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PLANT METABOLISM

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CONTENTS

UNITS AND ABBREVIATIONS

The following list is included for easy reference by the reader. Abbreviations are defined when first introduced in the text.

UNITS

μ	micron = 0·001 mm
μ1	0·000001 litre
mμ	millimicron = 0·001 μ = 10 Å
Å	Ångstrom unit = 0·1 mμ
g	gravitational force
g	gram
hν	quantum
kcal	kilocalories

ABBREVIATIONS

Á	anion
AMP	adenosine monophosphate, adenylic acid
ADP	adenosine diphosphate
ATP	adenosine triphosphate
Chl	chlorophyll
DNA	deoxyribonucleic acid
DPD	diffusion pressure deficit
DPN	diphosphopyridine nucleotide, co-enzyme I. More correctly, nicotinamide adenine dinucleotide and this, abbreviated to NAD, is now being widely used in research literature.

EMP	Embden–Meyerhof–Parnas pathway of respiration
ER	endoplasmic reticulum
ΔF	change in free energy of a reaction, if negative energy is released, if positive energy must be supplied.
FAD	flavin adenine dinucleotide
G–1–P	glucose–1–phosphate
G–6–P	glucose–6–phosphate
IAA	β–indolylacetic acid
iP	inorganic orthophosphate
K_s	Michaelis constant
OP	osmotic pressure
$\sim$P	high energy phosphate bond
PGA	3–phosphoglyceric acid
Q_{10}	temperature coefficient
RNA	ribonucleic acid
RuDP	ribulose diphosphate
SF	suction force
TCA	tricarboxylic acid (Krebs) cycle
TP	turgor pressure
TPN	triphosphopyridine nucleotide, co-enzyme II. More correctly, nicotinamide adenine dinucleotide phosphate (NADP).
UDP	uridine diphosphate
UDPG	uridine diphosphate glucose
UTP	uridine triphosphate
WP	wall pressure

PREFACE

THERE is first the book and then its readers. For whom then is this book intended? Primarily I have had in mind those studying biological sciences during their first year at a University or equivalent Institution. I have therefore felt able to assume that my readers have an elementary knowledge of organic chemistry and of biology such as is acquired in the United Kingdom by preparation for the Advanced Level Examinations of the General Certificate of Education.

The biochemistry of metabolic processes has purposely been presented only in outline. This means, of course, that the University student will need to develop a parallel knowledge of biochemistry. It also means, I venture to hope, that almost all of the chapters will be within the grasp of students in our Grammar Schools, particularly those in their second and third years in the Sixth Form.

Newer aspects of metabolic physiology are now being introduced and emphasised in the Advanced Level syllabuses of Biology and Botany. The book, therefore, may be of service to those whose task it is to develop the teaching of these revised syllabuses.

Might I also hope that trained biologists working in other fields may find here a not too forbidding account of the present status of our knowledge of the metabolism of plants and that some advanced University students may be stimulated to further reading by this new presentation of familiar material.

For the presentation and for all errors I take full responsibility. I am, however, very grateful to my colleagues, Drs. Helgi Opik and E. G. Brown who have criticised the manuscript during its prepara-

tion. May I also thank Patricia Phillips and Adéle Fishman who have typed the manuscript and Marlene Jones who has helped to prepare the text figures.

I am also grateful to the many publishers and authors who granted permission to use copyright material.

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Swansea

1

INTRODUCTION

> "*It is sure that if he can add to what the eye itself reveals, an adequate mental picture of the invisible molecular events which underlie the visible, the biologist will gain increased understanding of the behaviour of every living thing.*"
>
> F. Gowland Hopkins, Lecture on *The Influence of Chemical Thought in Biology* delivered at Harvard, 1936.

LIVING organisms are built up of molecules and while organisms remain alive they are centres of intense and complex chemical activity. Their growth, development, movements and reproductive activities are the outcome of these highly complex and organised chemical changes. Visible patterns of development arise out of the invisible patterns of chemical activity. The sum total of these chemical reactions of living organisms comprises their *metabolism*. This book describes and discusses some of the more important and most actively investigated aspects of the metabolism of plants, having in mind particularly the green flowering plant.

The study of the functioning of living organisms is usually referred to as their physiology and hence the study of the functioning of plants is known as plant physiology. If you look at some text-books of plant physiology, you will find that their contents are, in nearly every case, grouped under the two main headings: Metabolism and Growth. This separation of the study of growth from the study of metabolism reflects the different status of our knowledge of these two aspects of plant physiology. The phenomena of growth and development are, however, certainly the

expressions in time and in plant structure of the changing metabolism of the organism and our, at present, limited ability to interpret growth and development in this way is discussed in Chapter 8 of the present work.

The emphasis placed on chemical activities in the opening paragraph of this Introduction draws attention to a difficult problem of demarcation; the question of the distinction between metabolism and biochemistry. When biochemistry is concerned with the structure and chemical properties of isolated compounds of biological origin and when metabolism is interpreted as embracing the integrated chemical activities of the whole organism, these two aspects of biology are clearly working at different levels of complexity. However, our understanding of metabolism comes not only from the study of whole organisms and of the structure and physiology of their individual cells but from advances in biochemistry. The student of metabolism assumes as a guiding principle that the reactions observed in isolated enzyme systems reflect physiological events and are not artefacts of isolation. Nevertheless, he uses such biochemical knowledge with discretion when attempting to interpret the physiology of living cells. From this, it follows that as the biochemist goes on to study the behaviour of complex systems containing, sometimes, many enzymes and other biological molecules and as the student of metabolism becomes concerned with the sequences of individual chemical reactions which underlie such processes as respiration and photosynthesis, the two approaches come very close together. It is sometimes argued that the distinction between the physiologist and the biochemist can be drawn by saying that the physiologist is concerned with metabolism at the levels of the cell, tissue and organism, whereas the biochemist studies the metabolic spectra of subcellular systems ranging from the organelles of the cell (nucleus, chloroplast, mitochondrion, microsome) down to single enzymes and their specific substrates. It is, however, impossible to observe strictly such a boundary when developing a discussion of plant metabolism. Nevertheless, insofar as the distinction drawn above

emphasises that the metabolic physiologist is concerned with the interpretation of the activities of living cells, it will guide the emphasis developed in the present volume. This will impose a welcome restriction on the scope of our subject matter and give a useful but not excessive overlap with present and future introductory texts of plant biochemistry.

THE METABOLIC PROCESSES INVOLVED IN GERMINATION

> "*. . . enzymes played a most important part in all metabolic changes . . . for each reserve the protoplasm was able to call into existence an appropriate enzyme.*"
>
> R. J. Harvey-Gibson. *Outlines of the History of Botany*, 1919.

Starting our story with a living mature seed and by tracing very briefly the development from this of a new and independent plant, we can review the main aspects of plant metabolism. Such a review will provide a general background for the more detailed later chapters.

The essential structures of selected angiosperm seeds are illustrated in Fig. 1. The multicellular embryo is differentiated into embryonic shoot (plumule) and root (radicle) and is associated with storage tissue from which it will receive the organic food material essential for the early growth of these essential organs. The mature seed has a low water content (10–20%) and associated with this the dormant tissues have a very low rate of metabolic activity (for instance, the rate of respiration of the dry barley grain as measured by its rate of oxygen absorption at 22°C is of the order of $0{\cdot}06\,\mu l\,0_2$/g/hr). When such a seed is placed at a suitable temperature and in the presence of oxygen and water it germinates; its embryo awakens to active life and begins to grow into the young plant or seedling. The process of germination is initiated by a rapid uptake of water leading to a swelling of the seed tissues and a stretching of the

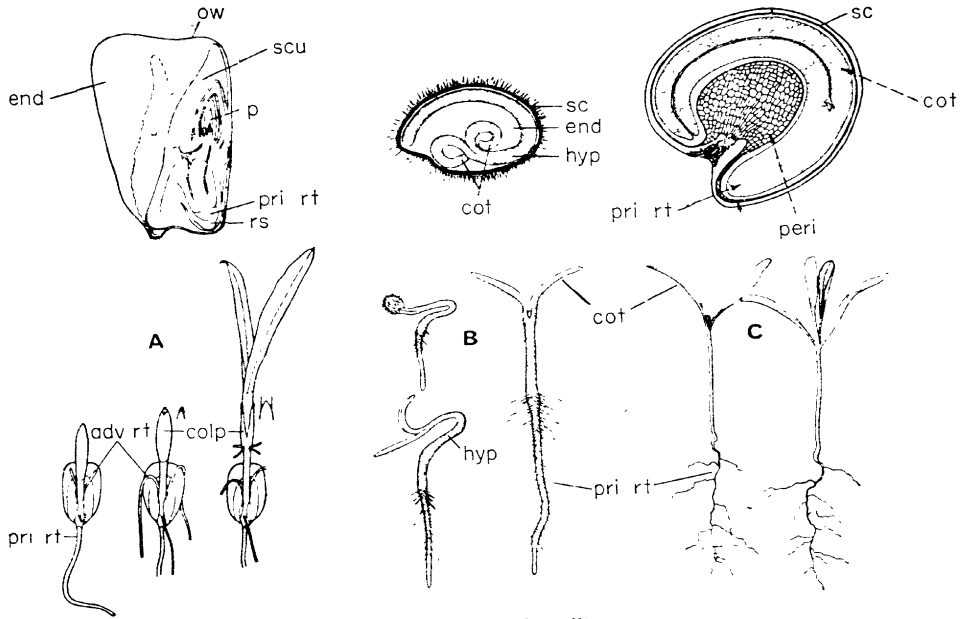

FIG. 1. Seeds and seedlings.

A *Zea mays* (maize) (germination after Avery).
B *Lycopersicum esculentum* (tomato).
C *Beta vulgaris* (beet) (seed structure after Bennett and Esau).

end=endosperm; cot=cotyledon; scu=scutellum; peri=perisperm; hyp=hypocotyl; p=plumule; pri rt=primary root; rs=root sheath; colp=coleoptile; adv rt=adventitious root; ow=ovary wall; sc=seed coat. (from H. E. Hayward. *The Structure of Economic Plants.* Macmillan, New York, 1938).

enclosing seed coat. The progress of this uptake of water with time for the seeds of barley, is shown in Fig. 2. The initial attractive force exerted by the "dry" seed for water may be very great indeed (500–1000 atmospheres) and involves a binding of water to

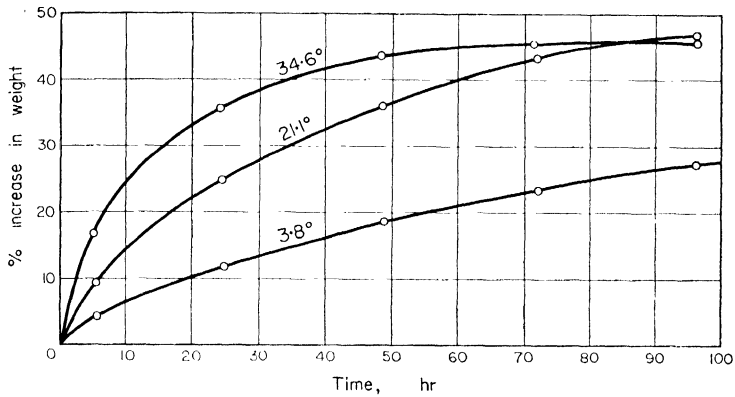

FIG. 2. The uptake of water at various temperatures by grains of *Hordeum vulgare* (barley), (after A. J. Brown and F. P. Worley. *Proc. Roy. Soc. B.*, **85**: 546, 1912).

organic molecules which can be compared to that exhibited by inorganic molecules for their water of crystallisation. This initial, *imbibitional* uptake of water leads to the liberation of a small amount of heat indicative of the loss of kinetic energy by the absorbed water molecules. As hydration of the cells proceeds, *osmotic forces* come into play and the forces motivating water uptake are of a lower order of magnitude (of the order of 10–30 atmospheres). This hydration of the tissues is associated with a rise in their metabolic activity first occurring in the radicle region of the embryo. The enhanced metabolic activity is indicated by an increased respiration rate (in contrast to the figure quoted above for "dry" barley grain, the oxygen uptake of the germinating barley grain is

of the order of $100\mu l \cdot 0_2/g/hr$). The relationship of respiration rate to moisture content in the oat grain is shown in Fig. 3.

In some grains, such as maize, the soluble sugar, sucrose, has been shown to be uniformly distributed in the dry embryo and reducing sugars like glucose cannot be detected in appreciable amounts until the embryo begins to elongate. Sucrose may, therefore, be the

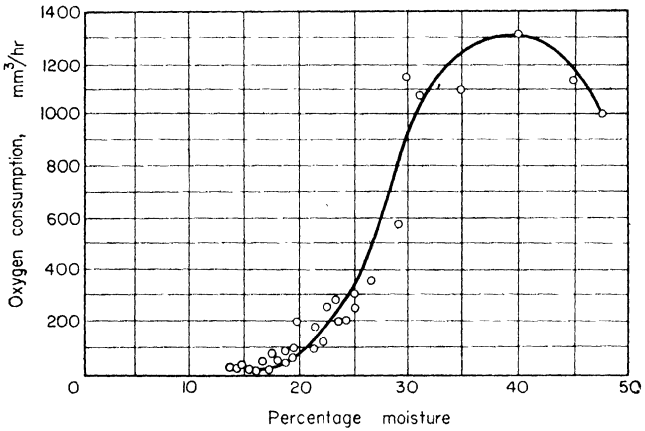

FIG. 3. The relationship between water content and the rate of respiration of germinating grains of *Avena sativa* (oat). Oxygen uptake determined at 25°C expressed as mm^3 per hour per 10g dry weight, (after A. C. Bakke and N. L. Noecker, 1933. *Iowa Agr. Expt. Sta. Res. Bul.* **165**: 319).

initial respiratory substrate involved in the rise in respiration rate. The activation of the metabolic process of respiration early in germination implies not only the availability of a respiratory *substrate* like sucrose in the embryo but the activation of the essential biological catalysts termed *enzymes*. These enzymes are protein molecules and hydration of these molecules is essential for their activity. Thus, respiratory enzymes are present preformed in the mature "dry" embryo and become active during the imbibitional phase of water uptake.

Now, following the uptake of water, the cells of the embryo become active, expand and even begin to elongate before the seed coat is ruptured or the main food reserves of the grain are mobilised. The food reserves stored in cotyledons or endosperm or less frequently in perisperm tissue are mainly in an insoluble form as polysaccharides (particularly as starch), as fats in the form of oil globules or as granules of protein. Their ultimate utilisation to promote the growth of the tissues of the embryonic root and shoot depends upon their conversion to soluble compounds and the transport of these compounds to the regions of cell expansion and cell division in the embryo. The conversion of insoluble food substances to simpler soluble compounds is another expression of enzymic activity. Some of the enzymes involved are present in the dry storage cells and are, like the respiratory enzymes, activated during the phase of rapid water uptake by imbibition. Other enzymes involved in the mobilisation of food reserves are, however, synthesised during the germination process. This can be illustrated by reference to the digestion of starch grains (which contain two polysaccharides; amylopectin and amylose, the latter usually constituting 15–35% of the grain). Probably in all, at least four enzymes are involved in the degradation of starch to the soluble monosaccharide, glucose. The two involved in the initial attack are *α-amylase* and *β-amylase* and their actions are, to an extent, complementary. α-amylase attacks both amylose and amylopectin to give rise to a complex mixture of molecules of lower molecular weight, called dextrins. β-amylase releases disaccharide units (maltose) from these compounds. Studies of α-amylase and β-amylase activity in germinating grains of *Avena sativa* show that the β-amylase is preformed in the grain and is activated during the initial rapid uptake of water. α-amylase development is, however, dependent upon synthesis of enzyme protein and this synthesis takes place in the endosperm cells in response to a stimulus from the germinating embryo. The observation that the embryo promotes enzyme formation in the endosperm is one of considerable interest for if we could fully understand the nature of the stimulus involved

it might be the starting point for a striking advance in our knowledge of the germination process. It would also contribute to our understanding of an important aspect of the physiology of multicellular organisms, that of how the reciprocal metabolic relationships between tissues are mediated. At present we have only an indication to suggest that the embryo cells supply to the endosperm cells, peptides (chains of amino acids) which could be then used as building blocks for the enzyme protein.

This discussion of the activities of the amylases enables us to draw attention to an important property of all enzymes. Proteins lose irreversibly their essential biological properties when heated. This denaturation of proteins, which involves a change in molecular architecture rather than in chemical composition, can also be brought about by irradiation in the ultra-violet, by short exposures to extremes of acidity and alkalinity or even by mechanical agitation in solution. Enzymes are, therefore, inactivated by heat; they are thermolabile. However, the exact temperature conditions which cause thermal denaturation are characteristic of each individual enzyme. Such a difference in the heat instability of the amylases was exploited in the researches mentioned above on the changing activity of these enzymes during germination. It was possible, by heating the seedling extract to 70°C for 20 minutes, to completely inactivate β-amylase without significantly reducing the α-amylase activity.

The germination of the embryo initiated by the utilisation of its own food constituents is now maintained by the larger supply of soluble compounds generated by enzyme activity in the storage tissues of the seed. Not only does the embryo stimulate the development of enzymes in the storage tissue but it directs the flow of soluble compounds from the storage cells to its own growing regions. The storage cells of the endosperm or cotyledons have a relatively low rate of respiration but by the action of their hydrolysing enzymes they become enriched with soluble food substances. The movement or translocation of these substances to the embryo is, therefore, movement from a region where they occur at high

concentration to a region where they are rapidly consumed in embryo metabolism. The storage tissue is a "source", the embryo a "sink" for these essential compounds. This, however, neither specifies the nature of the actual compounds which flow to the embryo nor the mechanism of the translocation process. The storage fats are probably attacked by "lipase" enzymes and the fatty acids so produced are converted to sugar. This raises the possibility that both storage polysaccharides like starch *and* fats are ultimately converted to the disaccharide, sucrose (cane-sugar) since there is very strong evidence that it is as sucrose that the carbon of carbohydrates is transported in the phloem of the conducting strands. In studies on barley germination there is the corroborative evidence that the embryo preferentially utilises sucrose for respiration and to build its cell walls.

The proteins of the storage cells are acted upon by a group of proteolytic enzymes to yield a mixture of free amino acids, together with the amides of glutamic acid and aspartic acid (glutamine and asparagine) and, probably, simple peptides (compounds composed of small numbers of amino acids linked together through peptide bonds). Whether all these simple organic nitrogen compounds are equally translocated is not known but the evidence does not point to a single transported compound as is the case for carbohydrates. Further, we know that the embryo cells rather than the storage cells contain the enzymes which promote the interconversions of amino acids required to produce the particular mixtures of amino acids involved in the synthesis of the proteins of the embryo cells. In quantitative terms the transport of carbohydrates and of soluble organic nitrogen from the storage cells is dominant. The storage tissues are, however, sources of other substances essential to the embryo, including phosphorus compounds and certain essential vitamins. Further, there is strong evidence that the growth hormones equally essential for the growth of the embryo are formed from precursors which are released from the storage cells early in the germination process. These growth hormones in their active form occur at the points of most active growth within the embryo and

may, in some way not yet understood, direct the flow of soluble food substances to these growth centres.

The exact nature of the translocation of organic solutes has yet to be satisfactorily worked out but current views on possible mechanisms involved will be discussed in a later chapter (Chapter 6, p. 157). Clearly, in the germinating seed food materials must pass from cell to cell within the storage tissues, be conducted within the vascular strands of the developing seedling and pass out from these strands to the dividing, expanding and differentiating cells of the growing regions. Longitudinal transport of organic solutes within the vascular strands takes place predominantly in the sieve tubes of the phloem. One very strong possibility is that within these special conducting units transport of materials is effected either by a mass flow involving the bulk of their fluid contents or by "channel" or "strand" flow of fluid and cell particles (these latter could be special "carrier" particles) within a mainly stationary fluid mass. There may well be, in the near future, a "break-through" in our understanding of the mechanism of sieve tube transport following improved techniques for the microscopic observation of living phloem tissue and the application of the electron microscope to the study of sieve tube structure. Entry of food materials into the phloem at points adjacent to the storage tissue and their removal in the regions of active growth of the seedling probably involves processes of secretion and absorption which demand energy derived from the respiration of living cells. Such movements of substances across cell boundaries often in directions opposed to concentration gradients and at speeds altogether more rapid than would occur by diffusion are often referred to as "*active*" movements. Work is done by the cell in promoting such "active" movements and in performing such work a redistribution of the energy released by respiration occurs. There is now an impressive body of evidence that the absorption and secretion by plant cells of both inorganic ions and organic substances are "active" processes in this sense. The movement of organic solutes *within* living tissues such as the growing regions of the young shoot and root of the seedling and

through the storage tissues of the seed probably, at some points, involves diffusion, at others, "active" transport across cell membranes, and at others, mass movement over longer distances by protoplasmic streaming within the cells. It remains a controversial question as to whether any flow of fluid occurs in the fine cytoplasmic strands (*plasmodesmata*) which interconnect the protoplasts of adjacent cells in all living plants.

The cells of the embryo are capable of increase in size (expansion growth) and differentiation; many of them are capable of cell division (mitosis). With the increase in their water content which occurs during the initial phase of germination this expansion growth commences and is soon reinforced by the formation of new cells, first at the apical growing point of the root and then at the corresponding growing region of the embryo shoot. These processes of cell division and cell expansion are dependant upon a release of energy and of essential small reactive molecules from the complex food substance (first of the embryo cells and later of the storage tissues). In *the process of growth* this energy and these reactive molecules, both of which arise from a degradation of complex food substances, participate in the synthesis of new cell material, particularly of the protein and lipid molecules essential to protoplast structure and of the complex polysaccharide and polyuronic acid molecules of the cell wall. The metabolism of the embryo cells, set in motion by the absorption of water, involves a multitude of interconnected chemical reactions. Some of the reaction sequences result in the degradation of complex food molecules (this aspect of metabolism is called *catabolism*), other sequences result in the synthesis of the unique molecules from which living structures are built (*anabolism*).

To illustrate the activation of metabolism which follows the uptake of water by the air-dry seed we quoted rates of respiration and expressed these in terms of oxygen absorption per unit of time. For most seeds oxygen is as essential as water for germination because of its requirement in the process of *respiration*. This catabolic process, whereby food materials are degraded and their energy

released, is an oxidative process. The storage carbohydrates, fats and proteins serve as substrates for respiration and are oxidised to yield as end products carbon dioxide and water. Oxygen absorption and carbon dioxide evolution proceed continuously at the respiratory centres within the living cells. During respiration, energy locked up within the structure of the food substance molecules is released. Some of this energy is dissipated as heat and this evolution of heat is most easily demonstrated by confining a mass of germinating seeds in a Dewar flask. Some of the released energy is, however, conserved because the degradation of the food substance molecules is linked at the respiratory centres to a simultaneous synthesis of certain energy-rich phosphorus compounds which act as mobile "power-houses" able, at other points in the cells, to "drive" the synthetic reactions of anabolism. Further, during the degradation of respiratory substrates many reactive intermediate compounds arise and some of these are withdrawn from the oxidative pathways of degradation to be used as "raw materials" for the synthesis of new essential organic molecules like the enzyme and structural proteins of the cell.

The swelling of all the living cells within the seed combined with the early extension growth of the embryo root results in the rupture of the seed coat. The young root emerges and quickly penetrates downwards into the soil. The rapid downward growth of the root anchors the seed and immediately creates an absorbing surface for the uptake of water and of essential inorganic ions from the soil. This is soon followed by the upward growth into the air of the young shoot and the opening of its first green leaves. The establishment of the root in the soil and the emergence into the light of the young shoot enables the seedling to draw upon its external environment for all its *essential nutrients*. The organic food materials of the seed, essential for the establishment of the young plant, are then no longer required for its further growth and development.

The carbon essential for the formation of all organic compounds is available to the green plant as the gas, carbon dioxide. This gas

is present in the air in low concentration (3 parts in 10,000 by volume) and its absorption by land plants takes place via their green leaves and young stems. Submerged water plants obtain their carbon dioxide already in solution from the water in which they live. Water is essential to all living organisms as the medium in which essential cell constituents dissolve and move and because the biological properties of many molecules like those of the proteins depend upon their association with water molecules and with hydrogen and hydroxyl ions. Water is also the source of hydrogen and oxygen atoms and these atoms are second only to carbon in importance in the architecture of organic molecules. In the land plants this water is absorbed mainly from the soil by the root system and transported to the shoot via the conducting elements of the xylem. The molecules of protein contain, in addition to carbon, hydrogen and oxygen, the elements nitrogen and sulphur. Many other essential organic molecules contain nitrogen and/or sulphur. The lipo-proteins involved in the formation of cell membranes and the nucleoproteins involved in the transmission of hereditary characters and in enzyme synthesis also contain phosphorus. Other phosphorus compounds, as already mentioned, play an essential part in the energy relationships of cells. Calcium is essential for the proper formation of the middle lamella. Magnesium is contained in the molecules of the chlorophylls, the essential photocatalysts of photosynthesis and also is implicated as part of certain enzyme systems. Potassium is an essential and mobile element within the plant. Active leaves and growth centres are usually rich in potassium. Although there is some evidence that it is involved in protein synthesis we do not know of essential potassium-containing organic molecules or of enzyme systems whose activity depends upon the presence of potassium. The essential elements discussed above are all required in differing but moderately large amounts for the growth and development of the green plant. There are, however, a number of other essential elements which are required in relatively very small amounts, as *micro* rather than as *macro*-nutrients. The recognised micro-nutrient elements are: iron,

manganese, zinc, copper, boron molybdenum and chlorine. It seems that in all cases these micro-nutrients are essential to plant growth because they function as essential constituents of particular enzyme systems. These essential macro-nutrient and micro-nutrient elements from nitrogen to molybdenum are absorbed as anions and cations from the soil or water in which the plant is growing.

Reference has already been made to the osmotic nature of the water uptake by the fully imbibed seed and osmotic forces are also involved in the absorption of water from the soil by the seedling root. Further discussion of the mechanism of water uptake by plant cells and of the movement of water through plant tissues will, however, be best deferred to the next chapter (p. 28) where the central theme will be the relationship between cell structure and cell function. Reference has also already been made to salt absorption, citing it as an "active" process, a process dependent upon the energy released in respiration. The "active" nature of salt absorption is emphasised by two important aspects of this process. Firstly, the absorption of anions and cations is *selective*; they are absorbed in amounts not determined by their relative concentrations in the soil or other nutrient solution. Secondly, the uptake of ions leads to their *accumulation*, to the development of internal concentrations of free ions within the cell often vastly in excess of their concentrations in the external solution. This selective accumulation of inorganic ions by the absorbing cells of the seedling root raises the whole question of how the energy released in respiration is harnessed to perform cellular work, in this particular case to power a number of selective "salt pumps". Later, when we explore further the inter-relationships between metabolic processes it will be appropriate to examine critically how far we really understand the mechanism of salt uptake by plant cells (Chapter 6, p. 157 *et seq.*).

The essential elements absorbed by the root are not only involved in its own metabolism, but are, in part, translocated either as free ions or as soluble organic compounds to the growing regions of the shoot, being metabolised most actively in its apical meristems and developing leaves. The root therefore plays its special role in

the nutrition of the whole plant. In turn and to an increasing extent, as the organic food reserves passed on from the parent plant are depleted, the development of the young plant becomes dependent upon the synthetic activity of its leaves and green stems, upon the primary synthesis of organic molecules from carbon dioxide and water by *photosynthesis*. Respiration has been described as an oxidative release of the energy locked up in the structure of organic molecules, such as in the molecules of sugars. Photosynthesis is, by contrast, a reductive process in which the energy-rich molecules of sugar are built up from carbon dioxide and water. The *overall* equation for both these processes can therefore be represented

$$\underset{\text{sugar}}{C_6H_{12}O_6} + 6O_2 \underset{\text{photosynthesis}}{\overset{\text{respiration}}{\rightleftharpoons}} 6CO_2 + 6H_2O \qquad (1)$$

Further, as clearly indicated by its name, the unique phenomenon in photosynthesis is not the assimilation of carbon dioxide, nor the synthesis of sugars but the utilisation for these processes of the radiant energy of the sun. This means that photosynthesis involves, in contradistinction to the other metabolic processes so far mentioned certain *photochemical* reactions, insensitive to temperature and mediated through a photocatalytic system. The chlorophyll pigments organised in special cellular structures called chloroplasts represent this system and give to photosynthetic tissues their green colour.

Study of the reactions involved in respiration has revealed that the oxidation of organic food substances is linked to the reduction of a special pyridinedinucleotide molecule (a co-enzyme) and that much of the available energy becomes concentrated in the reduced molecules of this compound. The subsequent regeneration of the co-enzyme by oxygen not only results in the formation of water (Eqn. 1) but is coupled to the simultaneous synthesis of an energy-rich phosphorus compound (a polyphosphate of the nucleotide, adenylic acid, the substance, *adenosine triphosphate*—abbreviated to **ATP**). This compound is one of those phosphorus compounds

which can act as a mobile "power house" and drive the synthetic reactions of anabolism. It is, therefore, of great interest that it has recently been shown, particularly in experiments with isolated chloroplasts, that the photochemical reactions of photosynthesis result in the synthesis of ATP and in the reduction of a pyridinedinucleotide co-enzyme. The energy trapped in ATP and the reducing power of the reduced co-enzyme then effect carbon dioxide assimilation and reduction by thermochemical (enzyme-activated) reactions which can occur in the absence of light (are "dark" reactions) and even in non-photosynthetic plant cells. There is a common energy currency in all plant cells, photosynthetic cells have a unique way of minting this currency.

Once its leaf area is sufficiently developed the young plant acquires the ability to grow and develop when supplied with its essential nutrients entirely in inorganic form. It becomes independent of the organic food reserves of the seed. The sugars synthesised in the chloroplasts become the primary sources of energy and of reactive intermediates for the synthesis of all the multitude of other organic molecules which go to make a living cell. From the leaves the sugars which are in excess of those required for the nutrition of the leaf cells pass via the sieve tubes of the phloem and in the form of the disaccharide, sucrose, to nurture the growing points of the shoot and to the root.

With the emergence of the shoot of the seedling into the air not only does photosynthesis become possible but there occurs an inevitable loss of water from the plant by evaporation, a process termed *transpiration*. Transpiration is not only a consequence of the exposure of the external surface of the shoot system to the air but is enhanced in magnitude by the development in the shoot surface of breathing pores (stomata) which facilitate the exchange of oxygen and carbon dioxide between the shoot tissues and the external air. The stomata are bordered by special cells (guard cells) whose size and form can change in a way which increases or decreases the pore area or closes the pore completely. This regulation of stomatal aperture is of importance both in the retention of

respiratory carbon dioxide for subsequent photosynthesis and for water conservation at times of drought.

If the water content of the actively metabolising leaf cells is not to fall significantly as a result of transpiration a continuous flow of water to the leaves must be set in motion; a "transpiration stream" must take place in the xylem strands stretching from the veins of the leaf down to the absorbing regions of the root. Such a transpiration stream will inevitably not only demand an enhanced water uptake by the root but will lead to transport of inorganic nutrient ions in the mass flow of liquid in the conducting cells of the xylem. A cut shoot set under conditions conducive to active transpiration absorbs water at the cut stem surface with considerable force, a suction pull for water is set up in the shoot system. The attraction for water develops in the leaf cells as water is evaporated from their surfaces into the air space system of the leaf tissue and is transmitted down through the continuous columns of liquid in the xylem vessels and tracheids. The removal of a plant from a water-saturated to a dry atmosphere leads to a marked enhancement of the rate of water absorption and to the force with which water is removed from the soil. Under conditions of active transpiration, plant roots may desiccate the soil to the point where it retains its small residual water content with a force as high as 15 atmospheres. There is strong evidence for the view that under such conditions of active transpiration, a *tension* is developed in the xylem fluid and that then the root acts in water absorption purely as a physical system, a wick or sponge, from which water is withdrawn by the transpiration of the leaf cells. Water absorption by the root under these conditions has, therefore, been described as a "passive" process.

However, during the passage of the seedling shoot through the soil its water supply cannot derive from transpiration. The same applies during the initial spring growth of perennating organs or during the early growth of the buds of shrubs and trees in the spring. Further, many seedlings and herbaceous plants when placed in a saturated atmosphere show a release of droplets of water from their leaves (the phenomenon of guttation). Under these conditions

there is evidence that the upward movement of water follows from the development of a positive pressure (a *compression*) of water in the xylem; a positive root pressure. Further, this positive root pressure is only demonstrable when the temperature and oxygen supply are favourable to root metabolism, supporting the contention that a positive root pressure is indicative of an "active" water uptake by the root.

By means of the above outline discussion of the physiology of germination and of seedling establishment, it has been possible to introduce or remind the reader of the major metabolic processes which proceed in the green plant. This enables us to pose most of the problems which will be considered in the present volume. This can perhaps best be illustrated by formulating a number of important questions which arise directly out of the present chapter and which will to some extent be answered in the subsequent chapters.

What is the significance of the fact that the reactions of living cells are catalysed by a large number of specific enzymes? What special properties of protein molecules make them able to play this unique catalytic role in plant metabolism? If enzyme proteins catalyse the synthesis and degradation of sugars and other carbohydrates, of fats and other lipids, of organic and amino acids, of vitamins and of the many other essential organic molecules of the cell, how then are the enzyme molecules themselves synthesised? If these enzymes control metabolism, is it through the controlled synthesis of enzymes that the genes of the nucleus control plant growth, form and development? Are compounds generally, always or never degraded and synthesised by the same chemical reactions? If there is a common pathway of synthesis and degradation what controls the direction of metabolism along such a pathway? As an example, and leaving aside the photochemical reactions, are the reactions involved in the photosynthesis of sugars the reverse of those in sugar respiration? How does the cell convert radiant into chemical energy in the process of photosynthesis? How exactly is energy conserved in the cell and transferred from energy-yielding to energy-absorbing reactions and with what efficiency is this

cellular redistribution of energy effected? If energy can be transferred from one chemical system to another in this way does this explain how cells perform the work which must be involved in such "active" processes as cell growth, growth movements, accumulation of solutes against concentration gradients, and rapid transport of organic molecules within the plant? Where do the reactions of metabolism take place within the cell and is cell structure capable of being interpreted in terms of the maintenance of orderly patterns of degradation and synthesis within the quite extraordinary complex chemical factory represented by the plant cell? What determines that some cells remain meristematic while others undergo enlargement and change in the several ways which give rise to the diverse types of tissue cells found in the body of the green flowering plant? What are the nutritive and other physiological interrelationships between the different tissue systems? Why is the duration of life of the multicellular plant finite or, phrased another way, can we explain in metabolic terms senescence and death?

The extent to which we can answer such questions and others which the thoughtful reader will formulate for himself, is the measure of our present understanding of plant metabolism.

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2

CELL STRUCTURE AND FUNCTION

"The vital processes of the individual cells form the first indispensable and fundamental basis for both vegetable physiology and comparative physiology in general." M. J. Schleiden (1838)

"We have seen that all organisms consist of essentially like parts, the cells . . . each cell . . . capable of developing independently if only there be provided the external conditions under which it exists in the organism. . . . The question as to the fundamental power of organised bodies resolves itself into that of the individual cells." Theodore Schwann (1839)

INTRODUCTION

THESE writings of the botanist Schleiden and the zoologist Schwann, from which our above quotations are taken, formed the first clear expression of the view that the properties of complex organisms are an expression of the separate activities of their cells, each cell living in the special environment created by the association together of the total cell population. This enunciation of the "cell theory" had, as its background, the brilliant researches of earlier microscopists right back to the first use of the term "cell" by Robert Hooke in 1665.

The early microscopists recognised that living material was built up from, or divided into, minute compartments or cells but not until the 19th century were microscopes of sufficient resolving power available for the living contents of cells to be observed.

Robert Brown first observed the nucleus in the epidermal cells of certain plants in 1831. The recognition of the general occurrence of a thin layer of mucilaginous material within the cell boundary and the use of the term *protoplasm* for this substance, which was only to be seen in life (and not like the cell boundary or wall also in death), followed from the gifted studies of Hugo von Mohl in the middle of the 19th century. Still further progress followed upon the manufacture and distribution of Abbé microscopes by Zeiss of Jena from around 1878. It was from studies with microscopes of this kind and by using basic aniline dyes as stains that Eduard Strasburger first described the chromosomes of the nucleus and their behaviour during the division of cells. Such microscopes made possible very rapid advances in our knowledge of the range of cell structure and form in plant tissues and laid the foundations of our present knowledge of nuclear and cytoplasmic structure as they can be seen with the ordinary light microscope with its resolving power of around 0·275 μ (2750 Å).

Cells in the living state have always been difficult objects to study microscopically because they and their contents are for the most part transparent to visible light. On the other hand, examination of fixed and stained cells always raises the problem of how far the structures seen correspond to structures in the living cell. Various extensions of optical microscopy have, therefore, been developed to overcome the lack of contrast within the living cell. The use of polarised light has enabled cell structures in which the molecules are highly orientated e.g. the layers of the cell wall, and the lamellae of plastids, to be recognised by virtue of their birefringence. Perhaps more important has been the development of the phase-contrast microscope by means of which such processes as nuclear and plastid division and the movement and change in form of bodies like mitochondria can be followed and photographically recorded in the living cell. In the phase-contrast microscope we use a monochromatic light source, reduce the intensity of the directly transmitted component of the light and retard the deflected beam to bring it a half wave-length out of phase with the transmitted light

and, thereby, through destructive or constructive interference, yield strong contrasts. The resolving power is not diminished, the contrast is dramatically increased.

One of the factors limiting resolution in the light microscope is the wave-length of visible radiation and, therefore, to increase resolution it is necessary to use radiation of shorter wave-lengths. This led Köhler to devise in 1904 an ultra-violet microscope involving a monochromatic source of ultra-violet radiation, lenses of fused quartz which will transmit radiation down to a wave-length of 2400 Å and photographic recording of the images. Such ultra-violet microscopes have, however, been used in cytological work not so much because of their increased resolution but because when combined with a microspectrophotometer they permit estimates of changes in the nucleic acid content (characteristically absorbing in the region of 2600 Å) in the nucleus and cytoplasm during cell division and differentiation.

The search for higher resolution has found its modern answer in the electron microscope whose resolution is more than 200 times greater than that attainable with light. The electron microscope uses a beam of high speed electrons focused by electromagnetic lenses. To be examined in the electron microscope the specimen must withstand evacuation (living material is excluded) and be unusually thin. To prepare ultra-thin sections, special microtomes are used which employ glass or diamond knives and the material, usually fixed in neutral osmic acid, must be embedded in a plastic which is polymerised after the monomer has penetrated the dehydrated cells. There is still a need to devise methods of dehydration and embedding which do not distort or disrupt cell structures. Nevertheless, the picture of cell structure revealed by electron microscopy can be accepted with growing confidence as it increasingly links up with knowledge obtained from other forms of microscopy and from studies of the chemistry and metabolic activity of fractions isolated from cell extract by high-speed centrifuging.

In this chapter we shall consider the structure of plant cells as revealed by light (resolving power down to $\simeq$ 3000 Å) and electron

(resolving down to $\simeq$ 15 Å) microscopy and consider what we can learn of the relationship of structure to function by observations on the intact cell and on cell "fractions" isolated by centrifuging.

GENERAL STRUCTURE OF PLANT CELLS

Many different kinds of cells make up the body of a flowering plant. All the various specialised cells, are however, derived by cell division and subsequent enlargement and differentiation from groups of meristematic cells situated at the apices of the root and shoot or making up the vascular and cork cambia of the larger root and shoot axes. The meristematic cells of the apical growing points are roughly isodiametric and frequently not more than 10μ in diameter, those of the cambia are small rectangles in transverse section but are elongated, often 100μ or more in length. Such cells have thin *cell walls*, rich in hemicelluloses and pectin. Within the cell wall the living material (the *protoplast*) consists of *cytoplasm* and a large *nucleus*, the latter occupying 2/3 to 3/4 of the protoplast volume. Minute *vacuoles* (rich in fatty or proteinaceous material), spherical or cylindrical *mitochondria* and *proplastids* can be distinguished by appropriate techniques in the ground mass of the cytoplasm (the *hyaloplasm*). Mature tissue cells, such as parenchymatous cells are derived from such cells. The marked increase in cell size (this may be several hundred fold) and the changes in protoplast and wall structure which lead to the development of a parenchymatous cell exemplify the processes of cell growth and differentiation. Further, since cells involved in water and salt absorption and in photosynthesis and food storage are of this kind their structure is of great interest to the physiologist.

Figure 4 shows some drawings of parenchymatous cells, in which the structures described below are labelled. The *cell wall* is composed of a framework of the polysaccharide cellulose. The water content of the wall accounts for 92–94% of its fresh weight. Associated with the cellulose (constituting perhaps 25% of the dry weight)

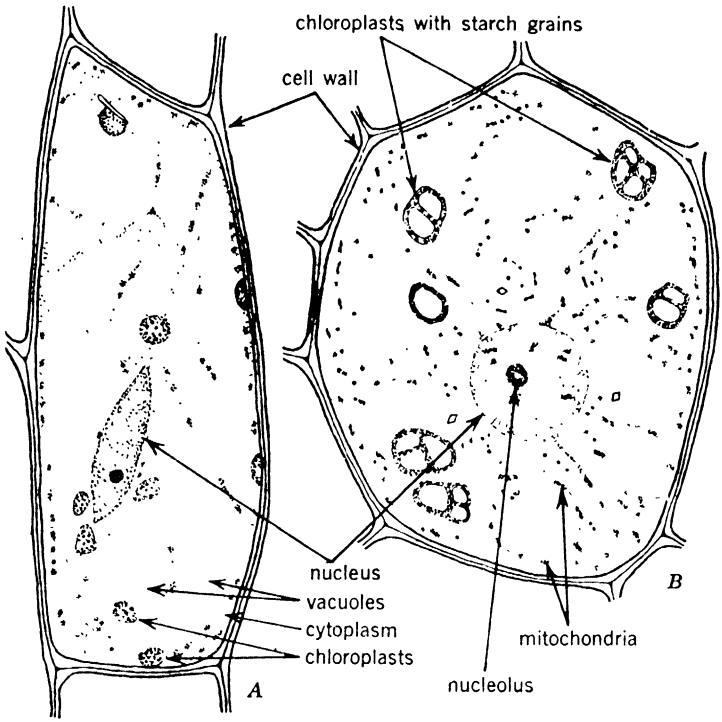

FIG. 4. Structure of parenchymatous plant cells. A, cell from the petiole of a sugar-beet leaf. It has vacuolated cytoplasm with mitochondria, chloroplasts and nucleus. B, starch sheath cells from young stem of tobacco, showing prominent starch grains in the chloroplasts. (Both × 1190), (from K. Esau. *Plant Anatomy*. John Wiley & Sons, Inc. New York, 1953).

are variable quantities of hemicelluloses, pectins, protein and fats. During the marked increase in size that accompanies the differentiation of a parenchymatous cell, the cell wall grows. The increase in cell wall area is accompanied by an increase in the amount of cell wall substance and there is usually a further increase in cell wall material after cell enlargement is complete.

The process of cell enlargement is associated with a massive uptake of water and as this takes place the vacuoles fill with fluid, become more prominent and ultimately fuse to give a large central vacuole which may occupy at least 90% of the total cell volume. The vacuolar fluid, often referred to as the cell sap, is a solution containing inorganic ions and soluble organic substances like sugars, organic acids and amino acids. The cytoplasm also increases in amount during the growth of the parenchymatous cell. This is indicated by the increase in protein content of the cell during the expansion phase of its differentiation. Nevertheless, the cytoplasm rarely occupies more than 5% of the total cell volume and typically takes the form of a thin layer (often not more than 5μ thick) immediately within the cell wall and surrounding the central vacuole. Some 95% of the fresh weight of cytoplasm is water, and some 3% is protein. On the assumption that one million can be taken as an average figure for the molecular weight of protein molecules, it has been calculated that a mature cell of the root cortex may contain some 10^8 protein molecules and at this concentration some 20 successive mono-molecular layers of protein could be present in the thickness of the cytoplasmic layer. The nucleus (8–10μ diameter) is contained in the cytoplasmic layer or suspended in the centre of the cell by trans-vacuolar strands of cytoplasm.

The lining layer of cytoplasm has a granular appearance due to the presence of various inclusions or cellular "particles". Some of these, the *mitochondria*, are spherical or cylindrical bodies with a maximum dimension up to 2μ and characteristically stained in the living cell by the "vital" stain, Janus Green B. Others of similar size but not usually stained by this dye are regarded as *proplastids*. These are the bodies from which it seems develop, during differentiation, the various forms of plastid, most important of which are the *chloroplasts* (in photosynthetic parenchyma) and the starch-forming *amyloplasts* (of storage parenchyma). As will be discussed later, mitochondria and plastids are centres of metabolic activity and can increase in number by division. They are constant features

of the cytoplasmic complex. By contrast, other inclusions are inactive and represent reserve food substances or by-products of cell metabolism. Examples of this type of inclusion are starch grains, protein "crystals" (aleurone grains) and minute oil droplets. These various inclusions are suspended in the optically clear groundmass of the cytoplasm, the *hyaloplasm*. This hyaloplasm, from its staining properties, is clearly rich in protein and shows physical properties (such as reversible sol $\rightleftharpoons$ gel change at constant temperature) corresponding closely with those of a hydrophilic colloidal system in which the disperse phase is composed of fibre-like units capable of weak chemical association.

The surfaces of contact between the cytoplasm and the cell wall and between the cytoplasm and the central vacuole seem to be almost free of visible inclusions and appear from staining reactions to be relatively rich in lipoidal (fatty) material. By appropriate treatment the fatty material at the vacuolar–cytoplasmic surface can, in some cases, be demonstrated by causing the formation of myelin processes extending into the vacuole—such myelin processes are strongly indicative of the presence at the interface of phospholipids like lecithin. By plasmolysis, followed by micromanipulative rupture of the cell wall, naked protoplasts can be obtained. When such naked protoplasts are immersed in potassium chloride solution the cytoplasmic layer can be dispersed. The result is to isolate the aqueous vacuole. This, however, does not immediately disperse and the spherical mass can be induced to swell or shrink by changing the osmotic pressure of the bathing solution. Dispersion, however, immediately follows the introduction of a fat solvent and minute oil droplets appear. The vacuole appears to be enclosed by a membrane (the *tonoplast*) which has semi-permeable properties, elasticity and an essential content of lipids. Experiments with dyes like acid fuchsin and aniline blue also suggest the occurrence of a membrane (the *plasmalemma*) at the cell wall–cytoplasm surface. These dyes do not readily penetrate cells and if injected into the vacuole or the lining layer of cytoplasm spread only within the limits of each phase. There seem to be barriers to their diffusion at

both cytoplasmic surfaces. Micromanipulation has also demonstrated the presence of a well-defined elastic membrane (the *nuclear envelope*) at the surface of the nucleus.

By grinding up (homogenising) plant cells in an appropriate medium (such as a buffered sucrose solution of appropriate strength) it is possible to obtain a *brei* in which nuclei, plastids and mitochondria can still be distinguished. By centrifuging such a brei at successively higher speeds we can sediment in turn the cell wall fragments, the nuclei, the plastids and the mitochondria. The behaviour of these isolated cytoplasmic inclusions strongly points to the presence at their surfaces of differentially-permeable, elastic, lipid-rich membranes. The cytoplasmic complex is rich in membranes and studies on the distribution within this complex of absorbed radio-active substances strongly suggests that these membranes restrict the movement into and within the cell of molecules and ions.

Cells in tissues are firmly cemented together by the *middle lamella*. Therefore, each protoplast is separated from its neighbouring protoplasts by the two intervening cell walls and the middle lamella between them. Nevertheless, in many tissues, the light microscope reveals the presence of many fine strands of cytoplasm (0·2–0·5 μ thick) crossing this wall system and maintaining cytoplasmic continuity between the tissue cells. These cytoplasmic strands or *plasmodesmata* (Fig. 5) are frequently concentrated together in certain areas and it is apparently at such sites that pits develop when the initially thin walls are thickened during subsequent differentiation. It has been calculated that a meristematic cell (about 20 μ long on each face) from the root of onion is probably connected with adjacent cells by some 20,000 cytoplasmic strands

THE OSMOTIC BEHAVIOUR OF CELLS

Work related to the phenomenon of osmosis dates back to the researches of the Abbé Nollet in 1748. Pfeffer was, however, the first to study quantitatively the movement of water into solutions

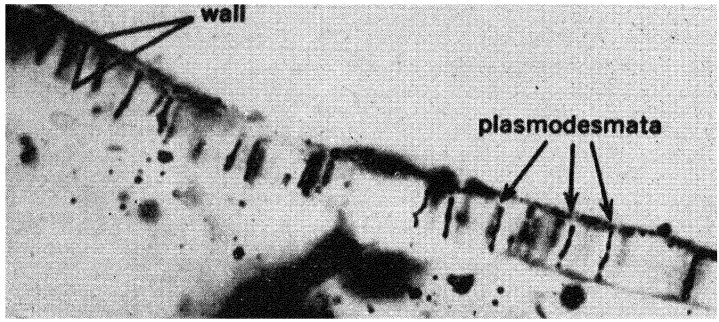

FIG. 5. Plasmodesmata in parenchymatous cells of *Solanum tuberosum* (potato) × 900, (From A. S. Crafts. *Plant Physiology*, **8**: 81, 1933).

of sucrose across copper ferrocyanide membranes, recording the hydrostatic pressures which were required to stop the osmotic flow of water in such a system. The data presented by Wilhelm Pfeffer in his book *Osmotische Untersuchungen: Studien zur Zellmechanik* published in 1877, showed that the hydrostatic pressures equal to and opposed to the osmotic pressures of the sucrose solutions were proportional to the concentrations of the sucrose solutions (i.e. proportional to $1/V$ where V is the volume of solution containing unit weight of sucrose) and proportional to the absolute temperature (T). The osmotic pressures of the sucrose solutions obeyed an equation analogous to the gas equation of Boyle; $PV = KT$. Later, in 1886, van't Hoff showed that if we use appropriate units (P in atmospheres, V in litres of solution containing one gram molecule of solute) then the K of the above equation is equal to the gas constant (R) and the equation $PV = RT$ applies to both gaseous and osmotic pressures. This enables us to define osmotic pressure thus: *the osmotic pressure* of a solution is equal to the gas pressure which the solute would exert if it were present as a gas, at that temperature, in a volume equal to the volume of the solution. Thus an "ideal" molar solution should at 0°C have an osmotic pressure of 22·4 atmospheres.

Pfeffer's system involved separation of the sucrose solution from water (the solvent) by a membrane allowing diffusion of water molecules but not of sucrose molecules and a membrane of this type was described as *semipermeable*. Further, the movement of water into the sucrose solution was prevented by a hydrostatic pressure applied to the solution; the osmotic pressure was revealed as a deficit of hydrostatic pressure, as a diffusion pressure deficit (*DPD*) of water in the sucrose solution. The osmotic pressure of the solution was the excess hydrostatic pressure which applied to the solution prevented osmosis (the net diffusion of water across the membrane) by raising the chemical potential (activity) of the water in the solution to that of pure water at the same temperature. This reduction of activity or "lowering of the pressure of water" in solutions is also reflected in the lowering of their vapour pressures (elevation of their boiling points) and depressions of their freezing points below that of the solvent and both these criteria (and particularly the latter) can be conveniently used for the determination of the osmotic pressures of solutions. The osmotic pressure in atmospheres at 0°C(P) is given by the equation

$$P = \frac{\Delta T}{1{\cdot}86} \times 22{\cdot}4$$

where ΔT is the observed freezing point depression (°C). Further, all these properties depend upon the number of solute particles per unit volume of solution (are colligative properties). Hence, solutions of electrolytes, depending upon their number of ions and percentage ionisation, have osmotic pressures in excess of solutions of non-electrolytes of equivalent molarity; an observation which led Arrhenius to develop the ionic theory of solutions.

The concept of the vacuolated parenchymatous plant cell as an osmotic system is based upon the classical researches of Pfeffer and of his contemporary, Hugo de Vries (working between 1871 and 1888) both of whom studied microscopically the responses of plant cells to externally applied solutions. When such cells are placed in solutions of sufficient strength the protoplasts decrease in volume

to such an extent that they shrink away from the cell walls; the cells are *plasmolysed.* Using cells of the red beet with their coloured vacuolar fluid, de Vries was able to follow this process accurately and detect the first evidence of separation of the protoplast from

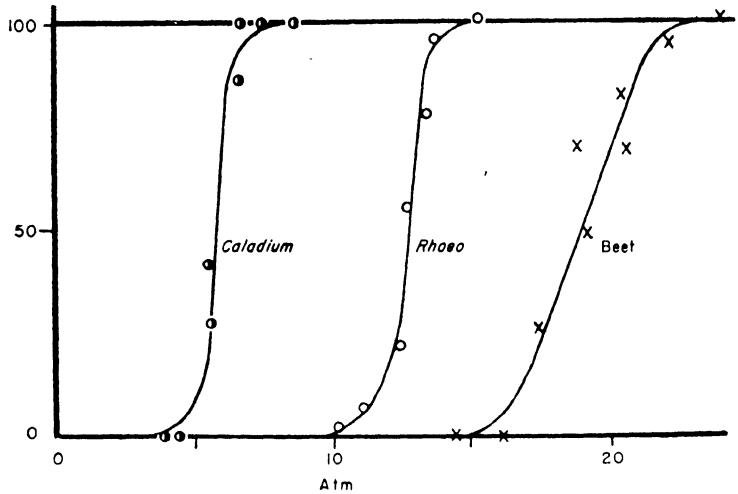

FIG. 6. Percentages of cells found plasmolysed (ordinate) in solutions of different osmotic pressures (Atm); *Caladium* petiole, *Rhoeo* leaf and beet root. The osmotic pressure bringing 50 per cent of the cells to plasmolysis brings the tissue to "limiting plasmolysis", (from T. A. Bennet-Clark, A. D. Greenwood and J. W. Baker. *New Phytologist,* **35**: 277, 1936).

the cell wall (the point of *limiting plasmolysis*). When working with uniform layers of tissue, the individual cells are not all quite equally susceptible to plasmolysis and, therefore, when dealing with such a population of cells, the solution which just plasmolyses 50% and just fails to plasmolyse the remaining 50% of the cells can be said to bring the tissue to the condition of "limiting plasmolysis" (Fig. 6). Such solutions are isotonic with the tissue and such isotonic solutions can be shown to be solutions of equal osmotic pressure. Therefore, the ability of solutions to withdraw water from cells is

a function of their osmotic pressures and when the plasmolysed protoplast ceases to decrease in volume (comes to equilibrium with the external plasmolysing solution) it can be postulated that the *DPD* of the solution is balanced by the *DPD* of the protoplast.

The phenomenon of plasmolysis indicates that the cell wall is permeable to (allows the diffusion of) both solute and solvent molecules and that the osmotic removal of water is one proceeding across membranes in the protoplast. Pfeffer referred to these as plasmatic membranes and visualised such membranes as occurring at both the outer surface of the protoplast and at the surface of the vacuole; de Vries used the terms ectoplast (we have referred to this membrane as the plasmalemma) and tonoplast. Clearly, to the extent that the protoplast behaves as an osmotic system one or both of these membranes must have semi-permeable properties.

When a cell is placed in a solution capable of plasmolysing it there occurs a decrease in cell volume prior to the onset of plasmolysis. Similarly, if a plasmolysed cell is transferred to water the protoplast first expands to fill the cell lumen and then an increase in cell volume occurs before the uptake of water ceases. The cell develops a state of turgor in which the protoplast presses against the cell wall (develops a *turgor pressure*) and the stretched elastic cell wall builds up a counter pressure (*a wall pressure*). The wall pressure builds up in the protoplast a hydrostatic pressure opposing the inward movement of water and when the cell is in equilibrium with water (is at full turgor) this wall pressure (*WP*) equals the *DPD* of the protoplast. At any point between limiting plasmolysis and full turgor, the osmotic *suction force* (*SF*) of the cell (also referred to as the DPD *of the cell*) is given by the equation

$$SF = OP_p - WP$$

where OP_p is the osmotic pressure of the fluid phase (predominantly vacuole) of the protoplast. Water will, therefore, enter or leave the cell according as to whether the osmotic pressure of the external solution is greater or less than the suction force. In land plants, the tissues are usually not fully turgid and the suction force of each

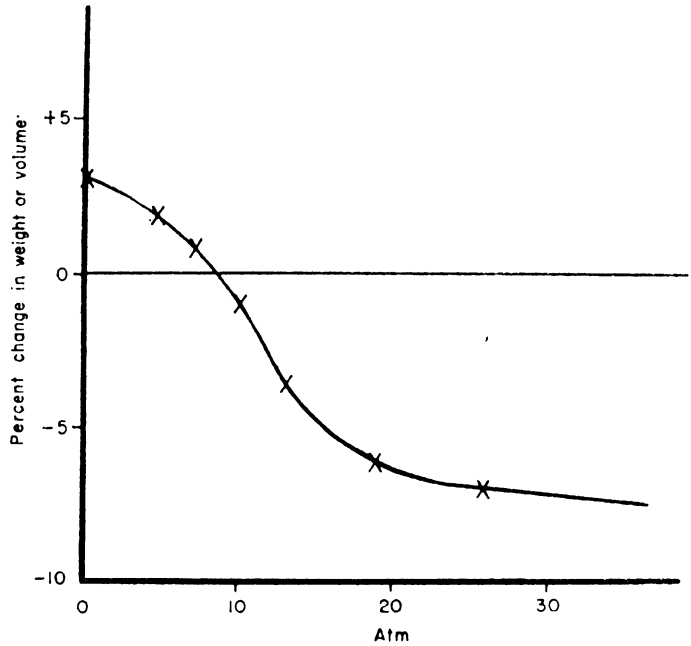

FIG. 7. Relationship between change in tissue or cell volume or weight from its initial (natural) value and the osmotic pressure of the solution in which it is immersed. The osmotic pressure in which there is no change in volume or weight gives the *DPD* (diffusion pressure deficit) or *SF* (suction force) of the cell or tissue, (from T. A. Bennet-Clark in *Plant Physiology* Vol. 2. pp. 105–192. 1959. Edited F. C. Steward. Academic Press, New York).

separate tissue can be determined by finding the osmotic pressure in which the tissue can be immersed without gain or loss in weight (or volume) (Fig. 7). Usually the value so determined cannot be regarded as the true suction force for the tissue *in situ* because within the plant the tissue will usually be either under compression by surrounding tissues (when the true value will be lower than that determined) or under tension (when the true value will be higher than that determined.)

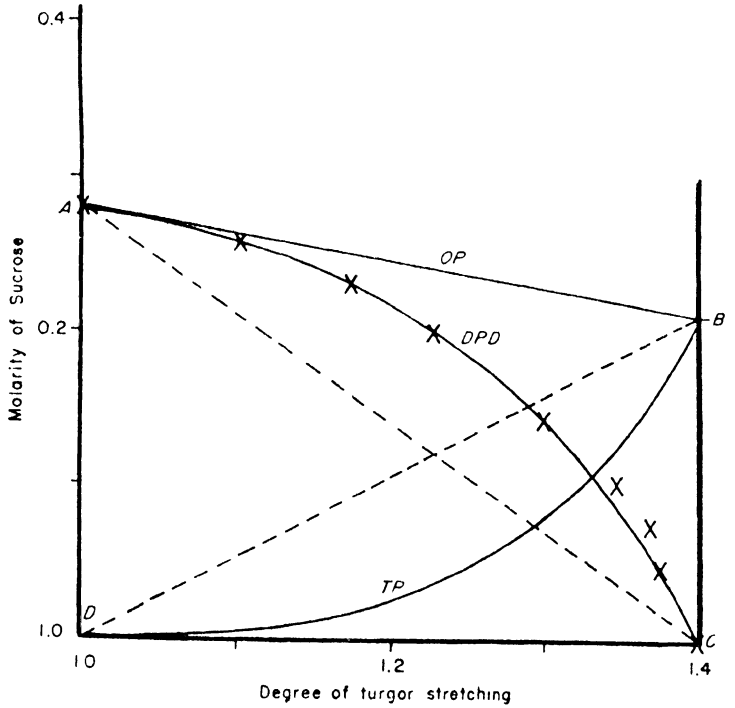

FIG. 8. Relationship between *DPD*, *OP* and *TP* in the cells of the alga *Nitella*. Volume of cell (*V*) at limiting plasmolysis is given the value 1·0 and volumes of cell up to full turgor are expressed as multiples of this. *DPD* values determined experimentally. *OP* calculated from *DPD* at limiting plasmolysis and increase in volume to full turgor. *TP* obtained by difference. The dashed lines *DB* and *AC* indicate the relationship between *TP* and *DPD*, respectively, which was assumed to exist before accurate measurements had been made, (from H. Tamiya. *Cytologia* (*Tokyo*) **8**: 542. 1938 via Bennet-Clark, 1959 as FIG. 7).

The osmotic concept of the water relations of the vacuolated cell as outlined above can be summarised in a diagram (Fig. 8) which depicts the changing values for cell volume (*V*), turgor or

wall pressure (*TP*), diffusion pressure deficit or suction force (*DPD*) and osmotic pressure of the protoplast (*OP*) as the cell absorbs water from the condition of limiting plasmolysis to that of full turgor.

The critical test which one should apply to this osmotic hypothesis is the demonstration that equations such as $DPD = OP_p - WP$ for the partially turgid cell and $DPD = OP_p$ for the plasmolysed cell are quantitatively obeyed. It is, however, technically very difficult to determine either the wall pressure or to obtain, for instance, an undoubtedly pure sample of vacuolar fluid. A number of workers have reported that the osmotic pressure of the solution causing limiting plasmolysis is considerably higher (0·2 to 5·0 atmospheres higher) than the osmotic pressure of the extracted vacuolar fluid. These findings suggest that the absorbing power of the cell for water may be greater than expected from the osmotic relationship and has led to the hypothesis that the cell augments osmotic forces by a mechanism of "active" water uptake. This implies that the force motivating water uptake is $OP_p + a$ where a is a secretion pressure brought about by some unknown mechanism involving the expenditure of cellular energy.

The picture of the cytoplasmic membranes as semipermeable, permitting diffusion of water molecules but not of solute molecules, arose from the apparent stability of plasmolysis when using as plasmolysing agents, solutions of sucrose or of single salts. However, as early as 1880 de Vries recorded that the plasmolysis of epidermal cells of *Rheo discolor* by glycerol and urea solutions was only temporary, the cells fairly quickly regaining turgor. Klebs in 1887 recorded a similar temporary plasmolysis of the cells of filaments of the alga, *Zygnema*, by urea solutions. Clearly, here there was evidence of the entry of solute molecules into the cells to enhance their OP_p and enable them to regain turgor. This subject of the permeability of cells to solutes was investigated in detail for the first time between 1890 and 1899 by Charles E. Overton at the University of Zurich and led to the realisation that many solutes can diffuse into cells and that substances with a high solubility in

lipids relative to water (a high oil/water partition coefficient) penetrated most rapidly. Later work, particularly by W. Ruhland, showed that not only lipid solubility but molecular size determines the permeating power of solutes to living cells. We have already referred to the evidence for the concentration of lipoidal material in cellular membranes. Their elasticity and their sieve-like properties as exposed by Ruhland's studies on permeability strongly suggest that proteins are also involved in the structure of cell membranes. Protein mono-molecular layers would confer elasticity and also act as molecular sieves. These classical studies on permeability established that many solute molecules can move across cell membranes by a diffusion process; that cell membranes were not semipermeable (in the sense of being permeable only to solvent molecules) but structures having highly characteristic permeability properties.

From considerations of the permeability of cell membranes one is led on to problems of the uptake and retention by cells of solute molecules and ions. This, however, forms the principal subject matter of Chapter 6. Here it is only necessary to emphasise that Overton clearly visualised that the interchange of solutes between the cell and its environment was regulated not only by resistances to diffusion (permeability in the narrow sense) but by an "active" transport of substances often in the opposite direction to that in which, from a purely physical standpoint, they would be expected to move. For this "active" transport mechanism, Overton used the term "adenoid" (gland-like) activity and it is with this dominant and active component that we shall be concerned when we come to discuss our knowledge of the accumulation by plant cells of essential nutrient ions.

THE FINE STRUCTURE OF CELLS

Bearing in mind the structure and osmotic behaviour of plant cells as revealed by the light microscope, consideration can now be given to the finer details of structure revealed by the electron

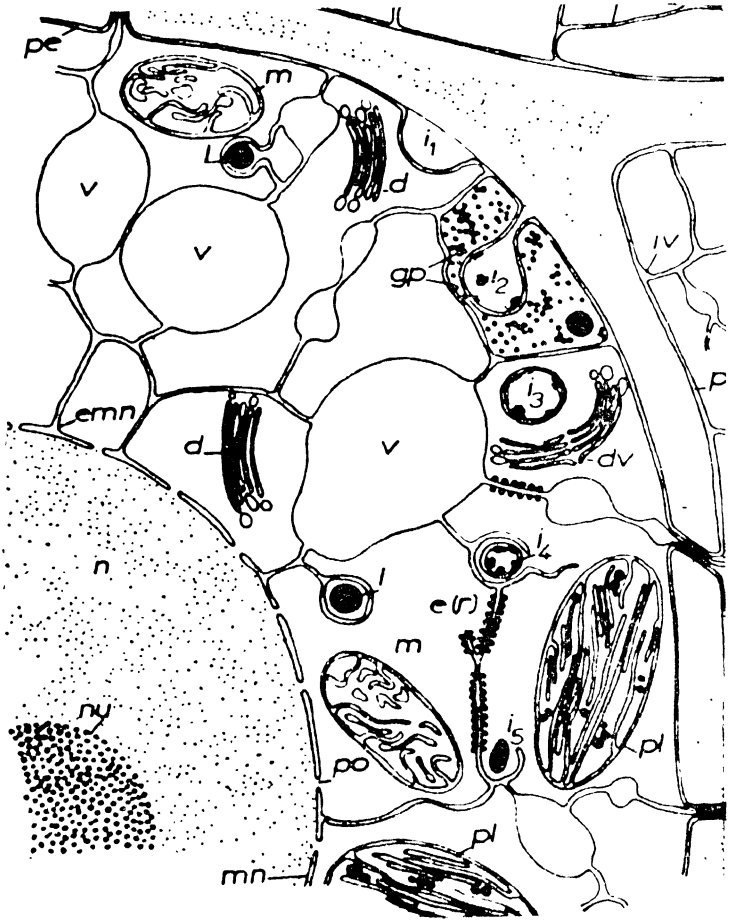

FIG. 9. Diagrammatic representation of structures in a meristematic plant cell as revealed by electron microscopy. n = nucleus, nu = nucleolus, mn = nuclear envelope, po = pore in nuclear envelope, emn = membrane of endosplasmic reticulum, v = vacuole, pe = plasmalemma, m = mitochondria, pl = plastid, d, dv = golgi structures, i_1 . . . = various inclusions, (from R. Buvat. *Ann. des Sc. Nat. Bot.* 11 série, **19**: 121, 1958).

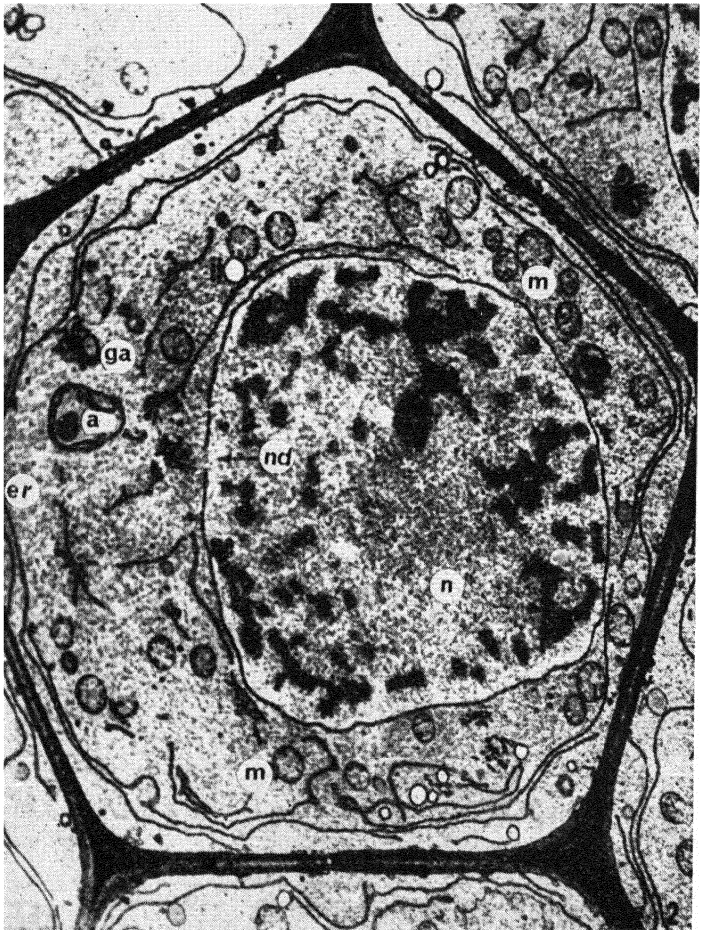

FIG. 10. Electron microscope (EM) picture of a transverse section of a root tip cell showing the nucleus (n), endoplasmic reticulum (er), mitochondria (m), golgi apparatus (ga), and an amyloplast (a). The point (nd) is a discontinuity in the nuclear envelope not a pore. The dark boundary region is the cell wall. Approx. ×6000, (from Whaley, Mollenhauer and Leech. *Amer. J. Bot.* **47**: 401, 1960.)

microscope (Fig. 9–14, 17, 21–23). These finer details of structure are particularly pertinent to our understanding of the localisation of metabolic events within the cell and of the nature of cell growth and differentiation.

The electron microscope resolves the optically clear hyaloplasm of the light microscope into two components. One, the matrix or ground substance, replaces the hyaloplasm of the light microscope in that, even in the electron microscope, no structural detail is revealed. The second is the *endoplasmic reticulum* (ER), a membrane-enclosed vesicular system appearing in thin sections of the cell as rounded, oblong or long slender vesicles (Figs. 12 and 13). In meristematic cells this system is clearly a continuum extending from the *nuclear envelope*, throughout the cytoplasm to the surface membranes and even through the cell walls. In differentiated cells the ER may be less prominent and may be represented only by apparently discontinuous vesicles. The ER usually shows a characteristic pattern of structure for each cell type; the ER structure and the metabolic activities of the cell are closely linked. Thus, in cells active in protein synthesis there occur on the outer surfaces of the ER membranes (Fig. 12) and sometimes free in the matrix numerous particles (ribosomes), often about 150 Å in diameter and rich in ribonucleoprotein (RNA-protein). These ribosomes, together with fragments of the ER are collected as the *microsome* fraction when a suitably prepared tissue homogenate is first cleared by centrifuging at 10,000*g* (to sediment mitochondria and larger cell inclusions) and then submitted to a much higher centrifugal force (about 100,000*g* for 1 hr or more). We shall refer to the metabolic activity of this microsome fraction in Chapter 5. By contrast, in differentiating cells in which the dominant metabolic activity is the synthesis of cell wall material the ER, particularly adjacent to the wall surface, will be relatively free of ER particles and the membranes appear quite smooth.

The ER membranes are rich in lipoprotein and about 5 mμ in thickness, each vesicle being bounded by a single membrane. The electron microscope reveals the nuclear envelope as consisting of

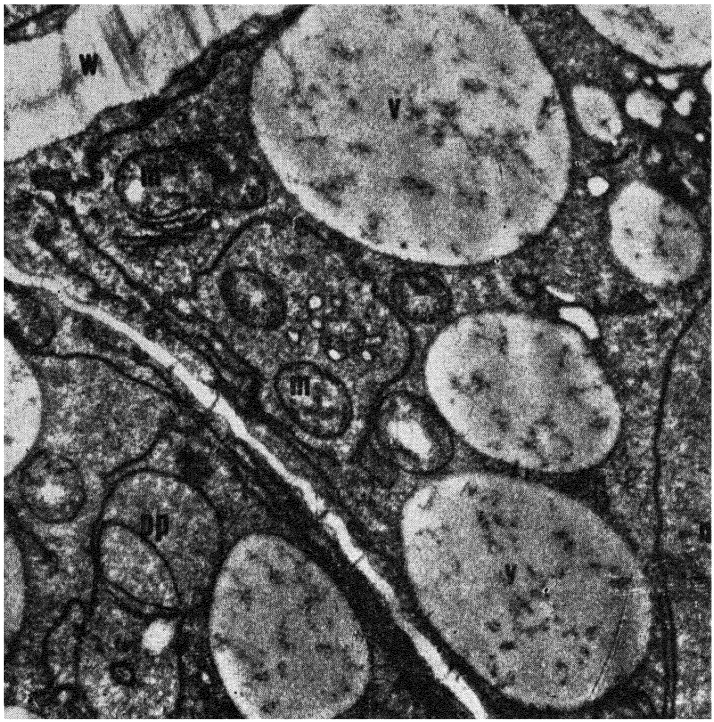

FIG. 11. EM picture of section of cells below the root promeristem where vacuolation is proceeding. Shows vacuoles (v), mitochondria (m), proplastid (pp), nucleus (n) and walls (w) with plasmodesmata. Approx. × 20,000 (from Whaley, Mollenhauer and Leech, 1960).

two membranes of similar structure and the outer of these two membranes has been shown to be continuous with the membranes of the ER, so that the space (20–40 mμ in width) between the two membranes of the nuclear envelope is continuous with the space system of the ER (Fig. 13). The ER offers a great area of contact with the matrix of the cytoplasm which in turn has continuity into the nucleus via pores (20–40 mμ diam.) in the nuclear envelope.

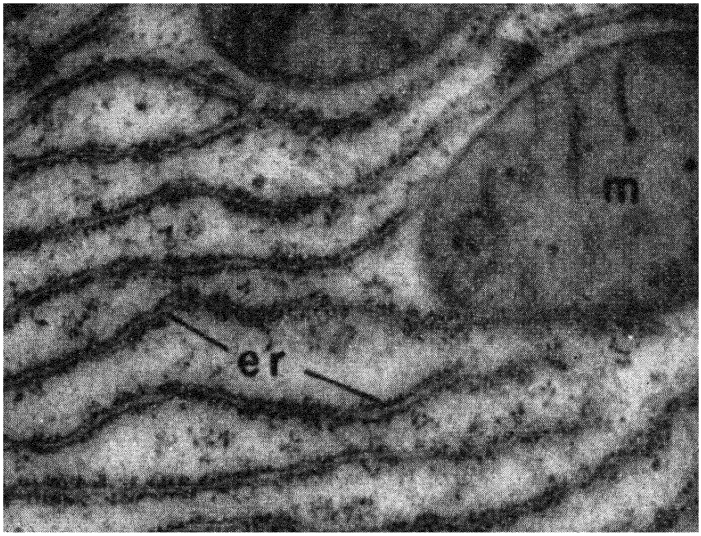

FIG. 12. Typical EM image of the endoplasmic reticulum (er) in which the outer surfaces are studded with microsomes. A few similar particles are seen in the cytoplasmic matrix. m mitochondrion. Approx. × 40,000 (from Keith R. Porter in *The Cell* Vol. 2. Edited by J. Brachet and A. E. Mirsky. Academic Press, New York, 1961).

While the functions of the ER are still uncertain there is evidence of its involvement in protein synthesis (microsome particles) and in intra-cellular transport and if enzymes are built into the ER membranes then it may be a major site of cytoplasmic activity, the location of the "soluble" enzymes of the cell.

There can also be distinguished in meristematic cells, particularly in the region of the cytoplasm in which the cell plate forms, *Golgi structures* (Figs. 10 and 13) composed of a stack of plate-like sacks bounded by membranes like those of the ER and associated with a group of more of less spherical vesicles. The metabolic role of the Golgi structures is uncertain, they are characteristic

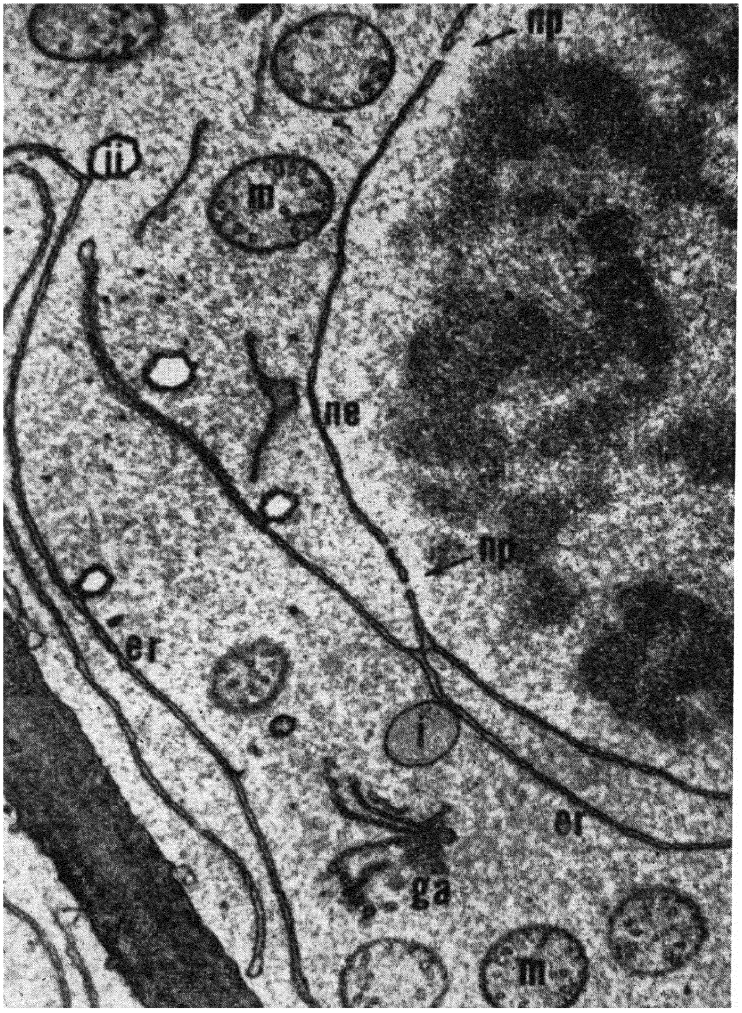

FIG. 13. Portion of a root meristematic cell as seen in the EM showing the double nuclear envelope (ne) with pores (np) and showing continuity of the outer nuclear membrane with the endoplasmic reticulum (er). m = mitochondria, ga = golgi apparatus, i and ii = inclusions. The cell wall runs across the left-hand bottom corner. Approx. × 17,000 (from Whaley, Mollenhauer and Leech, 1960).

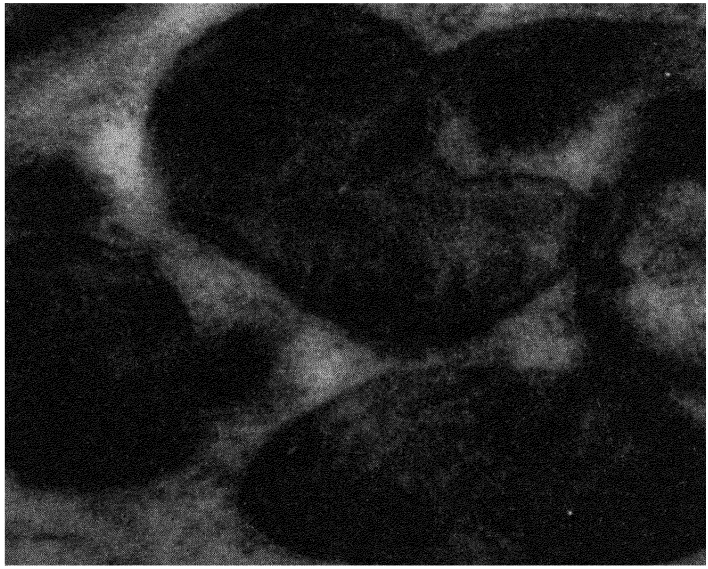

FIG. 14. Mitochondria of *Chlamydomonas reinhardi* as seen in the EM showing typical crista structure, × 60,000 (photo by Ruth Sager and G. E. Pallade from A. B. Novikoff in *The Cell* Vol. 2 as Fig. 12).

of meristematic cells, appear to increase in number during cell division and to disappear during the early stages of differentiation.

Sections of meristematic cells frequently reveal numerous small vacuoles, some of which appear to contain reservoirs of metabolites. As cells begin to expand these vacuoles become "empty" (presumably contain in life an aqueous solution) and are clearly enclosed in a membrane similar to the plasmalemma. The *plasmalemma* itself is seen in the electron microscope as a single-layered smooth and continuous membrane sometimes thrown into invaginations.

The *mitochondria* are seen by electron microscopy (Fig. 14) to be enclosed by an outer membrane and within this is an inner membrane which is infolded to form across the central matrix

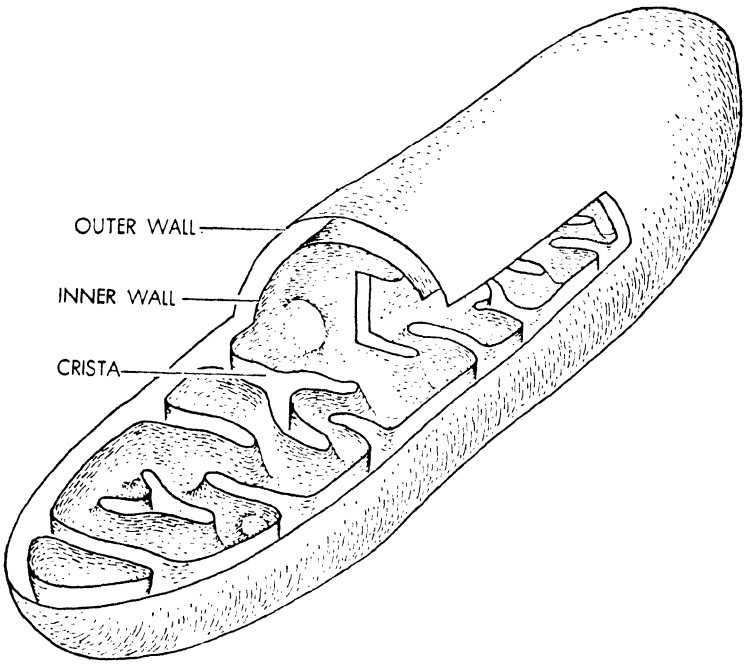

FIG. 15. Diagrammatic representation of the structure of a mitochondrion (after A. L. Lehringer. Scientific American Reprint 91. Sept. 1961).

baffles or *cristae* or to give rise to finger-like intrusions into the matrix (Fig. 15). The membranes are about 45 Å thick and the space between, in which the phospholipid content seems to be concentrated, is about 50 Å thick. The composition of dry mitochondria isolated by centrifuging is 40% protein, 25% phospholipid and 5% ribonucleic acid (RNA). When isolated mitochondria are burst about 60% of the protein is released in soluble form suggesting that the central matrix contains soluble protein. The mitochondria are distributed in the matrix of the cytoplasm and are often observed in contact with the ER or nuclear envelope. When cells are starved they shrink and when cells age they become disorganised. There

is strong evidence that mitochondria can divide and the new mitochondria then grow to full size. These cytoplasmic inclusions are centres of respiratory activity in the cell; when they are disrupted most of their metabolic activity is lost.

The amyloplasts (starch forming plastids) (Fig. 10), chromoplasts and chloroplasts arise from structures, *proplastids* (Fig. 11), similar in size to mitochondria but not readily stained with Janus Green B and containing internally an array of granules or vesicles (100–250 Å diam.) rather than defined cristae. In the cells of higher plants these proplastids are the units transmitted from cell to cell, increasing in numbers by division and from which the functional plastids develop during cell differentiation. The first step in the development of plastids is the development from the granules or vesicles of lamellae which are characteristically more linear and more precisely arranged than the cristae of mitochondria. Proplastids and the plastids to which they give rise are like mitochondria rich in lipo-protein and protein and have a small content of RNA.

The *chloroplast*, the centre of photosynthetic activity, contains the two chlorophylls *a* and *b* (usually in a ratio of about 3:1) together with the accessory carotenoids and xanthophylls. A typical analysis of dry chloroplasts is protein 40–50%, phospholipid 23–25%, RNA about 5%, chlorophylls *a* and *b* 5–10%, carotenoid 1–2%. The choloroplasts of higher plants are shaped like a plano-convex lense, approximately 5 μ in diameter and 2–3 μ thick. They lie in the cytoplasm with their broad sides parallel to the cell wall. The light microscope reveals the presence within the chloroplast membrane of a colourless *stroma* and green disc-like *grana* (Fig. 16). The electron microscope examination of thin sections has revealed details of the internal structure (Figs. 17 and 18). The outer membrane is double-layered (each layer 35–50 Å thick) and within this is the proteinaceous stroma which contains minute granules (50–250 Å diam.), larger osmium-staining droplets and starch grains ("photosynthetic starch"). Embedded in this stroma are double-membraned lamellae extending the width of the plastid

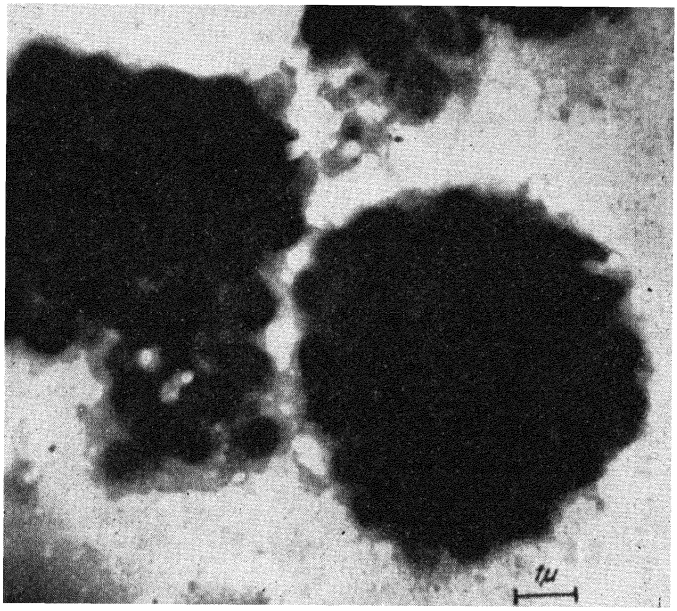

FIG. 16. Grana in isolated spinach chloroplasts (face view) (from S. Granick and K. P. Porter. *Amer. J. Bot.* **34**: 545, 1945).

and stacked one upon another. These lamella are differentiated into less dense thin *stroma lamellae* and more dense thicker *grana lamellae* of disc-like form. The grana are cylindrical piles (10–100) of these disc-like grana lamellae held together at their edges. The grana contain the chlorophylls associated with the protein membranes and the intervening lipid molecules. The number and size of the grana per chloroplast varies with species. In the chloroplasts of spinach there are, for instance, 40–60 grana, each 6000 Å in diameter and 800 Å thick. Present evidence allows us to postulate that the photochemical reactions of photosynthesis and the consequent photodecomposition of water and the production of

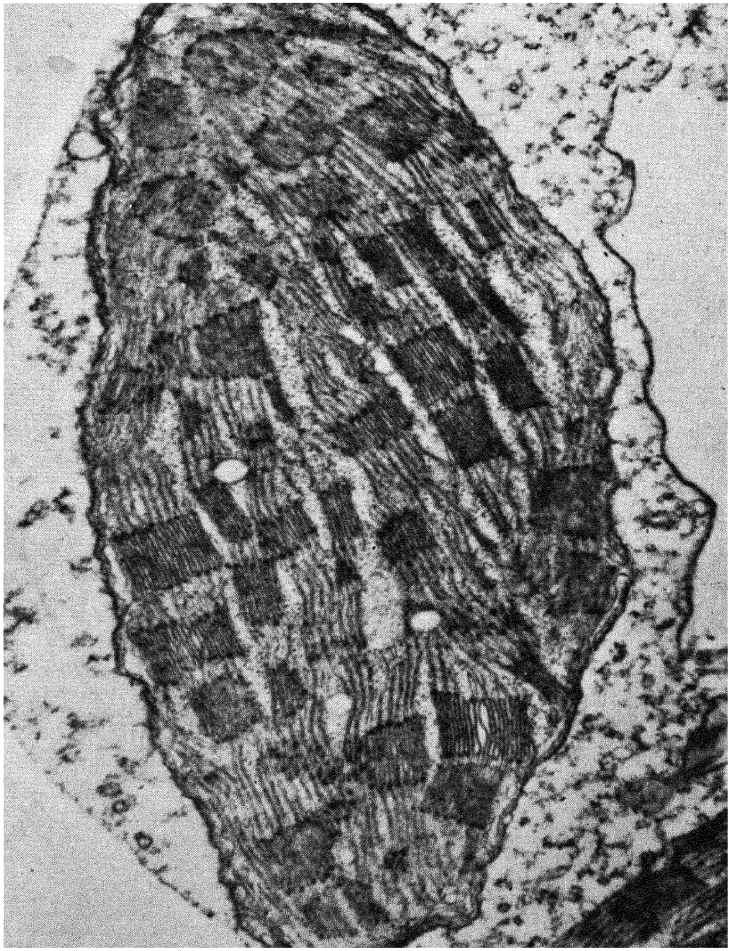

FIG. 17. The chloroplast of maize as seen in the EM. The dense areas which resemble stacks of coins are the grana built up of grana lamellae. The lamellae interconnecting the grana are stroma lamellae, ×30,000 (photo by A. E. Vatter from A. L. Lehringer. Scientific American, Reprint 91. Sept., 1961).

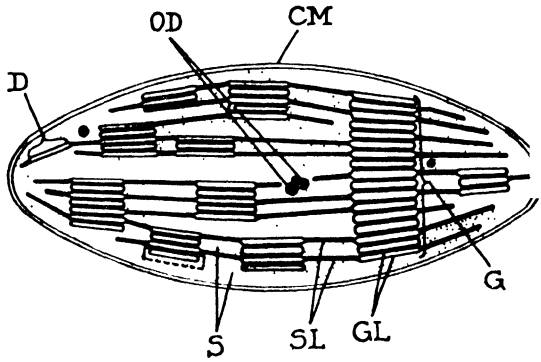

FIG. 18. Interpretation of the structure of a barley chloroplast (as von Wettstein). CM = chloroplast double membrane (each 35–50 Å thick); S = stroma; G = granum made up of a cylindrical pile of discs); SL = stroma lamella (each membrane is 20–30 Å thick); GL = grana lamella (each membrane is 40–60 Å thick); D = disk; OD = osmiophilic droplet. The possible arrangement of molecules in the dashed area is shown in Fig. 39 (from S. Granick in *The Cell* Vol. 2. p. 501 as Fig. 12).

oxygen occur in the grana, while the reactions whereby carbon dioxide is converted to starch occur in the stroma regions of the plastid.

The resting or interphase *nucleus* in electron micrographs shows the nuclear envelope, *chromatin* areas, *nucleoli* and intervening "structureless" *nucleoplasm* (Figs. 8 and 11). Present techniques have failed to reveal the details of the structure of the interphase chromosomes. Chemical analysis of whole nuclei show the presence, on a dry weight basis, of 70% protein, 3–5% phospholipid, 10% deoxyribonucleic acid (DNA) and 2–3% ribonucleic acid (RNA). The chromatin material, as might be expected, is rich in DNA (30–50% dry weight) and in basic proteins (proteins rich in basic amino-acids). The nucleoli are also rich in protein and contain nucleic acid, probably entirely RNA. The RNA of the nucleus differs in base composition from that of the cytoplasm (it has, for instance, a higher adenylic acid content). A number of

enzymes have been detected in nuclei although these do not include the respiratory enzymes. There are no enzymes which occur only or even mainly in the nucleus although some enzymes (e.g. DNA-ase) may be very active in the nucleus. Although the nucleus is relatively metabolically inert by comparison with the cytoplasm, nevertheless, the maintenance of active metabolism in the cytoplasm is dependant upon the presence of the nucleus. Enucleated cytoplasm more or less quickly becomes metabolically disorganised. We shall return again to this question of the nuclear-cytoplasmic relationship when considering protein synthesis, and particularly, the synthesis of enzyme proteins.

Modern knowledge of *cell wall* structure comes from detailed chemical analyses, X-ray defraction studies and work with the electron microscope. The dry primary cell wall contains hemicelluloses (up to 50%), cellulose (up to 25%) and smaller amounts of pectic substances, fats and protein. Hemicelluloses, cellulose and pectins are all polysaccharides with molecules built up from the linking together in chains of sugars or uronic acid residues. The morphology of cell walls is, however, *not* destroyed by extracting the hemicelluloses and pectins, indicating that the structural framework of the wall is built of cellulose. The molecules of cellulose consist of long chains of about 3000 glucose units and in the cell wall these molecules are associated parallel to one another to form *microfibrils*. In the microfibril the cellulose molecules are united laterally by weak chemical linkages (hydrogen bonds) (see Chapter 3, p. 59 and Fig. 25) and end-to-end by primary co-valent bonds. Each microfibril contains some 2000 cellulose molecules, has a diameter of 100–250 Å and a length of several microns. The cellulose molecules within the microfibril are much more closely associated in certain regions than in others. X-ray diffraction studies reveal that these regions of high association (the so called *micelles*) are 50–60 Å wide (i.e. involve about 100 molecules parallel to one another) and about 600 Å long (Fig. 19).

The microfibrils are themselves associated into *macrofibrils* (these may involve up to 400 microfibrils) which can be seen with the

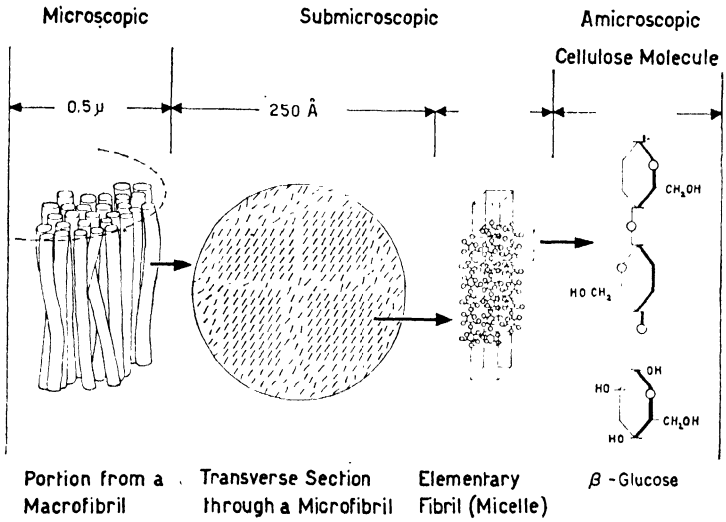

FIG. 19. Structural elements of cellulose involved in cell wall architecture (from K. Muhlethaler in *The Cell* Vol. 2. p. 85 as Fig. 12).

light microscope, particularly after appropriate swelling treatment. Since primary cell walls may contain up to 95% of water, the structural framework of microfibrils is a very "open" one probably occupying only 2.5% of the wall volume.

The primary cell walls of the meristematic cells have their microfibrils orientated predominantly in a direction transverse to the axis of the root or shoot. When cell expansion occurs there is an increase in cell wall material, a synthesis of cellulose and other cell wall constituents. Expansion at the outer surface of the cell wall causes stretching in the direction of most active cell extension and as this happens the microfibrils become re-orientated more and more along the longitudinal axis of growth. At the same time new layers of wall are laid down on the inner face of the wall and with their microfibrils in the original transverse direction. These, in turn, undergo re-orientation as growth proceeds so that at the end of the cell expansion phase there is a transition outwards in the wall from

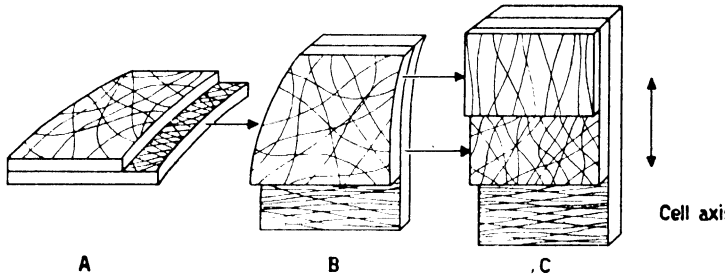

FIG. 20. Multinet structure of the cell wall of the cotton hair showing the orientation of the cellulose microfibrils in the successive wall layers laid down during elongation (after A. L. Houwink and P. A. Roelofsen. *Acta. Botan. Neerl*, **3**: 385, 1954).

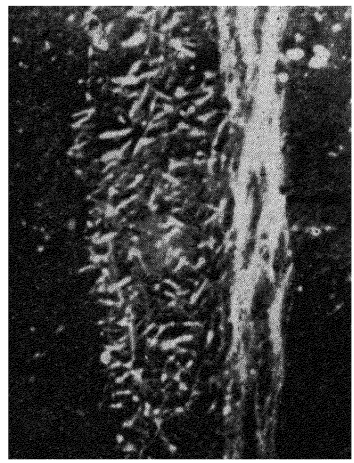

FIG. 21. Transverse section (EM) through the wall of epidermal cell of an onion root; the cell was situated about 1 mm from the tip. The arrow points towards the centre of the cell. The scale corresponds to 1μ, (by courtesy of G. Setterfield and S. T. Bayley. *Canad. J. Bot.*, **35**: 435, 1957).

transversely to longitudinally scattered microfibrils (Figs. 20 and 21). This type of cell wall growth is usually termed *multinet* growth and clearly involves the laying down of new wall material over the inner surface of the existing wall (growth by *apposition*). However, there is evidence, particularly from feeding expanding cells with sugars labelled with tritium (a radioactive isotope of hydrogen of mass 3), that although growth by apposition may sometimes account for the major increase in cell wall substance there is, nevertheless, some incorporation of new wall material throughout the whole thickness of the wall. Such incorporation of new wall substance into the existing framework is referred to as growth by *intussusception*.

Cell walls can continue to grow for a time after separation from contact with cytoplasm; cellulose synthesis must then occur *within* the wall. Certain electron micrographs have shown the presence within the cell wall of granular aggregations (cytoplasm?) at which microfibrils appeared to terminate and these have been described as "islands of synthesis", possibly associated in some way with plasmodesmata and other extensions of the ER into the cell wall. Growth by intussusception could occur by growth of cellulose microfibrils from such centres into the intervening wall. It is, however, perhaps even more likely that cellulose and other wall polysaccharides can arise *in situ* and remote from such cytoplasmic islands by the action of soluble enzymes. Thereby, intussusception growth could occur without disturbing the orderly layered arrangements of microfibrils developed by apposition.

It should be emphasised that we do not yet fully understand the mechanism whereby cellulose, or hemicelluloses or pectins are synthesised nor do we yet know whether the plant hormones (auxins) promote cell expanison by some direct action on the association together or primary synthesis of cell wall constituents. Furthermore, we do not understand fully what factors determine the regular orientation of microfibrils as laid down in each wall layer. Particularly, during wall thickening in fully expanded cells, the orientation may differ from layer to layer (Fig. 22). The

FIG. 22. Outermost lamellae of the wall of the alga *Valonia ventricosa*. EM picture at × 25,000 (from R. D. Preston. *The Molecular Architecture of Plant Cell Walls*. Chapman & Hall. London, 1952).

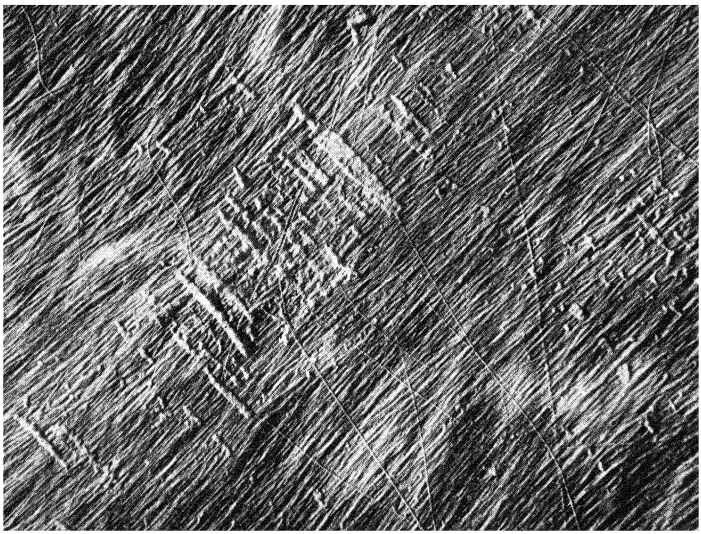

FIG. 23. Electron micrograph (×24,000) of the innermost surface of the cell wall of the alga, *Chaetomorpha melagonium*, showing cytoplasmic remnants arranged in files at approximately right-angles to the existing microfibrils of the inner face of the wall, i.e. in the direction which the microfibrils will take up in the wall layer next to be deposited. Note the few microfibrils which would form part of this new layer. (from Eva Frei and R. D. Preston. *Proc. Roy. Soc.*, *B* **154**: 70, 1961).

pattern for each layer seems to be determined by some pattern established at the cytoplasm-wall interface and which can be revealed prior to the actual development of the new layer of cellulose (Fig. 23).

PROLOGUE TO CHAPTER 3

The living cell is the territory of metabolism; the reactions of metabolism occur each at their allotted place in this territory. Though the cell is "small" the territory of the cell in terms of

molecules is vast and its pathways of metabolism (surfaces or membranes) are long and many in number. These are protein surfaces, and the catalysts of metabolism are the special proteins termed *enzymes*. Now if enzymes are built into membranes and these are distributed in patterns then we can begin to visualise the physical basis responsible for the organisation and regulation of chemical reactions in the cell. The rapid and efficient operation of enzyme systems with many enzymes participating in a regular sequence implies that the enzymes must be built into membranes in an orderly sequence, in the form of production lines. Studies of the fine structure of cells, therefore, seek to reveal the factory organisation and separate workshops in which metabolism proceeds. What of the production units—the individual enzyme proteins?

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3

ENZYMES—THE CATALYSTS OF METABOLISM

"It should be emphasized that the results obtained have, for the most part, been concordant with those furnished by isotopic and genetic studies on intact cells. There is, therefore, good reason to believe that, in general, the reactions observed in isolated enzyme systems do reflect physiological events and are not artefacts of isolation."

Bernard D. Davis, 1956 in *Enzymes, Units of Biological Structure and Function.* Academic Press, New York.

"Enzymes are wonderful substances—they consist of wonderful molecules. We do not know much about these molecules as yet, . . . more must be done in the field of molecular structure . . . leading to the complete structural determination—the location of every atom—of the molecules of many proteins."

Linus Pauling, 1956 in *Enzymes, Units of Biological Structure and Function.* Academic Press, New York.

INTRODUCTION

THE CHEMICAL reactions which constitute cellular metabolism proceed at a temperature only fractionally above that of the natural environment and the pH of cytoplasm is close to neutrality, neither markedly acid nor alkaline. Rapid reactions occur in cells between compounds which are quite stable in mixed aqueous solution. This intense chemical activity in living cells results from the activity of numerous specific catalysts or enzymes. Such substances were first extracted, in an active form, from living cells by the Buchners in

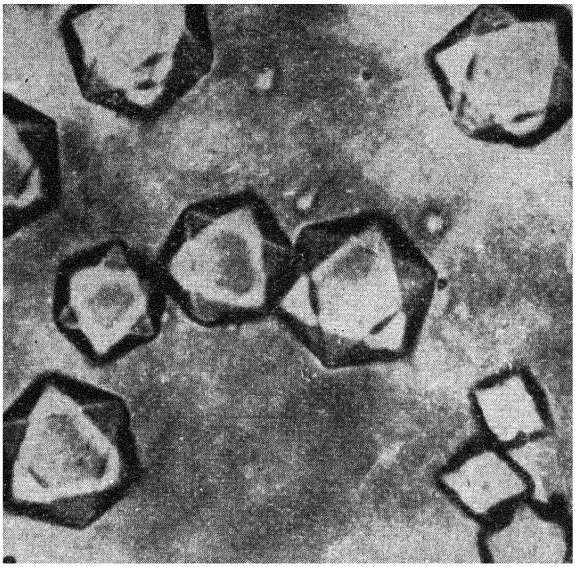


FIG. 24. Crystals of the enzyme urease. (from J. B. Sumner and G. F. Somers. *Chemistry and Methods of Enzymes.* Academic Press, New York, 1953).

1897. They made the discovery that when yeast is ground with sand, pressed and the expressed juice filtered, one obtains a clear liquid capable of rapidly fermenting sugar, of degrading sugar to carbon dioxide and ethanol. Fermentation, shown in Pasteur's elegant experiments to be the result of the activity of living cells is here being effected by a clear solution of cellular origin. The ability of the yeast-press juice to promote fermentation is destroyed by heat or by rendering the liquid markedly acid or alkaline. Its catalytic activity is unstable.

During the present century such catalytic solutions derived from living cells have been submitted to intensive chemical study. From them have been obtained, in increasing purity and in increasing number, the catalytic agents or enzymes. A milestone in this

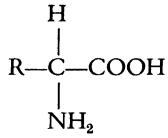
progress was the obtaining of an enzyme in crystalline form, the enzyme *urease* isolated from jack beans by Sumner in 1926 (Fig. 24). This crystalline urease of Sumner was the first convincing demonstration of the protein nature of enzymes. This enzyme also illustrates the often high specificity of these biocatalysts. Urease promotes the hydrolysis of urea (hence its name) to ammonia and carbon dioxide. It is inactive towards substituted ureas and to all compounds except urea. Another example of enzyme specificity is the finding that enzymes which have one optical isomer as substrate are inactive to the other isomer. Thus, the enzyme which oxidises glutamic acid, the enzyme *glutamic acid dehydrogenase*, is active only towards the L-isomer of glutamic acid. Here we are considering the oxidation of glutamic acid to the optically inactive α-ketoglutaric acid and ammonia. However, the reaction, in common with many biological reactions, is reversible and this enzyme, using α-ketoglutaric acid as the carbon skeleton, can effect an asymmetric synthesis giving only L(+) glutamic acid free from any of the D(−) glutamic acid.

THE PROTEIN NATURE OF ENZYMES

The consideration just mentioned leads us to consider the nature of proteins which are themselves composed of units (residues) of the L-amino acids. Proteins are molecules of great size (with molecular weights ranging from 10,000 to several million). When completely degraded by acid or enzyme hydrolysis they yield a mixture of amino acids. The number of different amino acids released and their relative proportions are characteristic of each protein. The questions therefore arise of how these amino acid units are linked together to form the protein molecules and of how far their molecular architecture explains their specific properties, including for the enzyme proteins their substrate and reaction specificity. Further, since enzyme proteins are giant molecules and the substrates of most enzymes are small organic

molecules, we must wonder whether substrate specificity is determined not by the structure of the whole protein molecule but by small reactive sites within it.

The α-amino acids have the general formula:



The α-carbon atom is bound to four different groups or atoms (hence the optical activity of all the α-amino acids except glycine where R = H). The R group is characteristic of each amino acid and may be aliphatic or aromatic and may contain only C and H atoms or may be basic (containing $—NH_2$ or =NH group(s)) or acidic (containing the —COOH group) or sulphur-containing. The α-amino group is basic, ionising thus

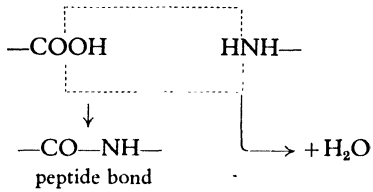
$$—NH_2 + H_2O \longrightarrow —NH_3^+ + OH',$$

the α-carboxyl group is acidic, ionising thus

$$—COOH \longrightarrow —COO' + H^+.$$

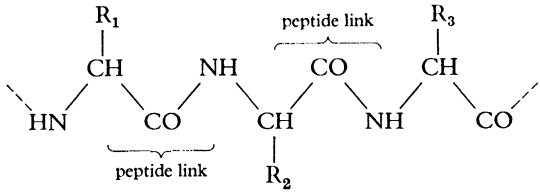
This means that amino acids are amphoteric compounds. At acid pH values the amino acid behaves as a base and is positively charged, at alkaline pH values it behaves as an acid and is negatively charged. At some intermediate pH (the iso-electric point) it has no net charge.

The amphoteric nature of α-amino acids is involved in their union together to build up the giant molecules of protein. The primary linkage is one formed by condensation of the α-amino group of one amino acid with the α-carboxyl of a second amino acid. The linkage is called a peptide bond and this arises thus:



In protein molecules, amino acids are arranged in chains (polypeptide chains) by such peptide bonds.

The carbon atom has four valencies and these make fixed angles with one another. The amino acids involved in polypeptide structure are all of the L series (have the same configuration as the reference compound, L-glyceraldehyde). This means that the polypeptide chain when represented in the single dimension of this page has the form:



There is a zigzag backbone and the R groups stick out from the plane of the backbone above and below, alternatively.

To understand how these polypeptide chains are arranged in a protein molecule it is necessary to consider the valency of hydrogen. When hydrogen loses an electron to become a proton or hydrogen ion or when it gains an electron to form a co-valent bond, it shows its primary valency of one. But the hydrogen atom can show a weak secondary valency (exhibit a co-ordination number of two) by accommodating four "valency electrons" in its shell. This secondary valency of hydrogen can be satisfied by sharing of a "lone pair" of electrons with an oxygen or a nitrogen atom and the bond so established is called a *hydrogen bond* (Fig. 25). Such hydrogen bonds are important in the building up of macromolecules such as those of protein.

Now the polypeptide chain depicted above is in the extended or β-form and in protein films (and therefore perhaps in the protein mono-layers of cell membranes) this form can be stabilised by hydrogen bonding between the carbonyl ($>C=0$) and imide ($HN<$) groups of the backbones of parallel orientated polypeptide

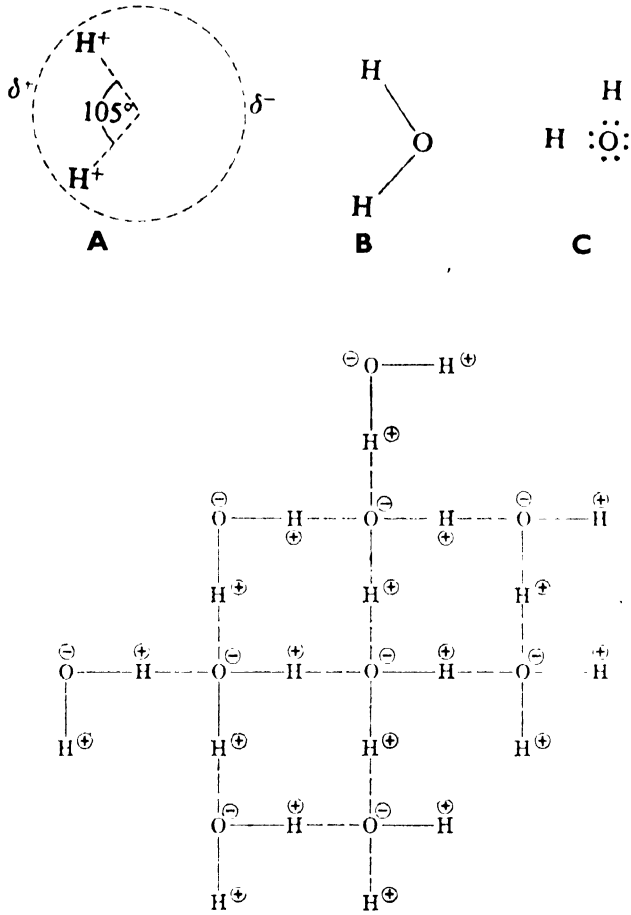


FIG. 25. The hydrogen bond.

A. Water molecule represented as two protons embedded in an oxygen atom; B. Conventional representation of the water molecule; C. "Electronic formula" for water; D. Association between water molecules by hydrogen bonding (diagrammatic and shown as in one dimension).

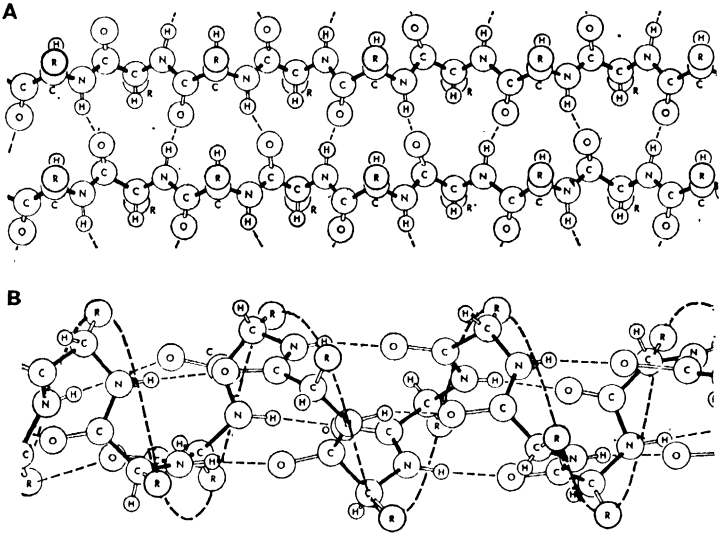


FIG. 26. Secondary structure of proteins. A. Polypeptide chains in the β-form associated to give a "pleated sheet" structure; B. Right handed α-helix structure of the polypeptide chain; Side chains of amino acid residues labelled R. Bold broken line traces the turns of the helix. Hydrogen bonds involved in associating β-chains and in stabilising the α-helix as less bold broken lines (between oxygen and hydrogen atoms). (from Paul Doty, Scientific American Reprint No. 7 September 1957).

chains to give a "pleated-sheet" structure (Fig. 26(A)). From the "primary" polypeptide unit of structure, a "secondary" structure has arisen.

However, another kind of "secondary" structure can arise because in the polypeptide chain there can be rotation around the bonds of the α-carbon atoms of the amino acid residues so that the chain can become thrown into a complex of coils. However, this coiling in nature is apparently not entirely random but is ordered and stabilised by hydrogen bonding. Thus the polypeptide backbone may be thrown into a regular spiral held rigid by hydrogen bonding between the imide group of one amino acid residue and

the carbonyl group three residue distances along the chain, and this occurs regularly down the spiral. The spiral or helix could, theoretically, be either right-handed or left-handed (Fig. 26(B)) but the L-configuration of the amino acids favours and leads to the *right-handed* helix being that present in protein molecules. It is this kind of "secondary" structure, involving folding of the polypeptide chains which seems to be of particular importance in the specific physical and biological properties of many proteins.

It is hoped that the structure of the protein α-helix, can be visualised from the description of its origin which has been given and from Fig. 26(B). The value of using a modern set of "atom models" to build up such a structure cannot, however, be over-emphasised. This is the only really satisfactory introduction to thinking about molecular architecture in three dimensions.

Now the α-helix is an elongated structure; a single helix containing say 300 amino acid residues would have a length of about 400 Å and a diameter of 10 Å. Since, however, there is evidence of α-helix structure in proteins whose molecules are globular it follows that short α-helices must be linked together, that the molecule can be visualised as built up of a stack of helical segments. This association seems to depend upon an association between the R groups of separate helices, and it is from such linkages between R groups that there arises the "tertiary" structure of protein molecules. The R group of the amino acid, cysteine, is $—CH_2—SH$ and two such groups can combine together, with the elimination of hydrogen to form the co-valent bond known as a disulphide linkage (—S—S—). Such disulphide linkages are very important in stabilising "tertiary" structure and the destruction of such bonds (for instance, by reduction) in enzyme molecules usually leads to loss of biological activity. This is so, for instance, in the enzyme, *ribonuclease*. "Salt" linkages can also arise by association between acidic R groups (those of aspartic and glutamic acids) and basic R groups (those of arginine and lysine) and "hydrogen bonds" can form between oxygen and nitrogen-containing R groups. Metal ions may also form co-ordinative complexes with R groups and

thereby link the α-helices together. The calcium present in the enzyme amylase may serve this function.

This discussion of protein structure reveals that the primary structure is built up from strong covalent peptide bonds but that weaker linkages, particularly "hydrogen bonds" are involved in the development of secondary and tertiary structure. The actual destruction of protein molecules usually requires strong chemical action such as the application of strong acids or alkalis and a high temperature. However, aqueous solutions of proteins are very unstable in their physical properties and important changes in "native" proteins (proteins in the form in which they occur in living cells) occur at temperatures above 40°C or when the pH is decreased below 3 or raised above 9. The changed protein ("denatured" protein) has lost its characteristic biological properties; in the case of enzyme protein it has lost its catalytic activity. The process of "denaturation" involves changes in secondary and tertiary structure. The techniques which have been gradually developed by trial and error for the isolation of enzymes are designed particularly to obtain the enzyme in its "native" rather than its "denatured" condition.

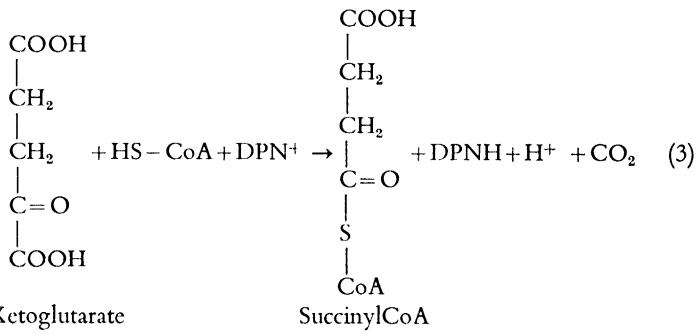
PROSTHETIC GROUPS AND COENZYMES

The enzyme, urease, crystallised by Sumner, is a "simple" protein in the sense that its molecules are entirely built up from amino acid residues. Many enzymes are, however, "compound" proteins in that the active enzyme molecule is made up of a protein moiety combined with some other molecule or atom and it is to this non-protein moiety that we give the name *prosthetic group*. The simplest prosthetic group is a metallic atom as exemplified by certain oxidising enzymes which are copper proteins (e.g. *ascorbic acid oxidase*). Other prosthetic groups are much more complex. For example, the *cytochromes*, so important in respiration, are iron porphyrin proteins; the *flavoproteins*, also involved in respiration, have as their prosthetic groups riboflavin (vitamin B_2) mono or

dinucleotides; and the *transaminases* and *amino acid decarboxylases* have pyridoxal phosphate (a derivative of vitamin B_6) as prosthetic group. These prosthetic groups are certainly part of the active centre of the enzyme molecule and in some cases we know that the prosthetic group undergoes a reversible chemical change as part of the mechanism of catalysis (see Chapter 5, p. 147). It is often very valuable in attempting to isolate an enzyme to submit the initial extract to dialysis (diffusion through a membrane of restricted pore size). The dialysis serves to separate the large protein molecules (too large to pass through the dialysis membrane) from small soluble molecules and ions. This often leads to inactivation of the enzyme, not by causing protein denaturation but by removing some dialysable co-enzyme or co-factor essential for enzyme activity. The term *co-factor* is usually reserved for inorganic ions such as the chloride ions essential for the action of the α-amylases and the Mg^{++} required for the enzymes promoting phosphate-transfer to and from adenosine polyphosphate. The term *co-enzyme* is used to describe organic substances which, like prosthetic groups, undergo chemical change as part of the catalytic process; such co-enzymes differ from prosthetic groups only in being readily separated from the protein moiety by dialysis. The classical example of a co-enzyme is co-zymase (also referred to as co-enzyme I or diphosphopyridine nucleotide, DPN, see Fig. 34 and footnote on p. 86), the detection of which by the biochemists, Harden and Young, will be discussed in Chapter 4. Another important example is co-enzyme A to which reference will again be made, both in regard to the respiratory breakdown of sugars and in regard to fat synthesis and degradation.

The cell constituent (metabolite) acted upon by an enzyme is usually termed its *substrate*, or where two cell constituents are caused to interact we talk of their being substrates. Enzymes are named either after the particular substrate upon which they act, e.g. *urease* whose specific substrate is urea, or according to the nature of the reaction they promote e.g. for phosphate-transferring enzymes the term *transphosphorylases*, or by a combination of both substrate and reaction-type, e.g. *triose phosphate dehydrogenase* (glyceraldehyde

phosphate dehydrogenase) which catalyses the removal of hydrogen from D-3-glyceraldehyde phosphate. The distinction between substrate and co-enzyme is often not immediately clear. Thus, *α-ketoglutaric acid dehydrogenase* oxidises this acid in presence of the two co-enzymes, DPN and co-enzyme A (represented in the equation as HS—CoA: see Fig. 34) thus:



The DPN is reduced, carbon dioxide is liberated and the product of the reaction is a compound between succinic acid and co-enzyme A. Now this succinyl-CoA is further transformed by a second enzyme system, the overall reaction of which is

$$\text{Succinyl}-\text{S CoA} + \text{ADP} + H_3PO_4 \rightarrow \text{ATP} + \text{succinate} + \text{CoA}-\text{SH} \quad (4)$$

Here then the distinction between substrates and co-enzymes can only be made in terms of the quantities involved. These enzyme systems oxidatively decarboxylate a mass amount of α-ketoglutaric acid to succinic acid. The small amount of co-enzyme A acts over and over again and the small amount of co-enzyme I (DPN) is constantly being re-oxidised by another set of enzymes and so acts over and over again to remove hydrogen from the α-ketoglutarate.

ENZYME ACTIVITY

Enzymes are catalysts: they enable the reactions of living cells to proceed at measurable velocities. Our understanding, imperfect as it is, of this catalytic activity of enzymes is founded upon the

study of the kinetics of the reactions *in vitro*. The isolated enzyme and its substrate(s) are allowed to interact under controlled conditions.

Firstly, the rate of an enzyme catalysed reaction is influenced by the concentration of the substrate. With a fixed amount of enzyme (E) there is, with increase in substrate (S) concentration, an increase in the velocity of the reaction until a "saturating" concentration of substrate is reached when the reaction proceeds with maximum velocity (V). From a quantitative study of the relationship between S and reaction rate (v) it has been concluded that many enzymic reactions involving a single substrate can be represented thus:

$$E + S \rightleftharpoons ES \xrightarrow{k} E + \begin{matrix}\text{reaction}\\ \text{product}\end{matrix} \qquad (5)$$

where ES is an enzyme–substrate complex or compound and k is the velocity constant of the reaction whereby the complex breaks up to give free enzyme and reaction product. The theoretical concept of ES is supported by independent direct evidence particularly from absorption data in the ultra-violet to indicate the formation of a compound between enzyme and substrate. In the reaction sequence above it is the velocity constant (k) which determines the maximum velocity (V) and this is achieved when all the enzyme is maintained as ES by the saturating concentration of S. The affinity of the substrate for its enzyme can be expressed as a dissociation constant of the ES complex and, in the simple case, this constant—the Michaelis constant (Ks)—can be determined because it equals the substrate concentration at which the rate of reaction is $0{\cdot}5\,V$ (half the maximum rate).

Enzyme-catalysed reactions like all thermochemical reactions increase in rate with rise in temperature. Below temperatures where denaturation of the enzyme begins to occur, enzymic reactions usually have a temperature coefficient (Q_{10}) between 2 and 3, i.e. the rate rather more than doubles for each 10°C rise in temperature.

The rate of all enzymic reactions is influenced by pH; most enzymes have a well defined pH optimum (pH at which they

are most active). This presumably occurs because pH determines the ionisation of the oxygen and nitrogen-containing R groups of the amino acids in the protein molecule and a particular state of ionisation of such groups at the active centre of the enzyme is necessary for peak activity. pH may have two other effects. If sufficiently high (alkaline) or low (acid) it may denature the enzyme protein. It may also alter the position of equilibrium of the reaction. This can be exemplified by the reactions catalysed by the dehydrogenase enzymes which have a pyridine nucleotide as co-enzyme. The oxidised form of the co-enzyme, diphosphopyridine nucleotide, can be represented as DPN^+, the reduced form as DPNH. The specific dehydrogenase which acts upon ethanol (the *alcohol dehydrogenase*) catalyses the following reversible reaction:

$$\underset{\text{ethanol}}{CH_3.CH_2OH} + DPN^+ \xrightleftharpoons{\text{dehydrogenase}} \underset{\text{acetaldehyde}}{CH_3.CHO} + DPNH + H^+ \quad (6)$$

H^+ is thus a product of the forward reaction and a change of pH of one unit is equivalent to a tenfold change in H^+ and will, therefore, alter the position of equilibrium since the equilibrium constant (K) is given by the equation

$$K = \frac{(CH_3CHO)\,(DPNH)\,(H^+)}{(CH_3CH_2OH)\,(DPN^+)}$$

The study of the inhibitors of enzymes has also contributed to our understanding of how enzymes act. Certain enzyme poisons such as carbon monoxide, cyanide and diethyl dithiocarbamate have been shown to inactivate enzymes by virtue of their high affinity for metals. Thus the inhibition of *cytochrome oxidase* by CO and CN^- is because these combine with the iron of the haem prosthetic group. Similarly, it is the affinity of dithiocarbamate for copper which makes it a powerful inhibitor of *ascorbic acid oxidase*. A second group of inhibitors combine with or oxidise sulphydryl groups (—SH in cysteine) and the activity of many enzymes (the

protein-degrading enzyme *papain*, the dehydrogenases and many others) depends upon some of the —SH groups in the protein being unsubstituted and reduced. The inhibitors which act in this way include iodoacetate, p-chloromercuribenzoic acid and the heavy metals (Cu^{++}, Hg^{++}).

The action of some inhibitors can be prevented or reversed by addition of more substrate; the extend of inhibition depends upon the ratio of inhibitor to substrate concentration. The classical example of such a relationship is the inhibition of the enzyme *succinic dehydrogenase* (this enzyme oxidises succinic acid) by malonic acid. The chemical similarity between substrate and inhibitor is clear from the formulas of succinic and malonic acids (see page 93).

In this case we know that the malonic acid combines with the enzyme protein at the site normally occupied by succinic acid (to give an enzyme–inhibitor complex, *EI*) but is not oxidised. Further, the concentration of succinic acid required to displace the malonate depends upon the relative affinities of the enzyme for substrate and inhibitor. If *Ks* and *Ki* are the dissociation constants, respectively, of *ES* and *EI*, then in the presence of a given concentration of inhibitor (*I*), the "apparent Ks", in practical terms the substrate concentration required to give half the maximum velocity ($0{\cdot}5V$) is increased to the value $Ks(1+(I)/Ki)$. Now this forms the basis of a practical test for this kind of competitive inhibition, a test to which we shall refer again when considering competition between ions in their uptake by plant cells (p. 163). This test depends upon the quantitative relationship between substrate concentration (*S*) and the velocity of the reaction (v). This is such that if we plot $1/v$ against $1/S$ (a double reciprocal plot) then we get a straight line with the intercept at $1/V$ (reciprocal of the maximum velocity) and of slope Ks/V. Then, if we had a competitive inhibitor the slope is altered to $Ks\dfrac{\left(1+\dfrac{(I)}{Ki}\right)}{V}$ but the intercept remains at $1/V$. This is in contrast to the action of a non-competitive inhibitor which does not

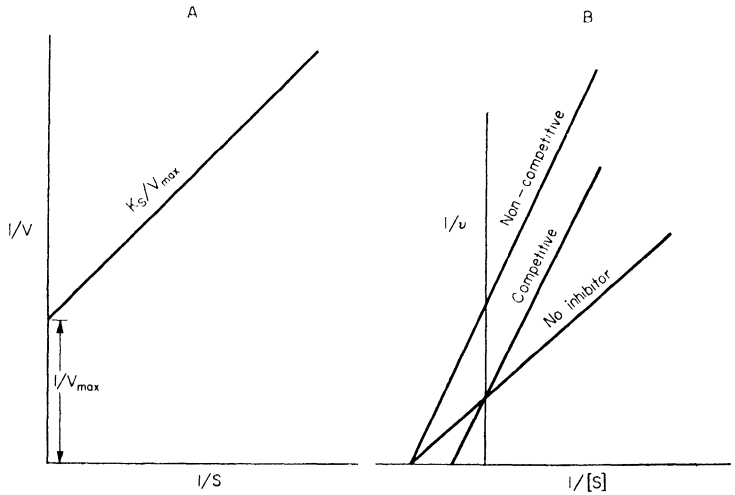


FIG. 27. Enzyme reaction kinetics. A. Plot of the reciprocal of the velocity of reaction $\left(\frac{1}{v}\right)$ against the reciprocal of the substrate concentration $\left(\frac{1}{S}\right)$. The value for the reciprocal of the maximum velocity shown. The slope corresponding to Ks/Vmax where Ks Michaelis constant; B. Similar plots to that in A but showing effect upon slope and value of the reciprocal of Vmax of adding a competitive and non-competitive inhibitor.

alter the slope but alters the maximum velocity and hence the value of $1/V$ as given by the point of intercept (Fig. 27).

HOW ENZYMES ACT

Most of the reactions which proceed in living cells are reversible reactions. A position of equilibrium is reached at which the forward and back reactions proceed at equal rate. Enzymes do not alter this position of equilibrium. What enzymes do is to speed up the reaction, they speed up equally both the forward and the back reaction.

In order to react, molecules must change from the passive to the "activated" state; the rate of reaction is determined by the number of molecules whose energy content renders them sufficiently activated. In a population of molecules all do not have the same energy content and by collisions between them this distribution is constantly changing. In a collision one molecule may have its energy increased at the expense of another molecule. If the temperature is raised the thermal agitation of the molecules is enhanced and a higher proportion will become "activated", in consequence the rate of reaction will increase. Some reactions have a high energy of activation, there is a high energy barrier impeding the reaction. Such reactions will only proceed at a measurable rate at high temperature. Many reactions known to occur in living cells will only proceed at high temperature in the absence of the appropriate enzyme. In the presence of the enzyme they proceed at temperatures compatible with life. What catalysts do, including enzymes, is to lower the energy of activation of reactions. The extra energy above the mean energy which the molecules have to acquire in order to react is reduced. Thus, it has been calculated that at 25°C the energy of activation of the acid hydrolysis of sucrose has the value 25,560 cal/mol. Whereas the energy of activation of sucrose hydrolysis in presence of the enzyme *saccharase* is at 25°C only 8700 cal/mol.

Any explanation, therefore, of how enzymes act must explain how they lower the energy of activation of the reaction, how they decrease the stability of their substrates or increase their potentiality to react with other molecules. Explanation of the remarkably specificity and activating properties of enzymes awaits elucidation of the details of the primary, secondary and tertiary structure of enzyme proteins and of enzyme-substrate compounds. This is a formidable task. However, work along these lines is actively proceeding. For instance, the structure of the *relatively* small molecules of the enzyme, *ribonuclease* (an enzyme which digests ribonucleic acid) has recently been almost completely elucidated. This has involved working out the amino acid sequence and folding of a polypeptide chain containing 19 different amino acid residues

and having a molecular weight of 14,000. Work designed to identify and characterise the site within the protein molecule at which it exerts it hydrolytic activity on the nucleic acid has also made some progress. Here, at the "active" site the substrate molecule is presumably placed under the particular molecular strain which will not only bring reactive groups into juxtaposition but reduce the additional energy required to break certain of its chemical bonds.

ENZYMES IN THE LIVING CELL

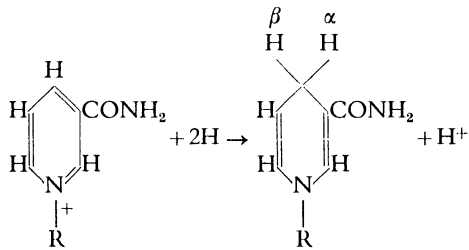
The purified enzymes of the biochemist are not artefacts but the separate catalytic agents of living cells. However, we must remember that within a cell each enzyme functions in the presence of a multiplicity of other enzymes and other protein molecules. Further, we do not know the precise location within the cell of any enzyme nor the concentrations of its substrate(s) or of interfering substances which are present at that location. This means that while the particular catalytic activities of many proteins are now known their quantitative role in metabolism is much less easy to assess. The importance of "enzyme teams" is emphasised by the discovery that cellular organelles or "particles" like plastids, mitochondria and microsomes have each a characteristic spectrum of enzymes and that the biochemical activities of such particles can be interpreted in terms of their enzymic complements. We shall have further occasion to stress the significance in metabolism of organised "multi-enzyme" units of this type.

There are, however, many enzymes which, because they do not seem to be contained in such cellular particles, are referred to as "soluble enzymes". Thus, the chemical reactions whereby the yeast cell converts glucose into ethanol and which seem to be identical with the first sequence of reactions in the normal respiration of plant cells are catalysed by such "soluble" enzymes. In the living cell such enzymes may, however, function as an "organised multi-enzyme" system which forms part of the endoplasmic

reticulum. The fact that they cannot be obtained in association may merely reflect our inability to preserve the endoplasmic reticulum in "cell-free" preparations. Thus, in alcoholic fermentation by the yeast cell, which occurs in the absence of oxygen, there is an oxidative reaction which is balanced by a reduction. These both involve dehydrogenase enzymes having the same co-enzyme, diphosphopyridine nucleotide. One of these reactions, that catalysed by *alcohol dehydrogenase*, has already been mentioned (p. 67; Eqn. 6.). The other reaction involves the oxidation of a triosephosphate by the enzyme *triosephosphate dehydrogenase*:

$$\text{Glyceraldehyde-3-phosphate} + H_3PO_4 + DPN^+ \rightleftharpoons \text{1,3-Diphosphoglyceric acid} + DPNH + H^+ \qquad (7)$$

The DPNH generated in this oxidation is utilised in the reduction of acetaldehyde to ethanol by the alcohol dehydrogenase. It is possible, since the co-enzyme is diffusable, that co-enzyme reduced by triosephosphate dehydrogenase has to migrate in solution to a "remote" site where the alcohol enzyme is located. From the speed of the reaction in the cell it is, however, much more likely that these two "soluble" enzymes do *in vivo* work at the same site. In support of this is the evidence of a difference in the steric specificity of the two enzymes for the DPN. It is the nicotinamide part of co-enzyme molecule which suffers oxidation and reduction by the removal or addition of hydrogen:



(8)

If one of the H atoms at the para position of the nicotinamide ring is replaced by deuterium (heavy hydrogen) then the C atom at this position becomes asymmetric. When this is done it is found

that hydrogen is removed from reduced DPN from one side of the nicotinamide ring by some dehydrogenases and from the other side by other dehydrogenasees. The alcohol enzyme by virtue of the side of the nicotinamide ring to which the hydrogen is added is defined as being α-specific. The *lactic dehydrogenase* which takes the place of the alcohol enzyme in mammalian muscle has the same specificity, whereas the *triosephosphate dehydrogenase* is β-specific. (Fig. 28).

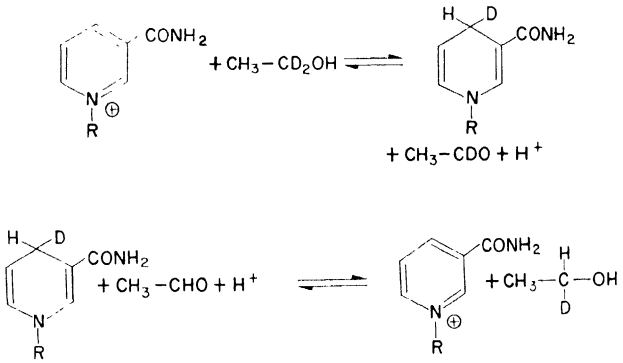


FIG. 28. The α-specificity of the alcohol dehydrogenase as demonstrated by using deuterium labelled alcohol as substrate and then recovering the deuterium by the reverse reaction. D = deuterium. R = ribose–pyrophosphate–ribose–adenine moiety of DPN (see Fig. 34).

Another very important aspect of enzymes in living cells is that cells which are performing different functions have different enzyme complements and activities. The contrasts between the metabolic activities of the absorbing cells of the root, the photosynthetic cells of the leaves, the living cells of the xylem and phloem, the secretary cells of nectaries and other glands, and the actively dividing cells of meristems reflect differences in enzymic activity within the different cell types. Similarly, the synthesis of alkaloids in some plants, of gums and mucilages in others, of tannins and resins in others and of particular polysaccharides (e.g. inulin) in still others, reflect differences in enzymic composition and activities

as between different species. These considerations indicate that genetic differences can be reflected in enzymic differences and raise the possibility that it is entirely through the control of enzyme synthesis and activation that the genes control physiology and development. They also raise the possibility that specialisation of structure and function within the organism is initiated by changes in the number and relative activity of enzymes within the incipient tissues. Great interest clearly attaches to the mechanism of protein synthesis, including the synthesis of enzymic proteins and of how the genes exert a control over this vital process.

In subsequent chapters we shall not only consider the action of further individual enzymes and of the multienzyme systems of chloroplasts, mitochondria and microsomes but the regulation of metabolism and differentiation through the controlled synthesis and activation and inhibition of enzymes.

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4

CATABOLISM

"*The existence of such anaerobic organisms is hardly in agreement with the old dogma 'no life without respiration' unless we assume, as is here done, that respiration includes all metabolic processes that involve a liberation of energy.*"

W. Pfeffer in *The Physiology of Plants.* Vol. 1. Translated by E. J. Ewart, Oxford, 1900.

"*Respiration in plants is taken to include all the phenomena of dissimilation, the characteristics of which are the breaking down of complex substances into simpler ones with a consequent release of energy.*"

W. Stiles and W. Leech in *Respiration in Plants.* Methuen & Co. Ltd., London, 1932.

INTRODUCTION

CATABOLISM embraces those processes which involve degradation of the chemical architecture of the complex organic molecules of cells. These processes result in the release of the potential energy of organic compounds and the major catabolic systems of living cells play an essential role in metabolism in that they release a substantial part of this energy in forms utilisable by the cell. Further the intermediate compounds arising during catabolism are often the starting materials for the synthesis of essential cell constituents.

The reactions involved in the oxidation of organic cell constituents by molecular oxygen constitute the catabolic process of *cellular respiration.* In this process, the tranfer of electrons from the organic molecules results in the reduction of oxygen to water

and the release of their carbon as carbon dioxide. This is the significance of the continuous uptake of oxygen and release of carbon dioxide by plant cells first experimentally demonstrated by de Saussure in 1804.

However, when the cells of higher plants are deprived of oxygen (placed under anaerobic conditions) the carbon dioxide release persists at least for some time and at least part of this carbon dioxide arises from a process of *fermentation*. Here, the electron acceptors are organic compounds (particularly pyruvic acid and acetaldehyde) and their reduction leads to the appearance in the cells of compounds like lactic acid and ethyl alcohol. Clearly, this fermentation which can occur in the cells of higher plants closely resembles the alcoholic fermentation of yeast first studied in detail by Pasteur in 1870 and the production of lactic acid in mammalian muscles under conditions of an oxygen deficit, a process studied in detail by Fletcher and Hopkins in 1907.

The hexose sugar, glucose, can serve as the starting point for either respiration or fermentation and the following equations are often used to contrast these two processes:

Respiration

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O \qquad \Delta F = -686 \text{ kcal} \tag{9}$$

Fermentation

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2 \qquad \Delta F = -54 \text{ kcal} \tag{10}$$

ΔF represents the change in free energy per gram-mol of substrate (glucose) and the negative sign of this value indicates that the reaction involves a *decrease* in free energy, i.e. a release of energy from the system.

The equations (Eqn. 9 and 10) indicate that both processes involve carbon dioxide evolution and decrease in dry weight of the cell. They also draw attention to the oxidative nature of respiration and to the much greater amount of energy released per molecule of glucose in this process.

These introductory paragraphs clearly prompt a number of interesting questions which will form the basis of our developing discussion of catabolism. At what rates do these catabolic processes proceed in plant cells and what are the factors which determine these rates? What substances other than glucose can and do regularly act as substrates for respiration? What are the chemical reactions involved and whereabouts in the cell do these reactions take place? How far do the reactions of fermentation still proceed in the cell when oxygen is available? How are these catabolic processes linked to the synthetic (anabolic) reactions of the cell and to other cellular processes which are energy-requiring?

RATES OF RESPIRATION

Rates of respiration can be calculated from measurements of carbon dioxide evolution and/or oxygen uptake, from recording rates of liberation of heat, or from measuring, in replicates of the experimental material, decreases in dry weight, in calorific value or in the content of specific food substances. The rate of respiration when calculated will be expressed by reference to unit time (e.g. per hour) and to unit amount of plant material (e.g. per unit fresh or dry weight, per single cell of the tissue, or per unit weight of cellular protein). The experimental conditions under which the determinations were made (temperature, oxygen supply, light or darkness, etc.) and the species, age and exact nature and previous nutritional history of the experimental plant material must also be precisely described.

Studies of respiration rate well illustrate the importance of expressing the rates of physiological processes in as many different ways as possible. Figures 29 and 30 show the oxygen uptake of young roots of maize expressed in different ways. When the respiration rate is expressed by reference to *fresh weight* the peak rate is in the region of most active cell division (0·5–2·2mm. behind the apex.) When, however, rate is expressed *per cell* or *per unit of protein nitrogen* the region of most active cell division has a minimal

rate of respiration while a high rate of respiration is recorded in the region of active cell elongation (2·2–7·0 mm behind the apex).

In the classical researches of Kidd, West and Briggs (1921) on the respiration of the sunflower, *Helianthus annuus*, it was found that while during the first 60 days of growth the total carbon

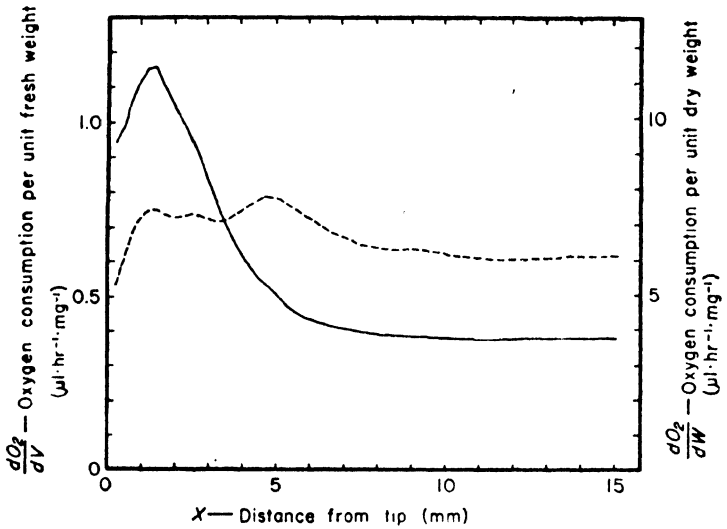


FIG. 29. Oxygen consumption (μ1 per hour) of seedling *Zea mays* (corn) roots at different distances from the tip. Solid curve per mg. fresh weight; broken curve per mg. dry weight. (from D. R. Goddard and W. D. Bonner in *Plant Physiology* Vol. 1A. Edited by F. C. Steward. Academic Press, New York, 1960).

dioxide evolved per plant per hour rose, there was from the onset of seedling growth and throughout the subsequent period a fall in the intensity of respiration expressed per unit of plant dry weight largely due to the increasing accumulation of inert dry matter in the form of cell wall materials. However, when the intensity of respiration is expressed per unit of protein nitrogen the rate is seen to increase with age probably indicating an increase in the rate of respiration per unit of cytoplasm as cells mature and senesce.

Goodwin and Goddard's studies of the oxygen uptake of the different tissues of the stem of *Fraxinus* again illustrate the importance of the units in which respiration rate is expressed. Per unit of fresh weight the cambium is the tissue with the most active respiration; however, when the rate is expressed per unit of

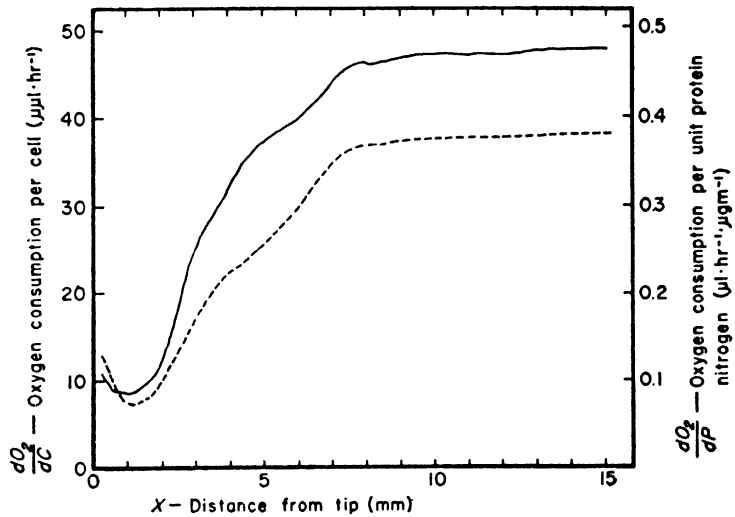


FIG. 30. Data as in Fig. 29 plotted by reference to cell number (solid curve) and to protein nitrogen (broken curve). Mitosis occurs from 0·7 to 2·2mm from tip, elongation is maximal at 4–5mm and ceases 9·0mm from root tip.

nitrogen the recently differentiated xylem is more active than the cambial tissue. Presumably, on a fresh weight basis, the respiratory activity of the xylem parenchyma and vascular ray cells is obscured by the presence in the xylem tissue of the mature and metabolically inert conducting cells.

Certain environmental factors markedly influence the rate of respiration. It is well known, for instance, that most tissues respire very slowly at or close to 0°C and that with rise in temperature there is a steady rise in respiration rate until we reach the

optimum temperature (temperature at which the maximum rate is recorded) somewhere between 30–40°C. Within the range 0–30°C the temperature coefficient may be fairly constant, and such temperature coefficient $\left(Q_{10} \text{ values} = \frac{\text{rate at } t+10°C}{\text{rate at } t°C}\right)$ frequently fall within the range 2·1–2·6. This means that the respiration process as a whole is responding like a thermo-chemical reaction to change in temperature (Chapter 3, p. 66). Above the optimum temperature the rate of respiration declines, particularly when the duration of exposure is prolonged. The deleterious effects of high temperatures are usually interpreted in terms of the denaturation of the essential respiratory enzymes.

A number of investigators have reported an enhanced rate of respiration in non-chlorophyllous tissues, e.g. *Vicia faba* roots following transfer from darkness to light. Activity was reported as being in the blue region of the spectrum (wave-lengths below 560 mμ) suggesting absorption of the light by carotenoid or flavanoid pigments. Further, even in chlorophyllous tissue, e.g. leaves, there appear to be stimulatory carry-over effects of illumination on the rate of respiration which cannot be explained in terms of enhanced levels of respiratory substrate produced by photosynthesis. In calculating rates of photosynthesis from measurements of carbon-dioxide uptake or oxygen evolution it is necessary to assume that the respiration rate is not directly influenced by light and uncertainty regarding this assumption has complicated attempts to determine the optimum efficiency of light energy utilisation in the photosynthesis of sugars.

The rate of oxygen uptake in tissues rich in respiratory substrates progressively increases as the external concentration of oxygen is raised from zero to 100%. This relationship is shown in a generalised form in Fig. 31. When respiration rate is measured by determining the rate of carbon dioxide evolution, a minimum rate (the extinction point=EP) is recorded at a low oxygen concentration (usually at some point within the range 1–9% oxygen depending on the tissue used) and below this oxygen tension the

carbon dioxide evolution rises steeply (Fig. 32) due to fermentation. The form of the carbon dioxide evolution curve illustrates the suppression of fermentation by oxygen (the Pasteur effect) but does not indicate the oxygen tension at which fermentation is completely suppressed; this can only be determined by simultaneous determinations of alcohol and lactic acid formation at these low levels of oxygen supply.

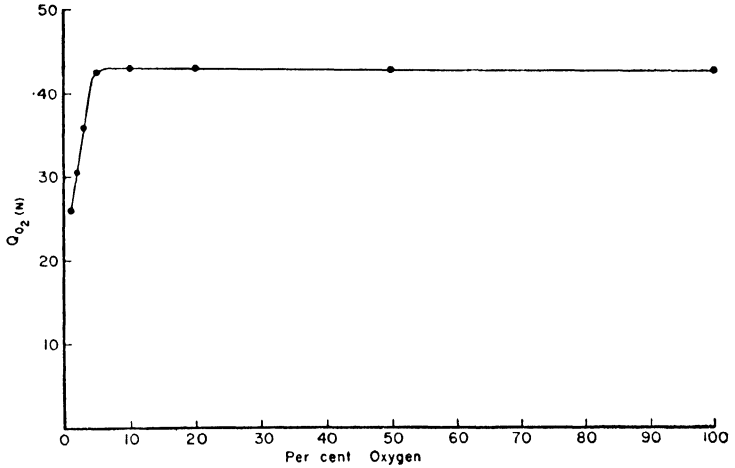


FIG. 31. The influence of the per cent oxygen upon oxygen uptake by the etiolated hypocotyl of the valentine bean (Results of Susan Smith and W. D. Bonner from Goddard and Bonner, 1960 as Fig. 29).

The rate of respiration may be controlled by the concentrations of respiratory substrates in the cells. A response curve similar to that shown in Fig. 31, where respiration rate is plotted against concentration of oxygen, can be obtained by inducing different levels of soluble sugar in potato tubers by storage at temperature within the range 1–10°C and then measuring their oxygen uptake at constant temperature. Further study of the soluble sugars present in the tubers shows that it is the content in the cells of the disaccharide, sucrose, which controls the rate of respiration. The

control of respiration rate by carbohydrate supply is also illustrated by measurements on the oxygen uptake of root tips derived from excised tomato roots, depleted of carbohydrate reserves by a period of culture in sugar-free solution and then supplied with sucrose at different concentrations (Fig. 33).

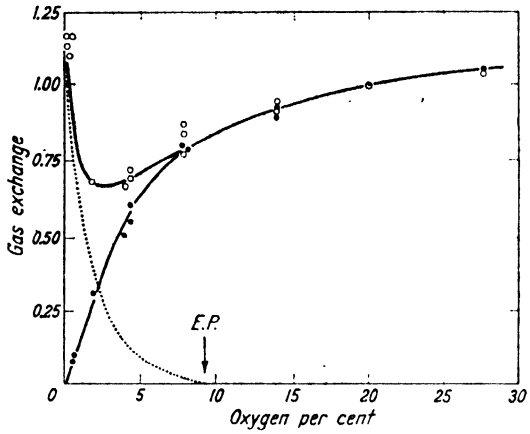


FIG. 32. The influence of the per cent oxygen upon the oxygen uptake (●) and the carbon dioxide evolution (0) of Bramley's Seedling apples. Dotted line shows the carbon dioxide evolution due to fermentation. E.P.=the oxygen tension at which fermentation is completely suppressed, (after Watson from W. O. James, *Plant Respiration*. Clarendon Press, Oxford, 1953).

Experiments on the respiration of detached leaves not only illustrate the control of respiration rate by availability of respiratory substrates but illustrate very clearly that not only carbohydrates but also proteins may be respired. This protein respiration involves protein hydrolysis to yield amino acids and amides, deamination of these amino acids and subsequent decarboxylation (leading to carbon dioxide release) and oxidation of the resulting organic acids.

In the overall equation (Eqn. 9. p. 76) used to express the respiration of glucose, the volume of carbon dioxide evolved is equal to the volume of oxygen absorbed. The fraction

$\frac{\text{volume of } CO_2 \text{ evolved}}{\text{volume of } O_2 \text{ absorbed}}$ is referred to as the respiratory quotient (R.Q.) and in the case of the respiration of glucose is unity. When fats are the respiratory substrates the R.Q. is 0·7, when proteins,

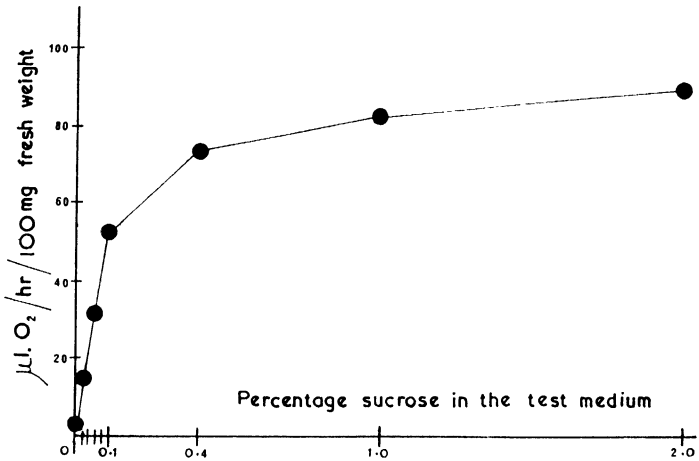


FIG. 33. Influence of the sucrose concentration in the external medium upon the rate of respiration of cultured tomato roots. The roots were grown in presence of 2 per cent sucrose, maintained in a sugar-free solution for 48 hr and then transferred to the test media and their oxygen uptake measured for 1 hr commencing 67 hr after transference to the test medium.

the R.Q is 0·8. When organic acids are oxidised R.Q. values greater than unity are obtained. For instance, the complete oxidation of malic acid according to the equation

$$\underset{\text{malic acid}}{\begin{matrix}CH_2.COOH\\ |\\ CHOH.COOH\end{matrix}} + 3O_2 \longrightarrow 3H_2O + 4CO_2 \qquad (11)$$

corresponds to an R.Q.=1·33. Values of R.Q. above untiy, particularly at low oxygen tensions, may also be indicative of the occurrence of fermentation.

CHEMICAL PATHWAYS OF RESPIRATION

One of the outstanding achievements of biochemistry has been the elucidation of the sequence of chemical reactions and the isolation of the enzymes which are involved in the alcoholic fermentation of sugars by yeast. This story had its beginning in one of the historic experiments in plant science, the preparation of "yeast press juice" by Hans and Edward Buchner in 1897 and the demonstration that this juice could promote an active fermentation of sucrose.

W. Pfeffer, influenced by the earlier studies of Pasteur, advanced in 1900 the view that respiration might proceed in two stages, a first stage corresponding to fermentation and a second stage involving the oxidation by oxygen of the products of fermentation. This concept was developed by two distinguished Russian plant physiologisists, V. I. Palladin (1922) and S. Kostychew (1927) who emphasised that the enzymes of fermentation occur in the cells of higher plants and that there is no evidnece to suggest that their activity is suppressed by oxygen. Further, knowing that ethyl alcohol is not itself readily oxidised by plant cells, they postulated that some precursor of alcohol in the fermentation sequence of reactions suffered rapid oxidation in the presence of oxygen. In support of Kostychew's hypothesis it is now known that (1) low concentrations of cyanide and of hydrogen sulphide by inhibiting the oxidative reactions of respiration lead to the accumulation of alcohol and acetaldehyde in plant tissue supplied with oxygen; (2) substances known to inhibit fermentation have been shown to inhibit respiration; (3) all the compounds known to be intermediates in fermentation can be detected in respiring cells. The "precursor of alcohol" which suffers complete oxidation to carbon dioxide and water in respiration is now known to be *pyruvic acid.* This contention is supported by a large body of evidence starting from the pioneer discovery by von Grab in 1921, using β-naphthylamine as a trapping agent, that pyruvic acid is formed during yeast fermentation prior to acetaldehyde and alcohol. This

was followed by the demonstration that the addition of 1-naphthyl-2-sulphonic acid to higher plant cells causes the accumulation of pyruvate by inhibiting its conversion to acetaldehyde by the enzyme carboxylase and by numerous experiments in which the cells of higher plants and animals have been shown to effect rapid oxidation of added pyruvic acid. We can now, therefore, develop our consideration of the chemical pathways of respiration by asking what reactions are involved in the formation of pyruvic acid and by what reactions this is oxidised by molecular oxygen.

The unravelling of the sequence of chemical steps involved in the formation and oxidation of pyruvic acid has followed from studies of the chemical activities of plant extracts and of the individual enzymes isolated from such extracts. Such studies have now yielded a very detailed picture of the biochemical reactions involved in respiration; have provided us with the essential biochemical foundation for a critical study of the process of respiration as it proceeds in the living cell. In this volume where our emphasis is on the metabolism of living cells a detailed consideration of the biochemistry of respiration or of any of the other vital aspects of metabolism would be inappropriate. Nevertheless, and just in so far as interpretation of cellular metabolism requires appreciation of the nature of the underlying chemical events we can properly draw upon the findings of biochemistry.

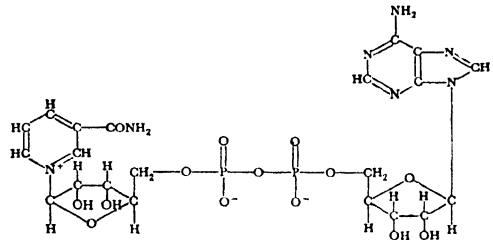
In 1908 the British biochemists, Harden and Young, when studying the alcoholic fermentation of glucose by "yeast pressed juice" found that the fermentation was augmented by the addition of inorganic phosphate. This enhancement was short-lived and if when the rate began to decline the fermentation liquor was boiled and then treated with uranium acetate, a sugar phosphate was precipitated. This was shown to be a diphosphate of fructofuranose and since the phosphate groups are attached to carbon atoms 1 and 6 of the sugar, is known as fructose-1-6-diphosphate. Subsequent biochemical work has shown that this compound arises whenever the primary substrate of respiration is glucose, fructose, some other simple sugar or a polysaccharide such as starch. The first stage

of carbohydrate respiration involves phosphorylation and interconversion of sugars to give this fructose-1-6-diphosphate.

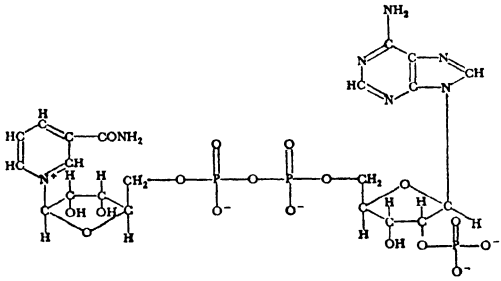
In 1934 it was shown that yeast and mammalian muscle contain an enzyme (aldolase) which splits this fructose-1-6-diphosphate between carbon atoms 3 and 4 to give two triose phosphates (glyceraldehyde-3-phosphate and dihydroxyacetone phosphate). The same enzyme was, in 1949, shown to be universally distributed in higher plants. Further, the two isomeric triosephosphates formed are interconvertible by means of a second enzyme, phosphotriose isomerase. This is important because it is only the glyceraldehyde-3-phosphate which is normally further metabolised in respiration.

Harden and Young, who revealed the importance of phosphorylation in fermentation, also showed that the "yeast press juice" (containing the mixture of enzymes known as zymase) could be separated by passage through a porous porcelain candle impregnated with gelatin into (1) a colloidal protein fraction which did not pass the filter and (2) a crystalloidal fraction stable to heat. Neither fraction alone promoted glucose fermentation but the activity was restored by mixing together the two fractions. Following the concept introduced by Bertrand as early as 1897, Harden and Young recognised the crystalloidal fraction as a co-ferment or *co-enzyme* which came, therefore, to be referred to as co-zymase. This co-enzyme was subsequently isolated in pure form and named diphosphopyridine nucleotide (or, for short, as DPN). This name has remained in use although it does not adequately indicate the nature of its two component nucleotides* (Fig. 34). Now DPN is a vital component of those enzyme systems called dehydrogenases which catalyse the oxidation of substances by removal from them of pairs of hydrogen atoms. The hydrogen removed from the molecule of the metabolite reduces the co-enzyme. DPN functions as the co-enzyme for a number of dehydrogenases each of which acts on a specific metabolite by virtue of the specificity of the

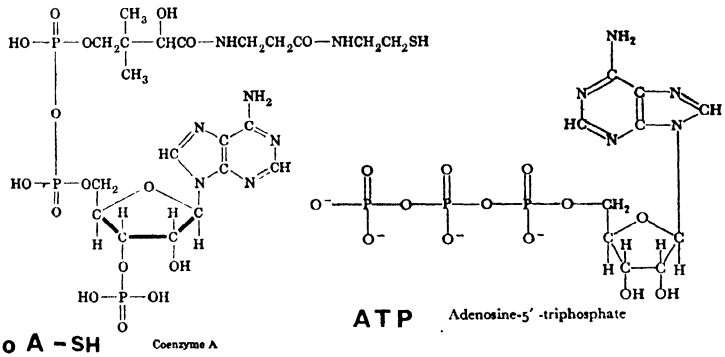
* In research literature this co-enzyme is now referred to as nicotinamide adenine dinucleotide (NAD).



DPN Diphosphopyridine nucleotide



TPN Triphosphopyridine nucleotide

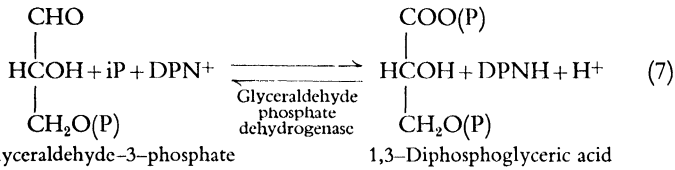


Co A – SH Coenzyme A

ATP Adenosine-5′-triphosphate

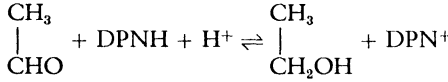
FIG. 34. The chemical structure of co-enzymes and of adenosine triphosphate.

catalytic enzyme protein. The glyceraldehyde-3-phosphate formed during fermentation is metabolised by being acted upon by one such specific dehydrogenase, the enzyme triosephosphate dehydrogenase. The action of this particular dehydrogenase is rather unique as the oxidation of the free aldehyde group of the triosephosphate to an acidic carboxyl group is coupled with combination of the carboxyl group with inorganic phosphate to give a diphosphoglyceric acid. The overall reaction can be represented thus: (as Chapter 3 p. 72)



Glyceraldehyde-3-phosphate — 1,3-Diphosphoglyceric acid (7)

In fermentation this oxidative step is equated with a balancing reduction in which the co-enzyme is re-oxidised. In yeast fermentation it is acetaldehyde which is reduced to ethyl alcohol by a second specific dehydrogenase (alcohol dehydrogenase), and the same co-enzyme (see Chapter 3, p. 67). These oxidation-reductions are reversible and here the dehydrogenase is acting "in reverse":



Acetaldehyde — Ethanol (6)

In respiration the reduced co-enzyme is oxidised by the intervention of molecular oxygen as will be discussed below.

Now the chemical steps between diphosphoglyceric acid and pyruvic acid are of interest in another direction. The diphosphoglyceric acid is first acted upon by one of a group of enzymes known as *transferases or kinases*, enzymes which catalyse the transfer of chemical groups from one molecule to another. The action of the kinase here is to produce mono (3)-phosophglyceric acid but the phosphate group removed does not appear as inorganic phosphate but is tranferred to an organic acceptor molecule, a molecule

of adenosine diphosphate (ADP) which is converted to the triphosphate (ATP) thus:

$$\text{1:3 diphosphoglyceric acid} + \text{ADP} \longrightarrow \text{3-phosphoglyceric acid} + \text{ATP} \qquad (12)$$

The 3-phosphoglyceric acid then undergoes an internal chemical change and releases a molecule of water to give a phosphopyruvic acid. The phosphopyruvic acid is now acted upon by a second kinase, transfers its phospho-group to a molecule of ADP (thereby converted to ATP) and pyruvic acid is formed. From a molecule of the hexose diphosphate, two molecules of the 3 carbon keto-acid, pyruvic acid are formed.

The pyruvic acid has now to be completely oxidised and on the assumption that the oxidative steps, like the oxidation of triosephosphate, each involve the removal of two hydrogen atoms, the overall reaction can be represented thus:—

$$CH_3.CO.COOH + 5O \longrightarrow 3CO_2 + 2H_2O \qquad (13)$$

or

$$\underset{\text{pyruvic acid}}{CH_3.CO.COOH} + 3H_2O \longrightarrow 5 \times 2H + 3CO_2 \qquad (14)$$

This latter method of representation (Eqn. 14) is justified because we know that five specific dehydrogenases each acting on a separate intermediate are involved and that the carbon of pyruvic acid is released as carbon dioxide by three reactions in each of which a specific *decarboxylase* enzyme splits off carbon dioxide from a carboxyl group. To initiate the oxidation the pyruvic acid has to be converted to "active acetate" and there has to be present in the cells a catalytic amount of another acid (oxalacetic acid) with which this can combine to give citric acid. The citric acid then undergoes the series of chemical changes which result in the reduction of DPN (dehydrogenase steps), the release of carbon dioxide (decarboxylase steps) and the regeneration of oxalacetic acid ready to combine with further "active acetate". It is this series of reactions which is known as the citric acid, tricarboxylic acid (TCA) or *Krebs cycle* (Fig. 35).

"Active acetate" is important; not only is it an essential intermediate in the oxidation of pyruvate arising from carbohydrate but also in the respiration of fats. The chemical nature of this "active acetate" (highly reactive acetate) was, for a time, an outstanding biochemical puzzle until Lipmann in 1946 obtained

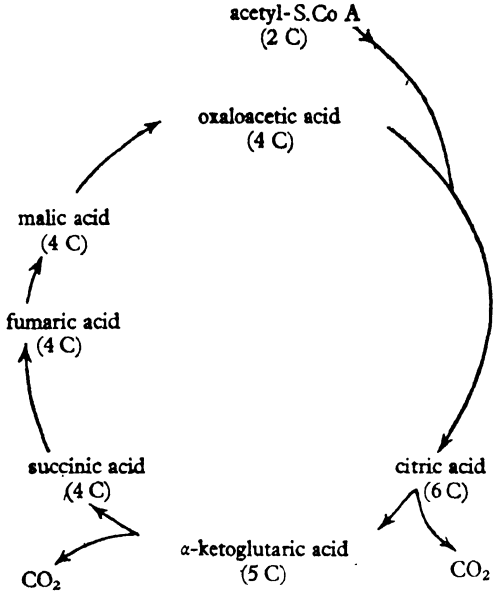
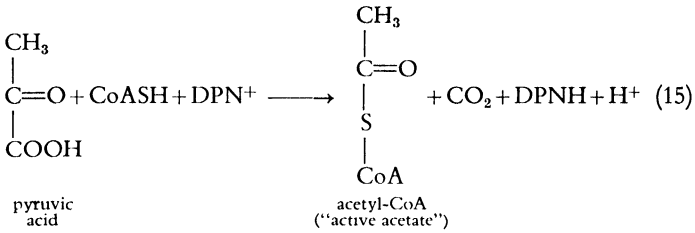


FIG. 35. The Krebs, tricarboxylic acid (TCA) or citric acid cycle. (After E. Baldwin, *The Nature of Biochemistry*, Cambridge University Press, 1962.)

evidence for and later isolated a co-enzyme of acylation now known as *Co-enzyme A* (Fig. 34) and which because it reacts through its sulphydryl group is usually abbreviated in biochemical equations to CoA-ASH. When, in 1951, Lynen demonstrated that the "active acetate" of yeast was acetyl-co-enzyme A the problem was posed of how pyruvate is converted to this compound. This has also proved a difficult problem and its unravelling has shown it

to be a complex process in which still other co-factors are involved. However, the overall equation for the oxidative decarboxylation which occurs can be represented:



This acetyl-CoA then transfers a "2C fragment" to oxalacetic acid to form the citric acid whose metabolism back to oxalacetate is effected by the Krebs cycle enzymes.

The oxidative decarboxylation of pyruvate and the subsequent dehydrogenase reactions of the Krebs cycle generate reduced DPN and the continuation of carbohydrate and fat respiration depends upon this reduced DPN being actively oxidised by molecular oxygen. This reduced DPN, like the reduced form of the other coenzyme TPN discussed below, is, however, stable in air and the oxidation of these co-enzymes depends upon the reaction sequence considered under the heading of "terminal oxidation".

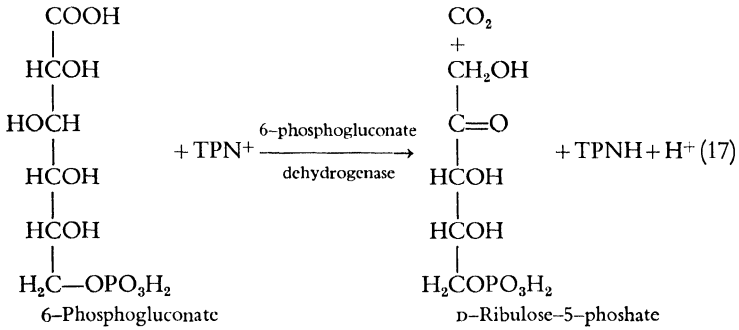
Many famous biochemists contributed to our present knowledge of fermentation and the sequence of reactions involved is often referred to as the *Embden–Meyerhof–Parnas* (**EMP**) *pathway* in particular recognition of the important contribution made by three German scientists. Once this biochemical sequence was worked out, from work particularly with yeast and muscle extracts, evidence quickly accumulated for the widespread and possibly universal occurrence of the intermediate compounds and enzymes concerned. This reaction sequence, coupled with oxidation of pyruvate via the Krebs cycle came to be regarded as *the* pathway of carbohydrate breakdown.

However, ever since the period 1932–7, it has been known that yeast and many other plant cells contain a dehydrogenase which

directly oxidises a monophosphate of glucose arising during the initial phosphorylation of sugars in respiration. Further, that the co-enzyme of this system differs from that of the other dehydrogenases so far mentioned; it is a *tri*-phosphopyridine nucleotide* (TPN). The action of this enzyme can be represented thus:

$$\text{glucose-6-phosphate} + \text{TPN}^+ \longrightarrow \text{phosphogluconate} + \text{TPNH} + \text{H}^+ \qquad (16)$$

Yeast and higher plant cells also contain a second enzyme which oxidatively decarboxylates the phosphogluconate thus:



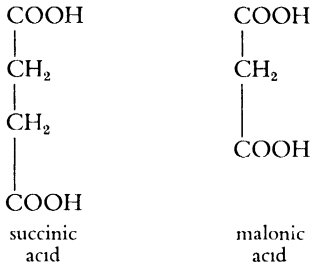
Subsequent research has demonstrated that the pentose (5 carbon sugar) phosphate so formed can suffer complete biological oxidation by a sequence of reactions, the overall effect of which can be represented thus:

$$\text{glucose-6-phosphate} + 12\text{TPN}^+ + 7\text{H}_2\text{O} \longrightarrow 6\text{CO}_2 + 12\text{TPNH} + 12\text{H}^+ \qquad (18)$$

This alternative route of carbohydrate breakdown, which involves as intermediates a number of pentose sugars, a 7-C sugar and a 4-C sugar which are also involved in photosynthesis, is usually referred to as the *pentose phosphate pathway* or *pentose phosphate shunt.*

* In research literature this co-enzyme is now referred to as nicotinamide adenine dinucleotide phosphate (NADP).

The elucidation of the biochemistry of the pentose pathway raises the question of its importance in carbohydrate breakdown relative to the EMP pathway. Evidence on this question comes from two approaches. The first arises from the finding that one of reactions of the Krebs cycle, the oxidation of succinic acid, is very specifically inhibited by another organic acid of similar structure, malonic acid.



The inhibiting action of the malonic acid depends not only upon its own concentration but also in the concentration of the natural substrate of the enzyme, the succinic acid. The inhibition is, therefore, described as competitive and is known to involve competition between the two acids for combination with the enzyme protein; the protein in combination with the malonic acid being inactive as a catalyst of the oxidation of succinate (see Chapter 3, page 68). Malonic acid, by inhibiting this reaction, inhibits the Krebs cycle sequence of reactions and blocks the oxidation of pyruvate by this pathway. Malonate is, in consequence, an inhibitor of respiration and it has been argued that the malonate-sensitive respiration is mediated by the Krebs cycle and the malonate-insensitive by an alternative pathway. However, this conclusion may be an over-simplification because the permeability of cells to malonate varies and is very sensitive to factors like pH and also the shutting down of the Krebs cycle may itself activate a pathway not normally operative at an appreciable rate.

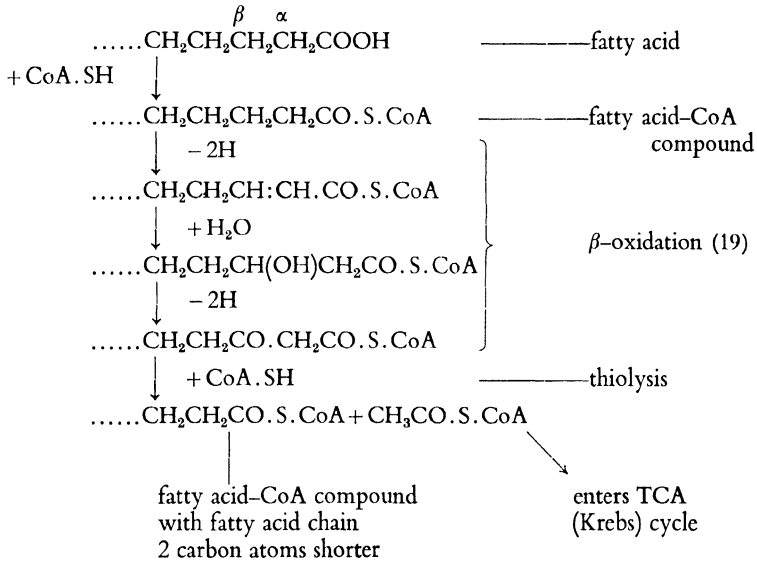
There is, however, another and rather interesting way of approaching this problem by the use of radio-active carbon (C^{14}).

This approach provides strong evidence that carbohydrate degradation in plant cells is in part, though apparently to a variable extent, via the pentose pathway. This involves the use as respiratory substrates of samples of glucose phosphate in which only the first carbon atom or only the 6th carbon atom of the sugar is enriched with radio-active carbon (the glucose molecule is not uniformly labelled but *specifically labelled on particular carbon atoms*). The carbon dioxide which is released immediately after supplying the glucose phosphate will come, in the pentose pathway, only from the carbon atom 1 of the sugar and only when this is radioactive should the carbon dioxide be labelled. By contrast, the interconversion of the triosephosphates (p. 86) in the EMP pathway should result in equally labelled carbon dioxide, whether C1 or C6 is labelled in the glucose sample. Thus, the extent to which the radioactivity of the carbon dioxide is reduced when the C^{14}_6 instead of the C^{14}_1 sugar is provided as substrate will be a measure of the contribution of the pentose pathway to the carbon dioxide evolved in respiration.

So far we have concentrated our attention upon the respiratory breakdown of carbohydrates. In a number of seeds the main food store is fat and the low R.Q. to be observed during the germination of such seeds is evidence of fat respiration. Since the classical studies by E. Schultze in 1876 of the chemical changes occurring during the germination of lupin seed we have known that proteins can also be respired. How far proteins are respired, except in a few such protein-rich seeds or under conditions of starvation and senescence, is, however, controversial. Further, our knowledge of the chemical reactions in the respiration of fats and proteins by higher plant cells is still very limited.

It has already been mentioned (p. 90) that "active acetate" (acetyl-CoA) arises not only from pyruvate but during fatty acid oxidation and that it is via this compound that the carbon of fats enters the Krebs cycle. The first step in fat break-down is a hydrolysis liberating the free fatty acid and glycerol. The glycerol probably undergoes phosphorylation and is oxidised to triose-

phosphate and hence enters the reactions of fermentation. The fatty acids appear to be activated by combination with CoA and then to undergo a process of β-oxidation:



Therefore, β-oxidation successively leads to the release of a two carbon fragment of the fatty acid chain which enters the main pathway of carbohydrate respiration as acetyl—CoA.

Protein respiration almost certainly involves first a hydrolytic breakdown by proteinases and peptidases to give free amino acids. Of the amino acids of plant cells, only glutamic acid is known to be actively oxidised by an enzyme universally distributed, the glutamic acid dehydrogenase:

$$\text{glutamic acid} + DPN^+ + H_2O \rightarrow \alpha\text{-ketoglutaric acid} + NH_3 + DPNH + H^+ \qquad (20)$$

The α-ketoglutaric acid is an intermediate in the oxidation of pyruvate by the Krebs cycle and, therefore, can enter the main

pathway of carbohydrate degradation. Plant cells also contain transaminase enzymes which catalyse the transfer of the amino groups of amino acids to keto acids. One of the most active of these enzymes in plant cells is that transferring amino groups to α-ketoglutaric acid (which is thus converted back to glutamic acid). Such a reaction can be represented thus:

$$\begin{array}{l}\alpha\text{-ketoglutaric} \\ \text{acid (amino} \\ \text{group acceptor)}\end{array} + \begin{array}{c}\text{amino} \\ \text{acid} \\ \text{(R)}\end{array} \longrightarrow \begin{array}{c}\text{glutamic} \\ \text{acid}\end{array} + \begin{array}{c}\text{keto acid} \\ \text{corresponding} \\ \text{to R}\end{array} \qquad (21)$$

It could, therefore, be that all the nitrogen of respired protein can' in this way, be canalised into the formation of glutamic acid and then be released as ammonia by the glutamic acid dehydrogenaese enzyme. We are not certain that this is the case; α-ketoglutaric acid, and oxalacetic acid (corresponding to the amino acid aspartic acid) and pyruvic acid (corresponding to alanine) are acids of the Krebs cycle and can, therefore, be oxidised by this pathway. However, we do not yet know how the other keto acids arising by transamination can be oxidised nor even, in many cases, whether they are normal cell constituents. There is, therefore, as yet no certain knowledge of the reactions by which many of the known amino acids of plant cells are oxidised during protein respiration.

TERMINAL OXIDATION IN PLANT CELLS

The oxidations involved in the respiration of carbohydrates, fats and proteins involve the transfer of hydrogen atoms and electrons and result in the reduction of the co-enzymes. Terminal oxidation involves transport of these hydrogen atoms and electrons to oxygen and a coupling of this with the creation of new chemical bonds in which most of the conserved energy of repiration is held in utilisable form.

Our present understanding, still very incomplete as far as plant cells are concerned, of the enzymic reactions which effect this transport has a long and interesting history. In 1886, MacMunn detected in many animal tissues pigments chemically related to

haemoglobin, capable of existing in both an oxidised and a reduced form and to which he assigned the role of enabling the tissues in which they occur to take up oxygen. This work of MacMunn was not at the time accepted but was later vindicated in a classical paper published in 1925 by Keilin under the title *On Cytochrome, a Respiratory Pigment Common to Animals, Yeast and Higher Plants.* In this paper Keilin not only described the characteristics of these pigments but obtained evidence that they formed a link between the dehydrogenases and oxygen. He was able to recognise three distinct cytochromes which he termed cytochromes *a*, *b* and *c*. These were iron-porphyrins combined with protein (hemoproteins) and in the oxidised form contained ferric (Fe^{+++}) and in the reduced from, ferrous (Fe^{++}) iron. The reduced forms of the cytochromes have distinct absorption bands in the visible spectrum; the oxidised forms diffuse poorly defined absorption spectra. If we take a flat-sided vessel containing a suspension of yeast between a bright source of light and a hand spectroscope and either keep the suspension under anaerobic conditions or add inhibitors like cyanide or sulphide or a reducing agent like hydrosulphite these absorption bands of the reduced cytochromes are clearly visible. If we admit oxygen to the anaerobic suspension these bands disappear as the cytochromes are oxidised by molecular oxygen.

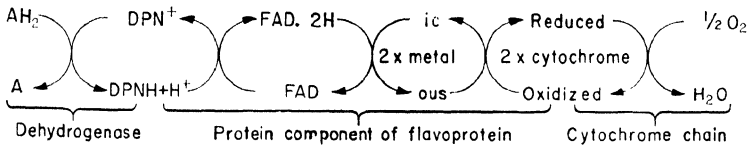
When Keilin succeeded in isolating cytochrome *c* from baker's yeast he found that it could be reduced by mild reducing agents but was then not reoxidised by shaking a solution in oxygen. Its oxidation immediately occurred, however, in the presence of living cells. This suggested the existence in living cells of an enzyme catalysing the oxidation of cytochrome c by oxygen. Now some years earlier the German biochemist, Otto Warburg, as a result of a series of brilliant researches started in 1918, had detected in all the living cells he had examined, an iron-containing oxygen-activating respiratory enzyme (*Atmungsferment*) and shown that the activity of this enzyme was inhibited by carbon monoxide and that this inhibition was reversed by illuminating the cells. In this connection it is interesting to recall that as early as 1897,

Haldane had shown that oxygen is displaced from hemoglobin (also a hemoprotein) by carbon monoxide and that the hemoglobin-carbon monoxide complex is unstable in light.

Warburg went on to demonstrate that the absorption spectrum of his respiratory enzyme indicated it to be a hemoprotein and in 1939 Keilin and Hartree separated from a cytochrome *a* preparation, a new cytochrome (cytochrome a_3 or *cytochrome oxidase*) which reacted directly with oxygen and was in its properties identical in every way with Warburg's respiratory enzyme. Cytochrome oxidase is unique in the rapidity of its reaction with oxygen and in its high affinity for this gas. This affinity for and reactivity with oxygen points to cytochrome oxidase as the last step in the transport of electrons and hydrogen ions to unite with oxygen and form water. The name cytochrome oxidase indicates that the enzyme catalyses a direct oxidation of its substrate (a cytochrome) by molecular oxygen and in this respect resembles the copper-protein enzymes, catechol oxidase first described by Bourquelot and Bertrand in 1895 and ascorbic acid oxidase discovered by Szent-Györgyi in 1931. These copper-protein enzymes have also been considered as possible terminal oxidases in plant respiration but the evidence that they function in this role is very controversial particularly in view of their relatively low affinity for oxygen. Further, while cytochrome oxidase appears to be universally found in respiring cells, these other enzymes have a wide but not universal distribution.

Substances which can exist in an oxidised or reduced form and which can interact can be arranged in order of their ability to oxidise or reduce one another. The highly reducing substances at one extreme of such a series have increasingly negative oxidation-reduction potentials (the standard hydrogen electrode having by definition a zero potential), the highly oxidising substances at the other end of the series have increasingly positive oxidation-reduction potentials. The span of oxidation-reduction potential involved in the electron transport system of respiration ranges from $-0{\cdot}32$ volts for the $DPN^+/DPNH$ system to $+0{\cdot}82$ volts for the oxygen

electrode. The path of hydrogen and electrons from the dehydrogenases to oxygen will be through a sequence of "carriers" each with a more positive oxidation-reduction potential. This enables us to say that electrons will flow from cytochrome *b* → cyt. *c* → cyt. *a* → cytochrome oxidase → oxygen. This poses the question of whether in the respiring cell this cytochrome complex directly oxidises the reduced co-enzymes. The answer is that ferric cytochrome *c*, the most extensively studied of the plant cytochromes, cannot oxidise the reduced form of DPN, so that presumably some hydrogen carrier is interposed between reduced co-enzymes and the cytochromes. In 1932, Warburg and Christian isolated a "yellow enzyme" (a flavoprotein) from yeast and such flavoproteins are now known to be universally distributed in living cells. These flavoproteins consist of protein in combination with flavin nucleotides and the nucleus of these nucleotides can undergo reversible oxidation and reduction; the molecule is colourless in the oxidised form and yellow in the reduced form. The reduced molecules are either slowly oxidised by oxygen or are quite stable in oxygen but they are very rapidly oxidised by ferric cytochrome *c* provided that the flavoprotein has been purified by a method which does not remove the iron or other heavy metal with which it is apparently associated in nature. Two of these flavoproteins are of particular interest to our present problem because one acts as a specific dehydrogenase for reduced DPN and the other for reduced TPN. These flavoproteins are, as might be anticipated, intermediate in oxidation-reduction potential between DPN^+/DPNH and the cytochromes. The hydrogen transport chain involved in the oxidation of reduced DPN in respiration can, therefore, be represented thus:



Clearly, provided each step in this transfer chain is a rapid reaction only catalytic amounts of flavoprotein and the cytochromes will be required to maintain a rapid uptake of oxygen.

THE LOCALISATION OF RESPIRATORY ENZYMES IN PLANT CELLS

The enzymes of fermentation are described as soluble enzymes because when cells are disintegrated in such a way that the nuclei, plastids and mitochondria are not disintegrated and then the suspension is centrifuged at very high speed (up to $100{,}000 \times g$ (the force of gravity)) these enzymes are present in the clear supernatant liquid. Of course, these enzymes may, in the living cell, be bound to the endoplasmic reticulum since this is not preserved in existing techniques used to disintegrate plant cells.

When from a suspension such as that described above the nuclei and plastids are sedimented by low centrifugal forces (up to $2000 \times g$) and then the suspension is spun at speeds giving a centrifugal force of about $10{,}000 \times g$ the mitochondria are sedimented. Such isolated mitochondria are capable of oxygen uptake and the oxidation of pyruvate and other acids of the Krebs cycle. They contain all the enzymes involved in the Krebs cycle and in terminal oxidation. Mitochondria are, therefore, the centres of respiratory activity. Our techniques of isolating mitochondria are still imperfect and the isolated mitochondria are unstable and quickly loose their oxidative and other metabolic activities. Because of this it is not yet possible to prove that the whole of the oxygen uptake involved in respiration is mediated by enyzmes located in the mitochondria but this may well be so.

CONSERVATION OF THE ENERGY LIBERATED IN RESPIRATION

Reference has previously been made to the formation of adenosine triphosphate from adenosine diphosphate at two steps in the sequence of reactions by which triosephosphate is converted to pyruvic acid.

This substance, adenosine triphosphate (ATP) (see Fig. 34), is a universal constituent in living cells and plays a central role in the conservation of energy and in the transfer of energy from catabolic to anabolic (synthetic) reactions.

The phosphate bonds of ATP can be broken hydrolytically under appropriate conditions and the free energy changes associated with the cleavage of each of the three phosphate bonds is as follows:

$$\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{phosphate} \quad \Delta F = -11{\cdot}5 \text{ kcal.} \qquad (23)$$

$$\text{ADP} + \text{H}_2\text{O} \rightarrow \text{AMP} + \text{phosphate} \quad \Delta F = -11{\cdot}5 \text{ kcal.} \qquad (24)$$

$$\text{AMP} + \text{H}_2\text{O} \rightarrow \text{adenosine} + \text{phosphate} \quad \Delta F = -2 \text{ kcal.} \qquad (25)$$

The molecule of ATP therefore contains two phosphate bonds which are "energy-rich" and one which is relatively poor in energy. We use the symbol ~P to represent a high energy phosphate radical so that the ATP molecule can be represented A–P ~P ~P where A represents the nucleoside, adenosine.

The synthesis of ATP from ADP in transfer reactions catalysed by specific kinases conserves energy released in the metabolism of triosephosphate to pyruvate. The reaction:-

$$\begin{matrix}\text{glyceraldehyde} \\ \text{3–phosphate}\end{matrix} + \text{DPN}^+ \rightarrow \begin{matrix}\text{3 phosphoglyceric} \\ \text{acid}\end{matrix} + \text{DPNH} + \text{H}^+ \qquad (26)$$

involves a large decrease in free energy and most of this would be lost as a positive heat of reaction. However, this reaction should be compared with the reaction catalysed by triosephosphate dehydrogenase (p. 88) which has a $\Delta F = +0{\cdot}4$ kcal. Part of the energy

released in the oxidation of triosephosphate is conserved by the simultaneous synthesis of the high energy phosphate bond in the carboxyl group of C atom 1 of the diphosphoglyceric acid (the instability of this 1 carboxyl phosphate group is indicative of its energy-rich nature). However, despite the instability of this carboxyl phosphate bond, its energy is conserved because in the presence of ADP and of the specific kinase, ATP is synthesised (Eqn. 12. p. 89). A similar conservation of the high energy content of the phosphate bond of phosphopyruvic acid is achieved by the formation of pyruvic acid being linked to the synthesis of ATP from ADP.

It thus follows that in the conversion of a molecule of fructose 1-6-diphosphate to 2 molecules of pyruvic acid, four of the terminal phosphate bonds of ATP are synthesised. However, ATP is, as already mentioned, also involved in synthetic reactions and when glucose is the respiratory substrate two molecules of ATP are degraded to ADP to form the fructose-diphosphate. The net gain in ~P during the fermentation of a gram mol. of glucose is, therefore, $2 \times 11{\cdot}5 = 23$ kcal. as phosphate bond energy. The equation (Eqn. 10) on p. 76) shows that the ΔF of fermentation is -54 kcal from which we can calculate that of the total energy released $23/54 \times 100 = 43\%$ is conserved as energy in ~P of ATP. Comparison of the equations for Respiration and Fermentation (Eqn. 9 and 10) leads us now to enquire whether the very much greater energy release in respiration is also conserved by ATP synthesis. Thus we are led to seek for reactions involved in pyruvate oxidation in which the energy released is conserved by simultaneous synthesis of ATP from ADP.

Using pigeon brain dispersions and heart muscle extracts, Ochoa, in 1944, obtained evidence that 6 molecules of inorganic phosphate were taken into organic combination per *molecule* of oxygen absorbed in respiration. Later work with mitochondria showed that a similar phosphate assimilation accompanied the oxidation of organic acids, including pyruvate. Mitochondrial preparations can also be used to study the quantitative synthesis of ATP during the oxidation of pyruvate by oxygen. To do this a rather interesting

technique has to be employed. Although the adenosine phosphates are so important in the energy relationships of the living cell they are present in cells in small amounts. Consequently, if ATP synthesis from ADP proceeds actively the small amount of ADP present is quickly depleted unless some mechanism by which the terminal phosphate bond of ATP is transferred in a further phosphorylation reaction is proceeding simultaneously and thereby constantly regenerating ADP. Reference has already been made (p. 102) to the phosphorylations of sugar which preceeds the formation of triosephosphate and to the involvement of ATP in these reactions. One of these phosphorylation reactions is catalysed by the enzyme, glucokinase and can be represented thus:

$$\text{glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP} \qquad (27)$$

If we take some recently isolated mitochondria, add some ADP to reinforce the natural content, glucose and glucokinase to trap the phosphate of newly formed ATP as glucose-6-phosphate, and an oxidisable substrate such as pyruvate or other organic acid of the Krebs cycle, it is possible to demonstrate active phosphorylation. Further, by measuring glucose-6-phosphate formation and oxygen uptake by the mitochondria one can work out what is called the P:O ratio, the molecules of phosphate incorporated into ATP per oxygen *atom* absorbed. This was first successfully carried out using animal mitochondria, but in work since 1953 by Laties and many others, active and efficient phosphorylation has been obtained with mitochondrial suspensions from higher plants. The most efficient suspensions of mitochondria have given P:O ratios above 3·0 and approaching 4·0 which corresponds well with Ochoa's estimates made earlier and implies that at least some 15 (5×3) $\sim$P can be synthesised per molecule of pyruvate oxidised (see Eqn. 13 and 14 for pyruvate oxidation on p. 89). It is interesting that many mitochondrial preparations have much lower efficiencies than those recorded above, the P:O ratios often approximating to 1·0, and that as mitochondrial suspensions "age" they loose their ability to effect phosphorylation while their oxidative activity as measured

by oxygen uptake and carbon dioxide release is unimpaired. A similar effect follows addition of dinitrophenol to cells or isolated mitochondria; this substance "uncouples" oxidation from phosphorylation, oxygen uptake is usually stimulated but no ATP synthesis takes place.

This brings us to the difficult question of the reactions involved in ATP synthesis in respiration. The reactions of the Krebs cycle have been very fully elucidated, both as regards the chemical changes involved and the changes in free energy occurring at each step. Only at one step (possibly the step operating when P:O ratios of unity are recorded with mitochondria) in this cycle is ATP synthesised. This is in the oxidation of α-ketoglutaric acid to succinic acid. In this reaction a compound between succinic acid and Co-A is formed (succinyl-S.CoA) and this reacts with ADP in the presence of inorganic phosphate thus: (see Chapter 3, p. 65).

$$\text{Succinyl-S.CoA} + \text{ADP} + H_3PO_4 \rightarrow \text{ATP} + \overset{\text{succinic}}{\underset{\text{acid}}{}} + \text{CoA-SH} \quad (4)$$

Incidentally, this is an interesting reaction because it shows us that the S-linkage binding CoA to metabolites is also an "energy-rich" bond; that it contains sufficient energy to power the synthesis of ATP from ADP and inorganic phosphate. Now, in the conversion of triosephosphate to pyruvate there are also two reactions (p. 102) involving ATP synthesis. Thus, we know of three *substrate phosphorylations* occurring in the complete oxidation of triosephosphate i.e. 6 molecules of ATP are synthesised by such reactions per molecule of glucose and, since two of these are consumed in the formation of fructose-1-6-diphosphate, there is a gain of $4 \times \sim P$ per glucose molecule by substrate phosphorylation.

Clearly, most of the ATP molecules formed in respiration are synthesised at some other part of the respiratory sequence than in either pyruvic acid formation or its oxidation in the Krebs cycle. Now there is a very large release of energy (decrease in free energy) involved in the oxidation of reduced DPN and this, calculated from the gap in oxidation-reduction potential between the DPN^+/

DPNH system and oxygen, comes out at some 52 kcal per g-mol. In line with this is the experimental observation that when reduced DPN is fed to mitochondria it is possible to obtain P:O ratios close to but usually slightly below 3·0. This means that for every two hydrogen atoms from reduced DPN transported along the terminal oxidation pathway to combine with one atom of oxygen there are up to three molecules of ATP synthesised. Since, to oxidise a molecule of glucose $12 \times 2H$ are transported and 12 atoms of oxygen absorbed, this indicates the synthesis of 36 ~P of ATP per molecule of glucose respired. It is this phosphorylation occurring in mitochondria and linked to the oxidation of reduced co-enzyme via the flavoprotein-cytochrome pathway which is termed *oxidative phosphorylation*.

From the above discussion it will be seen that some 40 ~P (36 by "oxidative" and 4 by "substrate" phosphorylation), each with an energy content of $\simeq 11$ kcal g-mol, are synthesised per g-mol of glucose respired and some 440 kcal therefore conserved as utilisable energy. Since the overall decrease in free energy envolved in the complete oxidation of a g-mol of glucose is 686 kcal (Eqn. 9, p. 76) this means that approx. 60% of this energy is trapped during respiration in a form which can be used in cellular metabolism for the synthesis of cell constituents or to perform work.

One of the major outstanding unsolved problems of respiration relates to the chemical mechanisms involved in oxidative phosphorylation. All we can say with certainty at the moment is that the changes in oxidation-reduction potential in three steps in the hydrogen and electron transport chain of terminal oxidation (in the steps successively between reduced DPN and flavoprotein, between cytochromes *b* and *c* and between cytochrome *a* and oxygen) are each sufficiently large to release energy in excess of that required to synthesise an ~P. What we do not yet understand is how these oxidation-reduction reactions are linked to ATP synthesis (see Chapter 9).

How far energy is conserved when sugar is degraded by the pentose pathway is at present unknown. Isolated mitchondria do not

oxidise reduced TPN. An enzyme (pyridine nucleotide transhydrogenase) is known which catalyses the reaction:

$$TPNH + DPN^+ \rightarrow TPN^+ + DPNH \qquad (28)$$

but the activity and distribution of this enzyme in plants is at present unknown. If plant cells cannot couple the oxidation of reduced TPN with phosphorylation then the significance in cellular metabolism of the pentose pathway is not clear. Although it may be proposed as a mechanism for the synthesis of pentose and other special sugars, we know of reactions whereby C_5, C_7 and C_4 sugars can be formed in cells without the intervention of the TPN-specific dehydrogenases which, in the pentose pathway, oxidise glucose-6-phosphate and phosphogluconic acid.

β-oxidation of fatty acids is coupled to the reduction of DPN and the acetyl-CoA enters the normal pathway of respiration mediated by the mitochondria. Further, there is experimental evidence for a high level of ATP synthesis associated with fat respiration. For instance, it has been calculated that in the respiration of one molecule of palmitic acid ($C_{16}H_{32}O_2$) there is net gain of 137 ~P of ATP, which corresponds to conservation of about 70% of the energy theoretically available from the complete oxidation of this fatty acid.

THE CONTROL OF RESPIRATION

Presumably, at full oxygen tension and optimum temperature and in the presence of abundant respiratory substrate, the rate of respiration is controlled by those chemical reactions whose rate puts a "brake" on the overall process, by reactions which act as "pacemakers". Isolated enzyme systems may be self-regulatory in their activity as instanced by the dehydrogenase which oxidises malic to oxalacetic acid. When in this system the oxalacetic acid concentration reaches 10^{-4}M it inhibits its own synthesis. Further, oxalacetic acid also inhibits another enzyme of the Krebs

cycle, the enzyme oxidising succinic acid. The concentration of oxalacetic acid, itself regulated by the rate of formation of acetyl-CoA with which it condenses, may, therefore, control the rate of cycling of the Krebs cycle. Again the rate of formation of glucose-6-phosphate by glucokinase action will depend upon the concentration of ATP and this, in turn, will depend upon the rate of ATP formation during the metabolism of triosephosphate in fermentation; the rate of glucose phosphorylation will be "geared in" to the rate of triosephosphate metabolism.

Reference has already been made to the stimulation of oxygen uptake which follows the application of dinitrophenol to cells or isolated mitochondria. The "uncoupling" of respiration from oxidative phosphorylation enhances the rate of the oxidative reactions of respiration. To maintain a high rate of oxygen uptake coupled with phosphorylation we have to add to mitochondrial preparations the ~P utilising glucose–glucokinase system and thereby maintain a high ratio of ADP:ATP. When cells are actively absorbing inorganic ions their rate of respiration is increased: this increased rate of oxygen uptake (the so-called "salt respiration") is due to the consumption of energy from ATP in the process of salt accumulation. The availability of the phosphate and energy-acceptor ADP is, therefore, clearly a controlling factor in the rate of respiration.

One of the most [illegible] longest known, and most difficult to explain examples of the regulation of carbohydrate catabolism is the observation, first made by Pasteur, that at low oxygen tensions the rate of respiration is strongly suppressed and the rate of fermentation increased and that under these conditions, and particularly at zero oxygen pressure, the rate of glucose breakdown can be four times that in air. This pronounced "sparing" action of oxygen upon the breakdown of sugars is known as the *Pasteur effect*. In this case, although the effect of oxygen upon the relative levels of ATP, ADP and inorganic phosphate is clearly involved, no simple biochemical explanation of the Pasteur effect is entirely satisfactory (see Chapter 6).

Synthetic processes proceeding in respiring cells not only consume the energy stored in ATP and other phosphorylated compounds but draw upon the intermediates of respiration. For instance, the enhanced rate of respiration of cells actively synthesising proteins is, in part, due to the utilisation in amino acid synthesis of organic acids which are Krebs cycle intermediates. It is clearly a major objective in the study of plant metabolism to understand the quantitative aspects of such inter-relationships between the metabolic processes of the cell.

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5

ANABOLISM

that this operation . . . begins only after the sun has for some time made his appearance above the horizon . . . that this operation of the plants is more or less brisk in proportion to the clearness of the day and the exposition of the plants . . . that this operation of plants diminishes towards the close of day, and ceases entirely at sunset; that this office is not performed by the whole plant, but only by the leaves and the green stalks; that even the most poisonous plants perform this office in common with the mildest and most salutary; that the most part of leaves pour out the greatest quantity of this dephlogisticated air from their under surface . . ."

Jan Ingen-Housz in *Experiments upon Vegetables, Discovering Their Great Power of Purifying the Common Air in Sunshine and Injuring it in the Shade and at Night.* Elmsly & Payne, London, 1779.

"*ATP is the main fuel of life produced in photosynthesis and oxidative phosphorylation. In both cases it is produced by an electric current, that is, the energy released by a 'dropping' electron.*"

A. Szent-Györgi in *Introduction to a Submolecular Biology* Academic Press, New York, 1960.

INTRODUCTION

THE term *anabolism* covers all those aspects of metabolism which involve the development of complex molecules from less complex molecules, of larger molecules from smaller molecules. These are the synthetic reactions of metabolism, the reactions whereby the primary nutrients, the inorganic ions, water and carbon dioxide required by green plants, are built up into a myriad of organic molecules.

The primary process of anabolism is *photosynthesis*, the process whereby sugars are synthesised, in green cells exposed to sunlight, from carbon dioxide and water. As earlier emphasised the unique aspect of this form of carbon assimilation is the conversion of light energy into the chemical energy of newly synthesised sugar molecules. It is this photosynthesis of sugar which maintains life in all its abundance on this planet, it is this process which has reduced the content of carbon dioxide in the earth's atmosphere to 0·03 per cent. and raised its oxygen content to 21 per cent.

It may well be that the first forms of life were colourless anaerobic micro-organisms dependent upon being bathed by a sea containing a great variety of complex organic compounds; that the first organisms were extreme *heterotrophs*. However, the continuation and evolution of life depended upon an event perhaps second only in improbability with the origin of life; depended upon some organism acquiring the ability to utilise as its primary source of energy the sun's radiation, depended upon the "invention" of photosynthesis. Thereby arose a new type of organism, a green plant, which was no longer dependent upon external organic matter but was an autotroph requiring only simple inorganic nutrients. This led to a separation of oxygen from its union with carbon; led to the first appearance of oxygen in the previously anaerobic atmosphere. The reductive process of photosynthesis provided the raw material for a new mechanism of energy release, the aerobic respiration of organic cell constituents. The sugar molecules elaborated by photosynthesis were at one and the same time the starting molecules (precursor molecules) for the synthesis of other organic molecules essential to life and the source of energy for such synthesis.

It is therefore, appropriate to open any discussion of anabolism with a consideration of the process of photosynthesis.

THE DISCOVERY AND GENERAL NATURE OF PHOTOSYNTHESIS

As early as 1772, Joseph Priestley described the power of plants to restore air vitiated by the burning of candles or animal life. Then in 1779 in the book *Experiments upon Vegetables, discovering their Great Power of Purifying the Common Air in Sunshine and Injuring it in the Shade and at Night*, Jan Ingen-Housz clearly established that the purifying activity of plants depended upon their exposure to light, that it involved the absorption of some constituent of the air and that during this process "dephlogisticated air" was evolved. By 1782 came the recognition by Jean Senebier that the constituent absorbed by plants from the air in light was "fixed air" and by 1796 the discovery of the nature of photosynthesis was almost complete for Ingen-Housz in his book *Food of Plants and Renovation of the Soil* published in that year clearly enunciated the view that plants acquire their carbon as "fixed air" which he recognised as carbonic acid, that this carbon is elaborated into organic matter in the light and that during the processes oxygen (previously referred to as "dephlogisticated air") is evolved. It remained for N. Théodore de Saussure in his book *Recherches chimiques sur la vegetation* published in 1804 to describe the quantitative experiments which had led him to the conclusion that both *water* and carbon dioxide are involved in the synthesis of organic matter by green plants in light.

Ingen-Housz recognised that photosynthesis was a property of the green parts of the plant, its leaves and green stems. The pigment complex conferring this green colour was termed chlorophyll in 1818 by Pelletier and Caventou and the importance of this complex stressed by Dutrochet in 1837 who regarded it as a veritable "elixir of life". Julius Sachs in 1862 first postulated starch as a direct product of photosynthesis and described the famous experiment in which if half of a leaf is effectively shaded and the other half exposed to light, then starch appears only in the illuminated half. Since the starch grains arose in the plastids, he regarded these

as the centres of photosynthesis. That the chloroplasts are also the centres of oxygen release in photosynthesis was elegantly demonstrated by Englemann (1882) by using oxygen-sensitive motile bacteria which migrated to the postion of the chloroplasts. Another famous nineteenth century German plant physiologist, W. Pfeffer (1873) showed that starch formation only occurred in illuminated leaves if the atmosphere contained carbon dioxide.

The general nature and importance of photosynthesis was established by the end of the nineteenth century. During the first 40 years of the twentieth century the chemistry of the chlorophyll pigments and their absorption spectra were worked out beginning with the classical studies of Willstätter and Stoll (1913–1918). During this period, the quantitative relationships between photosynthesis and temperature, light intensity and carbon dioxide concentration were also worked out, beginning with the classical studies of F. F. Blackman in 1905. These studies of Blackman showed that when photosynthesis is proceeding in well illuminated leaves receiving an ample supply of carbon dioxide then its Q_{10} is always above 2·0 whereas at low light intensities the Q_{10} approaches 1·0. Q_{10} is the temperature coefficient, the ratio of the rate of a "reaction" at x°C to its rate at $x-10$°C. For ordinary chemical reactions (thermochemical reactions) the Q_{10} is 2·0 or higher (see Chapter 3, p. 66: Chapter 4, p. 80), whereas photochemical reactions (reactions involving the absorption of radiant energy) are almost insensitive to temperature and, therefore, have Q_{10} values close to unity. From these observations, Blackman suggested that at low light intensities photosynthesis was limited by reactions involving light energy (photochemical or "light" reactions), whereas the response of photosynthesis to temperature at high light intensities indicated the occurrence of thermochemical reactions, which he designated, for contrast with the photochemical reactions, as "dark" reactions. In support of this concept the renowned German biochemist, Otto Warburg, showed that cyanide (a powerful inhibitor of enzymes with metal ions as prosthetic groups) was a powerful inhibitor of photosynthesis at high and a relatively

weak inhibitor at low light intensities. The cyanide inhibition of a "dark" reaction was, as expected, most marked at high light intensities when "dark" reactions would be limiting the rate of photosynthesis. At the same time, Warburg showed that at high light intensities, the rate of photosynthesis during a period of illumination was enhanced by intermittent illumination (by interposing dark intervals between the light flashes). A similar observation was later made by Emerson and Arnold (1932) and in their experiments it was shown that. using intense flashes of 10^{-5} second, the minimum dark interval permitting maximum oxygen evolution per light flash was 0·03 sec at 25°C and 0·4 sec at 1°C. These flashing light experiments again pointed to "dark" reactions which could be limiting and which, after a period of intense illumination, could "catch" up in a dark interval to permit again of the maximum rate of oxygen evolution in light. These observations formed the background for the generalisation of the nature of photosynthesis advanced by van Niel in 1930–31 in a survey of photosynthesis both in green plants and in certain purple sulphur bacteria. Van Niel suggested that all forms of photoreduction of carbon dioxide can be formulated:

$$CO_2 + 2H_2A \rightarrow \{CH_2O\} + H_2O + 2A \qquad (29)$$

In the case of the sulphur bacteria A = sulphur, in the case of green plants, A = oxygen. Photosynthesis is represented as an oxidation–reduction reaction in which carbon dioxide is reduced to the level of carbohydrate, represented $\{CH_2O,\}$ by 4 hydrogens from the reductant H_2A which, therefore, also yields 2A (2 atoms of sulphur or a molecule of oxygen according as to whether the reductant is hydrogen sulphide or water). Since, clearly the sulphur formed in bacterial photosynthesis must come from the H_2S then if this general formulation is correct the oxygen of photosynthesis must come entirely from water in green plants. Van Niel's equation for photosynthesis in the green plant is, therefore:

$$CO_2 + 2H_2O \rightarrow \{CH_2O\} + H_2O + O_2 \qquad (30)$$

and the introduction of the second molecule of water in the left-hand side of the equation is meaningful since there must be no less than two water molecules involved to release the molecule of oxygen.

Van Niel further postulated that the photochemical reactions of photosynthesis are concerned with the decomposition of the reductant, water. In its simplest form this would presumably involve four identical photochemical reactions:

$$4\{H_2O\} \xrightarrow{4h\nu} 4\{H\} + 4\{OH\} \qquad (31)$$

In this equation $h\nu$ represents a unit (a photon or quantum) of light energy, $\{H_2O\}$ represents water either as such or in some complex, $\{H\}$ represents the compound which is directly concerned in carbon dioxide reduction and $\{OH\}$ is the "peroxide" whose decomposition releases oxygen and which can be represented

$$4\{OH\} \rightarrow 2H_2O + O_2 \qquad (32)$$

The concept that the photochemical reactions are concerned with the photolysis of water received support from the demonstration in 1937 by R. Hill that he could isolate chloroplasts which although they had lost the ability to reduce CO_2 could, when and only when illuminated, both evolve oxygen and reduce ferric compounds or quinones. The Hill reaction was interpreted as a partial photosynthetic system, one in which the linkage between the primary reducing activity generated photochemically and the "dark" reduction of CO_2 had been broken. The concept that the reactions involved in carbon dioxide assimilation and reduction were the "dark" reactions of photsynthesis was supported by the demonstration, first made in 1935 by Wood and Werkman with bacteria, that CO_2 can be assimilated into organic form using the energy and "reducing activity" generated by respiration. The unique feature of photosynthesis was not the assimilation of CO_2 but the utilisation of light as the source of energy for this assimilation.

Despite these tentative steps towards an understanding of the

mechanisms involved in photosynthesis no real breakthrough in our understanding of its biochemistry came until immediately after the Second World War which brought with it the availability for biological research of isotopes including the radioactive isotope of carbon (C^{14}) and the mass isotope of oxygen (O^{18}). One of the first of the post-war experiments was the demonstration by S. Ruben in 1941 using, alternatively, CO_2 and H_2O labelled with O^{18}, that the oxygen evolved in photosynthesis comes from the H_2O, as postulated by van Niel, and *not* from the CO_2. But this is leading us on to the biochemistry of photosynthesis and it would be appropriate if first we considered further certain aspects of photosynthesis as it proceeds in the living leaf or algal cell.

INFLUENCE OF EXTERNAL FACTORS ON THE RATE OF PHOTOSYNTHESIS

It was a result of his studies of the influence, at constant temperature, of carbon dioxide concentration and light intensity that Blackman (1905) was led to enunciate the principles of *limiting factors* thus "when a process is conditioned as to its rapidity by a number of separate factors the rate of the process is limited by the pace of the slowest factor". Later and more accurate determinations showed that the curves relating rate of photosynthesis to light intensity or carbon dioxide concentration are of the form shown in Fig. 36. With fixed CO_2 concentration this means that there is a region where the rate of photosynthesis rises in a linear fashion with rise in light intensity (where light is a "limiting factor") but that with further increase in light intensity the curve begins to bend (some other factor is beginning to limit the rate) and beyond a certain point becomes parallel to the abscissa. At this point the system is light saturated and the rate is determined by limiting "dark" reactions. One very interesting aspect of this light curve is that it seems from the very beginning to be linear; light at very low intensities is used with an *efficiency* similar to that at all intensities up to the point when other factors begin to limit the rate.

This suggests that the amount of light being absorbed by the "chlorophyll complex" is linearly related to the intensity and that a fixed amount of carbon dioxide is assimilated (or oxygen evolved) per unit of light energy absorbed even though, at low intensities, only a small proportion of the chlorophyll molecules will, at any one time, be in an "activated" state (their energy enhanced by absorption of energy) (see p. 133).

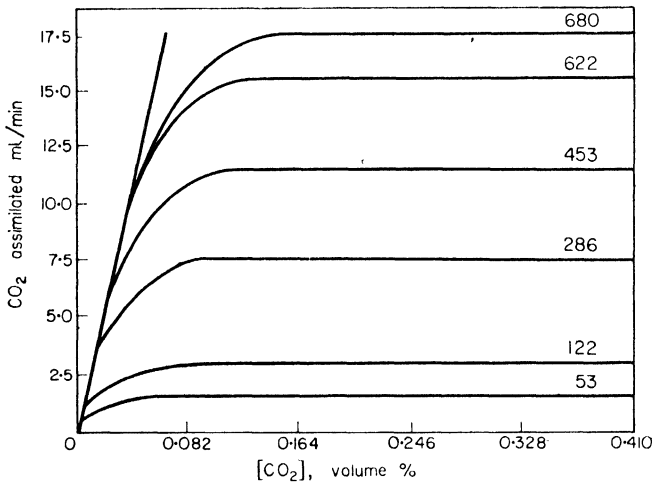


FIG. 36. Relationship of carbon dioxide concentration to the rate of carbon dioxide assimilation at various light intensities (shown against each curve in kerg/cm²/scc). Data for whole plants of *Triticum sativa* (wheat) after W. H. Hoover, E. S. Johnston and F. S. Brackett. *Smithsonian Inst. Publ. Misc. Collections*, **87**: No. 16. 1933 (from E. I. Rabinowitch. *Photosynthesis*, Vol. 2. Pt. 1. Interscience Pub. Inc. New York, 1951).

These studies have involved the use of artificial "white" light having a spectral composition similar to natural sunlight. Other workers have used monochromatic light (literally light of a single wavelength but in practice light within a narrow band of wavelength) from different regions of the visible spectrum in an attempt to assess which regions of the spectrum are utilised in photosyn-

thesis. In such studies it is necessary to know the extent to which the particular wavelength is absorbed and, if possible, to compensate for non-specific absorption (absorption other than by the pigments of the chlorophyll complex). It is also necessary to express the absorbed radiation in similar units irrespective of wavelength. One way of expressing "intensity" or absorbed radiation would be in the conventional terms of energy, such as ergs per cm^2 per sec. However, the radiant energy concerned in photosynthesis is involved in the photochemical reactions and in such reactions radiation is absorbed in units or "packets" of energy termed *quanta* (usually denoted $h\nu$*) and the energy value of these quanta varies with the wavelength (long wave radiation having a lower quantum value than short wave radiation). It is, therefore, preferable to express the amount of energy absorbed as a number of quanta and to express the efficiency of each wavelength in terms of the number of quanta absorbed for each molecule of carbon dioxide assimilated (as a *quantum efficiency*). This reveals, for instance, whether a quantum of red light is used with equal efficiency to a quantum of green, or yellow or blue light in photosynthesis. A typical result of such an experiment is hown in Fig. 37. In experiments of this kind, the major difficulty is to determine exactly the energy absorbed by the "chlorophyll complex" and, in consequence, there has been some disagreement between different laboratories as to the quantum efficiency of different wavelengths. It is clear, however, that all wavelengths of the visible spectrum up to about 700 mμ are utilised and that although the efficiencies are highest in the red and in the blue region there is, nevertheless a very significant utilisation between 640 and 490 mμ. These results are particularly interesting when compared with the absorption spectra of the individual pigments of the "chlorophyll complex" (Fig. 40, p. 124).

* E(energy of a quantum in ergs)$=h\nu$; where h=Planck's constant ($6{\cdot}61\times10^{-27}$) and ν (the frequency of the radiation)$=\frac{C}{\lambda}$, where C=velocity of light (3×10^{-10}cm/sec) and λ=wavelength in cm.

Under bright natural light photosynthesis is limited by the low carbon dioxide concentration (0·03 per cent.) of the atmosphere and the rate of photosynthesis (and, in turn, of growth) of most plants can be enhanced by increase in the concentration of carbon dioxide at least up to 1 per cent. There are two aspects of the

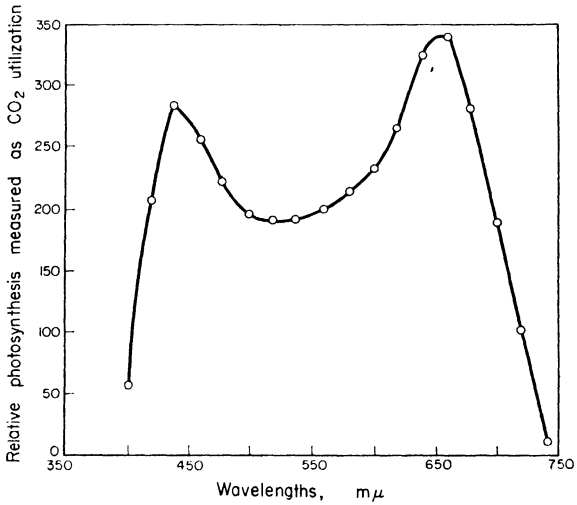


FIG. 37. Relative rates of photosynthesis of wheat in different wave lengths of light of equal intensity. (Data of W. H. Hoover, *Smithsonian Inst. Publ. Misc. Collections* **95**, No. 21, 1937; 11).

influence of carbon dioxide concentration upon the rate of photosynthesis which are particularly interesting. One is the very high rate of photosynthesis which can occur with the low natural carbon dioxide concentration of 0·03 per cent. This phenomenon is underlined by the ability of certain unicellular algae to lower the concentration of carbon dioxide by photosynthesis to a value as low as 0·001 per cent. Here we have a clear indication that the reaction bringing carbon dioxide into organic combination within the cells must have a very high affinity for carbon dioxide and that this

first formed product must be rapidly further metabolised. The second interesting aspect of the effect of carbon dioxide concentration is that cells which have been receiving a high carbon dioxide concentration when transferred to a low carbon dioxide concentration carry on photosynthesis initially less rapidly than those maintained continuously in a low carbon dioxide concentration. However, such cells soon develop again the capacity to utilise actively the low carbon dioxide concentration. This suggests that some enzyme system promoting the first step in carbon dioxide assimilation increases in activity at low carbon dioxide concentrations.

As already indicated, temperature has a marked effect upon the rate of photosynthesis under conditions where light intensity and carbon dioxide concentration are not limiting, and this observation led Blackman to postulate the occurrence of "dark reactions". For most plants, photosynthesis increases from just above 0°C to 35°C, although often at temperatures above 30°C the initial high rate is not maintained and this is markedly so above 35°C. One major factor in the decline in rate of photosynthesis at these high temperatures is the denaturation of enzyme proteins. It is, therefore, very interesting that some tropical plants can photosynthesise at temperatures above 40°C and that some algae, indigenous to hot springs, can grow at temperatures up to 89°C.

In measuring the relationship between temperature and photosynthesis or between the other external factors like carbon dioxide concentration and light intensity, it is important to remember that when we assess photosynthesis by oxygen evolution or carbon dioxide uptake we are, in fact, measuring *apparent photosynthesis.* The photosynthetic cells just like all other living cells, are carriyng on respiration and the gaseous exchange of aerobic respiration is the reverse of that of photosynthesis. Of course, in bright sunlight photosynthesis proceeds, in many leaves and green algae, at a very much higher rate than respiration. In cells of the unicellular alga, *Chlorella,* photosynthesis can proceed at 60 times the rate of respiration, and frequently photosynthesis can proceed at 10–30 times

the rate of respiration. However, for each plant or plant organ there is a light intensity which causes photosynthesis to just balance respiration, a light intensity called the *compensation point* at which gaseous exchange is zero. Clearly at light intensities not far removed from the compensation point a correction for the rate of respiration is quite essential in estimating the rate of photosynthesis. This is an important matter when trying to determine the quantum efficiency of photosynthesis because in such experiments low light intensities, associated with almost complete absorption, are used. The usual technique is to measure respiration immediately before and immediately after the period of exposure to light and to take the mean of these two values for the rate of respiration during the period of photosynthesis. Recent work, making use of the oxygen isotope O^{18}, strongly indicates that the rate of respiration is, in many cases, not markedly affected by the rate of the photosynthesis proceeding simultaneously in the cell except insofar as this results in a gradual accumulation of respiratory substrate and that any "light stimulation" of respiration over and above this is detected by a measurement of respiration rate immediately following the period of illumination (see Chapter 4, p. 80).

THE PHOTOSYNTHETIC APPARATUS

The pigments of the chlorophyll complex are contained in the chloroplasts. In higher plants the chloroplast is the site of photosynthesis as indicated by the coincidence between the presence of chloroplasts and the ability to carry on photosynthesis. Sachs demonstrated that the starch formed by photosynthesis first made its appearance as grains in the chloroplasts. The chloroplasts isolated by Hill clearly had the ability to develop reducting activity and evolve the oxygen of water. Later work, particularly by D. I. Arnon and his associates in the University of California, has shown that chloroplasts can be obtained which not only evolve oxygen but reduce carbon dioxide to carbohydrate.

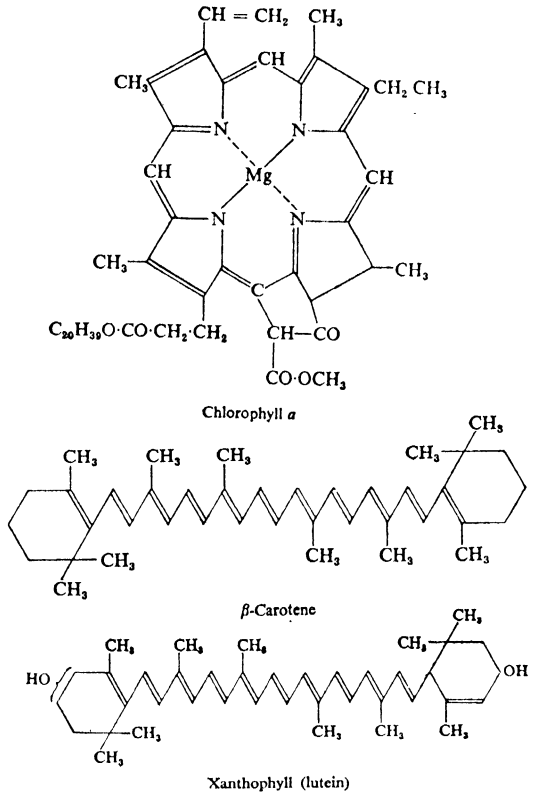


FIG. 38. Chemical structure of chlorophyll *a* (note the long phytyl side chain) and skeleton formulae of β–carotene and the xanthophyll, lutein.

The chloroplasts usually contain four pigments (Fig. 38), two green pigments, chlorophylls *a* and *b* which are magnesium porphyrin compounds, the orange β-carotene and the yellow lutein (xanthophyll). The molar ratio of chlorophyll *a* to chlorophyll *b* is often about 3:1, and the ratio of chlorophylls to carotenoids is often about 2:1. These pigments can be readily extracted from leaves,

other green tissues and algae by organic solvents and separated by adsorption chromatography. The pigments as obtained by solvent extraction differ in chemical stability, in absorption spectra and in fluorescence from the pigments *in situ* in the chloroplast. The present view is that *in situ* the chloroplast pigments are associated with protein and phospholipid. This concept is supported not only from studies of absorption spectra and fluorescence intensity but from studies of chloroplast structure in the electron microscope and of the chemical composition of isolated chloroplasts. One model which has been advanced of the arrangement of phospholipid, chlorophyll, carotenoid and protein molecules between the "grana" lamellae is shown in Fig. 39.

The absorption spectra of the two chlorophylls show two narrow absorption maxima, one in the red and one in the blue. β-carotene and lutein show a broad (2 peaked) absorption maximum in the blue (Fig. 40). The high absorption and efficiency of utilisation of red light in photosynthesis indicates the importance of the chlorophylls. The efficiency of blue light and of intermediate wavelengths indicates that light absorbed by the carotenoids is also utilised. This would fit in with the hypothesis, for which there is other evidence, that chlorophyll *a* is the direct photocatalyst in photosynthesis and that the other pigments of the chloroplast are effective in so far as they are able to transfer the energy they absorb to chlorophyll *a*. A close packing of the pigment molecules such as is envisaged in Fig. 39 would be essential for such transfers of energy to proceed efficiently.

THE PATH OF CARBON IN PHOTOSYNTHESIS

Our knowledge of the path of carbon in photosynthesis comes mainly from a series of brilliant researches initiated in 1946 by Melvin Calvin and his associates at the Lawrence Radiation Laboratory of the University of California. These workers made use of the radioactive isotope of carbon, C^{14}, from which they prepared

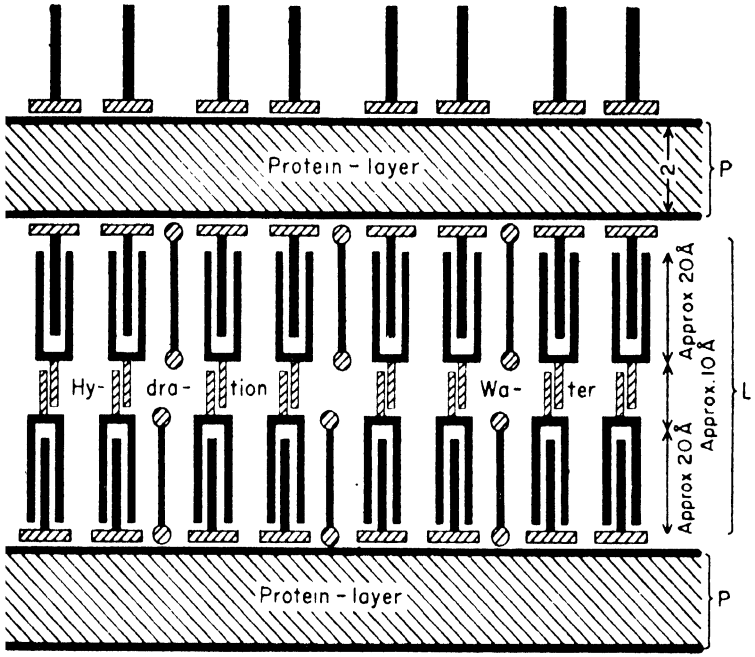


FIG. 39. Model of how the *phospholipid* (shown as a "tuning fork" with the fatty acid chains shown black and the phosphate-choline part shown hatched), *chlorophyll* (shown as a capital T in which the black long arm of the T represents the long phytyl side chain) and *carotenoid* (dumb-bell shaped) molecules may be arranged between the "grana" lamellae (protein) in the chloroplast. The black parts of the molecules are the predominantly carbon and hydrogen parts of the molecules (non-polar, lipophilic) and these are shown associated together. The hatched parts of the molecules contain, in addition, oxygen, nitrogen or phosphorus atoms and because of this associate with one another, with water or with protein (are polar or hydrophilic). (after B. Hubert. *Rec. trav. botan. Neerl.*, **32**: 323. 1936 and A. Frey-Wyssling. *Protoplasma*, **29**: 279, 1937).

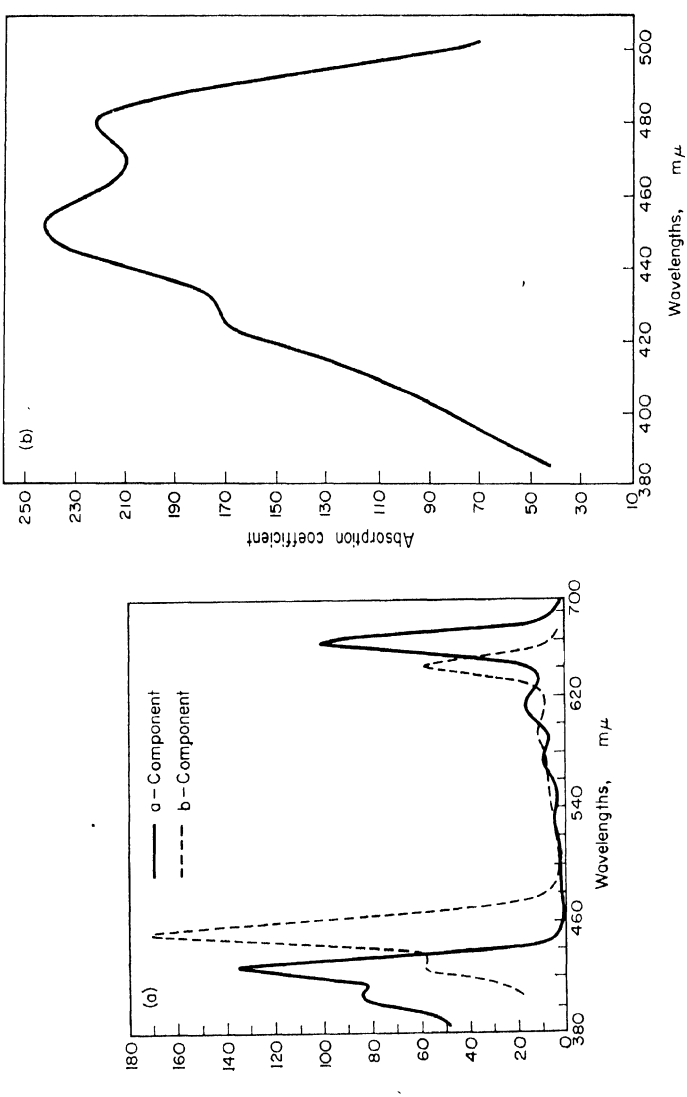


FIG. 40. Absorption spectra. A of chlorophyll *a* (solid line) and chlorophyll *b* (broken line). B of β-carotene.

radioactive carbon dioxide ($C^{14}O_2$) and then fed this to unicellular algae (*Chlorella pyrenoidosa* and *Scenedesmus obliquus*) and green leaves. After short periods of photosynthesis in $C^{14}O_2$ the cells were instantaneously killed and their enzymes denatured by plunging into boiling alcohol. The compounds into which the C^{14} had been incorporated by photosynthesis were then separated from the alcoholic extract. To achieve this separation of the radioactive

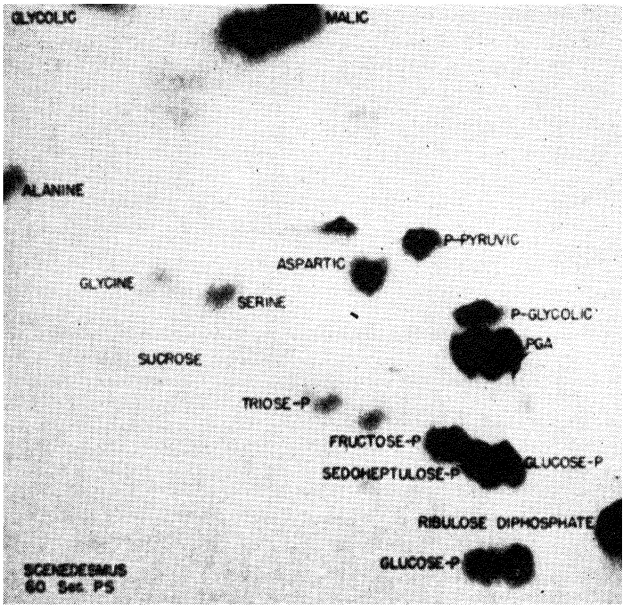


FIG. 41. Radio-autograph of a paper chromatogram of the alcoholic extract of cells of the alga *Scenedesmus* actively photosynthesising radioactive carbon dioxide $C^{14}O_2$. Radioactivity located by contact of the chromatogram with a photographic plate. Each radioactive spot labelled with its chemical composition (from J. A. Bassham and M. Calvin *The Path of Carbon in Photosynthesis*. Prentice Hall Inc., New Jersey, 1957).

carbon compounds and to identify them they used the technique of two-dimensional paper partition chromatography which had been developed by A. J. Martin and R. L. M. Synge in 1941. The compounds were located on the paper chromatograms, both by their

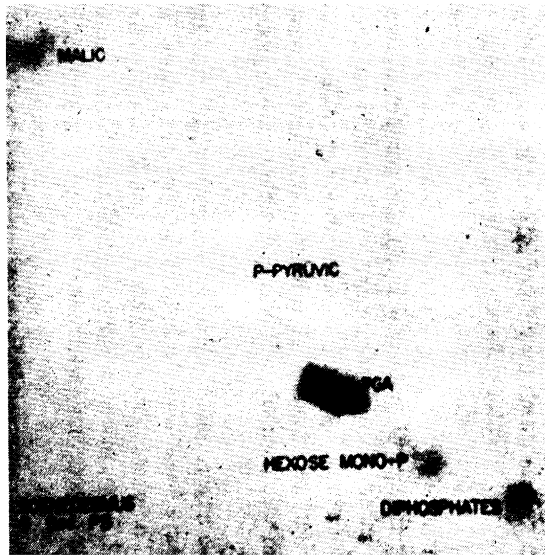
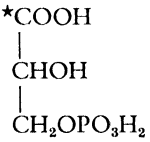


FIG. 42. Radio-autograph prepared as in Fig. 41. *Scenedesmus* cells assimilating CO_2 were, at zero time, supplied with $C^{14}O_2$ and extracted 5 *seconds* later. Almost the whole of the detected radioactivity is in phosphoglyceric acid (PGA), (via E. I. Rabinowitch, *Photosynthesis*, Vol. 2, Part 2. Interscience Pub. Inc., New York, 1956).

chemical reactions and their radioactivity. The individual compounds were then washed off the chromatograms and their identity confirmed by re-chromatography with known "cold" compounds. Degradation of the isolated radioactive intermediates by micro-

methods made it possible to determine the relative radioactivity of each carbon atom they contained. From such studies came first the identification of the intermediates between carbon dioxide as starting material and the end-products of photosynthesis which proved to be mainly sugars and amino acids. Then the degradation studies enable the sequence of the reactions to be confirmed. Finally, there followed the isolation of the enzymes which catalyse these "dark" reactions of photosynthesis. Appropriately the cyclic metabolic pathways thereby worked out are referred to as the *Calvin cycle*. The significance of these biochemical studies has subsequently been upheld by strong experimental evidence that in all photosynthetic organisms the greater part of the carbon dioxide assimilated in the chloroplasts passes through the Calvin cycle.

Within less than 1 minute the radioactivity of carbon dioxide can be picked up in sugar phosphates, phosphoglyceraldehyde, phosphopyruvic acid, phosphoglyceric acid (PGA), amino acids (particularly alanine and aspartic acid) and organic acids (particularly malic acid) (Fig. 41). By reducing the period of exposure to $C^{14}O_2$ to a few seconds it was possible to show that the first stable intermediate product of photosynthesis was PGA (Fig. 42) and that virtually all the C^{14} was located in the carboxyl carbon (marked *) of this compound.



3–phosphoglyceric acid

(PGA)

The PGA and the phosphoglyceraldehyde and the hexose phosphates which are quickly labelled in photosynthetic carbon dioxide assimilation are also intermediates in the EMP pathway of respiration (p. 88, 89; Eqn. 12 and 17). This immediately suggested that PGA might be the precursor of the hexose phosphate and

ultimately of starch by undergoing phosphorylation and reduction to give first 3-phosphoglyceraldehyde and then fructose-1-6-diphosphate by the equilibrium reactions which involve these compounds in respiration. This would require the chloroplasts to

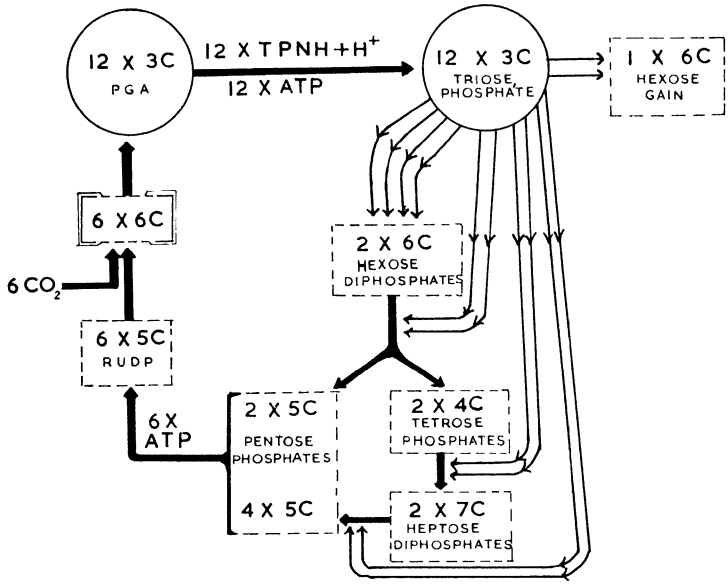


FIG. 43. Simplified version of the Calvin cycle showing the path of carbon dioxide assimilation in photosynthesis.

contain the necessary enzymes and to be capable of synthesising ATP and reducing the appropriate co-enzyme. This view that the hexose phosphates are thus derived from PGA was supported by the demonstration that the first formed hexose phosphates had their radioactivity almost entirely in carbon atoms 3 and 4 (the middle carbon atoms of the chain of 6) as would be the case if two triose units with radioactive aldehyde groups joined through these to give a hexose diphosphate.

The origin of the PGA in photosynthesis was not immediately clear. Calvin and his group first searched unsuccessfully for a 2-carbon compound which could be the acceptor of CO_2 to give PGA. Then A. A. Benson, working in Calvin's laboratory, showed that the chromatograms contained not only labelled hexose phosphates but also labelled 7-carbon and 5-carbon (pentose) phosphates including the 5-carbon ribulose-1-5-diphosphate (RuDP). This opened up the new possibility that the CO_2 combined with a pentose diphosphate to give a 6-carbon compound which then split to give 2 molecules of PGA. That this was, in fact, the case was demonstrated first by an experiment in which, after a period of $C^{14}O_2$ feeding, the light was switched off when PGA accumulated and RuDP disappeared completely and then by the complementary experiment in which the light was kept on but the $C^{14}O_2$ withdrawn when PGA disappeared and RuDP accumulated. RuDP is now accepted to be the compound with which carbon dioxide first combines in photosynthesis under the influence of the enzyme, *carboxydismutase* (first isolated in 1954). Further, the observation that PGA was the first stable intermediate product of photosynthesis indicated, and this has been confirmed by *in vitro* studies, that the 6-carbon compound formed by combination of CO_2 with RuDP is quite unstable and immediately undergoes cleavage to give two molecules of PGA.

By a patient study of the radioactive carbon labelling of the 7-carbon and 5-carbon sugar phosphates it was concluded that PGA was the starting material for their synthesis and, therefore, the precursor, not only of hexoses, but of RuDP. Further, it was possible to set out the reaction sequences by which these 7- and 5-carbon sugars must be formed. These reactions were at first hypothetical but subsequent work has led to the isolation from plant cells of enzymes which catalyse the postulated reactions. It is these sugar interconversions giving rise to RuDP together with the reactions whereby PGA gives rise to hexose that constitute the Calvin cycle.

It would be going beyond the scope of the present text to consider

these reactions in detail. The Calvin cycle is, however, presented in a simplified form in Fig. 43 (p. 128) and the following is the balance sheet of the path of carbon in photosynthesis:

$$
\begin{aligned}
&(a)\ 6\text{RuDP} + 6\text{CO}_2 \rightarrow 12\text{PGA}\\
&(b)\ 12\text{PGA} \xrightarrow[12\text{ATP}]{12\times(2\text{H})} 12\text{C}_3\ (\text{phosphoglyceraldehyde})\\
&(c)\ 2\text{C}_3 \rightarrow 1\text{C}_6\ (\text{net gain of hexose})\\
&(d)\ 4\text{C}_3 \rightarrow 2\text{C}_6\\
&(e)\ 2\text{C}_6 + 4\text{C}_3 \rightarrow 2\text{C}_5 + 2\text{C}_7\\
&(f)\ 2\text{C}_7 + 2\text{C}_3 \rightarrow 4\text{C}_5\\
&(g)\ 6\text{C}_5 \xrightarrow{6\text{ATP}} 6\text{RuDP}
\end{aligned}
\qquad (33)
$$

Thus the assimilation of six molecules of CO_2 leads to the net gain of a molecule of hexose and requires the energy supplied by 18 moles of ATP and the reducing activity of 12 molecules of reduced co-enzyme (Reactions 33(b), (g) and Fig. 44).

Reference has been made to the rapid labelling of certain amino acids and of malic acid. PGA gives rise to pyruvate and this is the precursor of malic acid and of the amino acid, alanine. Malic acid is the precursor of the labelled aspartic acid. The very rapid labelling of fats during the photosynthesis of unicellular algae probably follows from conversion of PGA, via pyruvate, to "active acetate" and utilisation of this, along with ATP and reduced co-enzyme molecules, for fatty acid synthesis.

THE CONVERSION OF LIGHT ENERGY INTO CHEMICAL ENERGY IN PHOTOSYNTHESIS

Carbon dioxide assimilation via the Calvin cycle requires a supply of reduced co-enzyme and of ATP. This suggests that the photochemical reactions of photosynthesis involve the conversion of electromagnetic energy into the chemical energy of the terminal

pyrophosphate bond of ATP and the reduced form of the co-enzyme.

Hill's early work with isolated chloroplasts of *Stellaria media* and *Lamium album* showed that when, and only when illuminated they evolved oxygen, that this oxygen came from water and that associated with this oxygen evolution the chloroplasts developed "reducing activity". However, this reducing activity not only failed to reduce carbon dioxide but appeared to be of very limited reducing potential. Then, in 1951, illuminated chloroplasts from other plants (particularly chloroplasts from spinach, Swiss chard and certain algae) were shown to be capable of reducing co-enzyme

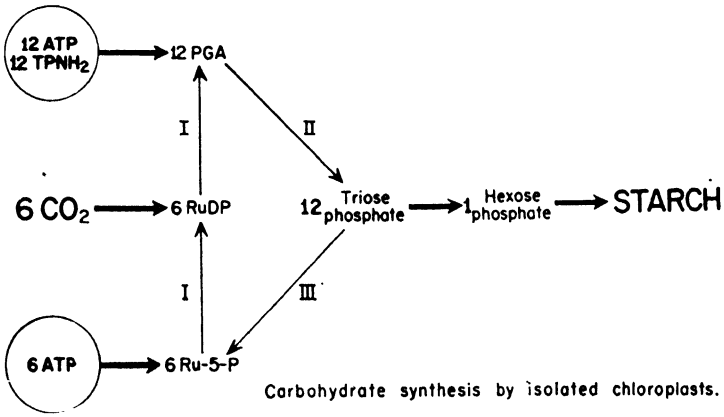


FIG. 44. Diagram summarising the path of carbon assimilation in chloroplasts (see also Eqn. 33). The assimilation can be divided into three phases. Phase I: The phosphorylation of ribulose monophosphate (Ru–5–P) to ribulose diphosphate (RuDP) which then accepts a molecule of CO_2 and is cleaved to 2 molecules of PGA (Eqn. 33 (g) and (a)). Phase II: PGA is reduced to triose phosphate (Eqn. 33 (b)). Phase III: triose phosphate acts as the precursor of both Ru–5–P and of hexose and starch. The reactions are driven by ATP and reduced TPN. The assimilation of 1 mole of CO_2 requires 3 moles of ATP and 2 moles of reduced TPN, (after D. I. Arnon in *Biological Structure and Function*. Edited by T. W. Goodwin and O. Lindberg. Academic Press, New York, 1961).

II (TPN) and, by adding an appropriate enzyme to the system, of assimilating carbon dioxide. The reaction used by Dr. Vishniac and Professor Ochoa to effect carbon dioxide uptake was that catalysed by their *malic enzyme*:

$$\underset{\text{pyruvic acid}}{CH_3CO.COOH} + C^{14}O_2 + TPNH + H^+ = \underset{\text{malic acid}}{HOOC.CHOH.CH_2.C^{14}OOH} + TPN^+ \quad (34)$$

Of course, this was still far short of full photosynthesis. However, in 1954, Professor D. I. Arnon and his colleagues at the University of California, showed that spinach chloroplasts properly prepared *could* carry out full photosynthesis, assimilating carbon dioxide and giving rise to the intermediates and end-products of the Calvin cycle. Clearly the whole of the photosynthetic apparatus was within the chloroplast. Here the photochemical acts were linked to the "dark" reactions of carbon dioxide assimilation presumably through the synthesis of ATP and the reduction of the co-enzyme of the chloroplast (TPN). Further, by illuminating a chloroplast preparation in absence of CO_2 but in presence of large amounts of ADP and inorganic phosphate and TPN^+, Arnon demonstrated oxygen evolution and the accumulation of ATP and reduced TPN ($TPNH + H^+$).

In mitochondria the oxidation of reduced co-enzymes results in ATP synthesis. It was, therefore, possible that ATP synthesis in the chloroplasts resulted from a similar oxidative phosphorylation in which part of the reduced TPN was re-oxidised. However, Arnon was able to demonstrate that isolated chloroplasts illuminated under anaerobic conditions and in absence of TPN synthesised ATP from added ADP plus inorganic phosphate and that during this process there was no oxygen evolution. The extent of this *photophosphorylation* was markedly enhanced by adding to the chloroplasts vitamin K (reported as a constituent of chloroplasts). This was followed by the demonstration that the chromatophores of the

photosynthetic bacterium, *Rhodospirillum rubrum* could similarly effect an anaerobic photophosphorylation. Here, in both cases, was a process of phosphorylation not linked to the metabolism of reduced co-enzyme, a process quite distinct from oxidative phosphorylation. This distinction has been emphasised by work with the red sulphur bacterium, *Chromatium*. This organism requires light for its growth and if provided with carbon dioxide as its source of carbon requires also the presence of hydrogen gas. It is an anaerobic organism. It contains an enzyme (hydrogenase) which catalyses the reduction of carbon dioxide by hydrogen gas. To synthesise its essential cell constituents it also requires ATP and this is the reason for its light requirement. Light functions in the economy of this organism, when supplied hydrogen gas, solely to effect an anaerobic photophosphorylation of ADP in which there is no associated formation of sulphur (in which no hydrogen or electron donor is involved).

The next step must be to visualise a "model" or "reaction scheme" for such phosphorylation. In oxidative phosphorylation the energy for the synthesis of the energy-rich bonds of ATP is released when electrons travelling in company with protons (as hydrogen atoms) drop from higher to lower energy levels as they move along the electron transport chain. The "falling" electron releases the power to attach phosphate to ADP. It is, therefore, possible for such "falling" electrons to be involved in photophosphorylation, the electrons travelling along a chain involving vitamin K and the cytochromes of the chloroplasts. What would be needed would be a source of high-energy electrons.

In photosynthesis the chlorophyll molecules absorb photons (quanta) and become *excited* molecules, molecules with more energy than the ground state energy (become, if you like, very "hot" and, therefore, very reactive molecules). Such molecules have a very short life and if they do not immediately participate in photochemical reaction they loose their energy, partly as heat and partly as radiation (as fluorescence or phosphorescence). It is of importance to know how much extra energy these "excited"

chlorophyll molecules contain because this will determine how much chemical "work" they can do. Now the chlorophyll molecule when it absorbs blue light is raised to the "excited" state known as the "second singlet state" (in which the energy excess is 65 kcal per molecule). However, such molecules are very unstable (their life is about 10^{-11} sec) and lose heat to give molecules at the "first singlet state" (41 kcals excess per molecule). Molecules at this "first singlet state" are also obtained when chlorophyll absorbs red light (which is also active in photosynthesis). Such molecules are also very short-lived (life approx. 10^{-9} sec) and undergo an internal conversion to the *triplet state* (31 kcals excess per molecule) the molecules of which have a longer life (10^{-2} sec). It is these activated "triplet state" chlorophyll molecules which by virtue of their longer life can most easily be involved in photochemical reactions.

Arnon has suggested that such activated chlorophyll molecules (Chl*) may expel their energy as an electron, (Eqn. 35)

$$\text{Chl}^* \rightarrow [\text{Chl}]^+ + e^- \qquad (35)$$

that this electron may be accepted by a suitable molecule (vitamin K_3) and then may dissipate its energy as it passes along an electron transport chain starting at vitamin K and involving the cytochromes of the chloroplasts. The electron having lost its excess energy would then return to unite with $[\text{Chl}]^+$ to give again the ground state chlorophyll, Chl. During this electron transport, ATP synthesis would occur just as ATP synthesis occurs during electron transport in oxidative phosphorylation. This scheme then involves a cyclic journeying of electrons from chlorophyll back to chlorophyll, the "falling" electrons generating ATP (Fig. 45). On the basis of this conception Arnon has used the term *cyclic photophosphorylation*.

We have referred above to the reduction of CO_2 through the action of hydrogenase in the sulphur bacterium, *Chromatium*. However, the photosynthetic sulphur bacteria, as van Niel showed, can use hydrogen sulphide or thiosulphate to reduce CO_2. Under

these circumstances light energy is also used for co-enzyme reduction and the amount of ATP synthesised per quantum is significantly less. Similarly when working with the chloroplasts of higher plants if TPN and chloride are added and if the external addition of vitamin K is omitted, then the chloroplasts evolve oxygen, TPN is reduced by the activity of a special chloroplast

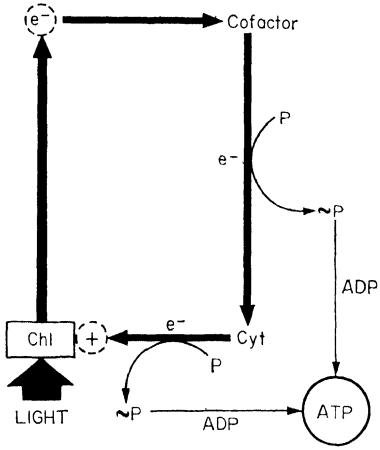


FIG. 45. Reaction scheme for *cyclic photophosphorylation*. e′ = electron; Chl = chlorophyll; cyt = cytochrome; co-factor = vitamin K_3 or other electron acceptor; ~P = high energy phosphate, (after D. I. Arnon as Fig. 44).

enzyme, TPN reductase, and a constant but lower rate of ATP synthesis proceeds. Under these circumstances the activity of the chloroplasts can be represented:

$$2TPN^+ + 2ADP + 2P + 2H_2O \rightarrow 2TPNH + H^+ + O_2 + 2ATP \quad (36)$$

Arnon contends that here is a second form of photophosphorylation in which oxygen is released from water, and has represented its mechanism by the "reaction scheme" as shown in Fig. 46. This scheme postulates that the electron "expelled" from chlorophyll in the primary photochemical act is accepted by TPN along with

an accompanying proton. The electron released by "activated" chlorophyll participates in TPN reduction and does not return to the chlorophyll molecule. Hence the designation of this process by Arnon as *non-cyclic photophosphorylation.* The electron deficit of the $[Chl]^+$ is, however, postulated as being made good by a reaction between either the hydroxyl ion (OH^-) or water and a

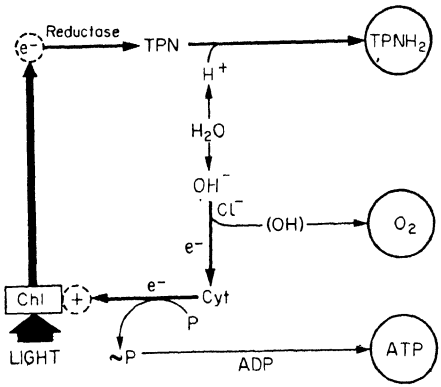


FIG. 46. Reaction scheme for *non-cyclic photophosphorlyation.* Key as Fig. 45. H_2O shown as source of H for TPN reduction and of the oxygen evolved. Cl' indicates essentiality of chloride for oxygen evolution. (after D. I. Arnon as Fig. 44).

cytochrome peculiar to the chloroplast. This cytochrome picks up the electron and in turn donates it to $[Chl]^+$ and it is in this transfer that energy is released for the combination of ADP with phosphate to give ATP. The discharged hydroxyl ion could then be the precursor of a "peroxide" and, in turn, of molecular oxygen. This scheme is very controversial because the particular cytochrome involved either as such or in association with chlorophyll must have a sufficiently high oxidising potential to drive the reaction

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^- \qquad (37)$$

Cytochrome f, the most oxidising cytochrome at present known to occur in chloroplasts has a potential far below that required.

It would seem that photochemical energy must go into this step as well as into the activation of the electron for TPN reduction. This is tantamount to saying that some photochemical act must be involved in a photolysis of the water molecule as postulated by van Niel (Eqn. 31, p. 114).

When working with isolated chloroplasts it is easy to suppress, apparently completely, either the non-cyclic or the cyclic processes of photophosphorylation. In the functioning chloroplast, however, it would seem that both these forms of photophosphorylation must proceed simultaneously for the non-cyclic process is essential for TPN reduction and the cyclic process is necessary to supplement the low yield of ATP in the non-cyclic process.

Reference was made in the opening paragraphs of this chapter to the possibility that the earliest forms of life were unicellular heterotrophs living in an atmosphere devoid of oxygen and probably rich in hydrogen. Such organisms would of necessity have generated their essential ATP by the inefficient process of fermentation. The first effect of the biological invention of chlorophyll molecules would then presumably have been to enable such organisms to effect a cyclic photophosphorylation, as can still be observed in the anaerobic bacterium, *Chromatium*. Thereby, they would use solar energy for ATP synthesis and hydrogen gas for CO_2 assimilation. The next evolutionary step could then have been the development of a non-cyclic photophosphorylation using a cytochrome capable of oxidising hydrogen sulphide or thiosulphate and thereby making, with solar energy, a new reductant for CO_2 assimilation. The final step in this hypothetical sequence of biochemical evolution would be the development of a system (a special cytochrome system?) with the higher oxidising potential which permitted the use of the ubiquitous substance, water, as an electron donor. This, in turn, by releasing molecular oxygen, made possible the evolution of aerobic respiration as an extension of the more limited and less energy-yielding reactions of fermentation.

Our understanding of the nature of photosynthesis has progressed a long way since the modern era in photosynthesis research was

initiated by van Niel in 1930. The most recent data regarding the structure of chloroplasts and the hypothesis of Arnon regarding the mechanisms of photophosphorylation are starting points for the next stage of this research. This will be concentrated upon further study of the photochemistry of chlorophyll *in vivo*, of the energy and space relationships between the separate pigments of the chlorophyll complex and of the relationship between chlorophyll *a* and the enzymes and co-factors which promote ATP synthesis and TPN reduction.

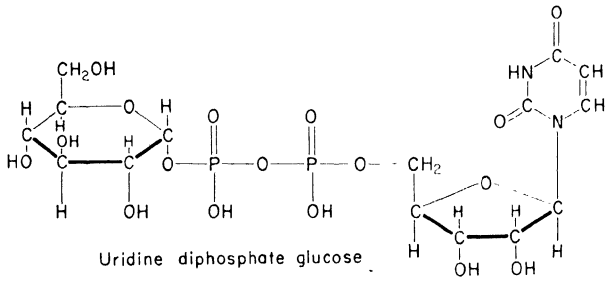
THE SYNTHESIS OF SUCROSE AND POLYSACCHARIDES

The monosaccharides produced in photosynthesis are partly consumed in respiration. They also serve as precursors, particularly of organic acids and fats and as building units for the synthesis of more complex sugars and polysaccharides.

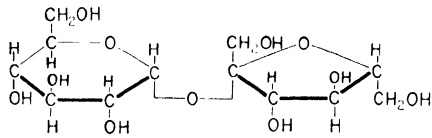
The glucose-6-phosphate which is one of the sugar phosphates arising in photosynthesis, can be converted to glucose-1-phosphate by the enzyme, phosphoglucomutase. The glucose-1-phosphate in the presence of the plant enzyme, pyrophosphatase, reacts with the nucleotide, uridine triphosphate (UTP) to give *uridine diphosphate glucose* (UDPG) thus:

$$\text{UTP} + \text{glucose-1-(P)} \rightleftharpoons \text{UDPG} + \text{pyrophosphate} \qquad (38)$$

This UDPG is an interesting and versatile compound.



It is the precursor of UDP-galactose, UDP-xylose and UDP-arabinose (sugars involved in the synthesis of the cell wall, polysaccharides known as galactans and pentosans). Both UDPG and UDP-galactose can suffer oxidation to the corresponding uronic acid derivatives involved in the synthesis of the hemicelluloses and pectins of the cell wall. UDPG is also involved in the synthesis of the widely distributed and very important disaccharide, sucrose (cane sugar).

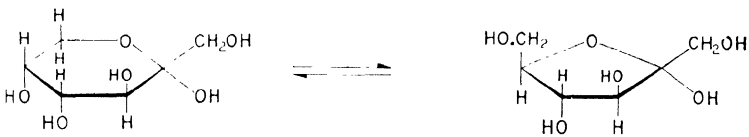


Sucrose

Higher plants have been shown to contain an enzyme which catalyses the reaction:

$$\text{UDPG} + \text{fructose} \rightleftharpoons \text{sucrose} + \text{UDP} \qquad (39)$$

This reaction is interesting in two ways. Firstly, the fructose unit in the sucrose molecule is a fructofuranose unit (5-membered ring). Fructose in solution is an equilibrium mixture containing a significant amount of such fructofuranose molecules.



(Pyranose form) α-D-fructose (Furanose form)

Secondly, the equilibrium in the reaction between UDPG and fructose favours sucrose synthesis since the back reaction in which sucrose reacts with UDP (uridine diphosphate) proceeds with an increase in free energy of 1 kcal ($\Delta F = +1$ kcal). This is in marked contrast to the hydrolysis of sucrose to free glucose and fructose

under the influence of invertase which proceeds with a *decrease* in free energy of 6·5kcal ($\Delta F = -6{\cdot}5$kcal) and which therefore in aqueous solution (water being one of the reactants) is virtually irreversible. The synthesis and hydrolytic breakdown of sucrose are therefore quite different reactions.

The polysaccharide, starch, is outstanding as a storage carbohydrate in plant cells. It occurs as grains 1–150 μ in diameter formed in the chloroplasts of photosynthetic cells or in the amyloplasts of colourless cells. Each storage starch grain shows a hilum or centre of initiation around which are deposited concentric layers of starch. Each layer represents the amount of starch deposited during 24 hours and the layers are often quite distinct because the starch deposited during the day is denser and more highly refractive than that deposited at night.

If starch is hydrolysed with acid it yields only glucose. When treated with the enzyme β-amylase it yields a dextrin (a polysaccharide unit giving a purplish colour with iodine in contrast to the blue-black reaction of starch) and the disaccharide, maltose. The disaccharide, maltose, consists of two glucopyranose units joined through a 1·4 α-linkage and the chemical evidence shows that starch molecules are built of chains of glucose units in which these 1·4 linkages are involved (Fig. 47).

When starch is heated in water to 60–80°C the starch grains swell and yield two components. One component (*amylose*) is water-soluble, is entirely degraded to maltose by the β-amylase and its molecules consist of unbranched spiral chains of glucose units (300–1000 units) linked by 1–4 α-linkages. The second component (*amylopectin*) is not soluble, yields both maltose and dextrin on treatment with β-amylase and has a *branched* chain structure due to the occurrence not only of 1:4 but also of 1:6 linkages between the glucose units (Fig. 47).

An enzyme (*glucosan-phosphorylase*) has been isolated from potato tubers and other plant tissues which synthesises the amylose component of starch from glucose–1–phosphate.

$$\text{Glucose–1–(P)} \rightleftharpoons \text{“amylose”} + H_3PO_4 \qquad (41)$$

This reaction will not proceed towards synthesis of polysaccharide unless a small amount of amylose or amylopectin is added as a "primer". Amylopectin is a better primer than amylose because due to its branched-chain structure it has more free non-reducing

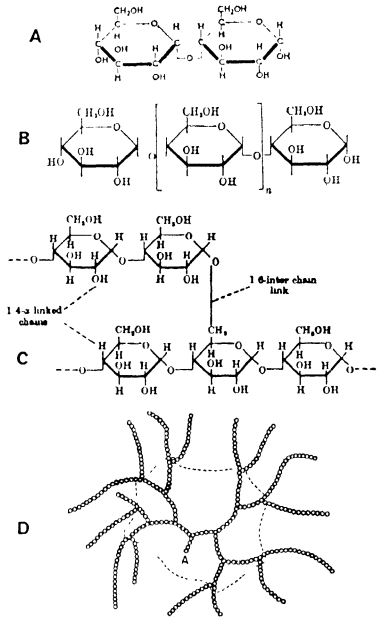
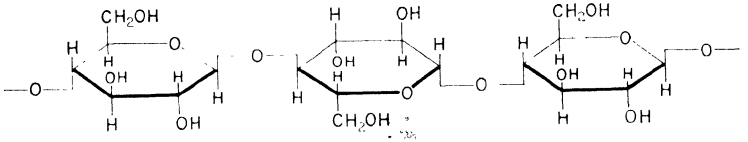


FIG. 47. The structure of starch. A = maltose. B = straight chain of amylose. C = branching by 1:6 linkages as in amylopectin. D = amylopectin. The dotted line shows inside it the dextrin-type molecule formed from amylopectin by the action of β-amylase.

groups. These serve as receptors for the glucose residues so that radiating out from the "primer" molecules "amylose" chains develop. The change in energy of the reaction is small; the energy of the ester-linkage between glucose and phosphate is similar to that of the glucosidic linkages in the polysaccharide, and hence there is a considerable polysaccharide synthesis when glucose-1-phosphate and "primer" are added to the enzyme.

A second enzyme, Q *enzyme*, involved in starch synthesis also occurs in the cells of the potato tuber. While the phosphorylase promotes the synthesis of straight chains of glucose units united by 1·4 α-linkages, the Q enzyme combines glucose units to such chains by 1:6 α-linkages. Q enzyme, in presence of glucose-1-phosphate will produce from amylose an amylopectin-like molecule, it is a "branching enzyme". The two enzymes working together and using glucose-1-phosphate as starting material simulate closely the starch synthesising system of the cell and can give rise to minute starch "grains".

Cellulose, the structural polysaccharide of the cell wall, (see Chapter 2, p. 48 *et seq.*) is, like starch, a glucosan but here the glucose units are in 1:4 β-linkage.



Cellulose

Unlike starch, it is completely resistant to hydrolysis by dilute acids and its hydrolysis, without carbonisation, to glucose is very difficult and involves the use of strong sulphuric acid at low temperature. During cell growth large amounts of cellulose are rapidly synthesised. Cellulose synthesis is, therefore, a very important aspect of the biochemistry of growth. Whereas, however, much is known of the mechanism of starch synthesis, of glycogen synthesis and of the synthesis of bacterial polysaccharides we know nothing of the mechanism of cellulose synthesis. This is a major gap in plant biochemistry; a breakthrough here would profoundly advance our knowledge of the regulation of cell growth and of cell wall growth and thickening.

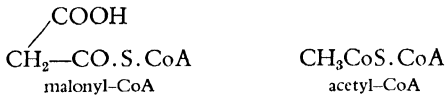
THE SYNTHESIS OF FATS

Fats are formed by the combination of fatty acids with the trihydric alcohol, glycerol. We are, therefore, here concerned with the synthesis of the fatty acids and then of the formation from them of fats.

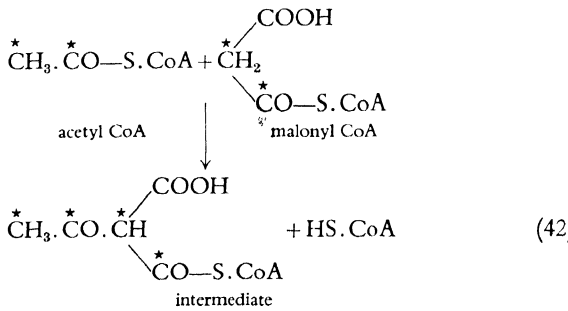
Reference has already been made (p. 94) to the involvement of co-enzyme A (CoA–SH) in the oxidative degradation of fatty acids to liberate units of acetyl–CoA ("active acetate") which can then enter the pathway of respiration via the Krebs cycle. Since the long carbon-hydrogen chains of fatty acids are almost always built up of an even number of carbon atoms (most frequently of 16 carbon atoms) the "active acetate" arising from fatty acid breakdown would obviously be a possible 2 C-unit from which such molecules could be synthesised. This raises the question of whether the route of synthesis is a reversal of the pathway of degradation. Two considerations should, however, make us hesitate before accepting this as a working hypothesis. Firstly, cells frequently do not follow the same pathway in building up and degrading complex molecules. This certainly applies to polysaccharides and proteins. Secondly, fatty acid oxidation takes place in the mitochondria whereas the evidence points to the microsomes or endoplasmic reticulum as being involved in fatty acid synthesis.

Recent work has resulted in the isolation of two enzyme-complexes and five co-factors (ATP, Mn^{++}, biotin, TPNH and carbon dioxide) essential for fatty acid synthesis. This system synthesised fatty acid from acetyl–CoA and all the carbon atoms of the fatty acid came from the acetyl-group. The puzzling feature was the necessity for carbon dioxide. However, by using only one of the essential enzyme-complexes along with ATP, Mn^{++}, biotin *and* carbon dioxide a compound was formed which in the presence of the second enzyme complex and TPNH gave fatty acid and simultaneously liberated carbon dioxide. The carbon dioxide was involved in the formation of an intermediate compound

and this was shown to be the co-enzyme A derivative of malonic acid, *malonyl-CoA*

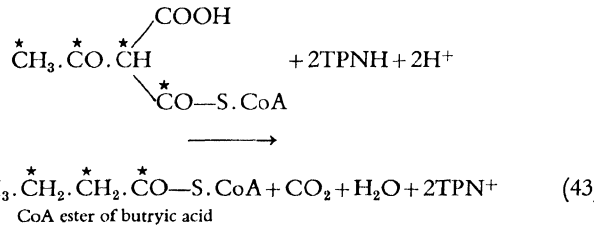


The highly reactive malonyl-CoA was formed by combination of the carbon dioxide with acetyl-CoA through the agency of the appropriate enzyme complex, ATP and the vitamin, biotin. The products of the second reaction were carbon dioxide, a CoA ester of a fatty acid with an even number of carbon atoms and reduced co-enzyme. Therefore, the first step in this second reaction sequence must be:



(42)

and this must suffer reconstruction thus:



(43)

(* carbon atoms of acetyl—CoA)

Now this reaction sequence is repeated, the malonyl-CoA now condensing with the butyryl-CoA, to give the CoA ester of a 6 C acid and so on until the full even-number chain length of 16 C atoms is reached.

Acetyl-CoA is therefore, both the product of fatty acid breakdown and the precursor for fatty acid synthesis. However, the condenstaion reactions described above and shown to be involved in this synthesis are achieved by the formation of the highly reactive, energy-rich intermediate, malonyl-CoA. This synthetic pathway may also be contrasted with the pathway of degradation in involving TPN instead of DPN (the co-enzyme of the dehydrogenases which effect β-oxidation), and Mn^{++} instead of Mg^{++} ions (see Chapter 4, p. 95, Eqn. 19). Whether the reversible reactions involved in β-oxidation are *also* of significance in fatty acid synthesis has yet to be satisfactorily established.

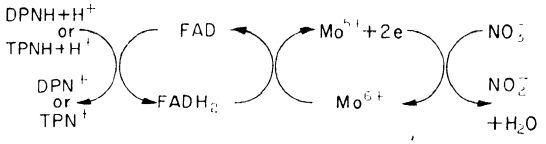
The synthesis of fats and phospholipids involves combination of fatty acids with each of the three alcoholic groups of glycerol (giving fats) or with two of these groups followed by combination of the third alcoholic group with a phosphoric acid-choline group (giving the phospholipid, lecithin) (Fig. 54). These reactions, unlike those of fatty acid synthesis, proceed actively in the mitochondria.

THE SYNTHESIS OF PROTEINS

Plants obtain their nitrogen by absorption of nitrate or ammonium ions from the soil. The assimilation of nitrate involves its enzymic reduction. The first step in this reduction is catalysed by the enzyme, nitrate reductase, for which molybdenum is an essential co-factor. The enzyme itself is a flavoprotein (a yellow enzyme), the prosthetic group of which is flavin adenine dinucleotide (FAD) and reduced co-enzyme (in higher plant DPN, in micro-organisms TPN) is the primary electron donor

$$NO_3^- + DPNH + H^+ \underset{\text{reductase}}{\overset{\text{nitrate}}{\rightleftharpoons}} NO_2^- + DPN^+ + H_2O \qquad (44)$$

The nitrate is reduced to nitrite by the transfer of hydrogen from reduced co-enzyme. This tranfer is not direct; the electron transport chain involved can be represented thus



Molybdenum is the direct electron donor effecting nitrate reduction, and the FAD prosthetic group completes the electron transport chain between reduced co-enzyme and nitrate. Similar but distinct reductases complete the stepwise reduction of nitrite

nitrite——→hyponitrite——→hydroxylamine——→ammonia

Iron and copper seem to be essential co-factors involved in nitrite reduction and there is evidence that the final step, the reduction of hydroxylamine, requires manganese. It is not clear whether nitrate always undergoes reduction to ammonia before the nitrogen is incorporated into organic nitrogen compounds. It may be that in some plants hydroxylamine is not reduced to ammonia by the hydroxylamine reductase but is acted upon by another enzyme to give rise to an organic oxime which is then reduced to an amino compound.

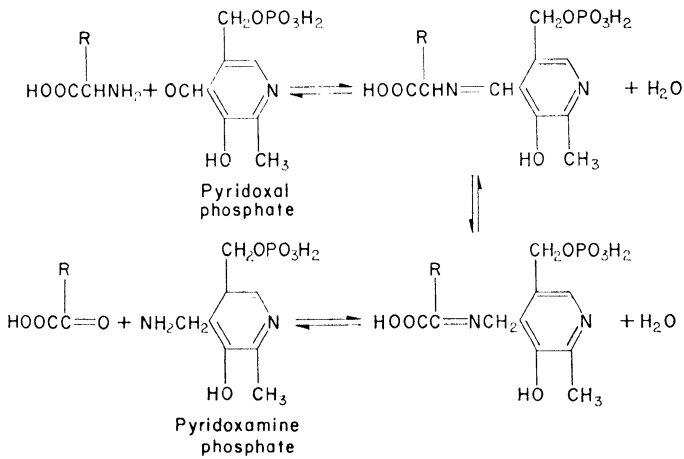
Feeding experiments with ammonium sulphate in which the nitrogen (at. wt. 14) is enriched with the heavy isotope of nitrogen (at. wt. 15, usually written N^{15}) show that the nitrogen of ammonium is most actively incorporated into newly synthesised L-glutamic acid, and to a lesser extent into L-aspartic acid. This incorporation into glutamic acid is catalysed by the universally distributed L-glutamic acid dehydrogenase (see Chapter 3, p. 57) which controls the reaction:

$$\alpha\text{-ketoglutaric acid} + NH_3 + DPNH + H^+ \rightleftharpoons \text{L-glutamic acid} + DPN^+ + H_2O \quad (45)$$

The existence of a corresponding enzyme promoting a reductive amination of oxalacetic acid to L-aspartic acid is uncertain.

Both α-ketoglutaric acid and oxalacetic acid are intermediates formed in the respiratory breakdown of sugars. Respiratory energy in the form of reduced molecules of co-enzyme, is involved in both nitrate reduction and the primary synthesis of amino acid molecules by the reactions discussed above.

Glutamic and aspartic acids can now function as precursors for the synthesis of a number of other amino acids through the action of a group of enzymes termed transaminases (amino-transferring enzymes). These enzymes have as their prosthetic group pyridoxal phosphate, a derivative of vitamin B_6 (pyridoxine). The pyridoxal phosphate accepts the amino group of the amino acid to become pyridoxamine phosphate and then transfers the amino group to other organic acids which are thereby converted to the corresponding amino acids. The following reaction illustrates the role of pyridoxal phosphate:



(47)

Transamination from glutamic and aspartic acids can lead to the synthesis of most of the amino acids required for protein synthesis (see Chapter 3, p. 57). Some amino acids are, however, almost

certainly synthesised by other reaction sequences: in a number of other cases it is doubtful whether transamination is involved in the *main* pathway of their synthesis. Nevertheless, these alternative routes of synthesis involve glutamic acid or amino acids known to be derived from it so that clearly this primary product of ammonium assimilation occupies a central position in amino acid metabolism.

The hydrolysis of proteins yields not only a mixture of amino acids but nearly always some ammonia. Part at least of this ammonia comes from amide residues (residues of glutamine and asparagine) present in the protein molecule. The free amides glutamine and asparagine often accumulate in cells, particularly when reserve proteins are being degraded and amino acids oxidised or following feeding with ammonium salts. These amides are not only to be regarded as stores of amide-nitrogen which can be readily used as a source of ammonia for amino acid synthesis but as units which are to be directly utilised in the synthesis of certain proteins. Study of the mechanism of glutamine synthesis suggests a further role of these amides. The synthesis of glutamine takes place under the influence of the enzyme, glutamine synthetase, and in the presence of magnesium ions by the equation:

$$\text{L-glutamic acid} + NH_3 + \text{ATP} \rightleftharpoons \text{L-glutamine} + \text{ADP} + \text{phosphate} \quad (48)$$

The synthesis of the amide group therefore involves the consumption of ATP. However, the reaction is reversible to a measurable extent so that energy stored in the amide group of accumulated glutamine could be re-utilised to synthesise ATP from ADP plus inorganic phosphate. The relatively high energy content of the amide group of glutamine may also explain why this compound functions as the donor for two of the nitrogen atoms incorporated into the purine ring during its synthesis.

The amide group and the peptide bond are compared below

—CO—NH—	—CO—NH—H
peptide bond	amide group

The involvement of ATP in amide synthesis therefore led to the speculation that ATP might also be involved in the synthesis of the peptide bonds of the protein molecule. If this is so, it would explain why respiratory energy is needed for protein synthesis and why inhibitors of oxidative phosphorylation are potent inhibitors of protein synthesis.

A number of other very interesting observations have led up to our modern concept of the mechanism of protein synthesis. Cells that are particularly active in protein synthesis are rich in cytoplasmic ribonucleic acid (RNA) and ribonucleo-protein (RNA-protein). Further, in a number of studies with intact cells it was found that their rate of protein synthesis varied directly as their nucleic acid content and that their synthesis of protein was strongly inhibited by ribonuclease (which degrades RNA) and also by chloramphenicol. More recently, a limited amount of protein synthesis has been shown to occur in preparations obtained by disrupting bacterial and higher plant cells provided these preparations are reinforced by the addition of an appropriate mixture of free amino acids and ATP. Such protein synthesis is prevented by adding ribonuclease or chloramphenicol. By using amino acids labelled with radioactive C^{14}, active incorporation of the free amino acids into protein can be demonstrated in such cell-free preparations and again this incorporation is inhibited by ribonuclease or chloramphenicol. This amino acid incorporation is also dependent upon adding ATP and is markedly enhanced by adding potassium ions; it probably indicates the synthesis of new protein molecules.

By disrupting cells in such a way as to liberate nuclei, mitochondria and microsomes, followed by separation of these "particles" by differential centrifuging it becomes possible to see how far these cellular structures are centres of protein synthesis. Nuclei, mitochondria and microsomes are all capable of incorporating amino acids into their proteins. The microsomes when supplied with amino acids, ATP, Mg^{++}, K^{+} and a "cytoplasmic fraction" not sedimented at $105{,}000 \times g$ are particularly active in protein

synthesis. The amino acids are first incorporated into the RNA-protein of the microsomes and later into its protein. Studies in the electron microscope of cells active in protein synthesis show the prominence in such cells of microsomes (Fig. 12, p. 40).

From such observations the hypothesis has been developed that several steps are involved in the synthesis of a protein molecule. In order to effect the synthesis of peptide bonds energy will be required; the amino acid units must be "activated". This activation involves ATP, and experimental evidence indicates that this is degraded during amino acid incorporation to AMP (adenosine monophosphate, adenylic acid). The requirement of a "cytoplasmic fraction" for protein synthesis by microsome preparations has led to the finding that its activity is due to its content of soluble RNA and that the amino acids can react with this RNA in solution and in the presence of ATP and that during this reaction AMP is formed. Further study of this essential RNA has shown that it is composed of as many specific types of RNA as there are natural amino acids involved in protein synthesis. The evidence now strongly supports the view that each amino acid reacts in the presence of ATP with its specific RNA to form a complex (Fig. 50) and that it is apparently in this form that each amino acid reaches the microsome. The RNA of the cytoplasm involved in the formation of these separate complexes with the separate amino acids is, therefore, called "*transfer* RNA".

The next stage, occurring within the microsomes, must presumably involve a controlled synthesis of peptide bonds between the amino acids to give rise to specific proteins; proteins which owe their specific biological properties, e.g. their activity as specific enzymes, to the sequences of the different amino acids along their polypeptide chains. Within the microsome we must seek the mechanism which ensures that the correct proteins are synthesised. This, in turn, clearly raises the problem of how the genes of the nucleus control the chemical events of the cell, in this case, of how the genes determine the kinds and the amounts of proteins synthesised at a site, like the microsome, which is removed from the

nucleus. Surely the key to this is the involvement of RNA and RNA-protein in protein synthesis.

Now the nuclear genes carried by the chromosomes are composed of the giant molecules of deoxyribonucleic acid (DNA), the nucleic acid which contains the sugar, deoxyribose. These giant molecules are built up of four kinds of nucleotides. The four nucleotides involved differ from one another in the nitrogenous bases which they contain and the four bases involved are adenine, guanine, thymine and cytosine. The nucleosides (base+sugar) are united together by phosphate groups to give chain-like polynucleotides in which the "backbone" consists of alternate residues of deoxyribose and phosphate groups. The four bases attached singly to each deoxyribose unit are the side groups of the polynucleotide chain. The DNA molecules consist not of a single polynucleotide chain but of two such chains twined round one another and held in position by hydrogen bonds to give a double spiral (Fig. 48). The hydrogen bonds occur between the bases; between the adenine units of one chain and the thymine units of the other chain and similarly between guanine and cytosine units of the adjacent polynucleotides. The reduplication of genes occurring during the interphase of mitosis can now be thought of as a duplication of the DNA-molecules of the chromosomes. This is thought to occur by the double spiral uncoiling and each half molecule acting as a template to make an exactly complementary nucleotide chain in the presence of a supply of the necessary deoxy-nucleotides plus the appropriate polymerase enzyme (Fig. 58, p. 198).

The unit of structure of RNA is a polynucleotide chain similar to that in DNA except that the sugar is ribose instead of deoxyribose and that the nitrogenous base, thymine, is replaced by uracil. Enzymic systems have been obtained from bacteria which synthesise RNA from its four nucleotide units supplied as triphosphates. These RNA synthesising systems only work if DNA is also present *and* the particular DNA added determines the chemistry of the RNA synthesised. This suggests that DNA can act not only as a template for its own synthesis but as a template for the synthesis

of RNA and that the RNA so synthesised will have the complementary sequence of bases along the length of the polynucleotide chain except that the complementary base to the adenine of the

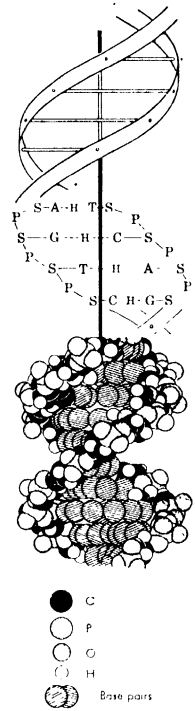


FIG. 48. The double helical structure of deoxyribonucleic acid (DNA). S = deoxyribose; A = adenine; C = cytosine; G = guanine; T = thymine. ···H··· = hydrogen bonding. (after C. Swanson in *The Cell* Prentice Hall, New Jersey, 1960).

DNA will be uracil (and not thymine) in the RNA molecule (Fig. 49). If the DNA molecules of the chromosomes act as templates for the synthesis of RNA molecules and if these RNA molecules migrate to the microsomes, the genetic message may be thus transferred to the centres of protein synthesis. For this

message to be translated into the structure of the proteins synthesised these *messenger* RNA molecules must control the sequence of amino acids in the polypeptide chains. If this is the case one would expect that experimental modification of RNA composition would lead to the synthesis of a "foreign" or abnormal protein.

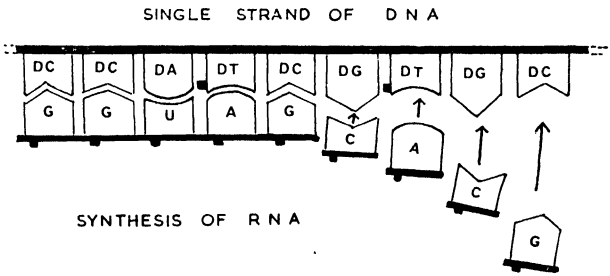


FIG. 49. Highly diagrammatic representation of a single strand of DNA acting as a template for the synthesis of a single strand of RNA (ribonucleic acid). A=adenylic acid, C=cytidylic acid, G=guanylic acid; U=uridylic acid, T=thymidylic acid. Prefix D=deoxynucleotide (redrawn from V. G. Allfrey and A. E. Mirsky in *How Cells make Molecules*. Scientific American Reprint No. 92. September 1961).

In support of this is the observation that bacteria fed 5-fluoro-uracil incorporate some of this foreign base where normally uracil occurs in RNA, and that such bacteria synthesise an abnormal enzyme and not the enzymic protein, β-galactosidase.

The hypothesis can now be formulated that within the microsome, the RNA molecules act as templates for protein synthesis and that the different forms of *transfer* RNA take up positions where their base sequence pairs with that of the template or *messenger* RNA and that then the amino acids become linked together like the closing of a zip-fastener (Fig. 50). Certainly the evidence is that in mammals the haemoglobin is so formed starting with the amino acid, valine, at one end of the chain and closing, bond after bond, until the 150 amino acid units have all been linked, a process taking about 1½ minutes. Once protein is synthesised, it and the "transfer

RNA" molecules now no longer complexed with amino acids must be freed from the RNA "messenger" template so that it can function again and again.

The concept of protein synthesis outlined above is clearly in many of its features still speculative. Much has yet to be learnt

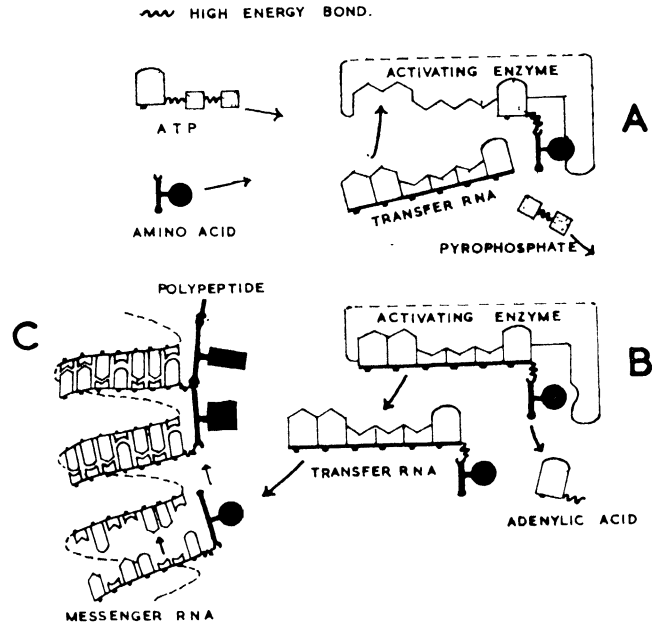


FIG. 50. Highly diagrammatic representation of the mechanism of protein synthesis.

A and B: formation of "activated amino acid" by combination of amino acid with ATP followed by linkage to specific "transfer RNA" and release from surface of the activating enzyme (based upon M. B. Hoagland, *Nucleic Acids and Proteins*. Scientific American Reprint No. 68, December 1959).

C. Each "transfer-RNA–amino acid complex" at its appropriate position on the "messenger-RNA" template. Amino acid linkage spreading downwards in the diagram (redrawn from V. G. Allfrey and A. E. Mirsky in *How Cells make Molecules*. Scientific American Reprint No. 92. September 1961).

and in this learning process some of the speculation will almost certainly be rejected or suffer considerable modification. What the most recent data has, however, for the first time made clear is the nature of the connection between the genes in the nucleus and the control of chemical structure and hence of function in the centres of metabolism sited in the cytoplasm.

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6

ABSORPTION, SECRETION AND TRANSLOCATION

"During the . . . first decades of the present century there was a widespread tendency to think of living cells as if they were simply aqueous spaces isolated from their environment by selectively permeable, but inert, membranes. Today we realize that this 'collodion-bag concept' was a flagrant oversimplification. The principal defect of this mode of thought was, of course, that it totally neglected the active transport processes."

Runar Collander in Cell Membranes: Their Resistance to Penetration and their Capacity for Transport in *Plant Physiology.* Vol. II. Edited by F. C. Steward, Academic Press, New York, 1959.

"What I do wish to stress is that solute movements in living cells are so intimately related to and dependent upon processes of metabolism that it should not be hoped that any real or hypothetical cell or membrane from which the factors of complex metabolism are absent, can go far in imitating the processes by which living cells absorb and accumulate solutes."

D. R. Hoagland in *Lectures on the Inorganic Nutrition of Plants.* Chronica Botanica Co., Waltham, U.S.A., 1944.

INTRODUCTION

Across the boundary between a living cell and its environment solutes continuously pass in both directions. When, during a given time interval, there is a net movement (*transport*) of a given solute into the cell we talk of *absorption* or *influx* of the solute and if this absorption leads to the establishment of a higher concentration inside than outside the cell we talk of solute *accumulation*. When the net movement is in the reverse direction we talk of solute

release or *efflux* and of the resulting *depletion*. In some cases and at some times such movements across cell boundaries appear to be controlled in direction and rate by physical forces such as diffusion, particularly diffusion across cell membranes allowing free movement of some molecules or ions and preventing or restricting the movement of others (across membranes showing selective permeability). Such movements are often referred to as *passive*, in that they tend to decrease the energy potential of the system. In other cases the solute movement is opposite in direction and/or different in rate from that expected from physical considerations. The movement is then a process involving consumption of cellular energy and is said to be *active*; such movement is often referred to as secretion. When solutes are transferred from cell to cell through a tissue or over the longer distances between plant organs we talk of such solute transport as *translocation*.

This dictionary-like opening to the present chapter will not only facilitate our subsequent discussion but is necessary because there is considerable variation between authors in the use of the terms italicised above.

SALT REQUIREMENTS OF PLANTS

The development of the technique of "water culture" by Sachs, Knop and others at the end of the nineteenth century demonstrated that plants have requirements for the elements, nitrogen, phosphorus, sulphur, potassium, calcium, magnesium and iron and that the sources of these elements in plant nutrition are their soluble salts present in the soil or water in which plants grow. The development during the present century of refinements in the "water culture" technique and improved methods for purifying inorganic salts has shown that other elements, the so called micro-nutrient elements boron, copper, manganese, molybdenum, zinc and chloride are also essential plant nutrients although required by cells in very small amounts. If any of these *essential elements* are only available in soluble form in inadequate amounts deficiency symptoms

develop as plant growth proceeds and if the deficiency is sufficiently severe death results. When available in excessive concentration these elements disturb normal growth and development, some of these elements being particularly toxic at high concentration. Thus boron, in the form of soluble borates is normally required at a concentration of not less than 0·05 parts per million but is markedly inhibitory to normal growth at a concentration of 1·0 parts per million.

The essentiality of most of the elements mentioned above could have been confidently predicted from the discussions we have developed in earlier chapters. Nitrogen and sulphur are constituent elements of proteins and many other cell constituents. The importance of phosphorous compounds such as nucleic acids, phospholipids and co-enzymes has been stressed several times. Magnesium is a constituent of the chlorophylls. Iron, copper, zinc, manganese and molybdenum are essential to the functioning of particular enzymes. In other cases, like that of calcium, their involvement in metabolism is less well understood and in still other cases, like potassium and boron, is almost completely obscure.

Analyses of the sap in the cell vacuoles and studies of the electrical conductivity of plant cells and cell fluids served during the first quarter of the present century to demonstrate that inorganic salts can be accumulated within plant cells to concentrations greatly in excess of their concentrations in the external environment and that this accumulation of salts was a selective process in that some elements were accumulated to a much greater extent than others Such observations made it clear that the uptake of inorganic salts or their ions could not be explained in terms solely of movement along a concentration gradient by diffusion. E. Overton, as early as 1895 had suggested that "adenoid activity", by which he meant metabolic activity, might be involved in solute absorption and W. Pfeffer, in his famous book, *The Physiology of Plants* published in 1900, visualised the possibility that chemical reactions might be involved in the movement of solutes into living cells. Now it was necessary to examine such ideas experimentally.

Hence from the early thirties onwards there occurred many studies of the quantitative aspects of salt uptake and of the factors which influence this process as it occurs in plant cells.

FACTORS CONTROLLING SALT ABSORPTION

Studies of salt uptake by single plant cells, isolated tissues and organs (particularly seedling roots) have served to emphasise that its characteristics are determined by the nature and metabolic state of the living material, its previous nutritional history and the conditions imposed by the experimental procedure. Surveying the many quantitative studies now available enables us to distinguish between (i) an initial, rapid and usually short duration change in the salt content of living cells whenever they are transferred to a solution of changed salt content, (ii) a continuing uptake leading to an overall and often pronounced accumulation of inorganic ions.

F. C. Steward, in 1937, referred to the first of these processes as *induced* absorption and considered that its characteristics corresponded with physical processes and was therefore, by our definition, a "passive" relationship. The long term uptake, usually involving accumulation of both anions and cations, and which he termed *primary* absorption is the process which has been shown in many recent studies to depend upon the metabolic activities of the living cells and which not only in this but in its other characteristics is clearly an "active" process. Further, if this distinction is valid we may expect rather different responses to environmental and nutritive variables according to the relative importance of induced (passive) or primary (active) absorption processes under the particular experimental conditions adopted by different workers. The virtue of the distinction drawn here is that it not only recognises the operation of both types of salt movement between the cell and its external environment but goes far to explain otherwise apparently contradictory results which in the past have been the subject of controversy between different laboratories.

The operation of two types of absorption is, for instance, illustrated by studies of the effects of temperature on the rate of salt uptake. In short term experiments (experiments of less than 2 hours duration) or when absorption proceeds at temperatures at or immediately above 0°C the Q_{10} for the influence of temperature upon rate of salt uptake is about 1·2, indicative of a physical process. At higher temperatures and over more extended periods the Q_{10} is of the order of 2–3 indicative of the rate being dependent upon thermochemical reactions; reactions of metabolism. Similarly in short-term experiments and with tissues of low metabolic activity (such as recently isolated slices of the storage tissues of beet or potato) the rate of uptake of ions may be linearly related to their external concentration and the equilibrium distribution of particular cations and anions between the external solution and the cell approach that corresponding to a diffusion equilibrium (a Donnan equilibrium) such as is established across a membrane permeable to inorganic ions but impermeable to other larger (metabolite) ions. The uptake then shows the characteristics of a "passive" process. By contrast, when the metabolism of the same tissue is "activated" by a prior washing in aerated water then the tissue acquires the ability in the presence of oxygen, and at a rate which may be determined by the oxygen concentration, to accumulate progressively both cations and anions over a prolonged period. Further, under these conditions the rate of this "active" accumulation may be independent of the external concentration over a wide range. Further, if the "activated" tissue is placed under anaerobic conditions it may again be shown to carry out a short-lived uptake of ions corresponding with the "passive" uptake of the initial resting tissue.

Studies along these lines have emphasised the significance in the salt nutrition of plants of the metabolically actuated component of ion uptake. Our attention therefore is directed towards experiments which have contributed to our knowledge of this process and to the theories advanced regarding the mechanism whereby metabolism controls ion accumulation. Such an "active" process

by definition involves the utilisation of metabolic energy and it is therefore not surprising that it is related to respiration. When respiration is inhibited by lack of oxygen or by using respiratory inhibitors there is a decrease in the rate of "active" ion uptake. The energy released in the oxidative reactions of respiration is conserved in a "useful" form by the simultaneous synthesis of high-energy phosphate bonds. The substance, dinitrophenol, at appropriate concentrations, "uncouples" the oxidative reactions of respiration from the synthesis of the energy-rich phosphates. Oxygen uptake is not inhibited, it may even be stimulated, but the released energy of the respiratory substrates is not conserved in a utilisable form. Such concentrations of dinitrophenol depress or completely inhibit "active" ion uptake.

The ions present in a solution affect the absorption of one another. In a complex solution such as is required to present all the essential nutrient ions, these interactions are of great complexity. However, quantitative studies of ion uptake from two-salt solutions have enabled interactions between pairs of cations and anions to be examined. Such work has shown that chemically related anions interfere with one another during absorption whereas more diverse ions do not. Thus, chloride uptake is reduced by bromide or iodide ions, sulphate uptake by selenate ions, phosphate uptake by arsenate ions. The alkali cations similarly "compete" with one another for absorption. Calcium ions compete with strontium but not with magnesium. Studies of the uptake of alkali cations from mixed solutions (with a common anion) illustrate ion interactions in a very interesting way. Firstly potassium ions are usually more rapidly absorbed than the other alkali cations (sodium, caesium, rubidium, lithium). This discriminative uptake is characteristic of "active" uptake and such selectivity is not marked under experimental conditions permitting only a physical or "passive" uptake. Secondly, the quantatitive effects of one cation upon the uptake of a second cation can be treated kinetically to see if there is a competitive inhibition of uptake similar to the competitive inhibition of enzyme reactions previously discussed

(p. 68). Thus, if we plot the reciprocal of the rate of K^+ uptake at several different external concentrations of K^+ against the reciprocal of the concentration of a second ion simultaneously present the straight lines will be parallel for a non-competitive interaction. If, however, there is a competitive interaction (resembling competitive inhibition) the straight lines will meet at a point corresponding to the reciprocal of the maximum rate (V) of K^+ uptake. Such studies (Fig. 51) have shown that K^+, Rb^+ and Cs^+ compete with one another whereas the inhibition of Rb^+ uptake by Li^+ is not competitive. Studied in this way cations and anions fall into a number of competitive groups. From the comparison of this competitive inhibition of uptake with the competitive inhibition of enzyme reactions if may be suggested that the "active" uptake of ions involves the formation of ion-complexes

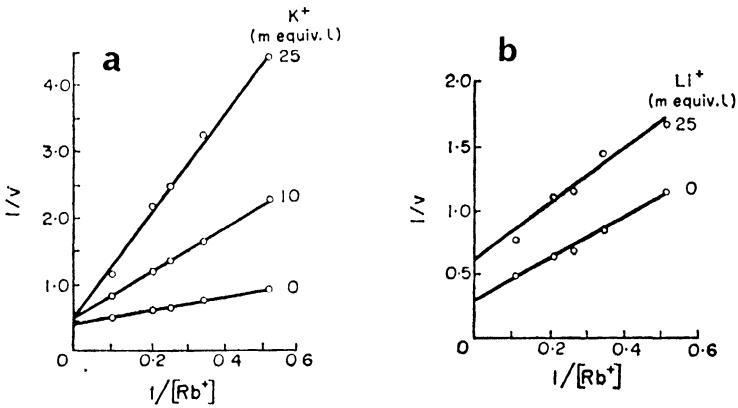

FIG. 51. Plots of the reciprocal of the concentration of rubidium ions [Rb^+] against the reciprocal of the rate of uptake of these ions $\left(\frac{1}{v}\right)$. Concentrations in m-equivalents per litre. Uptake rates in m-equivalents per gram of tissue per hour. (a) shows competitive inhibition of Rb^+ uptake by potassium ions, K^+ (b) shows non-competitive inhibition of Rb^+ uptake by lithium, Li^+, (after E. Epstein and C. E. Hagen. *Plant Physiology*, **27**: 457, 1952 from J. F. Sutcliffe *Mineral Salts Absorption in Plants*, Pergamon Press, Oxford, 1962).

comparable to substrate-enzyme complexes. This implies that the absorption of ions involves their chemical combination with substances located at the barrier preventing or restricting free diffusion of ions between the cell and its external environment. Further, it may be postulated that ions within each competitive group combine with the same cellular constituent at the diffusion barrier. Such studies therefore lead us on to enquire both as to the location and nature of the diffusion barrier and the nature of the chemical reactions which link metabolism with ion uptake.

THE SITES OF SALT ACCUMULATION WITHIN CELLS

By using radioactive ions it is possible to trace their rate and extent of penetration from an external solution into the cell walls, into the cytoplasmic phase and separately into cytoplasmic structures, particularly mitochondria (separated by centrifuging) and vacuoles. By previous feeding with radioactive ions followed by immersion of the cells in "cold" solutions it is possible to study (by determination of the labelling of the external solution) how far the absorbed ions can exchange with the external solution and to see if different parts of the total content of any chosen ion, exchange at different rates with the external solution. It is also easily possible to study how far "absorbed" ions can be rapidly or only slowly washed out of tissues and cells. Experiments along these lines with higher plant and algal cells have, in general, indicated that, for each ionic species, part can be very rapidly exchanged or washed out, part (in mature vacuolated cells by far the greater part) is strongly retained within the cells, and a third part is retained with an intermediate tenacity. Thus, for instance, in the large highly vacuolated cells of the alga, *Nitella*, a small part (about 0·1 per cent.) of its potassium exchanges very rapidly (50 per cent exchange is reached in 23 *seconds*), a second part (1–2 per cent.) is exchanged to this extent in 5 *hours*, and the third overwhelming larger part is exchanged to this extent only in about 40 *days*.

The region of the cell into which ions penetrate rapidly by diffusion and from which they are readily removed by washing has been referred to as the *free space*. The regions into which, by contrast, ions penetrate slowly, in which they accumulate and from which they are only slowly exchanged is then referred to as *non-free space*. The real difficulty comes in trying to identify the cell locations corresponding to "free space" and "non-free space". It is, however, generally agreed that ions enter the vacuoles slowly, that this entry is an "active" process and that the ions of the vacuolar sap account for most, if not all, of the very slowly exchanged ions of fully expanded cells (hence the reference by many authors to vacuolar non-free space). It is also agreed that ions rapidly diffuse into and out of the cell walls so that they constitute a free space. The difficulty comes in trying to assess the exchangeability of ions in the cytoplasm. Studies with isolated mitochondria have shown not only their ability to carry out active salt accumulation but indicate the mitochondria as part of the non-free space of the cells. It is particularly the exchange status of ions in the hyaloplasm which is still the subject of controversy although the balance of evidence is that the plasmalemma is a barrier to solute movement so that as a result of it and, perhaps, also of its associated endoplasmic reticulum, there is a cytoplasmic non-free space in which ions are retained although with less tenacity than following their accumulation into the vacuoles and mitochondria. The similarity of structure of all cytoplasmic membranes as revealed by electron microscopy also seems to fit in with this conclusion. The "active" uptake of ions therefore seems to involve their movement at a speed and in a direction not determined by the concentration gradient and across permeability barriers represented by the lipo-protein membranes of the cell.

THE LINKAGE OF METABOLISM WITH SALT ABSORPTION

The lipoprotein membranes of the cell act as diffusion barriers; they have a high resistance to passive penetration by ions. How then can we explain the rapid active movement of ions across such membranes, a movement which can proceed against the concentration gradient? One explanation is to postulate that the ions undergo reversible binding with some constituent of the membrane (this constituent is usually called the *carrier*). The ion is then visualised to pass across the thickness of the membrane, not as a free ion but as an ion-carrier complex (Fig. 52). This concept has the immediate attraction that it suggests an explanation of selectivity; selective absorption would reflect the abundance in the membrane and chemical affinities of the carrier molecules. Ions which compete with one another would be ions capable of combination with the same carrier but the affinities of the carrier for the separate ions of the group would differ. Selective uptake of potassium, as compared with other alkali metal cations, would reflect the high affinity of the carrier involved for potassium. The number of different carrier molecules would be equal to the number of groups of ions which can be shown to compete with one another in absorption.

If we accept the hypothesis that active ion uptake operates through carrier systems we are led to consider how the operation of such systems could depend upon energy released by metabolism. Referring to Fig. 52, we see that energy could be used for regeneration of the carrier ($X^- \longrightarrow X$), for promoting combination of the carrier with the ion ($X \longrightarrow XK$), for breakdown of this complex ($XK \longrightarrow X^- + K^+$) or for transport of the carrier-ion complex (XK) and/or of the carrier precursor (X^-) across the membrane. There are a disconcerting number of points at which the operation of the system could depend upon energy. Clearly, to be able to choose between these alternatives, to formulate the chemical reactions connecting metabolism with ion uptake and to really explain specificity of uptake we must know the exact chemical

nature of the carrier molecules. This we do not know though modern biochemical techniques should lead on the near future to the isolation of such carrier molecules if indeed they are the basis of active uptake.

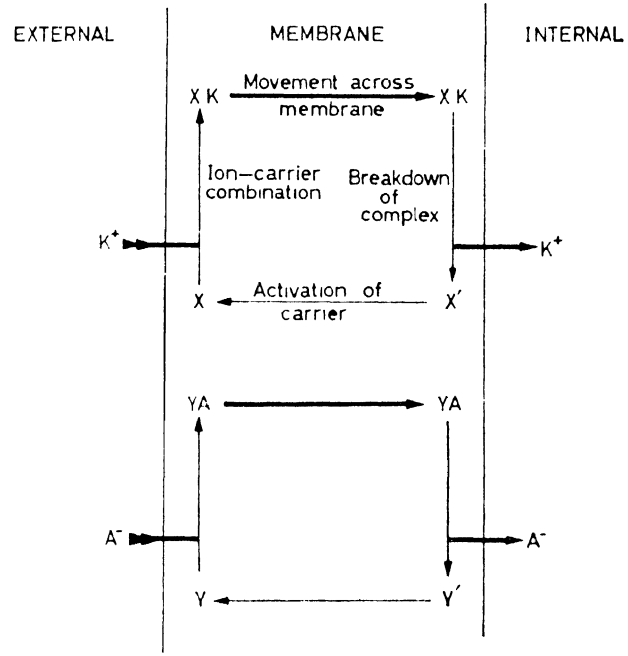

FIG. 52. The general concept of the operation of a carrier mechanism in ion uptake by cells. X and Y = carriers. X′ and Y′ = precursors of the carriers. XK and AY = carrier–ion complex. K^+ = cation, A' = anion (from H. E. Street. The Physiology of Roots in *Viewpoints in Biology* I. Butterworths, London, 1962.)

However, the mechanism of active ion uptake is being studied in a number of botanical laboratories and, therefore, as you might have expected, there is no lack of hypotheses regarding the chemical nature of carriers nor of how energy enters carrier systems. Such hypotheses merit the readers attention because they illustrate the

nature of original thought in plant physiology and challenge him (or her) to think how such hypotheses could be subjected to the test of experiment.

In Fig. 52, both a cation and an anion carrier are depicted; cation and anion uptake are visualised as involving *separate* carriers. However, not only is it possible to visualise amphoteric carriers (carriers capable of binding both cations and anions) but it could be that only the uptake of anions (or of cations) involves a carrier system. Thus, if anions are transported actively in this way, the cations might then move passively along the electrical gradient created by the accumulation of the negatively charged anions at the inner surface of the transport path. The Swedish botanist, Lundegårdh, was led to consider such a hypothesis from evidence he obtained that the outer surfaces of the absorbing cells of the root were negatively charged and might therefore be cation permeable but repel anions. Further, from studies of the rate of respiration during active salt uptake he concluded that the rate of oxygen uptake of the absorbing cells was the result of two component uptakes, one (the "ground respiration") unrelated to ion uptake and the second whose magnitude was determined by the rate of *anion* uptake (the "anion respiration"). He thus concluded that anion uptake was quantitatively geared to respiration and that cation uptake was a passive inward movement along an electrical gradient created by anion absorption. Then, in 1939, Lundegårdh found that the "anion respiration" of his roots was extremely sensitive to inhibition by cyanide and by carbon monoxide in the dark. From this he concluded that the anion carrier system was synonymous with the cytochrome system. At the exterior surface of the cell reduced cytochrome (p. 97) reacts with oxygen thus:

$$4Fe^{++}_{cyt} + 4H^{+} + O_2 \rightarrow 4Fe^{+++}_{cyt} + 2H_2O \qquad (49)$$

or considering only the oxidation of the cytochrome:

$$Fe^{++}_{cyt} \rightarrow Fe^{+++}_{cyt} + (\acute{e}) \qquad (50)$$

or if the cytochrome cations are associated with anions then the

release of an electron allows the cytochrome to pick up a further univalent anion thus:

$$\begin{Bmatrix} Fe^{++}_{cyt} \\ 2A^- \end{Bmatrix} + A^- \rightarrow \begin{Bmatrix} Fe^{+++}_{cyt} \\ 3A^- \end{Bmatrix} + (\acute{e}) \qquad (51)$$

Then the extra anion now associated with the oxidised cytochrome may be presumed to pass along the cytochrome electron-transport chain or move inwards by diffusion of the cytochrome-anion complex and be released at a point of lower oxidation potential, for instance, where cytochrome *b* reacts with flavoprotein. For this process to lead to anion uptake it is postulated that the release of the anion occurs in the inside of the diffusion barrier membrane. This hypothesis when first enunciated aroused considerable interest, not only because of Lundegårdh's personal reputation as a plant physiologist, but because it was clearly capable of experimental test.

The Lundegårdh hypothesis required that 4 monovalent anions and not more than 4 be absorbed per uptake of one O_2 molecule in "anion respiration" and that all anions stimulate respiration to the same extent per anion charge absorbed. Experiment showed that neither condition was fulfilled. It also became clear that one of the ions whose uptake most enhanced respiration was the cation, NH_4^+ and that respiration was enhanced when ion uptake is restricted to cation uptake as occurs when a root is surrounded by a moist cation exchange resin. This hypothesis also precludes an active uptake of ions occurring under anaerobic conditions following upon a period of active aerobic respiration; it is a mechanism which would not provide for any storage of the ability to promote active ion uptake. In this it is contrary to a number of experimental observations. Further, only one carrier is postulated for all anions and it therefore fails to explain how there is no competition in uptake, for instance, between halide ions and sulphate ions, nor between sulphate and nitrate. It equally leaves unexplained competition between cations. Finally, it faces the insuperable obstacle that the respiratory cytochrome system is

certainly almost entirely confined to the mitochondria of plant cells. The value of this untenable hypothesis was that it stimulated research into the mechanism of salt accumulation.

This exercise of examining critically hypotheses regarding the mechanism of active ion uptake can now be extended to some more recent suggestions regarding the chemical nature of carriers and of how such carrier mechanisms could be energy requiring. The mechanisms postulated all suggest that the carrier molecules are amphoteric (hence capable of binding both cations and anions), and thereby evade a major criticism of the Lundegårdh hypothesis. In 1952, R. J. Goldacre reported rhythmic movements of root hair vacuoles and suggested that they arise from ordered contraction and unfolding of protein molecules orientated in the vacuole membranes. A similar folding and unfolding of protein molecules was considered to motivate protoplasmic streaming. Goldacre suggested that such contractile proteins within the membrane could when they were in the unfolded form bind ions by free valencies exposed at the membrane surface. Contraction would draw these ions through the membrane and the act of contraction could lead to liberation of the ions as the free valencies of the protein become satisfied amongst themselves in the folded molecules (Fig. 53). Unfolding of the protein molecule would reset the trap. Knowledge of the behaviour of myosin, the contractile protein of muscle, suggests how respiratory released energy could work such a mechanism. The extended form of myosin is energy-rich and may contract spontaneously, the unfolding of the myosin molecule is linked with the simultaneous degradation of ATP to ADP. The ability of dinitrophenol to inhibit salt absorption implicates ATP as a primary source of energy in this process. This hypothesis is in accord with the importance of proteins in membrane structure, it clearly defines two states of the carrier interconverted by utilisation of ATP which seems now the most universal source of the energy involved in cellular work and it explains transport across the thickness of the membrane by making this a forceful displacement of the ions. It is difficult to devise experimental tests of this hypothesis.

However, it is opposed by one interesting experimental observation. If both the rhythmic movements in root hair vacuoles observed by Goldacre and protoplasmic streaming are dependant

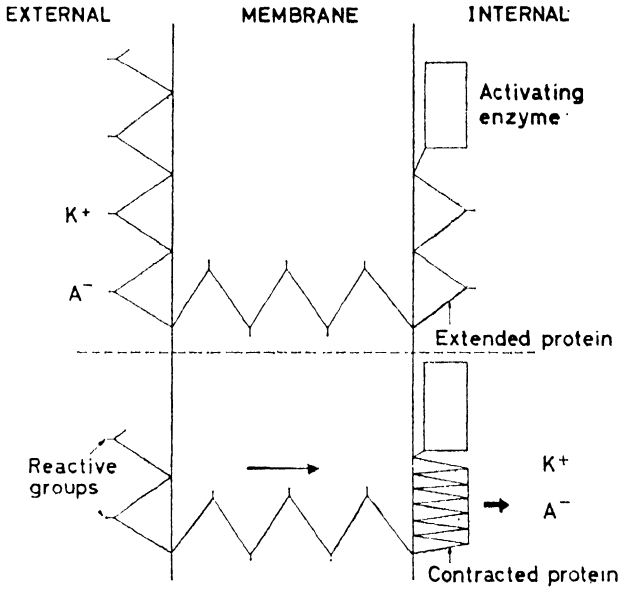

FIG. 53. Diagrammatic representation of the Goldacre concept of the functioning of a contractile protein as an amphoteric carrier of ions. *Above* the dotted line—"trap set", *below* intake and release into the cell of ions as a consequence of contraction of the protein fibre. Energy could be fed into the system to extend the protein fibre and re-expose reactive groups external to the membrane (to set the ion trap). (from H. E. Street as Fig. 52).

upon the folding and unfolding of protein molecules, as Goldacre himself contended, then any treatment which does not impair protoplasmic streaming would not be expected to inhibit salt uptake. However, in work with cells of the red beet and of the leaf of *Elodea canadensis*, it has been shown that concentrations of chloramphenicol, which do not inhibit either oxygen uptake or

protoplasmic streaming, do inhibit salt uptake and protein *synthesis*.

In 1956, Bennet-Clark suggested that the carrier could be a protein associated with the phosphatide, lecithin. This takes account of the fact that it is particularly lipo-proteins which are involved in the building of cell membranes and that certain enzymes seem to be located in cell membranes. On this hypothesis the phosphate group in the phosphatide is regarded as the active centre binding cations and the basic choline group as the anion binding centre (Fig. 54). The liberation of the ions into the cell is regarded as being effected at the inner surface of the membrane by decomposition of the lecithin by the enzyme, lecithinase. The regeneration of the carrier from phosphatidic acid and choline is

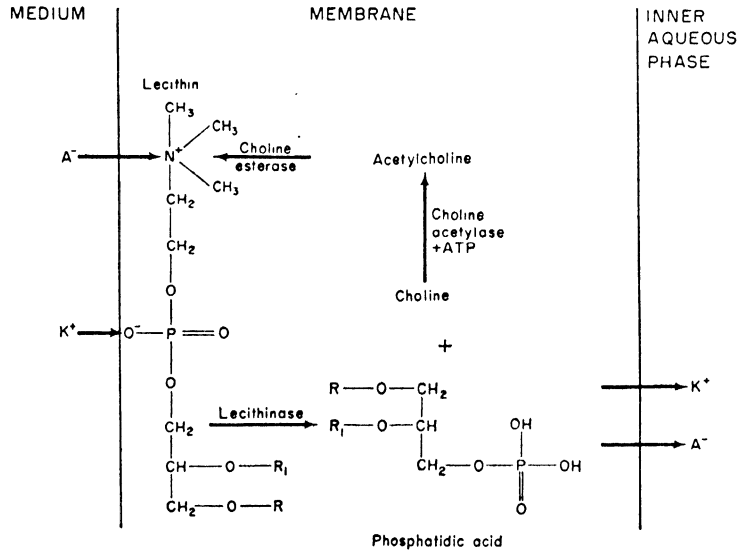

FIG. 54. Diagrammatic representation of the hypothesis of Bennet-Clark that a cyclic formation and breakdown of the phospholipid, lecithin within the membrane would enable it to act as an amphoteric carrier. Energy as ATP is consumed in lecithin synthesis. (from Sutcliffe as Fig. 51).

postulated as involving enzymes (choline acetylase and choline esterase) and ATP as source of energy. Experimental data in support of this hypothesis is at the moment lacking but clearly it faces the difficulty of demonstrating the presence in the cell membranes of the required number of distinct phosphatides to correspond with the number of known "competitive groups" of cations and anions.

The reference above to the inhibition of salt absorption by chloramphenicol, a specific inhibitor of protein syntheis, can serve to introduce one of the most interesting recent hypotheses. This arose from the observations that when the "dormant" cells of storage organs (potatoes, beets) are activated by washing in aerated distilled water then they develop not only a capacity for "primary" salt absorption but also for protein synthesis, and certain factors seem to affect these two processes in the same way. However the concept that there was a close functional relationship between these two processes seemed to face the difficulty that active ion uptake can take place into cells in which there is no net protein synthesis. However, studies involving the use of the mass isotope of nitrogen (N^{15}) have shown that in such cells protein is being rapidly broken down and resynthesised (there is a rapid protein "turnover"). This has led Steward and his co-workers to suggest that peptides in the cell membranes bind both cations and anions and that such peptides are protein precursors which are liberated and travel to the microsomes where, as protein synthesis takes place, the ions are liberated. Much along the same lines, Sutcliffe (1962) suggests that proteins are constantly entering into and being released from the structure of cell membranes. The released proteins carry ions to the ribonucleic acid templates of the endoplasmic reticulum and there, when protein and template combine "clusters" of ions are released. Such ions could attract to themselves water to form ion-rich vesicles and these could move in the cytoplasm and by bursting at the vacuole surface release ions to the central pool. Here, then, we have attempts to visualise an active mechanism of ion transport which explains the relationship of

salt uptake to protein turnover and incorporates ideas which flow from our rapidly growing knowledge of the fine structure of cytoplasm as revealed by the electron microscope (see Chapter 2).

THE TRANSLOCATION OF SALTS

From the evidence that cellulose cell walls are readily permeable to water and to salt ions it would seem that salts could travel through living tissues by diffusion in the cell walls. However, various factors (such as illumination) which tend to divert salts from the cytoplasm into the central vacuole seem to reduce markedly the export of salts to surrounding cells. This suggests that salt movement from cell to cell in a living tissue may involve the protoplasmic continuity of cells via plasmodesmata. Further, if the hypothesis of minute mobile ion rich vesicles is correct one might visualise that these can travel, not only across the cytoplasm to the central vacuole but through cytoplasmic connections into adjacent cells.

Considering the root, it would seem that salt movement across the cortex could proceed to the parenchyma surrounding the xylem conducting elements entirely within the cytoplasmic continuum (the symplast). Alternatively, movement as far as the endodermis could occur predominantly in the cell walls. At the endodermis the impermeability of the cell walls due to the Casparian strip would divert salts into the cytoplasm of the endodermal cells and living cells of the stele. This problem is not resolved, nor do we understand the mechanism whereby the living cells of the stele release salts into the xylem elements. It is, however, tempting to suggest that the efflux of salts from the living cells into the xylem is comparable to the release of ions into central vacuoles. Certainly, just as the ions are selectively accumulated in the vacuole so they seem to be selectively secreted into the young xylem vessels and tracheids and this secretion seems often to proceed against the concentration gradient. The consequence is that in the region of active absorption of salts by the root the

xylem sap differs in composition and is more concentrated than the external soil solution. From this region the salts are transported by the mass flow initiated in the xylem conducting elements by transpiration.

Although most nutrient elements are translocated as free ions in the xylem, analysis of xylem sap always reveals the presence of organic compounds. These include not only soluble sugars but organic compounds of nitrogen and phosphorous. The evidence indicates that such organic compounds are of quantitative significance in the movement in the xylem of at least the elements, nitrogen and phosphorus.

THE TRANSLOCATION OF ORGANIC COMPOUNDS

Extensive experiments involving ringing (removal of the tissues (bark) external to the xylem) demonstrated the importance of the "bark" in the longitudinal transport of organic substances, particularly of the sugars synthesised in the leaves. Knowledge of the anatomy of the bark dating back to Hartig's discovery of the sieve tubes in 1837, immediately focused attention upon these conducting elements of the phloem as the major pathway for the translocation of organic compounds. Subsequent work has confirmed that although organic substances occur in the xylem vessels and tracheids and hence are translocated in the transpiration stream, nevertheless, by far the most important channel for the translocation of carbohydrates, amino acids and other organic compounds is the sieve tube tissue.

We know the channel of translocation but the mechanism of the translocation process in the sieve tubes has proved a most puzzling problem. The nature of this problem can be illustrated by considering the translocation of the sugars, produced in the leaves by photosynthesis, to the developing storage organs like fruits and tubers. This carbohydrate moves as sucrose. In 1953, Kennedy and Mittler showed that the willow aphid feeds by inserting

its proboscis into a single sieve tube unit. The aphid can be anaesthetised and then cut free from its proboscis. The proboscis remains as a very fine tube leading down into the sieve tube and from this tube there exudes pure phloem sap. This contains about 10 per cent of sucrose and only traces of other sugars. This sucrose moves very rapidly in the sieve tube. This can be illustrated by quoting the value calculated in 1922 by Dixon and Ball for the rate of movement of sucrose along the stolon during the development of a potato tuber. From the duration of the development of the tuber, from measuring its carbohydrate content, from the overall cross sectional area of the sieve tubes in the stolon and by assuming that the sucrose moved as a 10 per cent solution they concluded that the sieve tube contents must travel at about 40 cm. per hour. This movement is far faster than could occur by diffusion of sugar molecules; it was calculated, for instance, that the rate of movement of sucrose in the phloem of the cotton plant proceeds at a rate 40,000 times faster than would be expected by diffusion. This inevitably leads us to considers whether the phloem sap flows in the sieve tube as water through a pipe; whether a mass flow of liquid occurs in the sieve tubes.

In 1930, the German botanist, E. Münch put forward this view in his *mass-flow hypothesis*. To illustrate his concept, Münch depicted an osmotic "model" in which a pressure-actuated mass flow of solution along a tube would occur (Fig. 55). In this model, spherical semi-permeable membranes enclose at A a solution of sugar and at C either a weaker solution of sugar or water and the two spheres are connected by a tube B. Now when the two spheres are immersed in water a greater hydrostatic pressure will develop by osmosis in A than in C and hence sugar solution will flow from A to C along B and water will be released through the pores of C into the surrounding water. This flow of sugar solution will continue until the concentrations of sugar in A and C are equal. To maintain the flow, sugar would have to be continuously added to A and withdrawn from C. Now, in relating this model to events in the plant, Münch postulated that A could represent

the ending of a file of sieve tube units in the photosynthetic tissue of the leaf, where sugar synthesised in the mesophyll cells could be continuously secreted into the sieve tubes. Then C would represent say the ending of the sieve tube system in a developing storage organ where sugar is being continuously withdrawn from the sieve tubes into the surrounding cells for storage or metabolic utilisation. B would be the continuous sieve tube connection between A and C. Here, then, an "active" movement of sugar into and at another level out of the sieve tube leads to a maintained difference in concentration at different levels and this, in turn,

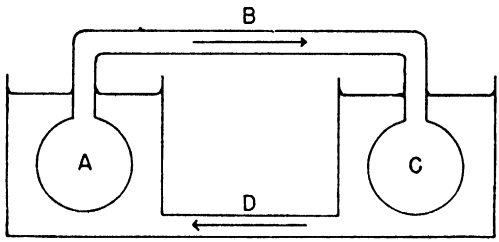

FIG. 55. Diagram of an osmotic model illustrating the principle of the Münch mass-flow hypothesis of translocation (for discussion see text).

leads to a mass flow of sap (sugar solution) in the sieve tube units from a region of high concentration to one where the sugar concentration is lower. The associated water movement would then presumably lead to release of water at the storage organ back into the xylem where it could enter the transpiration stream (D).

A short critical discussion of this Münch hypothesis will enable us to consider some of the current uncertainties regarding the mechansism of ploem transport. It can be shown, as expected from the Münch hypothesis, that a gradient of hydrostatic pressure experimentally induced in the xylem of a cut twig causes an enhanced transport of sugar in the phloem and along the direction of falling pressure in the xylem. This enhanced flow occurs without dilution of the sieve tube sap. In many trees a cut into the phloem causes an immediate release of sugary sap. There always seems to be an

immediate "expulsion" phase to this release of sap followed by a slower "bleeding". The initial expulsion is clearly suggestive of the rupturing of a system under considerable hydrostatic pressure. On other evidence it has been calculated that in willow the hydrostatic pressure in the sieve tubes can be as high as 30 atmospheres. Again there is strong evidence that sugar transport occurs from sites of higher to sites of lower sugar concentration and that mature sieve tubes can be plasmolysed (i.e. can act as osmotic systems).

The main doubts regarding the mass-flow hypothesis come from studies of sieve tube structure. The sieve tubes are divided transversely by the sieve plates and the "pores" of these plates seem to be blocked by the "slime plugs". Surely such sieve plates would prevent mass-flow of fluid in the sieve tube. Studies of sieve tube structure in the electron microscope by Professor Preston and his associates at Leeds support the view that the sieve plate pores are filled with dense cytoplasmic material. However, Mercer and his associates at the University of Sydney have advanced the view that the appearance of dense slime plugs in the pores of the sieve tube is an artefact. They describe the sieve tube as being lined within its longitudinal walls and on the sides of the sieve plate pores by a membrane which encloses a solution in which slime is dispersed. The particles of slime appear to be orientated along lines of flow at and in the sieve plate pores. The individual mature sieve tube units are figured by these workers as continuous with one another via *open* sieve pores so that the whole sieve tube acts as a conduit. They consider that the presence of the membrane within the longitudinal walls acts as a semi-permeable barrier so that the sieve tube is a plasmolysable osmotic unit. This and the open nature of the sieve plate pores are compatible with the mass-flow hypothesis. On this basis they suggest that one important role of the companion cells may be the active secretion into or absorption from the sieve tube of organic solutes and their lateral movement from or to the surrounding tissues.

More recently, Dr. R. Thaine, working in Surrey, has claimed to have observed by phase-contrast microscopy, active streaming

n fine transcellular strands running for long distances and passing unbroken through the sieve plates. Particles similar in size to mitochondria or small plastids are described as travelling along these transcellular strands and their movement is interpreted as that of particles being swept along in a flowing stream of liquid. These transcellular strands are considered by Thaine as indicating the nature of the flow occurring in sieve tubes. It is, however, extremely controversial whether the cross-sectional area of these

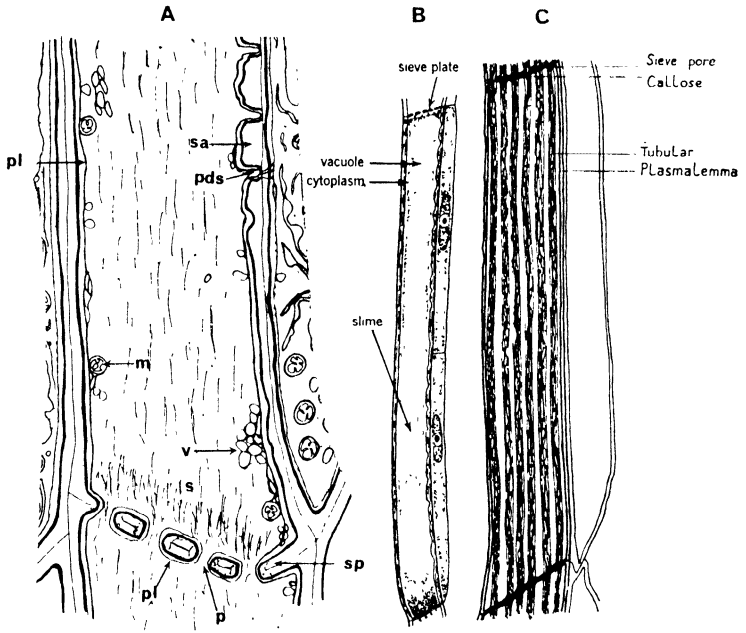

FIG. 56. Sieve tube unit structure as depicted by various authors. A. Sieve tube unit, with phloem parenchyma to left and companion cell to right as depicted by Duloy, Mercer and Rathgeber (*Aust. J. Biol. Sci.* **14**: 506, 1961). s.a. = sieve area, s.p. = sieve plate, p = pore, s = slime, pds = plasmodesmata, pl = plasmalemma. B. Mature sieve tube of *Cucurbita* with companion cell (from K. Esau. *Plant Anatomy*, John Wiley & Sons, Inc., New York, 1953). C. sieve tube unit of *Cucurbita* as depicted by Thaine (*J. Exp. Bot.*, **13**: 158, 1962).

strands and their rate of flow could account for the rate and mass of movement of solutes in the sieve tubes.

The present uncertainty of our knowledge of sieve tube structure is emphasised by the contrasted drawings of sieve tubes shown in Fig. 56 and taken from different authors.

The content of this chapter emphasises to the writer both the importance for the understanding of physiological processes of extending our knowledge of the fine structure of plant cells and the decisive role of "active" processes (processes consuming cellular energy) in the movement of solutes within and between cells.

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7

THE REGULATION OF METABOLISM

Most studies of the cell at a molecular level have had to be primarily qualitative, identifying its micro- and macro-molecular components and working out the metabolic interrelations that are so conveniently symbolised by arrows. But now that many biosynthetic pathways have become more or less completely known, it has become possible not only to describe flow rates but to analyse in detail the mechanisms that control them.

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INTRODUCTION

We have discussed the importance and underlying chemical reactions of various aspects of plant physiology. It is clear that these physiological processes are interrelated, that they are each facets of an integrated metabolism. However, to understand the metabolic activities of living cells we must inevitably first consider these facets in isolation; as separate multi-enzyme systems. This then serves as a basis from which we can move on to consider the interdependence of physiological processes. Finally, we must endeavour to uncover the control mechanisms by which unstable equilibria ("steady states") are maintained and changed to new equilibria (as cells grow and change their chemical activities during cell differentiation). It is in the study of such control mechanisms that we shall probably expose the causes of senescence and death.

It is a tenet of genetics that the metabolic activities and potentialities of cells depend upon and are limited by their inheritance. This implies that the factors controlling metabolism must be determined in their number and intensity by the inherited genes. In line with this concept is the extensive experimental evidence that the genes control the synthesis of the essential proteins of the cell including those which function as enzymes. Further, in view of the evidence that the nuclear genes are composed of DNA–protein then one way in which they could exert this function would be by determining the structure of a "messenger RNA" which, moving out from the nucleus could, in turn, confer upon the microsomes the ability to synthesise a particular enzymic or structural protein (see Chapter 5, p. 153). Any change in the structure of the genic DNA (*a mutation*) could then be reflected either in a change in the stability or specific activity of an enzymic protein or, if sufficiently profound, lead to the synthesis of biologically inactive protein. Such a change could manifest itself as the deletion of an enzyme having, if it occupied a sufficiently central position in metabolism, a lethal effect or, alternatively, inducing an absolute requirement for an external supply of some metabolite whose biosynthesis involves the lost enzyme. Mutants of this kind in which a particular enzyme is missing can be readily induced in certain micro-organisms (like the fungus *Neurospora*) by mutagenic agents like X-rays and the study of such biochemical mutants has not only established the role of the genes in controlling enzyme synthesis but also enabled a number of biosynthetic pathways in metabolism to be worked out. Work with micro-organisms has also shown that genes also control factors involved in solute absorption by cells. The factors concerned here are also probably "enzyme-like" and have hence been termed *permeases* (they could also be described as specific solute "carriers"—see Chapter 6).

Here then we have as a starting point to a discussion of the regulation of metabolism the evidence that enzyme synthesis is controlled by the genes. This control is exercised through the agency of "messenger RNA" molecules synthesised under the

direct control of the genes within the nucleus and then released into and exerting their effects within the cytoplasm, the site of synthesis of most, if not all, enzymes. This is, however, a qualitative concept. To understand its quantitative aspects we have to try to answer such questions as: what controls whether certain genes function and what controls the activity with which they function in enzyme synthesis? Once enzymes have been synthesised are there mechanisms which control their activity?; if self-regulatory systems lead to the establishment of "steady states" what factors disturb such balances to cause cells to change both qualitatively and quantitatively their metabolic activities?

INDUCTION AND REPRESSION OF ENZYME SYNTHESIS

In certain micro-organisms it has been possible to demonstrate the activity of certain enzymes under all tested environmental and nutritive conditions. Such enzymes have been described as constitutive. However, a number of these micro-organisms have been shown to be capable of developing the activity of additional enzymes if supplied with certain potential metabolites or molecules similar to such metabolites. Thus yeasts are capable of actively metabolising glucose by the activity of constitutive enzymes, but constitutive enzymes are not available which will metabolise other sugars such as galactose or arabinose. However, if these sugars are supplied the yeast quickly develops a capacity for metabolising them and this *adaptation* can take place without cell growth or division. The "adapted" cells show newly acquired enzymic activity. Such new enzymes whose activity builds up in response to the "inducing" molecules are termed *adaptive* enzymes. In such cases it is very difficult to prove that the enzymes concerned are absolutely absent before induction but we do know that the induction involves synthesis of new enzymic protein (and not an unmasking of the activity of preformed enzyme molecules).

In other cases the feeding of low-molecular weight metabolites can lead to almost equally dramatic reductions in enzyme activity. Thus, in certain bacteriea, the feeding of natural molecules like the amino acids, arginine, tryptophane and methionine or the pyrimidine, uracil may cause the loss of the activity of enzymes concerned in their biosynthesis. The enzyme whose activity is lost or markedly reduced is not necessarily that which completes the synthesis of the *repressor* molecule (i.e. not that catalysing the last reaction of the biosynthetic pathway). For instance, uracil can suppress, in certain strains of the bacterium, *Escherischia coli*, the activity of the enzyme *aspartate transcarbamylase* which promotes the interaction between aspartic acid and carbamyl phosphate, a reaction which is the first step in the reaction sequence involved in pyrimidine biosynthesis. Further, the experimental evidence indicates that the uracil acts as a repressor by inhibiting synthesis of the enzyme.

Here, then, we have examples of the regulation of metabolism by a promotion or suppression of *enzyme synthesis*. What is not yet fully understood is how "inducers" promote and "repressors" inhibit the synthesis of new enzyme protein. When two "inducer" molecules are supplied simultaneously and when the rate of protein synthesis is limited by nitrogen supply then it is possible to demonstrate competition between the systems synthesising the adaptive enzymes; in so far as one enzyme is synthesised the amount of the second enzyme formed is correspondingly reduced. Secondly, induction of an enzyme can sometimes be effected by a compound (chemically similar to the substrate) but not acted upon by the enzyme whereas other molecules which can act as substrates for the enzyme may be ineffective as inducers. Thus methyl-β-D-thiogalactoside is a powerful inducer of *β-galactosidase* in *E. coli* although inactive as a substrate whereas phenyl-β-D-thiogalactoside which can act as a substrate does not induce synthesis of the enzyme. Thirdly, enzyme induction can be a very rapid process, a very marked rise in enzyme content occurring within hours or even minutes. This, for instance, is the case with one of the very

few enzymes known, in the case of higher plants, to be adaptive, the enzyme, *nitrate reductase* (Chapter 5, p. 145). These observations have been regarded as indicating that the sites for the synthesis of adaptive enzymes are preformed in the cells but require activation, in which case inducers may function by releasing the enzyme protein from the RNA templates in the microsomes and thereby permitting further protein synthesis. Recent and very intensive study of enzyme induction and repression in bacteria has, however, led to the postulation of an alternative concept. This is that the chromosomes carry not only "structural" genes or gene groups (*operons*) which synthesise the molecules of messenger RNA and hence regulate protein synthesis but other genes ("regulator" genes) whos function is to synthesise a *repressor* which, either directly or after cytoplasmic modification, suppresses the functioning of the *operon*. Within this framework the induction of enzymes is interpreted as the consequence of the inactivation of the repressor substance by the inducer molecule, and repression of enzymes as involving cytoplasmic activation of the repressor substance.

There are some cases where micro-organisms adapt only very slowly to a metabolite, where the organism can be gradually *trained* over a number of cell generations either to metabolise a compound or overcome its inhibitory effect on growth and metabolism. For instance, the bacterium, *Bacillus lactis aerogenes* can be slowly trained to utilise glycerol and if fully trained can retain, at least for a time, the ability to utilise this source of carbon when grown in its absence and supplied with glucose. The alga, *Chlorella vulgaris*, can slowly develop resistance to the "anti-metabolite" selenomethionine and the resistance is due to enhanced activity of the enzymes reducing sulphate to the normal metabolite, methionine. Thus, resistance to selenomethionine once developed is retained in its absence. However, sulphur starvation or methionine feeding lead to a decrease in the activity of the sulphate-assimilating enzymes and, in consequence, loss of resistance to the "anti-metabolite". By growing a particular strain of *Chlorella vulgaris* in darkness we have slowly trained it to utilise galactose as an effective

carbon source and have shown that the "trained" cells have enhanced activity of the enzyme, *galactokinase* (the enzyme which promotes the formation of galactose-6-phosphate by transfer of phosphate from ATP). In this case the activity of the galactokinase quickly decreases when galactose is replaced by the readily utilisable sugar, glucose.

The fact that several generations of cells have to be traversed in this phenomenon of training raises the possibility that here we are selecting mutant cells, cells which have acquired by gene change an ability to synthesise a new enzyme. However, in the *Chlorella* studies described above the enzymes whose activity is enhanced are already present in low activity in the "normal" cells and the experimental evidence strongly supports the view that *all* the cells can be trained and that there is no selection of some mutant arising during culture of the organism. In these cases we are probably witnessing an increase in the number of enzyme-synthesising sites; we are eliciting a *greater* flow of the necessary messenger-RNA from the nucleus to activate more microsomal centres of synthesis. If this proves to be so then it will provide evidence that substances in the cytoplasm can not only initiate but enhance the functional capacity of genes.

INHIBITION AND ACTIVATION OF ENZYMES

The activity of any metabolic pathway will depend upon the availability of the precursor molecules and the activities of the separate enzymes involved. Thus, reaction pathways using common metabolites as starting materials will be in competition and each pathway will be limited in its rate by the pace of the slowest step in the reaction chain. Again the reaction pathway may be very sensitive to the concentrations of the reaction products. This may be due to the position of equilibrium in certain key reactions. Such a situation is exemplified by the dehydrogenase reactions of respiration. Unless the co-enzyme (DPN) is kept almost completely in the oxidised form (by the reactions of terminal

oxidation) the respiratory intermediates instead of being oxidised will suffer reduction. Again the enzymes in a biosynthetic or other metabolic reaction chain may be strongly inhibited by intermediates or by the essential end-product itself. Oxalacetic acid inhibits its own synthesis from malic acid by the *malic dehydrogenase*, it also strongly inhibits the enzyme, *succinic dehydrogenase*. Thus, oxalacetic acid, an intermediate in the Krebs cycle, is an inhibitor of two of the enzymes of the cycle. Oxalacetic acid is, during the active operation of the Krebs cycle, removed by reaction with acetyl-CoA to give citric acid. If, however, the supply of acetyl-CoA drops, then oxalacetate begins to accumulate and its further formation is checked by the inhibitory action of the oxalacetate on Krebs cycle enzymes. A similar situation can be seen in the synthesis of the amino acid isoleucine from aspartic acid, in which the amino acid, threonine, is an intermediate. One of the steps which precedes threonine formation is the phosphorylation of homoserine to homoserine phosphate by *homoserine kinase*. Threonine strongly inhibits this enzyme. In turn, threonine is deaminated to α-ketobutyric acid by *threonine deaminase* and this enzyme is strongly inhibited by the end-product of the biosynthesis, isoleucine. This type of control has been defined by Krebs (1957) as a *negative feed-back*, whereby a product of a reaction sequence inhibits one of the earlier reactions of the sequence, thereby slowing down the overall rate until the product is removed. Such *negative feed-back* mechanisms may operate in many of the reaction sequences of metabolism.

By contrast, the Calvin cycle in photosynthesis illustrates a *positive feed-back* mechanism. The concentration of Calvin cycle intermediates persisting in photosynthetic cells in the dark is very low. When photosynthesis commences the availability of ribulose diphosphate (RuDP) may limit the rate of CO_2 assimilation. However, the formation of phosphoglyceric acid (PGA) immediately raises the level of other intermediates in the cycle, including that of RuDP and this in turn speeds up the rate of CO_2 assimilation. Similarly, when cells are starved of respiratory substrate the ATP of the cells may be markedly depleted. The initial utilisation of

sugar may then be limited in rate by the rate of the phosphorylation reactions which involve ATP and lead to the formation of fructose diphosphate. However, once sugar again begins to enter the respiratory pathway oxidative phosphorylation proceeds and ATP is synthesised at a far greater rate than it is required for the initial phosphorylation reactions of respiration. The level of ATP therefore rises and the initial reactions proceed at a faster rate.

It is known that certain cell constituents are involved in the reversible activation and inactivation of enzymes. Thus, the enzyme *phosphoglucomutase* which catalyses the reaction:

$$\underset{\text{(G-1-P)}}{\text{glucose-1-phosphate}} \rightleftharpoons \underset{\text{(G-6-P)}}{\text{glucose-6-phosphate}} \qquad (52)$$

requires for activation a primer (catalytic) amount of glucose-1-6-diphosphate and the active catalyst is an enzyme phosphate in which a phosphate group is combined with a β-hydroxyl group of one of the constituent amino-acids, serine. The enzyme phosphate can transfer its phosphate to either G-1-P or G-6-P to give the diphosphate and when the diphosphate reacts with inactive enzyme a mixture of the monophosphates of glucose results. A catalytic amount of the glucose diphosphate is needed for the enzyme to interconvert massive amounts of the monophosphates. Some enzymes (particularly many of the enzymes involved in cellular respiration and proteolytic enzymes) are only active when certain sulphur-containing groups (thiol groups) in their molecules are in the reduced or sulphydryl form (—SH). One natural compound which seems to be of significance in maintaining the activity of such —SH enzymes is the tripeptide, glutathione (γ-glutamylcysteinylglycine) which reduces disulphide groups being itself simultaneously oxidised. It is interesting that the glutathione content of cells rises just before their division and is usually high in actively growing cells. The glutathione content of seeds, particularly of the embryo tissues, rises during germination and this may be an important factor involved in the activation of enzymes occurring during the early stages of germination (Chapter 1).

Whether there are natural inhibitors of enzymes distinct from the intermediates and end-products of reaction sequences, to which reference was made in discussing negative feed-back, is still a matter of controversy. Certain powerful inhibitors of growth and metabolism have been demonstrated to occur in dormant seeds, buds and storage organs and evidence has been obtained that the amounts of such inhibitors decrease during the natural breaking of dormancy. It has, however, proved difficult to identify these chemically and to obtain satisfactory evidence that they are responsible for the dormant state of the tissues.

Reference has previously been made (Chapter 5, p. 148) to the enzyme, *glutamine synthetase* which promotes the synthesis of the amide, glutamine. We find that some plant roots when grown in excised culture (see Chapter 8) contain a powerful inhibitor of this enzyme and that the content of this inhibitor is markedly increased when the roots are supplied with glutamine as their sole source of nitrogen. Here the inhibitor is quite distinct from the end-product of the reaction (glutamine) but its content in the cells rises in response to glutamine accumulation. This may indicate that where end-products or intermediates inhibit enzymes they may do so, not directly but by giving rise to or promoting the synthesis of inhibitor molecules.

It is a characteristic feature of metabolism that many reactions are coupled together through a common co-enzyme or energy carrier molecule. One obvious example of such coupling is the linkage between electron transport and ATP synthesis in the process of terminal oxidation (Chapter 4). It has been demonstrated that certain mitochondria cease oxygen uptake unless supplied with phosphate and ADP, and as soon as all the ADP has been converted to ATP the electron transport system stops. This explains how energy-consuming processes like salt accumulation, protein synthesis and cell wall growth by consuming ATP enhance respiration. Respiration rate can also be enhanced by dinitrophenol (DNP) which acts by uncoupling electron transport from phosphorylation. Electron transport proceeds at an enhanced rate and the energy

release is lost as heat. Students of animal physiology have long known that the first effect of DNP on animals was to raise their temperature to an abnormal level. What is of particular interest is that while the coupling between electron transport and phosphorylation is very tight in recently isolated mitochondria, this coupling loosens as the mitochondria "age", and that mitochondria from senescent cells also show a lower production of ATP per electron transported than do those from young actively metabolising cells. Further, an "uncoupler" can be isolated from "aged" mitochondria. It is therefore possible that breakdown in coupling can occur in cells during differentiation or senescence as a result either of the formation of uncoupling substances or of failure to maintain certain spatial relationships at the catalytic surfaces within organelles such as mitochondria.

CELL STRUCTURE AND THE CONTROL OF METABOLISM

Any consideration of coupling and uncoupling mechanisms naturally leads on to a consideration of the importance of cell structure and the control of metabolism. Studies of the activities and enzyme contents of nuclei, plastids, mitochondria and microsomes show clearly that these structures, separated from one another and from the hyaloplasm and endoplasmic reticulum by cellular membranes, are the centres of particular aspects of metabolism. This means that the flow between these centres and the concentrations within them of metabolites, co-enzymes and energy carriers are regulated by diffusion barriers. It is also clear from our knowledge of the fine structure and chemistry of these cytoplasmic "particles" that within them there is a further organisation and segregation of enzymes. Thus, in the chloroplasts, the photochemical reactions of the grana lamellae are separated from "dark" reactions occurring in the stroma. In the mitochondria the enzymes promoting oxidative phosphorylation and occuring in the cristae are separate from other mitochondrial enzymes. Further, from the

metabolic activities of disrupted chloroplasts and mitochondria, we have evidence that within these structures reaction chains are not only chains in the sense that one reaction produces the substrates for the next reaction of the chain but that the enzymes involved are spatially arranged at catalytic surfaces to form a reaction chain or production line in space. In consequence overall concentrations of metabolites in cells give no idea of effective concentrations at catalytic surfaces, surfaces where intermediates may undergo successive chemical changes without ever entering into aqueous solution.

Pasteur in his studies on yeast noted that when this organism is growing under anaerobic conditions it multiplies slowly, evolves a large amount of carbon dioxide and consumes a large quantity of sugar which it ferments to enthanol. Pasteur noted, however, that when oxygen is supplied less carbon dioxide is evolved and less sugar consumed although cell growth and division now proceed more rapidly. It is this action of oxygen in suppressing the fermentation of sugar and initiating its aerobic respiration which is usually referred to as the *Pasteur effect.* A satisfactory explanation of the mechanism of the Pasteur effect has only recently been advanced by Professor F. Lynen and his associates at the Universtiy of Munich and their work illustrates the importance of enzyme segregation in metabolic controls of this type. The oxidation of phosphoglyceraldehyde in both fermentation and respiration is controlled by the triosephosphate dehydrogenase (Chapter 4, pp. 88, 89) and this is a "pacemaker" reaction in sugar degradation. The reaction involves combination with inorganic phosphate and its subsequent transfer to ADP to form ATP. This reaction proceeds in the hyaloplasm (in the endoplasmic reticulum?), depends upon the availability of phosphate ions there and releases ATP into this phase of the cell. The generation of ATP in the hyaloplasm promotes glucose uptake probably through the hexokinase reaction (p. 103). When oxygen is supplied oxidative phosphorylation is initiated in the mitochondria, the amount of ATP synthesis is markedly enhanced, ADP and phosphate ions from the hyaloplasm move

into the mitochondria and there the ADP is almost entirely converted to ATP. As a consequence of this oxidative phosphorylation, the activity of phosphoglyceraldehyde oxidation is reduced by lack of phosphate ions and ADP in the hyaloplasm and glucose uptake is limited by the rate of release of ATP from the mitochondria. The suppression of fermentation is a consequence of the two effects.

The control of metabolism through such phenomena as the promotion and inhibition of enzyme synthesis and activity and through segregation of enzymes within the cell have been termed by Krebs "*primitive*" control mechanisms. Such mechanisms are found in all organisms from unicells to the most highly evolved plants and animals. The term "primitive" is used to distinguish these mechanisms from those mediated in both plants and animals by hormones and in higher animals additionally by the nervous system. The discussion of such mechanisms naturally leads on to the subjects of the next chapter, cell growth and differentiation in plants, processes in which hormones (plant hormones or phytohormones) are clearly involved.

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8

GROWTH AND DIFFERENTIATION

"I am a firm believer that without speculation there is no good or original observation." Charles Darwin in a letter to Alfred Russell Wallace.

"Morphogenesis, the science that treats of the cause and origin of form, is a field where many different disciplines meet. Morphology is obviously concerned in it, and physiology, embryology and genetics. Biochemistry and biophysics have an important place here as well. There is no field in biology which touches so many problems. This, I believe, is because we are dealing, in morphogenesis, with the basic phenomenon in biology—how protoplasm builds organized living systems." Edmund W. Sinnott, 1953, in *Growth and Differentiation in Plants.* Iowa State College Press, Iowa.

INTRODUCTION

It would be going beyond the scope of the present text to develop any general consideration of plant growth and development. Further, many of the phenomena of growth and development are not, in the present state of knowledge, interpretable in metabolic terms. One of the immediate and major tasks facing plant physiologists is the extension of our knowledge of the regulation of metabolism and, particularly, of energy-flow in the cell and in the multicellular organism so as to be able to interpret in these terms the phenomena of cell growth and differentiation and organ growth and development. Within the limits of this chapter little more can be attempted than to indicate in a very general way the kind of problems which immediately arise when one begins to probe in this direction.

MERISTEMS AND CELL DIVISION

The growth of plant organs is the outcome of cell division, enlargement of the new cells and their differentiation into the different kinds of tissue cells. These processes of cell division and growth are localised in *meristems*. The extreme apices of roots are occupied by primary meristems and in the older parts of the root system secondary meristems (cambia) give rise to additional vascular tissues and to protective layers of cork cells. Growth and morphology are the outcome of the activities of these meristems, and further the activity of each meristem influences the activity of other meristems (particularly those near to it) giving rise to *growth correlations*. For instance, while the main root apical meristem is active it retards the activity of the more recently initiated lateral root tip mersitems, a phenomenon usually referred to as *apical dominance*.

By preparing longitudinal sections of the root it can be seen that its extreme tip is occupied by a cap of cells which protect the permanently dividing tissue or *promeristem* from which the root cap cells and all the new cells which give rise to the primary tissues of the growing root have their ultimate origin. Some of the tissue cells arise directly from new cells initiated in the promeristem, others after these cells have undergone a limited number of further divisions. In some species it seems that the root promeristem is a group of dividing cells lying on the surface of a hemisphere. The cells on the proximal surface of the hemisphere (that surface furthest from the extreme tip) give rise to the cells from which are formed the stele, the cortex and the piliferous layer and sometimes also the outer cells of the root cap. The distal (apical) flat face of the hemisphere is covered by a plate of promeristem cells which give rise to the central cells of the root cap. The hemisphere itself is composed of a group of cells (500–1000 cells) which rarely divide and these together are usually therefore referred to as the *quiescent centre* of the meristem (Fig. 57). During root development there may be quite large changes in the number of

dividing cells and in the number of quiescent cells in the promeristem: thus the quiescent centre is absent or represented by a very few cells in young lateral roots and embryonic roots.

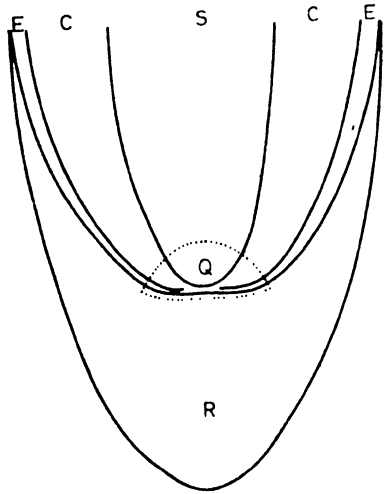


FIG. 57. Diagrammatic median section of the root apex of *Zea mays* (maize). The sites of the initial cells of the promeristem are indicated by dots. E = piliferous layer, C = cortex, S = stele, Q = quiescent centre R = root cap. (Drawn by Dr. L. Clowes from H. E. Street as Fig. 52).

Despite changes in promeristem size and in the cell division activity of its constituent cells, the whole apical meristem of the root is always an organised structure with a pattern in the arrangement of the cells and with cell division and cell enlargement proceeding in such a way that this pattern is not destroyed. The maintenance of pattern is the outcome of a control of the planes along which division walls are laid down in cell division and of a balance between rates of cell division on the one hand and the rates and directions of cell expansion on the other. The orientation of division walls seems to be determined by the shape of the spindle

arising during the nuclear division (mitosis) which precedes cell division (cytokinesis). Meristem size is either stable or changing with time according to the balance established between division rate and the rate at which the daughter cells mature to the point where they differentiate directly into the root tissue cells.

The discovery of *mitosis* around 1880, mainly as the result of the elegant studies of the botanist, Eduard Strasburger and the zoologist, Walther Flemming, was a landmark in the history of biology and combined with the discovery some fifteen years later of meiosis (reduction division) laid the basis from which modern knowledge of cytogenetics was to develop. The recognition that multicellular plants have localised growth centres or meristems where cells continue to divide and that these are distinct from regions where division ceases and cell behaviour is to be described in terms of expansion and differentiation leads us on to other very important aspects of nuclear behaviour and function. It poses such problems as what changes are involved in the preparation of nuclei to undergo mitosis and what naturally "triggers" this process to commence or inhibits it from occurring. To answer such questions it is necessary to consider what we know of the metabolism of the nucleus both during interphase and during mitosis.

The reduplication of the chromosomes, including reduplication of their genetic material (their DNA) takes place during *interphase* (in the "resting" nucleus) so that when the nucleus enters upon prophase each chromosome normally contains twice the material it contained at telophase. This also implies that synthesis of new chromosome material does not take place during mitosis. The chromosomes of the interphase nucleus are in the "extended" condition (in contrast to the highly spiralised "condensed" form of the chromosomes during mitosis) and it is while in this state that both their DNA and other constituents including the characteristic basis protein of the chromosomes (the histone) are doubled (as far as we can judge from experimental studies *exactly doubled*). It is at this stage also that the chromosomes exert their controlling influence on cell metabolism. The description "resting nucleus"

for the interphase nucleus therefore only has meaning in that it indicates that the nucleus is not involved in division.

The mechanism of the *replication of the DNA molecules* of the chromosomes is at present entirely a matter for speculation. It has been suggested that this could involve an unwinding of the double helix (Fig. 58) and the synthesis from the appropriate

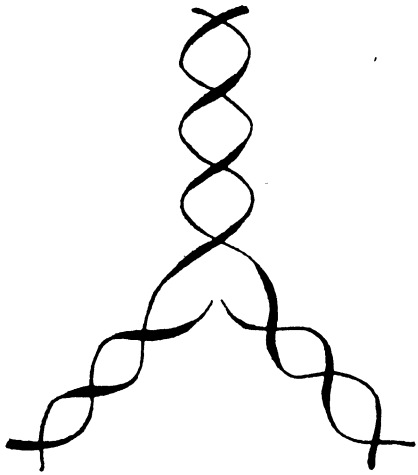


FIG. 58. Diagram of the replication of the DNA molecule.

precursors and under the influence of the appropriate enzyme(s) of a complementary spiral to each exposed single spiral to give finally two complete double helices (Fig. 48). This duplication of chromosomal material, as indicated by the doubling of the DNA content of the nucleus, must take place if division is to occur. A failure of this aspect of nuclear metabolism can be shown to occur in some cells which fail to divide. However, doubling of the chromosomal material when completed does not, of itself, cause the nucleus to enter the prophase of mitosis. Often DNA replication is completed long before prophase and there are many instances known where DNA replication goes on to give 4× or even a higher multiple of the telophase content without prophase

being initiated. For instance, by appropriate illumination and nutrition, cells of the alga, *Chlorella*, can be caused to grow large and their nuclei accumulate abnormally large amounts of DNA. Then on transfer to darkness these cells divide rapidly to give 16 smaller cells with the normal "telophase" DNA content. It is now recognised that many of the differentiated tissue cells of higher plants are polyploid although the cells in their meristems are uniformly diploid. In such polyploid cells DNA replication has continued without mitosis intervening to restore the normal DNA content. The substance, *kinetin* (6–furfurylaminopurine; see Fig. 63) discovered as a decomposition product of nucleic acid by Professor F. Skoog of the University of Wisconsin and subsequently shown to stimulate the division of certain plant cells, can, in certain cases, induce divisions in such polyploid plant tissue cells.

There is evidence that the centres (poles) from which the spindle develops are cytoplasmic structures and that they divide in the cytoplasm either before mitosis is complete or very early in interphase. Further, it is during interphase that the proteins which will go to form the spindle and its fibres are synthesised. This must mean that a very considerable part of protein synthesis in meristematic cells must be concerned with the *synthesis of the spindle proteins.*

One very interesting aspect of the metabolism of dividing cells is their very low rate of respiration during mitosis and the insensitivity of mitosis once initiated to inhibition by inhibitors of respiration. This means that the massive intracellular movements involved in mitosis can and normally do proceed without the cell simultaneously generating energy by respiration. This has led to the view that an "*energy reservoir*" is filled during interphase, as a result of respiration in the cytoplasm, and that this when filled is capable of powering the process of mitosis. The chemical nature of this energy reservoir is still uncertain. It does not seem to be the building up of a sufficient store of ATP although nuclei contain an ATP-ase (an enzyme which can split the terminal phosphate bond of ATP) and, therefore, ATP could be the immediate donor

of energy for mitosis. Two hypotheses have been put forward as to the nature of the reservoir, one that it is some unknown high-energy compound (possibly a compound with high energy S–bonds such as occur in co-enzyme A) whose breakdown could be linked to the synthesis of ATP from ADP; the other that the energy required is locked up in the protein molecules of the chromosomes and the spindle and which, therefore, have the ability to combine and contract when the signal is given (when the "trigger" is pulled to initiate mitosis).

Clearly, the changes during interphase which prepare the cell for mitosis involve the metabolic activities of the cytoplasm. If the interphase nucleus is the control tower for the metabolism of the cytoplasm, the latter is in turn the source of energy and of precursor molecules for the essential metabolic events which must proceed both within the nucleus and beyond it prior to division. The further elucidation of this two-way inter-relationship between the nucleus and the cytoplasm is a major objective of current research in cell physiology.

During the prophase the two most prominent events are the coiling and associated condensation of the chromosomes so that they become cytologically defined and the disappearance from the *nucleolus* (or nucleoli) of the RNA which is a prominent constituent of this structure during interphase. Often the nucleoli completely disappear as they become separated from the "nucleolar organiser" regions of the chromosomes during prophase condensation of the chromosomes. Then towards the end of prophase the nuclear envelope breaks down.

Certain changes which occur in the cytoplasm at this time may be related to the loss of nucleoli (particularly if these are important in the flow of RNA from nucleus to cytoplasm) and to the breakdown of the nuclear envelope. The nuclear envelope is, perhaps, the most constant feature of the membrane system which we call the endoplasmic reticulum (ER) (see Chapter 2) and associated with the breakdown of the nuclear envelope there is usually some disintegration of the ER which may become

reduced to a discontinuous system of vesicles confined to the periphery of the cytoplasm. These changes can be correlated with independent evidence that the synthetic activities of the cytoplasm are at their lowest ebb during mitosis. It has also been observed that there is a marked decrease in cytoplasmic viscosity, particularly at the time of the differentiation of the spindle and the build-up of —SH compounds in the cytoplasm which occurs during interphase is reversed during mitosis and again particularly during organisation of the spindle.

The *mitochondria* do not become enmeshed in the spindle but usually either aggregate around the poles or along the surface of the spindle and are fairly accurately divided between the daughter cells. It seems that in many plant cells there is a significant *decrease* in the number of mitochondria prior to cytokinesis (division of the cell into two daughter cells).

The disorganisation of cytoplasmic structure and function referred to above in describing mitosis is probably even more marked at the meiosis (reduction division) which leads to the origin of haploid pollen grains and ova. This would ensure the primacy of the nucleus in heredity. In those cases where there is evidence of maternal inheritance (cytoplasmic inheritance) this may reflect the incompleteness of cytoplasmic simplification so that some replicating cytoplasmic particles or *plasmagenes* (the identity of these with recognised cytoplasmic structures is uncertain) are carried over and function in the fertilised egg with the messages received from the diploid nuclei of the female parent.

At the conclusion of mitosis (*at telophase*) the nuclear membrane is reconstituted from the persistent vesicles of the ER and this seems to be catalysed in some way by the extension of the chromosomes during this period. If any chromosomes become detached from the main group they also become associated with ER vesicles and can form micronuclei. Again as the chromosomes extend nucleoli become again apparent at the "nucleolar organizer" sites of the chromosomes apparently by the aggregation there of newly synthesised nucleolar substance.

The cell as it exists at telophase and before cytokinesis has double the functional potentialities of the parent cell and is about to reconstruct in two separate cells a new cytoplasmic organisation. There has been a doubling of physiological potential (*a physiological reproduction*). In this connection, Professor D. Mazia of the University of California has pointed to the probable importance of the duplication of the nucleoli. It is the nucleolus which during mitosis has gone through a cycle from "*oneness*" to "*nothingness*" to "*twoness*" and it is with the reappearance of the nucleoli, and in time very closely correlated with this, that there is a reactivation of cellular metabolism and the initiation of a reconstruction of the ER. Probably the nucleolus is an active "middleman" between the genes and their expression in the cytoplasm.

The beginning of *cytokinesis* is nicely adjusted to telophase and the plane of division is normally exactly along the equator of the spindle. If the spindle is not centrally located, unequal daughter cells are produced as has been clearly shown in studies of the growth of *Spirogyra* filaments. The first evidence that cytokinesis is to occur is the appearance of a zone (the phragmoplast) across the equator of the spindle. This zone is rich in RNA and contains ER structures which have invaded the spindle and clustered in its equator. It is thought that it is from these ER structures that the plasmalemmae of the daughter cells are formed and that it is between these membranes that here is secreted the *cell plate* which becomes the middle lamella. At this stage the cytoplasm is clearly synthesising pectins and very quickly cellulose begins to be deposited by the daughter cells on either side of the cell plate to give two daughter cells, each with its own cellulose wall.

It not only accords with observation but would appear to be essential to the very persistence of the cells that normally cell growth must intervene between successive mitoses. Further, the evidence strongly supports the view that a nucleus is only capable of controlling a certain mass of cytoplasm and that when by growth this mass is attained then physiological reproduction becomes essential. This may also be expressed by saying that there is a

critical *nuclear/cytoplasmic ratio* which is not exceeded in cells which continue to divide. It is in line with this concept that the dividing cells of polyploids are often larger than those of the normal diploids. Attention may also, in this connection, be directed to the fact that cell expansion is a finite process both in extent and time even in cells which enlarge beyond the point where they can divide; also that tissue elements (such as vessel units, tracheids, fibres) which are developed by very marked and predominantly unidirectional cell expansion are dead units when fully differentiated (their growth leads to a breakdown of cellular organisation).

There is considerable evidence that the normal development of cells is for them to undergo *limited* growth and then divide and that cells which do not continue this life history are cells in which mitosis has become *blocked* in some way. This hypothesis is supported by the ability of many such non-dividing cells to resume mitosis if appropriately stimulated. Such stimulation is illustrated by the response of certain tissues to mechanical wounding or insect attack (some of the uninjured cells dividing to heal the wound or, in the case of insect attack, to form a gall) and by the plant tumours which result from the invasion of plant tissues by the Crown Gall bacterium. Explanted tissues can often also be induced to divide by transference to an appropriate nutrient medium supplemented with essential *growth factors*. One source of such growth factors which has proved particularly valuable in the initiation and maintenance of actively growing tissue cultures from such explants is the liquid endosperm of the coconut (*coconut milk*) whose normal metabolic function is to support the growth of the immature coconut embryo. A classical example of the chemical stimulation of cell division in mature expanded cells is the induction of an actively growing tissue culture from an explant of tobacco stem pith cells by the use of a culture medium supplying not only sugar and essential mineral ions but supplemented with appropriate concentrations of both kinetin (p. 199) and the plant growth hormone, β-indolylacetic acid (IAA) (p. 17). Further, in the tissue mass that develops there can develop organised meri-

stems giving rise to roots and shoots (Fig. 59). Examples such as this show that differentiation is a reversible process in that starting from mature tissue cells we can, by appropriate stimulation, obtain again cells whose structure and behaviour are identical, with the meristematic cells from which they were originally differentiated. Our consideration of the metabolism of mitosis has led us on to the problem of the physiological inter-relationship between the nucleus and the cytoplasm which is determinative not only in mitosis but in cell growth and differentiation.

CELL GROWTH AND DIFFERENTIATION

Behind the promeristem of the root tip is the region where cell enlargement and differentiation are the dominant processes. For instance, in the seedling maize root very few cell divisions are observed beyond 1·5 mm from the root cap and it is in the 2nd and 3rd mm from the root cap that the most active linear extension by cell expansion takes place. By 5 mm from the root cap most of the roots cells are fully expanded and already their differentiation into the root tissues is well advanced. As earlier described the meristematic cells are isodiametric, small, have a high nucleus to cell volume ratio and are filled with cytoplasm. During cell expansion, not only is the cell volume increased dramatically but the cells change in shape and develop vacuoles. The mature cortical cells of the root may have 20 or more times the volume of the promeristem cells. The process of cell enlargement is associated with the uptake of water leading first to the appearance of a number of small vacuoles and ultimately of a single central vacuole. This uptake of water which is the consequence rather than the cause of the cell expansion, is accompanied by large increases in cell dry weight, protein content and cell wall material. The expansion process involves real growth and some degree of differentiation (change towards specialised function). The time course of this growth

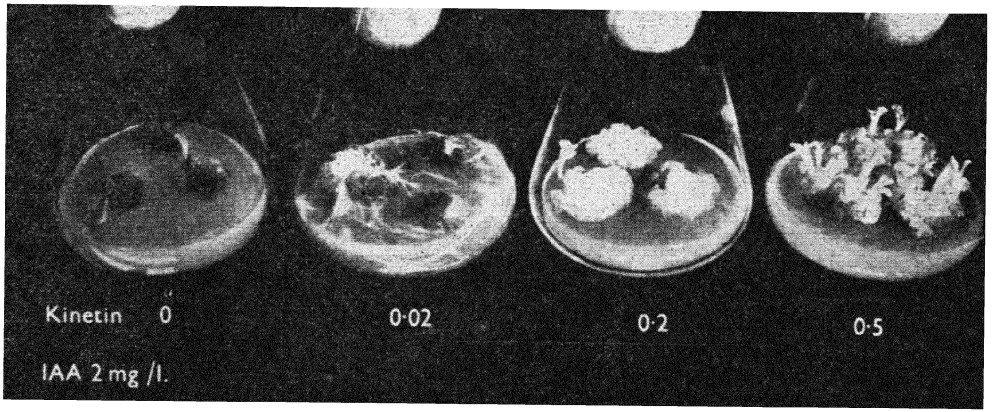


FIG. 59. The effect of kinetin (0–0·5 mg/l.) in the control of growth and organ initiation in tobacco callus cultured on a nutrient agar containing 2·0 mg/l. IAA. Extensive root formation at low kinetin concentration (0·02 mg/l.). Active callus growth at 0·2 mg/l., shoot formation at 0·5 mg/l. (From F. Skoog and C. O. Miller. *The Biological Action of Growth Substances* p. 118 *et seq.* Symposia of the Society for Experimental Biology. XI. Cambridge Press, 1957).

follows a characteristic pattern (Fig. 60); at first growth is slow, then it accelerates to reach and maintain a high velocity until again as the growth process reaches its completion, the rate quickly

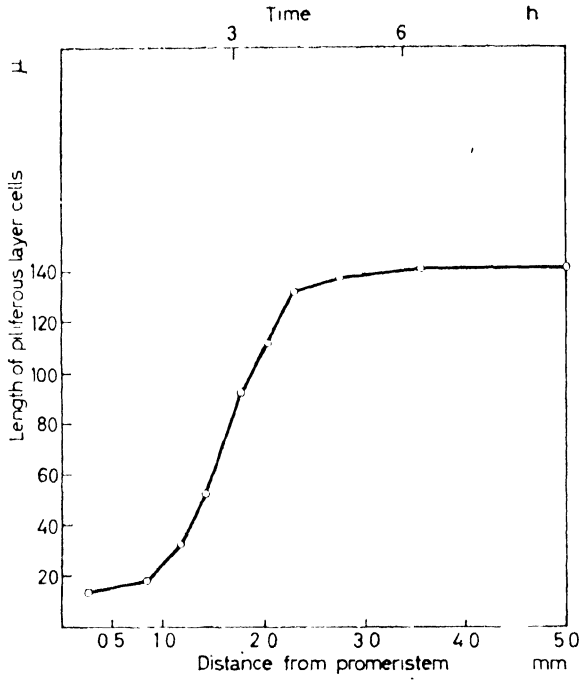


FIG. 60. Curve showing the course of elongation of the piliferous layer cells of excised tomato roots growing in a culture medium containing 1·5 per cent sucrose. (From H. E. Street as Fig. 52).

declines to zero. Studies on root hair development emphasise rather nicely the principle already enunciated that cells have a limited expansion potential. The longest cells of the piliferous layer are either without hairs or produce the shortest hairs, while the shortest piliferous layer cells produce the longest hairs. Each piliferous layer cell appears to have a certain capacity for growth which may be expressed in either a longitudinal or horizontal direction.

The cells as they expand and differentiate become part of the continuing pattern of arrangement of the primary root tissues. It could be that the primary tissues impose their pattern upon the differentiating cells by some apically directed influence. When, however, the embryo root is initiated or when root meristems arise in tissue culture masses it is clear that the root meristem precedes the existence of mature root tissues. Further, it has been shown that if root tips are cut off, re-oriented and then replaced on the root stump, the new tissues as they appear are out of line with those of the stump. It is in the apical millimetre or so of the root, in the region of active cell division that the tissue pattern is determined. The destiny of cells in the meristem is determined by their micro-environments in that region.

The cells as they differentiate into the functional tissue cells, diverge from the cells of the meristem and from one another in size, shape, structure and physiology. Cells initially "identical" in genetics and physiology embark upon separate pathways of differentiation. Professor R. Brown and his associates in the University of Edinburgh, using thin serial sections of living pea and bean roots, have revealed a changing pattern of enzyme activity during the process of cell expansion. Not only are there significant changes in the relative activities of certain enzymes per cell but also per unit of cellular protein. Further, it has been shown that although during the process of expansion the synthesis of new protein and of cytoplasmic RNA run parallel, the composition of the RNA changes. The proportion of purine to pyrimidine bases in the nucleic acid rises as the cells mature. There are, also, changes in the respiratory activity both per cell and per unit of protein and changes in the sensitivity of the respiration to inhibitors.

These observations on the changing enzyme activities of cells during differentiation prompted the distinguished American bacteriologist, Sol Spiegelmann to characterise differentiation as the contolled production of unique enzymatic patterns. This implies that studies on the induction and suppression of enzyme synthesis (see Chapter 7) could hold the key to understanding

differentiation. They cannot, however, at this stage in our knowledge go very far to explain differentiation primarily because we do not know whether such systems are involved in the control of the synthesis of all enzymes, do not understand the *mechanisms* of induction and repression and have no experimental evidence that these phenomena are the cause of the "switch overs" which occur during differentiation from one pattern of enzymes to another equally functional and perhaps more stabilised pattern associated with a different metabolic status. This highlights a major gap in our understanding of differentiation. Differences in the "environment" at different points in a meristem such as differences in carbon dioxide and oxygen tensions might well be expected to alter the relative concentrations of metabolites and this, in turn, could modify enzyme synthesis and the activities of enzyme molecules. What we cannot do is to describe how such changes can lead to differentiation in the right sense and to the right extent and over the requisite cell masses to give the functional tissue pattern of a plant organ. Further, and still accepting the reversibility of differentiation (particularly through cell division with its "disorganising" influence on the cytoplasm) we have also to explain the quite considerable stability of the different metabolic patterns characteristic of the different mature tissues of which a multicellular plant is built up.

Initially all the cells of a multicellular organism may be presumed to be genetically identical, being derived from the mitosis of the fusion nucleus of the ovum. Tissue and organ differentiation also proceed with a uniformity and precision which seems to rule out the involvement of genetic change (mutation) in differentiation. The ability of mature tissue explants to regenerate root and shoot meristems of normal size and function also argues for the genetic stability of the body cells. It is, however, known that two kinds of nuclear change can occur in cell differentiation. The first of these is the occurrence of *polyploidy*. While meristems and potentially meristematic tissues (for instance, the pericycle of the root from which lateral root meristems arise) remain uniformly diploid,

nevertheless, polyploidy is a widespread phenomenon amongst the cells of specialised tissues (root cortical and endodermal cells are frequently tetraploid, vessel units of the metaxylem often tetra- or octaploid). When explants, involving differentiated cells, are used to initiate tissue cultures and these, in turn, to initiate organised meristems then it is a general finding that despite the high degree of polyploidy in the explant, nevertheless, the meristems are built up of diploid cells. These observations suggest that the polyploidy which develops during differentiation is either a barrier to the division of such cells or modifies their behaviour so that they do not participate in meristem initiation. It may also be suggested that polyploidy has the biological significance, in large tissue cells, of extending the range of control of the nucleus. There is, however, *no* evidence that this tissue polyploidy is a cause rather than a consequence of differentiation or that it has any role in stabilising particular patterns of metabolic activity.

The second kind of nuclear change is usually designated *nuclear* (or *chromosomal*) *differentiation* and our knowledge of this comes mainly from three lines of work with animal cells. First we have nuclear transplantation experiments such as those brilliantly executed by Drs. T. J. King and R. Briggs at the Lankenau Hospital, Philadelphia. These workers transplanted nuclei from frog embryos at various stages of differentiation into enucleated frog eggs and found that nuclei from the early stages of development permitted normal animals to develop. However, nuclei transplanted from later in embryo development resulted in the development of abnormal embryos and these abnormalities were intensified as nuclei from older and older embryos were transplanted. Further, nuclei taken from the abnormal embryos continued to cause abnormal development when transferred to enucleated eggs. The conclusion was drawn from these findings that nuclei from differentiated embryo cells had altered capacities as compared with egg nuclei and that these differences were persistent despite repeated nuclear division, indicating strongly that the chromosomes themselves were "differentiated". Secondly, there is evidence of

chromosomal changes during differentiation from detailed microscopic studies of the giant chromosomes of the larval tissues of certain insects. The banding pattern of these chromosomes is modified during development by the appearance of enlarged areas called *Balbiani rings* and tissues and their stage of development can be correlated with the occurrence and distribution of these Balbiani rings. The Balbiani rings are often associated with fine loops which extend out laterally from the chromosomes and these loops can be shown to synthesise droplets of RNA which are probably released into the cytoplasm. The extent of these loops can change and they can form and regress, suggesting that the formation of the loops is related to the "activity" of the chromosome sites where they occur. Thirdly, we have the evidence that the tissues within certain animals have characteristic and different levels of RNA in their nuclei, and that the nuclear RNA content changes as the tissues differentiate.

These observations strongly suggest that the total gene complement may rarely, if ever, be fully and simultaneously committed in the control of metabolism; that the metabolism of the cell is controlled by those particular genes which are "activated" in its nucleus. One can then speculate that the interphase chromosomes may be at certain points along their length fully extended and actively involved in the transmission of messages to the cytoplasm and that other regions which are condensed or coiled may be inactive. Nuclear differentiation could then be visualised to involve relatively stable changes in the configuration and chemical composition of the chromosome threads corresponding to different gene activation patterns. Further, the agents determining these patterns could be molecules (RNA or protein molecules or fragments of such molecules) of cytoplasmic origin which become associated with the chromosomal DNA at certain but not at all loci along the chromosomes.

At this point we can try to see how far the discussion of the regulation of metabolism developed in Chapter 7 and the description given above of some of the changes which occur during cell

differentiation enable us to think about possible ways by which one metabolic pattern or "steady state" could be superseded by another and different pattern during differentiation. When one does this it is quickly realised that in the present primitive state of our knowledge in this field one can only construct hypothetical *model systems.* Such models must, of course, only invoke mechanisms of a kind that we have reason to believe operate in living cells and, further, they must be at least theoretically susceptible to experimental test (even if their critical experimental testing is difficult or impossible with existing experimental techniques). Models of this kind have been formulated by Jacques Monod and François Jacob of the Pasteur Institute, Paris and a very brief description of two of the simpler models they have put forward will illustrate this way of thinking about a physiological problem.

We have considered (Chapter 7) the situation where a product of a reaction chain (say the final product) inhibits the activity of an earlier enzyme in the chain (say the first enzyme in the sequence). The first model put forward by Monod and Jacob postulates *interaction between two metabolic pathways* by this mechanism. Considering two independent metabolic pathways giving rise to metabolites a, b, c, d and α, β, γ, δ they postulate that the enzymes (E_1 and $\acute{E}_1$) catalysing the first reaction in each pathway could be inhibited by the final product (d and δ) of the *other* pathway (Fig. 61). This is a "cross feed-back" where one of the two pathways provided it once has a head-start or a temporary metabolic advantage, will permanently inhibit the other. *Switching* of one pathway to the other could be accomplished by a variety of methods; for instance, by inhibiting temporarily any one of the enzymes of the active pathway. Systems of this kind, however, do not involve a change in the cells capacity to synthesise enzymes and, therefore, it would seem, cannot be part of the primary mechanism of differentiation.

The second model (Fig. 62) is based upon the concept of the role of "structural" and "regulator" genes in the induction and repression of enzyme synthesis (Chapter 7, p. 185) and involves two

reaction sequences interconnected in that each produces an inducer *of the other.* The two systems, not necessarily otherwise closely related in metabolism (they could be involved in widely different metabolic systems), are mutually dependent. One system automatically induces the other by inactivating the appropriate repressor substance. If a metabolic pattern was the outcome of a number

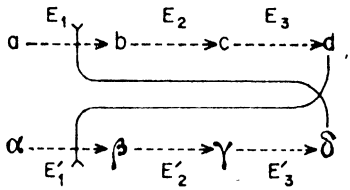


FIG. 61. Model I. The reactions along the two pathways a → b → c → d and α → β → γ → δ are catalysed by the enzymes E_1, E_2, E_3 and E'_1, E'_2, E'_3. Enzyme E_1 is inhibited by δ, the product of the other pathway. Conversely, enzyme E'_1 is inhibited by metabolite d, product of the first pathway (from J. Monod and F. Jacob in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, Vol. 26. Cold Spring Harbor, New York, 1961).

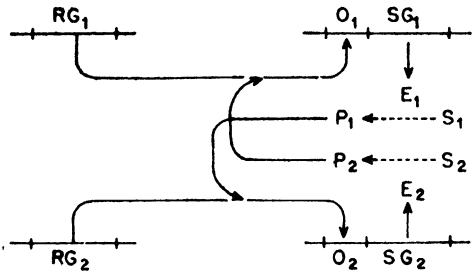


FIG. 62. Model II. Synthesis of the enzyme E_1 depends upon activity of the structural gene SG_1. The activity of this gene is suppressed by regulator gene RG_1 via a repressor molecule. The synthesis of enzyme E_2 is similarly controlled by the genes, SG_2 and RG_2. P_1 product of action of E_1 on substrate (S_1) suppresses the functioning of RG_2 and hence its presence induces enzyme E_2. P_2 can similarly induce formation of the enzyme E_1 (from J. Monod and F. Jacobs as Fig. 61).

of reaction sequences interconnected in this way, a "point" activation of one system would lead to the growth of the mutually dependent systems. Similarly, if we consider the opposite case where a reaction product of one reaction sequence acts as an activator of the repressor substance then the systems become mutually inhibitory. The "live" system inhibits the second system and the only way the second system can come into operation is by eliminating temporarily a substrate of the "live" system, and once activated it represses the first system.

HORMONES AND THE REGULATION OF GROWTH AND DIFFERENTIATION

We have so far concerned ourselves with considering cellular mechanisms which may operate in the control of cell growth and differentiation. However, in multicellular plants many adjacent cells often follow the same course of growth and differentiation to give rise to tissues and, further, these tissues differentiate as part of the development of organs like the shoot and root. It is in this relationship of the cell to its tissue environment and the tissue to its organ environment that we must look for the factors which initiate and determine the growth and direction of differentiation of the individual cells. A cell at a certain site in an organ is not only in a particular environment in regard to light, temperature, water, radiation and such factors but it is part of a protoplasmic continuum (a symplast) and hence imports and exports nutrients and metabolic products from and into this system.

Within a photosynthetic unicellular alga function mechanisms which control the integrated activities of its nucleus and cytoplasm and within its cytoplasm of the chloroplasts, mitochondria, microsomes, ER membranes and hyaloplasm. Within the multicellular organism *extra-cellular controls* determine the distribution and activity of centres of organised cell division (meristems), of organised cell expansion and of the specialised tissues concerned with photosynthesis, the bio-synthesis of particular metabolic products,

solute and water absorption, translocation and so on. Thus, superimposed upon the nutritive inter-relationships of the specialised structures *within* the cells we now have nutritive inter-relationships *between* cells fulfilling different and partial functions in the economy of the whole organism.

The multicellular organism presents us with a different kind of complexity to that presented by the algal unicell. The higher plant cell has a genetic equipment which differs in a very interesting way from that of the unicell. The fertilised ovum has the ability to develop into the whole multicellular autotrophic plant (it has, we say, *totipotency*). Many of the body cells of the developed plant retain this totipotency as has been demonstrated by tissue culture techniques. With appropriate nurture they also can generate the whole organism. However, neither the ovum nor the body cells can give an autotrophic unicell. The meristematic cell of a flowering plant is a highly heterotrophic cell only capable of maintaining its meristematic activity in a very special environment. Removed from this special environment it dies, moving out of it within the framework of the organism it differentiates in an exclusive way. Totipotency is expressed in the potentiality of the cell to differentiate in many different ways, but any one pathway of differentiation is to the exclusion of an alternative pathway. The emergence of the multicellular organism involves the determination and balanced development of cell lines along these many and complementary pathways of differentiation.

It is very easy in a general way to demonstrate nutritive inter-relationships between the separate organs and tissues of a plant. This was done long ago by ringing experiments and by defoliation which interrupted the flow of nutrient ions and metabolites. More recently such inter-relationships have been studied by such techniques as the growth in culture of isolated root tips and leaf primordia. For instance, the growth in culture of excised root tips has demonstrated the dependence of the root upon the shoot, not only for a supply of carbohydrate but for certain vitamins, particularly for vitamins of the B group which are known to

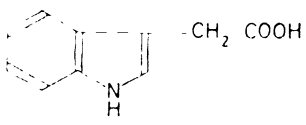
function as co-enzymes and prosthetic groups of essential enzymes. It is clear, however, that many root systems must have additional and, as yet, unknown nutrient requirements for a supply of essential nutrient ions, sugar and B vitamins only permits the roots of a very limited number of species to grow in culture. There are many aspects of the nutritive inter-relationships between plant organs which have yet to be elucidated. This is even more so when we come to the tissue level. We are very far from understanding qualitatively, let alone quantitatively, the extent to which the different tissue cells are dependent upon metabolites received from other parts of the organism or of understanding the totality of the contributions they separately make to the organism as a whole. Clearly, this interdependence of cells for metabolites means that tissues must interact during differentiation and one aspect of this interaction is that each line of differentiation has its own permissive nutrient and metabolite requirements. The tasks before plant physiologists in this field are not only to be able to describe in *quantitative* terms the input and output of metabolites from all the different tissue cells, not only to understand what determines the rates of movements of these substances between cells and tissues within the organsim but, by culturing isolated cells, to find out how far these substances permit of *or* determine (programme) differentiation itself. It is the great interest of these questions which motivates the intensive work now proceeding in a number of botanical laboratories to obtain growing cultures of separated higher plant cells (free-cell cultures) and to induce single isolated plant cells to divide, grow and differentiate.

The word, metabolite, is as all-embracing as its mother word, metabolism. Some metabolites can clearly be described as essential in that if the cell has lost the capacity for their biosynthesis its continuing life depends upon a supply from other cells. Such metabolites, since they enter into the metabolism of all cells might for that very reason not be the direct determinants in differentiation, although differentiation may profoundly affect the cells' ability to carry out their synthesis. Again, because there is strong evidence

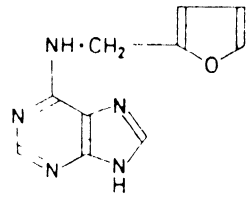
that *patterns of differentiation are determined in meristems* rather than under the influence of specialised tissues, it would seem to be in meristematic cells that the final steps take place in the synthesis of the *chemical determinants* of tissue differentiation. This, of course, is not to say that special precursor molecules are not synthesised outside of the limits of the meristems. For instance, leaves undoubtedly supply substances to the shoot meristems which are essential for the transformation of a vegetative into a flowering apex. Nor should this hypothesis be regarded as at variance with the strong experimental evidence that the maintenance of an active state of cell division is dependent not only upon essential metabolites but also upon determinant molecules synthesised in and then transported from mature tissue cells to the meristems.

Our knowledge of the role of the meristem in the chemical determination of growth and differentiation has its beginnings in the classical studies of tropisms and particularly of phototropism (the growth curvature of plant organs in response to and determined in direction by unilateral illumination). It was the experimental study of the phototropism of the coleoptiles of grass seedlings, initiated in 1881 by Charles Darwin, which led on to the discovery of the natural occurrence of the plant growth hormones now called *auxins*. This discovery is usually placed at 1928 when F. W. Went working in the Department of Botany of Utrecht University described how a chemical agent essential to the expansion growth of coleoptile cells could be collected from the coleoptile tips by diffusion into agar jelly. This work confirmed hypotheses which had gradually developed during the previous 15 years and which are exemplified by the following quotation from a research paper by A. Paál of the University of Budapest in 1919: "the tip is the seat of a growth-regulating centre. In it a substance (or mixture) is formed and internally secreted, and this substance, equally distributed on all sides, moves downwards through the living tissue. If the movement of this correlation carrier is disturbed on one side, a growth decrease on that side results, giving rise to curvature of the organ."

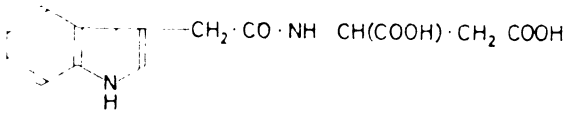
Subsequent work showed that the substance synthesised in the coleoptile tip and moving from there to initiate expansion in the cells below was β-indolylacetic acid (IAA), (Fig. 63) that the cells of the coleoptile could not embark upon cell expansion in the absence of an external supply of this substance and that it was effective in minute (catalytic) amount. IAA, by its activity at



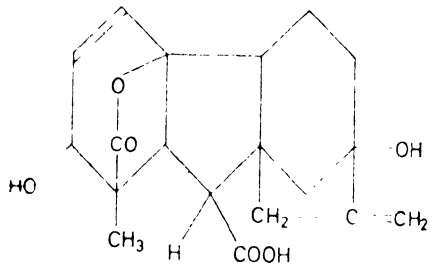
β-Indolylacetic acid
(IAA)



Kinetin (6–furfurylaminopurine)



Indolylacetylaspartic acid



Gibberellic acid

FIG. 63. Chemical structure of β-indolylacetic acid (IAA) and other growth regulators.

great dilution and by expressing a physiological effect at a site spacially removed from its site of synthesis clearly called to mind the animal hormones and hence was described as a plant growth hormone.

The new growth hormone clearly controlled the linear growth of the grass coleoptile and its tropic curvatures were the outcome of an unequal distribution of the hormone induced by various unilateral stimuli. Quickly, evidence accumulated that auxin was universally distributed in higher plants. Secreted by the apical meristems of both shoots and roots it controlled in these organs the expansion of the tissue cells. Further, its physiological activities were not confined to the control of cell expansion. It was implicated in apical dominance (the inhibition of lateral bud and lateral root development by the active apical meristem), in the initiation of adventitious roots at the base of stem cuttings and probably also in lateral root initiation, in the seasonal activity of the cambium in trees, in the retention or falling of leaves and flower buds, in flower development and in the initiation and continuance of fruit development. Auxin could inhibit or promote not only cell expansion but cell division. The reaction it produced varied both with the concentration of the auxin and the sensitivity of the tissue and this sensitivity or responsiveness of tissues to auxin clearly depended upon their kind and stage of development and their position within the organism. More recent experimental work has emphasised the role of auxin in organ initiation and, particularly in work with tissue cultures, shown that it is concerned in tissue differentiation. For instance, vascularisation (the development of strands of xylem elements) in growing tissue culture masses seems to take place only along lines where a sufficiently steep gradient of auxin concentration becomes established.

For a long time IAA remained the only hormonal factor known to exert a controlling influence in plant growth and development although even during this period there was obtained evidence that some of its physiological activities were dependent upon the simultaneous presence of catalytic amounts of other growth

factors, particularly of certain vitamins and purine bases. More recently, we have witnessed the isolation of certain metabolic products (*gibberellins*) from cultures of the fungus, *Gibberella fujikuroi* (the causative agent of the *bakanae* or "foolish seedling" disease of rice) and the demonstration that these substances were responsible for inducing in diseased rice plants the excessive elongation growth. These substances, particularly gibberellic acid (Fig. 63), were shown to markedly enhance internode elongation in other species, being active, for instance, against dwarf but not against tall varieties of the garden pea. The interest in these gibberellins was markedly increased when evidence was obtained that such compounds are natural constituents of flowering plants and that they are probably essential to cell expansion. This permitted the hypothesis to be advanced that when gibberellins promote cell expansion gibberellin is and auxin is not a "limiting factor" and, conversely, where auxin is essential for expansion then the cells are deficient in auxin but not in gibberellin. The gibberellins like auxin also have effects on cell division. For instance, there is evidence that gibberellin is involved in the initiation of cell division in the cambium and that the main effect of auxin in cambial activity may be to promote the differentiation of the products of cambial activity into xylem elements rather than to initiate the divisions. Following the discovery of the gibberellins came the description of the activity of kinetin as a cell division factor by Professor Skoog (see p. 199). Not only kinetin but a number of other related purines have this activity and Skoog has, therefore, coined the term *kinins* (from kinesis or division) for these compounds. No naturally occurring kinin has yet been isolated and identified from plant cells but there is much circumstantial evidence for the natural occurrence of such substances. Skoog, using isolated plugs of tobacco pith cells showed not only that both kinetin and auxin were essential for the formation of a growing tissue culture but that the relative concentrations of these two substances had determinative effects on the cultures, particularly in regard to their capacity to initiate root and shoot meristems (Fig. 59). In other

systems interactions between auxin, kinetin and gibberellic acid on growth by division and expansion and on cell differentiation have been exposed.

Clearly, not only auxin but kinins and gibberellins are candidates for consideration as determinants in growth and differentiation in higher plants. If we are to elucidate their role in the organism, what further information do we need and how may it be obtained? Well, first we need to know where and in what amounts they are synthesised, what concentrations of each reach and are established in the cells whose growth and differentiation is reflected in plant development, and how far, at different sites within the organism, can we correlate the relative and absolute concentrations of these substances with the patterns of growth and differentiation which occur. It must immediately be stressed here that there are very great technical difficulties in the way of obtaining this information. For the moment, therefore, it may well be that advances in this direction will come from the study of such problems in the apparently simpler systems represented by organ, tissue and free-cell culture. Of course, all studies of this kind run the risk that there may well be additional and, as yet, unknown substances which play an equally key role in the control of growth and differentiation.

This problem of the chemical identification of the natural regulators of growth and differentiation also faces formidable technical obstacles. Firstly, those regulators so far detected are present in extremely low concentration in plant cells. This precludes their recognition or estimation in plant extracts by chemical means. Hence their presence and their attempted purification have to be followed by sufficiently sensitive biological tests (bioassays). The specificity of these bioassays can, however, only be assessed against known compounds such as are available to the plant physiologist in pure form. One such bioassay for auxin activity depends upon the measurement of the longitudinal extension, in a suitable sugar-salt medium, of coleoptile segments (short segments cut from that region of the etiolated grass coleoptile which is capable of most marked elongation). These segments

show only very limited extension in the basic medium but addition of IAA (or of other "synthetic auxins") at appropriate concentration markedly enhances this extension. If such a test is used in a search for natural growth regulators it is those which similarly stimulate coleoptile segment extension which will be detected. It does not follow, of course, that the detected substances would resemble IAA in its other physiological properties and "fractions" of the plant extract which fail to promote or may inhibit the growth of the coleoptile segments may do so because they contain impurities masking the activity of any "auxins" they contain.

Secondly, we do not know the chemical form in which IAA and the plant gibberellins exert their activity in metabolism, although there is considerable circumstantial evidence that the action of IAA depends upon its combination with some "acceptor" (protein?) within plant cells. Therefore, the experimenter trying to detect and separate natural auxins is always in doubt as to whether chemical changes taking place during his extraction procedures may be destroying the natural compounds and creating chemical artefacts.

Consequent upon the development of paper partition chromatography (see Chapter 5, p. 126) and the demonstration that, with appropriate solvents, this technique can effectively separate known indole compounds, many workers have submitted plant extracts to this procedure in an attempt to separate out natural growth regulators. The chromatograms so obtained have usually been entirely indole negative to chemical reagents. However, when such chromatograms have been cut into sequential segments and the segments separately washed (eluted) with water or alcohol it has been shown, by bioassay of such eluates, that there are along the chomatograms a number of discrete regions showing growth-regulating activity. The chemical constituents occurring in these active regions of the chromatograms have hardly ever been positively identified, although frequently the evidence has strongly indicated their lack of identity with known indoles and the plant gibberellins. Such studies have, therefore, supported the view that

plant cells contain a number of un-identified substances which can give, apparently at very low concentration, positive responses in the biossays used to detect and estimate auxins and gibberellins. The significance of these substances, however, just cannot be assessed until they are isolated in pure form to permit of their wider biological testing and determination of their chemical structure. Clarification of this apparently very complex situation urgently needs the extraction and fractionation of plant extracts on a much larger scale than hitherto usually attempted in biological laboratories.

Another major approach to the role of the growth regulators is to try to trace their involvement in cell metabolism. This is only just beginning to be attempted for the gibberellins and kinins. The problem of the *mechanism of action* of IAA has, by contrast, been studied in many laboratories. So much is this so that one distinguished plant physiologist, Professor James Bonner of the California Institute of Technology, has remarked "It is really rather a disgrace to the profession of plant physiology that the nature of auxin action has not been revealed." However, the situation here is, in many respects, paralleled by almost equally unsuccessful work directed to finding out how the animal hormones work. As already stressed, these substances are effective and occur naturally at *very* low concentrations. Perhaps a bigger obstacle is revealed by the phrase "the probable subtlety of mechanism involved" which comes from a recent review in *The mechanism of action of auxin* by Professor A. W. Galston and W. K. Purves of Yale University. The micro-nutrient elements (see Chapter 6, p. 159) and the vitamins, which are similarly active in very low concentrations, act as prosthetic groups or co-enzymes (or parts of the co-enzyme molecules) of enzymes. However, very many attempts to demonstrate enzyme activation *in vitro* by auxin have yielded negative results. Quite recently some workers have published evidence that auxin activates the enzyme *pectin methyl esterase*. Further, this enzyme could be important in maintaining the plasticity of cell walls and hence promote cell expansion. However,

the claim that this enzyme is activated by auxin has been contested and amongst those who have supported the view that this is the "site" of auxin action there is a divergence of opinion as to whether auxin activates the enzyme, promotes synthesis of the enzyme or is involved in attaching the enzyme to the cell wall. IAA is therefore not yet a recognised co-factor or co-enzyme for any known enzyme.

Studies of the changes in composition and metabolic activity occurring in response to the auxin treatment of living cells have shown effects on many processes and cell constituents. These have included respiratory patterns, nucleic acid metabolism, permeability properties, cell wall plasticity, protoplasmic viscosity and protoplasmic streaming. The very multiplicity of the effects of auxin emphasises the difficulty of distinguishing primary from secondary effects. Some workers have stressed that the plants in which auxin is clearly a growth regulator are plants whose cell walls are composed of cellulose, hemicelluloses and pectic substances. Further, one of the most firmly established effects of auxin is that established by the German botanist, A. N. J. Heyn in 1930, that it enhances the plastic (irreversible) extensibility of cell walls. It may, therefore, be that our lack of knowledge of the biochemistry of the synthesis of cell wall constituents, and particularly of its structural framework of cellulose (Chapter 5, p. 142) is a major handicap to the elucidation of the mechanism of auxin action.

The indirect evidence that auxin enters into combination with some acceptor within the cells poses the question of whether IAA itself can be expected to activate any enzyme system. Clearly, to test critically whether auxin action involves the activation (or inhibition) of enzymes it is necessary to isolate from cells the *active form* of the auxin. IAA may, in this sense, simply be the diffusable precursor and the auxin response of cells depend upon their capacity to form from this the active molecular species. Certain auxin-protein complexes have, at various times, been isolated from plants but critical studies of the physiological activity and general occurence of such compounds have not been under-

taken. Similarly, certain conjugates between IAA and amino acids (for instance, the conjugate between IAA and aspartic acid Fig. 63) have been detected in plant cells following IAA-feeding and some evidence obtained that such conjugates are naturally plant constituents. However, more study is required to map their physiological properties and to find out if they are metabolised to larger molecules. Many studies of the natural auxins in plant extracts have been confined to separating the constituents preferentially soluble in ether as against in water (since this corresponds to the solubility of IAA). In those few investigations where the preferentially water-soluble substances (freed from sugars and other major metabolites) have been examined, chromatography has revealed substances highly active in auxin bioassays and giving chemical reactions for amino acids (although they were clearly not any of the known free amino acids). More recently excised root cultures have been shown to release, into the culture medium in which they are growing, a peptide of the indole, 5-hyroxytryptophane. This peptide is active in promoting the extension growth of coleoptile segments *and* also highly stimulatory to root growth. These somewhat isolated observations emphasise the importance of testing, both in bioassays and against isolated enzyme systems, the indole compounds of plant cells other than the free IAA.

The above discussion of the difficulties involved in assessing the significance and mode of action IAA and other growth regulators has not only taken us to the frontiers of knowledge but beyond into the confusions of no-man's land where we are groping in the dark for clues as to the topography. This has been necessary to illustrate the kinds of problems which face the plant physiologist as he attempts to move forward from the study of separate metabolic processes to the interpretation of the metabolic patterns of the whole organism and of how they develop during its life-history. This mention of life-history reminds us that we not only have to account for the growth and development of the organism in terms of the underlying metabolic events but also explain why multicellular plants become senescent and die; to understand what

it is in these patterns which must inevitably lead to their own ultimate disorganisation and destruction.

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9

A FURTHER DIMENSION

"*The language of current biochemistry is still that of letters and dashes which means that this science is still moving in the same molecular dimension as it was moving at its birth in the last century . . . dive into a new dimension, that of the submolecular or subatomic dimension of electrons, a dimension the happenings of which are dominated by quantum, or wave, mechanics . . . the atom is no more an indivisible unit but consists of a nucleus surrounded by a cloud of electrons with varying and fantastic shapes and it seems likely that the subtler phenomena of life consist of the changing shapes and distributions of these clouds.*"

Albert Szent-Györgyi in *Introduction to a Submolecular Biology* Academic Press, New York, 1960.

To approach the central problems of plant metabolism we have to extend our thinking into two opposite directions. We have, in the two chapters (7 and 8) which have just gone before, extended our thinking from the molecular level to the supramolecular level, right up to the level of organisation represented by the living multicellular plant. The other direction in which we have to probe is to the sub-molecular level where we are concerned with the behaviour of electrons. It is in this dimension that we can hope to understand more deeply the process of photosynthesis where we have to determine the excitation states to which electrons are raised in chlorophyll and other photosynthetic pigments by the absorption of photons, to follow the transfers of excitation energy between the pigments of the chlorophyll complex and to trace the ways in which the energy of excited electrons becomes converted into the energy of chemical molecules like those of ATP

and reduced TPN (see Chapter 5). It is also as electrons are sent down the oxidative chain from co-enzymes to flavoprotein to the sequence of cytochromes and, ultimately, to oxygen that most of the utilisable energy made available by respiration is canalised into the synthesis of the terminal phosphate bond of ATP by oxidative phosphorylation (see Chapter 4). Here, in these two vital processes of life we have problems which have so far proved resistant to elucidation by the methods of classical biochemistry. We also have the unsolved problem of how the energy of phosphate bonds is converted into work as exemplified in plant cells by osmotic work, the translocation of solutes, the accumulation of solutes, the movement of flagella, cytoplasmic streaming and the movements of chromosomes in nuclear division. Of course, reactions which proceed with a decrease in free energy liberate energy but to say this does not indicate how this energy is harnessed to do work; ATP can be identified as the fuel but the nature of the cellular work machine is obscure.

Reducing agents tend to export, oxidants to accept, electrons, and the potential or pressure of such electron transfers between reductant and oxidant (the Redox potential) is a measure of the energy change involved (one electron volt=23 kcal). In such reactions, involving organic molecules, it is usual for an electron pair to be transferred from one molecule to the other, two new closed shell molecules being formed. The two molecules then part, the one having become richer, the other poorer by two electrons. Both molecules have two states differing by two electrons. This situation is not altered by the discovery by L. Michaelis that "all oxidations of organic molecules although they are bivalent, proceed in two successive steps, the intermediate state being a free radical." However, this discovery does raise the question of whether electron transfer can occur between organic molecules which are not oxidising or reducing agents in the sense that they do not have two stable states differing by two electrons. Szent-Györgyi has recently drawn the attention of biologists to such systems, systems in which single electrons can be transferred between closely associated

molecules and has stressed the possible biological importance of such *charge transfer* within and between molecules.

For our present purpose we shall very briefly consider this form of electron transfer in order simply to show how it might serve as a mechanism relating electron migration to chemical bond synthesis, for instance might be the key to understanding what is involved in oxidative phosphorylation.

Charge transfer can take place between molecules sufficiently close that their electron clouds overlap and involves movement of an electron from a donor to a vacant orbital of an acceptor molecule. The molecules usually stay together, if they do separate they do so as free radicals with an unpaired electron. The electron transfer occurs without major loss of energy because it does not involve a rearrangement of molecular structure. If the accepting orbital is lower than the donating one, the transfer can occur spontaneously without any outside source of energy. Now, from the formation of tetradentate chelates by ATP with Mg. as the linking atom and from the rotary dispersion of ATP solutions it seems that the molecule of ATP is folded. Such folding could take place in the pentose unit of the molecule bringing the two terminal phosphate groups to be flat on the face of the adenine ring. Adenine is a good electron donor (an electron could come either from the π pool or from the lone pair on one of its N atoms). The P atom has unoccupied orbitals (*d*-orbitals) and there is evidence that this atom can serve as a reversible electron transmitter. Now, in order to synthesise ATP from ADP it is necessary to overcome the mutual repulsion of positive charges which stand in the way of the formation of the P—O bond between the terminal phosphate group and phosphate (see Fig. 34 for the formula for adenosine triphosphate). If, by charge transfer an electron from the adenine group moves to the P atom this repulsion will be changed to an attraction promoting ATP synthesis. Further, the electron donated to the phosphate chain can be released from electrostatic bondage if an electron of sufficient energy, released during oxidation enters the adenine at the appropriate orbital. The synthesis

of ATP has then been promoted by feeding in a high-energy electron which has moved by charge transfer to be released as a tired electron and this has been coupled with the synthesis of the terminal pyrophosphate bond. Here we have perhaps the explanation of why nature tied the high-energy pyrophosphate bonds to adenine and incorporated the pentose as a centre of folding. Here the adenine functions in a mechansim to promote ATP synthesis. Perhaps its same properties are also of significance in the transformation of bond energy into cellular work. But to consider this would be to carry speculation still further and the author has already exposed himself both here and elsewhere in this volume to the criticisms that he has indulged too much in speculation and over-simplification. However, if those of my readers for whom this book is primarily written are stimulated to continue *their* studies of plant metabolism to the point where they are able to assess critically my speculations and simplifications the purpose of the work will have been achieved.

The last two chapters of this book carry important lessons for those on the threshold of being trained as biologists. Chapter 8 emphasises the high order of the organised complexity of biological systems, a complexity which can only be fully appreciated by those who have studied living organisms by the many avenues of approach which have grown up during the history of biological science. There is no substitute in biology for the first-rate biologist. The present chapter (and many other points in this volume) should, however, also indicate the importance to biology of the concepts, not only of chemistry but also of physics, and seem to justify the prediction that future progress in our understanding of many biological problems will more and more depend upon the co-operative activities of biologists, biochemists and biophysicists. This does not mean that to become a biologist one must first become fully fledged as a chemist and as a physicist—the sciences are expanding too fast and life is too short for this. It does mean, however, that the biologist needs to be able to develop a common language with those whose primary training is in these disciplines.

He should therefore engage in the serious study of these physical sciences during his last years at school and during the early part of his university training.

These sentences are the epilogue to this short volume; it is hoped they are the prologue to some of you becoming the research and teaching biologists of the next generation.

> "*The basic texture of research consists of dreams into which the threads of reasoning, measurement and calculation are woven.*"
>
> A. Szent-Györgyi

MORE ADVANCED READING

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INDEX

Page numbers in italic refer to text figures. Page number followed by *et seq.* indicates the main discussion of the subject.