_2O) + H_2O + 2S \tag{2}$$

It is this comparison which has led to the hypothesis (11, 12) that the two reactions may be looked upon as examples of photochemical carbon dioxide reduction with different hydrogen donors, and that they would represent special cases of such a process which, in its most general form, could be expressed by the equation

$$CO_2 + 2H_2A \longrightarrow (CH_2O) + H_2O + 2A \tag{3}$$

On the basis of this equation photosynthesis obviously leads to oxygen production only when the function of hydrogen donor is fulfilled by $H_2O$; in all other cases the photosynthetic reaction, now better characterized as a photochemical carbon dioxide reduction, would depend upon the simultaneous dehydrogenation—and hence upon the presence—of some oxidizable substance $H_2A$.

The generalized equation (3) leaves open the possibility for the existence of photosyntheses in which neither $H_2O$ nor $H_2S$ serves as hydrogen donor. The complete oxidation of sulfide to sulfate by purple sulfur bacteria might be considered as furnishing examples of such reactions in which oxidizable sulfur compounds, intermediate between hydrogen sulfide and sulfate, were involved as hydrogen donors. Indeed, experiments with various types of purple sulfur bacteria showed that sulfur, sulfite, and thiosulfate can be used for photosynthesis (12), and the quantitative correlations are in close agreement with the equations:

$$1.5CO_2 + 4H_2O + S \longrightarrow 1.5(CH_2O) + 1.5H_2O + H_2SO_4, \tag{4}$$

$$CO_2 + 2H_2O + 2H_2SO_3 \longrightarrow (CH_2O) + H_2O + 2H_2SO_4, \text{ and} \tag{5}$$

$$2CO_2 + 5H_2O + Na_2S_2O_3 \longrightarrow 2(CH_2O) + 2H_2O + 2NaHSO_4 \tag{6}$$

In view of the chemical similarity of selenium and sulfur compounds it could be expected that oxidizable selenium compounds could also act as hydrogen donors in photosynthesis. Saposhnikov (15), in 1937, carried out experiments with purple sulfur bacteria in media containing selenium instead of sulfur. The results showed that in such systems a carbon dioxide reduction occurs concomitantly with an oxidation of selenium to selenate, in accordance with the equation

$$1.5CO_2 + 4H_2O + Se \longrightarrow 1.5\ (CH_2O) + 1.5H_2O + H_2SeO_4 \qquad (7)$$

The results represented in these various equations undeniably form a strong support for the usefulness of the generalized concept of photosynthesis. Nevertheless, it must be conceded that they could also be interpreted in a different way. The intimate connection of carbon dioxide utilization with the presence of oxidizable compounds might, namely, be attributed to the anaerobic nature of the organisms which would cause a cessation of a "normal" photosynthetic reaction with oxygen liberation as soon as the oxygen can no longer be disposed of by a respiratory process involving the oxidizable sulfur compounds. One could postulate a mechanism whereby the further production of oxygen is inhibited as soon as a small excess has accumulated by changing the redox potential in the cells, in line with the hypothesis advanced by Lipmann (16, 17) for an explanation of the Pasteur-effect (see also (18)). The completely negative result of tests for oxygen with luminous bacteria, both in suspensions of purple and of green bacteria, implies, however, that at no time can there be any excretion of oxygen outside the cell. Hence the interrelation of oxidation and photosynthesis must be an extremely close one. This point will be further discussed in connection with experiments on the kinetics of the bacterial photosyntheses.

## IV. Photosynthesis with Organic Hydrogen Donors and with Molecular Hydrogen

When the interpretation of the metabolism of the green and purple sulfur bacteria as a photochemical carbon dioxide reduction with hydrogen donors other than water was first proposed, it seemed not at all impossible that the behavior of the second group of purple bacteria, ordinarily found in media containing organic materials, could be considered as a counterpart to the former, in which the normal hydrogen donors would be represented by organic molecules (11, 12).

Such a possibility was not remote. Molisch's experiments on the favorable influence of light were, of course, not conclusive. But when it was demonstrated that representatives of this group of organisms behave in culture solutions much as do the purple sulfur bacteria, *viz.*, that development under strictly anaerobic conditions takes place only in the light, the analogy appeared plausible (19).

Quantitative experiments were, however, difficult to perform because the non-sulfur purple bacteria could not be grown in mineral media in which only one single, pure organic compound was present. The addition of complex

materials, peptone or yeast extract, was required for their development. Consequently the analysis of such cultures was too difficult. But when it was found that the purple sulfur bacteria can be cultured in mineral media to which, instead of oxidizable sulfur compounds, simple organic acids are added, a quantitative investigation of such cultures became realizable.

The notable results of such experiments, published by Muller (20), can be summarized as follows: In the first place growth appeared to be extremely profuse, considering the anaerobic conditions and the low concentrations of organic acids used. This soon became understandable when it was shown that in such systems the organic compound is almost completely converted into cell material. A careful search failed to produce evidence for the formation of anything but cell substance and some carbon dioxide. The amount of the latter depended in a regular manner upon the composition of the acid used; the higher the oxygen content of the organic compound, the larger the amount of carbon dioxide. *E. g.*, with acetic and lactic acid (53.3% oxygen) the total carbon dioxide production amounted to 13–14% of the weight of substrate utilized; for malic acid (59.6% oxygen) it rose to about 40%.

This behavior becomes understandable if one considers the chemical composition of the cell material produced. Analyses of purple bacteria (21) have shown that the over-all composition on a dry weight, ash-free basis corresponds to 55.7% C, 7.4% H, 15.1% O, and 11.8% N. This means that they represent organic matter considerably more reduced than carbohydrate. Hence, in the conversion of more oxidized substances some carbon dioxide must be "left over."

Substrates more reduced than acetic or lactic acid are also converted into cell substance. In such cases, however, the transformation always involves a simultaneous uptake of carbon dioxide. Distinct already in the case of propionic acid, this phenomenon assumes considerable proportions in the assimilation of butyrate. The average carbon dioxide uptake here amounts to 0.75 mole per mole of butyrate used.

Although these results can readily be interpreted on the basis of a photochemical carbon dioxide reduction with the aid of organic hydrogen donors, the experiments so far reported do not prove this point. Additional evidence can be adduced from the behavior of the bacteria in butyrate media with varying amounts of carbon dioxide. Muller has shown that cultures in butyrate media without special addition of sodium bicarbonate attain only very slight development. Unpublished experiments of the reviewer have yielded the significant result that, up to a certain limiting bicarbonate concentration which depends upon the initial butyrate concentration, the amount of butyrate decomposed by purple sulfur bacteria is a function of the quantity of carbon dioxide originally present. This demonstrates convincingly that butyrate utilization depends upon the presence of carbon dioxide. But the actual mechanism is not, of course, deducible from these experiments.

Meanwhile, Gaffron had been studying the metabolism of a non-sulfur purple bacterium identified as *Rhodovibrio parvus* Molisch. By using an entirely different experimental approach, the above-mentioned difficulties, due to the required addition of complex materials to the medium for culturing the organism, could be eliminated. The experiments were carried out with so-called "resting bacteria," grown previously in yeast extract media and suspended in solutions of known composition. The metabolism of such suspensions was measured with the manometric technique, which Warburg first had used for studies on photosynthesis of green algae. (For a discussion of methods see, *e. g.*, Gaffron (22, 23, 24).)

A suspension of *Rhodovibrio* cells in dilute bicarbonate solution, illuminated in an atmosphere of nitrogen or argon with 5% $CO_2$, does not cause a measurable gas exchange. If to such a suspension a fatty acid salt is added a rapid uptake of carbon dioxide ensues. In general the rate of this uptake remains practically constant as long as the fatty acid has not been completely decomposed. It ceases abruptly at the moment when the substrate has been utilized. No metabolism of fatty acids under anaerobic conditions could be detected in the dark.

Thus the results of these experiments are entirely analogous to those earlier described for cultures of purple sulfur bacteria. In the absence of an oxidizable substance photosynthesis cannot be detected; it starts as soon as such a substrate becomes available, and again ceases with its disappearance.

TABLE II

CARBON DIOXIDE UPTAKE BY SUSPENSIONS OF *Rhodovibrio parvus* FOLLOWING THE ADDITION OF THE SODIUM SALTS OF VARIOUS FATTY ACIDS, IN MOLES OF $CO_2$ PER MOLE OF FATTY ACID

| Substrate | Number of carbon atoms | Carbon dioxide uptake | |
|---|---|---|---|
| | | Total | Assimilated* |
| Acetic acid | 2 | 0.85–0.89 | −0.25–−0.11 |
| Propionic acid | 3 | 1.29–1.42 | 0.29–0.42 |
| *n*-Butyric acid | 4 | 1.30–1.43 | 0.30–0.43 |
| *i*-Butyric acid | 4 | 1.61 | 0.61 |
| *n*-Valeric acid | 5 | 1.62–1.90 | 0.62–0.90 |
| *i*-Valeric acid | 5 | 1.83 | 0.83 |
| Me.-Eth.-Acetic acid | 5 | 1.68–1.91 | 0.68–0.91 |
| *n*-Caproic acid | 6 | 1.90–2.34 | 0.90–1.34 |
| *i*-Caproic acid | 6 | 2.10–2.30 | 1.10–1.30 |
| Heptylic acid | 7 | 2.03–2.54 | 1.03–1.54 |
| Caprylic acid | 8 | 2.69–2.96 | 1.69–1.96 |
| Nonylic acid | 9 | 2.90–3.90 | 1.90–2.90 |

* The values in this column are only approximations; they have been derived from those in the column to the left.

In complete agreement with Muller's results is also the fact that Gaffron's search for organic excretory products was entirely negative; the only metabolic products that could be detected were the bacteria themselves.

Of particular importance is the fact that the technique described enabled Gaffron to investigate the fate of a large number of organic acids. He established that the absorption of carbon dioxide increased with the chain-length of the fatty acid; up to nonylic acid, $C_9H_{18}O_2$, this increase was fairly regular, for still higher members of the series the results became less reproducible, and more irregular (23, 24). Table II contains a summary of his results.

The measured carbon dioxide uptake in these experiments is the net result of two different processes. It has been stated above that no organic excretory products could be detected after the fatty acid had been utilized. The conversion of the substrate into bacterial substance, with the simultaneous uptake of carbon dioxide, also results, however, in the gradual accumulation of alkali in the medium, because it is the fatty acid, and not the sodium salt, that is ultimately transformed into organisms. The alkali accumulation causes a secondary, purely chemical uptake of carbon dioxide from the gas phase; when the latter contains 5% $CO_2$ the alkali will finally be present as bicarbonate. Assuming, for the sake of simplicity, that the newly formed cell substance be neutral, then the purely chemical carbon dioxide uptake would amount to approximately one mole per mole of fatty acid salt decomposed.* Thus the actual carbon dioxide assimilation (or production in the case of acetate) can be computed from the total uptake by subtracting one mole per mole of fatty acid salt decomposed. The values thus obtained are those in the last column of the table.

The trend of these figures is clear; but the actual data for each acid vary rather considerably. This led me, in 1935 (14), to suppose that the irregularities between fatty acids, first observed and stressed by Gaffron (24), might perhaps disappear with a further accumulation of experimental data. Since that time a large number of similar experiments have been carried out (unpublished), the results of which show that with cultures of *Spirillum rubrum* Esmarch, another member of the non-sulfur purple bacteria group, the remarkable discrepancy between acetate, propionate, and butyrate does not exist.

Table III summarizes the results.

* The exact amount will be somewhat less because the solution of a sodium salt of a fatty acid, in equilibrium with a gas phase containing 5% $CO_2$, must contain some of the base in the form of bicarbonate already.

TABLE III

SUMMARY OF DATA ON $CO_2$ PRODUCTION AND ASSIMILATION BY *Spirillum rubrum* ESMARCH WITH ACETATE, PROPIONATE, AND BUTYRATE, IN MOLES PER MOLE OF SUBSTRATE

| Substrate | No. of expts. | $CO_2$ production (+) or assimilation (−) | |
|---|---|---|---|
| | | Average | Range |
| Acetate | 48 | +0.205 | +0.148–0.276 |
| Propionate | 20 | −0.312 | −0.232–0.410 |
| *n*-Butyrate | 15 | −0.653 | −0.446–0.715 |

From all the existing data an average carbon dioxide assimilation of about 0.4 mole per "mole" of $\begin{matrix}|\\CH_2\\|\end{matrix}$ can be computed (cf. also (14)). This result makes it clear that in suspensions of non-dividing cells as well as in growing cultures the assimilation proceeds to products more reduced than carbohydrate. Since nothing more definite is known about the nature of these substances (see also later) it is at present most convenient to express the relationship by the approximate equation

$$2\left(\begin{matrix}|\\CH_2\\|\end{matrix}\right) + H_2O + CO_2 \longrightarrow 3(CH_2O), \text{ or} \tag{8}$$

$$2\left(\begin{matrix}|\\CH_2\\|\end{matrix}\right) + 4H_2O + CO_2 \longrightarrow 3(CH_2O) + 3H_2O \tag{8a}$$

In discussing Muller's results it was stated that, although the data could readily be interpreted in terms of a photochemical carbon dioxide reduction with organic hydrogen donors, they do not, in themselves, constitute definite evidence for such a concept. Gaffron's investigations, on the other hand, furnish an argument which decidedly points in that direction.

The development of this argument becomes even more convincing in view of the demonstrations that the purple bacteria can also assimilate carbon dioxide with molecular hydrogen. Roelofsen first showed this reaction for purple sulfur bacteria (25, 26); Gaffron soon afterwards reported it for sulfur-free forms ((24); see also French (27, 28)). The quantitative measurements of the amount of carbon dioxide reduced per mole of hydrogen vary somewhat, as could have been expected from the results obtained with the fatty acids, but in general they are in fair agreement with the approximate equation

$$CO_2 + 2H_2 \longrightarrow (CH_2O) + H_2O \tag{9}$$

Table IV represents the most important data.

TABLE IV

CARBON DIOXIDE ASSIMILATION BY PURPLE BACTERIA WITH MOLECULAR HYDROGEN

| Organism | Group | Ratio $H_2/CO_2$ | Investigator |
|---|---|---|---|
| *Rhodovibrio parvus* | Non-sulfur purple bacteria | 1.85–2.25 | Gaffron (24) |
| *Streptoc. varians* | Non-sulfur purple bacteria | 2.6 | Wessler and French (28) |
| *Streptoc. varians* | Non-sulfur purple bacteria | 2.2 –2.6 | van Niel (unpubl.) |
| *Chromatium* spec. | Sulfur purple bacteria | 2.4 | van Niel (21) |

There can be no doubt concerning the fundamental interpretation of this reaction. Inasmuch as hydrogen uptake is strictly limited to the presence and simultaneous disappearance of carbon dioxide, and does not proceed except in the light, the process must be regarded as a photochemical carbon dioxide reduction with hydrogen as the reducing agent. From unpublished results of culture experiments it appears, furthermore, that the non-sulfur purple bacteria are capable of performing the synthesis of their cell materials from carbon dioxide.* This having been established, one must admit that the actual assimilation of carbon dioxide in the presence of various fatty acids, particularly in view of the observed regularity (equations 8 and 8*a*), is likely to follow the same path. There is no reason to assume a fundamentally different mechanism for the production of the same compounds (bacterial cells) from carbon dioxide in the presence of hydrogen and in the presence of fatty acids. If it is then borne in mind that the degradation of the fatty acids has been established as an enzymatic "dark reaction" (see section VIII–X), and that there exists no evidence for such degradations being other than dehydrogenations, the evidence for the concept that the photosynthesis of purple bacteria in organic media is fundamentally a photochemical carbon dioxide reduction with organic hydrogen donors is fairly convincing.

Still, the evidence is only indirect. A convincing demonstration of this principle could be hoped for only if it were possible to find a system in which the organic substrate, during the metabolism of the purple bacteria, becomes converted into a product with the original carbon skeleton unaltered. This type of conversion is most frequently encountered in the microbial decomposition of alcohols and sugars (molds, acetic acid bac-

* These experiments have been carried out in mineral media containing such small quantities of yeast extract that in the absence of hydrogen no visible growth could be detected.

teria!). Foster (29) has recently discovered that secondary alcohols admirably meet this requirement. They are photosynthetically converted into the corresponding ketones, concomitantly with the assimilation of carbon dioxide. The reaction equation for the conversion of isopropanol into acetone:

$$CO_2 + 2CH_3CHOHCH_3 \longrightarrow (CH_2O) + H_2O + 2CH_3COCH_3 \qquad (10)$$

has been firmly established both by chemical analyses of growing cultures and by manometric measurements (Table V).

TABLE V

ASSIMILATION OF $CO_2$, DISAPPEARANCE OF ISOPROPANOL, AND FORMATION OF ACETONE IN ANAEROBIC CULTURES OF A STRAIN OF PURPLE BACTERIA DURING ILLUMINATION

| $CO_2$ uptake in moles | Isopropanol disappeared in moles | Acetone produced in moles | Molar ratios | |
|---|---|---|---|---|
| | | | Isopropanol: $CO_2$ | Acetone: Isopropanol |
| 15 45 | 34.19 | 35.00 | 2.22 | 1.02 |
| 24.10 | 48 85 | 51.70 | 2.02 | 1.06 |
| 38.40 | 77.00 | 78.45 | 2.00 | 1.02 |
| 39.10 | 82.90 | 82.50 | 2.12 | 1.00 |
| 45 70 | 90.20 | 91.65 | 1.97 | 1.02 |
| 56.00 | 110 40 | 113.00 | 1.97 | 1.02 |

This investigation thus furnishes the first incontrovertible and direct evidence for the role of an organic substrate exclusively as hydrogen donor for photochemical carbon dioxide reduction.

Does it follow that in the carbon dioxide assimilation with fatty acids and other organic substances the organic substrate is only hydrogen donor? Such a conclusion would seem premature. It is entirely conceivable that during the progressive dehydrogenation of the substrate intermediate products are formed which can and will be used directly in synthetic reactions. The occurrence of such processes has been made very probable by experiments to be discussed later.

Molisch's monograph on the purple bacteria leaves one with the impression that the best media for culturing the organisms are those that contain both peptone and glycerin or dextrin. Similar statements can be found in the publication by Schneider (30) on the physiology of the non-sulfur purple bacterium *Rhodobacillus palustris* Molisch. In view of these remarks one would be led to expect the purple bacteria, or at least the non-sulfur group, to show especially marked activity in the presence of sugars and glycerin. Yet this is far from true. Gaffron has stressed that,

for *Rhodovibrio*, organic acids seem to be the most readily attackable compounds. This holds true for all strains of both sulfur and non-sulfur purple bacteria which I have so far examined. The lack of agreement is primarily the result of studies with impure cultures; in such media as advocated by Molisch and Schneider a varied flora of non-photosynthetic bacteria develops with great rapidity, and under anaerobic or semi-anaerobic conditions these microorganisms convert the glycerin and carbohydrates chiefly into fatty and simple hydroxy acids. It is thus as a source of these acidic compounds rather than on their own intrinsic merit as foodstuffs that these substances are beneficial. In studies with pure cultures the favorable influence has not been noted.*

On the other hand, it is not true that the non-sulfur purple bacteria can use only fatty and simple substituted mono- and dibasic acids, as claimed by Gaffron. A large number of experiments with crude and pure cultures have been carried out during the past ten years. The results obtained all indicate that the ability to attack various groups of substances differs widely among the different species of the purple bacteria. This aspect of the physiology of the group will be dealt with in detail in another publication.

Even a strict separation of the sulfur and non-sulfur purple bacteria on the basis of the utilizability of oxidizable sulfur compounds for their photosynthetic process cannot be carried out. Several of the species of typically non-sulfur purple bacteria are capable, under the appropriate conditions, of oxidizing thiosulfate, and of developing in thiosulfate media. It is true that in all these cases no development has been obtained in entirely mineral media; the addition of small amounts of yeast extract, presumably as a source of growth factors, is required.

In general, however, a convenient subdivision of the purple bacteria into two main groups—for which Molisch in 1907 proposed the names *Thio-* and *Athiorhodaceae*—is possible. In addition, the photosynthetic bacteria also include the group of green bacteria. Up to the present time there is no evidence to show that the last-mentioned group is capable of using organic compounds instead of hydrogen sulfide for a photochemical carbon dioxide

* Schneider claims to have used a pure culture of *Rhodobac. palustris* for his investigation. Because his results were so much at variance with my own, I wrote to him for subcultures which were most kindly placed at my disposal. A microbiological analysis of these cultures soon made it clear that they were in no sense pure. They contained not only three different representatives of the non-sulfur purple bacteria (*Rhodospirillum*, *Rhodovibrio*, and *Rhodobacillus*), but in addition various species of lactic acid bacteria, aerobic and anaerobic spore formers, bacteria of the coli-aerogenes group, and *Pseudomonas* species. Thus the results of his investigation could easily be duplicated, but the interpretation becomes entirely different.

reduction. The chief characteristics of the three subgroups of photosynthetically active bacteria can be summarized briefly as follows (Table VI).

TABLE VI

SUMMARY OF CHARACTERISTICS OF THE THREE GROUPS OF PHOTOSYNTHETIC BACTERIA

| Organisms | Description of main features |
|---|---|
| Green bacteria | Green-colored bacteria, occurring in hydrogen sulfide media. Photosynthetic activity seems restricted to a photoreduction of carbon dioxide with hydrogen sulfide as hydrogen donor. Oxidation proceeds only to elementary sulfur. Other sulfur compounds and organic substances not used as hydrogen donors. Organic growth factors not required. |
| Sulfur purple bacteria (*Thiorhodaceae* Molisch) | Purple to red-colored bacteria, occurring primarily in sulfide-containing media. Capable of oxidizing various inorganic sulfur compounds to sulfate with the simultaneous photoreduction of carbon dioxide. Various organic substances, particularly the lower fatty acids, and some hydroxy and dibasic acids, can be used as hydrogen donors instead of $H_2S$. Some species can also use molecular hydrogen. Organic growth factors not required. |
| Non-sulfur purple bacteria (*Athiorhodaceae* Molisch) | Purple, red, or brown-colored bacteria, occurring principally in media containing organic compounds. Photochemical reduction of carbon dioxide with a large number of different organic substances; some species capable of using molecular hydrogen for photosynthesis. Although some species are also capable of oxidizing inorganic sulfur compounds to sulfate, growth is dependent on the presence of small amounts of complex organic materials, such as yeast extract, which presumably furnish necessary organic growth factors. |

It is entirely possible that organisms with characteristics intermediate between these groups can be found. This, however, is a problem for the systematist, and a more detailed discussion falls outside the scope of this review.

Summarizing the chief points of the discussion in this chapter, it may be stated that the evidence presented seems to justify the conclusion that the metabolism of the *Athiorhodaceae* closely resembles that of the *Thiorhodaceae*. Both groups of organisms are photosynthetic, but the former uses primarily simple organic substances as hydrogen donors for the photoreduction of carbon dioxide. The generalized equation for photosythesis

$$CO_2 + 2H_2A \longrightarrow (CH_2O) + H_2O + 2A$$

thus has been useful in an attempt at interpreting the metabolism of the purple bacteria in organic media.

## V. Objections to the General Concept of Bacterial Photosyntheses, and an Evaluation of the Evidence

In the preceding chapters the metabolism of the green and purple bacteria has been interpreted as a photosynthetic carbon dioxide reduction depending upon the simultaneous dehydrogenation of special hydrogen donors. It has been shown that the experimental results so far considered readily fit in with this hypothesis. Nevertheless, it must be admitted that during the past few years some objections have been raised, and different explanations have been proposed.

In 1936 Czurda (31) claimed to have demonstrated the photosynthetic production of oxygen by purple sulfur bacteria. The evidence presented for his claim consisted in the demonstration that in media containing both sulfide and leuco indigo carmin the dye was converted into the oxidized form after the cultures had been illuminated for some time and the sulfide had disappeared.

The experimental result can be undoubtedly interpreted as due to the production of oxygen by illuminated colonies of the purple bacteria. It is not, however, the only possible interpretation. In 1931 the same observation had already been reported (12), but it was then considered as the first example of photochemical carbon dioxide reduction with the aid of organic, easily oxidizable substances rather than as proof for the evolution of oxygen. Especially if it is borne in mind that the specific and extremely sensitive luminous bacteria method for the detection of oxygen has invariably yielded negative results, a decision between the two alternative explanations seems to favor the latter. This has, furthermore, been substantiated by experiments in which the reduced indigo carmin and the bacteria were kept spatially separated. Under such conditions a re-oxidation of the dye during illumination of the organisms could not be observed (21, 32). It should be mentioned that Czurda soon afterwards gave up his own interpretation in favor of the other (33).

A second claim for oxygen production was made by Nakamura (34) on even more indirect evidence. Having observed the development of *Rhodobacillus palustris* in the dark, but only in the presence of oxygen (see section IV), this investigator measured the respiration of resting bacteria both in the light and in darkness. It appeared that the rate of oxygen consumption was decreased by illumination. Nakamura's deductions from this result are as follows: Inasmuch as a certain amount of oxygen is needed for respiratory purposes, and the consumption of oxygen is diminished by illumination, it is clear that the difference is supplied by photosynthesis.

One thus finds in Nakamura's tables data concerning the rate of oxygen production, calculated from the difference in oxygen utilization in light and darkness.

It will by now be obvious that, although Nakamura's explanation is a possible one, it may by no means be considered as the only, or even as the most satisfactory one. The frequently reiterated objection of the failure to detect oxygen production by any direct method holds good also in this instance. The fact that a given substrate can be dehydrogenated in the dark with oxygen as the ultimate acceptor, while in the light carbon dioxide can fulfill this role (see section IV) is no evidence in favor of the production of oxygen in the light. If an aerobic bacterium is allowed to respire in the presence and in the absence of quinone, one generally finds that the rate of oxygen uptake is less where the second acceptor is also present. This is readily understandable, and no one would deduce from such observations that the organism produces oxygen from quinone. Yet the analogy is sound, because it is known that in the light, carbon dioxide can be used by purple bacteria as hydrogen acceptor.

Nakamura's interpretation implies that in the light the rate of photosynthesis, *i. e.*, "oxygen production," is smaller than the rate of oxygen consumption in the dark. Otherwise, oxygen production by illuminated purple bacteria should exceed its consumption, and thus be directly demonstrable. In a later section it will be shown, moreover, that ordinarily the rate of photosynthesis is limited by the substrate dehydrogenation. By special treatments photosynthesis can be made to proceed—only in the presence of oxidizable substances—much faster than corresponds with the rate of oxygen consumption in the absence of substrate. Many of Nakamura's studies were carried out with suspensions to which no substrate had been added. This shows sufficiently clearly, then, that photosynthesis, if it were not entirely dependent on the presence of extraneous hydrogen donors, should manifest itself by the liberation of oxygen in most cases, and that the evolution of oxygen would long ago have been established beyond a doubt.

At first sight it seems curious that in many instances this investigator did not observe a depression in the rate of oxygen uptake of *Rhodobacillus* suspensions when illuminated. This is particularly striking in the experiments with lower fatty acids (cf., *e. g.*, his Table 13). It must be pointed out, however, that these compounds are not utilized under anaerobic conditions in the light except in the presence of carbon dioxide. And in his experimental set-up this was excluded by the presence of potassium hydroxide in the containers in which the respiration was measured. With substances

such as malic and pyruvic acid a carbon dioxide production takes place (see section IV). Because the acids were used as potassium salts, this carbon dioxide will remain in the solution as carbonate and thus provide the organisms with a source of carbon dioxide. Even under these conditions, however, the photosynthetic mechanism of the bacteria in Nakamura's experiments did not display its maximum capacity. It has, namely, been ascertained (section VIII) that in suspensions of purple bacteria with strong respiratory activity the respiration is completely inhibited by proper illumination.

From these considerations it follows logically that the previously advanced concept of the photosynthetic metabolism of the purple bacteria is not in any way invalidated by Nakamura's results. They find a more coherent explanation on the basis of a competitive acceptor-action of both oxygen and carbon dioxide in the light.

Of an entirely different nature are the objections raised by Gaffron (35, 36) against the interpretation of the metabolism of the purple sulfur bacteria in the presence of organic substrates. His experiments led him to believe that these organisms do not use the organic molecule directly in the photoreduction of carbon dioxide, but that they can photosynthesize only with hydrogen sulfide, or other oxidizable, inorganic sulfur compounds. The actual disappearance of the organic substances, together with the growth of the bacteria in their presence, was accounted for in the following manner.

While in the light a photosynthetic reaction occurs according to the equation

$$2CO_2 + 4H_2O + H_2S \longrightarrow 2(CH_2O) + 2H_2O + H_2SO_4,$$

the bacteria would, in the dark, be able to reverse this process, just as the green plants in darkness carry out a respiration which is the exact opposite of their photosynthetic metabolism. If extraneous sources of organic matter are present, the decomposition with sulfate reduction of cell materials would be inhibited and replaced by a similar oxidation of the substrate. Thus the latter would be oxidized to carbon dioxide, at the same time giving rise to an equivalent amount of hydrogen sulfide. Also in the light the sulfate reduction would proceed under the influence of supplied organic matter, but here the sulfide would by photosynthesis be converted into sulfate, concomitantly with the formation of cell material from carbon dioxide. In this manner, therefore, the assimilation with organic substrates would bear no relation to that of the *Athiorhodaceae*, but be essentially the same as in the presence of hydrogen sulfide.

The experimental results of Muller naturally can be explained equally well along these lines as on the basis of the concept discussed earlier. Yet, the latter implies a close relationship in the metabolism of the two groups of purple bacteria, which, in case Gaffron's interpretation were accepted, would altogether disappear. Roelofsen, in reinvestigating this problem, could not confirm Gaffron's results (25, 26). This led him to the conclusion that the formation of hydrogen sulfide in the latter's experiments must have been caused by contaminating sulfate-reducing bacteria. In 1935 (14) I accepted Roelofsen's conclusion without further experimental evidence, as did also Bavendamm (37). However Gaffron's second publication on the subject (36) could not be so easily dismissed. In consequence new experiments were carried out (21) which have shown conclusively that the purple sulfur bacteria are unable to reduce sulfates, as had already been shown by Winogradsky (cf., *e. g.*, (38)). Furthermore, it appeared that the influence of sulfate on the assimilation of organic substances, as reported by Gaffron, is reproducible only within quite narrow limits of bicarbonate concentration of the solution in which the bacteria are suspended, and, under such circumstances, is also exerted by inorganic salts other than sulfate, which obviously cannot lead to a sulfate reduction. In short, this study demonstrated convincingly that Gaffron's explanation had to be abandoned in favor of the one proposing a direct dehydrogenation of the organic substrate whith carbon dioxide as acceptor.

Recently Nakamura has again claimed the occurrence of a sulfate reduction by *Thiorhodaceae* (39). The conclusion is based upon the demonstration that in an atmosphere of hydrogen the bacteria cause a diminution in pressure in the presence of sulfate, whereas such a pressure change was not observed with suspensions to which no sulfate had been added. Also the addition of elementary sulfur did not cause a hydrogen uptake. The last-mentioned fact is emphasized because suspensions of the non-sulfur purple bacterium *Rhodobacillus palustris* do cause an uptake of hydrogen in the presence of free sulfur.

It must be observed that the mere demonstration of hydrogen uptake in the presence of sulfate does not constitute a proof for the occurrence of sulfate reduction. No data are presented to show the formation of hydrogen sulfide. The only experiments which I had previously conducted in connection with the problem of sulfate reduction by sulfur purple bacteria were carried out with organic substrates, but not with hydrogen. The formation of hydrogen sulfide by these bacteria from molecular hydrogen had been shown to occur, but only with cells containing large amounts of sulfur globules, and in the absence of sulfate. This formation of hydrogen sulfide was interpreted by me as a phytochemical reduction, *i. e.*, a side path of the dark metabolism, rather than as a quantitatively important process. It is, therefore, astonishing that Nakamura failed to obtain evidence for hydrogen uptake by purple sulfur bacteria which, considering the culture conditions, should have contained sulfur globules. For the rest, also in his experiments, the rate of hydrogen uptake is quantitatively insignificant as compared with the normal rate of dark metabolism, or with the rate of hydrogen assimilation in the presence of carbon dioxide in the

light. However, it is of the same order of magnitude as the phytochemical reduction which I reported in 1936. This vast difference between the rates of photosynthetic hydrogen assimilation and of hydrogen uptake in the dark makes it clear that, whatever the dark reaction represents, it cannot be considered as an intermediate step in the photochemical utilization of hydrogen. And this becomes all the more evident if it is borne in mind that the latter proceeds independently of the presence or absence of sulfur compounds in the medium.

Although it is impossible to interpret Nakamura's recent results without new and much more complete experimental evidence, so much is certain that they do not in the least subtract from the validity of the statement that, at the present time, the most satisfactory concept of the purple bacteria photosyntheses is the one based on the generalized reaction equation of photosynthesis, in which organic substances as well as molecular hydrogen can play the role of hydrogen donors.

## VI. Consequences

The general survey presented in the foregoing sections has shown that, at the present time, there do not exist sufficient reasons for modifying the concept of photosynthesis as a photoreduction of carbon dioxide with a variety of hydrogen donors. This working hypothesis accounts better than any other so far advanced for the accumulated body of experimentally established data, and permits of an integrated interpretation of the multitude of facts.

It will now be shown to what extent the acceptance of this broad generalization leads to theoretical consequences which can readily be tested experimentally.

Prior to the characterization of the bacterial photosyntheses as a photoreduction of carbon dioxide with hydrogen donors other than $H_2O$, the photosynthetic mechanism of the green plants was generally considered as involving a reorganization, under the influence of light, of carbonic acid, more or less intimately combined with chlorophyll. For ten years the ingenious formulation of Willstätter and Stoll had been used in attempts to unravel the detailed mechanism of the process. But if one desires to look upon the various types of photosynthesis as fundamentally similar, and representing "variants" of a more general metabolic process, it would appear that the Willstätter-Stoll theory can no longer be accepted. The generalized reaction equation implies that the liberation of oxygen in green plant photosynthesis results from the dehydrogenation of

$H_2O$ rather than from the transformation of carbonic acid into formaldehyde-peroxide, followed by its decomposition.

Undoubtedly the tremendous influence exerted by Wieland's theory that respiration primarily consists of a dehydrogenation of the substrate has done much toward the change in outlook on the possible mechanisms of photosynthesis. Also the generalization of the Wieland concept to embrace all biochemical reactions with the resultant emergence of the science of "comparative biochemistry," due to the work of Kluyver, has meant a considerable advance in the development of biochemical thought. It is, therefore, not surprising to find that after 1930 the literature on green plant photosynthesis abounds with contributions in which the process is tacitly assumed to represent a reduction of carbon dioxide with hydrogen derived from water. As a result of this development it seems justifiable to undertake a re-evaluation of the photosynthetic mechanism. This seems all the more permissible since Gaffron has recently published experiments which have demonstrated a much closer relationship between the photosynthetic mechanism of green plants and of purple bacteria than had hitherto been suspected. In fact, one might paraphrase his findings by asserting that he has achieved an experimental modification of the behavior of green algae so that photosynthetically they have become purple bacteria (40, 41, 42).

For many years it has been known that green plant photosynthesis consists of two types of reactions: photochemical, or "light," and ordinary chemical, or "dark," reactions. By skillful measurements it has been possible to show that chlorophyll is one of the components of the photosynthetic mechanism necessary for the absorption of radiant energy. But a more penetrating analysis of the mechanism has been handicapped by the lack of knowledge concerning the active components, and by the impossibility of achieving a more or less normal photosynthetic process with cell extracts or ground-up materials. For these reasons a careful comparison between the various characteristics of green plant and bacterial photosyntheses, known to involve at least partly different systems, might make it possible to correlate differences and similarities in various processes with differences and similarities in the organisms. Thus, by considerations of a "comparative biochemical" nature, a somewhat better understanding of this most important biological reaction might be gained.

Much valuable information concerning green plant photosynthesis has been obtained through studies on the kinetics of the process. The recognition of "light" and "dark" reactions and the establishment of chlorophyll as the only essential light-absorbing pigment have resulted from experiments of this kind. Analyses of data concerning the dependence of the rate of photosynthesis on various environmental factors have led, furthermore, to the following important conclusions. First, the photochemical reaction must be a first order reaction; second, light absorbed by

different chlorophyll molecules can be "gathered" in some manner so as to cooperatively cause the reduction of a carbon dioxide molecule; and, third, the "dark" reaction is a composite of at least three different chemical mechanisms.

Experiments on the energetics of green plant photosynthesis have also contributed greatly to the trends of thought used in approaches to the intimate mechanism. Since the classical study of Warburg and Negelein (88, 89) numerous attempts have been made to describe the photosynthetic mechanism in terms of 3 or 4 one-quantum steps.*

Before dealing with the corresponding information on bacterial photosyntheses, we shall here consider the consequences of the adoption of the generalized equation for photosynthesis from a theoretical point of view. It appears reasonable to distinguish three photosynthetic "types:"

1. Green plant photosynthesis, in which oxygen is produced and $H_2O$ represents the typical hydrogen donor;
2. Photosynthesis of green bacteria, which proceeds only with the auto-oxidizable hydrogen donor $H_2S$, this being oxidized only to sulfur;
3. Purple bacteria photosyntheses in which a variety of oxidizable substances participate.

All three types have two elements in common; each deals with the photoreduction of carbon dioxide and takes place with absorbed radiant energy. From this comparison it might thus seem that there exists a close connection between the absorption of the radiant energy and the reduction of the carbon dioxide. Assuming, for the time being, that the photosyntheses of types 2 and 3 are also made up of "light" and "dark" reactions, one might be inclined to believe that the carbon dioxide is immediately involved in the light reaction.

However, this inference leads to an undesirable consequence. Although radiant energy is indisputably required for green plant photosynthesis, which represents an endothermic reaction with a storage of about 120 Calories in the products per mole of carbon dioxide converted, this is not true for some of the bacterial photosyntheses, certain of which are energetically practically neutral. This difference in energy relations results

* It seems inadvisable to quote the vast number of references to the original literature on green plant photosynthesis. The interested reader may be referred to such excellent treatises and reviews as Spoehr, "Photosynthesis," New York, 1926; Burk and Lineweaver, *Cold Spring Harbor Symposia*, **3** (1935); Emerson, *Ergeb. Enzymforsch.*, **5** (1936); Gaffron and Wohl, *Naturwissenschaften*, **24** (1936); Manning, *J. Phys. Chem.*, **42** (1938); Wohl, *New Phytologist*, **39** (1940). Only the relatively recent publications, immediately bearing on the present discussion, will be found in the Bibliography.

chiefly from the fact that only in the former is oxygen liberated. Regarding the oxygen liberation as the result of a dehydrogenation of water, it is clear that the most logical assumption would be that such a dehydrogenation is accomplished under the influence of absorbed radiant energy. This tends to ascribe to the photochemical reaction the function of acting on the hydrogen donor. Particularly Stoll (43, 44) has developed this possibility for green plant photosynthesis on the basis of the chemical structure of chlorophyll. However, the consequence of such an assumption would seem to be that the dehydrogenation of the oxidizable compounds in bacterial photosyntheses is also accomplished under the immediate influence of the pigment system activated by light. Such an assumption seems perhaps even less reasonable than the previously discarded one, because it is well known that many microorganisms are capable of bringing about the dehydrogenation of the same substances without the aid of absorbed radiant energy.

Hence these attempts at correlating the main features of the bacterial photosyntheses and the important facts bearing on the process in green plants appear to lead to an impasse. But it must be remembered that the generalized equation, so often used in the previous pages, only describes the over-all reaction and cannot make any pretense at suggesting a mechanism. Before a more satisfactory attempt can be made it will therefore be necessary to review the existing information on the kinetics and energetics of the "abnormal" photosynthetic reactions, and such additional data as may be helpful for an interpretation of the results.

## VII. Kinetics of the Bacterial Photosyntheses

The need for assuming that bacterial photosyntheses are also the result of both photochemical and dark reactions first became clearly apparent from Gaffron's data on the effect of temperature on the rate of carbon dioxide assimilation by the non-sulfur purple bacterium *Rhodovibrio parvus* (23). A change in temperature from 25° to 40° C. caused a nearly fivefold increase in the rate. So considerable a response to an increase in temperature can only be considered compatible with the occurrence of "dark" reactions in the photosynthetic process.

A similar behavior is exhibited by purple sulfur bacteria and by various other species of *Athiorhodaceae* in the presence of a variety of hydrogen donors (unpublished). The temperature optimum is, however, not always as high as for *Rhodovibrio*. This is only to be expected. Yet it deserves special mention that among the numerous strains investigated there are some with a maximum as low as 30° C.

That a photochemical reaction is involved follows logically from the observation that a carbon dioxide assimilation under anaerobic conditions does not occur in the dark. The effect of light intensity on the rate of photosynthesis has been studied to some extent by French (27, 45) and by Eymers and Wassink (46). The first-mentioned author used two different species of *Athiorhodaceae*, *Streptococcus varians*, and *Spirillum rubrum*, while the studies of Eymers and Wassink were conducted with *Chromatium* spec., a representative of the *Thiorhodaceae*.

The results are in good agreement; at low light intensity the rate of photosynthesis appears directly proportional to the energy supply. Strict linearity was not observed by French, especially with *Str. varians*, an observation which is (in all probability correctly) ascribed to the difficulty of measuring the rate of one isolated photosynthetic reaction.

As will be shown later on, nearly all purple bacteria, under anaerobic conditions in the dark, carry out a fermentation process in the course of which carbon dioxide and acidic substances are formed. During illumination in the absence of substrate these products are again removed by photosynthesis. What happens photosynthetically if simultaneously another known substrate is added depends largely upon the experimental conditions (*vide infra*). Hence at such low light intensities, where the photosynthetic process does not even proceed fast enough to compensate completely the fermentation reaction, the interpretation of the rate measurements in terms of a well-defined photosynthetic process is difficult.

These measurements with *Spir. rubrum*, *Str. varians*, and *Chromatium* have been restricted to very low light intensities. The following Table VII summarizes the results.

TABLE VII

RELATIONSHIP BETWEEN LIGHT INTENSITY AND RATE OF PHOTOSYNTHESIS BY PURPLE BACTERIA; DATA COMPILED FROM FRENCH, AND EYMERS AND WASSINK

| Organism | Highest recorded intensity in cal./cm.²/min. | λ in mμ | Rate-relation | Reference |
|---|---|---|---|---|
| *Spir. rubrum* | $1 \times 10^{-3}$ | 825–865 | Linear over entire range | French (45) |
| *Spir. rubrum* | $1 \times 10^{-2}$ | Not recorded | Linear over entire range | French (45) |
| *Str. varians* | $5 \times 10^{-2}$ | 700–900 | Linear over entire range | French (27) |
| *Str. varians* | $7 \times 10^{-2}$ | 425–560 | Linear over entire range | French (27) |
| *Str. varians* | $8 \times 10^{-2}$ | 425–680 | Linear over entire range | French (27) |
| *Str. varians* | $1.2 \times 10^{-3}$ | 852–894 | Linear (?) up to about $0.5 \times 10^{-3}$; flattens out at higher intensity | French (27) |
| *Chromatium* | $11.5 \times 10^{-2}$ | 589 | Perfectly linear up to $10 \times 10^{-2}$; at $11 \times 10^{-2}$ first indication of bending | Eymers and Wassink (46) |

This satisfactory establishment of a linear relationship between rate of photosynthesis and light intensity over the lower ranges shows the similarity in behavior of the light reaction in bacterial and green plant photosyntheses. However, none of these measurements had been carried out for light intensities considerably above the saturation point. The largest difference between an incident intensity which just saturates and highest intensity used covers only a twofold range (French; measurement in infrared light for *Str. varians*).

Yet the problem of the effect of relatively large incident intensities on the rate of photosynthesis, especially in the case of purple bacteria, is important in view of the different interpretations that have been placed on the rate-intensity curves for green plant photosynthesis. There the possibility has been considered that the gradual saturation could be the result of photo-oxidations, taking place at higher intensities, and thus masking the occurrence of an increased rate of photosynthesis. The lack of oxygen production by photosynthesizing purple bacteria, though obviously not completely eliminating the possibility of photo-oxidations or other secondary phenomena, lends emphasis to this point.

Several experiments of this sort have been carried out during the past few years, using a simplified set-up which did not allow of a computation of the absolute energy relations. Only relative intensities could be measured accurately, but the range could thus be greatly extended. Most of the experiments were carried out with *Streptococcus varians*, using a bank of ordinary incandescent bulbs at a varying distance from the vessels containing the bacterial suspensions. Also, by using different concentrations of cells for each experiment, the effect of mutual shading could be estimated, and only those results considered in which this influence was not apparent. In Table VIII some of the typical data are presented.

TABLE VIII

RELATION BETWEEN RATE OF PHOTOSYNTHESIS AND RELATIVE LIGHT INTENSITY IN SUSPENSIONS OF *Streptococcus varians*

| Relative intensity in arbitrary units | Rate of assimilation in cmm. $H_2$ per 100 min. | | |
|---|---|---|---|
| | Cell density, $N$ | Cell density, 1.5 $N$ | Cell density, 2 $N$ |
| 1.53 | 10.4 | 10.8 | 12 2 |
| 1.92 | 17.2 | 21.6 | 24.5 |
| 2.42 | 26.0 | 37.9 | 42.8 |
| 2.80 | 30.0 | 43.6 | 50.0 |
| 3.57 | 31.2 | 49.9 | 63.6 |
| 20.00 | 27.8 | 44.7 | 59.2 |

It appears that at the lowest light intensities used there is no proportionality between rate and number of cells. At a "relative intensity" of approximately 3.00 the strict

proportionality has become established; somewhat below this intensity also the relation between rate and intensity ceases to be rectilinear and rapidly the rate becomes entirely independent of the light intensity even when this has been increased to 20, *i. e.*, about seven times as high as the intensity corresponding to light-saturation under the experimental conditions.

The existence of a range over which strict proportionality holds good shows that the photochemical process corresponds to a first order reaction.

For green plant photosynthesis it has been shown that the maximum rate of photosynthesis occurs only at wave lengths corresponding accurately with the maximum absorption of chlorophyll. The purple bacteria possess, in addition to the red pigments which are responsible for the characteristic coloration of the cells, also a green pigment which is chemically closely related to chlorophyll (see section VIII). However, the absorption spectrum of this bacteriochlorophyll shows a considerable shift to longer wave lengths as compared with the green plant chlorophyll.

Now, it may be remembered that Engelmann (2) already claimed a photosynthetic activity for the purple bacteria in the infrared region of the spectrum. Dangeard (47), however, was the first to show conclusively that purple sulfur bacteria can be grown when supplied with radiant energy completely devoid of visible radiation. The same observation was reported later for *Athiorhodaceae* by Gaffron (24).

But these observations are not sufficient to demonstrate that it is only the energy absorbed by the green pigment which is effectively used in bacterial photosynthesis. Much more convincing in this respect are some experiments first reported by Eymers and Wassink (1936, referred to in (87)) on the growth of pure cultures of purple bacteria in a spectrum. Using a large grating spectrograph, Arnold has since carried out numerous similar experiments. A tube, filled with an appropriate agar medium, and uniformly inoculated with a pure culture of a purple bacterium, was exposed to the spectrum. After a sufficient period of exposure the bacteria grow in bands coinciding with the absorption bands of the green pigment (unpublished). However, an unamibiguous interpretation of these results is made difficult because the relative light intensity of the various spectral regions is not the same.

During his studies on the efficiency of purple bacteria photosynthesis, Roelofsen (26) made the observation that the quantum numbers for wave lengths 589 m$\mu$ were much smaller than those determined for 578 m$\mu$. He ascribed this difference to the larger absorption of the red pigments at the shorter wave length, and concluded:

"These facts justify the conclusion that the light absorbed by the (red pigments) is lost for the carbon dioxide assimilation, at least with the hydrogen donators at issue" (26, pp. 119–120).

In view of the fact that his determinations of the quantum number are open to some criticism (see section X) the result again is not conclusive.

This cannot, however, be said with respect to the studies of French (45) on the rate of assimilation by purple bacteria at various wave lengths. His results show conclusively that the photosynthetic activity of the organisms is a direct function of the light absorbed by the green pigment only. The curve representing the rate of assimilation as a function of the wave length is "in effect, the relative absorption curve of the photosensitizing pigment," and corresponds with that of the green pigment. Consequently, it may be concluded that also in this respect the photosynthetic mechanism of green plants and purple bacteria show a fundamental similarity; the participation of the red pigments in photochemical reactions seems hereby excluded.

A short consideration of the problem of phototaxis should here be added. The accumulations of purple bacteria in a spectrum, observed by Engelmann (2) and confirmed by Buder (6), have been interpreted as the result of phototactic responses. Particularly Buder's data show clearly that congregation of purple bacteria in a spectrum occurs wherever an absorption by any one component of the pigment system can be demonstrated. It is true that the accumulations coinciding with the absorption maxima of the green pigment are considerably denser than those caused by the absorption due to the red pigments, but the latter are so clearly marked, and their position is so accurately in agreement with the optical characteristics of the main red pigment, that one must conclude that phototactic responses can be caused by the carotenoids. French (45) has ventured the tentative explanation that all phototactic movements are conditioned by pigments of the carotenoid group, as seems also to be true for green algae (see, *e. g.*, Voerkel (48)), and that the accumulations at the absorption maxima of the green pigment might be caused by chemotactic responses to changes in the carbon dioxide concentration resulting from active photosynthesis in those regions. The careful and excellent studies on phototaxis of the purple bacteria by Schrammeck ((49), see also (50)) have been carried out with "white" light, and thus cannot help in explaining the anomalies in the situation. It is to be hoped that future experiments may furnish evidence which will aid in understanding the phototactic behavior of the purple bacteria.

Additional studies on the dependence of the rate of purple bacteria photosyntheses on various factors have dealt with the effect of concentrations, including *p*H, redox potentials, and nature of the specific hydrogen donors. Since most of these investigations have been conducted with the

manometric technique, the interpretation is sometimes open to serious criticism. Conclusions have often been drawn from the rate of observed pressure changes, apparently assuming that the pressure change is in all cases due exclusively to a photosynthetic carbon dioxide assimilation. This, however, is far from true. The oxidation of the specific substrates which act as hydrogen donors in nearly all instances induces changes in the reaction of the suspending medium which frequently give rise to an additional, but entirely secondary carbon dioxide uptake from the gas phase. In some few cases the resultant products are acidic, and cause the decomposition of bicarbonates in the medium with the liberation of gaseous carbon dioxide in amounts which may be considerably greater than the quantity of carbon dioxide that has actually been assimilated. Consequently such rate measurements are of value only if the chemical changes in the environment are taken into consideration (cf., *e. g.*, (14, 21, 46)).

The influence of *p*H on the rate of development of a purple sulfur bacterium has been reported by Tshesnokov and Saposhnikov (51, 52). They observed a difference in the optimum *p*H for development depending upon the nature of the hydrogen donor used. Thus, while with hydrogen sulfide the optimum for the particular strain used was close to *p*H 8.5–9.0 the development in media with sulfite and thiosulfate was best around *p*H 7.5. Again, in cultures with fatty acids optimum development occurred at *p*H 8.5, whereas with substituted and dibasic acids a shift toward the acid side (optimum *p*H 7.0–7.5) was noted.

The authors have considered these relations as indicating that the more oxygen is contained in the specific hydrogen donor the more does the optimum *p*H shift toward the acid side, and suggest that it is ultimately the redox potential of the medium which determines this behavior (53). Without denying this possibility, I wish to point out that an alternative explanation might well be preferable. I have pointed out before (12) that various purple bacteria are affected differently by the hydrogen ion concentration of the medium in combination with the sulfide concentration. The experiments suggested that the important factor was the concentration of undissociated hydrogen sulfide which, above a certain limiting concentration, would become toxic. Obviously, no such toxicity would be observable in a neutral to alkaline medium which contains thiosulfate or sulfite instead of sulfide; the dissociation constants of the corresponding acids are considerably greater. The same mode of reasoning can very well be applied to account for the behavior in the presence of organic acids. Again, the fatty acids are more toxic than the hydroxy or dibasic acids (21, 23) so that with a definite concentration of the acids a higher *p*H will

be required to offset the inhibitory effect of the undissociated acid molecules in the case of fatty than in the case of substituted acids.

In this connection the observations concerning the effect of neutral salts on the rate of assimilation in the presence of propionic acid by *Chromatium* suspensions are also of importance (21). The very slow assimilation in dilute (*m*/200) bicarbonate solutions was found to be markedly speeded up by the addition of various salts. Similar observations have since been made with suspensions of *Athiorhodaceae*. In these cases only the influence of the bicarbonate concentration has, however, been studied, so that the results may be entirely due to changes in *p*H of the medium. A remarkable agreement was noticed in the bicarbonate concentration which supported a maximum rate of assimilation for the sulfur purple bacteria in the presence of propionate and for the *Athiorhodaceae* in acetate solutions. In both cases a minimum of 300 cmm. of $CO_2$ present as bicarbonate in 2 cc. suspension was found to be necessary for optimum assimilation in an atmosphere containing 5% carbon dioxide (unpublished).

The dependence of the rate of assimilation with molecular hydrogen on the *p*H of the medium has been carefully studied by French for *Streptococcus varians*, and a striking optimum was observed at *p*H 7.8. Already at *p*H 7.1 the assimilatory activity of the organisms is greatly impaired; there is a rapid decrease in rate during the first 25 minutes. The peculiar increase with time at more favorable reactions will be dealt with later.

Provided that the composition of the suspension medium is satisfactory, the concentration of the substrate seems to play no role in the fate of photosynthesis. This follows from the fact that the addition of a given substrate generally results in the commencement of photosynthesis, the velocity of which does not change until the substrate has been completely utilized. The results obtained by Gaffron (24) have since been corroborated in hundreds of similar experiments.

When it comes to a comparison of the rates of photosynthesis in the presence of different substrates, the results are very much more difficult to interpret. This is chiefly due to the above-mentioned difficulty in realizing just what the observed pressure changes mean. A good example for discussion is furnished by the experiments of Roelofsen (26) who measured simultaneously the uptake of carbon dioxide by *Chromatium* suspensions in the presence of sulfide and sulfite. With the first substrate an extremely rapid uptake of carbon dioxide was observed; with sulfite it was considerably slower. From this and similar experiments with sulfur and thiosulfate Roelofsen concluded that the rate of carbon dioxide assimilation with these various substrates was substantially different, and that, *e. g.*, the

assimilation with sulfur is too slow to be measured. Taking into account the differences in the chemical conversions and the subsequent secondary, non-photosynthetic absorption or liberation of carbon dioxide by the medium, I have previously exposed the fallacy of these conclusions (14) and presented experimental evidence in favor of the viewpoint that the rate of assimilation with a number of sulfur compounds is essentially the same.

More complicated, however, are those instances in which photosynthesis of purple bacteria is studied in the presence of organic acids. Though it is true that also here the secondary uptake of carbon dioxide can be measured by occasional determinations of the total bicarbonate content of the medium, yet an evaluation of the actual carbon dioxide assimilation remains totally impossible. Because this is a general difficulty encountered in all studies on photosynthesis in the presence of organic substrates, it is necessary to explain the reasons in some detail.

The over-all result of the assimilation in the presence of acetate, propionate, and butyrate in bicarbonate media, and with carbon dioxide in the gas phase, can be roughly represented by the equations:

$$C_2H_3O_2Na + H_2O + CO_2 \longrightarrow 2(CH_2O) + NaHCO_3 \quad (11)$$

$$C_3H_5O_2Na + 1.5H_2O + 1.5CO_2 \longrightarrow 3.5(CH_2O) + NaHCO_3 \quad (12)$$

$$C_4H_7O_2Na + 2H_2O + 2CO_2 \longrightarrow 5(CH_2O) + NaHCO_3 \quad (13)$$

Disregarding the carbon dioxide taken up from the gas phase to form $NaHCO_3$, these equations would lead one to conclude that the carbon dioxide actually assimilated amounts to 0.0, 0.5, and 1.0 mole, respectively, in the transformation of acetate, propionate, and butyrate.

But it has been shown before that there are good reasons for assuming that the decomposition of organic compounds during photosynthesis by purple bacteria in reality consists of a hydrogen transfer from the substrate to carbon dioxide. In that case, equation (11) might more properly be written as a combination of two reactions:

$$C_2H_3O_2Na + 3H_2O \longrightarrow 8H + CO_2 + NaHCO_3 \quad (11a)$$

$$8H + 2CO_2 \longrightarrow 2(CH_2O) + 2H_2O \quad (11b)$$

Similarly, equations (12) and (13) then become:

$$C_3H_5O_2Na + 5H_2O \longrightarrow 14H + 2CO_2 + NaHCO_3, \quad (12a)$$

$$14H + 3.5CO_2 \longrightarrow 3.5(CH_2O) + 3.5H_2O, \text{ and} \quad (12b)$$

$$C_4H_7O_2Na + 7H_2O \longrightarrow 20H + 3CO_2 + NaHCO_3, \quad (13a)$$

$$20H + 5CO_2 \longrightarrow 5(CH_2O) + 5H_2O \quad (13b)$$

These equations show that the conversion of a mole of acetate, propionate, and butyrate requires the assimilation of 2, 3.5, and 5 moles of carbon dioxide.

However, it has also been pointed out that the dehydrogenation of an organic substrate must not necessarily proceed to completion. Indeed, the recent studies of Barker (54), Giesberger (55), Clifton, *et al.* (56, 57), and Winzler (58, 59) have made it extremely probable that the oxidative dehydrogenation of acetate and other organic compounds does not normally take place with the liberation of the theoretically possible amount of carbon dioxide, but that some intermediate product or products of the dehydrogenation are utilized immediately for synthesis of cellular materials. Their experimental results have been interpreted as representing biochemical oxidations which, in the case of the decomposition of acetate, can be approximately expressed by one of the following equations:

$$C_2H_3O_2Na + O_2 \longrightarrow (CH_2O) + NaHCO_3, \text{ or} \quad (14)$$

$$2C_2H_3O_2Na + 3O_2 \longrightarrow (CH_2O) + CO_2 + H_2O + 2NaHCO_3, \quad (15)$$

depending upon the nature of the organism.

Now, if the purple bacteria, in dehydrogenating acetate with carbon dioxide as acceptor, were to behave like the various organisms used in the above-mentioned studies on "oxidative assimilation," one might then expect a behavior according to one of the equations:

$$C_2H_3O_2Na + 2H_2O \longrightarrow (CH_2O) + 4H + NaHCO_3 \quad (16a)$$

$$4H + CO_2 \longrightarrow (CH_2O) + H_2O, \text{ or} \quad (16b)$$

$$2C_2H_3O_2Na + 5H_2O \longrightarrow (CH_2O) + 12H + CO_2 + 2NaHCO_3, \quad (17a)$$

$$12H + 3CO_2 \longrightarrow 3(CH_2O) + 3H_2O \quad (17b)$$

Any set of equations (11*a* and *b*), (16*a* and *b*), (17*a* and *b*) will give the same end-result, equation (11). The different sets do imply, on the other hand, a considerable variation in the quantity of assimilated carbon dioxide per mole of acetate. It follows, therefore, that the amount of carbon dioxide, photosynthetically assimilated, depends upon the mechanism of the acetate decomposition. The same considerations apply equally to an understanding of the photochemical decompositions of other organic compounds. Unless the mechanism of the decomposition is known one cannot determine by any method the photochemical carbon dioxide reduction in the presence of organic compounds. Yet, for an interpretation of the kinetic studies on bacterial photosynthesis, the accurate knowledge of the "carbon dioxide turn-over" is obviously a prerequisite.

In 1936 I made some observations (unpublished) which seemed to show

a possibility of determining this mechanism.* While studying the photosynthetic activity of purple sulfur bacteria in the presence of acetate and propionate it appeared that the ratio of the times required for the decomposition of equimolar amounts of the two acids was almost exactly 8:14. This is just what one would expect if the dehydrogenation proceeds according to equations (11*a* and *b*) and (12*a* and *b*). Comparisons with other fatty acids were excluded because the strain used failed to attack any of the higher fatty acids. The experiments were later continued with *Spirillum rubrum*, but with varying and unpromising results. In general, the rate of propionate decomposition was so much slower than that of either acetate or butyrate utilization that the suspicion arose that the dehydrogenation of the various fatty acids might be brought about by different enzyme systems.

Experiments on the photochemical carbon dioxide reduction by *Chromatium* in the presence of both propionate and thiosulfate had already shown that the dehydrogenation of the hydrogen donors may proceed simultaneously, so that the rate of carbon dioxide uptake in the presence of both compounds is the sum of those observed for each one separately (*e. g.*, Fig. 10, ref. (21)). The same phenomenon may be observed with suspensions of *Spirillum rubrum* in the presence of acetate, propionate, or both. However, the rate in the presence of both donors is not always quite so high; sometimes it appears only slightly higher than that of the most readily decomposed substance, and occasionally the latter is not increased at all by the simultaneous presence of propionate. But even in such cases it is perfectly clear that the decomposition of both acids takes place independently, because the high rate will persist for a period of time considerably longer than in the presence of acetate alone. Fig. 1 shows examples of the two extreme results in experiments of this sort.

The same behavior has also been noted with other *Athiorhodaceae*, and with mixtures of propionate and butyrate. Some species of the *Athiorhodaceae* are also capable of utilizing formate; for such organisms mixtures of acetate and formate have been found to exhibit the phenomenon. The simultaneous utilization of hydrogen and fatty acids has also been demonstrated.

* The term "mechanism" is here used in a somewhat different sense than is commonly implied. An actual knowledge of the enzyme systems involved, and of the mode of action of such substances, is not required for a computation of the "$CO_2$ turn-over," but merely a knowledge which will permit of a decision between such equations as (11), (11*a* and *b*), (16*a* and *b*), or (17*a* and *b*). These and similar equations thus are expressions of the meaning of "mechanism" at this place.

The simplest interpretation of these experimental results is that different enzymes are required for the dehydrogenation of the various fatty acids. By thus acting independently each can furnish the material to reduce carbon dioxide, and, if the system responsible for the last process is not saturated by one of the dehydrogenating functions, the higher rate of carbon dioxide uptake, even the sum of the two separately, may be obtained.

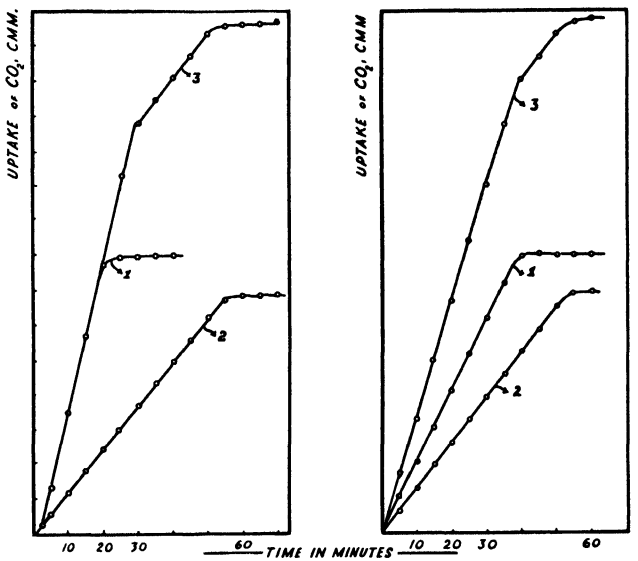



Fig. 1.—Two extreme cases of carbon dioxide uptake by illuminated suspensions of *Spirillum rubrum* in the presence of: (1) 0.1 cc. $M/10$ sodium acetate, (2) 0.1 cc. $M/20$ sodium propionate, and (3) 0.1 cc. $M/10$ sodium acetate plus 0.1 cc. $M/20$ sodium propionate. Substrates added at time 0.

Apart from evidence relative to the existence of enzymes specific for each one of a number of the lower fatty acids, these experiments have also led to the demonstration that the dehydrogenation of the hydrogen donors must be considered as a "dark" reaction (*vide infra*). It is thus evident that in cases where an increased rate of assimilation results from the addition of two different hydrogen donors the photochemical system is the one that is not saturated.

In section V we have discussed Nakamura's interpretation of the reduction in the rate of oxygen uptake by purple bacteria suspensions upon illumination. The above conclusion shows that Nakamura's explanation is untenable. It would imply that whenever the photosynthetic mechanism is not saturated by the hydrogen donor—and that should hold for many cases where the substrate is only slowly attacked—photosynthesis with oxygen production should occur.

Finally, it becomes clear that the simple determination of the time ratios cannot very well yield information concerning the number of moles of carbon dioxide that are reduced in the decomposition of different fatty acids if the latter is accomplished by different enzyme systems. In order for such experiments to be useful, a very different prerequisite has to be complied with: the photochemical system must be the rate-limiting factor in every case. By comparing the rate of assimilation in a medium where manometric measurements can be unambiguously interpreted in terms of carbon dioxide reduction with the time required for the complete decomposition of a known amount of an organic compound, one might be able to compute the carbon dioxide turn-over. So far, only a few experiments have been carried out, using the system hydrogen–carbon dioxide for reference. In this form the method can, of course, only be used with strains which can use molecular hydrogen. The data obtained show that the "mechanism" of assimilation in the presence of acetate can best be approximated by the previously given equation (11*a* and *b*); in other words, that the acetate is only partially dehydrogenated and that one-half of the molecule is never converted into $CO_2$ but immediately used for synthesis. In section VIII evidence for this reaction, but of an entirely different nature, will be presented. The earlier reported results with *Chromatium* species may have been fortuitous.

A few words remain to be said concerning those cases where rate measurements have shown a change in rate not accompanied by changes in the number of organisms. Two cases may be considered: (1) those in which a decrease in rate has been observed, and (2) those which have demonstrated an increased rate in the course of time.

The former are probably due to an injury suffered by the organisms. There is, however, one instance which requires special mention because it is not so easily explained. In trying different methods for the determination of the ratio in which hydrogen and carbon dioxide disappear during photosynthesis in suspensions of *Streptococcus varians* it was observed that the two-volume method, devised by Warburg for similar purposes, gave most erratic and incomprehensible results. This, ultimately, was

traced to the fact that when using dilute suspensions it is by no means immaterial how far the dilution is carried on. Thus the same number of organisms, but in different volumes of suspension medium, may show entirely different rates of metabolism under conditions that ensure adequate illumination. Particularly the fact that more concentrated suspensions do not show the irregularity of a gradual decrease in metabolic rate led to experiments with dilute suspensions in bicarbonate solution to which a boiled extract of the bacteria had been added. The decrease in rate was not observed, but a gradual increase here occurred. It is, however, not quite certain that in these experiments no increase in the number of organisms had occurred.

There are, however, most certainly cases in which an increase in the rate of photosynthesis in the presence of a given substrate occurs, and where this cannot be ascribed to the formation of new cells. Many as yet unpublished results of Foster have shown this behavior to be rather commonly associated with photosynthesis in the presence of alcohols, and, in a few cases, of hydrogen. Such results can at present best be ascribed to an adaptive enzyme formation in organisms not previously exposed to the particular substrate.

The mass of experimental data on the rate of carbon dioxide uptake during a short period immediately following the addition of a substrate shows that in general the maximum rate is not attained immediately, but is observable only after a period of some few minutes. This is reminiscent of the "induction period" in green plant photosynthesis, particularly in view of the fact that later substrate additions often fail to show this behavior. It seems, however, premature to place too much confidence on any interpretation at present.

## VIII. The Dark Metabolism of the Purple Bacteria

Under natural conditions a culture of photosynthetic bacteria receives light during only part of the solar day; even if their growth be limited to this period it is to be expected that during the interval of darkness they display some metabolism.

It has long been known that green plants respire in the dark. There are also many indications that this respiration continues during illumination. For an understanding of some aspects of photosynthesis it is necessary to have exact information as to the interrelationships of the photosynthetic and respiratory metabolism. Consequently it is appropriate to discuss briefly the dark metabolism of the purple bacteria.

In spite of an occasional statement in the literature purporting to show the development of purple sulfur bacteria in the dark, a clear-cut demonstration of such growth has never been published. This is not true with respect to the *Athiorhodaceae*. From the isolation of *Spirillum rubrum* by Esmarch in 1888 on, many microbiologists have cultured representatives of this group in the absence of light. Yet, all these instances deal with bacteria which are able to withstand oxygen tensions corresponding to those in air. It is necessary to remember that so far all *Thiorhodaceae* species which have been grown in pure culture have been found to be anaerobic, but that the group of non-sulfur purple bacteria comprises both aerobic and anaerobic types. The last-mentioned group contains intermediate degrees of tolerance for oxygen. Some strains of *Spirillum rubrum* behave like true anaerobes when freshly isolated, and can be grown only in complete absence of oxygen. By repeated transfers it has been possible to so modify the characteristics of these strains that ultimately they have become able to develop aerobically. Similar attempts with strains of *Rhodovibrio* have so far failed to produce the change; this type of bacterium has not yet been cultured except under anaerobic conditions.

The anaerobic purple bacteria of both groups should, therefore, carry out a fermentative metabolism in the dark. The aerobic species of the *Athiorhodaceae* may be expected to show an oxidative dark metabolism. Yet, from the numerous unsuccessful attempts to grow the anaerobic purple bacteria in the dark, using a large variety of culture media, it may be concluded that, whatever the fermentative metabolism consists of, it is not adapted to the production of all the cell constituents needed for the duplication of an organism.

Gaffron (35) was the first to report that suspensions of purple sulfur bacteria, under anaerobic conditions in the dark, form both gaseous and acidic products. Neither their nature nor their source was further investigated. He also demonstrated that the fermentation products can be rapidly used in photosynthesis. Roelofsen (26) confirmed Gaffron's observations, and furnished proof for the formation of both hydrogen and carbon dioxide by bacteria grown in the light in peptone media. Hydrogen is apparently not produced by organisms grown in a mineral, thiosulfate-containing culture solution. It appeared, however, impossible to affect the rate of fermentation by the addition of known substances so that the source of the fermentation products cannot be better defined than by referring to it as "cell substance." Roelofsen considered this "auto-fermentation" as the probable source of energy for the bacteria in darkness.

Also *Athiorhodaceae*, both the aerobic and anaerobic types, display a

similar auto-fermentation in the dark, though ordinarily of a considerably smaller magnitude than that of the *Thiorhodaceae* (24, 27, 45). It is, however, quite possible that, if more strains of the latter group are studied in this respect, the differences in the behavior of various species may appear to be as great as is now known for the representatives of the *Athiorhodaceae*. Again, the substrates for the fermentation process are unknown cell constituents. French (27) has shown that the rate of "auto-fermentation" can be greatly reduced by exposing the suspension without added substrates to air for some time.

The anaerobic decomposition of definite organic substances has been studied by Nakamura, who reported the decomposition of formate to hydrogen and carbon dioxide, and the production of hydrogen, accompanied by only small amounts of carbon dioxide, from dextrose. The other products of the anaerobic sugar decomposition were not investigated (60).

Of considerably greater interest are the results of studies on the metabolism of purple bacteria in the dark, in the presence of oxygen. Even suspensions of the normally anaerobic purple sulfur bacteria and of *Rhodovibrio* strains rapidly consume oxygen when exposed to air (24, 26, 34). An idea of the comparative magnitude of auto-fermentation and auto-respiration of sulfur purple bacteria may be gained from the following figures (unpublished experiments with *Chromatium*).

| | | |
|---|---|---|
| Aerobic | $O_2$ uptake | 70 cmm. per hour |
| | $CO_2$ production | 74 cmm. " " |
| Anaerobic | Gas and acid production | 72 cmm. per hour |
| | $CO_2$ production only | 34 cmm. " " |

Though quantitatively of the same order of magnitude, energetically the respiratory process must be approximately ten times as important. Hence it is questionable whether the "natural" dark metabolism of *Thiorhodaceae* is really fermentative rather than oxidative.

This becomes still more doubtful in view of Gaffron's experiments on the respiration of the anaerobic, non-sulfur purple bacterium *Rhodovibrio* (24). His results show that its rate of oxygen consumption in the dark is greatly increased by the addition of acetate, and that an oxidation of molecular hydrogen can also be observed in the dark. Thus it seems that the chief difference between aerobic and anaerobic types of purple bacteria is not so much a fundamentally different dark metabolism as a variation in the degree of tolerance for oxygen. This makes it more important than ever to attempt the culture of anaerobic types in the dark under reduced oxygen tension.

The respiration of aerobic types has been investigated particularly by Nakamura (34, 60, 61, 62), with *Rhodobacillus palustris*, in the presence of a large variety of substrates. From his results it is clear that particularly those compounds which are readily utilizable as hydrogen donors for the photosynthetic reaction, *i. e.*, the organic acids, cause an increase of the oxygen consumption over that of controls which carry on an auto-respiration. Carbohydrates do not influence the rate of oxygen consumption with the sole exception of dextrin—no doubt due to impurities other than carbohydrates in the preparation used. Beyond showing that certain compounds can be respired Nakamura's publications add little that can aid in understanding the process.

The results of numerous culture experiments have convinced me that the aerobic members of the *Athiorhodaceae* can all be grown in the dark in the same media and with the same variety of substrates which permit the culture of the organisms under anaerobic conditions in the light. Even those strains which can photosynthesize with molecular hydrogen can be cultured in media which—except for a small amount of yeast extract to provide growth factors—contain only inorganic compounds.

These facts lead to a most important conclusion. A non-illuminated culture can obviously decompose the substrate only by dehydrogenation reactions which are fully independent of photochemical processes. It thus seems reasonable to assume that the primary action of the organisms on the substrate—whether in the light or in the dark—is a dark reaction. Only for the transference of hydrogen to carbon dioxide in photosynthesis would the light then be necessary.

Unpublished experiments on the respiration of *Spirillum rubrum* and *Streptococcus varians* in the presence of mixtures of fatty acids have fully substantiated this hypothesis. As was pointed out in the previous section, the experiments on photosynthesis with mixtures of substrates had shown that the dehydrogenation of such closely related substances as acetic and propionic acids is accomplished with the aid of different enzyme systems. By comparing the oxygen consumption of the organisms in the dark with either one or both fatty acids present it could now be shown that the respiratory process exhibits the same characteristics as the photosynthetic one. Fig. 1 might equally well represent the consumption of oxygen in the dark as the carbon dioxide uptake in photosynthesis.

Thus one is forced to conclude that the decomposition of the substrate is a typical dark reaction, and that ordinarily it is this process of hydrogen transfer which limits both the rate of photosynthesis and that of respiration. A convincing demonstration of this principle is furnished by experi-

ments in which the decomposition of identical quantities of substrates was followed to completion, both in the dark in the presence of oxygen and in the light under strictly anaerobic conditions. The results have shown that *the substrate decomposition proceeds in both cases at exactly the same rate, and is completed after the same lapse of time.*

If, then, the rate of dehydrogenation of the substrate, and not the rate of transfer to the acceptor, limits the over-all rate of respiration and of photosynthesis, it should be possible to determine quantitatively the amount of hydrogen transferred by using oxygen as final acceptor (dark) and thus to compute the actual carbon dioxide reduction in photosynthesis with organic substances. So far, this procedure has been used only for a determination of the equations for acetate decomposition, and it has been shown that the decomposition of acetate by respiration proceeds according to the equation

$$C_2H_3O_2Na + O_2 \longrightarrow (CH_2O) + NaHCO_3 \qquad (18)$$

Inasmuch as this decomposition, with the transference of but four hydrogen atoms per mole of acetate, proceeds at the same rate as the anaerobic, photosynthetic decomposition, it is reasonable to believe that also photosynthesis with acetate involves the photochemical reduction of only one mole of carbon dioxide per mole of acetate. The following Table IX is presented as a sample experiment.

TABLE IX

DECOMPOSITION OF 0.1 CC. *M*/10 NA-ACETATE THROUGH RESPIRATION AND PHOTOSYNTHESIS BY *Spir. rubrum*

Cells deprived of reserve products by previous exposure of the suspension to air for 24 hours in absence of substrate. Auto-respiration negligible.

| | Found | Calculated from equation (18) |
|---|---|---|
| Oxygen consumed in cmm. | 211 | 224 |
| Total $CO_2$ production in cmm. | 232 | 224 |
| Increase in bicarbonate of suspension | 214 | 224 |

| | With $O_2$, dark | With $CO_2$, light |
|---|---|---|
| Time in minutes required for complete decomposition of substrate | 60 ± 2 | 60 ± 2 |

The experiments on acetate respiration by *Spir. rubrum* have furnished a most unexpected additional result; the oxidative decomposition is apparently dependent on the presence of carbon dioxide. Although the rate

of respiration in the absence of substrate is not appreciably affected by the absence of carbon dioxide, the oxygen uptake with acetate hardly exceeds the auto-respiration. Experiments with different phosphate mixtures, both in the presence and absence of carbon dioxide, have conclusively shown that the result is not due to a *p*H effect. Whether this behavior is characteristic only for *Spir. rubrum* or holds true generally cannot yet be decided; in view of Hes's demonstration of the necessity of carbon dioxide for methylene blue reduction by ordinary aerobic microorganisms (63, 64) the latter would not be surprising.

With this background it became also possible to study the interrelations between the respiratory and photosynthetic decomposition of added substrate by purple bacteria. In the green plants the respiration is generally the exact opposite of photosynthesis, so that a determination of both carbon dioxide and oxygen still does not make it possible to determine the extent to which respiration has occurred during photosynthesis.

The addition of acetate to suspensions of purple bacteria with quantitatively insignificant auto-respiration results in a rapid oxygen uptake in the dark, accompanied by the production of carbon dioxide. In the absence of oxygen, but in the light, a similar conversion of the acetate takes place, but here with a concomitant reduction of carbon dioxide. As an inspection of the equations below will show, the simultaneous determination of oxygen and carbon dioxide in experiments carried out in the light in the presence of both oxygen and carbon dioxide here permits of an accurate estimation of the extent to which each one of the reactions has occurred.

$$\text{Respir.:} \quad C_2H_3O_2Na + O_2 \longrightarrow (CH_2O) + NaHCO_3$$

$$\text{Photosynth.:} \quad C_2H_3O_2Na + H_2O + CO_2 \longrightarrow 2(CH_2O) + NaHCO_3.$$

So far, experiments have been limited to *Spirillum rubrum* using acetate as a substrate. The evidence is conclusive: In the light the decomposition proceeds completely through photosynthesis. No oxygen is used at all, and the change in total carbon dioxide content of the system is, within the limits of experimental accuracy, the same whether the suspensions are illuminated in air or in nitrogen. The uptake of oxygen in corresponding experiments in the dark is in agreement with equation (18). The results of one such experiment are summarized in Table X.

The complete suppression of respiration in suspensions of *Spirillum rubrum* by illumination cannot, however, be used as a conclusive argument to show that also in green plant photosynthesis respiration is ordinarily inhibited by illumination. It should be remembered that only in the

metabolism of the purple bacteria does the respiration as well as the photochemical carbon dioxide reduction depend upon the dehydrogenation of the substrate. In green plants the photosynthetic process is entirely independent of such dehydrogenation. The two systems are, therefore, not comparable. But the results show clearly that even if the purple bacteria can be made to grow in the dark, at the expense of a respiratory mechanism, it is still necessary to consider them as primarily photosynthetic organisms.

The dark metabolism of the green sulfur bacteria has not yet been investigated.

TABLE X

SAMPLE EXPERIMENT RELATIVE TO THE COMPLETE SUPPRESSION OF RESPIRATION OF ACETATE BY ILLUMINATION

| Conditions | Time required for complete decomposition of substrate, min. | Increase bicarbonate in cmm. $CO_2$ | Total increase in $CO_2$ of system in cmm. | Oxygen consumed in cmm. | Carbon dioxide uptake from gas phase |
|---|---|---|---|---|---|
| Dark | 70 ± 5 | 193 | 208 | 204 | ... |
| Light, nitrogen | 70 ± 5 | 197 | 51 | 0 | 146 |
| Light, air | 70 ± 5 | 196 | 51.5 | 0 | 144 5 |

## IX. The Pigment System of the Purple Bacteria

All the evidence presented so far has made it obvious that the bacterial photosyntheses involve the operation of enzyme systems of the same general nature as those that function in the oxidative degradation of substrates by non-photosynthetic organisms. These substrate-specific dehydrogenases may, in fact, be identical in various organisms; at present there is not sufficient experimental evidence to give up this simplest assumption. Nothing definite is known about the chemical nature of these enzymes of the purple bacteria. A more profitable approach to an understanding of their mode of action would seem to be afforded by a detailed study of such systems in readily available material.

The ability of the purple bacteria to produce specific enzymes in response to the presence of particular substrates is too general a microbiological phenomenon to merit special attention here.

From the point of view of biochemical equipment the outstanding characteristic of the photosynthetic bacteria is their well-developed pigment

system. The discussion in section VII has already indicated the important role of this system in the photosynthetic process.

Most bacteria with photosynthetic activity appear in clumps as pigmented masses with a color ranging from a light pink to a dark red or brown. The color of any one strain may vary considerably, depending upon the cultural conditions. Particularly striking are the color changes in cultures of the *Thiorhodaceae* when growing in the presence of sulfide. In young stages the growth appears as a chalky, light rose colored suspension. This characteristic aspect is due primarily to the occurrence of sulfur droplets in the cells; when, through lack of other oxidizable compounds, the sulfur becomes converted into sulfate, the color of the organisms changes to a deep burgundy red. It is not known, nor does it seem likely, that this color shift implies any changes in the pigments themselves. The addition of fresh sulfide to such cultures will cause, within a period of a few hours, the reappearance of the first-mentioned aspect, coinciding with the accumulation of new sulfur globules in the organisms.

Another marked variation in color is presented by some species of bacteria which under anaerobic conditions appear brown in the presence of air, however, typically red. Here the cause is not physical—*i. e.*, a change in refractility but is chemical. The pigment system of the anaerobic cultures is reduced with respect to that of aerobic ones. The shift from brown to red and *vice versa* can be demonstrated with the same culture many times (19). Some strains of brown bacteria never produce a red pigmentation; ordinarily they are dark brown when grown aerobically, and present a light olive-green to yellowish brown color under anaerobic conditions.

The red and brown color of purple bacteria cultures is due to the occurrence in the cells of two types of pigments. If moist bacteria are extracted with 80% methyl or ethyl alcohol a bluish green extract is obtained, leaving the organisms only faintly pink colored. A preliminary extraction of thoroughly air-dried bacteria with carbon bisulfide or chloroform furnishes a purplish red extract, in which case the residue is blue-green in color.

Experiments of this type have formed the basis of most of the later work. They were first systematically performed by Molisch, although the occurrence of both red and green pigments in the bacteria had been demonstrated many years earlier by Arcichovsky, Ewart, and others. Molisch characterized the red pigment as a carotenoid, and even recognized, on the basis of evidence obtained from absorption spectra measurements, the occurrence of two different red pigments in two different species of purple bacteria. Beyond this point our knowledge of the pigments themselves was not advanced until

about 1934 when various workers began to publish independently on the chemical constitution of the purple bacteria pigments.

The green pigment, which Molisch had called "bacteriochlorin," was reported by Schneider (65) to be a true chlorophyll, closely related to, but not identical with, green plant chlorophylls *a* and *b*. It was established that the molecule represents a pyrrol pigment with a porphine-nucleus containing a magnesium atom in chemical combination. The empirical formula was determined as $C_{55}H_{72}O_6N_4Mg \cdot 1H_2O$, although the exact number of hydrogen atoms was not proved. From the presence of six oxygen atoms per molecule Schneider concluded that the bacterial pigment, for which the much more satisfactory name "bacteriochlorophyll" was proposed, belongs to the chlorophyll *b*-series.

Subsequent studies by H. Fischer, *et al.*, have greatly contributed to our knowledge of the constitution of the bacteriochlorophyll (66, 67). By chemical degradation through bacteriopheophytin, and pheophorbide, and treatment of the latter with hydriodic acid at high temperatures the bacteriochlorophyll can be converted into oxo-pheoporphyrin *a*5. This compound, in turn, had previously been obtained as a derivative of chlorophyll *a*, so that bacteriochlorophyll is considered as belonging to the chlorophyll *a* series. The occurrence of an extra oxygen atom in the bacterial pigment is due to the presence of an acetyl group in the place where both chlorophyll *a* and *b* contain a vinyl group.

By treatment of bacteriochlorophyll or -pheophytin with "chlorophyllase" from leaves the molecule is hydrolyzed with the production of phytol and bacteriochlorophyllid or -pheophorbide. A quantitative study of the reaction with bacteriopheophytin has furnished results in excellent agreement with those yielded by a similar study on pheophytin from leaves. Thus it seems justifiable to conclude that the bacteriochlorophyll molecule is a phytol-ester with the phytol in a position corresponding to that which it occupies in the leaf chlorophylls.

Chemical analyses by Fischer, *et al.*, have made it probable that bacteriochlorophyll contains two more hydrogen atoms per molecule than chlorophyll *a*. The exact position of these hydrogens has not yet been determined.

Fischer has proposed the structural formula I for bacteriochlorophyll. For the sake of ready comparison, formula II, representing chlorophyll *a*, is included (Fig. 2).

The exact positions of the double bonds are uncertain. Stoll and Wiedemann (68) have expressed the opinion that it does not seem feasible to attempt too seriously to fix this position. The physical concepts favor the acceptance of resonance in a continuous conjugated double bond system. As an aid in realizing the chemical relations, however, the above formulae probably represent a very close approximation.

Whereas the green plants in general contain both chlorophyll *a* and *b*, the purple bacteria have not been shown to possess more than one bacteriochlorophyll. Schneider had made some claims for the existence of two different green pigments, but Fischer, *et al.*, have been unable to obtain convincing evidence. Also many attempts to separate different bacteriochlorophylls by chromatographic analysis (unpublished) have been completely negative.

The present evidence strongly supports the idea that all purple bacteria, *Thio-* as well as *Athiorhodaceae* species, contain the same bacteriochlorophyll. Unpublished results, mostly obtained in collaboration with E. Wiedemann, have shown the identity of the crystalline methylpheophorbides from a large variety of species. (See also Stoll and Wiedemann (68).) Spectroscopic evidence published recently by Wassink, Katz, and Dorrestein is in complete agreement with this contention (69, 70); see also (71).

I. Bacteriochlorophyll. II. Chlorophyll *a*.

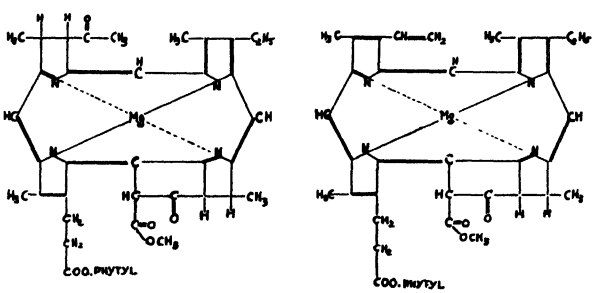


Fig. 2.

Bacteriochlorophyll is extremely unstable, especially in the light. Fischer, *et al.*, report the same behavior for bacteriochlorophyllid. This makes it practically impossible to determine the bacteriochlorophyll content of purple bacteria without a partial destruction of the substance. By taking advantage of the relatively great stability of the bacteriopheophytin a method has been worked out which permits of a rapid quantitative estimation of the pigment in the modified form (71).

The pigment fraction that can be extracted from purple bacteria with chloroform, carbon disulfide, etc., has been shown to contain various substances belonging to the carotenoid group. Isolation of a crystalline substance from *Spirillum rubrum* and its subsequent chemical analysis led van Niel and Smith to the conclusion that this "spirilloxanthin" would be

a carotenoid of the formula $C_{48}H_{66}O_3$ and containing 15 double bonds per molecule (72). This compound is the most abundant of the red pigments in *Spir. rubrum.* The presence of other carotenoids in the crude extracts was shown by chromatographic analysis, but none of these pigments was obtained in sufficient quantity to permit analysis. Karrer, *et al.* (73, 74), soon afterwards reported the isolation from both *Rhodovibrio* and *Thiocystis* of a substance which, according to its absorption spectrum, should be identical with spirilloxanthin. It was designated as rhodoviolascin. Chemical analysis led the authors to propose an empirical formula $C_{42}H_{60}O_2$ and a structure containing 13 double bonds and 2 methoxyl-groups. The original interpretation has undergone a number of modifications, and the constitution is as yet uncertain. A number of other carotenoid pigments have also been isolated or detected, but on the whole the chemistry of these substances has not yet been worked out satisfactorily (cf. also (75)). In the form of extracts or chemically pure compounds the red pigments are somewhat more stable than the bacteriochlorophyll, though most of them seem to be easily oxidized.

In sharp contrast to the photosensitivity of extracted bacteriochlorophyll and the limited stability of the carotenoid pigments when isolated from the bacteria, the pigment system in the cells is remarkably resistant to light, temperature, and oxygen. Pure cultures of purple bacteria maintain their characteristic color for years, and from previously dried cell material bacteriochlorophyll and red pigments can be isolated in an apparently unchanged form after five years, even when the dry powder has been exposed to light and air. This indicates that the pigments are present in the cells in a "protected" form, probably as chemical compounds.

Another set of observations points in the same direction. Many investigators, from Molisch on, have noted that the characteristic absorption bands of the bacteria themselves do not coincide in their positions with those of the isolated pigments, but are shifted, usually toward the longer wave lengths. In green plants similar shifts had also been observed, and it was particularly Lubimenko (76) who, on the basis of his experiments, advanced the idea that the pigments in the intact photosynthetic apparatus are not free but occur in chemical combination with proteins. Experiments with purple bacteria pigments convinced Lubimenko that the same concept applies to them (77). The same conclusions were reached by Lévy, *et al.* (78), in a study of the pigment system of *Chromatium Okenii.*

The systematic and careful investigations of Mestre (79, 80) did much toward establishing this view for the leaf pigments. Stoll, in 1935, introduced the term "chloroplastin" to describe the probable compound of

protein and chlorophyll, soon afterwards obtained by Stoll and Wiedemann, (68) and by Smith (81).

Water-soluble pigments of complex constitution were first obtained from purple bacteria by Lévy, *et al.* (78), but the actual demonstration of a pigment-protein complex was furnished by French (82, 83, 84). Both the stability and absorption characteristics of such water-soluble complexes agree with those of the pigments in the intact organism. As Lubimenko (77) and Lévy, *et al.* (78), had already made probable, the complexes contain both the green and the red pigments in protein combinations. Protein fractions containing either the green or the red pigment alone have not been separated. Both pigment types may, therefore, be combined with the same protein or occur in some still larger complex. It is quite conceivable that the complexes contain lipoidal groups or substances in addition to the pigments and protein, as has been assumed for the leaf pigments by Baas-Becking and co-workers (cf., *e. g.* (85, 86)). French has introduced the name "photosynthin" for the water-soluble pigment complex of the purple bacteria.*

The absorption spectra of the aqueous pigment solutions of purple bacteria show, particularly in the infrared region, certain characteristic differences between different bacterial species. This is in agreement with the results obtained with measurements of the absorption spectra of the living bacteria (69, 70, 84), and also with the bands in which the bacteria develop in a spectrum (Arnold, unpublished). Inasmuch as the carotenoid pigments do not show absorption at wave lengths above about 550 m$\mu$, these differences cannot be due to the presence of different *red* pigments. The green pigment alone is responsible for the absorption of infrared rays. Since the bacteriochlorophyll, isolated from purple bacteria with different absorption spectra, does not appear to be different either chemically or spectroscopically, the conclusion has been drawn that the observed differences in spectral characteristics are due to a combination of one bacteriochlorophyll with different protein carriers in the various organisms. For data concerning the absorption spectra the reader is referred to the original literature, particularly (6, 45, 67, 69, 70, 71, 72, 77, 84).

The pigment system of the green bacteria is virtually unknown. Except for a few old data on absorption spectra by Monteverde and Metzner, obtained with material admittedly impure, no measurements were reported until 1939, when Katz and Wassink published measurements on the infrared absorption of pure culture material, both of the organisms and of the extracted green pigment (69). The spectrum of the bacteria themselves shows two bands, one of which is very faint. The extract also gives indications of the two bands, but, as in the case of the purple bacteria, markedly

* Concerning the nomenclature of this and similar compounds see, especially, G. Mackinney, *Ann. Rev. Biochem.*, 9, 459–490 (1940).

shifted to the shorter wave lengths. In fact, the main band nearly coincides with that of green plant chlorophyll.

Fischer, *et al.* (67), p. 21, write, in connection with the chlorophyllous pigment of the green bacteria:

"Es gelingt verhältnismässig leicht, bei diesen Chlorobakterien Sauerstoffentwicklung im Licht und damit Assimilationstätigkeit nachzuweisen."

It is not clear whether this statement refers to new observations of the authors. As has been pointed out before, the luminous bacteria method has, in my hands, so far yielded entirely negative results. On the basis of some occasional observations Fischer has advanced the interesting hypothesis that the green pigment (Metzner's "bacterioviridin") might represent 2-acetyl-chlorophyll *a*. The evidence is yet, however, too scant to support this concept.

While van Niel and Muller (19) failed to find carotenoid pigments in green sulfur bacteria, Katz and Wassink have presented some reasons for believing that the older observation was incorrect. By shaking an alcohol extract of the bacteria with petroleum ether a yellow petroleum ether layer was obtained showing strong absorption in the blue region, and upon drying furnishing a substance which colors blue with sulfuric acid.

Green plant chlorophyll displays a beautiful red fluorescence, especially in solution, less conspicuous in the plant. The bacteriochlorophyll shows, in solution, at best a very weak fluorescence. Vermeulen, *et al.* (87), have elucidated the reason for this behavior: the fluorescence of bacteriochlorophyll solutions is strong, but mostly invisible (fluorescence maxima at 700 and 800 m$\mu$. Just as the absorption spectrum of the pigment is shifted toward the shorter wave lengths upon extraction, so also do the fluorescence bands of extracts occur at shorter wave lengths; the fluorescence band of the green pigment in the intact cells is situated at 930 m$\mu$. The spectral composition of the fluorescence light is independent of the wave length of the incident light. Measurements on the dependence of the yield of fluorescence on the composition of the exciting irradiation are not conclusive because, as the authors have rightly emphasized, the fluorescence radiation is, at least in part, absorbed by the bacterial cells. Hence a lower yield can be expected where, due to a relatively small absorption of incident light, the penetration into the suspension is greater. On the whole, however, the evidence indicates that the fluorescence yield is constant. The experiments also showed a considerably smaller absolute fluorescence yield than for *Chlorella*. The latter was determined as being around

0.15% of the incident energy, in the case of *Chromatium* it was only 0.005%.

These fluorescence studies—the only ones so far published for purple bacteria—are of considerable importance in connection with the possible mechanisms of photosynthesis, as will be pointed out in the next section.

## X. The Energetics of the Bacterial Photosyntheses

The classical researches of Warburg and Negelein (88, 89) on the efficiency of green plant photosynthesis have exerted a profound influence. Their demonstration that this reaction can proceed with a high energetic yield has formed the basis for most subsequent speculations on its mechanism. Concepts have been rejected because they implied consequences that seemed irreconcilable with thermodynamic considerations, just as mechanisms were proposed because they seemed to comply best with the now so familiar quantum number computed by Warburg and Negelein.

Without entering into the details of this problem the major aspects can be outlined as follows. The photochemical conversion of carbon dioxide and water in green plants results in the formation of products in which chemical energy has been stored up to the extent of about 112 Cal. per mole of carbon dioxide assimilated. In order to make the reaction proceed normally, energy must, therefore, be available in some other form. It is supplied by the absorption of light quanta, and Warburg and Negelein calculated from their determinations of the amount of photosynthesis produced by a measured number of light quanta that green plants can reduce a molecule of carbon dioxide with four quanta of absorbed radiant energy. This number was found to be independent of the wave length of light used. The absorbed energy exceeds the minimum requirements by only a small fraction. If one supposes the photosynthetic reaction to proceed in steps, extra energy is necessary for the formation of intermediate products, and it can be calculated that the energy supplied by four quanta of red light is so small that many of the postulated mechanisms for photosynthesis become thermodynamically impossible. For a more detailed discussion of this phase reference is made to the important contributions by Wohl, Franck, and others (90, 91, 92).

Although the experimental results of Warburg and Negelein have been supported by a number of later investigations, the recent excellent studies of Emerson and Lewis (93) have shown that not too much reliance should be placed on the computations of earlier workers. The important point is that, although the actual measurements of Emerson and Lewis agreed closely with the best of previously reported ones, their interpretation of the data is different. It was discovered that these measurements do not apply to the—implicitly assumed—reaction equation of photosynthesis. Under conditions which yield the above-mentioned quantum number the reaction measured manometrically is complicated by a second process dur-

ing which the gas produced is not oxygen, but carbon dioxide. By measuring photosynthesis in such a way that this carbon dioxide production could be eliminated or determined separately so that the necessary correction could be applied to the results, it was established that the smallest number of quanta which permit the reduction of a molecule of carbon dioxide is at least twice as large as that previously assumed.*

It should at once be pointed out that these conclusions gain considerable support from efficiency determinations made by entirely different methods than those used by Warburg, Emerson, and others. Thus Arnold, who first measured the efficiency calorimetrically, obtained results from which a quantum number eight can be computed ((94), and numerous unpublished experiments). Similar results, some obtained by still other methods, have been reported by Manning, Farrington Daniels, *et al.* (95, 96, 97, 98). In consequence, many of the arguments that were considered valuable or even conclusive a year ago have lost much of their force.

From the point of view of energetic relationships the bacterial photosyntheses offer a problem which, in many respects, differs widely from that of ordinary photosynthesis. The need for special hydrogen donors and their dehydrogenation during the photosynthetic process brings into play a source of chemical energy. In many cases the extent of this is thermodynamically almost sufficient to make the over-all reaction possible without the supply of radiant energy. It might thus be expected that accurate information concerning the quantum number in bacterial photosyntheses could furnish a sound basis for the evaluation of hypothetical mechanisms.

Roelofsen was the first to take up this problem (26). Determinations of the light energy absorbed by a suspension of *Chromatium* simultaneously with measurements on the effect of the illumination on the metabolism led him to the statement:

"... these results point to the necessity of four quanta per molecule of carbon dioxide and certainly one may conclude from it that the number of one quantum, ... required thermodynamically, is entirely insufficient."

Nakamura, who attempted a similar determination with *Rhodobac. palustris*, is even more positive in his conclusions:

"Berechnet man die Quantenzahlen nicht aus den Mittelwerten, sondern aus den.... Höchstwerten, so ist die Zahl der pro Molekül Kohlensäure verbrauchten Quanten im

* These results were presented by Emerson at a lecture during the A. A. A. S. meetings at Seattle, Wash., June, 1940.

Infrarot 4.5–5.0. Berücksichtigt man hier die Versuchsfehler..., so könnte man wohl sagen, dass die Zahl der pro Molekül Kohlensäure verbrauchten Quanten im Infrarot 4 sein kann, also dieselbe Ziffer wie 0.Warburg sowie Schmucker bei der Photosynthese von grünen Pflanzen festgestellt haben" ((34), p. 224–225).

The experimental evidence obtained by Roelofsen and Nakamura is, however, entirely inadequate to allow of any such conclusions. The determinations of the absorbed energy and of manometric changes may be entirely dependable; the calculation of quantum numbers is, however, impossible, because the experiments cannot decide the fundamentally important question of how much carbon dioxide had been reduced.

Roelofsen's and Nakamura's experiments were carried out with suspensions of purple bacteria in the absence of a specific substrate. In the first case the reason for this is clear enough: the low light intensity, required to insure complete absorption, together with the necessity for using dense suspensions, gave rise to a gas production ("auto-fermentation") even in the light. The addition of substrates did not alter this behavior; hence no substrates were added, and photosynthesis was determined by measuring the difference in gas production in darkness and during illumination. This procedure will, no doubt, give an idea of the extent to which the fermentation reaction is suppressed or counteracted by the irradiation. But the chemical nature of the photosynthetic reaction remains completely unknown. Hence it is impossible to even surmise the magnitude of actual carbon dioxide reduction in these experiments (cf. also the discussion pertaining to the carbon dioxide "turn-over" in photosynthesis with organic substrates in sections VII and VIII).

It is not clear why Nakamura used suspensions in the absence of substrate and in the presence of oxygen. From the difference in oxygen consumption in the dark and in the light he computed the "oxygen production" caused by photosynthesis. It has already been pointed out (sections V, VII, and VIII) that the interpretation is highly questionable. Since here also any knowledge of the chemical reactions that occur in the dark and in the light is lacking, it is obvious that the computation of a ratio of absorbed quanta/number of molecules of carbon dioxide reduced is utterly impossible. It should here be stated once and for all that an efficiency determination can yield definitive information only if the carbon dioxide "turn-over" during the period of assimilation is determinable. At the present time this implies that organic substrates cannot be used as hydrogen donors unless the "mechanism" of the decomposition is fully understood. So far, this is true only for isopropanol (29), and, with restrictions, for acetate. Unfortunately, the rate of photosynthesis with isopropanol seems

much too slow to offer reasonable prospects for good efficiency measurements.

The investigation of French (27) has been conducted in such a manner that the above objections have been largely avoided. Photosynthesis and light absorption were simultaneously determined for a suspension of *Str. varians* in the presence of molecular hydrogen. The uptake of hydrogen under anaerobic conditions takes place only with the concomitant reduction of carbon dioxide. Here, then, the amount of carbon dioxide assimilation is directly measurable.

For the computation of the quantum number French assumed a ratio of hydrogen/carbon dioxide utilization of 2.0, based upon Gaffron's experimental results with *Rhodovibrio* (24). An actual determination of this ratio for *Str. varians* (28) showed it to be somewhat larger. A comparison of the number of quanta absorbed and carbon dioxide molecules assimilated leads to a quantum number of about 5 for this reaction.

In the later publication (28) French also refers to quantum number determinations with *Spir. rubrum* in a butyrate medium, reported earlier (45). In this instance the considerations given above apply, however, so that it seems justified to omit a further discussion here.

Carbon dioxide reduction with molecular hydrogen represents a reaction which theoretically requires no energy. Thus French's results show that the process is thermodynamically much more inefficient than green plant photosynthesis. This may, however, not be a fair way of presenting the situation. It is entirely possible that the bacterial photosyntheses comprise *photochemical* reactions which are quite as efficient as those in green plants, but whose efficiency is apparently lessened by the simultaneous oxidation of the substrate. This would become apparent if it could be shown that photochemical carbon dioxide reductions with different hydrogen donors by purple bacteria always involves the same number of quanta, regardless of the chemical energy supplied by the hydrogen donor. This most important phase has not yet been attacked systematically. It seems likely that an attempt in this direction will require an introductory study of the "carbon dioxide turn-over" in the presence of various organic substrates, because the number of inorganic hydrogen donors is too small to allow of adequate comparisons. Essential is the inclusion of systems which theoretically require a significant energy supply.

One such system is the first stage of the photochemical reduction of carbon dioxide in the presence of thiosulfate. The thiosulfate is first oxidized to tetrathionate according to the equation

$$CO_2 + 4Na_2S_2O_3 + 3H_2O \longrightarrow (CH_2O) + 2Na_2S_4O_6 + 4NaOH \qquad (19)$$

Following this initial conversion the further oxidation of polythionate to sulfate occurs.

The occurrence of reaction (19) has been reasonably well established (21, 35). It requires the supply of 92 Cal. per mole of carbon dioxide, so that the reaction is energetically very close to green plant photosynthesis.

Eymers and Wassink have made a study of the quantum yield of this process. With both infrared (850–800 mμ band, isolated from the light of a caesium lamp) and yellow (590 mμ, sodium lamp) irradiation they reported the absorption of 12–18 quanta for the reduction of one carbon dioxide molecule. (Smallest numbers 8.8 at 590 mμ; 9.2 at 800–850 mμ.) This would show that the efficiency of the conversion of radiant into chemical energy by *Thiorhodaceae* is of the same order of magnitude as by green plants, and considerably less than was claimed by Warburg and Negelein for the latter.

Nevertheless, these results are not entirely free from objections. Also Eymers and Wassink had to contend with the fact that their suspensions showed a considerable metabolism in the dark. Their figures for carbon dioxide assimilation are consequently the result of two processes: assimilation with thiosulfate and assimilation with the unknown products of fermentation. It is not possible to determine how much each of these two, probably independent, reactions contributes to the total carbon dioxide turn-over, and hence the quantum numbers are subject to this uncertainty. On the whole, the good agreement between the computed results of a large number of determinations with different light intensities makes their data look convincing. On the other hand, it cannot be denied that the tables show a distinct tendency for the quantum numbers to become smaller as the difference between light and dark metabolism becomes more pronounced. This indicates that the assimilation with the organic hydrogen donors (auto-fermentation products) plays a definite role in the final outcome of the results, but the magnitude of the effect cannot well be estimated. At present the difficulty due to an unknown interference of an equally unknown process can be avoided only if it is possible to use organisms with a negligible dark metabolism. Since to all appearances this eliminates the *Thiorhodaceae* that have so far been studied, the possibility of using non-sulfur purple bacteria should be more carefully considered. Some species at least can oxidize thiosulfate completely to sulfate, and it is more than likely that these organisms could be used to advantage for another attack on the problem of the efficiency of purple bacteria photosyntheses which thermodynamically require much energy.

Also in the experiments of French the unknown influence of a dark me-

tabolism on the photosynthetic process must be considered. However, a comparison between the magnitude of auto-fermentation and photosynthesis shows that the effect of the former can have been only very small.

A report on the determination of the quantum number of *Thiorhodaceae* photosynthesis with the startling result that a value of one was found during photosynthesis with sulfide (53) can only be mentioned here. The experiments have not been published in sufficient detail to permit of a judicious evaluation.

The present information concerning the energetics of bacterial photosyntheses leaves much to be desired. It seems that even for reactions which thermodynamically require little if any energy a considerable input of radiant energy is still necessary. But whether any regularities exist in the relation between the number of quanta and the energetic requirements of the reaction cannot yet be decided.

## XI. The Chlorophyll-Carbon Dioxide Ratio and Outlook on the Mechanism of Photosynthesis

Photosynthesis proceeds normally with light absorbed by the chlorophyllous pigments. From the kinetic studies it has appeared that the absorption of light quanta must be considered as a first order reaction. The discussion of the energetics has shown that more than one, probably even more than four quanta must be absorbed to make possible the reduction of a single molecule of carbon dioxide.

The relatively high efficiency of photosynthesis at weak light intensity implies that, even although the absolute rate may be low, the absorbed quanta are used as efficiently as in stronger light. In dim light the possibility is negligible that any one chlorophyll molecule would be hit by more than one quantum during the short space of time which it takes for photosynthesis to become measurable. It follows logically that quanta absorbed by different chlorophyll molecules can be used cooperatively for the reduction of a carbon dioxide molecule.

In the course of their studies on the kinetics of green plant photosynthesis in intermittent light Emerson and Arnold (99, 100) arrived at the astonishing conclusion that the number of chlorophyll molecules exceeds the number of carbon dioxide molecules reduced in a single flash by a factor of about 2500. This implies that if a few out of every 2500 pigment molecules absorb a quantum each, the radiant energy can be so integrated as to permit the reduction of one carbon dioxide molecule. The phenomenon is not limited to green algae; Arnold and Kohn (101, 102) have ex-

tended the observations to plants belonging to four different divisions of the plant kingdom and found the situation similar in all cases. Many other observations have led to the same conclusion (cf., *e. g.* (103, 104, 105)).

Various interpretations of this curious relationship have been suggested. Emerson (106) considers it likely that the rate of photosynthesis in flashing light of high intensity is limited by an essential catalyst which is present in an amount only about 1/2500 of that of the chlorophyll. Optical models which would allow of the rapid transference of absorbed energy through a "block" of spatially arranged pigment molecules have been discussed ably by Wohl (104), who has published the most recent review of this problem.

In purple bacteria photosynthesis also Arnold has shown the existence of a large discrepancy between the number of bacteriochlorophyll molecules present and the number of carbon dioxide molecules reduced per flash (unpublished.) These studies were carried out with a *Rhodospirillum* species in the presence of acetate. By assuming the reaction to proceed according to the equations:

$$C_2H_3O_2Na + 2H_2O \longrightarrow (CH_2O) + 4H + CO_3HNa,$$

$$4H + CO_2 \longrightarrow (CH_2O) + H_2O,$$

for which evidence has been presented in previous sections, the results show a ratio chlorophyll/carbon dioxide of about 400/1. The difference with the ratio in green plants is, however, considerable. This might be ascribed to the existence of a different mechanism, or to a difference in the arrangement of the pigment molecules. The absence of visible chloroplasts in purple bacteria would make the latter assumption not irrational. A study of the ratio in blue-green algae, which share with the green plants the fundamental similarity in photosynthetic mechanism, but, like the purple bacteria, lack chloroplasts, thus seems important.

On the other hand, there are at present some indications that the interpretation of the high ratio may have to be sought in an entirely different direction. McAlister (107) was the first to present evidence that the ratio might be unity. The same conclusion has been reached independently by Arnold (unpublished) and Franck (private communication through H. Gaffron) who have discovered that the data on the rate of photosyntheses in intermittent light make it necessary to assume the occurrence of a new kind of dark reaction which proceeds at considerably slower rate than the so-called "Blackman-reaction" which has been measured by previous workers. This reaction becomes observable when intermittent illumination is brought about by means of rotating sectors with a fixed ratio between the open and closed area. In this event a change in the relative length of

the dark period can only be achieved by simultaneously changing the duration of the period of illumination. Under these conditions the increase in the length of the period of darkness is vastly greater than corresponds with the theoretical requirements. The disagreement can readily be explained by the assumption of a second type of dark reaction which proceeds at a rate about 1000 times slower than the Blackman-reaction.

Arnold has observed a similar discrepancy in the rate of photosynthesis of purple bacteria in flashing light. The second dark reaction again proceeds at a slower pace than the dark reaction which ordinarily limits the rate of photosynthesis at high light intensity. But the ratio between the times necessary for completion of the two reactions is here about 250:1.

Since the chlorophyll-carbon dioxide ratio is computed from the absolute amount of photosynthesis per flash of light and the period of the dark reaction, these findings indicate that, as already claimed by McAlister on the basis of fundamentally different evidence, the actual value of this ratio may be close to unity.

Does this imply that in photosynthesizing cells carbon dioxide combines with chlorophyll or with a chlorophyllous compound in the ratio 1/1? This is, of course, not at all necessary. It seems much more reasonable to assume a mechanism for photosynthesis in which such a chemical combination is superfluous. If carbon dioxide reduction were to take place at a chlorophyll complex, the number of quanta necessary for the reduction of one molecule would also have to combine at this one place. The successive absorption of eight or more quanta by one such complex at low light intensity cannot occur during the short period following illumination after which photosynthesis observably starts out. In this connection the calculations of Kohn should be consulted (108)!

The non-participation of chlorophyll itself in the actual carbon dioxide reduction process is also indicated by the results obtained by Wassink, *et al.* (87, 109, 110, 111), in studies on the relationship between assimilation and fluorescence.

Another reason for wishing to divorce the carbon dioxide reduction from the chlorophyll molecule itself is derived from considerations of a "comparative biochemical" nature. Since 1936 it has become firmly established that the reduction of carbon dioxide is effected by many organisms in whose metabolism radiant energy plays no role. In that year four independent investigations were published which demonstrated this conclusively. Barker (112) showed the methane fermentation to be a process in which carbon dioxide acts as the final hydrogen acceptor during the dehydrogenation of organic compounds, and becomes reduced to meth-

ane; Wieringa (113, 114) discovered a bacterium capable of converting molecular hydrogen and carbon dioxide anaerobically into acetic acid; Woods (115) demonstrated that *B. coli* reversibly produces formic acid from hydrogen and carbon dioxide; and Wood and Werkman (116) made it extremely probable that propionic acid bacteria, during the fermentation of glycerol, convert carbon dioxide into succinic acid. The subsequent studies of the last-mentioned authors have left no doubt as to the occurrence of a process in which carbon dioxide is built into a larger molecule in the dark during a typically catabolic fermentation reaction (117, 118). The recent studies of this and similar reactions with radioactive carbon dioxide and the stable carbon isotope $C^{13}$ have given adequate support to the concept that *the ability of organisms to reduce carbon dioxide, without the supply of radiant energy, is a very general phenomenon indeed* (119, 121, 122, many unpublished results of Carson, Ruben, and Foster).

It might be objected that the ability of many organisms to reduce carbon dioxide in the dark cannot be used as an argument to propose the same mechanism in green plants. But Gaffron (124) has recently shown that algae which can photochemically reduce carbon dioxide in the presence of hydrogen are also capable of reducing carbon dioxide in the dark in the presence of both hydrogen and oxygen. This implies, therefore, that one must either assume the presence of different enzyme systems in the same organism, both capable of reducing carbon dioxide, or be willing to accept temporarily the simpler hypothesis that the carbon dioxide reduction itself is a dark reaction. It would appear that there is much in favor of the latter.

Is this case the function of the light would logically seem to be concerned with the dehydrogenation of the second component of the system: the water. This is most agreeable because the decomposition of this compound with the evolution of oxygen is an energy-requiring reaction. The liberation of oxygen is most easily conceivable as the result of the decomposition of a peroxide. Gaffron's investigations (125) on the effect of poisons on oxygen production have made it probable that a catalase-like enzyme is here involved. The photochemical reduction of carbon dioxide by algae in the presence of hydrogen also tends to show that the oxygen production is the result of a dark reaction.

The simplest hypothesis to account for the facts so far discussed can be expressed by the following scheme

$$H_2O + h\nu \longrightarrow \underset{\text{reduction of } CO_2}{H} + \underset{O_2}{OH}$$

This does not imply, as has been supposed by some critics, that the water molecule is split into a hydrogen atom and a hydroxyl radical. The scheme represents only the essence of the photochemical reaction. It is almost certain that enzyme systems are involved which, by binding ("activating") the water and by serving as hydrogen and hydroxyl acceptors, make the reaction thermodynamically possible. Whether chlorophyll is an integral part of the enzyme system or plays the role of a photosensitizer is for the present purpose unimportant.

A mechanism such as the above fits in well with the observations that the light functions in a first order reaction. It has the additional advantage that an absorbed quantum causes only one type of photochemical reaction: the formation of a hydrogenated system which, by subsequent hydrogen transfer, results in the reduction of carbon dioxide.

This is not entirely specific. Warburg has shown the occurrence of a photochemical nitrate reduction, and unpublished results of the author have demonstrated the occurrence of a photochemical reduction of hydroxylamine by algae. These facts find a ready explanation on the basis of the simple scheme in which the photochemical reaction is only responsible for the production of special hydrogen donors. Their further fate depends upon the surrounding elements.

Green plant photosynthesis is thus considered as a complex of photochemical and dark reactions in which the former consists of a photodecomposition of water, with the aid of chlorophyll and enzymes of unknown nature. One series of dark reactions proceeds from here by transferring hydrogen to the ultimate acceptor ($CO_2$, nitrate, hydroxylamine, etc.), while a second series results in the formation of a peroxidic compound and its decomposition with the liberation of oxygen. The acceptance of the hypothetical series of steps in each dark reaction helps somewhat in accounting for the failure to produce photosynthesis with extracts of green cells, even, for any length of time, with intact chloroplasts.

At first sight it appears as if the above scheme would violate the earlier expressed or implied similarities of green plant and bacterial photosyntheses. It has been shown that in the purple bacteria the dehydrogenation of the specific hydrogen donors, necessary for the occurrence of photosynthesis, is very probably caused by dark reactions. However, it should be realized that the generalized equation for photosynthesis expresses exclusively the dependence of carbon dioxide reduction upon the dehydrogenation of the $H_2A$ compound, and not a mechanism. There is no reason for supposing that the photochemical reaction in purple bacteria differs fundamentally from that assumed to occur in green plants. But one dis-

similarity must be kept clearly in mind. The photosynthetic processes of the purple bacteria occur normally at considerably longer wave lengths than that in green plants, and this means that the available energy per quantum is smaller in the former case. Thus the two products, the "hydrogenated" and the "hydroxylated" systems cannot, together, represent the same energy level as in plants, and this, in turn, may render it impossible for the hydroxylated system to become rearranged into a peroxidic compound. The logical consequence is that only where a peroxide is formed can the system, by the splitting off of oxygen, regenerate itself spontaneously; where this does not happen, the "hydroxyl-accepting" system becomes oxidized and can be regenerated only by an additional reduction process. It is here that the dependence of bacterial photosyntheses upon the presence of appropriate hydrogen donors becomes understandable in a different sense. In the absence of such donors the reaction could proceed until the available amount of "hydroxyl acceptor" had been exhausted by becoming oxidized. The dehydrogenation of the additional reducing substance here becomes a means by which the OH-accepting system can go back into circulation. In all those cases where the actual photochemical reaction and the carbon dioxide reduction proceed rapidly, the rate of photosynthesis is then determined by the rate at which this reduction process takes place.

The following equations, simplified in the extreme by leaving out all the enzyme systems involved, and by representing only the carbon dioxide reduction to its supposed final state, give an idea of the two types of reactions.

Green plants

$$\begin{cases} 4(H_2O + h\nu \longrightarrow H + OH) \\ 4H + CO_2 \longrightarrow (CH_2O) + H_2O \\ 2(2OH \longrightarrow \text{peroxide} \longrightarrow H_2O + O_2) \end{cases}$$

Purple bacteria

$$\begin{cases} 4(H_2O + h\nu \longrightarrow H + OH) \\ 4H + CO_2 \longrightarrow (CH_2O) + H_2O \\ 2(2OH + H_2A \longrightarrow 2H_2O + A) \end{cases}$$

One other point should be made clear. The carbon dioxide is here pictured as being reduced as such, and to carbohydrate. This is not necessarily true. It may well be—and Ruben, *et al.* (126), have presented evidence in favor of this idea—that the "final acceptor" enters into the system by enzymatically becoming combined with an organic compound already present. The reaction discovered by Wood and Werkman in which a four-carbon organic acid is formed from a three-carbon substance and carbon dioxide may serve as a most interesting example of such reactions. The actual reduction process, therefore, may well involve a substance different from carbon dioxide. It would thus become possible to obtain various reduction products with the aid of various organisms and systems.

Were carbon dioxide itself the immediate hydrogen acceptor, one would certainly expect formic acid to be the first reduction product. The mere fact that among the purple bacteria can be found many strains which are incapable of using formic acid either as hydrogen donor, or more important, as hydrogen acceptor in the presence of appropriate hydrogen donors (unpublished) speaks decidedly against the direct reduction of carbon dioxide.

It is admitted that the "mechanism" here presented hardly deserves the name, and that it is too sketchy to allow of experimental verification. Nevertheless, it serves a useful purpose, because it implies that many biochemical reactions, which so far have seemed to bear no relation to the photosynthetic process, may be closely integrated with the latter. The attempt to correlate the many diversified facts uncovered in a variety of fields of biochemical endeavor cannot, in itself, be claimed to furnish direct evidence. But it should be remembered that Kluyver, in the preface to the first treatise on "comparative biochemistry" (127), has defended the attitude here adopted in the following words:

> A study of the chemical activities of microorganisms reveals all the advantages which may be derived from "comparative biochemistry." Although this line of study has not as yet been much developed, it may in future win the same significance for biochemistry as "comparative anatomy" has already long ago attained for anatomy. . . .Biochemists as a rule are unwilling to accept the thesis, maintained in these lectures, that the evidence for the occurrence of a special intermediate stage in a biochemical process can only be of an indirect nature. From this point of view it follows that one can only judge the probability of a supposed reaction mechanism by coordinating all experimental data available.

Nor is the scheme in any sense "new." It has gradually grown out of a first, rough hypothesis, presented in 1935 (14, 32) through further experience, and particularly through the valued intercourse with many colleagues, among whom I especially wish to mention W. Arnold, J. Franck, H. A. Spoehr, and H. Gaffron. That the fundamentals can be found in the 19th century literature is certain; few things in science are entirely original, and even those, in the end, appear more or less closely connected with what has gone on before.

As a simple working hypothesis it seems to present some advantages; its value can only be judged by future developments.

### Bibliography

1. Th. W. Engelmann, *Pflügers Arch. ges. Physiol.*, **30**, 95–124 (1883).
2. Th. W. Engelmann, *Botan. Ztg.*, **46**, 661 ff. (1888).
3. S. Winogradsky, *Ibid.*, **45**, 489 ff. (1887).
4. S. Winogradsky, "Zur Morphologie und Physiologie der Schwefelbakterien," Leipzig, 1888.

5. H. Molisch, "Die Purpurbakterien nach neuen Untersuchungen," Jena, 1907.
6. J. Buder, *Jahrb. wiss. Botan.*, **58**, 525–628 (1919).
7. J. Buder, *Naturwissenschaften*, 1920, 261–268.
8. McG. Skene, *New Phytologist*, **13**, 1–17 (1914).
9. W. Bavendamm, "Die farblosen und roten Schwefelbakterien des Süss- und Salzwassers," Jena, 1924.
10. A. J. Kluyver and H. J. L. Donker, *Chem. Zelle Gewebe*, **13**, 134–190 (1926).
11. C. B. van Niel: "Contrib. to Marine Biol.," Stanford Univ. Press, 1930, 161–169.
12. C. B. van Niel, *Arch. Mikrobiol.*, **3**, 1–112 (1931).
13. W. Bavendamm, Kultur der am Kreislauf des Schwefels beteiligten Bakterien. "Abderhaldens Handb. d. biol. Arbeitsmethoden," Abt. XII, Teil 2, 1934, 483–546.
14. C. B. van Niel, *Cold Spring Harbor Symposia*, **3**, 138–150 (1935).
15. D. I. Saposhnikov, *Microbiology (U. S. S. R.)*, **6**, 643–644 (1937).
16. F. Lipmann, *Biochem. Z.*, **265**, 133–140 (1933).
17. F. Lipmann, *Biochem. Z.*, **268**, 205–213 (1934).
18. J. C. Hoogerheide, "Bijdrage tot de kennis van de reactie van Pasteur." Dissert., Delft, 1935.
19. C. B. van Niel and F. M. Muller, *Rec. trav. botan. Néerland.*, **28**, 245–274 (1931).
20. F. M. Muller, *Arch. Mikrobiol.*, **4**, 131–166 (1933).
21. C. B. van Niel, *Ibid.*, **7**, 323–358 (1936).
22. H. Gaffron, "Abderhalden's Handb. d. biol. Arbeitsmethoden," Abt. XI, Teil 4, 101–160, 1929.
23. H. Gaffron, *Biochem. Z.*, **260**, 1–17 (1933).
24. H. Gaffron, *Ibid.*, **275**, 301–319 (1935).
25. P. A. Roelofsen, *Proc. Koninkl. Akad. Wetenschappen. Amsterdam*, **37**, 660–669 (1934).
26. P. A. Roelofsen, "On Photosynthesis of the *Thiorhodaceae*," Dissert. Utrecht, 1935.
27. C. S. French, *J. Gen. Physiol.*, **20**, 711–735 (1937).
28. S. Wessler and C. S. French, *J. Cellular Comp. Physiol.*, **13**, 327–334 (1939).
29. J. W. Foster, *J. Gen. Physiol.*, **24**, 123–134 (1940).
30. E. Schneider, *Beitr. Biol. Pflanz.*, **18**, 81–115 (1930).
31. V. Czurda, *Arch. Mikrobiol.*, **7**, 110–114 (1936).
32. C. B. van Niel, *Bull. Assoc. Dipl. de Microbiologie, Nancy*, Nr. **13** (1936).
33. V. Czurda, *Second Internat. Congr. Microbiol., Rept. of Proceedings*, London, 470–471 (1937).
34. H. Nakamura, *Acta Phytochim.*, **9**, 189–234 (1937).
35. H. Gaffron, *Biochem. Z.*, **269**, 447–453 (1934).
36. H. Gaffron, *Ibid.*, **279**, 1–33 (1935).
37. W. Bavendamm, *Ergeb. Biologie*, **13**, 1–54 (1936).
38. S. Winogradsky, *Ann. Inst. Pasteur*, **3**, 1–12 (1889).
39. H. Nakamura, *Acta Phytochim.*, **11**, 109–125 (1939).
40. H. Gaffron, *Nature*, **143**, 204 (1939).
41. H. Gaffron, *Am. J. Botany*, **27**, 204–216 (1940).
42. H. Gaffron, *Am. J. Botany*, **27**, 273–283 (1940).
43. A. Stoll, *Naturwissenschaften*, **20**, 955–958 (1932).
44. A. Stoll, *Ibid.*, **24**, 53–60 (1936).

45. C. S. French, *J. Gen. Physiol.*, **21**, 71–87 (1937).
46. J. G. Eymers and E. C. Wassink, *Enzymologia*, **2**, 258–304 (1938).
47. P. A. Dangeard, *Le Botaniste*, **14**, 1–224 (1921–1926); **19**, 1–418 (1927).
48. S. H. Voerkel, *Planta*, **21**, 156–205 (1933).
49. J. Schrammeck, *Beitr. Biol. Pflanz.*, **22**, 315–380 (1935).
50. B. Strzeszewski, *Bull. Intern. Acad. Sc. de Cracovie*, Ser. **B**, **1913**, 416–431 (1914).
51. W. A. Tshesnokov and D. J. Saposhnikov, *Biochimia (U. S. S. R.)*, **1**, 63–74 (1936).
52. W. A. Tshesnokov and D. J. Saposhnikov, *Ibid.*, **1**, 157–164 (1936).
53. D. J. Saposhnikov, *Ibid.*, **2**, 181–197 (1937).
54. H. A. Barker, *J. Cellular Comp. Physiol.*, **8**, 231–250 (1936).
55. G. Giesberger, "Beiträge zur Kenntnis der Gattung *Spirillum* Ehbg." Dissert., Utrecht, 1936.
56. C. E. Clifton, *Enzymologia*, **4**, 246–253 (1937).
57. C. E. Clifton and W. A. Logan, *J. Bact.*, **37**, 523–540 (1939).
58. R. J. Winzler and J. P. Baumberger, *J. Cellular Comp. Physiol.*, **12**, 183–211 (1938).
59. R. J. Winzler, *Ibid.*, **15**, 343–354 (1940).
60. H. Nakamura, *Acta Phytochim.*, **10**, 211–218 (1937).
61. H. Nakamura, *Ibid.*, **10**, 259–270 (1938).
62. H. Nakamura, *Ibid.*, **10**, 297–311 (1938).
63. J. W. Hes, *Nature*, **141**, 647–648 (1938).
64. J. W. Hes, *Ann. fermentations*, **4**, 547–558 (1938).
65. E. Schneider, *Hoppe-Seyler's Z. physiol. Chemie*, **226**, 221–254 (1934).
66. H. Fischer and J. Hasenkamp, *Liebigs Ann. Chem.*, **515**, 148–164 (1935).
67. H. Fischer, R. Lambrecht, and H. Mittenzwei, *Hoppe-Seyler's Z. physiol. Chemie*, **253**, 1–39 (1938).
68. A. Stoll and E. Wiedemann, *Fortschr. Chem. organ. Naturstoffe*, **1**, 159–254 (1938).
69. E. Katz and E. C. Wassink, *Enzymologia*, **7**, 97–112 (1939).
70. E. C. Wassink, E. Katz, and R. Dorrestein, *Ibid.*, **7**, 113–129 (1939).
71. C. B. van Niel and W. A. Arnold, *Ibid.*, **5**, 244–250 (1938).
72. C. B. van Niel and J. C. H. Smith, *Arch. f. Mikrobiol.*, **6**, 219–229 (1935).
73. P. Karrer and U. Solmssen, I. *Helv. Chim. Acta*, **18**, 1306–1315 (1935); II. *Ibid.*, **19**, 3–5 (1936), etc.; V. *Ibid.*, **23**, 460–463 (1940).
74. P. Karrer, *Ibid.*, **18**, 25–27 (1935).
75. E. Schneider, *Rev. faculté sci. univ. Istanbul*, **1**, 74–80 (1936).
76. V. Lubimenko, *Rev. gén. botan.*, **39**, 547–559, 619–637 (1927).
77. V. Lubimenko, *Jour. Soc. Bot. de Russie*, **6**, 107–119 (1921).
78. R. Lévy, G. Teissier, and R. Wurmser, *Ann. physiol. physicochim. biol.*, **1**, 298–311 (1925).
79. H. Mestre, "Contrib. to Marine Biology," Stanford Univ. Press, 1930, 170–187.
80. H. Mestre, *Cold Spring Harbor Symposia*, **3**, 191–209 (1935).
81. E. Smith, *Science*, **88**, 170–171 (1938).
82. C. S. French, *Ibid.*, **88**, 60–62 (1938).
83. C. S. French, *J. Gen. Physiol.*, **23**, 469–481 (1940).
84. C. S. French, *Ibid.*, **23**, 483–494 (1940).
85. B. Hubert, "The Physical State of Chlorophyll in the Living Plastid." Dissert., Leiden, 1935.

86. L. G. M. Baas-Becking, *6th Internat. Botan. Congr., Proceedings*, Vol. II, 265–266 (1935).
87. D. Vermeulen, E. C. Wassink, and G. H. Reman, *Enzymologia*, **4**, 254–268 (1937).
88. O. Warburg and E. Negelein, *Z. physik. Chem.*, **102**, 235–266 (1922).
89. O. Warburg and E. Negelein, *Ibid.*, **106**, 191–218 (1923).
90. K. Wohl, *Ibid.*, **37**, 105–147, 169–230 (1937).
91. J. Franck, *Chem. Rev.*, **17**, 433–442 (1935).
92. J. Franck and K. F. Herzfeld, *J. Chem. Phys.*, **5**, 237–251 (1937).
93. R. Emerson and C. M. Lewis, *Am. J. Botany*, **26**, 808–822 (1939).
94. W. A. Arnold, "Investigations in photosynthesis." Thesis, Harvard Univ., 1935.
95. W. M. Manning, J. F. Stauffer, B. M. Duggar, and F. Daniels, *J. Am. Chem. Soc.*, **60**, 266–274 (1938).
96. W. M. Manning, C. Juday, and M. Wolf, *Ibid.*, **60**, 274–278 (1938).
97. J. L. Magee, T. W. Dewitt, E. C. Smith, and F. Daniels, *Ibid.*, **61**, 3529–3533 (1939).
98. H. G. Petering, B. M. Duggar, and F. Daniels, *Ibid.*, **61**, 3525–3529 (1939).
99. R. Emerson and W. Arnold, *J. Gen. Physiol.*, **15**, 391–420 (1932).
100. R. Emerson and W. Arnold, *Ibid.*, **16**, 191–205 (1932).
101. W. Arnold and H. I. Kohn, *Ibid.*, **18**, 109–112 (1934).
102. H. I. Kohn, "A study of certain components of the photosynthetic mechanism in green plants." Thesis, Harvard Univ., 1935.
103. H. Gaffron and K. Wohl, *Naturwissenschaften*, **24**, 81–90, 103–107 (1936).
104. K. Wohl, *New Phytologist*, **39**, 33–64 (1940).
105. W. Arnold, *Cold Spring Harbor Symposia*, **3**, 124–127 (1935).
106. R. Emerson, *Ergeb. Enzymforsch.*, **5**, 305–347 (1936).
107. E. D. McAlister, *J. Gen. Physiol.*, **22**, 613–636 (1939).
108. H. I. Kohn, *Nature*, **137**, 706–707 (1936).
109. E. C. Wassink, D. Vermeulen, G. H. Reman, and E. Katz, *Enzymologia*, **5**, 100–109 (1938).
110. L. S. Ornstein, E. C. Wassink, G. H. Reman, and D. Vermeulen, *Ibid.*, **5**, 110–118 (1938).
111. E. C. Wassink and E. Katz, *Ibid.*, **6**, 145–172 (1939).
112. H. A. Barker, *Arch. Mikrobiol.*, **7**, 404–419 (1936).
113. K. T. Wieringa, *Ant. van Leeuwenhoek*, **3**, 88–89 (1936).
114. K. T. Wieringa, *Ibid.*, **3**, 263–273 (1936).
115. D. D. Woods, *Biochem. J.*, **30**, 515–527 (1936).
116. H. G. Wood and C. H. Werkman, *Ibid.*, **30**, 48–53 (1936).
117. H. G. Wood and C. H. Werkman, *Ibid.*, **34**, 7–14 (1940).
118. H. G. Wood and C. H. Werkman, *Ibid.*, **34**, 129–138 (1940).
119. H. G. Wood and C. H. Werkman, *J. Biol. Chem.*, **135**, 789–790 (1940).
120. S. R. Elsden, *Biochem. J.*, **32**, 187–193 (1938).
121. S. F. Carson and S. Ruben, *Proc. Nat. Acad. Sci.*, **26**, 422–426 (1940).
122. H. A. Barker, S. Ruben, and M. D. Kamen, *Ibid.*, **26**, 426–430 (1940).
123. H. A. Barker, S. Ruben, and J. V. Beck, *Ibid.*, **26**, 477–482 (1940).
124. H. Gaffron, *Science*, **91**, 529–530 (1940).
125. H. Gaffron, *Cold Spring Harbor Symposia*, **7**, 377–384 (1939).
126. S. Ruben, W. Z. Hassid, and M. D. Kamen, *J. Am. Chem. Soc.*, **61**, 661–663(1939).
127. A. J. Kluyver, "The chemical activities of micro-organisms." London, 1931.

# UNTERSUCHUNG ENZYMATISCHER PROZESSE IN DER LEBENDEN PFLANZE

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## I. Allgemeine Vorstellungen über die Wirkung der Enzyme in lebenden Zellen

Die Lehre von den Enzymen entwickelte sich anfänglich unter dem Einfluss von Anregungen, die biologischen Fragestellungen entstammten, welche den Stoffumsatz in Organismen betrafen. In Anbetracht der grossen Empfindlichkeit der Enzyme gegenüber verschiedenen Einwirkungen chemischer, physikalischer und physikalisch-chemischer Art bot indessen das Studium der Enzyme in den Geweben und besonders im intakten Organismus erhebliche Schwierigkeiten die in Zusammenhang standen mit der komplizierten und unbestimmten Zusammensetzung des natürlichen Mediums, in dem die Enzyme ihre biologischen Funktionen verrichten.

Dies gab den Anlass zur Entwicklung zahlreicher Methoden für die präparative Darstellung der Enzyme und für ihre Reinigung von Begleitstoffen. Die Anwendung solcher gereinigter Präparate ermöglichte es den Forschern, ihre Experimente unter übersichtlicheren Bedingungen durchzuführen und auf diese Weise den Ablauf enzymatischer Reaktionen und ihre Beeinflussung durch verschiedene Faktoren zu verfolgen. Auf diesem Wege gelang es nicht nur über viele wichtige Besonderheiten enzymatischer Reaktionen Aufschluss zu gewinnen, sondern auch die chemische Natur einer Reihe von Enzymen zu ergründen.

Gerade die Erfolge der Arbeit mit isolierten Enzympräparaten haben indessen dazu geführt, dass sich die Enzymforschung weiter und weiter von den Fragen der Biologie entfernte und zu einem selbstständigen wissenschaftlichen Fach wurde.

Man rechnete anfänglich darauf, dass das Studium der Enzyme im künstlichen, vereinfachtem Milieu es gestatten würde, die erzielten Resultate für die Klarstellung der physiologischen Rolle der Enzyme bei den Lebensäusserungen der Organismen zu verwerten. Diese Hoffnung ist aber in manchen Fällen nicht in Erfüllung gegangen. Zwischen dem Verhalten der Enzyme in künstlichen Gemischen und in lebenden Organismen besteht ein wesentlicher Unterschied, der die Auswertung des "rein enzymologischen" Versuchs zur Lösung physiologischer Probleme erschwert. In künstlichen Systemen ist nämlich die Wirkung der Enzyme fast immer *einseitig* (z.B. hydrolytisch), während die enzymatischen Vorgänge in lebenden Zellen in der Regel *leicht reversibel* sind. Daher sind auch die Gesichtspunkte bei der Beurteilung der Enzymwirkungen grundsätzlich verschiedene, je nachdem ob es sich um Wirkungen in vitro oder in vivo handelt. Während nämlich die Wirkung eines Enzympräparats an der Geschwindigkeit des Substratumsatzes (z.B. der Hydrolyse) gemessen wird, und die Einflusse verschiedener Agenzien auf das Enzym eben von diesem Standpunkt aus beurteilt werden, ist für das Verhalten eines Enzyms in der lebenden Zelle weniger seine Gesamtquantität kennzeichnend als das Verhältnis der Geschwindigkeiten seiner synthetisierenden und hydrolysierenden Wirkungen, welches die *Richtung* des enzymatischen Prozesses im Organismus bestimmt, und eben dieser Gesichtspunkt ist für die Beurteilung verschiedener Wirkungen auf die Enzyme in der Zelle massgebend.

Die Bedingungen, von denen die Geschwindigkeit und die Richtung enzymatischer Reaktionen in den Zellen abhängen, fesselt schon seit langem die Aufmerksamkeit der Forscher. Es tauchte vor Allem die Frage nach den Ursachen auf, denen zufolge in der Zelle Enzyme und ihre nicht abge-

bauten Substrate nebeneinander bestehen können, z.B. Amylase und Stärke, proteolytische Enzyme und Eiweisskörper, u.s.w.

Da bei Berührung von Enzymen mit den entsprechenden Substraten in homogener wässeriger Lösung praktisch vollständige Spaltung der Substrate erfolgt, wurde angenommen, dass die Enzyme und die Substrate in der lebenden Zelle räumlich voneinander getrennt sind. Hieraus erwuchs die Vorstellung von der Ungleichmässigkeit oder Heterogeneität des natürlichen Mediums, in dem die Enzyme ihre Wirkungen ausüben. Diese bereits von F. Hofmeister (1) geäusserte Anschauung wurde später durch W. Palladin (2) und anderen Autoren ausgebaut. Deutlicher und exakter konnte die Frage auf grund der Arbeiten von A. Oparin und seinen Mitarbeitern (3) formuliert werden, die in zahlreichen Modellversuchen zeigen konnten, dass die Adsorption von Enzymen an Eiweissniederschlägen in manchen Fällen die hydrolytische Wirkung der Enzyme aufhebt. Aus diesen Befunden zog A. Oparin den Schluss, dass die Regulierung der hydrolytischen Wirkung der Enzyme in den Zellen nicht allein durch räumliche Trennung von Substraten und Enzymen zustandekommt, sondern auch durch inaktivierende Adsorption der Enzyme auf den Strukturgebilden des Plasmas. Es ist aber einleuchtend, dass die regulierenden Einflüsse in lebenden Zellen nicht nur in einer Verstärkung oder Schwächung der Hydrolyse bestehen, sondern auch die synthetischen Funktionen der Enzyme berühren.

In Übereinstimmung mit der van't Hoff'schen Gleichung verläuft die Hydrolyse der Substrate enzymatischer Reaktionen desto vollständiger, je höher der Wassergehalt des Reaktionsgemisches ist; durch Ausschaltung von Wasser aus dem System muss umgekehrt die Synthese begünstigt werden (4). In einer Reihe von Arbeiten mit Enzympräparaten gelang es tatsächlich, durch Anwendung wasserfreier Medien die synthetisierende Wirkung der Enzyme zur Äusserung zu bringen. Es unterliegt keinem Zweifel, dass diese Gesetzmässigkeiten auch die Richtung der enzymatischen Reaktionen in der lebenden Zelle bis zu einem gewissen Grade beeinflussen.

Wenn man aber die lebende Zelle, die 70–80% Wasser enthält, als ein homogenes Medium betrachtet, so ergeben sich von vornherein äusserst ungünstige Bedingungen für enzymatische Synthesen. Man ist daher zu der Annahme genötigt, dass in der Zelle trotz ihres Wasserreichtums bestimmte "trocknere" Bezirke vorliegen, in denen die Bedingungen für enzymatische Synthesen geeigneter liegen. Wir gelangen auf diese Weise wiederum zur Vorstellung von der Heterogenеitat der Zelle und einer hiermit verknüpften Regulierung der intrazellulären Enzymwirkungen.

Alle diese Erwägungen wurden von A. Oparin in seiner in den Jahren 1935–37 aufgestellten Theorie in Betracht gezogen (5). Diesen Vorstel-

lungen gemäss, ist ein Teil des Enzyms in der lebenden Zelle in Lösung vorhanden und übt in diesem Zustand einseitige hydrolysierende Wirkung aus. Der andere Teil ist dagegen an wasserarmen plasmatischen Strukturgebilden adsorbiert und ist nur in synthetischer Richtung wirksam. Demgemäss wird die Synthese und die Hydrolyse des Substrats in der lebenden Zelle durch ein und dasselbe Enzym vollzogen, obgleich beider Prozesse voneinander räumlich getrennt sind.

Die Strukturgebilde des Protoplasmas müssen auch dadurch die enzymatischen Synthesen begünstigen, dass die Ausgangsprodukte der Synthese in hohen Konzentrationen an ihnen angereichert werden können. Verschiedenartige Modellversuche des Autors dieser Theorie, sowie anderer Forscher (vgl. z.B. H. Marston (6), A. Kusin und O. Bogdaschevskaya (7) u.a.) bestätigen die Annahme, dass heterogene Beschaffenheit des Mediums und die hiermit verbundene Konzentrierung und Adsorption der Substanzen tatsächlich die hydrolytische Wirkung von Enzymen vermindert und das Zustandekommen von Synthesen begünstigt. Indirekt wird diese Theorie auch durch die Tatsache gestützt, dass in Geweben mit hoher synthetischen Enzymaktivität, z.B. in reifenden Samen, Zuckerrübenwurzeln, Kartoffelknollen und vielen anderen, der überwiegende Anteil der Enzyme in adsorbiertem Zustand vorliegt.

Zu ähnlichen Vorstellungen gelangen auf anderem Wege die Cytologen, die durch direkte Beobachtung der Bildung und des Abbaus der Zellsubstanzen feststellen konnten, dass die synthetischen Funktionen nicht diffus über die ganze Zelle verstreut, sondern vielmehr an bestimmte Zentren gebunden sind, und zwar an die Mitochondrien, Chondriosomen und deren Abarten—die Plastiden (vgl. E. Horning (8), A. Kurssanov (9)). Schliesslich berechtigt auch die chemische Analyse der Plastiden, die einen erhöhten Gehalt an wasserfreier lipoider Phase aufweisen (s. z.B. W. Menke (10)) und bedeutende Enzymmengen (K. Linderstrøm-Lang und H. Holter (11)) enthalten, diese Gebilde als geeignete Zentren für enzymatische Synthesen in der Zelle zu betrachten. Ebenso geeignet für die enzymatischen Synthesen, kann auch die an Lipoiden reiche Oberschichte des Protoplasmas betrachtet werden (A. Kurssanov (9)).

Neben der Erforschung der Adsorption als eines regulierenden Prinzips der Enzymwirkungen in der Zelle, wurde das Problem auch vom Standpunkt der Energiebilanz aus bearbeitet. Aus dem Vergleich der Verbrennungswärmen geht hervor, dass die synthetisierten Produkte in der Regel energiereicher sind als die Ausgangsstoffe. Allerdings sind die Differenzen im Falle hydrolytischer Enzyme gewöhnlich nicht gross, aber dennoch hinreichend um die Frage nach dem Ursprung der Energie für die enzymatischen Synthesen zu rechtfertigen. Es haben daher manche Forscher dieser Seite des Problems ernstliche Beachtung gewidmet (vgl. z.B. H. Borsook

(12), A. Blagowestschenski (13)). Der Grundgedanke dieser Forschungsrichtung ist der, dass die Energie gleichzeitig ablaufender exothermer Reaktionen, z.B. Oxydationen, auf die enzymatischen Synthesen übertragen wird. In manchen Versuchen, die mit gelösten Enzympräparaten oder Gewebsbreien angestellt wurden, konnten enzymatische Synthesen tatsächlich durch das Ablaufen einer exothermen Reaktion im Medium begüngstigt werden (J. Abelous und H. Ribaut (14), A. Blagowestschenski und K. Nikolaev (15) u.a.). Diese Vorstellungen lassen sich auch gut auf die lebende Zelle anwenden, in der einzelne enzymatische Oxydationsreaktionen oder der Atmungsprozess im Ganzen als Energiequellen dienen können.

Ohne die Möglichkeit der Beteiligung von Oxydationsvorgängen an der Steuerung reversibler Enzymwirkungen in Abrede zu stellen, können wir jedoch vermuten, dass diese Vorgänge nicht die einzigen Energiequellen in den Zellen darstellen.

Als weitere, sehr wesentliche Energiequelle für enzymatische Synthesen kommen verschiedene zusätzlichen Energieformen in Betracht, z.B. Adsorptionen, osmotische Druckdifferenzen u.dgl. die im lebenden Protoplasma durch stetigen Energieaufwand bei anderen Prozessen erzeugt und unterhalten werden.

Es kann zurzeit kaum bezweifelt werden, dass die Struktur des lebenden Protoplasmas keine passive Zustandsform der Zellsubstanz ist, sondern ein aktiv ablaufender Prozess, für den ununterbrochene Energiezufuhr erforderlich ist (vgl. E. Bünning (16), W. Stiles (17) u.a.). W. Lepeschkin weist darauf hin, dass im lebenden Protoplasma die wichtigsten Substanzen —Proteine und Lipoide—zu labilen chemischen Verbindungen zusammengefügt sind (Vitaide), die beim Absterben der Zelle unter Abgabe von Wärme und strahlender Energie verfallen (18). Die eigenartige chemische Zusammensetzung des lebenden Protoplasmas geht auch aus einer Arbeit von F. Vles und M. Gex (19) hervor, in der die Verschiedenheit der Absorptionsspektra des lebenden und des toten Zellinhalts nachgewiesen wurde.

Schliesslich äussert sich der energetische Zustand des lebenden Protoplasmas in ununterbrochener Zirkulationsbewegung und in der Bildung zahlreicher flüssiger Strukturen, deren Existenz nur bei stetigem Energieaufwand möglich ist. Die Besonderheit der Struktur des lebenden Protoplasmas sind in den cytologischen Untersuchungen von A. Guilliermond (20) deutlich zutage gebracht. Besonderer Beachtung verdient auch die Tatsache, dass die Eiweissmoleküle, die normalerweise globulären Aufbau haben (D. Talmud (21) in der lebenden Zelle in gestreckter Form vorliegen (W. Lepeschkin (18), J. Lengmur (22)) und sich folglich in Bezug auf ihren

Energieinhalt von dem zustand des inerten Eiweisses sehr stark unterscheiden.

Es folgt, hieraus, dass der reine Vergleich der Verbrennungswärmen einzelner Substanzen noch lange nicht ausreichend ist zur Beurteilung der Energiebilanz von Reaktionen, die sich in derart komplizierter Umgebung abspielen, wie sie das lebende Protoplasma darstellt, denn die Energieniveaus der Reaktionsteilnehmer können durch die vitale Struktur des Protoplasmas tiefgreifend geändert werden. Infolgedessen können unter den betreffenden Verhältnissen nicht nur die Hydrolysen, sondern auch die Synthesen exotherme Vorgänge darstellen.

Es lässt sich zurzeit schwerlich entscheiden, ob die intravitale Struktur des Plasmas unmittelbar durch die Energie der Atmung aufrecht erhalten wird, wie W. Lepeschkin (23), K. Paech (24), W. Stiles (17) und andere annehmen, oder ob dies auf dem Wege mehr oder weniger komplizierter Energieumwandlungen in irgendwelchen Oxydations-Reduktions-Systemen erfolgt.

Es erscheint jedoch jetzt schon der Schluss berechtigt, dass Struktur und Energie inbezug auf enzymatische Synthesen in den Zellen zwei Aspekte ein und derselben Erscheinung darstellen (A. Kurssanov (25)).

Es folgt hieraus, dass die theoretischen Vorstellungen A. Oparin (5), denenzufolge die Reversibilität enzymatischer Reaktionen auf Phänomenen der Adsorption von Substraten und Enzymen im heterogenen Medium beruhen, eigentlich auch den Begriff der Energie umfassen, die für die Bildung und Aufrechterhaltung der Strukturgebilde im lebenden Protoplasma erforderlich ist.

## II. Bestimmung der Aktivität von Enzymen in lebenden pflanzlichen Geweben

Die Verfahren zur Bestimmung von Enzymen in den Geweben von Tieren oder Pflanzen beruhten bis in die letzte Zeit hauptsächlich auf der Methode *autolytischer Gemische.*

Das Prinzip dieser Methode besteht in der Freilegung der Zellfermente durch möglichst vollständige mechanische Zerstörungen der Gewebe, wonach in der zerriebenen Masse die Geschwindigkeit der enzymatischen Spaltung verschiedener Substanzen gemessen wird. Da hierbei die Struktur des lebenden Protoplasmas und damit auch die Reversibilität der Enzymwirkungen gewöhnlich vollständig verloren geht, sind die autolytischen Gemische eher stark verunreinigten Enzympräparatoren als lebenden Zellen gleichzusetzen. Es nimmt deshalb nicht Wunder, dass es bei weitem nicht immer gelingt, auf diese Weise erhaltene Resultate mit der Richtung und dem allgemeinen Charakter der physiologischen Tätigkeit lebender Organismen in Einklang zu bringen.

Noch bedenklicher sind die Verhältnisse, die man beim Arbeiten mit autolytischen Gemischen schafft, indem man durch spezielle Massnahmen "optimale" Bedingungen für die Wirkung der Enzyme zu sichern bestrebt ist. Der Zusatz von Wasser zu dem zerriebenen Material, die Einstellung des *p*H und der Temperatur auf Werte, die für das betreffende Enzym optimal sind, der Zusatz von Antiseptica u.s.w., stellen Bedingungen her, die sogar in Bezug auf einseitige Enzymwirkung vollständig von den in der lebenden Zelle vorherrschenden abweichen. Die Kompliziertheit des Bildes wird noch dadurch gesteigert, dass die Enzyme infolge des Zerreibens der Gewebe mit manchen Substanzen in Berührung kommen, die in der lebenden Zelle von den Enzymen räumlich getrennt waren und die ihre Wirkung auf diese oder jene Weise zu beeinflussen vermögen. Hierher gehören zum Beispiel die Gerbstoffe, deren Anwesenheit in pflanzlichen Geweben mitunter das Arbeiten mit autolytischen Gemischen infolge vollständiger Inaktivierung der Enzyme gänzlich vereitelt. *Die nach der Methode der autolytischen Gemische ermittelten Enzymaktivitäten sind demzufolge in hohem Masse konventionell.*

Obige Betrachtungen führen zwar zu dem Schluss, dass die Methode der autolytischen Gemische für Beobachtungen über die physiologischen Vorgänge in Zellen wenig geeignet ist; es wäre indessen falsch, diesem Verfahren jegliche Bedeutung absprechen zu wollen, denn in manchen Fällen, zum Beispiel wenn es lediglich gilt, das Vorhandensein eines gewissen Enzyms im untersuchten Objekt festzustellen, kann die Methode der autolytischen Gemische gute Dienste leisten. Von Bedeutung ist die Methode auch zur Prüfung der enzymatischen Aktivität industriell verarbeiteter Materialien, zum Beispiel bei der Beurteilung der Backfähigkeit von Mehl, des Verzuckerungsvermögens von Malz, der Enzymaktivität in gerollten Teeblättern u.dgl. In allen diesen Fällen wirken die Enzyme unter annähernd denselben Bedingungen wie in autolytischen Gemischen. In ähnlicher Weise sind schliesslich Bestimmungen der Enzymaktivität in den Verdauungssäften von Tieren und insektenfressenden Pflanzen auszuführen, insofern die physiologische Rolle der von den Zellen sezernierten Enzyme in einseitiger hydrolytischer Wirkung besteht.

Was die intrazellulären Enzyme betrifft, so lässt sich das Studium ihrer physiologischen Rolle im Organismus nur dann mit Erfolg betreiben, *wenn die Intaktheit der Zellen gesichert ist.*

In dem Bestreben, die Lücken in unserer Kenntnis der intrazellulären enzymatischen Prozesse bis zu einem gewissen Grad auszufüllen, wurden von mir seit 1934 Studien über die Wirkung der Enzyme in lebenden Pflanzenzellen durchgeführt.

Die erste Aufgabe, deren Lösung zur Verwirklichung dieser Absicht erforderlich war, betraf die Ausgestaltung von Methoden zur quantitativen Bestimmung der Aktivität von Enzymen in lebenden Geweben.

Zu diesem Zwecke machten wir von der Methode der Vakuuminfiltration Gebrauch, die früher von A. Virtanen, J. Björksten (26), und K. Mothes (27), zur Unterrsuchung des Eiweissstoffwechsels der höheren Pflanzen angewendet worden ist. Der Vorteil dieser Methode gegenüber anderen Methoden zur Einführung verschiedener Substanzen in das pflanzliche Objekt besteht in der Geschwindigkeit und Gleichmässigkeit, mit der es gelingt, in den Geweben der Pflanze Lösungen jener Substanz anzureichern, deren Umwandlung untersucht werden soll. Darum wird in Vakuuminfiltrationsversuchen die Geschwindigkeit der enzymatischen Reaktionen nicht durch Mangel an Substrat verzögert.

Eine andere Bedingung, die zu beachten ist, wenn man den Messungen der Geschwindigkeit enzymatischer Prozesse in der Zelle quantitativen Wert verleihen will, besteht darin, die Dauer der Versuche so kurz abzumessen, dass die Umwandlungen der eingeführten Substanzen während der Versuchszeit nicht zum Abschluss kommen. Diese letztere Bedingung wurde in früheren Untersuchungen öfters vernachlässigt; infolgedessen erhielt man für die Geschwindigkeiten enzymatischer Prozesse beim Umrechnen pro Zeiteinheit Werte, die so gering waren, dass sie auf keinen Fall mit der tatsächlichen Geschwindigkeit des Ab- und Aufbaus der Substanzen in der lebenden Pflanze in Vergleich gesetzt werden konnten.

Unter Berücksichtigung dieser Anforderungen gelang es uns, quantitative Messungen der Enzymaktivitäten in lebenden Pflanzen durchzuführen.

Zu diesem Zweck wurden Methoden entwickelt für die Bestimmung der hydrolysierenden und synthetisierenden Wirkung der Invertase (Saccharase) (A. Kurssanov (28)), der synthetisierenden Wirkung der Proteasen (A. Kurssanov und K. Bryuschkova (29)), sowie der phosphorylierenden und dephosphorylierenden Wirkung der Phosphatasen (A. Kurssanov und N. Kryukova (30), A. Kurssanov (31)). Ferner haben B. Rubin und N. Syssakyan (32) von denselben Prinzipien ausgehend, eine Methode zur Messung des Oxydations- und Reduktionsvermögens lebender Pflanzengewebe ausgearbeitet.

Die hydrolysierende Wirkung der Invertase wird gemessen an der Geschwindigkeit der Spaltung von ins lebende Gewebe infiltrierter Saccharose, die synthetisierende Wirkung nach der Geschwindigkeit der Synthese dieses Disaccharids aus einem Gemisch von Fructose und Glukose.

Zahlreiche nach dieser Methode ausgeführte Versuche ergaben, dass für Invertase in Pflanzen ein bestimmtes, mehr oder weniger konstantes Ver-

hältnis zwischen den Geschwindigkeiten der synthetisierenden und hydrolysierenden Wirkung besteht, solange die äusseren Bedingungen und der Innenzustand der Zellen konstant gehalten werden. Dieses Verhältnis, nach dem wir die *vorherrschende Richtung* des enzymatischen Prozesses beurteilen, kennzeichnet die Einstellung der Pflanze oder ihrer einzelnen Organe auf vorzugsweise Speicherung von Saccharose oder von Monosen.

Bei künstlicher Verschiebung des normalen Verhältnisses zwischen Monosen und Saccharose durch Infiltration von Blättern mit einer Lösung der betreffenden Zucker setzt sofort, in Übereinstimmung mit dem Massenwirkungsgesetz eine einseitige Invertasewirkung ein, die auf Wiederherstellung des gestörten Gleichgewichts gerichtet ist. Diese Wirkung dauert so lange an, bis die ursprüngliche Gleichgewichtslage wiederhergestellt ist. (A. Kurssanov (28), s. Abb.1.)

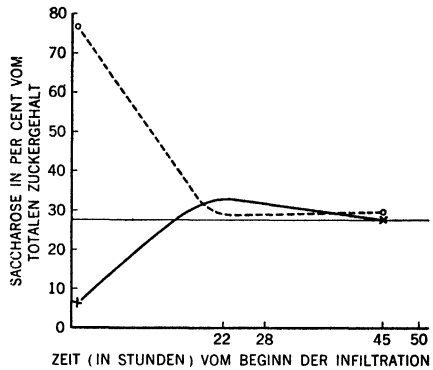



Fig. 1.—Restitution des gestörten Zuckergleichgewichts in Blättern von *Cyclamen persicum*.
1. Nach Infiltration mit Saccharose;
2. Nach Infiltration mit Monosen;
3. Kontrollproben ohne Infiltration.

Folglich ist das hier beschriebene Verfahren zur quantitativen Bestimmung der synthetisierenden und hydrolysierenden Enzymwirkungen in lebenden Geweben eigentlich ein Verfahren zur Messung der Geschwindigkeit, mit der der Enzymapparat der betreffenden Pflanze imstande ist, das gestörte Gleichgewicht wieder herzustellen.

In Tabelle I sind einige Befunde angeführt, die die Aktivität und die Wirkungsrichtung der Invertase in verschiedenen Pflanzen veranschaulichen.

Wie aus diesen Beispielen ersichtlich ist, sind sowohl die absolute Aktivität der Invertase wie die Richtung ihrer Wirkung bei einzelnen Pflanzen sehr verschieden. Es ist ferner zu beachten, dass die hier mitgeteilten Werte, besonders für Blätter, nur für einen gewissen durchschnittlichen Zustand der Gewebe typisch sind. Im folgenden soll gezeigt werden, dass das Verhältnis zwischen Synthese und Hydrolyse in den Zellen unter gewissen Bedingungen starke Änderungen erfahren kann, die mit bestimm-

TABELLE I

SYNTHETISIERENDE UND HYDROLYSIERENDE WIRKUNG DER INVERTASE IN LEBENDEN GEWEBEN EINIGER PFLANZEN

(In mg. Invertzucker pro 1 Stunde und 1 gr. Trockensubstanz)

| Lfd. nr. | Pflanzliches Objekt | Invertaseaktivität | | Verhältnis: Synthese/-Hydrolyse | Literaturangabe |
|---|---|---|---|---|---|
| | | Synthese | Hydrolyse | | |
| 1 | Erbsensamen (milch-reif) | 78.4 | 0.0 | ~ | B. Rubin und O. Lutikova (33) |
| 2 | Zuckerrübe (*Beta vulgaris*) Wurzel | 133.5 | 4.2 | 31.4 | A. Oparin (5) |
| 3 | Zichorie (*Zychorium intibus*) Blätter | 9.7 | 3.7 | 2.62 | A. Kurssanov (28) |
| 4 | Tee, junge Blätter (*Camellia thea*) | 16.1 | 9.0 | 1.79 | A. Kurssanov (81) |
| 5 | Zuckerrübe (*Beta vulgaris*) Blätter | 12.6 | 8.4 | 1.50 | A. Kurssanov (28) |
| 6 | Banane, Blätter (*Musa japonica*) | 23.3 | 16.1 | 1.44 | A. Kurssanov (28) |
| 7 | Farn (*Scolopendrium vulg.*) | 9.2 | 7.9 | 1.16 | A. Kurssanov (25) |
| 8 | Zitrone, Blätter (*Cytrus limonium*) | 3.2 | 3.0 | 1.07 | A. Kurssanov (25) |
| 9 | Chinabaum, Blätter (*Cynchona Ledgeriana*) | 7.2 | 9.3 | 0.77 | A. Kurssanov und N. Kryukova (35) |
| 10 | Mandarine Blätter (*Cytrus unschiu*) | 4.2 | 5.6 | 0.75 | A. Kurssanov (25) |
| 11 | Weizen Blätter (Hordeiforme 7434) um die Zeit des Staudens | 11.4 | 20.4 | 0.56 | N. Syssakyan (85) |
| 12 | Zyklamen Blätter (*Cyclamen persicum*) | 5.8 | 15.2 | 0.38 | A. Kurssanov (28) |
| 13 | Apfelsine (Schale der reifen Frucht) | 1.0 | 5.0 | 0.20 | A. Kurssanov und N. Kryukova (37) |
| 14 | Tomate (*Solanum lycopersicum*) unreife Frucht | 2.2 | 63.3 | 0.03 | A. Kurssanov und N. Kryukova (37) |
| 15 | Kohl, Sorte Nr. 1 Zwei-Blättchen Stadium | 0.0 | 4.0 | 0.00 | B. Rubin und E. Arzichovskaya (38) |

ten Phasen der physiologischen Tätigkeit des Organs in Zusammenhang stehen.

Wenn man berücksichtigt, dass es beim Arbeiten mit 0.1–0.2 mol Zucker-

lösungen gelingt, etwa 50 bis 150 mg. Zucker pro 1 gr. Trockengewicht in die lebenden Gewebe zu infiltrieren, so lässt sich bei annähernd bekannter Geschwindigkeit des enzymatischen Prozesses die richtige Expositionsdauer leicht feststellen. Gewöhnlich beträgt sie in unseren Versuchen nicht mehr als 2–3 Stunden. In den Fällen wo die Wirkung der Invertase besonders intensiv ist, muss die Versuchsdauer bis auf 30 oder sogar 15 Minuten gekürzt werden.

Die hohe Geschwindigkeit, mit der die Umwandlungen der infiltrierten Zucker im lebenden Gewebe erfolgen, lässt vermuten, dass die enzymatischen Prozesse in diesen Versuchen nicht von der Permeabilität des Plasmas abhängen, sondern sich vielmehr bereits an der äusseren, der Zellulosemembran zugewandten Oberfläche des Protoplasten abspielen. In einer Reihe von Versuchen (A. Kurssanov und N. Kryukova (39), A. Kurssanov (25)), wurde gezeigt, dass die Geschwindigkeit des Eindringens der infiltrierten Saccharose durch die Zellulosemembranen die Geschwindigkeit der Hydrolyse dieses Zuckers in den Zellen der betreffenden Pflanze um das Mehrfache übertrifft. So wurde, zum Beispiel, in Versuchen an Blättern von Cyclamen persicum gefunden, dass die Geschwindigkeit des Eindringens von Saccharose aus dem Intercellularraum in die Zellen 29 mg. in 1 Stunde pro 1 gr. Trockengewicht der Blätter entspricht, während die Geschwindigkeit der Hydrolyse des Disaccharids 12–15 mg. beträgt. Diese und einige andere Versuche führen zu dem Schluss, dass die Permeabilität der Zellulosemembranen für die Zucker die Geschwindigkeit ihrer enzymatische Umwandlung in den Zellen nicht einschränkt. Was das weitere Eindringen der Zucker ins Innere des Protoplasten anbelangt, so ist die Geschwindigkeit dieses Prozesses eine viel geringere; in den Blättern von Hydrangea hortensis beläuft sie sich beispielsweise auf Werte von etwa 4–5 mg. Glukose pro Stunde.

Die synthetisierende Wirkung der *Proteasen* wird quantitativ gemessen durch die Geschwindigkeit, mit der Eiweisskörper und Peptone aus einem in die lebende Pflanze infiltriertem Aminosäuregemisch gebildet werden.*

In Tabelle II sind Werte angeführt, die die Geschwindigkeit der Synthese von Eiweiss aus Aminosäuren in einigen Pflanzen kennzeichnen.

Aus der Tabelle ersieht man, dass das Hauptprodukt der Synthese Eiweiss ist, während Peptone in viel geringerer Menge gebildet werden.

Beschränkt man die Versuchsdauer derart, dass die Umwandlung der

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* Zu diesem Zweck dienen gereinigte Hydrolysate, die durch Säurehydrolyse von tierischen oder pflanzlichen Proteinen bereitet werden.

TABELLE II

SYNTHETISIERENDE WIRKUNG DER PROTEASEN IN DEN GEWEBEN VERSCHIEDENER PFLANZEN

(In mg. N pro 1 g. Trockengewebe)

| Lfd. nr. | Pflanzliches Objekt | Eingeführter Aminostickstoff (N mg. pro 1 g. Trockensubstanz) | Proteinasen (Eiweisssynthese) | | Polypeptidasen (Peptonsynthese) | | Gesamtsynthese pro 1 Stunde |
|---|---|---|---|---|---|---|---|
| | | | in 15 min. | umgerechn. f. 1 Stunde | in 15 min. | umgerechn. f. 1 Stunde | |
| 1 | Erbse (12-tägige Keimlinge) | 10.9 | 8.0 | 32.0 | 0.6 | 2.4 | 34.4 |
| 2 | Hafer (12-tägige Keimlinge) | 9.2 | 5.0 | 20.0 | 0.4 | 1.6 | 21.6 |
| 3 | Weizen (11-tägige Keimlinge) | 9.7 | 4.7 | 18.8 | 0.8 | 3.2 | 22.0 |
| 4 | Zichorie (Blätter) | 11.2 | 4.3 | 17.2 | 0.2 | 0.8 | 18.0 |
| 5 | *Cyclamen persicum* (Blätter) | 16.0 | 2.8 | 11.2 | 0.6 | 2.4 | 13.6 |
| 6 | Champignon (Fruchtkörper) | 31.1 | 6.4 | 25.6 | ... | ... | ... |

infiltrierten Aminosäuren nicht bis zu Ende vorschreitet (gewöhnlich 15–20 Minuten), so ist die Geschwindigkeit der beobachteten Eiweisssynthese sehr hoch, viel grösser als die Synthesegeschwindigkeiten, die sich auf Grund von langdauernden Versuchen errechnen lassen. Wenn mann z.B. die Werte der Eiweisssynthese in Blättern in den Versuchen von W. Pearsall und M. Billimoria (40), die 72 Stunden oder länger dauerten, auf 1 Stunde und 1 g. Trockengewicht umrechnet, so entspricht die Synthese Werten von 0.002 bis 0.065 mg. Stickstoff, d.h., sie ist um ein Vielfaches geringer als die Geschwindigkeit des innerhalb kurzer Zeitspannen gemessenen synthetischen Prozesses.

Was die hydrolytische Wirkung der Proteasen betrifft, so ist es uns bisher noch nicht gelungen, dieselbe in lebenden Zellen zu messen. Die hydrolytische Aktivität der Proteasen ist indessen sogar im zerriebenen Gewebe äusserst schwach, wo doch die synthetisierende Enzymwirkung praktisch ausgeschaltet ist.

So, zum Beispiel, beträgt nach den Befunden mehrerer Autoren (41, 42, 25) die durchschnittliche Geschwindigkeit der Eiweisshydrolyse in vegetativen Pflanzenorganen nur 0.1–0.3 mg. N pro 1 Stunde und 1 g. Trockengewicht.

Es dürfte daher auch in den normal funktionierenden Organen höherer Pflanzen die hydrolytische Aktivität der Proteasen um das Vielfache geringer sein als die synthetische Aktivität. Diese Annahme ist desto wahrscheinlicher, als sie in Einklang steht mit dem bedeutenden Überwiegen von Eiweissstoffen gegenüber deren Abbauprodukten in lebenden Zellen (A. Kurssanov (25)).

Die Bestimmung der Phosphorylierungsgeschwindigkeit beruht auf der Infiltration der lebenden Pflanzengewebe mit 0.58 mol. Lösung von anorg. Phosphat ($NaH_2PO_4$). Die durchschnittliche Versuchsdauer beträgt 30 Min. Im Laufe dieser Zeit wird ein Teil des eingeführten Phosphats bereits an organische Substanzen gebunden, wobei hauptsächlich Zuckerphosphate gebildet werden. Die Dephosphorylierungsaktivität wird bestimmt auf Grund der Geschwindigkeit der Abspaltung von Phosphat aus organischen Phosphorsäureestern, mit denen man das Gewebe infiltriert hat (s. Tabelle III).

TABELLE III

SYNTHETISIERENDE UND HYDROLYSIERENDE WIRKUNG DER PHOSPHATASEN IN EINIGEN PFLANZEN

(Umgerechnet in mg. Phosphor pro 1 g. Trockengewicht der Pflanze in 1 Stunde)

| Lfd. nr. | Pflanzliches Objekt | Phosphorylierung | Dephosphorylierung von | | | |
|---|---|---|---|---|---|---|
| | | | Phytin | Glycerinphorphorsäure | Glucosemonophosphat | Fructosediphosphat |
| 1 | Zichorie (Blätter) | 4.36 | 0.70 | 0.98 | 0.55 | 0.50 |
| 2 | Zichorie (Wurzeln) | 1.25 | 0.12 | 0.22 | 0.22 | 0.14 |
| 3 | Hafer (10-tägige Keimlinge) | 2.26 | 1.56 | 0.60 | 0.80 | 0.21 |
| 4 | Weizen (12-tägige Keimlinge) | 2.18 | 1.08 | 0.75 | 0.52 | 0.27 |
| 5 | Erbse (15-tägige Keimlinge) | 2.16 | 1.41 | 0.58 | 0.60 | 1.00 |
| 6 | Hanf (16-tägige Keimlunge) | 0.96 | 1.20 | 0.84 | 0.60 | 0.90 |
| 7 | Cyclamen repsicum (Blätter) | 0.60 | ... | 0.50 | 0.37 | 0.22 |
| 8 | Crinum sp. (Blätter) | 2.30 | 0.80 | 1.24 | 0.76 | 0.82 |
| 9 | Pappel (junge Blätter) | 8.40 | ... | 3.21 | ... | ... |

Ausser dem Summenwert für die Gesamtsynthese kann man durch getrennte Bestimmung der Phosphorsäureester nach einem zu diesem Zweck ausgebauten Schema (31) die synthetisierende Wirkung der einzelnen Phosphatasen messen. Die Versuche ergaben, dass in den Pflanzen bei Infiltration mit anorganischem Phosphat gleichzeitig verschiedene Phosphorsäureester gebildet werden, unter denen gewöhnlich die Hexosemonophosphate an erster Stelle stehen (A. Kurssanov und N. Kryukova (43)).

Die Messung des Oxydations- und Reduktionsvermögens lebender pflanzlicher Gewebe nach B. Rubin und N. Syssakyan (32) beruht auf der Geschwindigkeit der Oxydation von Ascorbinsäure bzw. der Reduktion ihrer reversibel oxydierten Form bei Infiltration des lebenden Pflanzengewebes mit den betreffenden Substanzen. In Tabelle IV geben wir einige auf diesem Wege ermittelten Werte wieder.

TABELLE IV

OXYDATIONS- UND REDUKTIONSFÄHIGKEIT EINIGER PFLANZEN, GEMESSEN AN ASCORBINSÄURE

(In mm.$^3$ 0.001 mol 2,6-Dichlorphenolindophenollösung, umgerechnet pro 24 Stunden und 10 g. frischer Blätter)

(Nach B. Rubin und N. Syssakyan)

| Lfd. nr. | Pflanzliches Objekt | Oxydationsvermögen | Reduktionsvermögen | Verhältnis Oxydation/ Reduktion |
|---|---|---|---|---|
| 1 | Kohl (Sorte Amager) | 19.2 | 9.6 | 2.0 |
| 2 | Cyclamen persicum | 8.0 | 1.6 | 5.0 |
| 3 | Hydrangea Hortensia | 36.2 | 12.8 | 2.8 |
| 4 | Zea Mais | 2.6 | 2.0 | 1.3 |

Für die Bestimmung der Wirkung anderer Fermente in der lebenden Pflanze stehen uns gegenwärtig noch keine vollendete Methoden zur Verfügung. Es ist aber ohne Weiteres einleuchtend, dass das Prinzip, das die Grundlage der oben beschriebenen Methoden bildet, auch in Bezug auf andere enzymatische Prozesse angewendet werden kann.

Obwohl die mannigfache Enzymausrüstung der lebenden Zelle mit den oben besprochenen Enzymen keineswegs erschöpft ist, ermöglichen es bereits diese Beispiele der Erforschung, einer Reihe von allgemeinen Fragen näherzutreten.

Eine der Richtungen, denen die weiteren Untersuchungen folgten, bestand im experimentellen Studium der Bedingungen und Gesetzmässigkeiten, die die Reversibilität der enzymatischen Reaktionen in den Zellen beherrschen; eine andere Richtung hatte die eingehendere Erforschung der physiologischen Rolle der Enzyme zum Ziel.

Die bisher erzielten Erkenntnisse sind noch weit davon entfernt, alle aufgeworfenen Fragen zu entscheiden. Dennoch können bereits auf Grund der zurzeit vorliegenden Befunde, unseres Erachtens, die gegenwärtig vorherrschenden Anschauungen bis zu einem gewissen Grade vertieft und in einigen Fällen revidiert werden.

## III. Bedingungen, von denen die reversible Wirkung der Enzyme in lebenden Zellen abhängt

Die theoretischen Vorstellungen über die Faktoren, die die reversible Wirkung der Enzyme in der Zelle bedingen, sind, wie oben bemerkt, hauptsächlich auf Grund von Modellversuchen und von gewissen allgemeinen Beobachtungen aus entwickelt worden. Die direkte experimentelle Prüfung der einzelnen Leitsätze dieser Theorie wurde durch den Mangel an geeigneten Methoden erschwert. Indem wir von der Bestimmung der synthetisierenden und hydrolysierenden Invertasewirkung in lebenden Geweben Gebrauch machten, konnten wir eine Reihe von neuen Tatsachen feststellen, durch die die Bedeutung der Strukturgebilde und die Adsorptionserscheinungen im Protoplasma für enzymatische Synthesen belegt wird. Andererseits wurde festgestellt, dass Oxydationsprozesse in den Zellen das Zustandekommen enzymatischer Synthesen fördern.

Es wurde vor Allem nachgewiesen, dass Invertase, die in der Form von Fermentpräparaten das typische Beispiel eines einseitig hydrolytisch wirkenden Enzyms darstellt, in den Geweben höherer Pflanzen tatsächlich sowohl die Hydrolyse wie die Synthese der Saccharose bewerkstelligt (A. Kurssanov (44)). Dieser Schluss ergab sich aus Versuchen, in denen sich nicht allein die Hydrolyse, sondern auch die Synthese von Saccharose in Blättern durch infiltrierte Hefeinvertase verstärken liess (Tabelle V). Diese Ergebnisse sind ein entscheidender Beweis gegen die Vorstellungen mancher Forscher über die Verschiedenheit der synthetisierenden und hydrolysierenden Enzyme—Vorstellungen, die auch inbezug auf Invertase verfochten worden sind (vgl. z.B. H. Collin (45), L. Berczeller (46) u.a.).

TABELLE V

BESCHLEUNIGUNG DER SYNTHESE UND HYDROLYSE DER SACCHAROSE IN DEN BLÄTTERN EINIGER PFLANZEN UNTER DEM EINFLUSS VON HEFEINVERTASE

| Lfd. nr. | Pflanzliches Objekt | Menge d. infiltrierten Invertase (mg. pro 1 g. Trockengewicht) | Invertaseaktivität in den Blättern (in mg. Invertzucker) | | Zunahme der Aktivität auf Kosten der Hefeinvertase | |
|---|---|---|---|---|---|---|
| | | | Synthese | Hydrolyse | Synthese | Hydrolyse |
| 1 | Cyclamen persicum | ... | 3.3 | 13.9 | 6.4 | 35.9 |
| | | 2.4 | 9.7 | 49.8 | | |
| 2 | Primala sp. | ... | 6.1 | 3.5 | 2.2 | 1.8 |
| | | 2.0 | 8.3 | 5.3 | | |

Es wurde ferner nachgewiesen, dass die infiltrierte Hefeinvertase in verschiedenen Pflanzen in sehr ungleichem Masse synthetische Wirksamkeit erlangt. Während nämlich gewisse Pflanzen 8–9 mal soviel Hefeinvertase in synthetisierenden Zustand versetzen können als ihr Eigengehalt an dem Enzym beträgt (Crinum sp.), besitzen andere Pflanzen diese Fähigkeit in viel geringerem Ausmass. Schliesslich gibt es, wie B. Rubin zeigen konnte, auch solche Pflanzen, die garnicht imstande sind infiltrierte Invertase in den synthetisierenden Zustand zu versetzen (Kohl, Sorte Nr. 1). Charakteristisch ist indessen, dass in solchen Pflanzen auch die eigene Invertase keine synthetische Wirkung ausübt. Man kann daher annehmen, dass die Zahl der Stellen, an denen dieses oder jenes Enzym in den Zellen adsorbiert werden kann, oder auch die Adsorptionskraft selbst, von Pflanze zu Pflanze in ziemlich weiten Grenzen variiert. Es sei ferner hervorgehoben, dass die Fähigkeit oder Unfähigkeit der Zelle, infiltrierter Invertase synthetisierende Wirkung zu verleihen, noch gar nichts über das Verhalten der Zelle gegenüber anderen Enzymen besagt, denn es können in den betreffenden Zellen bei vollständig fehlender synthetisierender Invertasewirkung synthetische Prozesse anderer Art vor sich gehen. Wir müssen daher annehmen, dass die Adsorption der Enzyme an den Strukturgebilden der Zelle eine *selektive Adsorption* ist.

Falls die Synthese tatsächlich durch Enzyme bewirkt wird, die am Protoplasma adsorbiert sind, so muss die Zerstörung der adsorbierenden Strukturen oder die Verminderung ihrer Oberflächenwirkung den Übergang der Enzyme in Lösung zur Folge haben und dementsprechend zu Verstärkung der Hydrolyse führen. Andererseits muss eine Steigerung der Adsorptionskräfte in der Zelle eine Verschiebung in umgekehrter Richtung herbeiführen. Diese Folgerungen wurden einer experimenteller Prüfung unterzogen. Vor Allem wurde versucht, die Gebilde, an denen die Invertase ihre synthetische Funktion verrichtet, zu zerstören, ohne die Zellen abzutöten (A. Kurssanov (44)).

Da die Plastiden und Chondriosomen,—die vermutlichen Zentren enzymatischer Synthesen,—ein Eiweisstroma besitzen, wurde auf verschiedene Weise versucht, den proteolytischen Abbau in den Blättern verschiedener Pflanzen zu steigern. Einerseits wurden die Blätter zu diesem Zweck in einer Stickstoffatmosphäre gehalten. Dies verursachte einen Abbau der Blattproteine und gleichzeitig eine starke Verschiebung des ursprünglichen Gleichgewichts zwischen synthetischer und hydrolytischer Invertasewirkung zugunsten der Hydrolyse. In anderen Versuchen wurde proteolytischer Abbau in den Blättern durch Infiltration der Zellen mit Papain und Cystein erzielt. Hierbei wurde ebenfalls neben gesteigerter

Proteolyse und Anhäufung von Eiweissabbauprodukten eine Hemmung der synthetischen Invertaseaktivität beobachtet.

Tabelle VI enthält die Ergebnisse einiger Versuche, die den besprochenen Zusammenhang veranschaulichen.

TABELLE VI

VERSCHIEBUNGEN DER REVERSIBLEN INVERTASEWIRKUNG IN BLÄTTERN VON CYCLAMEN PERSICUM IM ZUSAMMENHANG MIT PROTEOLYTISCHEM ABBAU

| Lfd. nr. | Art der Einwirkung | Amino N in mg. pro 1 g. Trockengewicht der Blätter | Invertaseaktivität | |
|---|---|---|---|---|
| | | | Synthese | Hydrolyse |
| 1 | Kontrollpflanzen | 1.8 | 4.6 | 18.1 |
| | Pflanzen nach 48 St. Aufenthalt in Stickstoffatmosphäre | 3.4 | 1.2 | 22.3 |
| 2 | Kontrollpflanzen | 1.5 | 6.1 | 19.2 |
| | Mit 0.05% Papain und 0.1% Cystein infiltrierte Pflanzen | 3.1* | 1.0 | 27.7 |

*Unter Abzug des Cystein-N.

Diese Resultate bestätigen die Vorstellung von der Bedeutung der Adsorption für die enzymatische Synthese und verleihen zugleich der Annahme Wahrscheinlichkeit, dass die Adsorption der Invertase in den Zellen an Körpern von Eiweissnatur erfolgt.

In letzterer Zeit erschien eine Arbeit von S. Raabe (47), welche tierische Esterasen zum Objekt hatte. Unter Anwendung des gleichen Prinzips wie in unseren Experimenten, hat Raabe tierische Gewebe mit Glutathion injiziert und auf diese Weise den proteolytischen Abbau gesteigert. Dies hatte einen starken Anstieg der hydrolytischen Esteraseaktivität in allen Organen des Tieres zur Folge. Ähnlich wie wir, erklärt der Verfasser dieses Ergebniss durch den Abbau von Eiweisskörpern und den Übergang adsorbierter Esterase aus dem synthetisierenden Zustand in den hydrolysierenden.

Aus diesen Versuchen kann gefolgert werden, dass in den Zellen nicht nur Invertase, sondern auch Esterase an Proteingebilden adsorbiert ist.

Ein anderer Weg zur Klarstellung der Rolle von Adsorptionserscheinungen bei der reversiblen Enzymwirkung in lebenden Zellen besteht in Beeinflussung der Oberflächeneigenschaften des Protoplasmas und der darin enthaltenen Gebilde. Derartige Einflüsse müssen naturgemäss die Verteilung der Enzyme zwischen Lösung und adsorbierenden Oberflächen verändern. Vorausgesetzt, dass die angewendeten Agenzien keine direkte Wirkung auf die Aktivität des Enzyms ausüben, wird diese Neuverteilung

spiegelbildliche Veränderungen der reversiblen Enzymwirkung herbeiführen. Als Mittel zur Veränderung der Oberflächenaktivität der Zellstrukturen wurden Narcotica verwendet (A. Kurssanov und N. Kryukova (48)). Die Versuche ergaben, dass die Einwirkung von Aetherdämpfen oder Aethylen in verschiedenen Konzentrationen auf die lebenden Zellen zu starken Verschiebungen spiegelbildlicher Art in der reversiblen Wirkung der Invertase führt (s. Fig. 2).

Stellt man diese Verschiebungen in Vergleich mit den durch verschiedenen Dosen der Narcotica ausgelösten Veränderungen des physikalisch-chemischen Zustands der Zelle, so drängt sich die Folgerung auf, dass die spiegelbildliche Verschiebung des enzymatischen Gleichgewichts nach seiten der Synthese bei schwacher Narkose dem Stadium entspricht, bei dem die oberflächenaktiven Eigenschaften des Plasmas gesteigert sind.

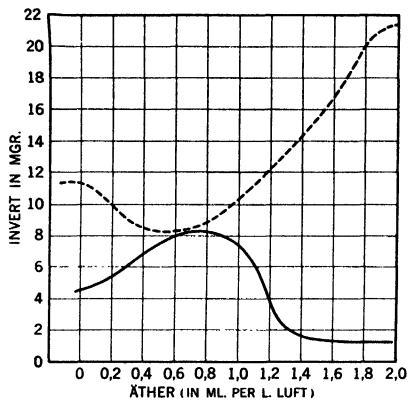



Fig. 2.—Einfluss von Aethernarkose auf die reversible Invertasewirkung von Blättern von *Cyclamen persicum*.

Die zweite Verschiebung, in der hydrolytischen Richtung, entspricht dem Stadium tiefer Narkose, bei der das Cytoplasma verflüssigt wird und seine oberflächen-aktiven Eigenschaften weitgehend einbüsst. Eine Verschiebung der reversiblen Invertasewirkung veranlasst auch die Infiltration von Phenylurethan (0.003–0.006 mol) in das lebende Pflanzengewebe. Durch dieses Narcoticum werden, nach O. Warburgs (49) Angabe, die Zentren von Oberflächenreaktionen besonders stark „blockiert"; wie unsere Versuche zeigen, hemmt es zugleich die synthetisierende Wirkung der Invertase.

Beim Vergleich der vorherrschenden Richtung der Enzymwirkung mit der Intensität der Zellatmung lässt sich in manchen Fällen eine Parallelität zwischen der Abmessungsenergie und der Geschwindigkeit der enzymatischen Synthese verzeichnen. Jedoch kommen auch Abweichungen von diesem Zusammenhang vor und sogar antiparallele Beziehungen, so z.B. beim Abkühlen der Pflanzen auf −5°, wo trotz starker Abnahme der Atmung die synthetisierende Wirkung der Enzyme bedeutend gesteigert is (s. A. Kurssanov, N. Kryukova, und A. Morosov (50) u.a.). Es darf daher wohl

nicht angenommen werden, dass die Energie der Atmung unmittelbar die Geschwindigkeit der enzymatischen Synthesen begrenzt. Eine gewisse Kopplung dieser Prozesse erscheint indessen sehr wahrscheinlich. Ein konstantes Verhältnis besteht, wie die Untersuchungen von B. Rubin (51) gezeigt haben, zwischen der Aktivität der Peroxydase und der synthetisierenden Invertasewirkung in den Zellen. Vor kurzem konnte N. Djatschkov in unserem Laboratorium dies durch direkte Versuche belegen, indem er zeigte, dass Infiltration von Blättern mit einer Peroxydase-Lösung das Verhältnis zwischen Saccharose-Synthese und -Hydrolyse stark zugunsten der Synthese verschiebt. Dabei wird das Auftreten des synthetischen Prozesses durch Peroxydase stimuliert sogar in jenen Blättern, welche normalerweise nur hydrolytische Wirkung zeigen. Hierher gehören auch die Beobachtungen von A. Kurssanov und N. Kryukova (25), denzufolge die Infiltration des pflanzlichen Gewebes mit kleinen Dosen von $H_2O_2$ die synthetisierende Wirkung der Invertase bedeutend steigert. Schliesslich haben B. Rubin und E. Arzichovskaya (38), sowie N. Syssakyan und A. Kobyakova (52) eine Parallelität zwischen der Richtung der Invertasewirkung und dem Oxydations-Reduktions-Vermögen des Gewebes festgestellt.

Alle diese Ergebnisse sprechen dafür, dass die Oxydationsreaktionen einen günstigen Einfluss auf die synthetisierende Wirkung der Enzyme in der Zelle ausüben. Andererseits stehen sie in gutem Einklang mit den Vorstellungen über den innigen Zusammenhang zwischen Energie und Protoplasma-Struktur.

## IV. Die physiologische Rolle der Enzyme

Die Lehre von den Enzymen entwickelte sich, wie erwähnt, hauptsächlich in der Richtung der Erforschung der Natur dieser Substanzen und des Mechanismus ihrer Wirkung. Die Untersuchungen der letzten Jahre über die reversible Wirkung der Enzyme haben es indessen ermöglichst, auch die physiologische Rolle der enzymatischen Prozesse im Organismus näher kennen zu lernen. Diese Möglichkeiten sind noch lange nicht erschöpft aber die Ergebnisse, die auf einigen Gebieten bereits erzielt wurden, lassen erhoffen, dass es gelingen wird, auf diesem Wege manche biologische Probleme zu lösen, die mit der Entwicklung der Pflanzen und ihrem Verhalten gegenüber exogenen Einflüssen zusammenhängen.

Nachstehend wird ein allgemeiner Überblick über die Arbeiten in dieser Richtung gegeben um die Wege der praktischen Anwendung der Lehre von den enzymatischen Prozessen in der lebenden Zelle auf die Lösung biologischer Probleme näher zu kennzeichnen.

### 1. *Die enzymatischen Prozesse in den Zellen in ihren Beziehungen zu den Art- und Sortenverschiedenheiten der Pflanzen*

Die moderne Pflanzensystematik beruht hauptsächlich auf äusseren morphologischen Merkmalen. Die chemische und besonders die biochemische Systematik ist noch ganz mangelhaft ausgebaut, obwohl schon mehrmals versucht wurde, chemische Merkmale der Pflanzen zur Aufstellung von Verwandschaftsbeziehungen zwischen einzelnen Gruppen heranzuziehen.

Da die chemische Tätigkeit der Zellen in mancher Hinsicht von der Geschwindigkeit und Richtung enzymatischer Prozesse abhängig ist, steht zu erwarten, dass auch die enzymatischen Reaktionen charakteristische Artmerkmale der Pflanzen darstellen.

Arbeiten in dieser Richtung, die im Laufe einer Reihe von Jahren von B. Rubin (53) durchgeführt wurden, haben gezeigt, dass das Verhältnis der synthetischen zur hydrolytischen Invertasewirkung, d.h. die vorherrschende Richtung der Enzymwirkung ein Merkmal darstellt, das für die betreffende Sorte oder Varietät einer Pflanze charakteristisch ist (festgestellt an Zwiebeln, Kohlsorten und anderen Gemüsearten). Besondere Beachtung verdient hierbei die Tatsache, dass ein hohes Verhältnis von Synthese zu Hydrolyse zugleich ein Merkmal guter Lagerfähigkeit der betreffenden Sorte und ihrer Resistenz gegen Mikroorganismen ist. Niedrige Werte dieses Verhältnisses sind hingegen charakteristisch für schlecht haltbare, zu Pilzerkrankungen neigende Gemüsesorten.

Auch für die Beurteilung der Früh- oder Spätreife einiger Sorten stellt das Verhältnis Synthese:Hydrolyse ein ebenso stabiles Merkmal dar. Dabei entspricht in der Regel das Vorherrschen der synthetischen Wirkung in den Zellen den spätreifen Sorten, während stärkere Hydrolyse stets für frühreife Sorten charakteristisch ist (festgestellt an Gemüsen und Obstbäumen). Demnach kann die Untersuchung der Enzymprozesse in den Zellen auch der Diagnose wirtschaftlich wichtiger Eigenschaften der Pflanzen dienlich sein.

Dabei ist jedoch zu beachten, dass die Konstanz des Verhältnisses Synthese:Hydrolyse, die diesen Arbeiten zu Grunde gelegt wurde, nur bei Prüfung der Pflanzen unter ganz bestimmten Bedingungen gültig ist. Dieses Verhältnis ist beständiger in Organen mit verlangsamter Lebenstätigkeit, zum Beispiel, in ruhenden Früchten, Wurzeln, Knollen u.s.w.

Dagegen weisen aktiv vegetierende Pflanzen, häufige und ziemlich starke Verschiebungen des enzymatischen Gleichgewichts auf, die sowohl durch Umweltfaktoren wie durch endogene Ursachen bedingt sind.

### 2. *Speicherung von Vorratsstoffen und allgemeine Ertragsfähigkeit der Pflanzen als Resultat reversibler enzymatischer Reaktionen*

Die Arbeiten zahlreicher Autoren haben gezeigt, dass die Intensität der Photosynthese, und mithin die allgemeine Ertragsfähigkeit der Pflanzen, in starkem Masse abhängig sind von der Geschwindigkeit, mit der

die primären Assimilationsprodukte aus den assimilierenden Organen fortgeschafft werden (s. z.B. A. Kurssanov (54)). Dies kann nicht nur durch Abtransport zu anderen Organen erfolgen, sondern auch an Ort und Stelle, infolge der enzymatischen Umwandlung einfacher Zucker in komplizierte Produkte, zum Beispiel in Stärke oder Rohrzucker.

Untersuchungen über die reversible Invertasewirkung in lebenden Blättern haben gezeigt, dass die synthetisierende Wirkung bei manchen Pflanzen so stark ist, dass sie sogar in Perioden ziemlich intensiver $CO_2$-Assimilation ausreicht, um die rasche Umwandlung der Monosen in Saccharose zu besorgen.

Ähnlich wie Invertase üben wahrscheinlich auch Amylase und einige andere Enzyme ebenso intensive synthetische Wirkungen aus, so dass die Beseitigung der primären Assimilationsprodukte aus dem Bereich des Assimilationsprozesses leicht in dieser Weise erfolgen kann. Immerhin sind die assimilierenden Organe meist ungeeignet für die Ablagerung grosser Mengen von Vorratsstoffen; deshalb ist das Hauptmittel der Entfernung von Assimilaten aus den Blättern doch der Abtransport, dessen Geschwindigkeit weitgehend von der Konzentration der einfachen Zucker in den Blättern abhängt.

Von diesem Standpunkt aus müssen die Verhältnisse für den Abtransport besonders günstig sein, wenn in den assimilierenden Zellen der hydrolytische Prozess den synthetischen überwiegt und wenn in den Speicherungsorganen das entgegengesetzte Verhältnis besteht. Es wirkt aber, wie gesagt, die Anhäufung der primären Produkte in den Blättern stark hemmend auf die Photosynthese und damit natürlich auch auf den Gesamtprozess der Zuckerspeicherung. Daher kann ein *statisches* Übergewicht der hydrolytischen Enzymwirkung in den Blättern schwerlich die Erhöhung des Gesamtertrags der Pflanzen fördern.

Es erscheint eher wahrscheinlich, dass die Tätigkeit des Blatts im Laufe des Tags mit periodischen Änderungen der Richtung enzymatischer Reaktionen einhergeht, so dass bald die hydrolytischen, bald die synthetischen Reaktionen das Übergewicht haben.

Diese Annahme fand ihre Bestätigung in direkten Bestimmungen der reversiblen Invertasewirkung in den Blättern der Zuckerrübe im Laufe von 24 Studen (B. Rubin und O. Lutikova (55) (vgl. Fig. 3, S. 351)).

Es ergab sich, dass die synthetisierende Wirkung der Invertase im Laufe des Tages bedeutende Änderungen erfährt, indem sie zwischen 12 und 18 Uhr maximale Werte erreicht und während der Nacht bedeutend schwächer wird. Daneben nimmt während der Tagesstunden die hydrolytische Wirkung merklich ab, so dass in dieser Periode die Synthese überwiegt;

Nachts ist dagegen der hydrolytische Prozess vorherrschend. In einer anderen Arbeit zeigten B. Rubin und N. Syssakyan (56), dass das Verhalten der Invertase in den Blättern von Baumpflanzen eine ähnliche Periodizität aufweist.

Schliesslich ist es N. Syssakyan und A. Kobyakova (57) gelungen in einer kürzlich veröffentlichten Arbeit einen direkten Zusammenhang nachzuweisen zwischen der Intensität der Photosynthese und dem relativen Übergewicht der synthetisierenden Invertasewirkung in Tabakblättern.

Aus allen diesen Befunden geht deutlich hervor, dass der enzymatische Apparat der Blätter aktiven Anteil am gesamten Prozess der Zuckerspeicherung nimmt, indem er während der Stunden maximaler Photosynthese die Assimilate in Reserveform überführt und bei Dunkelheit Bedingungen für den raschen Abtransport schafft.

Zweifellos sind die Reize, die diese Veränderungen bedingen, bis zu einem gewissen Grad durch den periodischen Wechsel von Tag und Nacht verursacht; jedoch besteht auch unabhängig hiervon in den Zellen der Blätter eine eigene Periodizität. Dies beweisen die Versuche von B. Rubin, E. Arzichowskaya, N. Spiridonova, und O. Lutikova (58) an tagsüber verdunkelten und die Nacht hindurch künstlich belichteten Zuckerrüben. Diese Versuche ergaben, dass der enzymatische Apparat der Blätter bei solchen Pflanzen, wenigstens in den ersten Tagen, seinen ursprünglichen Rhythmus beibehält, d.h. am Tage energischer synthetisiert und bei Nacht hydrolysiert, obwohl diese Veränderungen mit den im Versuch geschaffenen Belichtungsverhältnissen nicht in Einklang stehen.

In Tabelle VII sind diese Beziehungen durch das Verhältnis Synthese/Hydrolyse veranschaulicht, dessen Werte um so geringer sind, je weiter die vorherrschende Richtung des enzymatischen Prozesses zugunsten der Hydrolyse verschoben ist.

TABELLE VII

ÄNDERUNGEN DER RICHTUNG DER INVERTASEWIRKUNG IN ZUCKERRÜBENBLÄTTER IM LAUFE DES TAGES BEI NORMALER UND UMGEKEHRTER FOLGE DER TAGESZEITEN

| Beobachtungsstunden | Belichtungsverhältnisse | Normaler Tag Synthese/Hydrolyse | Belichtungsverhältnisse | Umgekerter Tag Synthese/Hydrolyse |
|---|---|---|---|---|
| 0 | Dunkelheit | 0.24 | Licht | 0.26 |
| 6 | | 0.13 | | 0.58 |
| 12 | Licht | 0.44 | Dunkelheit | 0.72 |
| 18 | | 0.70 | | 0.94 |
| 24 | Dunkelheit | 0.08 | Licht | 0.35 |

Die Ursachen, auf denen diese innere Periodizität beruht, wurden bis zu einem gewissen Grad durch die Versuche von N. Syssakyan und A. Kobyakova (59) klargestellt. Diese Autoren konnten zeigen dass eine Störung der normalen Grössenverhältnisse zwischen der $CO_2$ assimilierenden Oberfläche und den Organen, die die Assimilationsprodukte verbrauchen, weitgehende Verschiebungen in der bevorzugten Richtung der enzymatischen Prozesse in den Blättern herbeiführt. Insbesondere verursacht Entfernung der Blüten und Früchte eine Stimulierung der synthetischen Wirkung der Invertase in den Blättern, während Einschränkung der Blattoberfläche bei verschonten Blüten und Früchten eine Steigerung des hydrolytischen Prozesses zur Folge hat, infolge deren ununterbrochener Abtransport aus den Blättern einsetzt.

Es kann daher angenommen werden, dass die Reize für die periodische Verschiebung von Synthese und Hydrolyse in den Blättern nicht nur von den Perioden ihrer photosynthetischen Tätigkeit ausgehen, sondern auch im innigsten Zusammenhang stehen mit dem Bedarf der anderen Organe an plastischem Material.

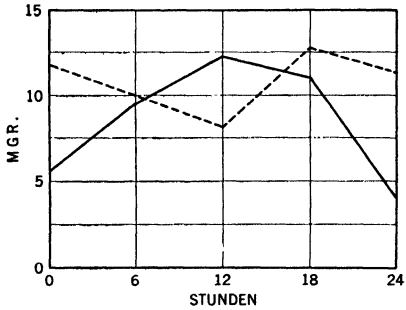



Fig. 3.—Anderungen der reversiblen Invertasewirkung in den Blättern der Zuckerrübe mit der Tageszeit. — — — — -Synthese, .....-Hydrolyse (Nach B. Rubin und O. Lutikova).

Sollte die Existenz einer endogenen Periodizität bei den Pflanzen in weiteren Untersuchungen Bestätigung finden, so wäre es möglich von diesem Standpunkt aus Untersuchungen über den Photoperiodismus zu unternehmen, indem man Unterschiede zwischen Pflanzen langen und kurzen Tags aufzustellen versuchte auf Grund der tagsüber erfolgenden Änderungen der Enzymreaktionen und ihrer Übereinstimmung oder Nicht-Übereinstimmung mit den Tag- und Nachtperioden.

Was die Bahnen betrifft, auf welchen die Fortbewegung plasmatischer Materialien in der Pflanze erfolgt, so findet nach den Befunden von N. Syssakyan in den Siebröhren und in dem dieselben umgebenden Parenchym nur hydrolytische Enzymwirkung statt; dies bestätigt die Anschauung, dass die plasmatischen Substanzen in der Form einfacher Verbindungen transportiert werden.

Schliesslich ist für die Ablagerung der Vorratsprodukte, die gewöhnlich

in speziellen Organen der Pflanze erfolgt, ein starkes und beständiges Übergewicht synthetisierender Wirkung der betreffenden Enzyme erforderlich. In Bezug auf die Zuckerrübe wurde dies von B. Rubin und O. Lutikova (61) bestätigt. Diese Autoren konnten feststellen, dass die synthetisierende Wirkung der Invertase in Rübenwurzeln desto stärker ist, je höheres Zuckerspeicherungsvermögen die betreffende Sorte aufweist. Gesteigerte hydrolytische Invertasaktivität in den Wurzeln steht, wie A. Oparin (62) gezeigt hat, in umgekehrtem Verhältnis zum Zuckergehalt und ist eher ein typisches Merkmal der durch starkes Wachstum gekennzeichnete Wurzeln der Futterrübe.

### *3. Die vorherrschende Richtung der enzymatischen Prozesse im Zusammenhang mit der Entwicklung der Pflanze*

Der Ablauf des Lebenszyklus der Pflanze steht in Zusammenhang mit einer Reihe aufeinanderfolgender Veränderungen ihrer biochemischen und physiologischen Tätigkeit. In dieser Beziehung stellen die Samenkeimung und die erste Entwicklungsperiode der neugebildeten Keimlinge ein besonders intensives Entwicklungsstadium der Phanerogamen dar.

Es ist deswegen begreiflich, dass die Untersuchung der Enzyme in keimenden Samen schon lange eifrig betrieben wird. Die Bestimmung der Aktivität der Enzyme in autolytischen Gemischen konnte indessen kein allseitiges Bild ihrer Tätigkeit vermitteln und zeigte nur eine Steigerung der hydrolytischen Prozesse während der Keimungsperiode an.

A. Kurssanov und K. Bryuschkova (68) unternahmen Bestimmungen der synthetisierenden und hydrolysierenden Invertasewirkung in lebenden Samen des Weizens und einiger anderer Pflanzen und zeigten, dass die Invertase in noch nicht gekeimten Samen sehr starke synthetisierende Wirkung äussert, während die Hydrolyse so gut wie vollständig ausbleibt. Die Keimung geht mit einer spiegelbildlichen Verschiebung dieses Gleichgewichts einher, infolge deren nach 5–6 Tagen in allen Teilen des Samens die hydrolytische Reaktion entschieden vorzuherrschen beginnt. Besonders stark ausgeprägt ist diese Verschiebung im Endosperm, in welchem die Invertase um diese Zeit ihre synthetisierende Funktion vollständig einbüsst. Das Ergrünen der Keimlinge und das hiermit verbundene Einsetzen der Photosynthese führt sogleich zu einer Verstärkung der synthetischen Invertasewirkung in den grünen Zellen—ein Hinweis auf den innigen Zusammenhang zwischen diesem Enzym und der Speicherung und Wanderung der Assimilate. Es ist ferner sehr charakteristisch, dass die Proteinasen in etiolierten Keimlingen rasch ihre synthetisierende Wirkung einbüssen und sie beim Ergrünen wieder erlangen. Man kann daher

vermuten, dass der Enzymapparat in den Keimlingen beim Übergang derselben von der heterotrophen zur autotrophen Entwicklung in manchen Beziehungen eine wesentliche Umgestaltung erfährt.

Ähnlich verhalten sich die Enzyme bei der Öffnung von Blattknospen. Durch Versuche von A. Kurssanov und K. Bryuschkova (64) wurde gezeigt, dass die Invertase ruhender Fliederknospen während der Winterzeit gar nicht hydrolysiert und eine ziemlich starke Synthese auszuüben vermag. Ferner wurde nachgewiesen, dass die Wirkung der Invertase noch vor Beginn der Frühjahrs-Vegetation eine starke Verschiebung nach Seiten der Hydrolyse erfährt. Die Entwicklung der Knospen zu jungen Sprossen und ihr Ergrünen geht, ebenso wie bei den Samenkeimlingen, mit Auftreten der synthetisierenden Invertasewirkung einher, die funktionell mit dem Einsetzen der Photosynthese verknüpft ist. Was die Proteinase anbelangt, so verhält sie sich ebenfalls in den aufbrechenden Knospen annähernd ebenso wie in Samenkeimlingen. Die grosse Ähnlichkeit im Verhalten der Enzyme in aufbrechenden Knospen und in keimenden Samen berechtigt zu der Folgerung, dass der Übergang der Enzyme aus dem nahezu einseitig synthetisierenden Zustand in den hydrolysierenden für pflanzliche Gewebe charakteristisch ist, die von der Ruhe zu aktiver Vegetation übergehen.

Erscheinungen, die den bei der Samenkeimung stattfindenden entgegengesetzt sind, kann man während der Samenreifung erwarten.

Die Untersuchungen von A. Kurssanov und K. Bryuschkova (65) über das Verhalten der Enzyme in lebenden Weizensamen während der Reifung zeigten, dass bereits wenige Tage nach der Befruchtung ein Zustrom von Invertase und Proteinase zum Fruchtknoten zu beobachten ist. Da die hinzuströmenden Enzyme in gelöstem Zustand vorliegen, äussern sie zunächst nur eine einseitige, sehr aktive hydrolytische Wirkung. Mit dem Übergang von der Milch- zur Wachsreife beginnt eine Adsorption der aufgenommenen Fermente an den Strukturgebilden der Zellen. Infolgedessen kommt die hydrolytische Wirkung fast vollständig zum Stillstand und die Synthesereaktionen gelangen zu besonders starker Entfaltung. Diese Periode entspricht der intensiven Speicherung von Vorratsstoffen in den reifenden Samen. Das letzte Stadium der Reifung ist schliesslich gekennzeichnet durch Absinken der synthetisierenden Enzymaktivität ohne Zunahme der hydrolytischen Wirkung. Die Ursache dieses Phänomens kann entweder die starke Entwässerung der Gewebe sein, oder ein Übergang der Enzyme in den inaktiven Zymogenzustand.

Hohe synthetische Enzymaktivität in der Periode der Samenreifung

beobachteten auch B. Rubin und O. Lutikova (33), die mit verschiedenen Erbsensorten arbeiteten.

Vergleicht man alle diese Ergebnisse mit den Befunden, die bei Versuchen mit zerriebenen Samen erhoben wurden, so stellt sich heraus, dass dem sogenannten Zymogenzustand der Enzyme, mit dem in älteren Arbeiten die inaktive Form gemeint wurde, bei Versuchen mit intakten Samen eine intensive synthetisierende Wirkung der Enzyme entspricht. Wenn man beachtet, dass die Bildung der sogenannten Zymogene in den Samen mit der Adsorption der Enzyme an Eiweisskörpern zusammenhängt (vgl. z.B. T. Chrzaszcz und J. Janicki (66)), so kann man annehmen, dass zwischen der inaktivierenden Adsorption der Enzyme in den Zellen und jener Adsorption, infolge deren die Enzyme synthetisierende Aktivität erlangen, nahe Beziehungen bestehen.

Die Änderungen der vorherrschenden Richtung der enzymatischen Prozesse in den Zellen hören nicht nach der Keimung der Samen auf, sondern sie dauern im Laufe der ganzen späteren Entwicklung der Pflanze fort. Durch die Arbeiten von N. Syssakyan (67) und besonders von B. Rubin und O. Lutikova (55) an Zuckerrüben wurde nachgewiesen dass die reversible Invertasewirkung in den Blättern, abgesehen von dem Tagesrhythmus, mit dem Alter eine Verschiebung zugunsten der hydrolytischen Prozesse erfährt. In einer soeben in unserem Laboratorium abgeschlossenen Untersuchung von G. Harebawa über die Enzyme der Teeblätter wurde gezeigt, dass die Richtung der Invertasewirkung auch bei dieser Pflanze in älteren Blättern mehr hydrolytisch wird als in jüngeren.

In den Versuchen von Pearsall und Billimoria (40) an lebenden Blättern wurde ferner der Nachweis erbracht, dass auch die synthetisierende Wirkung der Proteinase mit fortschreitendem Alter der Blätter abnimmt. Die Untersuchungen von A. Kurssanov und K. Bryuschkova (68) an Blättern verschiedenen Alters beim Hafer liessen gleichfalls eine Abnahme der synthetischen Aktivität der Enzyme von den höher gelegenen Blättern zu den tieferen erkennen. In der erwähnten Arbeit wurde ferner nachgewiesen, dass die Tätigkeit der Enzyme in jedem Blatt in dessen individuellem Lebenslauf bestimmte Veränderungen durchmacht. Für Proteinase z.B. äussern sich diese Veränderungen in einer eingipfeligen Kurve mit maximaler synthetischer Aktivität um die Mitte der Lebenszeit des Blattes (vgl. Fig. 4).

Aus der Abbildung ist ersichtlich, dass die Blätter, je nach der Zeit ihres Auftretens an der Pflanze ihren individuellen Entwicklungslauf in verschiedenen Fristen durchmachen. Trotzdem sind die in jedem Blatt, betrachtet aus seiner Höhenlage, fortschreitenden Veränderungen ähnlicher Art. Dies dauert solange, bis die Entwicklung der

Fortflanzungsorgane der Pflanze einsetzt. Zu dieser Periode (Fig. 4, 25 Juni) geht die synthetisierende Wirkung der Proteinase sowie der Invertase vollständig verloren, und die hydrolytischen Vorgänge erlangen das Übergewicht. Die Analyse dieser Erscheinung führt zu dem naheliegenden Schluss, dass die physiologische Bedeutung einer solchen Verschiebung darin besteht, die Vorratsstoffe zu mobilisieren und den Reproduktionsorganen zuzuleiten. Die um diese Zeit zu beobachtende Erschöpfung der Blattvorräte lässt diese Anschauung plausibel erscheinen. Gleichzeitig verschwindet ein beträchtlicher Anteil der Enzyme aus den Blättern—ein Hinweis darauf, dass die Enzyme zusammen mit den Vorratsstoffen zum in Entwicklung begriffenen Blütenstand wandern. Der Zustrom hydrolytisch aktiver Enzyme zu den Fruchtknoten nach der Befruchtung steht gleichfalls in Einklang mit diesem Enzymschwund in den Blättern. Nach Abschluss der Blüte (Fig. 4, 5.Mai) geht der gesamte Enzymgehalt in den Blättern wieder steil in die Höhe, wobei insbesondere die synthetischen Prozesse zunehmen. Dieser Anstieg entspricht der allgemeinen Verstärkung der Nährfunktion der Blätter in der Periode der Samenreifung.

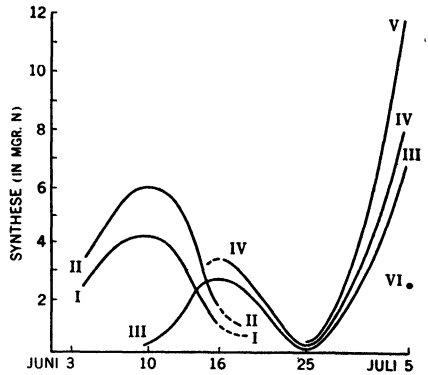



Fig. 4.—Synthetisierende Wirkung der Proteinasen in Haferblättern verschiedenen Alters. Abszisse-Daten der Probeentnahme; Ordinate -Werte der Synthesewirkung. Die Zahlen an den Kurven bezeichnen die Reihenfolge der Blätter von den untersten zu den obersten.

Das Ende des Lebenszyklus der Pflanze oder ihrer einzelnen Organe ist gekennzeichnet durch eine Verstärkung der hydrolytischen Prozesse in den Zellen. So verlieren bei Pfirsichbäumen im Herbst, nach den Befunden von V. Nilov und O. Pavlenko (69), die Enzyme in den vergilbenden Blättern gänzlich ihre synthetischen Funktionen. Nach unseren Beobachtungen erfolgt diese Verschiebung noch lange vor dem Laubfall. Daher erreichen in den Blättern jene Prozesse rechtzeitig ihr Ende, die mit dem Abbau und Abtransport gewisser Produkte aus dem abfallenden Laub zu den überwinternden Organen zusammenhängen.

Das Studium der reversiblen Enzymwirkungen in reifenden fleischigen Früchten lehrte, dass der Verlauf der Reifung auch hier weitgehend von der Lage der enzymatischen Gleichgewichte abhängig ist.

Über diese Frage ist bisher nur eine einzige Untersuchung von A. Kurssanov und N. Kryukova veröffentlicht worden (37). Die Autoren bestimmten die reversible Wirkung der Invertase in den Früchten von Tomaten, Gurken, Mandarinen, Apfelsinen und

Zitronen während der letzten Reifungsstadien. Die hauptsächlichen und besonders typischen Veränderungen, die sich hierbei ergaben, waren folgende: ein Anstieg des Verhältnisses Synthese:Hydrolyse am Anfang des Gelbwerdens der Früchte und eine starke Verschiebung des enzymatischen Gleichgewichts nach Seiten der Hydrolyse um die Zeit des Erweichens des Fruchtfleisches. Wenn man annimmt, dass letztere Verschiebung in den reifenden Früchten nicht die Invertase allein betrifft, sondern auch für andere Enzymgruppen gilt, so kann man den Schluss ziehen, dass das Erweichen des Fruchtfleisches, sowie dessen vollständige Mazerierung auf späteren Stufen der Reife, mit einseitig hydrolytischen Enzymwirkungen zusammenhängt, die eine eigenartige intravitale Autolyse der Gewebe verursachen.

In derselben Arbeit wurde gezeigt, dass der Zusatz von Aethylen zwecks Beschleunigung des Heranreifens grüner Früchte die Ausbildung der hydrolytischen Prozesse bedeutend verstärkt und beschleunigt. Wir können folglich den Schluss ziehen, dass die Rolle des Aethylens bei der künstlichen Fruchtreifung zum Teil auf das Verschieben der enzymatischen Gleichgewichte beruht, die das Aethylen als oberflächenaktive Substanz herbeiführt, indem es den adsorbierten Anteil der Enzyme von den Strukturgebilden der Zellen verdrängt (vgl. A. Kurssanov und N. Kryukova (48)).

Dieser Gesichtspunkt lässt sich offenbar auch auf andere Einwirkungen übertragen, durch die die Reifung von Früchten beschleunigt werden kann. Dies gilt insbesondere für Aether und Chloroform (H. Gore (70)), sowie für Aethylalkohol (S. Soldatenkov (71), G. Rakitin (72)) als oberflächenaktive Substanzen.

*4. Einfluss der Aschenelemente auf die enzymatischen Prozesse in Pflanzen*

Der eingreifende Einfluss, den die Mineralstoffernährung auf die gesamte Entwicklung und die physiologischen Funktionen der Pflanzen ausüben, veranlasst uns manche Aschenelemente als wichtige Teilnehmer des Stoffwechsels zu betrachten. Indessen sind unsere Kenntnisse über die biochemische Rolle der einzelnen Elemente, trotz der Fortschritte auf dem Gebiete der praktischen Anwendung mineralischer Düngemittel, in mancher Beziehung sehr mangelhaft. Insbesondere ist die Frage des Einflusses der Aschenelemente auf die Geschwindigkeit und die Richtung enzymatischer Reaktionen in den Zellen noch fast unerforscht.

Die Anwendung der Methoden zur Bestimmung der Enzymwirkungen in der lebenden Pflanze ermöglichte es, die Rolle der Phosphorsäure bei der Saccharose-Synthese eingehender klarzustellen. Es gelang ferner, über den Einfluss des Kaliums auf die reversible Wirkung der Invertase in den Zellen Aufschluss zu gewinnen. Zweifellos können auch andere für das Leben der Pflanze unentbehrliche Aschenelemente die Geschwindigkeit

und die Richtung enzymatischer Reaktionen auf diese oder jene Weise beeinflussen. Es ist dabei sowohl eine direkte Beteiligung des betreffenden Aschenelements an der enzymatischen Reaktion denkbar (wie im Fall des Phosphors bei der Saccharose-Synthese) als auch eine indirekte Einwirkung auf das Enzymsystem über Änderungen, die das Aschenelement (z.B. Kalium) in anderen Prozessen und speziell im physikalisch-chemischen Zustand der Plasmakolloide veranlassen kann.

In dieser Übersicht werden nur jene Arbeiten besprochen, die den Phosphor und das Kalium betreffen; von den übrigen Elementen, deren Einfluss auf die Reversibilität der Enzymreaktionen noch ungenügend erforscht ist, soll hier nicht die Rede sein.

Der Gedanke, dass Phosphorylierungsreaktionen beim Aufbau der Saccharose beteiligt sind, wurde erstmalig von A. Oparin und A. Kurssanov (73) geäussert. Nach diesen Vorstellungen wird durch die Anlagerung eines Phosphorsäurerests an die Alkoholgruppe am sechsten Kohlenstoffatom des Fructosemoleküls die Furanoseform dieses Zuckers stabilisiert, d.h. jene Form, die unmittelbar an der Saccharose-Synthese beteiligt ist. Die Frage der Bedeutung der Phosphorylierung für die Saccharose-Synthese wurde später auch von anderen Forschern diskutiert, so z.B. von Burkard und C. Neuberg (74) und W. Hassid (75), die Hexosephosphorsäureester aus pflanzlichen Geweben isoliert haben. Die Begünstigung der Zuckerspeicherung in den Pflanzen durch Phosphatdüngung ist ferner schon längst aus der agronomischen Literatur bekannt. Eine direktere Bestätigung fand diese Auffassung jedoch erst in den Versuchen von N. Syssakyan (67, 76). Dieser Autor unternahm vergleichende Bestimmungen der Geschwindigkeit der Saccharose-Synthese in den Blättern von Zuckerrübe und Zichorie bei Aufzucht der Pflanzen mit verschiedentlich dosierter Phosphaternährung. Unter Anwendung der Vakuuminfiltrations-Methode zur Bestimmung der synthetisierenden und hydrolysierenden Invertasewirkung konnte er nachweisen, dass die Synthese von Saccharose aus Monosacchariden bei Mangel an Phosphorsäure stark herabgesetzt ist. Sehr wahrscheinlich ist, dass in allen Teilen der Rübenpflanzen bei Phosphatmangel anstelle von Saccharose ein anderes Disaccharid gebildet wird, das der Maltose ähnlich, aber mit ihr nicht identisch ist (N. Syssakyan (76)). Die relative Resistenz dieses Zuckers gegenüber Säurehydrolyse ist ein Anzeichen der Abwesenheit von Furanoseringen im Molekül und ein indirekter Beweis für die Beteiligung der Phosphorsäure bei der Stabilisierung der Furanoseform. In einer Arbeit von A. Kurssanov und N. Kryukova (43) wurde diese Frage eingehender untersucht. In einer Reihe von Versuchen konnte gezeigt werden, dass eine

bestimmte Beziehung zwischen der Phosphorsäure und den Zuckern in den Zellen besteht. Dies ergab sich aus der Tatsache, dass die durch Infiltrierung von Monosen in den Blättern hervorgerufene Saccharose-Synthese stets von der Bildung einer gewissen Menge von Hexosemonophosphaten in den Zellen begleitet ist.

Andererseits veranlasst auch die Infiltrierung mit $NaH_2PO_4$, die zur Phosphorylierung eines Teils der Zucker führt, die Synthese einer gewissen Menge von Saccharose aus dem in den Zellen vorgebildeten Vorrat an Monosacchariden. Diese Verhältnisse veranschaulicht der in Tabelle VIII wiedergegebene Versuch.

TABELLE VIII

ZUSAMMENHANG ZWISCHEN DER SYNTHESE ORGANISCHER PHOSPHORVERBINDUNGEN UND DER SACCHAROSE-SYNTHESE IN ZICHORIE-BLÄTTERN

| Infiltrierte Substanz | Synthese (pro 1 Stunde und 1 g. Trockengewicht) | |
|---|---|---|
| | Organischer P (in mg.) | Saccharose, mg. |
| Glucose + Fructose | 464 | 18.3 |
| $NaH_2PO_4$ | 743 | 4.4 |
| Glucose + Fructose + $NaH_2PO_4$ | 836 | 25.7 |

Vergleichende Bestimmungen der Phosphorsäureester in Blättern von Zuckerrüben, die auf normalem Nährboden, bzw. bei Phosphatmangel gezüchtet worden waren, zeigten, dass es die Bildung von Hexosemonophosphaten ist, die bei Phosphatmangel die stärkste Verminderung aufweist. Infiltrierung von $NaH_2PO_4$ in solchen Pflanzen führt zu gesteigerter Hexosemonophosphatbildung und stellt daneben sofort die normale synthetische Aktivität der Invertase in den Zellen wieder her. Diese Versuche berechtigten zu der Annahme, dass die Schwächung der synthetisierenden Wirkung der Invertase bei Phosphathunger unmittelbar mit dem Mangel an Hexosemonophosphat in Zusammenhang steht. Geht man davon aus, dass im Saccharosemolekül die Fructose in Furanoseform vorliegt, so kann man vermuten, dass es die Phosphorylierung gerade dieses Zuckers ist, die für die Saccharose-Synthese notwendig ist. Neue Beweise dafür, dass die Phosphorylierung eine der Stufen des Saccharoseaufbaus darstellt, bringen die Versuche von N. Kryukova (77), in denen die Phosphorylierung in lebenden Zellen mittels Monojodacetat (0.005 M) oder Natriumfluorid ($^1/_2$ M) gehemmt wurde. Durch Infiltration der Blätter verschiedener Pflanzen mit Lösungen dieser Gifte konnte N. Kryukova eine vollständige Hemmung der Phosphorylierungsreaktionen

ohne sichtbare Schädigung der Zellen erzielen. Es ergab sich, dass die Saccharose-Synthese in den Pflanzen hierbei stark vermindert ist, während die hydrolytische Wirkung der Invertase keine Veränderung aufweist (vgl. Tabelle IX). Es sei dabei hervorgehoben, dass die Atmungsintensität und der R. Q. in den ersten Stunden nach Einführung der Gifte keine wesentlichen Änderungen aufwiesen.

TABELLE IX

SYNTHESE UND HYDROLYSE DER SACCHAROSE IN WEIZENBLÄTTERN BEI AUSSCHALTUNG DER PHOSPHORYLIERUNGSREAKTIONEN

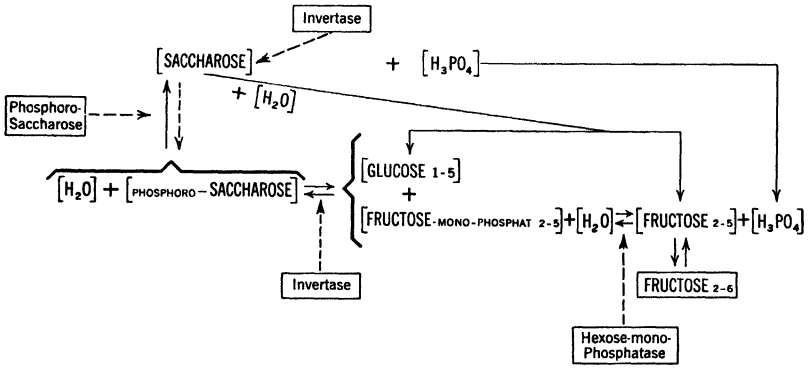
(Nach N. Kryukova)

| Phosphorylierung in mg. P pro 1 Stunde und 1 g. Trockengewicht | | | Invertaseaktivität in mg. Invertzucker pro 1 Stunde und 1 g. Trockengewicht | | | |
|---|---|---|---|---|---|---|
| Kontrollen | | $CH_2JCOOH$ | Synthese | | Hydrolyse | |
| | | | Kontrolle | $CH_2JCOOH$ | Kontrolle | $CH_2JCOOH$ |
| 1 | 2.22 | 0.00 | 17.6 | 1.3 | 27.1 | 31.3 |
| 2 | 1.46 | 0.00 | 14.1 | 0.0 | 34.0 | 33.0 |

Wichtig für die Beurteilung der Rolle der Phosphorylierung beim Kohlenhydratstoffwechsel der Pflanzen, und speziell bei der Saccharose-Synthese, sind Befunde über den Gehalt an Phosphatsäureestern in aktiv vegetierenden Geweben. Die von uns durchgeführten Bestimmungen und Berechnungen ergaben z.B., dass das Gehalt an Hexosemonophosphaten in Hafer- und Weizen-Keimlingen 18–19 mg. pro 1 gr. Trockensubstanz der Pflanze beträgt, in Lupinenkeimlingen—25 mg., in Hanfkeimlingen—11 mg., in Zuckerrübenblättern –9.7 mg. u.s.w. Demzufolge machen die phosphorylierten Zucker einen recht ansehnlichen Prozentsatz aus, und zwar etwa 15–25% vom gesamten Monosengehalt. Davon ist ungefähr die Hälfte auf Rechnung von Fructosemonophosphat zu setzen. Nach den in unserem Laboratorium erzielten Befunden von A. Nijeradse ist der Gehalt an phosphorylierten Zuckern in jungen Blättern besonders hoch und nimmt in alten Blättern beträchtlich ab. So z.B. entfallen in jungen Teeblättern fast 35% des gesamten Monosengehalts auf Hexosemonophosphate, während dieser Anteil in alten Blättern 3.5% nicht übersteigt. Daneben sinkt auch die Fähigkeit zur Synthese von Saccharose, wie G. Harebava zeigte, von den jungen zu den alten Teeblättern.

Unter Berücksichtigung der Gesamtheit der früher erhobenen experimentellen Befunde lässt sich die Bildung und der Abbau der Saccharose in den Zellen durch folgendes Schema veranschaulichen:

**Schema des enzymatischen Aufbaus und Abbaus der Saccharose nach A. Kurssanov und N. Kryukova (43)**




Aus diesem Schema folgt, dass drei verschiedene Enzyme an der Synthese der Saccharose beteiligt sind: (1) Hexosemonophosphatase, (2) Invertase und (3) Phosphosaccharase. Das Schema beruht auf der Reversibilität der enzymatischen Reaktionen, deren Richtung durch das Massenverhältniss der Reaktionsteilnehmer gesteuert wird. Naturgemäss gestalten sich diese einfachen Beziehungen in den Zellen viel komplizierter infolge der Heterogenität des Plasmas und der damit zusammenhängenden räumlichen Lokalisation der Substanzen. Trotzdem wird, wie experimentell nachgewiesen, durch die zusätzliche Einführung eines der Komponenten dieses Systems (z.B. eines der Zucker) in das lebende Gewebe, ein Prozess in letzterem ausgelöst, der auf die Restitution des gestörten Gleichgewichts gerichtet ist (vgl. Fig. 1). Obigem Schema gemäss, muss auch die Einführung von zusätzlichem anorganischem Phosphat in das System zur Phosphorylierung einer gewissen Menge von Fructose (2, 5) führen. Hierdurch wird das zweite, durch Invertase katalysierte Gleichgewicht gestört, und es muss auch in diesem Fall eine Umkehr einsetzen, die auf die Restitution des Gleichgewichts, d.h. auf die Synthese von Saccharose (über Phosphorsaccharose) gerichtet ist.

In unseren Versuchen (s. Tabelle VIII) konnte derartiges Verhalten tatsächlich nachgewiesen werden.

Als irreversibel erscheint in diesem Schema die durch die Invertase bewirkte Hydrolyse der Saccharose. Da aber die Produkte der Hydrolyse dieses Disaccharids mit Phosphorsäure ein System beweglicher Gleich-

gewichtsreaktionen bildet, die wieder zur Bildung von Saccharose führen, ist die gesamte Kette von Umwandlungen eine geschlossene. Obwohl die Synthese und die Hydrolyse der Saccharose nach diesem Schema auf verschiedenen Wegen erfolgen, ist der Prozess infolgedessen im Ganzen reversibel.

Weitere Untersuchungen in dieser Richtung werden wahrscheinlich dazu verhelfen, die räumliche Anordnung der Einzelreaktionen dieses Schemas in der Zelle näher zu definieren, und ihren Zusammenhang mit dem strukturell-energetischen Zustand des Plasmas zu ergründen.

Was die Rolle des Kaliums anbelangt, so wird nach den Befunden von A. Kurssanov und N. Kryukova (39) durch Infiltration dieses Elements in der Form von KCl die synthetisierende Wirkung der Invertase in den Blättern von Cyclamen persicum merklich angeregt und die hydrolysierende Wirkung geschwächt. Eine recht starke Verschiebung der reversiblen Invertasewirkung nach der synthetischen Seite beobachtete auch A. Scherbakov (78), der Erbsenblätter mit Lösung von KCl und $K_2SO_4$ infiltrierte.

Vor Kurzem wurden diese Befunde in einer Arbeit von N. Syssakyan und B. Rubin wieder bestätigt, die an Blättern des Apfelbaums und der Erdbeere durchgeführt wurde. Diese Autoren haben ferner nachgewiesen, dass KCl auch die synthetisierende Wirkung der Proteinase steigert.

Besonderer Beachtung verdient in den Versuchen von N. Syssakyan und B. Rubin die Tatsache, dass die Verstärkung der synthetischen Tätigkeit der Enzyme unter dem Einfluss von KCl besonders deutlich zutage tritt bei Temperaturen, die unterhalb 0° liegen. Da die Abkühlung des Plasmas gewöhnlich mit bedeutenden Veränderungen seines physikalisch-chemischen Zustands einhergeht, kann angenommen werden, dass der Einfluss des Kaliums nicht unmittelbar die Wirkung der Enzyme betrifft, sondern mit den Veränderungen in Zusammenhang steht, die dieses Element in den kolloidalen Eigenschaften des Plasmas auslöst, vor Allem mit der Steigerung der Fähigkeit, Wasser zu binden.

### 5. *Einfluss der Temperatur auf die Wirkung der Enzyme in lebenden Zellen*

Im obigen wurde gezeigt, dass die vorherrschende Richtung der enzymatischen Reaktionen in den Zellen, sowohl im Verlauf des Lebenszyklus der Pflanze, wie im Zusammenhang mit den Bedingungen ihrer Luft- und Mineralstoffernährung bedeutende Veränderungen aufweist. Noch stärkere Verschiebungen erleiden die enzymatischen Reaktionen bei Überschreitung der normalen Existenz-Bedingungen, z.B. bei starken Temperaturschwankungen oder bei Störung des Wasserhaushalts der Zellen. In

gewissen Fällen sind diese Verschiebungen in physiologischer Beziehung als Anpassungsreaktionen zu bewerten, in anderen Fällen erfolgen sie als Resultat der Unfähigkeit des Organismus, seine enzymatische Tätigkeit in Übereinstimmung mit den veränderten Bedingungen zu verrichten. Die Schädigung und sogar der Untergang der Pflanze, die unter diesen oder jenen ungünstigen Bedingungen stattfinden können, sind in Wirklichkeit öfters nicht durch die unmittelbare Einwirkung eines gewissen Umweltfaktors auf die Pflanze verursacht, sondern durch die unter solchen Verhältnissen erfolgende Dekompensation des Enzymapparats.

Studien von A. Kurssanov, N. Kryukova, und A. Morosov (50) über den Einfluss der Temperatur auf die reversible Invertasewirkung in den Blättern verschiedener Pflanzen haben gezeigt, dass die Abhängigkeit des enzymatischen Prozesses von der Temperatur in den lebenden Zellen in ganz anderer Weise zum Ausdruck kommt als in Versuchen mit Enzympräparaten (s. Fig. 5).

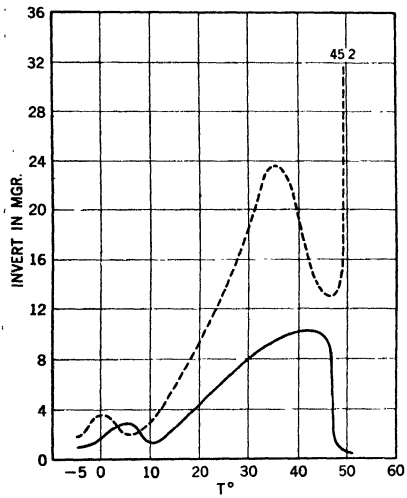



Fig. 5.—Einfluss der Temperatur auf die reversible Wirkung der Invertase in den Blättern von *Cyclamen persicum*. ( ——— -Synthese, .....-Hydrolyse.)

Aus den Kurven auf Fig. 5 ist ersichtlich, dass die Beziehung zwischen Temperaturerhöhung und Invertaseaktivität nur in dem Intervall zwischen −10 und +35° C. im Allgemeinen dem entspricht, was man an Enzympräparaten zu beobachten pflegt. Allerdings sind auch in diesem Intervall die Temperaturquotienten ($Q_{10}$) andere als die für reine Präparate ermittelten, doch bleibt die allgemeine, der Invertase wie anderen Enzymen zukommende Tendenz zum Absinken der $Q_{10}$-Werte beim Übergang von tieferen zu höheren Temperaturen auch in der lebenden Pflanze erhalten.

Setzt man die Pflanzen niedrigeren Temperaturen aus (−10 bis −5°), so steigt in ihnen die Aktivität der Invertase sowohl in Bezug auf Hydrolyse wie auf Synthese wieder an. Diese Besonderheit, die als *Kältemaximum* bezeichnet wird, erwies sich als ein sehr konstantes Phänomen, das nicht nur dem Zyklamen, sondern auch anderen Pflanzen eigen ist.

Eine weitere Eigentümlichkeit des Verhaltens der Invertase in lebenden Zellen ist die Depression ihrer hydrolytischen Wirkung, die bei +40 bis +45° (bei *Cyklamen persicum*) einsetzt. Die Ursache dieser Anomalien beruhen darauf, dass die Temperatur in den Zellen nicht allein auf die Aktivität der einzelnen Enzyme einwirkt, sondern auch auf viele anderen Äusserungen der Zelltätigkeit. Wohlbekannt ist speziell, dass der physikalischchemische Zustand der Kolloide bei extremen Temperaturen starken Veränderungen unterliegt H (F. F. Nord, vgl. z.B. L. Holzapfel und F. F. Nord (80)).

Deswegen lässt sich z.B. das Kältemaximum vielleicht mit der Abnahme der Viskosität der Plasmakolloide in Zusammenhang bringen die in den Zellen nach Angabe von D. Rubinstein (86) gewöhnlich 5–10° oberhalb des Auftretens von Eiskristallen im Plasma zu beobachten ist. Was die vorübergehende Abnahme der hydrolytischen Wirkung bei 40–45° betrifft, so hängt sie vielleicht zusammen mit beginnender Koagulation der thermolabilsten Kolloide und mit Adsorption eines Teils der hydrolytisch wirkenden Invertase an denselben. Stärkeres Anwärmen der Zyklamenblätter (bis auf +50°) hat ihr Absterben zur Folge. Bei der Zerstörung der vitalen Strukturen hört dabei die synthetisierende Wirkung der Invertase in den Zellen auf, und es stellt sich einseitige hydrolytische Wirkung en.

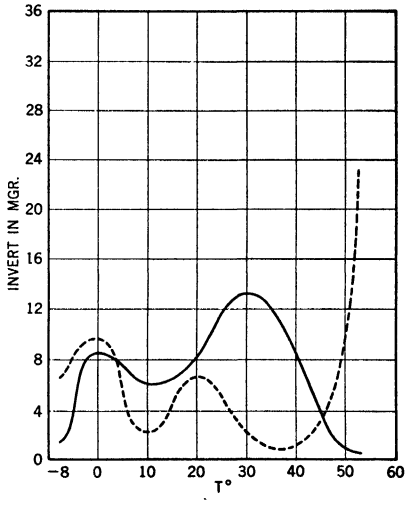



Fig. 6.—Einfluss der Temperatur auf die reversible Wirkung der Invertase in Blättern von Fragaria vesca, die unter Schnee geflückt worden waren.

Zweifellos haben alle diese Veränderungen auch eine bestimmte physiologische Bedeutung. Besonders das Aufleben der enzymatischen Tätigkeit in den Zellen bei Temperaturen, die nahe dem Nullpunkt gelegen sind, steht offenbar in Beziehung mit der Überwinterungs-Resistenz der Pflanzen. Es konnte tatsächlich experimentell nachgewiesen werden, dass das Kältemaximum viel stärker bei winterfesten Exemplaren ausgeprägt ist als z.B. bei Treibhauskulturen (vgl. Fig. 5 und Fig. 6).

Nach den an Blättern von Obstpflanzen durchgeführten Untersuchungen von N. Syssakyan und B. Rubin (79) ist das Weiterbestehen hoher Enzymaktivität bei niedrigen Temperaturen ein Kennzeichen kältefester Sorten. Dabei konnten die genannten Autoren zeigen dass die Aktivitätssteigerung beim Abkühlen auch der Proteinase eigen ist.

Ausserdem zeigten A. Kurssanov und N. Kryukova an verschiedenen, für niedrige Temperaturen sehr empfindlichen Varietäten des Chinabaums, dass Abkühlung von deren Blättern auf +2 bis 0° zur irreversibler Verschiebung des enzymatischen Gleichgewichts nach seiten der Hydrolyse führt, als Folge deren weitgehende Destruktion des Zellinhalts einsetzt.

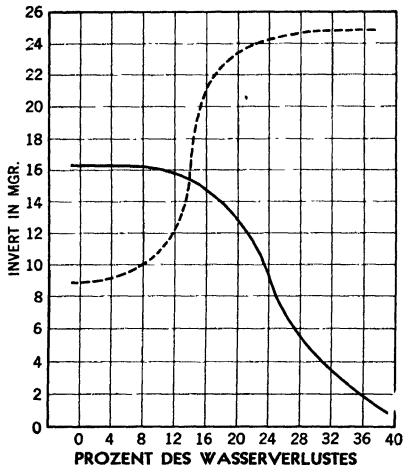



Fig. 7.—Verschiebung der reversiblen Invertasewirkung in jungen Theesprossen beim Darren. (————————-Synthese, .....-Hydrolyse.)

Diese Befunde berechtigten uns zu der Folgerung, dass die Schädigung und der Untergang der Pflanzen unter dem Einfluss verschiedener Temperatureinwirkungen nicht nur durch direkte Zerstörung des Protoplasmas infolge seiner thermischen Koagulation oder der Bildung von Eis verschuldet sind, sondern dass sie in erheblichem Masse bestimmt werden durch Verschiebungen der enzymatischen Prozesse, die Veränderungen der Temperaturbedingungen in der Pflanze hervorrufen.

### 6. *Wasserhaushalt und Dürre-Resistenz*

Bestimmungen der reversiblen Invertasewirkung in der welkenden Pflanze wurden erstmals von A. Kurssanov (81) vorgenommen, der an jungen Teesprossen (*Camelia Thea*) arbeitete. Die Versuche hatten den Zweck, die biochemische Bedeutung der Operation des Darrens von Teeblättern klarzustellen, die in den Fabriken bei der Teebearbeitung angewendet wird. Es wurde festgestellt, dass das Verwelken mit einer Abschwächung der synthetisierenden Wirkung der Invertase und einer nahezu spiegelbildlichen Zunahme der hydrolytischen Wirkung einhergeht (s. Fig. 7).

Eingehendere Beleuchtung fand diese Frage in den Arbeiten von N. Syssakyan und seinen Mitarbeitern (36, 82, 83, 84, 85), die dem Studium der inneren Ursachen gewidmet waren, von denen das verschiedene Verhalten der Pflanzen gegenüber Wassermangel abhängt.

Die Resultate der hauptsächlich an verschiedenen Weizensorten durchgeführten Untersuchungen zeigten, dass allen Pflanzen, bei einem bestimmten Grad des Verwelkens, eine starke Verschiebung der reversiblen Enzymwirkungen nach seiten der Hydrolyse eigen ist. Während jedoch diese Verschiebung bei dürrefesten Weizensorten erst nach Verlust von 45–50% des Wasservorrats eintritt, verlieren die Sorten, die gegen Dürre nicht resistent sind, bereits bei einem Wasserdefizit von 20–30% ihre Synthesefähigkeit. Beachtet man dabei, dass diese Gesetzmässigkeit nicht nur für die Carbohydrasen gilt, sondern auch für Proteinasen (N. Syssakyan und A. Kobyakova (82)) und wahrscheinlich für andere Enzyme, so liegt die Folgerung nahe, dass der Untergang der Pflanze beim Verdorren durch eine allgemeine Dekompensierung der biochemischen Tätigkeit verschuldet wird, infolge deren die hydrolytischen Reaktionen starkes Übergewicht im Vergleich zu den Synthesen erlangen.

In diesen Zustand versetzt, sind die Pflanzen naturgemäss nicht mehr zu weiterem Wachstum und Stoffspeicherung befähigt, sondern sie unterliegen einer Art von intravitaler Autolyse, bei der nicht bloss die Vorratsstoffe, sondern auch konstitutive Substanzen zerstört werden. Sehr wichtig ist der Umstand, dass die Verschiebungen der enzymatischen Gleichgewichte beim Einwelken anfänglich reversibler Art sind; beim Erreichen eines bestimmten Entwässerungsgrades aber, der als *kritische Grenze* bezeichnet wird (N. Syssakyan und A. Kobyakova) büsst der Enzymapparat die Fähigkeit zur Restitution des normalen Verhältnisses zwischen Synthese und Hydrolyse ein. Infolgedessen schreiten sogar bei Wiederherstellung der Wasserversorgung in der Pflanze einseitige hydrolytische Prozesse fort, die nach einiger Zeit zum Untergang der Pflanze führen.

Interessante Feststellungen über den inneren Zustand der Pflanze beim Einwelken ergaben sich in den Versuchen derselben Verfasser über die Wirkung der Phosphatasen in welkenden Blättern (84). Nach diesen Befunden kommt es bei starkem Einwelken in den Blättern zu einer erneuten Verstärkung der Phosphorylierungsreaktionen. Während aber normale Blätter durch die vorwiegende Bildung von Hexosemonophosphaten gekennzeichnet sind, findet in dem gewelkten Blatt ausschliesslich die Synthese von Hexosediphosphat statt; dies ist allem Anschein nach ein Anzeichen der Abnahme des Oxydationspotentials in den gewelkten Blättern und der Ausbildung von anaeroben Abbauprozessen.

## V. Zusammenfassung

Obwohl in der Frage der Regulierung enzymatischer Prozesse in der lebenden Zelle noch keine hinreichende Klarheit erzielt ist, halten wir es für möglich, einige allgemeine Grundsätze auszusprechen, die wir als gut begründet betrachten:

(*a*) Die Synthese und Hydrolyse einer Substanz in der lebenden Zelle wird durch ein und dasselbe Enzym bewerkstelligt.

(*b*) Je nach dem physikalisch-chemischen und energetischen Zustand des Plasmas und der in diesem eingeschlossenen Gebilde (Kerne, Plastiden, Mitochondrien u.s.w.), verteilen sich die Enzyme in den Zellen zwischen mikroheterogener wässeriger Lösung und den Strukturgebilden, an denen die Enzyme adsorbiert werden.

(*c*) In wässriger Lösung üben die Enzyme hydrolysierende und in adsorbiertem Zustand synthetisierende Wirkung aus. Obwohl beide Wirkungsrichtungen mit demselben Enzym zusammenhängen, sind sie demnach in der Zelle räumlich voneinander getrennt.

(*d*) Das Verhältniss der Gewchwindigkeiten der synthetisierenden und der hydrolysierenden Wirkung, oder die *vorherrschende Richtung* des enzymatischen Prozesses in der Zelle, hängt ab von dem quantitativen Verhältniss zwischen strukturgebundenem und gelöstem Enzym, sowie von dem Angebot adäquater Energie für die Synthese.

(*e*) Konstante physikalisch-chemische und energetische Verhältnisse in der Zelle vorausgesetzt, verteilen sich die Ausgangs- und Endprodukte der enzymatischen Reaktion in der Zelle entsprechend dem Verhältnis zwischen den Geschwindigkeiten der synthetisierenden und hydrolysierenden Enzymwirkungen. Jede Störung des erreichten Gleichgewichts zwischen den Reaktionsteilnehmern (z.B. durch Neubildung oder Fortschaffung eines dieser Produkte) führt unter diesen Bedingungen, in Übereinstimmung mit dem Massenwirkungsgesetz, zur Ausbildung eines einseitigen enzymatischen Prozesses, der auf die Restitution des gestörten Gleichgewichts gerichtet ist, d.h. auf die Umwandlung des überschüssigen Reaktionsteilnehmers in den im Unterschuss vorliegenden.

(*f*) Bei Änderung der physikalisch-chemischen und energetischen Bedingungen in der Zelle kann das Verhältnis zwischen den Geschwindigkeiten der synthetischen und hydrolytischen Enzymwirkungen Verschiebungen in der einen oder der anderen Richtung erfahren. Dementsprechend ändert sich auch die Verteilung der reagierenden Substanzen. Deswegen sind Änderungen des inneren Zustands der Zelle immer mit mehr oder weniger regen Umwandlungen der Inhaltsstoffe der Zelle verbunden.

(*g*) Verschiebungen in der vorherrschenden Richtung der Enzymwirkung in den Zellen können sowohl durch innere Ursachen, wie durch den Einfluss von Umweltfaktoren auf den inneren Zustand der Zellen veranlasst sein. Dabei sind die Veränderungen in den einen Fällen solcher Art, dass sie zu gleichsinnig gerichteter Verschiebung aller oder der meisten enzymatischen Prozesse führen (z.B. beim Welken u.dgl.); in anderen Fällen sind die Veränderungen mehr spezifisch und betreffen nur irgend einen bestimmten Enzymprozess.

(*h*) Der Stoffwechsel im Organismus beruht in der Hauptsache auf der individuellen Regulierung der enzymatischen Reaktionen, bei der die einzelnen enzymatischen Prozesse in den Zellen inbezug auf Geschwindigkeit und Richtung verschiedene Veränderungen erfahren können. Die Voraussetzung für die individuelle Regulierung der enzymatischen Prozesse sind einerseits in der *selektiven* Adsorption von Substraten und Enzymen an den Strukturgebilden der Zellen zu suchen, andererseits in besonderen Energieübertragungen.

(*i*) Jeder Pflanzenart und -Sorte ist bei gleichbleibenden inneren und äusseren Bedingungen ein konstantes Verhältnis zwischen Synthese und Hydrolyse eigen, welches demnach ein Artmerkmal der Pflanze darstellt.

(*k*) Die Entwicklung der Pflanze, die Bedingungen ihrer Ernährung und ihre Wechselbeziehungen mit der Umwelt verursachen in ihren einzelnen Organen wesentliche Änderungen der vorherrschenden Richtung der Enzymreaktionen. Zum Teil stellen diese Änderungen die direkten physiologischen Funktionen der Enzyme im Organismus dar und sind reversibler Art, indem sie unter dem Einfluss äusserer Einwirkungen oder periodischer Änderungen des inneren Zustands der Zellen wiederkehren. Eine andere Gruppe von Veränderungen bilden die irreversiblen Verschiebungen nach seiten der Hydrolyse, die mit fortschreitendem Alter der Pflanze, sowie unter dem Einfluss ungünstiger Existenzbedingungen eintreten (z.B. bei weitgehendem Verwelken, bei tiefen Temperaturen u.s.w.); solche Verschiebungen führen zum Untergang der Pflanze.

### Literaturverzeichnis

1. F. Hofmeister, "Die chemische Organisation d. Zelle," Braunschweig, 1901.
2. W. Palladin, *Fortschr. d. Naturwiss.*, **1** (1910).
3. A. Oparin, *Ergeb. Enzymforsch.*, **3**, 57 (1934); A. Oparin und A. Kurssanov, *Biochem. Z.*, **209**, 181 (1929), **256**, 190 (1932); A. Oparin und S. Manskaya, *Ibid.*, **260**, 170 (1933); A. Oparin, S. Manskaya, und J. Glasunov, *Ibid.*, **272**, 317 (1934); A. Oparin, S. Manskaya, und M. Magaram, *Ibid.*, **265**, 21 (1933); A. Oparin und S. Riskina, *Ibid.*, **252**, 8 (1932).
4. H. van't Hoff, *Z. anorg. Chem.*, **18**, 1 (1898).

5. A. Oparin, *Enzymologia*, **4**, 13 (1937); *Biochimia* (*U. S. S. R.*), **2**, 135 (1937); *Iswestia Akad. Nauk U. S. S. R.* (Ser. biol.), **1937**, 1733.
6. H. Marston, *Biochem. J.*, **17**, 851 (1923).
7. A. Kusin und O. Bogdaschevskaya, *Biochimia* (*U. S. S. R.*) **5**, 339 (1940).
8. E. Horning, *Ergeb. Enzymforsch.*, **2**, 336 (1933).
9. A. Kurssanov, *Iswestia Akad. Nauk U. S. S. R.* (Ser. biol.), Nr.4, 669 (1936).
10. W. Menke, *Hoppe-Seyler's Z. Physiol. Chemie*, **257**, 43 (1938).
11. K. Linderstrøm-Lang und H. Holter, *Ergeb. Enzymforsch.*, **3**, 309 (1934).
12. H. Borsook, *Ergeb. Enzymforsch*, **4**, 1 (1935).
13. A. Blagowestschenski, *Trudi Moskovsk. Doma Utschen.* (1940), Moskva.
14. J. Abelous und H. Ribaut, *Compt. rend. soc. biol.*, **52**, 543 (1900).
15. A. Blagowestschenski und K. Nikolaev, *Biochem. Z.*, **276**, 368 (1935).
16. E. Bünning, *Fortschr. Botan.*, **7**, 159 (1938).
17. W. Stiles, *Nature, London*, **142**, 879 (1938).
18. W. Lepeschkin, "Kolloidchemie d. Protoplasma," Leipzig, 1938.
19. F. Vles und M. Gex, *Compt. rend. soc. biol.*, **98**, 853 (1928); *Arch. phys. biol.*, **11**, 1 (1934).
20. A. Guilliermond, *Protoplasma*, **16**, 291 (1932).
21. D. Talmud, *Doklady Akad. Nauk U. S. S. R.* (1938).
22. J. Lengmur, Uspechi Chimii (1939).
23. W. Lepeschkin, *Protoplasma*, **28**, 175, 529 (1937).
24. K. Paech, *Planta*, **24**, 529 (1935).
25. A. Kurssanov, "Reversible Wirkung d. Fermente in lebenden Pflanzenzellen. U. S. S. R.," S. 233 Verlag Akad. Nauk U. S. S. R., Moskva, 1940.
26. J. Björksten, *Biochem. Z.*, **225**, 1 (1930); *Planta*, **11**, 1, 75 (1930).
27. K. Mothes, *Ibid.*, **19**, 117 (1933); Flora, **28**, 58 (1933); *Ber. botan. Ges.*, **(B) 51**, 31 (1933).
28. A. Kurssanov, *Biochimia* (*U. S. S. R.*), **1**, 269 (1936).
29. A. Kurssanov und K. Bryuschkova, *Ibid.*, **3**, 569 (1938).
30. A. Kurssanov und N. Kryukova, *Ibid.*, **3**, 529 (1938).
31. A. Kurssanov, *Ibid.*, **3**, 467 (1938).
32. B. Rubin und N. Syssakyan, *Doklady Akad. Nauk U. S. S. R.*, **15**, 487 (1937).
33. B. Rubin und O. Lutikova, *Ibid.*, **27**, 34 (1940).
34. A. Oparin, *Biochimia* (*U. S. S. R.*), **2**, 135 (1937).
35. A. Kurssanov und N. Kryukova, *Ibid.*, **4**, 562 (1939).
36. N. Syssakyan, *Ibid.*, **3**, 94 (1938).
37. A. Kurssanov und N. Kryukova, *Ibid.*, **3**, 201 (1938).
38. B. Rubin und E. Arzichovskaya, *Ibid.*, **2**, 952 (1937); *Doklady Akad. Nauk U. S. S. R.*, **27**, 64 (1940).
39. A. Kurssanov und N. Kryukova, *Biochimia* (*U. S. S. R.*), **2**, 674 (1937).
40. W. Pearsall und M. Billimoria, *Ann. Bot.*, **2**, 317 (1938).
41. K. Paech, *Planta*, **24**, 78 (1935).
42. A. Smirnov, "Physiologo-Biochim. osnowi Obrabotki tabaka," 1933, Krasnodar (russ.)
43. A. Kurssanov und N. Kryukova, *Biochimia* (*U. S. S. R.*), **4**, 229 (1939).
44. A. Kurssanov, *Ibid.*, **1**, 411 (1936).
45. H. Collin, *Compt. rend.* **170** (1916); *Rev. gén. botan.* (1916).

46. L. Berczeller, *Biochem. Z.*, **84**, 37 (1917).
47. S. Raabe, *Ibid.*, **299**, 141 (1938).
48. A. Kurssanov und N. Kryukova, *Biochimia* (*U. S. S. R.*), **2**, 720 (1937).
49. O. Warburg, *Biochem. Z.*, **103**, 188 (1920), *Hoppe-Seyler's Z. physiol. Chemie*, **102**, 245 (1922); O. Warburg und E. Negelein, *Biochem. Z.*, **173**, 257 (1921).
50. A. Kurssanov, N. Kryukova, und A. Morosov, *Iswestia Akad. Nauk U. S. S. R.* (Ser. biol.), **1938**, 51.
51. B. Rubin, "Biochem. Grundlag. d. Bewahrens d. Gemüse," Moskva, 1939.
52. N. Syssakyan und A. Kobyakova, *Biochimia* (*U. S. S. R.*) **5**, 301 (1940).
53. B. Rubin, *Ibid.*, **1**, 467 (1936); *Iswestia Akad. Nauk U. S. S. R.* (Ser. biol.), **1937**, 1775.
54. A. Kurssanov, *Planta*, **20**, 535 (1933); **22**, 240 (1934); A. Kurssanov und M. Kasakova, *Trud. Zentr. Institut Sach. Prom.*, **1**, 3 (1933); A Kurssanov, W. Blagowechenski, und M. Kasakova, *Bübl. Soc. Natur Moscou* (Ser. biol.), **42**, (2), 171 (1933).
55. B. Rubin und O. Lutikova, *Biochimia* (*U. S. S. R.*), **2**, 423 (1937).
56. B. Rubin und N. Syssakyan, *Ibid.*, **4**, 210 (1939).
57. N. Syssakyan und A. Kobyakova, *Ibid.*, **5**, 301 (1940).
58. B. Rubin, E. Arzichowskaya, N. Spiridonova, und O. Lutikova, *Iswestia Akad. Nauk U. S. S. R.* (Ser. biol.), (1940).
59. N. Syssakyan und A. Kobyakova, *Biochimia* (*U. S. S. R.*), **6**, Nr.1 (1940).
60. N. Syssakyan und A. Kobyakova, im Druck.
61. B. Rubin und O. Lutikova, *Biochimia* (*U. S. S. R.*), **2** 423 (1937).
62. A. Oparin, *Ibid.*, **2**, 135 (1937).
63. A. Kurssanov und K. Bryuschkova, *Ibid.*, **4**, 566 (1939).
64. A. Kurssanov und K. Bryuschkova, *Ibid.*, **5**, Nr.4 (1940).
65. A. Kurssanov und K. Bryuschkova, *Ibid.*, **5**, Nr.6. (1940).
66. T. Chrzaszcz und J. Janicki, *Biochem. Z.*, **285**, 47 (1936); *Biochem. J.*, **30**, 342 (1936).
67. N. Syssakyan, *Biochimia* (*U. S. S. R.*), **1**, 301 (1936).
68. A. Kurssanov und K. Bryuschkova, *Ibid.*, **5**, 188 (1940).
69. V. Nilov und O. Pavlenko, *Biochemia* (*U. S. S. R.*), **5**, 33, 41 (1940).
70. H. Gore, Bur. of Chem., U. S. Dep. Agr. *Bull.* **141** (1911); **155** (1912).
71. S. Soldatenkov, *Doklady Akad. Nauk U. S. S. R.*, Nr.7, 571 (1935); S. Soldatenkov und M. Kubli, *Ibid.*, Nr.2, 85 (1934).
72. G. Rakitin, *Iswestia Akad. Nauk U. S. S. R.* (Ser. biol.), 1129 (1935); 743 (1936) und andere.
73. A. Oparin und A. Kurssanov, *Biochem. Z.*, **239**, 1 (1931).
74. J. Burkard und C. Neuberg, *Ibid.*, **270**, 229 (1934).
75. W. Hassid, *Plant Physiol.*, **13**, 641 (1938).
76. N. Syssakyan, *Iswestia Akad. Nauk U. S. S. R.* (Ser. biol.), 309 (1938).
77. N. Kryukova, *Biochimia* (*U. S. S. R.*), **5**, Nr.5 (1940).
78. A. Scherbakov, *Ibid.*, **3**, 417 (1938).
79. N. Syssakyan und B. Rubin, *Doklady Akad. Nauk S. S. S. R.*, **25**, 300 (1939).
80. L. Holzapfel und F. F. Nord, *Ber.*, **71**, 1217 (1938); *Biodynamica*, **3**, Nr. 57 (1940).
81. A. Kurssanov, *Biochemie d. Theeindustrie*, **1**, 32 (1935).

82. N. Syssakyan und A. Kobyakova, *Biochimia* (*U. S. S. R.*), **3**, 796 (1938).
83. N. Syssakyan und A. Kobyakova, *Ibid.*, **4**, 202 (1939).
84. N. Syssakyan und A. Kobyakova, *Ibid.*, **5**, 225 (1940).
85. N. Syssakyan, "Biochem. Charakterist. d. Dürreresist.," Verlag Akad. Nauk U. S. S. R. Moskva, 1940.
86. D. Rubinstein, "Physiko-chemische Grundlage d. Biologie," Verlag Biomedgis, Moskva, pp. 357, 524, 1932.

# DIE VERDAUUNG BEI DEN NIEDEREN VERTEBRATEN*

VON H. J. VONK

*Utrecht*

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* Hierunter werden verstanden die Kaltblüter (besser Poikilothermen), ferner—Fische, Amphibien und Reptilien.

## I. Unterschiede in der Verdauung zwischen Vertebraten und Invertebraten

Bevor wir zu dem eigentlichen Thema übergehen, wird es gut sein einiges vorauszuschicken über die Unterschiede in der Verdauung der Vertebraten und Invertebraten. Wir betrachten hierzu die Art der verdauenden Enzyme, ihre Verteilung im Darmkanal, die Resorption und die Fortbewegung der Nahrung.

Die Enzyme, die Eiweiss, Kohlenhydrate und Fette im Verdauungskanal spalten, zeigen bei Invertebraten und Vertebraten eine ziemlich weitgehende Übereinstimmung. Eine Ausnahme macht Pepsin, das nur bei Vertebraten und niemals bei Invertebraten gefunden wird. In Übereinstimmung hiermit wird auch Salzsäure, welche den für die Wirkung des Pepsins notwendigen niedrigen Säuregrad (Optimum *p*H 2) zustandebringt, soweit bekannt, bei keinem Invertebraten in den Darmkanal abgeschieden. Für die Spaltung des Eiweisses sind ferner bei Vertebraten Trypsin und Erepsin nötig, welche beide nach Untersuchungen der letzten Jahrzehnte (1) aus einer ziemlich grossen Anzahl von Teilenzymen bestehen. Diese Teilenzyme wurden auch bei den Invertebraten, welche hierauf untersucht wurden, gefunden (2). Die Anwesenheit von Trypsin wurde einwandfrei festgestellt bei *Maja squinado* (3) und bei *Sepia officinalis* (4). Es ist nicht unwahrscheinlich, dass neben diesen Enzymen im Darmkanal der Invertebraten Fermente abgesondert werden, welche vergleichbar sind mit den Gliedern der proteolytischen Enzymsysteme, die in den inneren Geweben der Vertebraten gefunden werden (Rosén (5)). Mittels einer Vorbereitung durch eine reduzierende Substanz, ist die Kleidermotte (*Tineola biselliella*) imstande das für andere Tiere unangreifbare Keratin zu verdauen (6).

Die Amylasen und Maltasen im Invertebratendarmkanal stimmen mit denjenigen der Vertebraten nahezu überein. Ebenso wie die Amylase der letzteren, muss die der Invertebraten durch Salzionen aktiviert werden (im Gegensatz zu der Pflanzenamylase). Merkwürdig ist aber, dass bei ziemlich vielen Invertebraten (besonders bei der Weinbergschnecke, *Helix pomatia*, anderen Schnecken und Crustacea) Carbohydrasen vorkommen, welche die verschiedensten $\beta$-Hexoside spalten können (besonders Cellobiose) (7). Weiter, dass bei den gleichen Tieren eine Cellulase im Darmkanal gefunden wird, welche (wenn auch langsam) Cellulose und noch besser Hemicellulosen angreift (8). Auch ein Chitinverdauendes Ferment ist bei diesen Tieren vorhanden. Diese Fähigkeit zur Verdauung von Cellulose, Chitin und $\beta$-Sacchariden scheint bei den Vertebraten gänzlich verloren gegangen zu sein. Die Verdauung von Cellulose geschieht hier im Coecum und Dickdarm durch Bakterien.

Bekanntlich ist die Spezifität bei Lipasen weniger ausgeprägt als bei den übrigen Verdauungsenzymen. Bei einigen Invertebraten scheinen die Lipasen mehr den Charakter von Esterasen zu haben (Krüger (9)). Sonst ist wenig über konstante Unterschiede oder Übereinstimmung zu sagen.

Über die Absonderung der Enzyme ist hervorzuheben, dass bei weitaus den meisten Invertebraten die Verdauungsenzyme in einem einzigen Saft zusammen vorkommen und an der gleichen Stelle gemeinsam abgesondert werden. Bei den Tieren welche eine Mitteldarmdrüse haben, ist diese das sezernierende Organ. Bei den Vertebraten dagegen hat eine Differenzierung in der Abscheidung der Enzyme stattgefunden. Das Sekret der Speicheldrüsen (Säugetiere) enthält nur Amylase,* der Magensaft Pepsin, Lab und Lipase, der Pankreassaft Amylase, Lipase und Trypsin, der Darmsaft hauptsächlich Erepsin und disaccharidspaltende Fermente. Es ist also hier eine Kette von Enzymen vorhanden, welche nacheinander ihre Verdauungsarbeit erfüllen. Diese Einrichtung dient wohl dazu, einer Überschwemmung des Blutes mit Spaltungsprodukten der Nahrung vorzubeugen. Die ausgiebigere Nahrungsaufnahme und schnellere Verdauung machen dieses notwendig.

Als besondere Verdauungsorgane treten bei den Vertebraten auf: der Magen, das Pankreas und die Leber mit der Gallenblase (letztere kann auch fehlen). Der Magen dient neben der Absonderung ihrer speziellen Fermente und deshalb als vorverdauendes Organ, bei vielen Tieren auch als Speicherorgan. Es kommt dann bei manchen Tierformen zur Ausbildung von verschiedenen Magenabteilungen oder wenigstens Magenzonen. Die von der Leber abgesonderte Galle macht eine umfangreichere Fettverdauung möglich. Die gallensauren Salze fördern nämlich durch Emulgierung die Lipasewirkung und durch Lösung (wahrscheinlich kolloidal) der Fettsäuren die Fettresorption.

Grosse Unterschiede zeigen die zwei Gruppen in Bezug auf die Resorption. Die Protozoën nehmen ihre Nahrung direkt in ihr Inneres auf (Phagozytose) und verdauen sie intrazellulär. Diese Art der Nahrungsaufnahme bleibt noch bei manchen Invertebratengruppen bestehen (10). Dies ist z.B. der Fall bei den Spongien, bei den Coelenteraten, den niederen Würmern und einem Teil der Mollusken. Bisweilen wird dann die Beute durch eine oberflächliche Vorverdauung für die Phagozytose vorbereitet. Die höheren Invertebratengruppen zeigen diese Phagozytose nicht mehr (z.B. Insekten, Crustacea, höhere Würmer, Cephalopoden). Auch bei den Vertebraten findet eine derartige Phagozytose im Darmkanal nicht statt

* Wenigstens bei Mensch, Affen, Schwein und Rodentia; bei anderen Säugern fehlt eine Amylase im Speichel nahezu.

(nur bei jungen Mäusen hat von Möllendorf sie noch beobachtet). Bei dem Vorläufer der Vertebraten Amphioxus aber, spielt die Phagozytose merkwürdigerweise bei der Verdauung eine grosse Rolle (Barrington (11), van Weel (12)).

Bei den Vertebraten sind die resorbierenden Organe viel schärfer von den sezernierenden differenziert, als bei den niederen Tieren. Bei den Invertebraten, wo eine Mitteldarmdrüse existiert (alle Mollusca, Crustacea, Arachnoidea) dient dieses Organ und manchmal sogar die gleiche Zellart, sowohl der Sekretion als der Resorption. Bei den Vertebraten werden die Enzyme in besonderen Drüsen sezerniert (Magendrüsen, Pankreas, Darmdrüsen). Die Resorption findet nur im Darme statt und zwar hauptsächlich im Dünndarm. Bei den Vertebraten findet die Resorption im Darme statt unabhängig vom osmotischen Druckgefälle und unter Verbrauch von Energie. Bei bestimmten Invertebraten verhält sich der Darm wie eine Diffusionsmembran, ohne Polarität (*Helix pomatia* (13)). Allerdings ist hier das Verhältnis zwischen dem Umfang der Resorption in Darm und Mitteldarmdrüse nicht bekannt. Ob eine Resorption durch letzteres Organ unter Energieverbrauch und unabhängig vom Diffusionsgefälle stattfindet, wissen wir nicht.

Die Fortbewegung der Nahrung findet bei Invertebraten entweder durch Darmbewegung statt oder mittels Zilien, die entweder direkt die Nahrungspartikeln in Schleim eingehüllt fortbewegen, oder dass sie einen Wasserstrom erzeugen, welcher die Teilchen fortbewegt. Solche Wasserströme dienen auch vielfach der Atmung. Bei den Vertebraten trifft man eine Fortbewegung durch Zilien nur noch an bei den Cyclostomata (auch bei dem Vorläufer der Vertebraten Amphioxus).* Bei allen höheren Gruppen geschieht sie durch rhythmische Kontraktionen des Darmes (Peristaltik).

## II. Vorbemerkungen über die Anatomie des Verdauungskanals bei den niederen Vertebraten

**Speicheldrüsen** (14).—Hauptsächliche Funktion des Speichels ist diejenige eines Gleitmittels, das trockene Nahrung schlüpfrig macht. Daneben kann er (bei manchen Säugetieren) Verdauungsfermente enthalten. Wenn schlechtschmeckende oder scharfe Stoffe in den Mund gebracht werden, wird ein wasserreicher Speichel abgesondert. Die Speicheldrüsen werden in muköse und seröse Drüsen eingeteilt, je nachdem sie Schleim (Muzin) oder ein schleimloses, etwas Eiweiss enthaltendes Sekret liefern. Sie können auch gemischt sein.

Die Fische besitzen keine Speicheldrüsen. Die Absonderung von Schleim geschieht durch in Mundhöhle und Oesophagus zerstreute Zellen. Speicheldrüsen treten erst

* Auch bei Reptilien findet sich ein Flimmerepithel im Oesophagus. Es ist aber fraglich ob dieses zur Fortbewegung der Nahrung dient.

bei Amphibien auf, sei es als echte aber kleine Drüsen, oder Drüsenfelder (auf der Zunge). Daneben finden sich zerstreute Schleimzellen und seröse Drüsenzellen. Die Drüsen sind fast alle mukös. Auch bei Reptilien finden sich Schleimzellen, Drüsenfelder und echte Speicheldrüsen. Sie sind teils muköser, teils seröser Natur. Zu den Speicheldrüsen gehören auch die Giftdrüsen der Schlangen (15).

**Einteilung des Darmkanals** (16).—Der Darmkanal kann in Vorderdarm, Mitteldarm und Enddarm eingeteilt werden. Wir werden jetzt besprechen, welche Entwicklung diese verschiedene Abschnitte bei Fischen, Amphibien und Reptilien erreicht haben.

**Fische.**—Der Vorderdarm besteht hier aus Mund (Munddarm), Kiemendarm, Oesophagus und Magen. Bei den Cyclostomata ist noch kein Magen zu unterscheiden; Magendrüsen und Pepsin fehlen. Ebenso fehlt diesen Tieren ein echtes Pankreas. Bei den Jugendformen ist eine Gallenblase vorhanden, welche in den Darm ausmündet. Merkwürdigerweise verschwindet diese bei den erwachsenen Formen, auch hat hier die Leber keine Verbindung mit dem Darm mehr. Die Petromyzontes haben im Vorderdarm ein Flimmerepithel, womit vielleicht die Nahrung fortbewegt wird.

Bekanntlich wird bei den höheren Vertebraten das Pepsin in den Hauptzellen, die Salzsäure in den Deckzellen der Magendrüsen gebildet (17). Bei den Fischen findet man Magendrüsen, aber keine Differenzierung in Haupt- und Deckzellen (18). Es wird Pepsin und Salzsäure abgesondet. Übrigens gibt es verschiedene Fischgruppen bei denen der Magen fehlt (vgl. Fig. 1). Hier findet man auch kein Pepsin und kein HCl. Die Ausführgänge von Pankreas und Gallenblase münden dann direkt hinter dem Oesophagus. (Da der Darm hier erweitert ist, wird bei Unkenntnis dieser Verhältnisse dieser Darmabschnitt in der physiologisch-chemischen Literatur bisweilen für einen Magengehalten (19).) Dieses Fehlen des Magens findet man u.a. bei der Gruppe der Karpfen (Cyprinoiden) (20). Oft ist der Magen der Fische V-förmig gelagert, so dass man ihn in eine weitere Pars cardiaca und eine engere Pars pylorica einteilen kann. Bei manchen Gruppen kommt es zur Bildung eines Magenblindsackes, der sich bis zum letzten Körperdrittel erstrecken kann.

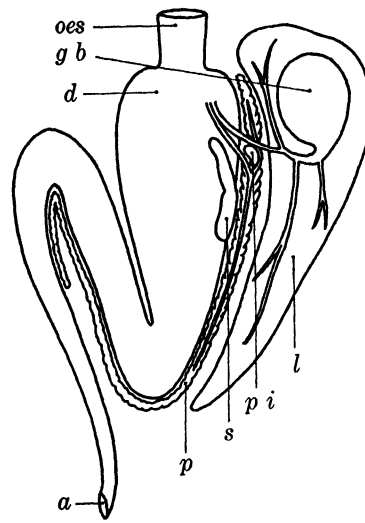



Fig. 1.—Anatomie des Darmkanals eines magenlosen Fisches (*Fundulus heteroclitus*). *oes* Oesophagus, *d* Darm, *a* Anus, *g.b.* Gallenblase, *l* Leber, *p.i.* Langerhanssche Inseln, *s* Milz, *p* Pankreas. Man beachte, dass die Ausfuhrgänge von Gallenblase und Pankreas kurz hinter dem Oesophagus in den erweiterten vorderen Darmteil münden (nach Babkin und Bowie).

Der Mitteldarm ist meist einfach gebaut und seine Teile sind oft wenig voneinander differenziert: In vielen Fällen kann man Mittel- und Enddarm unterscheiden, die dann durch eine deutliche Klappe, die Valvula Bauhini, von einander getrennt sind. Merk-

würdig ist, dass echte mehrzellige Drüsen nach den Angaben der Histologen dem Mittel- und Enddarm vollkommen fehlen. Es wird nur die Anwesenheit von Schleimzellen erwähnt. Eine Ausnahme bildet die Gruppe der Gadiden, wo durch eine Reihe von Untersuchern das Vorkommen von echten Lieberkühnschen Drüsen, wie sie für die übrigen Wirbeltiere charakteristisch sind, nachgewiesen wurde. Bei den Selachiern ist bekanntlich der Mitteldarm mit der grossen Spiralklappe versehen.

Darmzotten (d.h. fingerförmige etwa 0.5 mm. hohe Ausstülpungen, wie sie bei den höheren Vertebraten die Darmwand in unzählbarer Anzahl bedecken) fehlen den Fischen. Dagegen kommen als Oberflächenvergrösserung wohl Falten vor, welche oftmals Netze auf der Darmwand bilden.

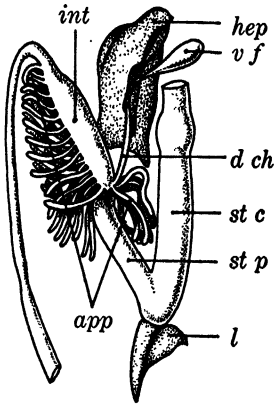



Fig. 2.—Vorderdarm und vorderer Mitteldarm eines Magenfisches (*Trutta lacustris* = Seeforelle). *stc* Cardiateil des Magens, *stp* Pylorusteil des Magens, *l* Milz, *int* Mitteldarm, *app* Appendices pyloricae, *hep* Leber, *vf* Gallenblase, *d ch* Ductus choledochus (nach Bütschli).

Weit mehr Besonderheit als Mittel- und Enddarm selbst, zeigen die ihnen anhängenden Gebilde. Gleich hinter dem Pylorus befindet sich bei sehr vielen Fischarten eine Anzahl eigentümlicher Ausstülpungen, die sogenannten Appendices pyloricae (vgl. Fig. 2). Sie fehlen den Selachiern, sowie den magenlosen Fischen (vgl. Fig. 1) und auch einer grossen Anzahl von Magenfischen unter den Teleostiern. Ihre Zahl kann wechseln von 1 bis über 900; sie können jede für sich, oder mehrere zugleich in den Darm ausmünden; ihre Wandungen (das Epithel einbegriffen) sind aufgebaut wie beim Mitteldarm. Bei den Gadiden kommen also auch in den Appendices die Lieberkühnschen Drüsen vor. Bei Acipenser ist die ganze Masse der Appendices fest zusammengewachsen, jedoch wird das Organ niemals drüsenartig.

Die Leber der Fische ist stark gelappt, eine Gallenblase ist vorhanden, und der Ductus choledochus mündet in geringer Entfernung vom Pylorus. Zusammen mit ihm oder in seiner Nähe mündet der Ausführgang des Pankreas. Diese Ausführöffnungen befinden sich vor, zwischen oder hinter den Appendices. Der geringe morphologische Wert, den man diesen Punkten, ihrer inkonstanten Lage wegen, beimessen kann, ist Ursache, dass man in neuerer Zeit die Unterscheidung eines Zwischendarms (Bursa entiana) vom Pylorus bis zum Duct. chol. aufgegeben hat.

Das Pankreas hat man längere Zeit hindurch bei vielen Fischen vermisst. Bei den Selachiern kannte man ein deutliches und kompaktes Pankreas, und Cuvier stellte die Theorie auf, dass die Appendices pyloricae überall da vorkommen, wo ein Pankreas fehlt. Es hat bis 1873 gedauert,* ehe durch die Arbeiten von Legouis (21) und später von Laguesse (22) gezeigt wurde, dass allen Fischen ein Pankreas zukommt, auch denen, welche Appendices besitzen. Das Organ ist aber kein kompaktes Gebilde, sondern es

* Wenn man von der unbeachtet gebliebenen Erwähnung des Organes durch Steller in einem nachgelassenen Werk (etwa 1830) absieht.

ist in viele feine Stränge zerteilt, die den ganzen Darm entlang sich verzweigen. Es folgt den Blutgefässen und dringt bei einigen Formen mit ihnen in die Leber ein, was Krukenberg veranlasste, von einem Hepatopankreas zu sprechen; mit Unrecht aber, denn die Organe vermischen sich gar nicht, obwohl sie einander durchdringen.

**Amphibien.**—Bei dieser Gruppe ist der Oesophagus kurz. Der Magen ist ein dickwandiges meist gerades Rohr. Er besitzt Fundus- und Pylorusdrüsen. Nach Kranenburg (18) findet man schon hier eine Differenzierung der Drüsenzellen in Haupt- und Belegzellen, obzwar weniger deutlich wie bei den höheren Vertebratengruppen. Bei

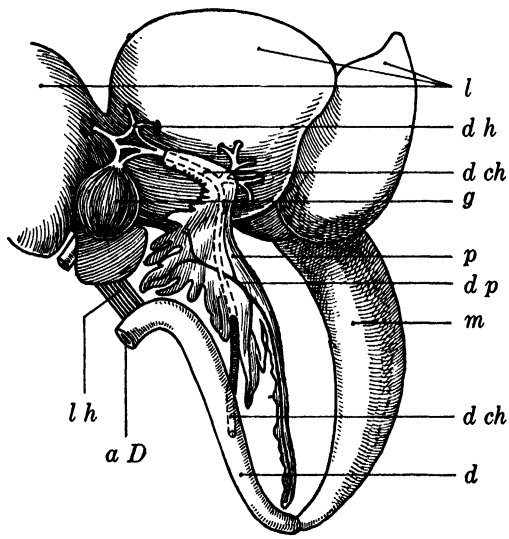



Fig. 3.—Magen-Duodenalschlinge beim Frosch. *l* Leber, *dh* Ductus hepatici, *p* Pankreas, *dp* Ductus pancreatici, *m* Magen, *dch* Ductus choledocus, *d* Duodenum, *aD* abgeschnittener Darm, *lh* Ligamentum hepato-duodenale, *g* Gallanblase, *dc* Ductus cystici (nach Ecker).

der Gattung Rana (und auch bei Proteus) findet man auch im Oesophagus (besonders im unteren Teil) eine grosse Anzahl ziemlich verzweigter Oesophagusdrüsen von welchen bei Rana festgestellt wurde, dass sie Pepsin absondern (2).

Der Darm ist relativ zum Körper länger als bei den Fischen und lagert sich in einer Anzahl von Schlingen. Der vordere Teil des Dünndarmes trägt den Namen Duodenum. Er wendet sich vom Pylorus gleich nach vorn und biegt dann auf der Höhe des Mageneinganges wieder nach hinten. Magen und Duodenum bilden also eine Schlinge, worin der grösste Teil des Pankreas liegt (vgl. Fig. 3). Der Enddarm ist kurz und mündet in die Kloake.

Eigentliche Darmzotten fehlen bei den Amphibien (nur wurden zottenähnliche Gebilde mit einem zentralen Lymphgefäss bei Salamandra maculata beschrieben (3)). Wohl kommen Leisten und blattähnliche Falten vor. Auch fehlen eigentliche Drüsen. Das Epithel besteht aus resorbierenden Zylinderzellen, wo zwischen auch Becherzellen (Schleimzellen) vorkommen.

**Reptilien.**—Der Oesophagus geht ohne scharfe Grenze in den Magen über. Bei einigen Gruppen (Krokodilen und Cheloniern) finden sich im Oesophagus Drüsen. Besonders bei *Testudo graeca* ist dies der Fall. Physiologisch scheinen diese Drüsen nicht untersucht worden zu sein. Bei Cheloniern ist der Oesophagus mit nach hinten gerichteten Stacheln besetzt. Der Magen der Reptilien ist höher differenziert und zeigt mehr Verschiedenheit als derjenige der Amphibien.

Bei Krokodilen hat der Magen einen kleinen deutlich abgegrenzten Pylorusteil, beim grössten Teil des Magens aber ist die Muscularis sehr dick. Die Muskeln gehen von zwei flachen Sehnenplatten aus. Der Magen ähnelt dadurch dem der Vögel. Doch ist bekanntlich der Muskelmagen der Vögel der pylorische Teil, während ihr Drüsenmagen dem Fundusteil der Säugetiere homolog ist. Bei den Krokodilen aber gehört die verdickte Muscularis zum Magenteil der mit dem Fundusteil der Säuger vergleichbar ist. Dieser muskulöse Magenteil der Krokodilen hat ein gewöhnliches drüsiges Epithel, wogegen der Muskelmagen der Vögeln bekanntlich an der Innenseite mit einem hornartigen Sekretionsprodukt bedeckt ist und keine Pylorusdrüsen hat.

Der Mitteldarm ist im allgemeinen kurz, nimmt aber bei Krokodilen und besonders bei Schildkröten beträchtlich an Länge zu. Nicht immer geht diese grössere Länge parallel mit der Aufnahme pflanzlicher Nahrung (4). Ein Duodenum ist mehr oder wenig deutlich zu unterscheiden. Vielfach trifft man, wie bei den Amphibien, eine Magenduodenalschlinge an. Bisweilen ist ein Coecum entwickelt. Die Schleimhaut des Darmes zeigt ursprünglich in der Entwicklung ein Netz von Falten, wovon später nur die Längsfalten übrig bleiben. Darmzotten kommen selten vor. Der Enddarm ist meist kurz und oft weiter als der Mitteldarm. Er endet in der Kloake (23–25).

## III. Allgemeines über die Verdauung der niederen Vertebraten

Einige Fragen über die Verdauung der Kaltblüter lassen sich, der Übersichtlichkeit wegen, besser im allgemeinen Teil behandeln als bei der Besprechung der einzelnen Gruppen. Diese Punkte betreffen die Identität der Enzyme, die Temperaturempfindlichkeit der Enzyme, die Reize der Sekretion und die Resorption.

**Identität der Enzyme.**—Es erhebt sich die Frage, ob die Enzyme der Kaltblüter vielleicht eine Art Mittelstellung einnehmen zwischen denjenigen der Invertebraten und der Säugetiere, oder praktisch mit den letzteren übereinstimmen. Von Scheunert (26) wurde z.B. für das Pepsin der Fische eine solche Mittelstellung angenommen.

Mit den modernsten Methoden der Enzymforschung (Adsorption und Kristallisation) wurde diese Frage bis jetzt noch nicht bearbeitet. Grösstenteils ist die relativ geringere Organmenge, welche man sich von unseren

Objekten verschaffen kann, daran schuld. Trotzdem ist wohl nicht daran zu zweifeln, dass die Enzyme der niederen Vertebraten mit denen der Säuger nahezu identisch sind.* Nur in ihrer Beständigkeit gegen Temperatur scheinen sie einigermassen davon abzuweichen (Siehe den nächsten Abschnitt).

Am ausführlichsten wurde diese Frage von Vonk (27) untersucht, besonders für die Enzyme der Fische. Dabei gelang es für *Acanthias vulgaris* nach der Methode von Pekelharing (28) (welche in letzterer Zeit auch Northrop als Basis für die Bereitung seines kristallisierten Pepsins gedient hat) tatsächlich ein kräftig wirksames Pepsinpräparat zu gewinnen. Das Prinzip dieser Methode ist, durch Selbstverdauung möglichst viel Eiweiss zum Verschwinden zu bringen. Die Salzsäure und die entstandenen Albumosen und Pepton werden darauf wegdialysiert. Bei noch schwach saurer Reaktion fällt dann ein Eiweisskörper aus, der sehr starke Enzymwirkung zeigt und wahrscheinlich Träger des Enzyms ist. Der Pepsingehalt des Haifischmagens ist nicht besonders hoch, überdies können bei dem langen Transport (Konservierung in 0.35% HCl) von Helgoland ab nach Utrecht beträchtliche Mengen verloren gegangen sein. Aus 2 kg. Frischgewicht an Schleimhaut wurden 100 mg. trockenes Pepsinpräparat erhalten. Eine noch weitere Reinigung durch Lösen in Salzsäure und erneute Dialyse (ein Verfahren, welches mehrmals wiederholt werden kann) musste der geringen Pepsinmenge wegen, unterbleiben. Das Präparat wurde nun verglichen mit einem ebensoweit gereinigten Präparat aus Schweinemagen. Es wirkt etwa 5 mal langsamer als dieses und etwa 25 bis 30 mal schwächer als ein weitgehend nach Pekelharing gereinigtes Präparat aus Schweinemagen. Der fünffache Unterschied in der Wirkung zwischen den Präparaten von Acanthias und Schweinepepsin ist wahrscheinlich Beimischungen zuzuschreiben. Es scheint sich nicht um einen wesentlichen Unterschied zu handeln. Geselschap (29) hat auch zwischen Präparaten von Schweine- und Hundepepsin Unterschiede in der Wirksamkeit gefunden. Weit wichtiger ist es, dass man beim Haifisch in derselben Weise wie bei Säugern zu hochwirksamen Präparaten kommen kann. Das Optimum für die Pepsinwirkung wurde für das Acanthiaspräpaparat bei *p*H 2.29 (Anfang des Versuchs) und *p*H 2.44 (Ende) gefunden. Für Schweinepepsin fand Ringer (30) 2.05 (Anf.) bis 2.41 (Ende). Weiter untersuchte Vonk (31) an Glyzerinextrakten der Schleimhaut die *p*H Optima beim Hecht, beim Frosch und bei einer Schildkröte.† Diese Optima lagen alle in der Nähe von *p*H 2 bei Anwendung von Glykokoll-Salzsäure Puffer. Diese Beobachtungen wurden ergänzt von Mennega (32), welche folgende Werte für verschiedene Tierarten fand: Hecht 2.40, Frosch 2.0, Testudo graeca 1.85, Barsch 2.0, Reiher 2.40, Falke 2.40. Substrat war hier Fibrin und Fleischpulver (bei letzterer Bestimmung der Wirkung durch Formoltitration). Die Übereinstimmung des Wirkungsoptimums für alle untersuchten niederen und höheren Vertebraten ist also recht auffallend und weist auf eine sehr nahe Verwandschaft aller dieser Pepsine hin. Zwar kann nach den Untersuchungen von Willstätter und seiner Schule das *p*H Optimum eines Enzyms durch Reinigung und Anwesenheit verschiedener Beimi-

* Es werden nur die Säuger für den Vergleich benutzt und nicht die Vögel, weil die Fermente der letzteren nur sehr wenig bearbeitet wurden.

† Methode: Kolorimetrische Bestimmung der Wirkung auf gefärbte getrocknete Fibrinkörner.

schungen erheblich verschoben werden. Doch weicht die Stellung des Pepsins mit seinem Optimum bei so hohem Säuregrad, von allen andern bekannten Enzymen ab. Nach den neueren Definitionen (vgl. Oppenheimer (33)) ist Pepsin eine Proteinase, welche nur auf Eiweisskationen wirkt, Trypsin ist nur wirksam auf Eiweissanionen, die Papainasen und Kathepsine wirken nur auf Eiweissstoffe im isoëlektrischen Zustande (34). Es sind keine Versuche bekannt, in welchen durch Zusätze Trypsin, Papainasen oder Kathepsin ein so niedriges *p*H-Optimum erteilt werden kann, wie das des Pepsins. Zweifelsohne sind also alle Proteinasen der Magenschleimhaut und der Magensäfte bei höheren und niederen Vertebraten echte Pepsine.

Dass die Eiweissverdauung durch das Enzym des Fischmagens Albumosen und Peptone und keine Aminosäuren als Endprodukte liefert, wurde schon in der älteren Literatur genügend festgestellt. Unter den Autoren, welche sich hieran beteiligten, seien genannt Richet, Van Herwerden, Weinland, Sullivan, und Yung. Diese Literatur ist ausführlich von Biedermann in *Winterstein's Handbuch der Vergleichenden Physiologie* zusammengefasst worden. Sie wird, soweit nötig, bei der speziellen Besprechung der Fische zitiert werden.

Für das Trypsin von *Acanthias vulgaris* fand Vonk, dass es gereinigt werden konnte, indem man einen wässerigen Extrakt von Haifischpankreas bis *p*H 4 ansäuerte; es entsteht ein Niederschlag, welcher das Enzym mitreisst. Dieser Niederschlag kann *in vacuo* getrocknet und aufbewahrt werden. Wieder bei *p*H 8 gelöst, wirkt er kräftig auf Fibrin. In dieser Weise hatten Michaelis und Davidsohn (35), ein wirksames Präparat aus Pankreasextrakt vom Schwein erhalten. Sie meinten anfänglich reines Trypsin in Händen zu haben, aber Hammarsten wies nach, dass es sich um ein Nucleoproteid handelt, das bei seinem isoëlektrischen Punkt von 3.5–4 ausfällt und das Trypsin adsorbiert. Da das Produkt aus Acanthiasextrakt nicht durch Darmschleimhaut aktiviert zu werden braucht (während das bei Glycerinextrakt wohl der Fall ist), hat hier eine Selbstaktivierung stattgefunden, wie sie in wässerigen Extrakten vom Pankreas der Säuger stattfindet. Dies ergab sich auch hieraus, dass nach einigen Tagen der chloroformhaltige wässerige Extrakt eine starke Tryptophanreaktion gab, so dass starke Autolyse stattgefunden hatte. Das *p*H-Optimum liegt genau an derselben Stelle für beide Trypsinarten. An dem wässerigen Extrakt wurde durch Wirkung auf Fibrin festgestellt, dass dieses u.a. Albumosen, Peptone, Leucin und Tyrosin als Spaltungsprodukte liefert. In allen Punkten zeigt das Acanthiastrypsin also Übereinstimmung mit Säugertrypsin.

Auch die Lipase von Acanthias wurde von Vonk (36) mit der Säugerlipase verglichen. Rosenheim (37) erhielt ein aktives Präparat von Säugerlipase, indem er ein Glycerinextrakt des Pankreas zehnfach mit Wasser verdünnte und etwas ansäuerte. Der lipasehaltige Niederschlag wurde nochmals mit Wasser gewaschen. Dasselbe Verfahren liess sich auch auf einen Glycerinextrakt von Acanthiaspankreas anwenden. Sowohl der

ursprüngliche Extrakt als die Lösung des Niederschlages wurden in ihrer fettspaltenden Wirkung stark durch Magnesiumchlorid aktiviert, wie es auch Pekelharing (38) für Säugerlipase gefunden hatte. Soweit untersucht, stimmt die Lipase des Haifisches *Acanthias* also mit der Säugerlipase überein.

Die Amylasen und Maltasen der Fische stimmen in ihren Optima überein mit denen der Säugetiere, besonders darin, dass das Optimum der Maltase viel flacher ist als das der Amylase.* Alle diese Optima liegen bei etwa *p*H 7. Dasselbe fand Wolvekamp (39) für die Schildkröte *Testudo graeca*. Dass die Amylasen durch Salze aktiviert werden müssen, wurde von Vonk (*l. c.*, 1927) für Frosch und Karpfen (Pankreas) festgestellt. Auch dieses deutet auf Übereinstimmung mit Säugeramylase hin; die pflanzlichen Amylasen werden nicht durch Salze aktiviert.

**Temperaturempfindlichkeit der Enzyme.**—Es wurde oben erwähnt, dass die Enzyme der Fische in ihrer Beständigkeit gegen Temperatur von den Säugerenzymen abzuweichen scheinen. Diese Tatsache beruht schon auf älteren Beobachtungen. Alle diese älteren Autoren sind sich einig, dass bei niederen Temperaturen das Fischpepsin relativ besser verdaut als das Säugerpepsin. Über den genauen Zusammenhang zwischen Wirksamkeit und Temperatur herrschen zwei oder drei verschiedene Meinungen. Nach der ersten (von Fick und Murisier und von Hoppe-Seyler), erreicht das Fischpepsin schon bei etwa 15° seine maximale Verdauungskraft und es bleibt dann diese Wirksamkeit unverändert bis 40°; nach der zweiten von Luchau und Krukenberg hat es sein Optimum bei 40°, wie das Säugerferment, und würde die relativ grössere Wirksamkeit bei 0°, verglichen mit dem Säugerpepsin den einzigen Unterschied ausmachen; die Untersuchungen sind aber nie von einem Autor bei sehr verschiedenen Temperaturen ausfegührt worden, sodass die Möglichkeit offen bleibt, dass das Optimum der Wirkung (für eine bestimmte Versuchsdauer) zwischen 15° und 40° liegt.

Rákoczy (Hecht- und Hundepepsin (40)) hat sich auch dieser Frage zugewandt. Er benutzt ein Pepsinpräparat aus getrockneter pulverisierter Magenschleimhaut mit Thymolzusatz. Aus Hechtmagen wurden Infuse erhalten, welche einige Male stärker sind als die aus Hundemagen (bei niederer Temperatur verglichen). Das Optimum des Säuregrades liegt für Hecht- und Hundepepsin bei $^1/_{20}$ *N* HCl (bei 15–16°). *p*H-Messungen wurden bei diesen Versuchen leider nicht ausgeführt, ebensowenig wird erwähnt, für welche Zeitdauer der Versuche diese Wahrnehmung gilt. Bei diesem Säuregrade wird bei 39° nach zweistündigem Stehen die Wirkung des Hechtpepsins

* Das Optimum der Säugermaltase wurde bestimmt von Vonk, van Nieuwenhoven und Noordmans (noch unveröffentlicht).

mit der Zeit geringer, die des Hundepepsins nicht (wie lange fortgesetzt ?). Um gleiche Bedingungen der Konzentration an Beimischungen der Extrakte herzustellen, wird jedem Hechtinfus ein gleiches Volumen gekochtes Hundeinfus zugesetzt und umgekehrt die Lösungen werden verdünnt mit einer gekochten Mischung der Infuse zu gleichen Teilen. Die Pepsinwirkung wird kolorimetrisch gemessen durch Einwirkung auf getrocknetes Karminfibrin. Hält man die Infuse gleiche Zeiten lang bei 39° und bei verschiedener Acidität, dann macht sich von einer Acidität höher als $^1/_{40}$ *N* HCl an eine vernichtende Wirkung der Temperatur beim Hechtinfus geltend, nicht aber beim Hundeinfus. Wie lange die Versuche fortgesetzt wurden, wird nicht erwähnt; nach längerer Zeit würde bei dieser Temperatur doch auch wohl das Hundepepsin an Kraft verlieren müssen, und sich so der Unterschied bloss als ein Unterschied in der *Schnelligkeit* der Vernichtung manifestieren. Durch die beschriebene Verdünnungsmethode kann der Unterschied in Resistenz keinen zufälligen (z.B. anorganischen) Beimischungen zugeschrieben werden. Er muss auf Eigenschaften des Enzyms selbst oder von als Träger fungierenden Substanzen beruhen. Auch Hammarsten hat in einer Publikation von 1908 (41) über die Identität von Pepsin und Chymosin einige Versuche mit der Schleimhaut des Hechtmagens ausgeführt und Resultate erhalten, die mit denjenigen von Rákoczy übereinstimmen.

Wenn man derartige Unterschiede in der Resistenz gegen Temperatur (oder andere Einflüsse) feststellen will, ist die beste Versuchsanordnung dass man die Enzyme eine bestimmte Zeit der Einwirkung dieses Einflusses aussetzt und darauf die Wirkung bestimmt. Die Unterschiede werden am deutlichsten sein, wenn die Zeit der Einwirkung des eventuell schädlichen Einflusses lang ist im Vergleich zu der Zeit der Wirkungsbestimmung der Fermente.

In dieser Weise wird ein Unterschied eher zu finden sein als bei blossem Vergleich der Temperaturoptima. Bestimmt man nur letztere, so soll wenigstens die Versuchsdauer so lang sein, dass ein Unterschied in der Temperaturempfindlichkeit sich äusseren kann, und nicht kürzer als die physiologische Verweildauer der Nahrung im betreffenden Darmabschnitt. Nicht alle in dieser Beziehung ausgeführten Untersuchungen genügen diesen Anforderungen.

Eine Untersuchung bei der diese Bedingungen mehr oder weniger eingehalten wurden, ist die von Pjatnitzky (42). Er findet für eine Zeitdauer von 10 St. das Temperaturoptimum für Froschpepsin bei 45–50°, für Menschenpepsin bei 50–55°. Die Pepsine von Mensch und Frosch sind bei seiner Versuchsdauer verschieden in Resistenz bei 65°, 60°, und 55° (hier verringert sich aber der Unterschied). Das menschliche Pepsin wird bei 70°, das Froschpepsin bei 65° zur Hälfte vernichtet.

Müller (43) hatte keine Unterschiede zwischen Frosch- und Hundepepsin feststellen können. Seine Versuche sind nicht beweisend, da die Versuchsdauer für Bestimmung des Temperaturoptimums zu kurz ist. Mardaschew (44) fand für das Temperaturoptimum des Pankreasex-

traktes vom Frosch 50°; Vergleiche mit Säugerenzym wurden nicht ausgeführt. Dieses Optimum ist nach ihm "normal," aber die Versuchsdauer von 40 min. ist zu kurz um Unterschiede mit Säugertrypsin festzustellen. Koschtojanz und Korjuïjeff (45) verglichen die Resistenz des Trypsins verschiedener Fische mit Hundetrypsin. Hier war tatsächlich die Bestimmungszeit (1 Stunde) kurz, verglichen mit den Vernichtungszeiten. Tabelle I zeigt die erhaltenen Resultate. Das Fischtrypsin

TABELLE I

RESISTENZ VERSCHIEDENER TRYPSINE. DIE EXTRAKTE STEHEN WÄHREND DER ANGEGEBENEN ZEIT BEI *p*H 8.04 BEI 60°. DARAUF WIRKUNGSBESTIMMUNG BEI 40°. ZUSAMMENGESETZT NACH DATEN VON KOSCHTOJANZ UND KORJUÏJEFF

| Zeit der Vernichtung in Stunden | 0 | 24 | 48 | 72 | 96 | Tierart |
|---|---|---|---|---|---|---|
| Erhaltene Aktivität in % von der des Hundetrypsins | 100 | 51.4 | 37.7 | 22.6 | 19.1 | Barsch |
| | 100 | 56.4 | 23 | 12.8 | 8 6 | Hecht |
| | 100 | 20.8 | 16 | 7.5 | 5 | *Gadus calliaris* |
| | 100 | 82 | 69 8 | 60 | 50.2 | Hund |

zeigt sich bei dieser richtigen Versuchsanordnung weit weniger resistent gegen diese Temperatur als das Hundetrypsin. Merkwürdig ist, dass das Ferment von *Gadus calliaris*, der im Eismeer lebt, wiederum eine geringere Resistenz hat, verglichen mit dem Enzym von Barsch und Hecht, welche auf niedrigerem Breitengrad vorkommen.

Chesley (46) hat die Amylasen des menschlichen Speichels, einer Fischart (*Brevoortia tyrannus*) und eines Reptils (*Terrapena carolina*) mit einander in ihrer Resistenz gegen die Temperatur verglichen. Tatsächlich nimmt ihre Resistenz in der genannten Reihenfolge ab. Chesley weist besonders darauf hin, dass alle andere Faktoren: *p*H, Salzkonzentration und Verdauungszeit genau gleich gehalten werden müssen, will man nicht zu fehlerhaften Resultaten kommen. Aus alledem geht hervor, dass die Fermente der Kaltblüter weniger resistent gegen die Temperatur sind als die der Wärmblüter, oder richtiger gesagt, letztere zeigen in ihrer Resistenz eine Anpassung an die höhere Körpertermperatur. Wenn nun Koschtojanz und Korjuïjeff auf Grund ihrer Versuche über die Temperaturresistenz behaupten, dass die Enzyme der höheren und niederen Vertebraten nicht identisch sind, so meine ich, dass diese Schlussfolgerung viel zu weit geht. Die Hauptsache ist, dass die Magenproteinase bei den niederen,

wie bei den höheren Wirbeltieren ein Pepsin ist, die Pankreasproteinase ein Trypsin (und nicht z.B. ein Kathepsin) u.s.w.

**Sekretion.**—Bei den Kaltblütern wurde bis jetzt vorwiegend die Magensekretion untersucht. Die Untersuchung ist schwierig, da es bei diesen Tieren bis jetzt nicht gelang, Dauerfisteln anzulegen, wie bei Säugetieren. Besonderheiten folgen bei den einzelnen Gruppen, doch lassen sich einige Haupttatsachen hier zusammenfassen.

Bei den Säugern unterscheidet man drei Phasen bei der Absonderung des Magensaftes: die erste oder reflektorische, die zweite oder chemische, die dritte oder Darmphase. Zu der ersten Phase gehören sowohl die Sekretion als Folge der blossen Wahrnehmung des Futters (durch Gesicht und Geruch: psychische S.) als die, welche stattfindet durch die Berührung des Futters mit der Schleimhaut des Mundes. Beide Erscheinungen sind reflektorischer Art. In der zweiten Phase wird Magensaft abgesondert wenn Extraktivstoffe oder Abbauprodukte des Futters die Magenschleimhaut berühren. Der Mechanismus ist wahrscheinlich hormonaler Art (Magensekretin), ist aber doch auch der Tätigkeit des Zentralnervensystems untergeordnet. Die dritte Phase, bei der Reize vom Darme aus Magensaftabsonderung veranlassen, ist relativ unwichtig. Die efferenten Nerven, welche die Magensekretion bewirken, sind Äste des Nervus vagus.

Es wurde nun zuerst von Smirnov (47) für den Froschmagen festgestellt, dass eine psychische Sekretion fehlt und dass Durschneiden des N. vagus am Halse die Sekretion nicht aufhebt. Der Reiz für die Sekretion ist mechanischer Art: Einbringen indifferenter Stoffe, wie Stückchen Kork oder Gummi, ruft Magensaftabscheidung hervor. Stoffe, wie Pilocarpin und Acetylcholin, welche die gleichen Erscheinungen hervorrufen wie Vagusreizung, veranlassen beim Frosch keine Magensaftabsonderung. Reizung des sympathischen Systems oder Injektion von Adrenalin verursachen dagegen diese Sekretion (Friedmann). Andere Autoren, besonders Babkin, fanden, dass für Fische die Verhältnisse ebenfalls sehr von denen bei den Säugern abweichen. Es besteht also ein bemerkenswerter Unterschied zwischen der Regulation der Sekretion des Magensaftes bei den Säugern einerseits und bei Fischen und Amphibien andererseits. Die Reptilien wurden hierauf noch nicht untersucht.

**Resorption.**—Es wurde in Abschnitt I hervorgehoben, dass erhebliche Unterschiede bestehen zwischen der Resorption der Invertebraten und der Vertebraten. Ein derartiger Unterschied lässt sich zwischen niederen und höheren Vertebraten nicht feststellen. Niedere Vertebraten wurden aber in dieser Hinsicht wenig untersucht und dann war noch meistens Zweck der Untersuchung die Aufklärung allgemeiner Fragen auf dem Gebiete der

Resorption. Da im Rahmen der "Advances" die Betrachtung der Enzyme und ihrer Absonderung im Vordergrund der Betrachtung steht, werden wir derartige Arbeiten bei jeder Gruppe kurz behandeln.

## IV. Die Verdauung bei den Fischen

**Die Nahrung der Fische.**—Über die spezielle Nahrung der verschiedenen Arten kann man aus den Fischereizeitschriften und Brehms Tierleben mancherlei Tatsachen entnehmen. Eine zusammenfassende Übersicht findet sich ausserdem bei Biedermann (16), eine solche aus letzterer Zeit bei Rauther (48) und eine ausführliche Aufzählung der Tatsachen in der Arbeit von Eggeling (49). Da wir uns hauptsächlich mit der enzymatischen Verdauung bei den Fischen beschäftigen werden, kommt es für uns nur darauf an zu wissen, zu welchen der grossen Gruppen, nämlich der Karnivoren, der Omnivoren oder Herbivoren, die behandelten Arten gerechnet werden müssen. Die Einteilung, welche die Fischereiliteratur gibt, in Raubfische und Friedfische, ist eine rein praktische: die Raubfische nähren sich von so grossen Beuteobjekten, dass sie der Fischerei schaden; die Friedfische schaden der Fischerei nicht, können aber ebenso ausgesprochene Karnivoren und Räuber sein und sind es auch meistens. Denn die überwiegende Mehrzahl der Fische sind echte und ausschliessliche Karnivoren. Daneben gibt es wenige Omnivoren, wie den Karpfen und einige andere Cyprinoiden; reine Pflanzenfresser sind sehr selten. Vielleicht ist *Box boops* (Mittelmeer) die einzige Form, von der mit Sicherheit gesagt werden kann, dass sie rein herbivor ist. Auch für andere Arten der Gattung *Box* gilt wachrscheinlich dasselbe. Rauther unterscheidet noch eine besondere Gruppe der Schlammfresser (Ilyophagen). Es gehören dazu Tiere, welche organische Zerfallstoffe und dazwischen vorkommende kleine Pflanzen und Tiere fressen. Diese Fische kann man auch zu der Gruppe der Omnivoren rechnen, welche Rauther nicht speziell erwähnt. Die weitere Unterteilung der Karnivoren, welche Rauther gibt, ist für uns weniger von Interesse, da doch über alle diese Untergruppen keine physiologische Untersuchungen vorliegen.

**Sekretion.**—Die Magensekretion bei den Fischen wurde in neuerer Zeit studiert von Dobreff (50) für Haifische (meist Scyllium) und von Babkin (51) und Mitarbeitern für einige Roggenarten.

Dobreff heberte (nach Weinland's Methode (52)) den Magensaft der Haifische mittels einer in den Magen eingeführten Glassonde aus. Als chemische Vagusreizung wurde benutzt intramuskuläre Injektion von Pilocarpin, Acetylcholin und Histamin. Keiner dieser Stoffe gab eine erhöhte Magensekretion. Auch Atropin (vaguslähmend) erhöht nicht die Sekretion. Die Sekretion des sauren Magensaftes findet (in geringer Mengem) beim Hungern kontinuierlich statt, was bei anderen Wirbeltieren nicht der Fall ist. Es findet keine Absonderung von "Appetitsaft" statt: aus Exemplaren von Scyllium, welche im Aquarium während 20 Minuten versucht hatten Beute zu fangen, konnte nicht mehr Magensaft ausgehebert werden, als aus ruhigen hungernden Tieren. Dobreff hat aber nicht

gefunden, welcher Reiz dann bei Fütterung die Magensaftsekretion in der Tat veranlasst.

Babkin, Chaisson, und Friedmann (51) untersuchtenn die Magensekretion bei zwei Roggenarten. Auch sie fanden, dass im Hunger kontinuierlich eine kleine Menge sauren Saftes abgeschieden wird. Reizung des Vagus (indirekt) oder des sympathischen Systems verursacht keine Sekretion. Ebensowenig Injektion von Histamin oder Adrenalin. Mechanische Reizung der Magenschleimhaut ruft keine erhöhte Absonderung von Magensaft hervor (beim Frosch ist dies wohl der Fall, vgl. später). Vernichtung des Rückenmarks (und damit Eliminierung des sympathischen Systems) verursachte eine "paralytische" Sekretion. Injektion von Adrenalin hebt diese auf. Indirekt steht die Sekretion also unter dem Einfluss des sympathischen Systems. Welche Reize des Futters nun eigentlich die starke Erhöhung der kontinuierlichen Hungersekretion veranlassen, wurde nicht gefunden. Die Autoren vermuten, dass bei den Elasmobranchii der Zustand der Gefässe grossen Einfluss auf die Sekretion hat.

Von der älteren Literatur sei noch die Arbeit von Weinland (53) erwähnt, der ein merkwürdiges Resultat bei Rochen erhielt, bei denen der Magensaft bald sauer, bald alkalisch reagieren kann. Die Absonderung von Säure und Alkali steht hier unter dem Einfluss von Sphinkteren, die besonders an den Magenvenen vorkommen. Bei geschlossenen Sphinkteren entsteht alkalisches, bei geöffneten saures Sekret. Allerdings wurden diese Resultate nur bei künstlicher Gefässverengung, welche der Injektion von *Secale cornutum* folgte, erreicht. Im alkalischen Sekret soll eine Diastase vorkommen. Eine Nachprüfung dieser Resultate ist wünschenswert.

Etwas mehr Arbeit ist in neurerer Zeit auf das Studium der Pankreasabscheidung der Fische verwendet worden. So untersuchte Babkin (54) die Pankreassekretion bei drei Roggenarten. Es findet im Hunger eine kleine kontinuierliche Sekretion statt von 0.02 cm.$^3$ pro Stunde. Einbringen von 0.36% HCl ins Duodenum verursacht Sekretion. Werden Spaltungsprodukte der Pepsinverdauung bei gleichem Säuregrad eingebracht, so ist die Sekretion erhöht, verglichen mit der Absonderung, welche auf den Säurereiz allein stattfindet. Die Latenzzeit für das Auftreten dieser Sekretion ist beim Hunde $^1/_2$–5 Min., für den Roggen 30 Minuten. Die Sekretion beim Hunde dauert $1^1/_2$–2 St., beim Roggen mehr als 4 Stunden. In dieser Sekretionsperiode sonderte der Roggen 0.43 cm.$^3$ ab, ein Hund auf gleiches Gewicht umgerechnet 34.5 cm.$^3$ Auf Sekretineinspritzung reagiert der Roggen sehr gut. Das vagusreizende Mittel Pilokarpin löst aber keinerlei Sekretion aus. Die humorale Regulierung der Pankreassekretion geschieht wie beim Säugetier, eine Sekretion unter dem Einfluss des N. vagus, welche beim Säugetier gerade den fermentreichen

Saft ergibt, findet hier nicht statt (ebensowenig wie bei der Magensekretion der Fische).

Babkin und Bowie (55) untersuchten weiter die Sekretion bei einem magenlosen Fische (*Fundulus heteroclitus*, vgl. Abb. 1). Da dieser Fisch ziemlich klein ist, wurde der Darminhalt nach Fütterung auspipettiert. Bei Hungertieren ist nur sehr wenig Saft im Darm vorhanden, die Gallenblase ist gefüllt. Fütterung mit Muskelfleisch oder Milch ruft Absonderung des Pankreassaftes und der Galle hervor, worauf der Darminhalt ein *p*H von 8 bis 9 annimmt. Nach 12 St. findet man die Gallenblase ganz zusammengefallen. Die Absonderung der Galle kann nicht durch mechanische Reize hervorgerufen werden. Ebensowenig durch Einbringen von HCl ins Duodenum oder durch Verabreichung von Atropin *per os*, wohl aber durch Einbringen von Pilokarpin. Die Unterschiede zwischen der Sekretion bei den Fischen und den Säugetieren betreffen also hauptsächlich die Magensekretion.

**Die Magenverdauung der Fische.**—Schon frühzeitig wurde erkannt, dass die Vorgänge im Fischmagen denjenigen der Magenverdauung bei den Säugern sehr ähnlich sind (Richet, Yung, Fick, Murisier, Hoppe-Seyler, Luchau, Krukenberg (56)). Reinen Magensaft hat man bis jetzt fast noch nicht gewonnen, was man durch Ausheberung erhielt war immer vermischt mit Nahrungsresten. Van Herwerden (57) erhielt den Saft dadurch, dass sie den Magen von *Scyllium stellare* ausspülte und dann den nachher abgesonderten Saft sammelte. Als Salzsäure berechnet war der Säuregehalt 0.08–0.1%. Da keine Reizung stattgefunden hatte, muss aber bezweifelt werden ob man es hier mit echtem Magensaft zu tun hat. Für das *p*H von ausgehebertem Saft mit Nahrungsresten dieser Tierart fanden van Herwerden und Ringer (58) 1.69 (elektrometrisch). Dieses niedrige *p*H wies schon auf eine anorganische Säure hin und diese Autoren bewiesen, dass hier überwiegend Salzsäure vorhanden war. Es wurde bei ihrer Analysenmethode besonders auf die Anwesenheit von Seewasser und Eiweissstoffen als mögliche Fehlerquellen geachtet, Weinland (52) war mit unzulänglicher Methodik zum Resultat gekommen, dass eine organische Säure vorhanden sei. Vonk (59) hat für den Mageninhalt des Haifisches *Acanthias vulgaris* auf der Höhe der Verdauung *p*H-Werte von 2.32 bis 3.22 beobachtet; für den Hecht sind die Werte höher: 4.92–5.97. Diese höheren Werte beim Hecht hängen zusammen mit dem Gesamtablauf der Magenverdauung, wovon später die Rede sein wird. Die titrierbare Säurenmenge ist bei Selachii (wenigstens bei den Haifischen) grösser als bei Teleostei (60). Schon die erwähnten älteren Autoren fanden, dass chsi aus der Magenschleimhaut ein Enzym extrahieren liess, das—wie die

Magenextrakte von Säugetieren—bei stark saurer Reaktion auf native Eiweissstoffe spaltend wirkt und als Verdauungsprodukte Albumosen und Peptone lieferte. Bei den Fischen kommen keine Haupt- und Belegzellen vor. Bei den Säugern produzieren erstere das Pepsin, letztere die Salzsäure. Hier ist eine Differenzierung der Zellen der Magendrüsen eingetreten, welche den Fischen fehlt. Es ist also bemerkenswert, dass die Produktion von Pepsin und Salzsäure nicht an das Vorhandensein dieser bestimmten Zellarten gebunden ist. Die Eigenschaften des Fischpepsins wurden schon im allgemeinen Abschnitt (III) eingehend besprochen.

Wir haben nur noch zu besprechen, wie der Verdauungsprozess im Magen vor sich geht. Bekanntlich ist das *p*H-Optimum des Pepsins etwa 2, und wurde das *p*H des Mageninhaltes bei Acanthias zu 2.3–3.2, für den Hecht (und andere Knochenfische) zu 4.9–5.9 gefunden. Das Pepsin ist aber bei *p*H 4 schon an der oberen Grenze seiner Wirksamkeit. Wie kann es bei letzteren Tieren wirken? Es lag nahean zunehmen, dass eine oberflächliche Schicht der Beute einen viel niedrigeren Säuregrad erreichen könnte, als die halbverdauten Massen des Mageninhaltes, deren *p*H man mit einer Wasserstoffelektrode bestimmen kann. Die Erfindung der Glaselektrode, womit das *p*H an beliebigen Stellen einer Oberfläche gemessen werden kann, bot die Gelegenheit diese Verhältnisse zu untersuchen (Mennega (61)).

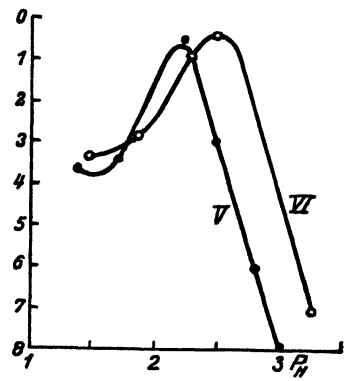



Fig. 4.—pH-Optimum der Magenextrakte (V und VI) von zwei Hechten. Abszisse: pH, Ordinate: Enzymwirkung (willkürliche Skala, bei 8 kleinste, bei O höchste Wirkung).

Der Hecht frisst seine Beute indem er sie am Kopfende fasst und darauf verschluckt, sodass der Kopf der Beute im hinteren Magenende, der Schwanz im vorderen oder sogar noch im hinteren Oesophagusteil leigt. Nach zufälligen Beobachtungen schätze ich, dass der Hecht eine Beute bis zu einem Drittel oder Viertel seines Körpergewichtes aufnehmen kann. Bei grosser Beute dauert es drei bis vier Tage ehe der Magen sich wieder entleert hat. Öffnet man die Bauchwand und den Magen etwa 12 bis 24 Stunden nach der Nahrungsaufnahme, so findet man den Kopf der Beute stark angegriffen, ebenso wie den Vorderteil der Beute (welche also im hinteren Magenteil liegt). Die Schuppen sind dort völlig verschwunden, das Fleisch ist unter der Oberfläche weich. Der Schwanzteil der Beute kann noch ganz intakt sein. Die Zone der Verdauung stimmt ungefähr überein mit der Magenzone, welche Magendrüsen enthält und woraus man Pepsin extrahieren kann. Mennega bestimmte nun das *p*H an verschie-

denen Stellen des Mageninhaltes. Eine Abbildung der erhaltenen Ergebnisse findet man in *Erg. Enzymforsch.*, 8 (1939), auf S. 83, Fig. 14 *A*. Man sieht hierauf, dass an verschiedenen Stellen Werte von 2.7, an zwei anderen solche von 2.6 und sogar 2.4 erreicht werden. Aus nebenstehender Fig. 4 ist zu ersehen, dass das Pepsin des Hechtes bei diesen Säuregraden noch sehr gut wirksam ist. Die Optima auf der Figur wurden von zei verschiedenen Tieren erhalten; sie liegen nicht genau an derselben Stelle, was durch Einflüsse von Beimischungen verursacht werden kann. Die Figur gibt von der möglichen Wirksamkeit des Pepsins bei *p*H 2.7 einen zu geringen Eindruck: um eine deutliche Optimumkurve zu erhalten wurde die Verdauungszeit kurz (30 min.) gewählt. Bei längerer Verdauungszeit wird die Wirkung bei *p*H 2.7 im Vergleich zum Optimum günstiger sein. Das verflüssigte Eiweiss muss nun durch die Peristaltik des Magens dem Pylorusteil zugeführt werden. Dort wurde beim Hecht ein höherer *p*H-Wert (2.96) beobachtet. Trotzdem wird hier das Pepsin wohl weiter wirken können, da nach Ringer (62) das Optimum für Azidalbumin und Albumosen viel breiter ist (etwa gleiche Wirkung von *p*H 1–4 als für natives Eiweiss. An einer Stelle wo an der Oberfläche der Beute das *p*H 2.60 betrug, war er 2 mm. tief 2.80, 3 mm. tief schon 4.70. Tatsächlich nimmt also nur eine dünne Schicht den fur die Verdauung günstigen *p*H-Wert an.* Wenn mehrere kleinere Beuteobjekte aufgenommen werden, so werden die Unterschiede zwischen Oberfläche und Innerem natürlich weniger ausgeprägt und auch schwieriger zu bestimmen. Das ist nach Mennega der Fall beim Barsch. Die Verdauung geht hier auch schneller vor sich.

Van Herwerden (63) stellte bei einigen Selachiern und Teleostiern eine Lipase in der Magenschleimhaut fest. Es ist aber zweifelhaft, ob sie abgesondert wird. Dieselbe Autorin und früher schon Redeke (64) zeigten, dass nach der Verdauung Fettröpfchen im Oberflächenepithel der Magenschleimhaut zu finden sind. Bekanntlich werden im Säugermagen fast keine Verdauungsprodukte resorbiert. Van Slyke und White (65) fanden, dass bei einem Haifisch in den ersten 6 Stunden der Verdauung etwa 24% des Eiweisses verschwanden, abgesehen von der Menge, welche in den Darm übergegangen war (es kann aber hier schon Resorption stattgefunden haben!). Eine gewisse Resorption im Haifischmagen ist also nicht ganz unwahrscheinlich.

Bayliss (66) fand bei *Pleuronectes plastessa* (der Scholle) keine Lipase im Magenextrakt. Auch vermisste sie Amylase. Die Angaben (Weinland) über das Vorkommen von Amylase im Magensaft von Roggen scheinen mir recht zweifelhaft.

Für das Verständnis der obenbeschriebenen Vorgänge der Verdauung einer Beute in aufeinanderfolgenden Schichten, wäre es erwünscht, etwas von den Magenbewegungen zu wissen. Bei Säugern sind diese stark im Pylorusteil (Knetung), relativ schwach im Fundusteil (keine Knetung, nur Transport). Hierüber ist aber bei Fischen sehr wenig

* Die gleichen Erscheinungen wurden von Mennega beobachtet für fleischfressende Amphibien und Reptilien (darüber später) und auch für Säugetiere. Bei Vögeln sind die Verhältnisse noch unklar. Vgl. Vonk, *Erg. Enzymforsch.*, 8 (1939).

bekannt. In einer Arbeit von Babkin und Mitarbeitern (67) wurde hauptsächlich der Einfluss des Nervensystems auf die Magenbewegungen bei Roggen studiert. Der Magen dieser Tiere hat eine wohlentwickelte sympathische und parasympathische Innervation. Merkwürdigerweise ruft sowohl Reizung vom Sympathicus als vom Vagus Magenbewegungen hervor. Erstere veranlasst sogar stärkere Magenbewegungen als letztere. Es ist hier also keine Rede von einer antagonistischen Innervation, im Gegensatz zu den Säugern, wo Vagusreizung Bewegung (und Sekretion) und Sympathicusreizung Hemmung der Bewegung (und der Sekretion) hervorruft. Die Hemmung durch das sympathische Nervensystem scheint sich erst während der Evolution der Vertebratenreihe entwickelt zu haben. Bei Amphibien persistiert noch der motorische Effekt des Sympathicus auf den Magen. Weiter ist noch merkwürdig, dass die Reizung beider Nervenarten, welche bei den Fischen Magenbewegungen hervorruft, keine Sekretion veranlasst, wie oben ausgeführt wurde.

**Darmverdauung der Fische.**—Die Sekretion des Pankreassaftes wurde schon oben besprochen. Ebenso die Versuche über die Identität des Trypsins des Haifisches. Es leuchtet ein, dass Arbeiten von Autoren, welche noch nicht die moderne Methoden der *p*H-Messung und der Pufferung von Verdauungsgemischen benutzen konnten, jetzt nur noch einen sehr beschränkten Wert haben. Sie wurden von Biedermann (*l. c.*, 1911) schon ausführlich zusammengefasst, sodass ich mich hier auf die neueren Arbeiten beschränken kann.

Polimanti (68) untersuchte die Verteilung der Enzyme über den Darmkanal bei *Scyllium canicula*, *Box salpa*, und *Conger vulgaris*. Im Dünndarm wurde Enterokinase gefunden, ihre Menge nimmt von vorn nach hinten ab. Erepsin wurde nach Vernon kolorimetrisch bestimmt. Seine Menge nimmt in caudaler Richtung zu, wie das auch bei Säugetieren der Fall ist. Die anderen Darmenzyme wurden nicht untersucht und ebensowenig die Enzyme des Pankreas. Über die gesamte Verteilung der Enzyme gibt die Arbeit also keinen Aufschluss. Bodansky und Rose (69) haben Glycerin-Wasser-Extrakte der Appendices pyloricae von *Lutjanus aya* untersucht. Sie fanden ein schwaches Optimum der Gelatinespaltung an der sauren Seite, ein stärkeres an der alkalischen Seite, aber auch dort ist die proteolytische Wirksamkeit nur gering. Da quantitative Vergleichungen mit dem Pankreas nicht angestellt wurden, kann diese Wirksamkeit der Appendices von adsorbiertem Pepsin des Magens und Trypsin des Pankreas herrühren. Stärke wird vom Darmextrakt ziemlich gut gespalten. Disaccharasen fehlen. Ohne das Pankreas untersucht zu haben, schliessen die Autoren, dass in den Appendices diejenigen Enzyme vorkommen, welche meistens mit dem Pankreassaft der Vertebraten abgesondert werden. Offenbar war den Autoren die Existenz des Teleosteerpankreas unbekannt und sie haben gar nicht bewiesen, dass die gefundenen Enzyme wirklich von den Appendices abgesondert werden.

Vonk (70) hat versucht die Verteilung der Enzyme über den Darmkanal bei verschiedenen Tieren mit moderneren Methoden festzustellen und die

Enzymkonzentrationen bei verschiedenen Fischen miteinander zu vergleichen. Die *p*H Bestimmungen geschahen elektrometrisch, die Bestimmung der Enzymwirkungen meistens mittels Titrationsmethoden. Um die Enzymwirkungen richtig vergleichen zu können (und auch um die Enzyme besser zu identifizieren, vgl. Abschn. III) wurden zuerst die Optima bestimmt. Diese sind:

| | | |
|---|---|---|
| Karpfen: | Pankreasamylase | 6.25 (Optimalzone 6.0–6.5) |
| Hecht: | Pankreasamylase | 7.0 |
| Karpfen: | Pankreasmaltase | 6.6 (Zone 6.0–7.5) |
| Acanthias: | Trypsin | 8.2 |
| Karpfen: | Erepsin | 8.7 |

Die Verhältniszahlen für die Enzymwirkungen sind für die Carbohydrasen zusammengefasst in Tabelle II. Aus dieser Tabelle lassen sich einige Schlussfolgerungen ziehen, erstens über den Enzymgehalt der Organe mit Rücksicht auf die Lebensweise der Tiere. Zweitens über die biologische Bedeutung der Verteilung der Enzyme über den Darmkanal.

TABELLE II

VERHÄLTNISZAHLEN $\left(\text{QUOTIENTE } \frac{\text{ZÄHLER}}{\text{NENNER}}\right)$ ZWISCHEN DER ENZYMWIRKUNG VON ÜBEREINSTIMMENDEN EXTRAKTMENGEN VERSCHIEDENER FISCHORGANE

Die erste Horizontalreihe der Tabelle bildet die Zähler, die erste Vertikalkolumne die Nenner des Quotienten. *Am.* = Amylase, *Ma.* = Maltase, *C*, = Cyprinus (Karpfen), *E.* = Esox (Hecht), *Ac.* = Acanthias (Haifisch), *S.* = Schwein, *P.* = Pankreas, *D.* = Darm, *G.* = Galle. Der Treffpunkt der vertikalen und horizontalen Reihen unter bzw. neben den Symbolen liefert die gewünschte Zahl.

| | Zähler | | | | | |
|---|---|---|---|---|---|---|
| | | *Am.C.P.* | *Am.E.P.* | *Am.C.D.* | *Ma.C.P.* | *Ma.S.D.* |
| Nenner | *Am.E.P.* | 375–1500 | ... | 16 | .. | ... |
| | *Am.C.D.* | 50–100 | 1/16 | ... | .. | ... |
| | *Am.E.D.* | 2000–3000 | 2–3 | 32–48 | .. | ... |
| | *Am.Ac.P.* | 375–1500 | 1 | 16 | .. | . . |
| | *Am.C.G.* | 50–100 | 1/16 | 1 | .. | ... |
| | *Ma.C.P.* | 10–20–25 | ... | ... | .. | 1/7 |
| | *Ma.C.D.* | ..... | ... | ... | 20 | 3 |

Bei dem omnivoren Karpfen kommt im Pankreas ungefähr die tausendfache Menge an Amylase vor wie beim karnivoren Hecht und Haifisch;

auch im Darm des Karpfens findet sich bedeutend viel mehr Amylase als beim Hecht. Maltase konnte im Pankreas und im Darm des Hechtes bei verschiedenster Reaktion gar nicht nachgewiesen werden, während sich beim Karpfen eine ziemlich grosse Menge im Pankreas und eine kleinere im Darm findet. Die Wirkung auf Glykogen ist im Pankreasextrakt vom Acanthias eben so gross wie die auf Stärke. Ein spezielles Glykogen spaltendes Ferment gibt es deshalb nicht. Die Trypsinwirkung des Pankreas ist beim Hecht und beim Haifisch ungefähr 8 fach stärker als beim Karpfen; für Erepsin im Darm ist der Unterschied etwas geringer. (Diese Zahlen wurden nicht in die Tabelle aufgenommen.) Der Unterschied zwischen Omnivoren und Karnivoren in der Menge der Enzyme ist also besonders gross für die Carbohydrasen, viel weniger für die Proteasen.

Über die Verteilung der Enzyme über den Darmkanal lehrt uns Tabelle II, dass beim Karpfen 50–100 mal mehr Amylase im Pankreas vorkommt als im Darm. Dieses stimmt überein mit den Verhältnissen bei den Säugetieren. Während aber dort die Maltase, welche die Kohlenhydratverdauung beendet, hauptsächlich in der Darmwand gefunden wird, ist dies bei dem Karpfen und dem Hecht nicht der Fall. Beim Karpfen findet man im Pankreas rund 25 mal mehr Maltase als im Darm. Die Arbeitsteilung des Darmkanals in der Produktion der Carbohydrasen ist also beim Karpfen noch nicht so weit durchgeführt wie bei den Säugetieren. Bei letzteren wird der Organismus vor Überschwemmung des Blutes mit Kohlenhydraten behütet durch die Verteilung einer (an jeder Stelle geringen) Menge der Maltase über die ganze Darmlänge. Auch beim Karpfen ist eine derartige Überschwemmung nicht zu befürchten: die Menge der Amylase im Pankreas ist 25 mal grösser als die der Maltase. Aus Tabelle III sehen

TABELLE III

| | Eiweiss | Pepton | Dipeptid |
|---|---|---|---|
| Nichtaktivierter Glycerin-Pankreasextrakt | − | − | − |
| Aktivierter Glycerin-Pankreasextrakt | + | ++ | |
| Darmextrakt | − | + (nur wenig) | + |

Hierin bedeutet + Spaltung, − Nichtspaltung, ++ erhöhte Spaltung.

wir, dass die Verteilung der Proteasen über den Darmkanal übereinstimmt mit der bei den Säugetieren.

Es wurde von Vonk hervorgehoben, dass diese abweichende Verteilung der Carbohydrasen bei den Fischen vielleicht zusammenhängt mit dem Fehlen von Lieberkühnschen Drüsen im Darmkanal. Falloise (71) hatte aus einem aufgeschnittenen und gefrorenen Darm zwei Gewebsschichte

isoliert und extrahiert. Die obere enthielt Erepsin und sollte keine Lieberkühnsche Drüsen enthalten, die untere enthielt hauptsächlich Disaccharasen und in dieser Schicht sollten sich die meisten Lieberkühnschen Drüsen befinden. Van Genderen und Engel (72) haben diesen Versuch wiederholt mit der viel exakteren Technik von Linderstrøm-Lang. Es stellte sich dabei heraus, dass von einem Vorkommen der Disaccharase speziell in den Lieberkühnschen Drüsen keine Rede war, sodass jetzt auch der Zusammenhang zwischen dem Fehlen dieser Drüsen und der Maltase im Fischdarm äusserst zweifelhaft ist. Wir sahen bei der Besprechung der Magenverdauung, dass zwar bei höheren Vertebraten Pepsin und Salzsäure je von besonderen Zellen produziert werden, dass aber der Fischmagen trotz des Fehlens dieser speziellen Zellen auch zur Produktion von Pepsin imstande ist. Es scheint also das Vorkommen bestimmter Enzyme niemals an spezielle Zellarten gebunden zu sein.

Aus Tabelle II ist weiter noch zu sehen, dass auch die Galle eine nicht unbedeutende Enzymwirkung hat. Auch Babkin hat die gleiche Tatsache gefunden für die Galle der von ihm untersuchten Tiere. Bei Säugetieren ist diese Erscheinung nicht bekannt.

Schlottke (73) hat neuerdings die Verdauung beim Karpfen weiter untersucht. Ausser der Darmwand und dem Pankreas, wurde auch der Darminhalt auf die Konzentration der Enzyme geprüft. Bei Fütterung wird reichlich Trypsin in den Darm abgegeben. Bis zum Ende der Verdauung kann es dort nachgewiesen werden. In der Schleimhaut des Darmes tritt es erst im Verlaufe der Verdauung auf. Die maximale Menge findet sich dort später als im Darmlumen. Die Auffassung von Vonk, dass das Trypsin im Pankreas produziert wird, und in der Darmschleimhaut nur adsorbiertes Trypsin vorkommt, besteht also zurecht. Der Gehalt an Polypeptidase ist in der Darmschleimhaut grösser als im Darminhalt. Dipeptidase findet sich hauptsächlich in der Schleimhaut, nahezu nicht im Inhalt. Nach Schlottke soll die Polypeptidase also teilweise intrazellulär wirken, die Dipeptidase wirkt gänzlich intrazellulär. Natürliches Futter wird schneller verdaut als künstliches. Für letzteres werden aber grössere Mengen Enzym abgesondert. Im natürlichen Futter wirken wahrscheinlich die Enzyme, welche darin anwesend sind, durch Autolyse mit.

Eine Verschiebung des Verhältnisses zwischen der Amylase- und der Proteinasekonzentration im Darminhalt, als Anpassung an die Art des Futters (tierisches oder pflanzliches) konnte nicht festgestellt werden. Ebensowenig ist dies bei den Säugern der Fall. Verschiedene Futterarten veranlassen dort zwar Unterschiede in der Menge der Sekrete, aber eine Änderung im Verhältnis der Enzyme des Saftes zueinander gibt es auch dort nicht.

Schlottke* hat diese Untersuchungen auch auf andere Fischarten ausgedehnt (Quappe, Flussbarsch, Regenbogenforelle). Auch dort stammt das Trypsin aus dem Pankreas. Bei *Lota* wird mehr Amylase und Maltase im Pankreas gefunden als im Darm. Dies stimmt wieder überein mit den Befunden von Vonk über die Verteilung der Amylase und Maltase beim Karpfen. Bei den Forellen sind Proteinase und Dipeptidase in besonders hoher Konzentration vorhanden. Trotzdem verläuft die Verdauung nicht schneller wie bei anderen Fischen, wohl aber gründlicher. Nur bei *Trutta* wurde eine Lipase im Pankreas gefunden. Bei den andern untersuchten Fischen stammt diese aus der Nahrung. Wenn Appendices vorhanden sind, tritt die Nahrung immer in diese ein, sowohl wenn die Schläuche weit, als auch wenn sie eng sind. Grössere Teilchen können nicht eindringen; die Frage, ob es eine besondere Einrichtung gibt, welche dies weiten Appendices verhindert, wurde nicht beantwortet. Im Extrakt der Appendices findet sich eine grössere Enzymmenge als im Inhalt des Mitteldarmes; besonders gilt dies für Proteinase. Eine gewisse sezernierende Rolle scheint also nach diesen Untersuchungen den Appendices zuzukommen.

Auch Chesley (74) hat die Konzentrationen der Enzyme verschiedener Fischarten mit einander verglichen. Die Totalmenge der Enzyme hängt zusammen mit der Aktivität der betreffenden Tierart: sie ist am grössten für die aktiven Fische und nimmt mit abnehmender Aktivität ab. Dies gilt am meisten für Trypsin, am wenigsten für Pepsin. *Brevoörtia tyrannus*, welche fettreiche Nahrung frisst, hat keine höhere Lipasemenge als Tiere, welche eine weniger fettreiche Nahrung aufnehmen. Der Besitz einer grossen Lipasemenge scheint aber zusammenzuhängen mit dem Bau des Pankreas: bei kompakten Pankreas ist die Lipasemenge gross. Dies würde übereinstimmen mit den Resultaten von Vonk, welcher eine grosse Lipasewirkung im kompakten Haifischpankreas und eine geringe im diffusen Pankreas des omnivoren *Cyprinus* fand. Weiter findet Chesley, dass bei Tieren mit kompakten Pankreas die Enzyme, die in ihr vorkommen, hauptsächlich auf dieses Organ beschränkt sind. (Der Befund Vonks, dass das diffuse Pankreas von *Cyprinus* sehr reich an Amylase ist, stimmt aber hiermit nicht überein). Chesley findet, dass bei Fischen mit kompaktem Pankreas, die Appendices wenig Enzyme enthalten, und dass bei Tieren mit diffusem Pankreas (und wenig Enzymen darin) die Appendices ziemlich viel Enzyme enthalten. So würde doch die alte Meinung (welche schon Cuvier äusserte), dass Pankreas und Appendices einander gewisser-

* Die bezüglichen Arbeiten von Schlottke werden nach den Manuskripten zitiert von Rauther (*l. c.*). Die Arbeiten waren aber bei der Fertigstellung meines Manuskriptes noch nicht in Z. f. Fischerei erschienen.

massen vertreten könnten eine gewisse Begründung haben. Doch ist wohl das Tatsachenmaterial noch bei weitem nicht gross genug, um daraus sichere Schlüsse zu ziehen. Die Wand der Appendices stimmt in ihrem Bau genau überein mit dem Bau der Darmwand. Das Organ ist niemals drüsenartig. Es kommt ihm bei der Kürze des Fischdarmes auch sicher wohl eine bedeutende Rolle als Oberflächenvergrösserung des Darmes für die Resorption zu.

Bayliss (75) untersuchte die Verdauung bei der Scholle. Sie bestätigte das Vorkommen der verschiedenen Enzyme, welche in den schon besprochenen Untersuchungen anderer Forscher bei den Fischen gefunden wurden. Besonders beschäftigte sie sich mit dem Vorkommen von Enzymen in der Galle. Sie fand hierin bei der Scholle hauptsächlich Trypsin und Polypeptidase. Besonders stark äussert sich die Trypsinwirkung, wenn man der Galle aus der Gallenblase Darmextrakt zugesetzt hat. Mackay (76) hatte gefunden, dass die Galle aus der Gallenblase von Fundulus* frei von Enzymen war, wenn das Pankreasgewebe, welches die Blase umgibt, durch kurzes Eintauchen der Blase in Bouinsche Fixierflüssigkeit abgetötet worden war. Dieses Experiment wurde von Bayliss bei der Gallenblase der Scholle wiederholt und bestätigt. Auch wenn man die Galle mit einer Kanüle derart auffängt, dass sie nicht mit der Wand der Gallenblase in Berührung kommen kann, ist sie frei von Proteasenwirkung. Bayliss vermutet, dass durch die teilweise Verwachsung des diffusen Pankreas mit der Gallenblase, letztere einen Weg bildet, durch welchen das Pankreassekret abgesondert werden kann. Sie suchte diese Vermutung zu beweisen durch Injektion von Pilokarpin und erwartete dann die Konzentration des Trypsins in der Galle erhöht zu finden. Dies war aber nicht der Fall. Es beweist aber auch nicht die Unrichtigkeit ihrer Vermutung, denn wir haben mehrfach gesehen, dass die Verdauungsorgane der Fische auf Injektion von Pilokarpin nicht sezernieren, da die Sekretion nicht durch Vagusreizung angeregt wird. Nach den Experimenten von Mackay und Bayliss scheint es mir recht wohl wahrscheinlich, dass die Enzyme in der Galle aus dem Pankreas stammen, zumahl, da sie bei anderen Tiergruppen, deren Pankreas nicht mit der Gallenblase verwachsen ist, in der Galle fehlen.

Offenbar hat Bayliss nicht versucht Pankreasgewebe frei zu präparieren, was, bei der Untersuchung anderer Fischarten, wie wir gesehen haben, möglich ist. Sie untersuchte die Fermente der Leber, vermischt mit dem Pankreas, und fand darin Trypsin und Kathepsin und nur wenig Amylase. Bayliss bezweifelt, dass die Enzyme des Pankreas in den Darm abgesondert

* Babkin und Bowie (*Biol. Bull.*, **54** (1928), 254) hatten schon gefunden, dass darin Protease, Lipase und Amylase vorkommen.

werden können, da sie keine Ausführgänge gefunden hat. Obgleich diese bei *Pleuronectes* noch unbekannt sein mögen, kann ihre Existenz schwerlich bezweifelt werden, da sie bei vielen anderen Fischen tatsächlich vorhanden sind (vgl. die Ausführgänge des Pankreas von *Fundulus* auf Fig. 1). Der einzige Weg der Pankreassekretion führt nach ihr über die Gallenblase. Der Leber- (und Pankreas-) extrakt enthält nur wenig Lipase, die Darmwand aber ziemlich viel. Das *p*H des Darminhaltes ist 7.5–8.0 (meist 8.0). Ob diese Werte ganz richtig sind, muss bezweifelt werden, da sie mit der Chinhydronelektrode gemessen wurden, welche bei Anwesenheit von viel Eiweisstoffen besonders bei *p*H-Werten höher als 7 keine zuverlässige Werte liefert.

Weiter seien noch erwähnt die Untersuchungen von Beauvalet (77), Karpewitch (78), und Mann (79), welche keine neuen Gesichtspunkte lieferten. Ishida (80) hat bei einigen magenlosen Seefischen neben den gewöhnlichen oben besprochenen Enzymen Amygdalase, Salicinase und Lichenase gefunden. Auch Schlottke fand Lichenase, die sonst bei Vertebraten nicht vorkommt. Bestätigung dieser Befunde muss abgewartet werden.

Eine kurze Bemerkung muss schliesslich noch gemacht werden über die Verdauung bei den magenlosen Fischen. Ihnen fehlt, wie wir oben sahen, das Pepsin und die Salzsäure. Die Eiweissverdauung muss hier also ausschliesslich durch Trypsin, Erepsin und ihre Teilenzyme stattfinden. Nach den aus der Enzymchemie bekannten Tatsachen, kann auch auf diese Weise das Eiweiss völlig abgebaut werden. Auf dieselbe Weise wird auch von allen Invertebraten das Eiweiss verdaut und aus der Pathologie ist bekannt, dass sogar der Mensch nach Magenresektion ohne Absonderung von Pepsin und Salzsäure am Leben bleiben kann. Es ist denn auch nicht nötig anzunehmen (wie dies Schäperclaus (81), zitiert bei Wunder (82), tut), dass bei den magenlosen Fischen die Wirkung des Pepsins und der Salzsäure von den Enzymen der Beute übernommen wird. Zweifellos wird die Autolyse der Beute (wie auch bei allen Fischen mit Magen) die Verdauung beschleunigen. Es ist aber nicht einzusehen, wie sie im Falle der magenlosen Fische die Verdauung *mehr* beschleunigen kann als bei den Fischen mit Magen. Da—wie wir sahen—die Verdauung im Magen Schicht für Schicht vor sich geht, wird auch dort die Autolyse der inneren Organe keineswegs aufgehoben, ausgenommen in den äusseren Schichten, wo gerade die Pepsinverdauung vor sich geht.

Die grosse Bedeutung natürlicher Nahrung neben künstlicher, hängt auch zusammen mit dem Bedarf der Tiere an Vitaminen, wie Hämpel (83) ausgeführt hat.

Es sei noch erwähnt, dass Barrington (84) die Eiweissverdauung bei der Larve (Ammocoete) vom *Lampreta planeri*, welche zu den Cyclostomata gehört, untersucht hat. Diese Tiere haben keinen Magen und kein Pankreas. Die Eiweissverdauung geschieht durch ein tryptisches Enzym (Optimum 7.5–7.8), welches im vorderen Darmteil abgesondert wird. Hiermit wurden die Befunde von Alcock (85) wiederlegt, welche 1899 mit unzureichender Methodik trotz des fehlenden Magens eine peptische Verdauung zu finden meinte.

**Ausnutzung der Nahrung und Resorption.**—Die Ausnutzung der Nahrung ist natürlich besonders für die Fischzucht von hohem Interesse. Wir können hier nur kurz ihren physiologischen Aspekt behandeln. Eine der besten und gründlichsten Untersuchungen ist noch immer diejenige von Van Slyke und White (86) am Haifisch *Scyllium canicula*. Sie fütterten die Fische mit einer bestimmten Fleischmenge und töteten sie nach 6, 12, 24, 48, und 72 Stunden. Die Teile des Darmtraktus wurden voneinander abgebunden und ihr Inhalt zentrifugiert. Sodann bestimmte man den Stickstoffgehalt des ungelösten Eiweisses nach Kjeldahl und von der erhaltenen Flüssigkeit den Aminostickstoff nach Van Slykes gasometrischem Verfahren. Zugleich wurde der Totalstickstoffgehalt und der Aminostickstoff nach totaler Hydrolyse der Flüssigkeit (ausgeführt durch zweitägiges Kochen mit 20% HCl) bestimmt. Der Quotient vom $NH_2$ nach totaler Hydrolyse und des $NH_2$ der Digestionsflüssigkeit gibt dann ein Mass für die mittlere Länge der Peptidketten in dieser Flüssigkeit. Es stellte sich nebenbei heraus, dass die Flüssigkeit des Darmes einen erheblichen Ureumgehalt hatte. Es wurde nachgewiesen, dass dieser nicht als Produkt der Verdauung, oder als zurückgeflossener Kloakeninhalt (Urin) aufzufassen war, sondern dass der Harnstoff aus der Galle stammte. In dieser war 1.7% N vorhanden, wovon 72.3% als Ureum.

Es verliefen 2 bis 3 Tage, ehe die Verdauung einer Fleischmenge, mit 1.3–2 g. N, beendet war. Nach den ersten 6 Stunden war im Magen neben Eiweiss viel gelöster Stoff; im Darme war nur sehr wenig Inhalt. Da in diesem Stadium der totale N-Gehalt des ganzen Darmkanals nur ungefähr 75% der verfütterten Menge betrug, schliessen die Autoren, dass 25% im Magen resorbiert wurde. (Ich weiss nicht, ob dieser Schluss ganz zulässig ist: es könnte in den ersten Verdauungsstunden auch allmählich Mageninhalt in den Darm übergehen, um dort fast momentan resorbiert zu werden.) Die Grösse der Peptone in der durch Zentrifugieren erhaltenen Flüssigkeit war die von Pentapeptiden. Der Gehalt an freien Aminogruppen dieser Peptone war etwas höher als der von Witte-Pepton. In der Zeit von 6–12 Stunden findet ein reichlicher Übergang des verdauten und unver-

dauten Eiweisses in den Darm statt: es findet sich nach 12 Stunden 30–45% des Gesamtstickstoffs im Darmtraktus; Pepton im Magen von der Grösse eines Tripeptids, im Darm etwas weiter gespalten. Nach 24 Stunden ist 40–70% des N verschwunden; 65 bis 85% des N im Magen ist in gelöstem Zustande. Das Magenpepton ist im Di- und Tripeptidstadium. Weiter gespalten wird es im Magen nicht. Nach 3 Tagen ist entweder aller N verschwunden oder wenigstens bis auf 10% des verfütterten N; dieser Rest ist fast ganz löslich. Die Peptidgrösse im Darm ist fast nicht weiter verringert als die nach 24 Stunden im Magen. Nach meiner Meinung wird das damit zusammenhängen, dass das Erepsin (dem nach den Untersuchungen von Waldschmidt-Leitz und Mitarbeitern die Aufgabe der Di- und Tripeptidspaltung zukommt) vorwiegend intrazellulär arbeiten soll, wie einige Autoren mutmassten.

Van Slyke und White vergleichen dann ihre Resultate mit denen von Schmidt-Mülheim, Abderhalden und London c.s. am Hunde. Die Hälfte vom N ist beim Hunde schon nach $^1/_2$ Stunde im Darm und in 5 Stunden ist der Magen leer. Der Temperaturunterschied bei Hund und Haifisch beträgt 20°; das kann theoretisch einen 4–9 fachen Unterschied in der Reaktionsgeschwindigkeit ausmachen. Tatsächlich geht die Verdauung beim Haifisch etwa achtmal langsamer. Auch bei Säugetieren kommt nach den genannten Autoren ein Teil des Eiweisses in ungelöstem Zustande in den Darm an. "It appears probable that cleavage proceeds as far in the canal of the fish as in that of the dog," meinen van Slyke und White. Die grösseren Unterschiede im Zustand des Speisebreis, die sich in den verschiedenen Teilen des Hundedarmkanals ergeben, würden durch die grössere Komplikation dieses Darmtraktus erklärt werden müssen.

Die Verdaulichkeit verschiedener Futtermittel wurde von Wohlgemuth (87) bei der Regenbogenforelle untersucht. Die Tiere wurden während einer bestimmten Zeit gefüttert und dann nach $1^1/_2$–8 Stunden getötet. Als Kriterium für die Verdaulichkeit wählte er die Zeit, nach der der Magen leer gefunden wird und den Zustand des Futters im Magen. Am besten werden Daphnias verdaut und dann, der Reihe nach: Culexlarven, Garneelen, Fischfleisch, Milz, Leber. Die Daphniden werden zweimal schneller verdaut als die Leber. Die Verdauung der natürlichen Nahrung geht also am schnellsten vor sich. Ihre lockere Lagerung wird besseres Eindringen der Enzyme ermöglichen und auch werden die eigenen Enzyme der Beuteobjekte durch Autolyse mitwirken.

Eine weitere Frage betrifft die Beziehung zwischen Temperatur und Verdauungsgeschwindigkeit. Es ist bekannt, dass Karpfen mehr fressen und besser wachsen bei höheren als bei niedrigeren Temperaturen. Hathaway

(88) fand, dass wenn diese Fische aus Wasser von 20° in solches von 10° gebracht wurden, die Nahrungsaufnahme bis auf ein drittel zurückgeht. Werden sie wieder auf 20° gebracht, so wird die ursprüngliche Höhe der Nahrungsaufnahme wieder erreicht. Offenbar muss die Verdauung bei höheren Temperaturen schneller verlaufen. Dies ist natürlich auch zu erwarten, da die wechselwarmen Tiere die Temperatur der Umgebung annehmen und dementsprechend ihre Fermente schneller oder langsamer wirken können. Auch auf die ganze Lebenstätigkeit der Tiere wird die Temperatur einwirken und damit auf die Geschwindigkeit, womit die Verdauungssäfte sezerniert werden, und auf die Resorptionsgeschwindigkeit (vgl. hierzu auch eine Arbeit von Riddle (89), welche im Abschnitt über Amphibien besprochen wird). Diese Beziehungen zwischen Temperatur und Verdauungsgeschwindigkeit hat Scheuring (90) studiert bei *Cobitis fossilis* (Schlammpeitzger). Bei mässiger Fütterung setzt dieses Tier seine Faeces in einem Male ab. Der Zeitverlauf zwischen der Nahrungsaufnahme und der Defäkation wird hier als Mass für die Geschwindigkeit der Verdauung genommen.

Die folgenden Zeitdauern wurden in der Weise für die Verdauung gefunden:

| Temperatur | 7° | 8° | 10° | 14° | 19° | 20° |
|---|---|---|---|---|---|---|
| Zeit in St. | 36–46 | 35 | 25–35 | 20 | 8–12 | 7–10 |

Es ist hiermit aber n. m. M. nicht bewiesen, dass die der Nahrung entzogenen nützlichen Stoffe gerade diesen Zahlen umgekehrt proportional sind. Bei höherer Temperatur wird mehr Nahrung aufgenommen als bei niedrigerer. Es wurde nicht festgestellt, ob die Ausnutzung der kleineren Menge bei tiefen Temperaturen besser oder schlechter ist als die der grösseren Menge bei höherer Temperatur.

Tatsächlich hat dann auch Mann (91) später gefunden, dass die Höhe der Stickstoffausnutzung von Temperaturveränderungen unabhängig ist, die Verdauungszeit dagegen von der Temperatur im Sinne der R. G. T.-Regel abhängt. Es wurde in den Versuchen über die Ausnutzung der N-Gehalt einer bestimmten verfütterten Nahrungsmenge ermittelt und ebenso in der gewogenen abgesetzten Kotmenge. Der Kot wird immer gut zusammenhängend und vollkommen von einer Schleimschicht umgeben abgesetzt, sodass Auslaugen durch das Aquariumwasser nicht möglich war. Durch besondere Versuche wurde dies bestätigt. Die Stickstoffsubstanz wurde von Plötzen, Karauschen und Schleien geringer ausgenutzt als von Barschen, Regenbogenforellen und Stichlingen. Erheblich ist der Unterschied aber nicht. Mann führt ihn darauf zurück, dass die drei erstgenannten

Arten Fische ohne Magen sind, die drei letztgenannten Fische mit Magenverdauung. Was die durchschnittliche Ausnutzung verschiedener Nährtiere durch alle untersuchten Fischarten zusammen betrifft, findet Mann die folgende Reihenordnung: *Siphlweus* 91.55%, *Tubifex* 90.4%, *Chironomus greg.* 90.17%, *Anodonta* 89.58%, *Unio* 87.09%, *Carcinogammarus* 86.41%, *Daphne* 85.09%, *Chironomus plum.* 80.14%, *Anabolia* 80.55%, *Planorbis* 76%. (In der Zusammenfassung werden zum Teil nicht unerheblich abweichende Zahlen gegeben.) Die genannten Unterschiede sind ziemlich gering, ein Grund für die verschiedene Ausnutzung konnte nicht gegeben werden. Chitinhaltige Beutetiere haben eine längere Verdauungszeit nötig als chitinfreie Nahrung. Der Abbau der Stickstoffsubstanz geschieht bei Magenfischen mit gleichbleibender Geschwindigkeit, bei magenlosen Fischen ist sie in den ersten Stunden nach der Nahrungsaufnahme grösser als später. Dies rührt wohl daher, dass bei Magenfischen die Verdauungsgeschwindigkeit durch die Magenverdauung limitiert wird.

Die eigentliche Resorption der Eiweissstoffe wurde von Cohnheim (92) untersucht. Er füllte den Darm zweier magenloser Fische (*Crenilabrus pavo* und *Labrus festivus*) mit einer Peptonlösung und hängte ihn in Ringerlösung, welche defibriniertes Blut der Tiere enthielt. Während des Versuchs wurde Sauerstoff durch diese Lösung geleitet. In dieser Flüssigkeit zeigen die Därme gute Bewegungen. Nach dem Ende des Versuches, wurde die Aussenflüssigkeit durch Aufkochen mit einigen Tropfen Essigsäure enteiweisst, und im Filtrat der N nach Kjeldahl bestimmt. Im Filtrat wurde viel Stickstoff gefunden, aber keine oder nur sehr schwache Biuretreaktion. War letztere vorhanden, so hatte auch im Versuch die Darmbewegung aufgehört. Cohnheim schliesst, dass das Pepton nicht als solches die Darmwand passiert, sondern im gespaltenen Zustand. Die Reaktionen auf bestimmte Aminosäuren waren schwach oder negativ. Es konnte aber eine gewisse Menge Ammoniak gefunden werden (etwa $^1/_3$ der totalen N-Menge). Cohnheim schloss hieraus, dass in der Darmwand schon Desaminierung stattfindet, eine Erscheinung, welche auch bei den Säugern beobachtet wurde. Der Rest des N muss aber als Aminosäure vorhanden gewesen sein. Dass die Reaktionen auf bestimmte Aminosäuren nicht gelangen, liegt wohl daran, dass von jeder von ihnen die Menge natürlich sehr gering ist. Methoden um diese Säuren durch den $NH_2$—N zu bestimmen waren damals noch nicht im Gebrauch.

Die Fettresorption bei den Fischen wurde bisher nur histologisch untersucht. Bei der Beschreibung der Magenverdauung erwähnten wir schon, dass Redeke und Van Herwerden Fettröpfchen im Magen von *Elasmobranchii* gesehen hatten. Green (93) fand Fettresorption in Magen und Darm des *Salmes*. In letzterer Zeit wurde die Fettresorption der Scholle (*Pleuronectes platessa*) ausführlich von Dawes (94) untersucht. Es wurde besonders für eine gute Fixierung der untersuchten Gewebsschnitte gesorgt. Eine neue Kombination bekannter Fixiermittel wurde angegeben, welche

bei diesem Versuchsobjekt die besten Resultate gibt. Den Tieren wurde natürliches Futter (Mytilus oder Nereis) verabreicht, und in einzelnen Versuchen wurde noch eine Emulsion von Olivenöl hinzugesetzt. Nach einer Mahlzeit sieht man überall im Darmkanal Fettröpfchen im Epithel auftreten, auch im Magen. Im Enddarm ist ihre Anzahl aber sehr gering. Wie bei den Säugern sind in dem Kutikularsaum der Zelle niemals Fettkügelchen zu sehen; sie treten erst in einiger Entfernung von der Zellfront auf. Das Auftreten der Fettröpfchen im Magenepithel ist etwas unregelmässig. Wenn die Tiere 6 Tage gehungert hatten, war bei den meisten das Magenepithel nicht frei von Fettröpfchen, obgleich weniger Tröpfchen darin zu finden waren als nach Fütterung. Wie man sich die Fettresorption im Fischmagen vorstellen muss, ist noch etwas unklar. Man hat in der Magenschleimhaut eine Lipase gefunden und wahrscheinlich wird diese auch sezerniert. Ihr Optimum wurde nicht bestimmt. Für die Magenlipase der Säugetiere liegt es aber hoch: bei 5–6. Wenn das Optimum für die Magenlipase der Fische die gleiche Lage hat, so kann man sich eine Spaltung im Magen schwerlich vorstellen, da wir sahen, dass die Schichten, in welchen das Pepsin und die Salzsäure vordringen, einen sehr niederen *p*H annehmen. Vielleicht könnte man eher an eine Spaltung durch die Enzyme der Beute selbst denken; da im Inneren der Beute das *p*H lange Zeit hoch bleibt, muss auch diese Autolyse längere Zeit stattfinden können. Hierauf würden Dawes, Versuche mit Olivenöl hinweisen können: gerade nach Verabreichung dieses Öls war die Menge der Fettkügelchen im Magenepithel auffallend gering, während es im Darme sehr gross war. Das Fett, welches sich in der natürlichen Nahrung befindet, ist schon emulgiert, sodass es auch darum wahrscheinlich dort besser gespalten und vom Magenepithel aufgenommen werden kann.* Das verfütterte Öl braucht aber zu seiner Emulgierung die Galle, welche auch die Fettsäuren, die nach der Spaltung entstehen, lösen kann. Wenn die Scholle mit kurzen Zwischenzeiten frisst, so passiert das Futter den Darmkanal viel schneller als bei einmaliger Fütterung und es wird auch nicht so viel Fett im Magen aufgenommen. Da eine mehr oder weniger kontinuierliche Futteraufnahme auch in der Natur wohl die Regel sein wird, darf man n.m.M. der Fettresorption im Magen keine allzuhohe Bedeutung beimessen.

Mackay (95) verdanken wir einige Beobachtungen über die Galle der Fische, besonders über ihr *p*H. Die Wasserstoffionenkonzentration der Galle ist ziemlich niedrig: *Fundulus heteroclitus* *p*H 6.8–7.0; *Zoarces anguillaris* 5.4–6.2; *Raja erinacea* 5.4–7.6; *Cottus groenlandicus* 5.4–5.8;

* Verschiedene Untersucher sahen aber im Haifischmagen Ölmassen.

*Clupea harengus* 5.8. Die Beobachtungen dieses Autors über den Enzymgehalt der Galle wurden schon erwähnt.

In der Galle von Haifischen wurde von Hammarsten (96) besondere Gallensäuren gefunden: die Scymnolschwefelsäuren. Sie liefern bei Hydrolyse Schwefelsäure und Scymnol: $C_{27}H_{46}O_5$. Von physiologischem Gesichtspunkte wäre es interessant zu untersuchen, ob diese Produkte einen solchen Einfluss auf die Verdauung und die Resorption der Fette haben, wie die anderen Gallensäuren.

## V. Die Verdauung bei den Amphibien

Die Amphibien sind alle karnivor und Raubtiere. Die Nahrung (vgl. *Brehms Tierleben*) besteht aus Insekten und andern kleinen Tieren, be. grösseren Formen auch wohl aus kleinen Fischen, oder aus Amphibieni Vielfach wird die Insektennahrung von den landbewohnenden Amphibien mit der klebrigen Zunge gefangen. Grössere Beuteobjekte werden von den landlebenden Amphibien vielfach besprungen. Die Larven leben von sehr kleinen Tieren, welche sie mit Schlamm gemischt aufnehmen. Die Zähne sind meist klein und fehlen bei einigen Formen.

**Sekretion.**—Der erste, welcher die Sekretion bei Amphibien untersuchte war Smirnov (47). Er hat bei Fröschen Magenfisteln angelegt; zwar entwickelte sich, wenn viel Verlust von alkalischem Schleim bei breiter Fistelung stattfand, zwischen dem fünften und dem zehnten Tage nach der Operation eine tödliche Hautnekrose, aber er hat auch Tiere mit Fisteln erhalten, die 6–12 Monate am Leben blieben. Der Magensaft wird erst 40 bis 50 Minuten nachdem die Nahrung verschluckt worden ist, abgesondert; eine psychische Sekretion fehlt also. Das Zentralnervensystem wird hier bloss für das Aufsuchen der Nahrung, nicht für die Vorbereitung des Magens auf ihren Empfang benutzt. Nach Verschlucken von Kork und Gummi wird immer Magensaft sezerniert. Durchschneidung des Vagus am Halse hat keinen Einfluss auf die Sekretion. Dagegen veranlasst Reizung des Sympathicus Sekretion. Die Magensäure wirkt beim Frosch wie bei den höheren Tieren als Regulator für den Übergang des Speisebreies aus dem Magen in den Dünndarm.

Friedman (97) hat die Sekretion der Salzsäure untersucht. Wie Smirnov, fand auch er, dass der Vagus nahezu keinen Einfluss hierauf hat. Die Sekretion der Säure steht aber unter dem Einfluss vom sympathischen Nervensystem. Reizung des Splanchnicus und Injektion von Adrenalin (sympathicotrop) verursachen Sekretion, aber Reizung des Vagus und Injektion von Pilocarpin und Acetylcholin (vagotrop) hatten keinen Effekt.

Smirnovs Beobachtung, dass mechanische Reizung der Magenschleimhaut mit Glasskugeln und Stückchen Gummi Sekretion veranlasst, wurde bestätigt. Smirnov meinte, dass letztere Erscheinung entweder nur dem Auerbachschen Plexus zugeschrieben werden müsse, oder durch einen Reflex verursacht wird, welcher nur durch die sympathischen Ganglien an der äusseren Seite des Magens verläuft. Friedmann meint aber auf Grund seiner Versuche, dass hier eine Reflexwirkung im Spiel ist, welche durch eine gewisse Stelle des Rückenmarkes verläuft. Weiter hat Friedmann (98) die Sekretion von Pepsin beim Frosch untersucht. Das Tier wird geöffnet, der Magen wird am oesophagealen und am pylorischen Ende abgebunden, sodass Mischung des Sekretes vom Oesophagus und vom Magen nicht auftreten kann.* Nerven und Blutgefässe bleiben intakt. Die abdominale Wand wird vernäht. In dieser Weise bleiben die Tiere sieben Wochen am leben. Im Winter findet keine Sekretion statt.

Werden Glasskugeln in den Magen gebracht, so findet Sekretion statt, sowohl bei intaktem als bei zerstörtem Zentralnervensystem. Auch im Oesophagus wird durch Reizung mit Glasskugeln Pepsin abgesondert, aber dazu muss das Rückenmark intakt sein. Ob eine derartige Reizung des Oesophagus auch Magensekretion hervorruft, ist sehr zweifelhaft. Auch für die Sekretion des Pepsins haben vagotrope Stoffe keinen Effekt, und sympathicotrope Stoffe positiven Effekt. Der Effekt von Histamin ist positiv für die Sekretion von Säure im Magen, aber negativ für die Absonderung von Pepsin im Magen. Für die Sekretion von Pepsin durch die Oesophagusdrüsen ist aber Histamin ein starker Reiz. Die Regulierung dieser Sekretion im Oesophagus muss also eine ganz andere sein als die im Magen.

In der Hauptsache wird also die Sekretion von Säure und Pepsin bei Amphibien parasympathisch reguliert, im Gegensatz zu den Säugetieren.

Auch Tinbergen (99) kam ungefähr zum gleichen Resultat: es fehlt psychische Sekretion, chemische Reize im Magen haben keinen Einfluss, mechanische dahingegen einen sehr starken Einfluss. Wolvekamp (100) meint, dass vielleicht nach längerer Zeit chemische Reize noch wirksam sein könnten.

**Die Magenverdauung der Amphibien.**—Über die Magenverdauung der Amphibien liegen viel weniger Untersuchungen vor, als über diejenige der Fische. In Abschnitt III wurden schon einige Versuche behandelt, welche zeigen, dass das Pepsin wahrscheinlich mit dem der Säugetiere identisch ist

* Beim Frosch und anderen Amphibien wird nämlich im Oesophagus durch besondere Drüsen Pepsin sezerniert. Vgl. später bei der Behandlung der Magenverdauung.

(mit der Beschränkung aber, dass der Einfluss der Temperatur auf beide nicht ganz gleich ist).

Schon 1876 hat von Swiecicki (101) in einer gründlichen Untersuchung die merkwürdige Tatsache festgestellt, dass beim Frosch im Oesophagus besonders gebaute Drüsen vorkommen, welche reichlich Pepsin sezernieren. Diese besonderen Drüsen kommen vor vom Anfang des Oesophagus bis in den kardialen Magenteil. Es sind verzweigte tubulöse Drüsen, mit zylindrischen Zellen. Die Ausfuhrgänge münden in Querrichtung in den Oesophagus und in die Cardia. Die Sekretion des Pepsins ist im Oesophagus viel grösser als im Magen. Die der Säure findet aber nur im Magen statt. Dieselben Tatsachen stellte von Swiecicki in seiner Arbeit fest für folgende Tiere: *Pelobates fuscus*, *Hyla arborea*, *Bufo variabilis* und einige Tritonarten. Kurze Zeit nachher wurden diese Untersuchungen bestätigt von Langley (102).

Über die Frage ob ein oder zwei Zellarten in den Magendrüsen vorkommen, ist man sich noch nicht einig. Nach Friedmann ist der Pepsingehalt des Magensaftes hoch (eben so hoch wie beim Menschen und 5 mal höher wie beim Hund). Dies weicht also ab von den Angaben von v. Swiecicki, dass im Magen fast kein Pepsin vorkommen soll. Vonk (103) fand für das Verhältnis der Pepsinmengen in Magenextrakten von Hecht, Schwein, Frosch, und Acanthias (Haifisch) die folgenden Zahlen: 15:4:2:1. Kenyon (104) fand dagegen, dass die Magenextrakte von allen von ihm untersuchten Fischen, Amphibien, Reptilien und Säugern nahezu gleiche Pepsinwirkung zeigten. Der Hecht, welcher nach Vonk eine viel höhere Pepsinmenge in seiner Magenschleimhaut besitzt wie alle andere untersuchten Tiere, hatte nach Kenyon sogar eine etwas schwächere Wirkung wie die anderen von ihm studierten Tiere aufwiesen. Diese Resultate von Kenyon sind sicher unrichtig. Die Ursache hiervon ist die von ihm benutzte Methode für die Bestimmung der Proteinasen. Als solche hat er nämlich verwendet die Bestimmung der Tyrosinmenge, welche bei der Wirkung der untersuchten Proteinasen (auch Pepsin!) entsteht. Diese Tyrosinmenge wurde gemessen mittels einer Methode von Folin und Denis.* Diese Methode wurde aber von ihnen, soviel ich sehen konnte, nirgends für die Bestimmung von Proteinasen empfohlen. Es ist dann

* O. Folin und W. Denis, *J. Biol. Chem.*, 12 (1912), 239 und 245. Zwar wird durch die kolorimetrische Tyrosinreaktion auch Tyrosin in Peptidform angezeigt, aber die Autoren haben nicht festgestellt, bei welcher Länge der Peptidkette die Reaktion negativ wird und es ist dann auch unbekannt, ob bei der Pepsinwirkung Peptide entstehen, welche diese Reaktion zeigen. Ausserdem zeigen einige andere Stoffe die Reaktion; sie ist nur spezifisch für Tyrosin im Vergleich zu andern *Aminosäuren*.

auch schon sehr lange bekannt, dass bei der Einwirkung von Pepsin auf Eiweiss gar keine Aminosäuren entstehen. Wenn trotzdem bei der Wirkung der Extrakte auf Eiweiss Tyrosin entsteht, so muss das dadurch verursacht sein, dass Gewebspeptidasen mitextrahiert wurden, welche die Verdauung weiter führen. Die Konzentration dieser Peptidasen wird natürlich viel geringer sein als die des Pepsins. Verwendet man nun die gebildete Tyrosinmenge zur Beurteilung des Pepsingehalts von der Magenschleimhaut, so wird die entstandene Menge dieser Aminosäure begrenzt werden durch die Menge der gleichzeitig anwesenden Peptidasen. Kenyons Versuche beweisen also nur, dass in den von ihm untersuchten Extrakten die Mengen der mitextrahierten Peptidasen ungefähr gleich sind, was auch keineswegs unwahrscheinlich ist. Wie aber der Autor schreiben konnte, dass mit der von ihm benutzten Methode gerade die Anfangsgeschwindigkeit der Wirkung des Pepsins gemessen wird, ist unbegreiflich. Auch für die Bestimmung des Trypsins, wofür er sie verwandte, gelten diese Bemerkungen, wenn auch in etwas geringerem Masse. Nur für die Bestimmung des Erepsins ist sie vielleicht brauchbar.

Früher hatte auch Riddle (105) schon mit der Methode von Mett gefunden, dass die Verdauungsgeschwindigkeit im Magen verschiedener poikilothermen Vertebraten sehr ungleich ist. Bei dem von ihm untersuchten Fisch *Amia calvia* (Kahlhecht) ist sie am grössten, dann folgen einige Amphibien (u.a. eine Ranaart) und schliesslich eine Schildkrötenart. Die Methode von Mett ist brauchbar, aber sicher nicht ideal.* Die Wirkung des Enzyms wird hier stark limitiert durch die beschränkte Oberfläche, welche mit dem Ferment in Berührung kommt, bzw. durch die Diffusionsgeschwindigkeit des Enzyms in dem Substrat. Zum Vergleich verschiedener Enzymmengen ist sie dann auch wenig geeignet, etwas besser vielleicht für den Vergleich der Wirkung einer Proteinase bei verschiedenen *p*H-Werten. Da jetzt so viele modernere und exaktere Methoden benutzt werden können, ist es nicht klar, warum diese Methode noch immer bevorzugt wird in vielen Untersuchungen, welche sich mit der Sekretion der Proteinasen befassen.

Riddle hat besonders die totale Verdauungsgeschwindigkeit verschiedener Kaltblüter bei verschiedenen Temperaturen untersucht. Bei mittleren Temperaturen ist "$Q_{10}$" für die Verdauungsgeschwindigkeit für alle Tierarten etwa 2.6 (Die kleinen Mettschen Röhrchen wurden in den Magen der Tiere geschoben, welche nach bestimmten Zeiten getötet wurden).

* Es wird hierbei ein Glasröhrchen, in welchem man einen Eiweissstoff hat koagulieren lassen, in das Verdauungsgemisch gebracht.

Die Minimumtemperatur, bei welcher noch Verdauung stattfindet, ist verschieden; für Amia ist sie z.B. 3°, für den Frosch 10°, bei den untersuchten Schildkröten noch etwas höher. Der Einfluss der Temperatur auf die Enzymwirkung und auf die Sekretion des Enzyms wurde nicht getrennt untersucht. Da das Pepsin sogar bei 0° noch etwas wirksam ist, war bei diesen Untersuchungen der Einfluss der Temperatur auf die Sekretion wahrscheinlich massgebend.

Auch bei Amphibien (Frosch) untersuchte Mennega (106) den Säuregrad im Magen bei der Verdauung. Die Tiere wurden mit Brot oder mit gehacktem Fleisch gefüttert. Nach Fütterung von Brot reagiert der ganze Mageninhalt stark sauer: *p*H 2.18. Bei Fütterung von gehacktem Fleisch besteht ein Unterschied in dem *p*H zwischen Oberfläche und tieferen Schichten: an der Oberfläche wurde gemessen: Cardia 3.25, Fundus 2.31, Pylorus 2.20. Da das *p*H-Optimum des Froschpepsins nach Vonk bei 1.50, nach Pjatnitzky zwischen 1.60 und 1.90 liegt, wirkt also das Pepsin beim Frosch an der Oberfläche der Nahrung ziemlich in der Nähe des Optimums. Bei der natürlichen Ernährung des Frosches wird die Säure leichter zwischen den kleinen Beuteobjekten durchdringen als bei Fleischfütterung. Wahrscheinlich ist dann das *p*H im ganzen Mageninhalt ziemlich günstig für die Pepsinverdauung. Vonk (103) hatte früher höhere Werte (2.2 bis 3.7) für den Mageninhalt des Frosches gefunden. Diese Messungen wurden mit der Wasserstoffelektrode ausgeführt, wozu der Mageninhalt mit etwas Wasser verdünnt werden musste. Auch wurden diese Versuche in einer ungünstigen Jahreszeit (November) ausgeführt. Delrue (107) fand für das *p*H vom reinen Magensaft des Frosches 4.2–4.8. Diese Werte können nicht richtig sein: wäre das *p*H des Magensaftes so hoch, so könnten an der Oberfläche des gefütterten Fleisches oder Brotes niemals so niedrige Werte vorkommen, wie Mennega gefunden hat. Das *p*H des Saftes kann höchstens 2.2 sein.

Untersuchungen, wie die hier angeführten, wurden schon in 1905 durch Grützner bei Fröschen ausgeführt (108). Er beobachtete dabei, dass der Mageninhalt nach Fütterung sauer auf Lackmus reagierte, mit Ausnahme einer Zone bei dem Oesophagus. Bestimmungen der Wasserstoffionenkonzentration wurden aber noch nicht ausgeführt, da die Methode in der Physiologie noch nicht benutzt wurde.

Die Magenbewegungen beim Frosch wurden zuerst mittels Röntgendurchleuchtung studiert von Balthazard und Roux (109). Sie fanden, dass—wie beim Hund und beim Menschen—zweierlei Magenbewegungen unterschieden werden können, diejenigen des Fundus und diejenigen der Pars pylorica. Der grössere Fundusteil weist kaum sichtbare Kontrak-

tionen auf, welche wohl nur dazu dienen flüssige oder verflüssigte Stoffe nach dem Pylorusteil zu transportieren. Der kleine Pylorusteil zeigt kräftige peristaltische Bewegungen, welche die Nahrungsmittel kneten und schliesslich in den Dünndarm befördern.

Grützner (110) fand, dass am Oesophagus- und Pylorusende des Froschmagens eine Rings- und Längsmuskelschicht vorhanden ist, während es in der Mitte des Magens fast nur eine Ringschicht gibt. Er gab 4 hungernden Fröschen einen Ballen Nahrung, bestehend aus "Semmelweichel,"* welches mit etwas Milch und ein wenig chemisch reinem Lackmus durchknetet worden war. Nach 12–15 Stunden wurden die Tiere getötet. Nahezu immer sah er in der Gegend des Pylorus "gewaltige Einschnürungen, welche mit ziemlicher Geschwindigkeit dem Darme zu wandern." Die durchknetende Wirkung, welche diese Bewegungen ausüben, zeigte er an einem der Länge nach durchschnittenen Magen. Am unteren Pylorusende ist der Inhalt am meisten durchfeuchtet und gemischt. Aus alledem ergibt sich, dass die im Fundusteil an der Oberfläche verdauten Schichten durch leichte Magenbewegungen dem Pylorusteil zugeführt werden, wo sie auch mechanisch verdaut werden. Ganz vergleichbar mit dem Vorgang bei den karnivoren Säugetieren ist der Prozess beim Frosch nicht, weil hier schon im Oesophagus Pepsin ohne Salzsäure abgesondert wird.

Was die Innervation der Magenbewegungen betrifft, lieferten Untersuchungen von Dixon (111), Langley und Orbeli (112), Itagaki (113), und Yüh (114) das Resultat, dass der Vagus bisweilen einen erregenden, bisweilen einen hemmenden Einfluss auf die Magenbewegung hat. Sympathicusreizung veranlässt aber immer Bewegung des Magens. Der Einfluss des sympathischen Systems auf die Magenbewegung herrscht also hier noch vor.

**Darmverdauung bei den Amphibien.**—Hierüber wurde sehr wenig gearbeitet. Kenyon (115) untersuchte mit der oben (bei der Magenverdauung) besprochenen Methode die Verdauung in Pankreas und Darm von *Necturus maculosus*. Der Pankreasextrakt wirkt kräftig auf Hühnereiweiss. Der Darmextrakt garnicht. Die Erepsinmenge im Darm stimmt ungefähr mit derjenigen des Hundes überein. Die Amylase im Pankreas von *Necturus* war ziemlich stark, die Wirkung war etwa die Hälfte von derjenigen vom "Hepatopankreas" des Karpfens (es scheint beim Karpfen die Leber mitextrahiert worden zu sein). Im Darmextrakte ist die Wirkung viel geringer. Invertase und Lactase kommen im Darmextrakte nicht vor. Auf das Vorkommen von Maltase wurde nicht geprüft. (Es

* Geweichtes Brot.

ist merkwürdig, dass bei vielen vergleichend-physiologischen Untersuchungen über die Verdauung vielfach nach Invertase gesucht wird und die Untersuchung der biologisch viel wichtigeren Maltase vernachlässigt wird.)

Bei seinen Untersuchungen über die Verdauung der Fische hat Vonk (*l. c.*) auch über die Darmverdauung des Frosches einige Versuche angestellt. Es wurden die Diastasezahlen nach Wohlgemuth für Pankreas und Darm bestimmt. Pro g. Pankreasgewebe ist D 39° 24 h. = 4000 bei Anwesenheit von NaCl zur Aktivierung und 500 ohne Zusatz von Kochsalz. Für das Pankreas des Karpfens (mit NaCl Zusatz) wurde 3200 gefunden. Diese Werte für Rana sind also recht hoch. Bei der genaueren Bestimmung durch Zuckertitration verhielt sich die Wirkung des Pankreas von Cyprinus zu derjenigen des Pankreas von Rana ungefähr wie 3 odor 4:1, also für Rana etwas ungünstiger. Die Amylase im Darmextrakt von Rana ist ungefähr 50 mal schwächer als im Pankreas. Ein vorläufiger Versuch lehrte, dass im Pankreas von Rana eine kleine Maltasemenge nachgewiesen werden kann. Diese fehlte im Darm (unveröffentlichte Nachprüfung in unserem Institut hat diesen Versuch bestätigt). Die Verteilung von Amylase und Maltase ist also beim Frosch die gleiche wie beim Karpfen und steht im Gegensatz zu den Verhältnissen bei den Säugern. Der *p*H im Darm ist nach Mennega (*l. c.*): Duodenum 7.84 ± 0.26, Dünndarm 7.89 ± 0.28, Enddarm 8.05 ± 0.22 (Glaselektrode).

Die Trypsinwirkung im Pankreas von Rana wurde ungefähr gleich derjenigen des Karpfens gefunden und etwa 6–8 mal schwächer als beim Hecht und beim Haifisch (*Acanthias*). Die Wirkung auf Glycylglycin ist im Darmextrakte von Rana $^3/_4$ von der beim Karpfen. Es geht also aus diesen Versuchen von Kenyon und Vonk hervor, dass bei *Necturus* und beim Frosch im Darmkanal die gleichen Enzyme wirksam sind wie bei den Säugern. Etwas überraschend ist die starke amylolytische Wirkung des Pankreasextraktes beim Frosch, der doch ein Karnivor ist. Allerdings ist diese Amylase nicht ohne Bedeutung, da sie das in der Nahrung vorhandene Glykogen zu spalten vermag. Es besteht aber kein Grund zur Annahme, dass die mit der Nahrung zugeführte Glykogenmenge hier grösser wäre als bei anderen Karnivoren. In diesem Zusammenhang muss noch erwähnt werden, dass Junold (116) im Speichel vom Frosch Amylase gefunden hat (*Rana temporaria, R. esculenta, Pelobates fuscus*). Der Speichel wurde gesammelt auf Seidenpapier, das in den Mund gebracht wurde. Bei Anuren gibt es 3 kleine Drüsenkomplexe:

1. Gl. intermaxillaris (unpaarig).
2. Rachendrüse (paarig).
3. Zungendrüse.

Bei Extraktion wurde in allen diesen Geweben Diastase gefunden, bei *R. esculenta* mehr als bei *R. temporaria* und *Bufo*. Diese Tatsache ist biologisch noch unverständlicher als die grosse Amylasenmenge im Pankreas des

Frosches. Denn das Glykogen kommt in den *Organen* der Beutetiere vor und diese müssen wohl zuerst mehr oder weniger verdaut sein bevor es von Amylase angegriffen werden kann.

**Resorption.**—Auch hierüber liegen nur wenige Untersuchungen vor. Jordan und Begemann (117) fanden, dass beim Frosch im Darme echte Resorption nachgewiesen werden kann. Bei operierten Tieren, deren Darm mit bluthypo-oder-isotonischen Lösungen gefüllt worden war (Darm abgebunden) fand man nach 2–3 Tagen den Darm ganz leer. Diese Tatsache ist deshalb wichtig, weil bei gewissen Invertebraten der Darm bloss als Diffusionsmembran fungiert oder (*Astacus*) semipermeabel ist. Es war deshalb wichtig festzustellen, dass der Darm der Amphibien sich ganz wie der der höheren Vertebraten verhält. Versuche *in vitro* ergaben, dass Glukose gut resorbiert wird, für Saccharose ist der Darm aber wahrscheinlich impermeabel. Sie ist erst 4 Stunden nach Anfang des Versuchs in der Aussenflüssigkeit nachzuweisen, während auf Glukose schon nach $1^1/_2$ Stunden reagiert werden kann. Die Autoren nahmen an, dass nach 4 Stunden der Darm im Absterben begriffen war. Lässt man den Froschdarm Eisenverbindungen aufnehmen und untersucht man nachher die Darmwand histologisch (wobei das Eisen als Berlinerblau sichtbar gemacht wird), so findet man das Eisen als Körnchen zusammengeballt in Vacuolen. Dies ist typisch für echte Resorption. Der durch NaCl abgetötete Darm zeigt diese Erscheinung nicht, das Eisen ist dann diffus über die ganze Zelle verteilt. Letzteres Bild zeigte auch der Darm von Helix, wenn er in lebendigem Zustand mit Eisenverbindungen gefüllt und nachher mikroskopisch untersucht wurde.

Westenbrink und Gratama (118) untersuchten die Resorption verschiedener Monosen durch den Froschdarm. Bekanntlich werden bei Warmblütern die Monosenarten mit sehr ungleicher Geschwindigkeit vom Darme aufgenommen. Es zeigte sich, dass dieses auch beim Frosch der Fall ist. Nachstehende Übersicht gibt die Resultate einer Versuchsreihe der genannten Autoren.

| | Ratte | Taube | Frosch |
|---|---|---|---|
| *d*-Galaktose | 108 | 115 | 58–74 |
| *d*-Glukose | 100 | 100 | 53–64 |
| *d*-Fructose | 42 | 55 | 27–37 |
| *d*-Mannose | 15 | 33 | 36–37 |
| *l*-Xylose* | 13 | 33 | 22–36 |
| *l*-Arabinose | 2 | 16 | 12–13 |
| *d*-Xylose | ... | ... | 7 |

* *l*-Xylose ist der natürlich vorkommende, rechtsdrehende Zucker.

Die Verhältniszahlen sind willkürlich gewählt; zu einem Vergleich der Resorptionsgeschwindigkeit der gleichen Zuckerart bei den drei verschiedenen Tieren können sie deshalb nicht dienen. Auch beim Frosch werden also Galaktose und Glukose am schnellsten resorbiert. Die Resorptionsgeschwindigkeiten der drei darauffolgenden Zuckerarten sind beim Frosch untereinander ungefähr gleich. Fructose nimmt also nicht—wie bei den Warmblütern—eine Mittelstellung ein. Die Resorptionsgeschwindigkeit der *l*-Arabinose ist für alle untersuchten Tiere am geringsten. Da also die Verhältnisse in der Hauptsache für höhere und niedere Vertebraten gleich sind, schliessen die Autoren, dass, auch der Mechanismus der Resorption wahrscheinlich derselbe sein wird. Dieser Mechanismus wird von Verzár und Mitarbeitern (119) durch die Phosphorylierung erklärt, welche die Zucker in der Darmwand erleiden sollen. Westenbrink und Gratama halten hiervon wenig für bewiesen.

## VI. Die Verdauung bei den Reptilien

**Nahrung.**—Die Reptilien sind alle karnivor und Raubtiere, mit Ausnahme von den Landschildkröten, einigen Süsswasserschildkröten und einigen Eidechsen. Viele Raubtiere unter ihnen verschlingen ihre Beute als Ganzes, nur wenige, besonders Krokodile und einige Schildkröten können sie durch Beissen zerkleinern.

Über die Verdauung dieser Tiere wurde noch weniger gearbeitet als bei den Amphibien. Zum Teil liegt das wohl daran, dass sie als Versuchstiere weniger bequem zu handhaben sind. Experimentelle Eingriffe sind bei ihen schwieriger: bei den Schlangen durch ihre Beweglichkeit, bei den Krokodilen durch ihre Gefährlichkeit und die Härte ihrer Haut und auch Schildkröten sind schwierig zu öffnen. Die Eidechsen liessen sich am leichtesten bearbeiten. Über die Sekretion ist nichts bekannt, sodass wir gleich zur Magenverdauung übergehen können.

**Magenverdauung.**—Über die Eigenschaften des Pepsins vergleiche man wieder Abschnitt III. Für die Versuche welche Kenyon (120) über das Pepsin von den von ihm untersuchten Reptilien ausgeführt hat, gelten dieselben Bermerkungen, welche über seine Methode bei der Besprechung der Amphibien gemacht wurden. Seine Resultate, dass bei allen von ihm untersuchten Kalt- und Warmblütern, die Pepsinkonzentration in der Magenwand gleich ist, können unmöglich richtig sein. Nach Langley (121) kommt im Oesophagus der von ihm untersuchten Schlange (*Coluber natrix*) kein Pepsin vor.

Die Säureverteilung im Magen einer Ringelnatter wurde von Mennega (106) untersucht. Die Beute wird bekanntlich als Ganzes aufgenommen. 24 Stunden nach dem

Verschlingen war die Verdauung schon ziemlich weit fortgeschritten. Von den Hinterbeinen waren nur die Tibiae übrig. Auf der Oberfläche wurden *p*H-Werte von 2.14 bis 2.80 gemessen. An einer Stelle, wo an der Oberfläche der *p*H 2.53 betrug, war dieser 2 mm. tiefer schon auf 3.32 gestiegen. Im Pylorusteil hatte der Brei einen *p*H von 3.22. Auch hier sind die Verhältnisse also ungefähr dieselben wie bei den übrigen von Mennega untersuchten Tierarten. Wird der *p*H kürzere Zeit (12 St.) nach dem Verschlingen eines Frosches gemessen, so ist die Haut zu einer schleimigen Flüssigkeit verdaut (*p*H 3.85). Der niedrigste *p*H, der dann gemessen werden konnte, war 3.20 auf dem Oberschenkel. Im Fundus war das *p*H an der Oberfläche der Beute 3.56–4.04, einige mm. tiefer 5.60. Es ist wahrscheinlich, dass die schleimige Haut des Frosches sehr viel Säure bindet.

In der gleichen Arbeit wurde das *p*H des Darminhaltes bei der Ringelnatter gleich hinter dem Pylorus zu 5.7 und 3 cm. weiter zu 6.7 bestimmt. Caudalwärts nimmt das *p*H zu: 10 cm. hinter dem Pylorus ist er 6.88 und 15 cm. 7.59.

**Darmverdauung und Resorption.**—Diese wurden von Wolvekamp (122) bei *Testudo graeca* und *Emys europaea* untersucht. Die erstere ist omnivor, die zweite ein Raubtier. Das *p*H-Optimum der Pankreasamylase von Testudo liegt bei 6.5. Die Menge der Amylase im Pankreas von Testudo ist ziemlich gross; Emys hat ungefähr die Hälfte, was für ein Raubtier sehr viel ist. Dieselbe Erscheinung fanden wir beim Frosch. Auch in der Darmschleimhaut von Emys befindet sich viel Amylase, sodass Wolvekamp meint, dass diese dort auch abgesondert wird.

Anfänglich wurde keine Maltase in Pankreas und Darm gefunden. Auspräparierte Därme wurden nun mit Lösungen von Rohrzucker und Maltose gefüllt. In der Aussenflüssigkeit des mit Rohrzucker gefüllten Darmes konnte nach $1^1/_2$ St. Reduktion von Fehlingscher Lösung gezeigt werden, welche nach $2^1/_2$ St. zugenommen hatte. Es hatte also reduzierender Zucker die Darmwand passiert. Nach dem Versuch mit Maltose war die totale Reduktion von Aussen- und Innenflüssigkeit zusammen grösser als die der Innenflüssigkeit vor dem Versuch. Es hatte also Transport von Zucker nach der Aussenflüssigkeit stattgefunden, und wenigstens teilweise als Monose. Ein Teil der Aussenflüssigkeit wurde nun mit 4 N Salzsäure gekocht und die Reduktion vor und nach dem Kochen bestimmt. Es fand keine Zunahme der Reduktion statt, sodass aller Zucker in der Aussenflüssigkeit Glukose gewesen sein muss. Durch diese Versuche wurden also erstens die Impermeabilität des Darmes für Disaccharide bewiesen (wie beim Säugetier), zweitens das Vorkommen von Maltase in der Darmwand. Durch Verdauungsversuche mit grösseren Extraktmengen als in den anfänglichen Versuchen, wurde nun das Vorkommen von Maltase im Pankreas und im Darm noch bestätigt. Die Menge der Maltase war im Darmextrakt grösser als im Pankreasextrakt. Dieses Verhalten kommt

also überein mit dem bei den Säugern und nicht mit dem Zustand bei den Fischen.

Kenyon (120) fand im Pankreasextrakt von allen der von ihm untersuchten Reptilien gute Trypsinwirkung. Er untersuchte folgende Arten: *Chelydra serpentina* L., die Schnappschildkröte, *Chrysemys belli* und *Chrysemys cinera* (Schmuckschildkröte) und *Pituophis sayi* (Schlange, Bull snake). Alle sind nord-amerikanische Arten. Die Trypsinmengen im Pankreas dieser Tiere sind nicht sehr verschieden. Alle sind denn auch Raubtiere. Sehr wenig Trypsin wurde in den Darmextrakten gefunden; die Darmwand der Schnappschildkröte enthielt eine grosse Menge Erepsin, auch die der Schlange zeigte gute Erepsinwirkung, die anderen Arten wurden darauf nicht untersucht. Für die Prüfung der Erepsinwirkung wurde als Substrat Casein gebraucht; bei den älteren Untersuchungen über Erepsin meinte man gefunden zu haben, dass neben Eiweissabbauprodukten auch das leicht verdauliche Casein von diesem Enzym angegriffen wird. Nach Untersuchungen aus der Willstätterschule ist dies aber unrichtig: wenn Casein von Darmextrakten angegriffen wird, ist immer eine kleine Trypsinmenge vorhanden, welche die Verdauung einleitet. Die hier vorgenommene Bestimmung des Erepsins (wobei dann noch die Wirkung von Aminopeptidase und Dipeptidase zusammengefasst wird), ist also nach moderneren Ansichten nicht mehr als einwandfrei zu betrachten. Die Menge des im Darme vorhandenen Trypsins wird die Wirkung limitieren. Trotzdem gibt die Methode für biologische Zwecke doch wohl einen gewissen Eindruck der Erepsinwirkung.

Im Pankreas aller dieser Tiere war eine ziemlich grosse Amylasemenge vorhanden. Auch die Darmwand zeigte Amylasewirkung, wenn auch etwa 4 mal weniger. Invertase im Darm wurde nicht gefunden. Der Darm der Schnappschildkröte enthielt eine geringe Menge Maltase. Die anderen Tiere wurden darauf anscheinend nicht untersucht (auch nicht das Pankreas).

## VII. Zusammenfassung

Es ist merkwürdig, dass die Untersuchung der Verdauung bei den niederen Vertebraten längere Zeit sehr vernachlässigt wurde, während diese Tiere eingehend untersucht wurden in Bezug auf die Physiologie von Herz, Nerven und Muskeln. Seit etwa 1920 hat sich dies aber geändert, sodass wir jetzt einen gewissen Überblick über die Verdauungserscheinungen dieser Tiere haben, obgleich dieser, besonders für die Amphibien und Reptilien nur auf die Untersuchung sehr weniger Formen beruht.

Bei den niederen Vertebraten kommen die gleichen Verdauungsorgane vor wie bei den höheren. In den Magendrüsen der Fische gibt es keine Differenzierung in Haupt- und Belegzellen (erstere sondern bei den Säugern Pepsin, letztere Salzsäure ab). Die Frage ob bei Amphibien und Reptilien ein oder zwei Zellarten in den Magendrüsen vorkommen, ist noch unentschieden. Bei den Fischen fehlt bei der Gruppe der Cyprinoiden und bei verschiedenen anderen Formen der Magen und damit die Absonderung von Pepsin und Salzsäure.

Grosse Abweichungen von den Verhältnissen bei den höheren Wirbeltieren finden wir bei der Regulierung der Sekretion der Verdauungssäfte. Bei den Säugetieren veranlasst Reizung des Vagus Sekretion und Bewegung von Magen und Darm, Sympathicusreizung hemmt diese Vorgänge. Bei den Fischen haben sowohl das parasympathische als das sympathische System einen motorischen Effekt. Reizung keines der beiden Systeme veranlässt aber Sekretion. Auch eine chemische (hormonale) Regulierung der Sekretion konnte bei Fischen und Amphibien nicht gefunden werden. Bei Amphibien veranlasst mechanische Reizung der Magen- (und Oesophagus-) schleimhaut Sekretion von Säure und Pepsin. Auch Reizung des Sympathicus hat einen sekretorischen Effekt. Fischen und Amphibien fehlt eine psychische Sekretion. Trotzdem wichtige Tatsachen gefunden wurden, ist die Regulierung der Sekretion bei den niederen Vertebraten noch keineswegs klar.

Der Verdauung im Magen der niedere Vertebraten unterscheidet sich was die Verteilung der Säure, die Fortbewegung der verdauten Nahrung, die Knetung im Pylorusteil betrifft, nicht von Verhältnissen bei den Säugern.

Es kommen bei den kaltblütigen Vertebraten die gleichen Enzyme vor wie bei den Warmblütern. Die Fermente der letzteren zeigen aber eine grössere Resistenz zu schädlichen Temperatureinflüssen. Die Verteilung von den Enzymen zwischen Pankreas und Magen ist bei Fischen und Amphibien, abweichend von den höheren Vertebraten, derart, dass Maltase neben Amylase im Pankreas gefunden wird und im Darm keine Maltase vorkommt. Bei Fischen kommen in der Galle nicht unbeträchtliche Mengen von Verdauungsenzymen vor, welche wahrscheinlich aus dem Pankreas stammen. Beim Frosch und anderen Anuren wird im Oesophagus durch besondere Drüsen die Hauptmenge des Pepsins abgesondert, die Salzsäure wird aber im Magen sezerniert. Übrigens ist die Verteilung der verschiedenen Enzyme in der Verdauungsorganen die gleiche wie bei den höheren Vertebraten.

Soweit wir nach den spärlichen Untersuchungen etwas über die Resorp-

tion aussagen können, unterscheidet sich diese nicht von derjenigen bei den Säugern.

### Literaturverzeichnis

1. Zusammenfassungen in: R. Willstätter, "Untersuchungen über Enzyme," I und II, Berlin, 1928; Nord-Weidenhagen, *Handbuch der Enzymologie*, Leipzig, 1940. J. H. Northrop, "Crystalline Enzymes", New York, 1939; C. Oppenheimer, "Die Fermente und ihre Wirkungen," I und II (1925–26). Supplement I (1926) und II (1939). Kurze Übersicht bei H. J. Vonk, *Biol. Rev.*, **12**, 245 (1937).

2. J. J. Mansour-Bek, *Z. vergleich. Physiol.*, **17**, 153 (1932); *Ebenda*, **20**, 343 (1934).

P. Krüger, *S. B. Preuss. Akad. Wiss.*, **26**, 1 (1929); *Ergebn. Physiol.*, **35**, 538 (1933).

Zusammenfassung bei H. J. Vonk, *Biol. Rev.*, **12**, 245 (1937).

3. J. J. Mansour-Bek, *Z. vergleich. Physiol.*, **17**, 153 (1932).

4. C. Romijn, *Arch. néerl. zool.*, **1**, 373 (1935).

5. B. Rosén, *Z. vergleich. Physiol.*, **21**, 176 (1935).

6. K. Linderstrøm-Lang und F. Duspiva, *Z. physiol. Chem.*, **237**, 131 (1935).

7. H. Bierry, *Biochem. Z.*, **44**, 402, 415, 426, 446 (1912).

8. W. Biedermann und P. Moritz, *Pflügers Arch.*, **73**, 219 (1898); P. Karrer und Mitarbeiter, Zahlreiche Arbeiten in *Helv. Chim. Acta*, von 1923 bis 1933.

9. P. Krüger, *S. B. preuss. Akad. Wiss.*, **26**, 1 (1929); *Ergebn. Physiol.*, **35**, 538 (1933).

10. Vgl. H. J. Jordan, "Allgemeine Vergleichende Physiologie der Tiere," 1929.

C. M. Yonge, *Biol. Rev.*, **12**, 87 (1937).

11. E. J. W. Barrington, *Phil. Trans. Roy. Soc.*, (B) **228**, 269 (1937).

12. P. B. van Weel, *Pubbl. Staz. Zool. Napoli*, **16**, 221 (1937).

13. H. J. Jordan und H. Begemann, *Zool. Jahrb. Abt. allg. Zool. u. Physiol.*, **38**, 565 (1921).

H. J. Jordan, *Handb. d. norm. u. path. Physiol.*, (Bd. 4) 1929, 167.

14. Vgl. C. Fahrenholz in Bolk c. s. *Handb. vergl. Anat. Wirbelt.*, **3**, 115 (1937).

15. Vgl. M. Phisalix, "Animaux vénineux et vénins," Paris, 1922.

16. Allgemeine Übersicht bei W. Biedermann in *Handb. d. vergl. Physiol.*, Bd. **2**, Tl. 1 (1911).

17. Letzte ausführliche Untersuchung darüber bei H. Holter und K. Linderstrøm-Lang, *Z. physiol. Chem.*, **226**, 149 (1934).

18. Übersicht bei W. R. H. Kranenburg, *Arch. musée Teyler (Haarlem)* (Sér. II), **7**, 245.

19. So z.B. bei F. Haurowitz und W. Petrou, *Z. physiol. Chem.*, **144**, 68 (1925).

20. Weitere Angaben in Oppel: *Lehrb. d. vergl. mikr. Anat. d. Wirbelt.*, **1** (1896) und Pernkopf u. Lehner in Bolk c. s. *Handb. vergl. Anat. Wirbelt.*, **3**, 349 (1937).

21. P. Legouis, *Ann. de sc. nat., zool.*, **17** und **18** (1873).

22. E. Laguesse, *Compt. rend.*, **112**, 440 (1891); *Journ. d. l'anat. et physiol.* Année, **30** (1894).

23. H. v. Swiecicki, *Pflügers Arch.*, **13**, 444 (1876).

24. Nach Leoschin, vgl. Biedermann, *l. c.* 16, S. 1353.

25. Jacobshagen, E., *Jen. Zs. Naturwissensch.*, **56** (1920).

26. A. Scheunert, *Handb. d. Biochemie*, 1909, 3.Teil, 2. Hälfte.
27. H. J. Vonk, *Z. vergleich. Physiol.*, **5**, 445 (1927); *Ebenda*, **9**, 685 (1929).
28. C. A. Pekelharing, *Z. physiol. Chem.*, **22**, 233 (1896); **35**, 8 (1902); Ders. u. W. E. Ringer, *Ebenda*, **75** (1911).
29. L. J. Geselschap, *Z. physiol. Chem.*, **94**, 205 (1915).
30. W. E. Ringer, *Z. physiol. Chem.*, **95**, 195 (1915); *Arch. néerl. physiol.*, **2** (1918).
31. H. J. Vonk, *Z. vergleich. Physiol.*, **9**, 685 (1929).
32. A. M. W. Mennega, Dissertation, Utrecht, 1938.
33. C. Oppenheimer, "Die Fermente und ihre Wirkungen," Supplement II (1939) und zwar S. 836, 784, 894 und I§§ 461, 598.
34. Vergleich dazu Vonk, *Z. physiol. Chem.*, **198**, 201 (1931).
35. L. Michaelis und H. Davidsohn, *Biochem. Z.*, **30**, 481 (1911).
36. H. J. Vonk, *Z. vergleich. Physiol.*, **5**, 445 (1927).
37. O. Rosenheim, *J. Physiol. (Brit.)*, **40** (1910).
38. C. A. Pekelharing, *Z. physiol. Chem.*, **81**, 355 (1912).
39. H. P. Wolvekamp, *Z. vergleich. Physiol.*, **7**, 454 (1928).
40. A. Rakoczy, *Z. physiol. Chem.*, **85**, 349 (1913).
41. O. Hammarsten, *Ibid.*, **56**, **18** (1908).
42. N. P. Pjatnitzky, *Ibid.*, **203**, 10 (1931).
43. H. Müller, *Pflügers Arch.*, **193**, 214 (1922).
44. S. R. Mardaschew, *Biochem. Z.*, **273**, 321 (1934).
45. Ch. S. Koschtojanz und P. A. Korjuïjeff, *Fermentforschung*, **14**, 202 (1935); P. A. Korjuïjeff, *Ebenda*, **15**, 152 (1938).
46. L. C. Chesley, *Biol. Bull.*, **66**, 330 (1934).
47. A. J. Smirnov, *Physik. med. ges. Jekaterindor* 1920 (Russ.) Zit. n. *Rona's Ber. Ges. Physiol.*, **13**, Ders. *Milit. med. Journ. d. IX. Armee* 1920 (Russ.) Zit. n. Rona ii.
48. M. Rauther, in Bronn's *Klassen u. Ordnungen d. Tierreichs*, Bd. **6**, 1. Abt., Buch, 6. Lief. (1940).
49. H. Eggeling, *Jen. Z. Naturw.*, **43**, 417 (1907).
50. M. Dobreff, *Pflügers Arch.*, **217**, 220 (1927).
51. B. P. Babkin, A. F. Chaisson, und M. H. F. Friedman, *J. Biol. Board Can.*, **1**, 251 (1935).
52. E. Weinland, *Ibid.*, **41**, 275 (1901).
53. E. Weinland, *Ibid.*, **41**, 35 und 275 (1901).
54. B. P. Babkin, *Biol. Bull.*, **57**, 272 (1929).
55. B. P. Babkin und D. J. Bowie, *Ibid.*, **54**, 254 (1928).
56. Diese ältere Literatur wird vollständig von Biedermann, *l. c.* S. 1088 ff. zitiert.
57. M. A. v. Herwerden, *Z. physiol. Chem.*, **56**, 453 (1908).
58. M. A. v. Herwerden und W. E. Ringer, *Ibid.*, **75**, 290 (1911).
59. H. J. Vonk, *Z. vergleich. Physiol.*, **5**, 445 (1927); **9**, 685 (1929).
60. M. A. v. Herwerden, *Z. physiol. Chem.*, **56**, 453 (1908).
61. A. M. W. Mennega, Dissertation, Utrecht, 1938 (Holländisch). Vgl. auch: H. J. Vonk und A. M. W. Mennega, *Acta neerld. Physiol.*, **8**, 27 (1938), und H. J. Vonk, *Erg. Enzymforsch.*, **8**, 55 (1939).
62. W. E. Ringer, *Kolloid-Z.*, **19**, 253 (1916).
63. M. A. van Herwerden, *Z. physiol. Chem.*, **56**, 453 (1908).
64. H. C. Redeke, *Tijdschr. Ned. Dierk. Ver.*, **6**, 292 (1899); *Anat. Anz.*, **17**, 146 (1900).

65. D. D. van Slyke und G. F. White, *J. Biol. Chem.*, **9**, 209 (1911).
66. L. E. Bayliss, *J. mar. biol. Ass.* (Plymouth), **20** (1931).
67. B. P. Babkin, M. H. F. Friedman und M. E. MacKay-Sawyer, *J. biol. Board Canada*, **1**, 239 (1935).
68. O. Polimanti, *Biochem. Z.*, **38**, 113 (1912).
69. M. Bodansky und W. Rose, *J. Physiol.* (Amerik.), **62** (1922).
70. H. J. Vonk, *Z. vergleich. Physiol.*, **5**, 445 (1927).
71. A. Falloise, *Arch. int. de Physiol.*, **2**, 299 (1904/05).
72. H. van Genderen u. Chr. Engel, *Enzymologia*, **5**, 71 (1938).
73. E. Schlottke, *S. B. Abh. Naturf. Ges. Rostock*, (3) **7** (1938).
74. L. C. Chesley, *Biol. Bull.*, **66**, 133 (1934).
75. L. E. Bayliss, *J. mar. biol. Ass. U. K.*, **20**, 73 (1935/36).
76. M. E. Mackay, *Biol. Bull.*, **56**, 24 (1929).
77. H. Beauvalet, *Compt. rend.*, **196**, 1437 (1933); *Compt. rend. soc. biol.*, **112**, (1933).
78. A. Karpewitch, Ref. in *Ber. Biol.*, **41**, 342 (1937).
79. H. Mann, "S. B. Ges. naturf. Fr.," Berlin, 1935.
80. J. Ishida, *Annot. zool. Jap.*, **15** (1935).
81. W. Schäperclaus, "Lehrbuch der Teichwirtschaft," Berlin, 1933.
82. W. Wunder, *Handb. der Binnenfischerei Mitteleuropas*, Bd. IIB, Physiologie der Süsswasserfische Mitteleuropas, Stuttgart, 1936.
83. O. Hämpel, *Z. Fischerei*, **24**, 1 (1926); *Ebenda*, **25**, 477 (1927).
84. E. J. W. Barrington, *Proc. Royal Soc.* (B), **121**, 221 (1937).
85. R. Alcock, *J. Anat.*, **33**, 612 (1899).
86. D. D. Van Slyke und G. F. White, *J. Biol. Chem.*, **9**, 209 (1911).
87. R. Wohlgemuth, *Allg. Fischereiztg.* N.F., **30**, 271 (1915).
88. E. S. Hathaway, *Ecology*, **8**, 428 (1927).
89. O. Riddle, *J. Physiol.* (Amerik.), **24**, 447 (1909).
90. L. Scheuring, *Z. Fischerei*, **26**, 231 (1928).
91. H. Mann, *Ibid.*, **33**, 231 (1935).
92. O. Cohnheim, *Z. physiol. Chem.*, **59**, 239 (1909).
93. C. W. Green, *J. Physiol.* (Amerik.), **30** (1912); *Bull. U. S. Bur. Fish.*, **33** 1913).
94. B. Dawes, *J. mar. biol. Ass. U. K.*, **17**, 75 (1930).
95. M. E. Mackay, *Biol. Bull.*, **56**, 8 und 24 (1929).
96. O. Hammarsten, *Z. physiol. Chem.*, **24**, 322 (1897).
97. M. H. Friedman, *J. Cell. Comp. Physiol.*, **5**, 83 (1934/35).
98. M. H. Friedman, *Ebenda*, **10**, 37 (1937).
99. L. Tinbergen, *Arch. néerl. zool.*, **4**, 364 (1940).
100. H. P. Wolvekamp, *Natuurw. Tijdschrift*, **22**, 165 (1940).
101. H. von Swiecicki, *Pflügers Arch.*, **13**, 444 (1876).
102. J. N. Langley, *Philos. Transact.*, **172**, 663 (1881).
103. H. J. Vonk, *Z. vergleich. Physiol.*, **9**, 685 (1929).
104. W. A. Kenyon, *Bull. V. S. Bur. Fisheries*, **41**, 181 (1925).
105. O. Riddle, *J. Physiol.* (Amerik.), **24**, 447 (1909).
106. A. M. W. Mennega, Dissertation, Utrecht, 1938.
107. G. Delrue, *Compt. rend. soc. biol.*, **105**, 42 (1930).
108. P. Grützner, *Pflügers Arch.*, **106**, 463 (1905).

109. J. Ch. Roux u. V. Balthazard, *Arch. d. physiol.*, **10**, 85 (1898).
110. P. Grützner, *Pflügers Arch.*, **83** (1901).
111. W. Dixon, *J. Physiol.* (Brit.), **28**, 57 (1902).
112. J. N. Langley und L. A. Orbeli, *Ibid.*, **41**, 450 (1910).
113. M. Itagaki, *Jap. J. Med. Sci. III. Biophysics*, **1**, 105 (1930).
114. L. Yüh, *Ebenda*, **2**, 25 (1931).
115. W. A. Kenyon, *Bull. V. S. Bur. Fisheries*, **41**, 181 (1925).
116. J. Junold, Inaugural-Dissertation, Leipzig, 1933.
117. H. J. Jordan und H. Begemann, *Zool. Jahrb.*, **38** (1921), *Abt. allg. Zool. u. Physiol.* S. 565.
118. H. G. K. Westenbrink und K. Gratama, *Arch. néerl. d. physiol.*, **22**, 326 (1937).
119. F. Verzár, "Absorption from the Intestine," 1936.
120. W. A. Kenyon, *Bull. U. S. Bur. Fisher*, **41**, 181 (1925).
121. J. N. Langley, *Philos. Trans. R. C.*, **172**, 663 (1881).
122. H. P. Wolvekamp, *Z. vergleich. Physiol.*, **7**, 454 (1928).

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