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THE PRINCIPLES OF PLANT BIOCHEMISTRY

PART I

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THE PRINCIPLES OF PLANT BIOCHEMISTRY

PART I

by

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PREFACE

THIS book, which deals only with the higher plants, is intended for the student and for the beginner in research. As far as possible, the sections have been treated comprehensively. Even so, unless the volume be enlarged to an unreasonable size, the results of all investigators on these particular lines cannot be discussed. Additional names, therefore, are included in the bibliographies, to provide the student with a wider field for reading purposes.

The problems here considered are, in the main, those concerned with the biochemistry of the sugars and the nitrogen compounds; the former when they take part in cell-wall formation and in respiration, the latter chiefly in connection with the synthesis and the breakdown of protein. Oxidising systems are also included, but only such as are known to be characteristic of the above-mentioned group of plants.

I take this opportunity of recording my sincerest thanks to Mr F. F. Blackman, F.R.S., and to Professor Sir F. G. Hopkins, F.R.S., for their help with portions of the manuscript.

It is planned to publish later a second volume dealing with other sections of the subject of plant biochemistry.

M. W. O.

Biochemical Laboratory, Cambridge,
Botany School, Cambridge,
Low Temperature Research Station, Cambridge.

September, 1930

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CARBOHYDRATES OF THE HIGHER PLANTS

MONOSACCHARIDES

Trioses.
Methyltetroses. **Apiose.**
Pentoses. *l*-**Arabinose**, *d*-**xylose**, *d*-**ribose.**
Methylpentoses. *l*-**Rhamnose.**
Hexoses. (Aldoses.) *d*-**Glucose**, *d*-**mannose**, *d*-**galactose.**
(Ketoses.) *d*-**Fructose.**
Heptoses. **Sedoheptose, mannoketoheptose.**

DISACCHARIDES

Maltose, (**cellobiose**), **gentiobiose.**
Sucrose, melibiose.
Primeverose, vicianose, strophantobiose.

TRISACCHARIDES

Gentianose, raffinose, melicitose.

TETRASACCHARIDES

Stachyose.

POLYSACCHARIDES

Pentosans. **Araban, xylan.**
Pentosan-hexosans. **Pectic substances, gums, mucilages.**
Hexosans. **Cellulose, galactans, mannans, starch, inulin.**

CHAPTER I

THE SUGARS

Types of Sugar in the Plant

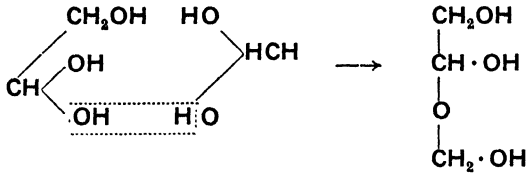
If the list on the preceding page is examined, it will be seen that the sugars naturally occurring in the plant are chiefly limited to a few members of the pentoses and hexoses, of which types of sugar many of the possible isomeric forms are known. There are grounds, it would appear, for believing that it is inherent in the structure of certain of the sugars to be more stable than others in a complex of interacting chemical systems, of which the plant is the outward expression. For it has been shown by Nef (see Spoehr, 82) that when formaldehyde is allowed to condense *in vitro* in presence of lead hydrate, many condensation products arise, and, if the mixture is left until the reactions attain equilibrium, the final products are almost entirely derivatives of the pentoses and hexoses, such bioses, trioses and tetroses as are formed being only transient stages; condensation, moreover, does not proceed farther to heptoses and octoses. The results are equally striking, as compared with the natural systems, if one considers the hexoses alone; in a glucose series at equilibrium under similar treatment there are found about equal quantities of aldoses and ketoses; of the aldoses, there is about five times as much glucose as mannose. Further, in a galactose series, *d*-galactose forms over 90 % of the sugar present.

These facts discovered by Nef undoubtedly throw light on the apparent restriction of sugars in the plant to a few members of this group. They show that in solution in the presence of impurities, certain of the sugars and, of the same sugar, certain isomers are more stable than others and tend to be formed to a greater extent.

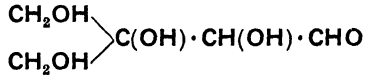
Monosaccharides

As evidence for the presence of the **trioses** in the plant, there is little to be said. Buston & Schryver (13) have isolated a crystalline, non-reducing substance from the leaves of the Cabbage (*Brassica oleracea*), which can be regarded as a triose. They suggest that it may

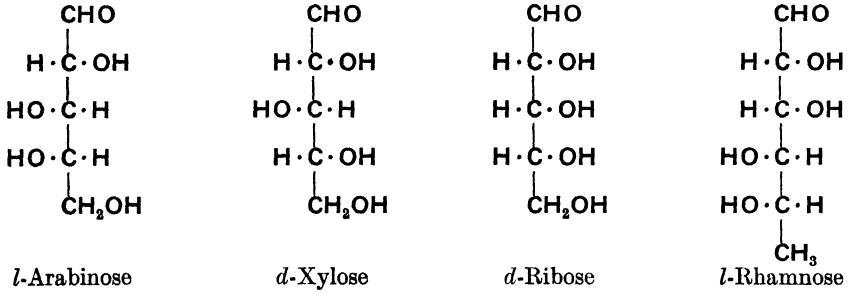
be formed by the condensation of hydrates of glycollic aldehyde and formaldehyde:



Methyltetroses. An oxymethyltetrose, apiose (Vongerichten & Müller, 87) has been obtained by hydrolysis of the glucoside, apiin, present in the Parsley (*Apium Petroselinum*). Apiin is hydrolysed into apiose and a flavone, apigenin. The sugar has probably the constitution:



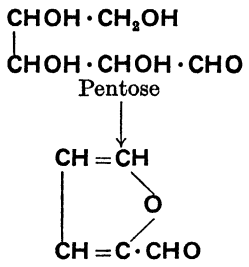
Pentoses and **methylpentoses.** (A methylpentose is a pentose in which one of the hydrogens of the primary alcohol group is replaced by methyl.) Of these sugars, the following representatives are found in the plant: *l*-arabinose, *d*-xylose, *d*-ribose and *l*-rhamnose.



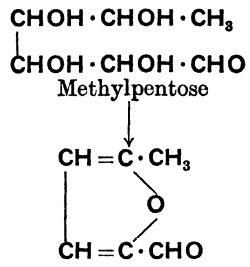
l-Arabinose *d*-Xylose *d*-Ribose *l*-Rhamnose

It is possible that small quantities of free pentoses are present in the tissues. As polysaccharides (the pentosans), usually together with condensation products of other sugars, the pentoses are very widely distributed in gums, mucilages, pectic substances and in cell-walls, both lignified and unlignified; they are frequently found also in the form of glucosides (see p. 15). There is no evidence that methylpentoses occur free.

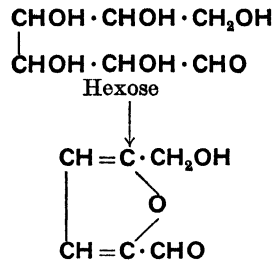
The detection of pentoses rests largely on colour reactions obtained with furfuraldehyde, a product of decomposition of these sugars when heated with hydrochloric acid. Under the same conditions, the methylpentoses produce methylfurfuraldehyde, and the hexoses, hydroxymethylfurfuraldehyde (Cunningham & Dorée, 23):



Furfuraldehyde



Methylfurfuraldehyde



ω-Hydroxymethylfurfuraldehyde

These aldehydes give coloured condensation products with various phenols, such as phloroglucinol, α-naphthol and thymol; also with aniline and aniline acetate. As hydroxymethylfurfuraldehyde tends to decompose into laevulinic and formic acids on heating with dilute acids, the colour reaction is given to a less extent with hexoses, and thus becomes more or less specific for pentoses.

There is, however, another class of compounds of which glucuronic and galacturonic acids are members, which produce furfuraldehyde under the same conditions (see below), and which may therefore introduce complexities. A specific colour reaction for these acids with napthoresorcinol, however, may be used to give an indication of their presence.

The estimation of pentoses rests on their decomposition with hydrochloric acid, and the fixation of the furfuraldehyde on heating with hydrochloric acid as an insoluble phloroglucide. Methylpentoses give a methylfurfuralphloroglucide, and hexoses, a hydroxymethylfurfuralphloroglucide; the two latter phloroglucides can be separated from furfuralphloroglucide by their solubility in strong alcohol. By this method, pentoses can be estimated, either as free substances in plant extracts or as polysaccharides. The presence of hydroxymethylfurfuraldehyde derived from hexoses, unless these are present in considerable quantities, does not give rise to appreciable error in pentose estimations; but its presence may render estimations of methylpentoses of doubtful value. In dealing, however, with results from complex mixtures containing pentoses, methylpentoses and hexoses, great caution is required.

More significant may be the errors introduced by the presence of galacturonic and glucuronic acids. Since the former, as will be seen later, occurs as a constant component of the pectic substances widely distributed in the cell-wall, both acids may possibly be found, either free or as simple conjugated products, in the plant. Hence it will be seen that the estimation of pentose (and pentosan) content, which

has often formerly been based on total furfuraldehyde obtained on distillation with acid, may well be open to criticism.

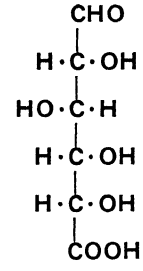
Davis, Daish & Sawyer (31) and Davis & Sawyer (32) claim to have estimated free pentoses to the extent of 0·3–1% (dry weight) in leaves of the Mangold (*Beta vulgaris* var.), Turnip (*Brassica campestris*), Carrot (*Daucus carota*), Potato (*Solanum tuberosum*), *Tropaeolum majus* and Sunflower (*Helianthus annuus*). Spoehr (81) also found free pentoses to the extent of about 0·3% (dry weight) in the axes of *Opuntia phaeacantha*. The claim rests on the fact that when the sugars from the alcoholic extract of a large quantity of material are fermented by Yeast, an unfermentable residue is left. This residue was found by Davis and his co-workers to give values for pentoses, estimated, on the one hand, by reduction of copper, and, on the other hand, by furfuralphloroglucide, which were, approximately, concordant. This occurrence of pentoses has been questioned by Colin & Franquet (22) who employed a qualitative test, namely, a green colour produced by condensation of pentose with β-naphthol in the presence of strong sulphuric acid. They used, as a source of possible pentoses, the alcoholic extract of leaves of the Potato and Beet. This gave no pentose reaction with β-naphthol, and, at most, on distillation with hydrochloric acid, a trace of furfuraldehyde which was estimated colorimetrically by means of the orcinol reaction. Colin & Franquet, therefore, doubt the existence of free pentoses to the extent claimed by Davis, Daish & Sawyer.

The origin of the pentoses is uncertain, though it has generally been maintained that they do not arise in photosynthesis, but from the hexoses as primary products of oxidation, and then remain inactive (de Chalmot, Bib. II, 12, 13). This author bases his conclusions on data which show an increase of pentose and pentosan content in Wheat seedlings germinated in the dark. Spoehr (81), however, found a considerable decrease of pentose and pentosan content in the same material under similar conditions. In view of the errors that may have arisen in the methods of estimation, owing, for instance, to the presence of pectic substances, no great reliance can be based on these values as evidence.

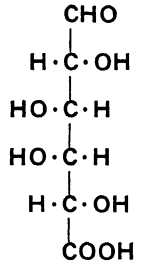
On the assumption that free pentoses are present in the leaves, Davis, Daish & Sawyer found the variation during the day to be small (see Tables VII, VIII and IX, pp. 34–36). From a comparison of pentose and pentosan values with hexose values throughout 24 hours in Mangold leaves, they are inclined to think that the

pentoses arise from the hexoses, and the pentosans from the pentoses. The same criticism as to validity of results can be applied in this case also.

In spite of lack of concordance in experimental data, a definite and satisfactory suggestion has nevertheless been made by de Chalmot as to a possible mode of origin of the pentoses. He points out that the aldehyde group of a hexose, such as glucose or galactose, may in the plant be fixed by union with some other group. This being so, oxidation may take place at the end group —CH_2OH, with the formation of glucuronic and galacturonic acids:

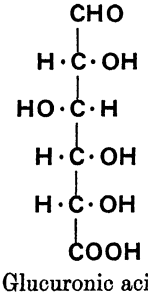


Glucuronic acid

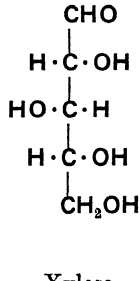


Galacturonic acid

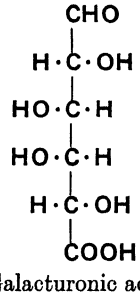
Glucuronic and galacturonic acids (sometimes, as a type, termed "uronic" acids) are water-soluble, strongly reduce alkaline copper solutions and give the reactions for pentoses. They decompose on heating with acid, giving rise to pentoses and carbon dioxide[1]:



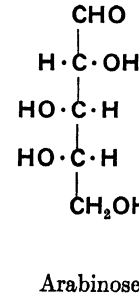
Glucuronic acid



Xylose



Galacturonic acid



Arabinose

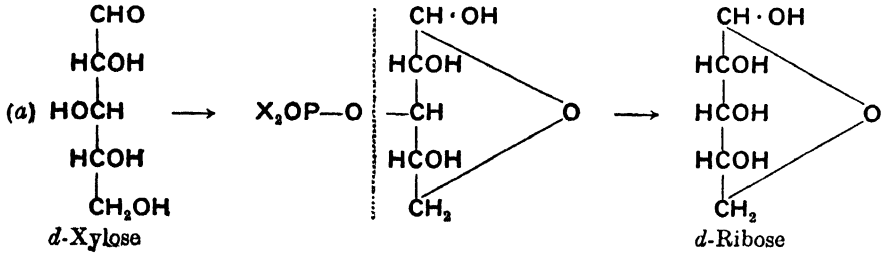
A further point of interest in this connection was noted by de Chalmot, namely, that in the plant glucose often occurs associated with xylose, and galactose with arabinose (see Pectic Substances). Spoehr claims to have isolated glucuronic acid from the plant of *Opuntia phaeacantha*. Galacturonic acid, moreover, is a component of the widely distributed pectic substances.

[1] Spoehr (81) has suggested that the above reaction may take place in sunlight.

Spoehr (81) further elaborates this conception in connection with *Opuntia*. He suggests that in the abnormal and restricted metabolism of this xerophyte there is a low concentration of free hexoses, owing to the fact that conditions of low water content and high temperature lead to the synthesis of hexose polysaccharides. Since respiration proceeds, however, without marked diminution, he further suggests that oxidation of hexoses in the polysaccharide complex takes place on the lines suggested above with the production of pentoses (as pentosans) in greater proportion than is usual. The characteristic of pentosan polysaccharides is that they are mucilaginous and retain water, and, certainly, abundant mucilage in the cell is an outstanding characteristic of *Opuntia phaeacantha* and probably other Cactaceae. Hence, as Spoehr points out, there is a correlation between increased production of pentosans, resulting in a water-retaining mucilage, and xerophytism. The subject will be considered fully in the section on hemicelluloses.

There is little to be said in detail of the individual pentoses.

d-**Ribose** forms part of the molecule of nucleic acid, a constituent of the nuclear material of plants. A reaction by which it may be derived from xylose has been suggested (Haworth, 42; see also Galactose). If the hydroxyl group (*a*) should become attached to a residue, such as phosphoric acid, by condensation with elimination of water:



then, in subsequent hydrolysis, a change of position of the groups may be brought about by a Walden inversion. In the formation of nucleic acids, it should be noted that phosphoric acid is involved.

l-**Arabinose** forms a component to a large extent of some gums, such as cherry gum and gum arabic. It is also found in the pectic substances.

d-**Xylose** is present, in varying amounts, in woody tissues as a condensation product.

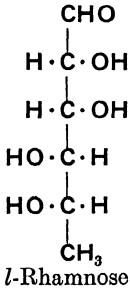
Of the methylpentoses, *l*-**rhamnose** is the best known represen-

tative in the higher plants. It occurs in several glucosides of the flavone, quercetin:

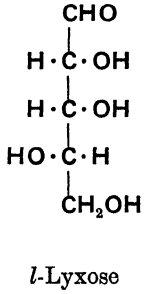
Glucoside	Components	Plant
Quercitrin	Rhamnose + quercetin	Quercitron bark
Rutin	Rhamnose + glucose + quercetin	*Ruta graveolens* and others
Xanthorhamnin	2 mols. rhamnose + galactose + rhamnetin (quercetin monomethyl ether)	Persian berries
Fustin	Rhamnose + fisetin	*Rhus cotinus*

The best known of the above are quercitrin and xanthorhamnin; the latter is the colouring matter of the fruits (Persian berries) of a species of *Rhamnus* which are used in dyeing.

l-Rhamnose is the methyl derivative of *l*-lyxose which is unknown.



l-Rhamnose



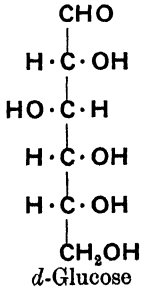
l-Lyxose

The methylpentoses give most of the reactions of the pentoses. On heating with acids, they produce methylfurfuraldehyde instead of furfuraldehyde. It is difficult to suggest a mode of production for rhamnose in the plant.

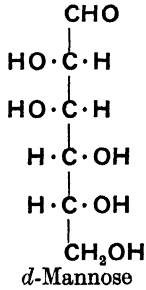
Though evidence points to the origin of the pentoses as that indicated in the preceding pages, the possibility of their direct formation as products of photosynthesis should be borne in mind.

In regard to the existence of the unstable γ-forms of the pentoses, see p. 8.

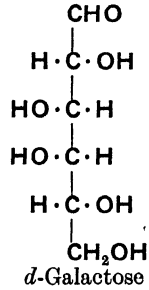
Hexoses. Of these sugars, the following representatives are found in the plant: *d*-glucose, *d*-mannose, *d*-galactose and *d*-fructose:



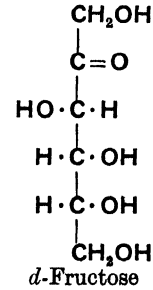
d-Glucose



d-Mannose



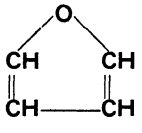
d-Galactose



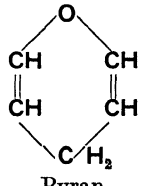
d-Fructose

d-**Glucose.** This sugar is apparently universally distributed in the plant, though in some tissues it may be present in low concentration. It is possibly the first sugar formed in photosynthesis; on this question, however, other views have been held (see p. 48).

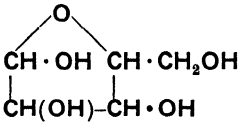
An important point to be borne in mind is that in solution the greater part of glucose is present as an oxide (lactone) structure. The usual form of glucose is now considered to have a 6-membered ring (Haworth, 42), but there is evidence for the existence of a 5-membered ring representing a specially active or labile form (γ-glucose). Not only is this true for glucose, but extensive researches (Haworth, 42) have shown that arabinose, xylose, lyxose, galactose, fructose and mannose also exist in these two forms. A revision of nomenclature and structural formulae has therefore been suggested, namely that the unstable γ-sugars should be regarded as derivatives of furanose, and the normal stable sugars as derivatives of pyranose. Glucose is thus termed gluco-pyranose, and γ-glucose, gluco-furanose; arabinose becomes arabo-pyranose, and γ-arabinose, arabo-furanose. The structural formulae are represented thus:



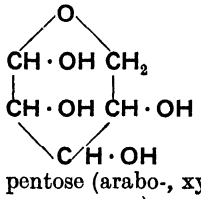
Furan



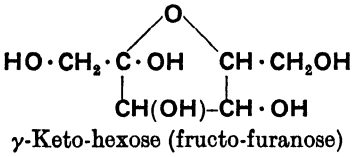
Pyran



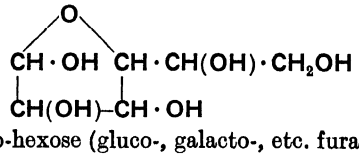
γ-Pentose (arabo-, xylo-, etc. furanose)



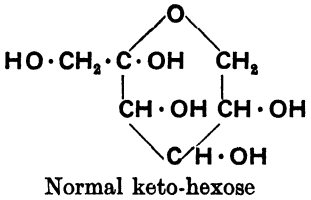
Normal pentose (arabo-, xylo-, etc. pyranose)



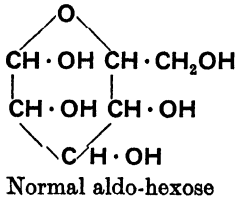
γ-Keto-hexose (fructo-furanose)



γ-Aldo-hexose (gluco-, galacto-, etc. furanose)



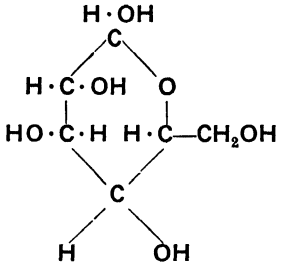
Normal keto-hexose (fructo-pyranose)



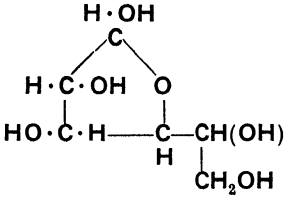
Normal aldo-hexose (gluco-, galacto-, etc. pyranose)

Except in the case of γ-fructose, which will be mentioned later, there is no direct evidence for the existence of the furanose series in the plant, though it is more than probable that these sugars are formed at certain stages in metabolism. The matter has been brought under consideration at this point in connection with glucose, since this sugar (together possibly with fructose) plays a most important part in carbohydrate metabolism. In regard to the pentoses, galactose and mannose, the question is more remote, since it is by no means certain that these sugars occur free to any extent in the plant cell.

In view of this latest modification, we may represent the two forms of *d*-glucose thus:



Normal form



Labile or γ-form

d-Glucose plays a very important part in plant metabolism. Some of the main types of reaction of which it is capable may be represented as decomposition, transformation, condensation and oxidation.

These aspects will now be summarised shortly:

A. Decomposition in respiration.

B. Transformation to fructose, mannose and galactose.

C. Condensation: 1. With other glucose molecules to form (*a*) cellobiose, (*b*) maltose and (*c*) gentiobiose. (Indirectly, also, through such disaccharides, to polysaccharides, such as cellulose and starch.) 2. With other sugars to form sucrose, vicianose, raffinose, etc. 3. To form the benzene ring. 4. To form the pyrone and pyrane rings of flavone and anthocyanin pigments. 5. With other compounds as glucosides.

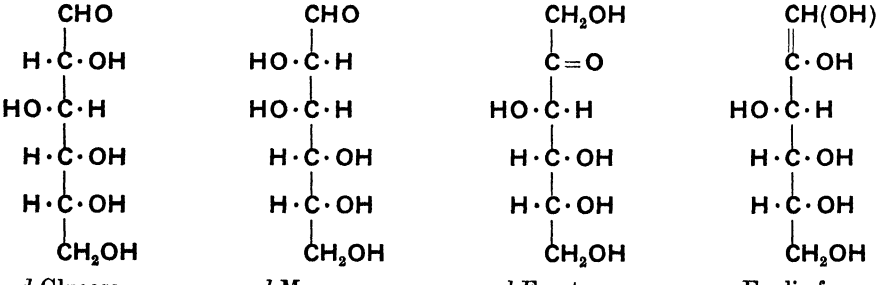
D. Oxidation to glucuronic acid.

A. *Decomposition.* It appears certain that a hexose is the direct substrate for respiration, but whether this is glucose or fructose or both is an open question. It is suggested (Blackman[1]) that an

[1] Analytic Studies in Plant Respiration. III. *Proc. R. Soc.*, 1928, **103** B, 491.

activation of hexose, possibly to the less stable 5-membered ring form, constitutes a preliminary to the initial stages of respiration. There is, moreover, evidence (Bodnár[1]) that a phosphate ester may be formed before the hexose molecule is subjected to the series of changes, probably catalysed by oxido-reductases of a special type, which results in intermediate products of respiration, such as methylglyoxal, lactic acid, acetaldehyde, pyruvic acid and glycerol. There is also evidence (Blackman, *loc. cit.*) that, if the respiratory changes are completed aerobically, a certain proportion of these intermediate products is resynthesised, possibly to the initial substrate. The final products of aerobic respiration of a hexose are carbon dioxide and water. Under certain conditions, of which the specific nature is unknown and which are realised in ripening fatty seeds and in woody tissues of certain trees in winter, intermediate products of decomposition, similar to those produced in normal respiration, may be formed and condensed to glycerides of fatty acids, the fats. A reversal of the whole process may subsequently occur under other conditions with the formation of sugar from fat. Synthesis of fat, involving this divergence from the main line of metabolic decomposition in respiration, is, to a less extent, common to all tissues. The synthesis of organic acids, such as malic, citric and tartaric acids, may be yet another line of modification of the metabolism of the intermediate products of respiration.

B. *Transformation.* It has always been considered possible that *d*-glucose may be transformed in the plant into both *d*-fructose and *d*-mannose through the common enolic form, this transformation being easily accomplished *in vitro* in alkaline solution:



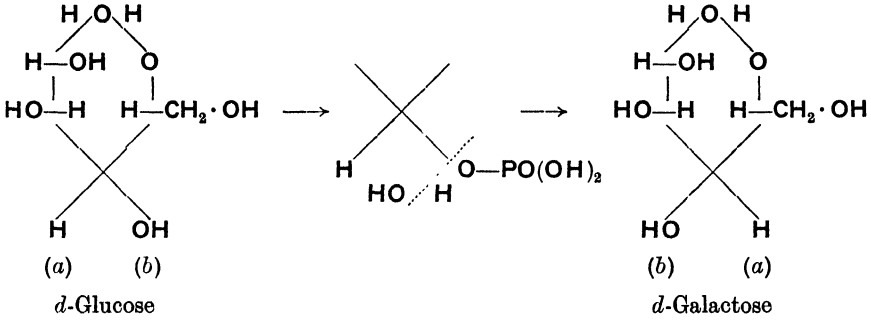
d-Glucose *d*-Mannose *d*-Fructose Enolic form

There may also be an interconversion of the γ-forms of these

[1] Biochemie des Phosphorsäurestoffwechsels der höheren Pflanzen. I. *Biochem. Zs.*, 1925, **165**, 1.

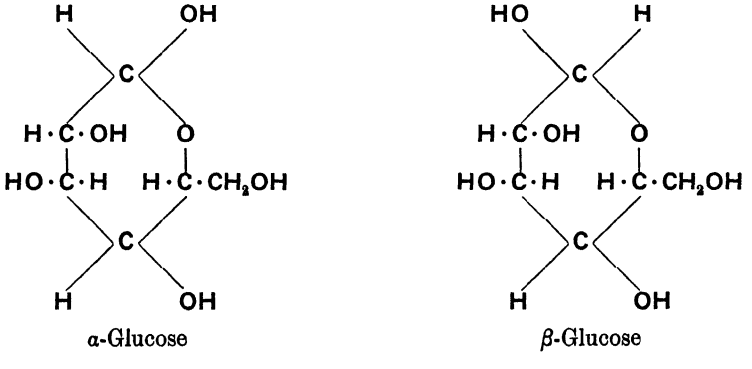
sugars. *d*-Fructose, however, may arise *de novo* quite independently of glucose.

A recent suggestion has been made as to the conversion of *d*-glucose into *d*-galactose in the plant. It is supposed that the hydroxyl group (*b*) becomes attached to a residue, such as phosphoric acid, by condensation with elimination of water. In subsequent hydrolysis of such a compound, a change of position of the groups (*a*) and (*b*) is now held to be involved (Walden inversion), and glucose is thereby converted into galactose:

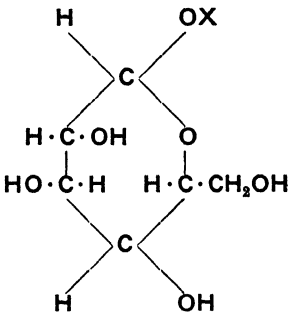


C. *Condensation.* *d*-Glucose readily condenses through the hydroxyl groups with many substances, including other sugars. The hydroxyl groups involved are usually those attached to the first, fourth and sixth carbon atoms of the molecule.

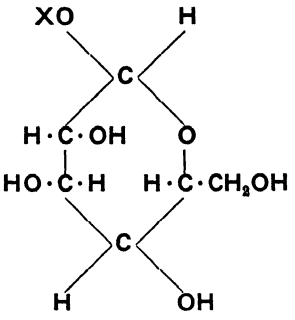
As the terminal carbon atom of *d*-glucose is asymmetric, condensation products with the hydroxyl group attached at this point may exist in two isomeric forms:



Such compounds are termed glucosides and are differentiated as α-glucosides and β-glucosides:



α-Glucoside



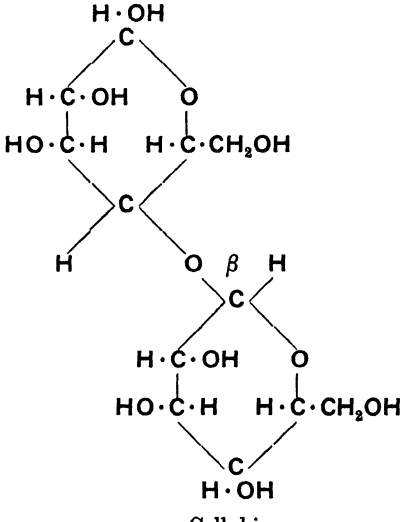
β-Glucoside

Condensation on this plan may take place with either another glucose molecule, some other sugar, or a substance of an entirely different nature (see section 5, p. 14).

A series of condensations which lead to products of special interest will now be considered. Some of these reactions are known to take place in the plant; others are, at present, only in the realm of speculation.

1. Glucose may condense with itself in three ways:

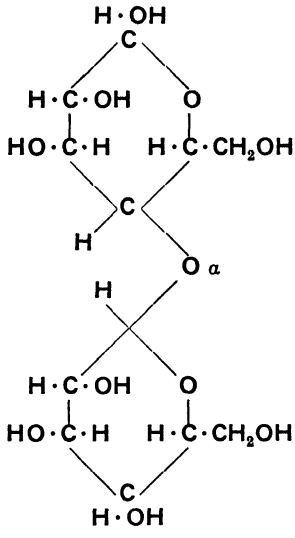
(*a*) To form a disaccharide, cellobiose, which is a β-glucosido-4-glucose:



Cellobiose

Cellobiose does not itself occur in the plant, but one of the suggestions for the constitution of the cellulose of the cell-wall is that it may be built up of glucose molecules on this plan of condensation (see p. 68), so that β-glucose would be the unit of the cellulose complex.

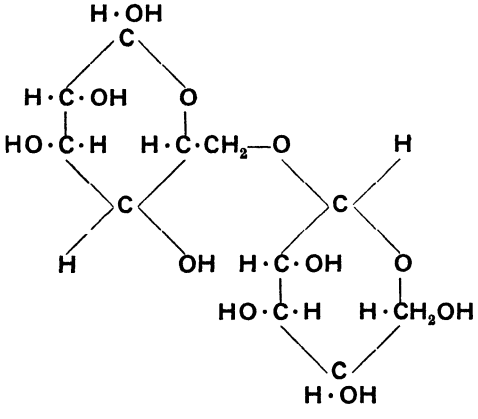
(*b*) To form the disaccharide, maltose, which is an *α*-glucosido-4-glucose:



Maltose

In a similar way it has been suggested that a condensation of *α*-glucose units on the plan of maltose *may* give rise to the major portion of the starch grain.

(*c*) As a *β*-glucosido-6-glucose. This constitutes the disaccharide, gentiobiose, which occurs as a component of amygdalin and other compounds:



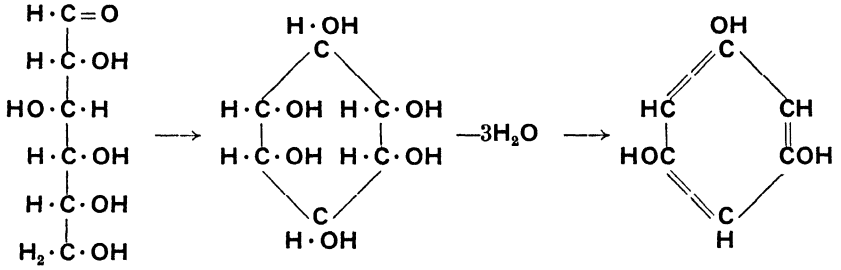
Gentiobiose

2. Of the condensation products with other sugars, the most important, from a biochemical aspect, is undoubtedly that with *d*-fructose, namely, sucrose or cane-sugar (see p. 18). The inter-

relationships of the hexoses with sucrose in the plant will be considered later.

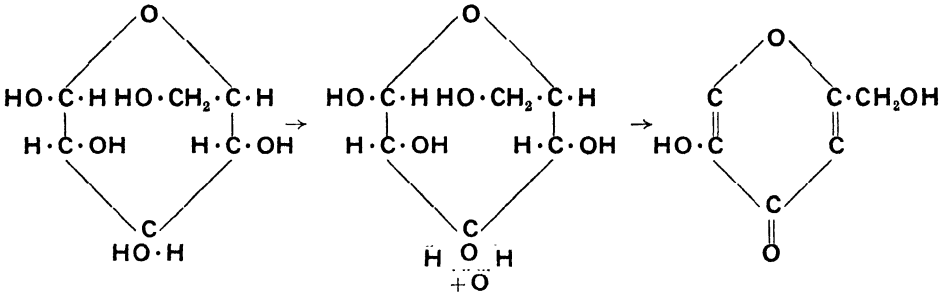
For condensation products with other sugars, see vicianose, etc.

3. It has been suggested that the benzene ring, which must be synthesised *de novo* in the green plant, since it is not absorbed, may arise directly from sugar by an aldol condensation as follows:



There is some evidence that the amount of the aromatic compounds formed in the plant is dependent on the supply of soluble carbohydrate. The benzene ring may, however, be produced, not as above, but by the condensation of a 3-carbon compound.

4. It has been suggested that a simple change in the 6-membered hexose ring may give rise to the pyrone and pyrane nuclei of the flavone and anthocyanin pigments:



A further condensation of the hexose ring with certain hydroxybenzoic acids might then be a possible line of synthesis of the pigments themselves.

5. Finally, there is the formation of the large group of glucosides, that is, using the word in the widest sense, compounds of glucose with substances other than sugars. The hydroxyl of the sugar molecule is replaced by members of very different groups of substances. In Table I is given a selection of glucosides illustrating

the various types of compounds with which the sugar may be combined.

TABLE I

Glucoside	Products of hydrolysis
Coniferin	Glucose + coniferyl alcohol (alcohol)
Salicin	Glucose + salicylic alcohol (alcohol)
Arbutin	Glucose + quinol (phenol)
Prunasin	Glucose + *d*-mandelonitrile (aldehyde)
Sambunigrin	Glucose + *l*-mandelonitrile (aldehyde)
Helicin	Glucose + salicylaldehyde (aldehyde)
Isoquercitrin	Glucose + quercetin (flavonol)
Cyanin	Glucose + cyanidin (anthocyan)
Aesculin	Glucose + aesculetin (oxycoumarin)
Sinigrin	Glucose + allyl isothiocyanate + $KHSO_4$ (mustard oil)
Indican	Glucose + indoxyl

It should, however, be realised that the glucoside type of condensation may take place with other sugars, such as pentoses, methylpentose and galactose; or with di- or trisaccharides which are themselves condensation products of either pentoses, hexoses, or both together; or, occasionally, the sugar may be of a rarer type, as, for instance, the case of apiose.

Table II gives examples of these condensation products with other sugars:

TABLE II

Glucoside	Products of hydrolysis
Quercitrin	Rhamnose + quercetin
Rutin	Rhamnose + glucose + quercetin
Xanthorhamnin	2 mols. rhamnose + galactose + rhamnetin
Fustin	Rhamnose + fisetin
Vicianin	Glucose + arabinose + *d*-mandelonitrile
Idaein	Galactose + cyanidin
Amygdalin	Gentiobiose + *d*-mandelonitrile
Apiin	Apiose + apigenin

The classification of many heterogeneous substances as glucosides is, in a sense, an artificial one, though the union with sugar confers more or less uniformity on dissimilar substances in respect of such properties, as solubility, ready power of crystallisation, etc.

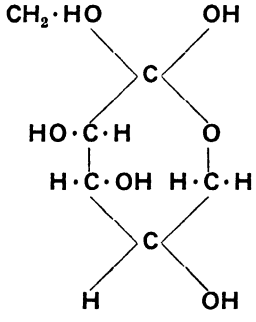
D. *Oxidation.* The fate of glucose when oxidation to glucuronic acid takes place will be considered in detail in Chapter II (see p. 75).

In regard to the metabolism of glucose in the plant, it will be considered together with that of fructose and sucrose, since the three sugars are intimately associated (see p. 46).

d-**Mannose**, as suggested above, may arise from *d*-glucose by transformation. It has not been detected uncombined in the plant. It is widely distributed as a condensation product, mannan (see Chap. II, p. 71). How or where the transformation from *d*-glucose to *d*-mannose takes place is unknown.

d-**Galactose**, again, like *d*-mannose, may arise from *d*-glucose by transformation. It, also, does not occur in the free state. A suggestion has been made (see p. 11) as to how *d*-glucose may be converted into *d*-galactose through combination with a third substance. Galactose is frequently found as a condensation product, galactan (see Chap. II, p. 71).

d-**Fructose** or **laevulose**. This sugar has probably much the same distribution as *d*-glucose. As free sugar it is present in the 6-membered ring form:



In cane-sugar, however (see below), the 5-membered ring, γ-form, is condensed with glucose. A certain amount of investigation has been made on the proportion of *d*-glucose and *d*-fructose found uncombined in the tissues. Details will be given when the interrelationships between the various sugars are considered (see p. 50).

Heptoses. Two heptoses have been, so far, found to occur in the plant, namely **sedoheptose**, from a Stonecrop (*Sedum spectabile*), and **mannoketoheptose**, from the Avocado Pear (*Persea gratissima*).

Disaccharides

First will be considered those formed from glucose alone.

Maltose. (The constitution has already been given, see p. 13.) This sugar is one of the products of hydrolysis of starch. Yet, even from starch-containing tissues, it appears to be often absent. Brown & Morris (12) maintained, on the basis both of the isolation of maltosazone and the hydrolysis with maltase, that considerable

quantities of maltose are present in leaves of *Tropaeolum*. Other observers (Ahrns, 1; Campbell, 14; Schroeder & Horn, 73; Tollenaar, 86), too, have indicated a value for maltose by increase of reduction, in appropriate extracts, after hydrolysis with dilute hydrochloric or sulphuric acid. Davis, Daish & Sawyer (31) and Davis & Sawyer (33), using the method of fermentation of all sugars other than maltose with maltase-free yeasts, failed to find maltose, not only in the leaves of the Mangold, which is a sugar plant, but also in the leaves of the Potato, *Tropaeolum*, Turnip, Carrot, Sunflower (*Helianthus annuus*), Dahlia, *Arum maculatum* and Vine (*Vitis vinifera*), which are starch plants.

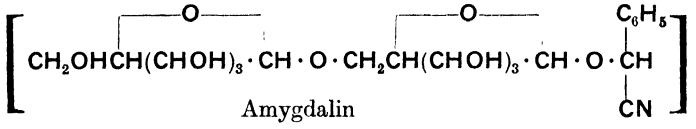
It is suggested by these same authors that the maltose found by Brown & Morris was produced by hydrolysis of starch by diastase during the drying of the leaves before estimation of the sugars. They quote several facts in confirmation of this point of view as follows. The values obtained by Brown & Morris for the starch in *Tropaeolum* leaves at the end of a sunny day ranged from 2·9 to 7·4 % (dry weight). Davis & Daish, however, obtained much higher values (as much as 17·6 %). Even if the value for maltose (of Brown & Morris) is added to that for starch as, for example, in Table III (p. 23), column 3, the total, 9·92, is still lower than the values of Daish & Sawyer. The latter further point out that Kluyver has also determined the maltose and other sugars in *Tropaeolum* leaves by the method of fermentation with special strains of Yeast. Kluyver, too, found very low values (3·2 % of total sugars) for maltose as compared with those of Brown & Morris (27·5 % and, even, in another case, 55·6 % of total sugars) in leaves gathered under similar conditions, though in regard to the other sugars, their respective values were very similar. Kluyver and Davis & Sawyer used leaves dried very much more rapidly than those of Brown & Morris, and found either very little (K.) or no (D. & S.) maltose present. Davis & Sawyer conclude, therefore, that maltose, when found in either small or larger quantities, is formed in the drying process. In the living leaf, moreover, they consider that maltose is speedily hydrolysed to glucose by the maltase which is present in the leaves.

Values for maltose, based on hydrolysis with acids, which have been given by many observers are open to suspicion, for, even if basic lead acetate is used to remove tannins and glucosides, not necessarily all glucosides are precipitated by this reagent, and therefore, on hydrolysis, these may lead to increase of sugar. In any case,

values for maltose in presence of cane-sugar, obtained by heating with dilute hydrochloric acid or sulphuric acid, are open to error, as fructose is largely destroyed by this treatment. Hydrolysis with 2·44 % hydrochloric acid, for instance, leads to the destruction of 31 % of fructose.

Gentiobiose (Armstrong, 5) as already explained (see p. 13) is a β-glucosido-6-glucose. It occurs in the trisaccharide, gentianose, and also, in combination with a mandelonitrile as the glucoside, amygdalin, in several genera of the Rosaceae. The decomposition of amygdalin takes place in three stages involving the following reactions in which two enzymes, amygdalase and prunase (together emulsin), also found in the plant, are involved. The first of these reactions is a splitting of the linkage between the two sugar molecules, the reaction being catalysed by the enzyme, amygdalase:

$$C_{20}H_{27}NO_{11} + H_2O = C_{14}H_{17}NO_6 + C_6H_{12}O_6$$



Amygdalin

In the second reaction the remaining glucose molecule is set free, the reaction being catalysed by the enzyme, prunase:

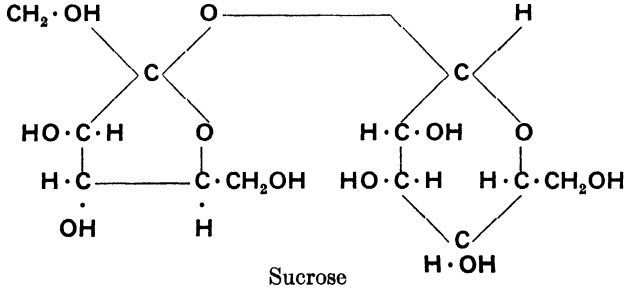
$$C_{14}H_{17}NO_6 + H_2O = C_8H_7NO + C_6H_{12}O_6.$$

Finally, the mandelonitrile is decomposed into prussic acid and benzaldehyde:

$$C_8H_7NO = C_6H_5CHO + HCN.$$

Next will be considered the disaccharides formed from glucose and other sugars.

Sucrose. This disaccharide is very widely distributed in the plant in all organs. In leaves it is almost invariably present in greater amount than the hexoses. Recent work has shown that sucrose is a condensation product of glucose and the 5-ringed lactone form of fructose (γ-fructose), and has the constitution

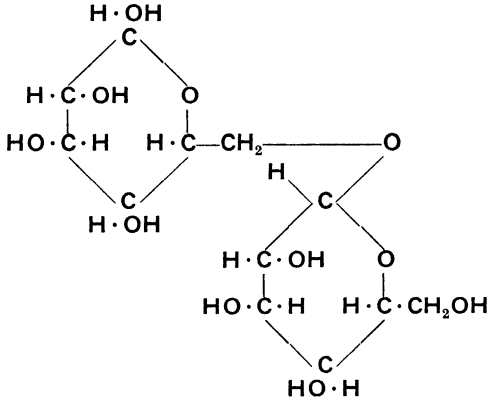


Sucrose

It is not yet known whether the α- or β-form of either hexose is involved in the linkage.

The biochemical significance of sucrose in the plant is discussed in connection with the hexoses (see p. 46).

Melibiose does not occur as such in the plant, but is only formed in the partial hydrolysis of raffinose. It is a condensation product of glucose and galactose:



Melibiose

Several disaccharides are known which are condensation products of a hexose and a pentose (Armstrong, 5):

Primeverose occurs in a number of glucosides. These are primeverin, primulaverin (*Primula officinalis*), gentiacaulin and monotropin (*Monotropa Hypopitys*, *Betula lenta*, *Spiraea Ulmaria*, *S. Filipendula*, *S. gigantea*).

Vicianose occurs in the glucosides, vicianin from *Vicia angustifolia* and gein from *Geum urbanum*. The sugars concerned are said to be glucose and arabinose.

Strophantobiose occurs in the glucoside, strophanthin, found in seeds of species of *Strophanthus*. It gives, on hydrolysis, mannose and rhamnose.

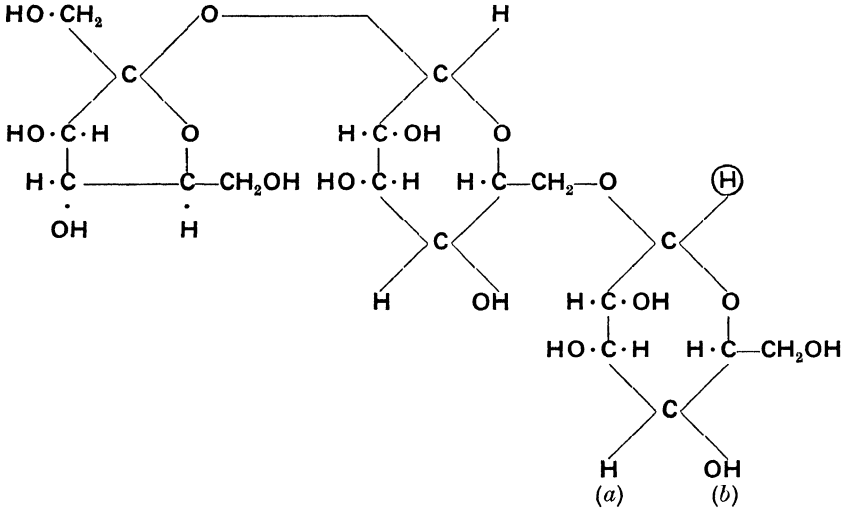
Trisaccharides

These may consist of various sugars (Armstrong, 5).

Gentianose is a non-reducing trisaccharide found in the roots of *Gentiana lutea*. On hydrolysis, it gives, with dilute acids, a mixture of fructose and glucose; with emulsin, a mixture of sucrose and glucose;

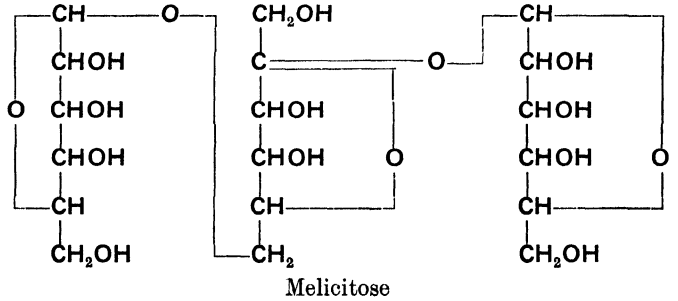
or, on the other hand, with invertase, a mixture of fructose and gentiobiose. Its constitution, therefore, is considered to be that given below.

Raffinose is also a non-reducing trisaccharide found in the Sugar Beet, Barley grains and seeds of the Cotton Plant (*Gossypium herbaceum*). On hydrolysis, it gives, with acid, a mixture of fructose, glucose and galactose; with emulsin, a mixture of sucrose and galactose; and, finally, with invertase, melibiose and fructose. The constitution set out below has been adopted.



Glucose [in gentianose]
Galactose (*a*, *b* and (H) inverted) [in raffinose]

Melicitose occurs in exudations (manna) from certain plants, namely in Briançon manna from *Larix europaea*, Persian manna from *Alhagi maurorum* and in manna from *Pseudotsuga Douglasii*. It does not reduce Fehling's solution, and, on hydrolysis, it gives two molecules of glucose and one of fructose:



Melicitose

Tetrasaccharides

Stachyose has been extracted from tubers of *Stachys tubifera*. It is a non-reducing sugar which, on hydrolysis, gives one molecule of glucose, one of fructose and two molecules of galactose.

Analyses of Sugars in the Plant

The interrelationships of the mono-, di- and polysaccharides have been chiefly studied in the leaf, since it is in this organ that primary synthesis of carbohydrates takes place. Quantitative analyses of sugars, starch, etc., have been made by several investigators, using, as material, occasionally the same species, but more often quite different plants. The chief of these investigations are:

1893. Brown & Morris. Leaves of Garden Nasturtium (*Tropaeolum majus*).
1898. Went. Internodes of Sugar Cane (*Saccharum officinarum*).
1911. Parkin. Leaves of Snowdrop (*Galanthus nivalis*).
1912. Campbell. Leaves of Mangold (*Beta vulgaris*).
1912. Ruhland. Leaves of Sugar Beet (*Beta vulgaris*, var. *Rapa*).
1912. Deleano. Leaves of Vine (*Vitis vinifera*).
1914. Colin. Leaves and root of Sugar Beet.
1916. Davis, Daish & Sawyer. Leaves of Mangold and Potato (*Solanum tuberosum*).
1917. Gast. Leaves of *Cucurbita* and other plants.
1918. Kylin. Leaves of Tulip (*Tulipa sylvestris*) and other plants.
1919. Spoehr. Vegetative axes of *Opuntia phaeacantha*.
1922. Shroeder & Horn. Leaves of *Tropaeolum*.
1923. Weevers. Leaves of Maple (*Acer Negundo*) and other plants.
1924. Ahrns. Leaves of *Tropaeolum* and other plants.
1924. Miller. Leaves of Maize (*Zea Mays*) and Sorghum (*Andropogon Sorghum*).
1925. Tollenaar. Leaves of Tobacco (*Nicotiana Tabacum*).
1926. de Wolff. Tubers of Potato.
1928. Evans. Fruit of Apple (*Pyrus Malus*).
1928. Haynes & Archbold. Fruit of Apple.
1928. Mason & Maskell. Vegetative organs of Cotton Plant (*Gossypium herbaceum*).
1928. Keulemans. Leaves of *Tropaeolum*.

In such estimations, the sources of error are considerable. Apart from those inherent in the methods of estimation, many errors arise from treatment and extraction of material. Some of these have been detected and eliminated by the later investigators. Changes in the material used may be produced by relatively slow drying as in the case of *Tropaeolum* (Brown & Morris) where maltose may have been formed by the action of diastase. Hence, in more recent cases,

material has been extracted with boiling alcohol. In regard to errors in actual method, many workers, for instance, have estimated maltose by hydrolysing the extract of sugars with dilute hydrochloric or sulphuric acid and measuring the increase of reducing power. Davis & Daish (30) claim that fructose may be almost entirely destroyed by this method, so that values by difference would be useless. Again, Davis, Daish & Sawyer have estimated pentoses and pentosans in leaves without taking into account the presence of uronic acids in pectins or possibly free in the cell. Further, extracts of sugars from the plant are frequently mixtures of sugars, namely, glucose, fructose and, possibly, pentoses in unknown proportions. The methods for estimation, on the other hand, are adapted for solutions of pure sugars or mixtures in known proportions.

In addition, a serious source of error, probably, is that due to variation in the standard (dry or fresh weight) to which the estimations are referred. Appreciable decomposition and loss may take place in some tissues on drying at 100° C.,[1] and this may not be uniform. Usually, values for sugar content are given as percentages of dry weight, whereas important deductions might be made if data were produced to enable the concentration of various sugars to be calculated.

Finally, errors of sampling may be introduced owing to lack of uniformity in the material employed; usually insufficient data are given to enable any correction to be made for these variations. In conclusion, it may be said that, on the whole, a rapid and simple method is preferable to a slow and complex one, since the material and extracts are less likely to be altered. One of the most important points is the selection of a suitable standard to which the values obtained in estimation may be referred.

The results of some of the above investigators will now be briefly described. Others will be mentioned during the consideration of special points.

Brown & Morris (12), using leaves of *Tropaeolum*, estimated the relative amounts of starch, glucose, fructose, maltose and sucrose under various conditions. The probability that the maltose estimated was artificially produced has already been considered. It is also very doubtful, in the face of later estimations (Davis, 29), if the

[1] **Archbold, H. K.** Chemical Studies in the Physiology of Apples. VIII. Further Investigations of the Methods of determining the Dry Weight of Apple Pulp. *Ann. Bot.*, 1928, **42**, 29–38.

values obtained by these authors for fructose are reliable. Brown & Morris estimated all the above-mentioned carbohydrates in the normal leaf as compared with detached leaves kept in water, both in insolation and in darkness. Table III represents their results as published.

TABLE III. Analyses of carbohydrates in leaves of

Percentage of dry wt of leaf	Expt. 1			Expt. 2		Expt. 3 (after sunny day)			
	(*a*) Picked from plant at 5 a.m.	(*b*) Detached leaves in water, insolated till 5 p.m.	(*c*) Picked from plant at 5 p.m.	(*a*) Picked at 9 a.m.	(*b*) Picked at 4 p.m.	Picked and dried at once		Picked and placed with petioles in water, in dark 24 hours	
						(*a*)	(*a'*)	(*b*)	(*b'*)
Starch	1·23	3·91	4 59	3·24	4·22	3·69	5·43	2·98	0·91
Sucrose	4·65	8·85	3·86	4·94	8·02	9·98	7·33	3·49	3·35
Glucose	0·97	1·20	0·00	0·81	0·00	0·00	0·00	0·58	1·34
Fructose	2·99	6·44	0·39	4·78	1·57	1·41	2·11	3·46	3·76
Maltose	1·18	0·69	5·33	1·21	3·62	2·25	2·71	1·86	1·28
Total sugars	9·79	17·18	9·58	11·74	13·21	13·64	12·15	9·39	9·73

Their observations were as follows: starch accumulates during exposure to light, and decreases in darkness. In detached and insolated leaves, since no translocation can take place, the total sugars increase, the increase being in sucrose and fructose. When leaves are detached and kept in the dark, the total sugars decrease considerably, the loss being in sucrose, but not in fructose. Finally, the amount of glucose shows no important change.

Their deductions are that sucrose, and not glucose (since it does not increase under such conditions), is the first sugar of photosynthesis; and that glucose is preferably used in respiration and metabolic processes, and, hence, fructose accumulates.

Went (90) made an extensive series of analyses of the sucrose and total hexose content of the consecutive internodes of the stem of the Sugar Cane, plants of different ages being used. For analysis, a stem was divided into its respective internodes; these were numbered, commencing from the base; each was weighed and minced, an aliquot portion being then taken for extraction (alcohol and water). Sucrose was estimated polarimetrically, and hexoses by Fehling's solution. The results were expressed as percentages of fresh weight (also as weight of tissue residue obtained after extraction and of total sap of an internode).

As an example, the values for sugars in the stem of one plant are given in Table IV; Fig. 1 shows the graphic representation of these values for four different plants.

TABLE IV. Sugars in Internodes of Sugar Cane (Went)

No. of internode. 1 = oldest member	Length of internode (cm.)	Wt of internode (gm.)	% sucrose of weight	% hexose of weight	Residue – sugars % of wt	% sucrose of residue	% hexose of residue	% sucrose in sap	% hexose in sap
1–10	7·5	18·0	5·0	0·5	20·6	24·3	2·4	6·4	0·6
11–14	7·5	36·0	5·8	1·9	13·2	44·0	14·4	6·7	2·2
15–16	10·0	57·0	4·2	2·9	11·9	35·3	24·4	4·8	3·3
17–18	8·0	53·5	2·1	2·9	11·8	17·8	24·5	2·4	3·3
19–20	12·5	65·0	0·6	2·9	11·1	5·4	26·1	0·7	3·2
21–27	15·5	38·0	0·0	2·3	8·1	0·0	28·4	0·0	2·5

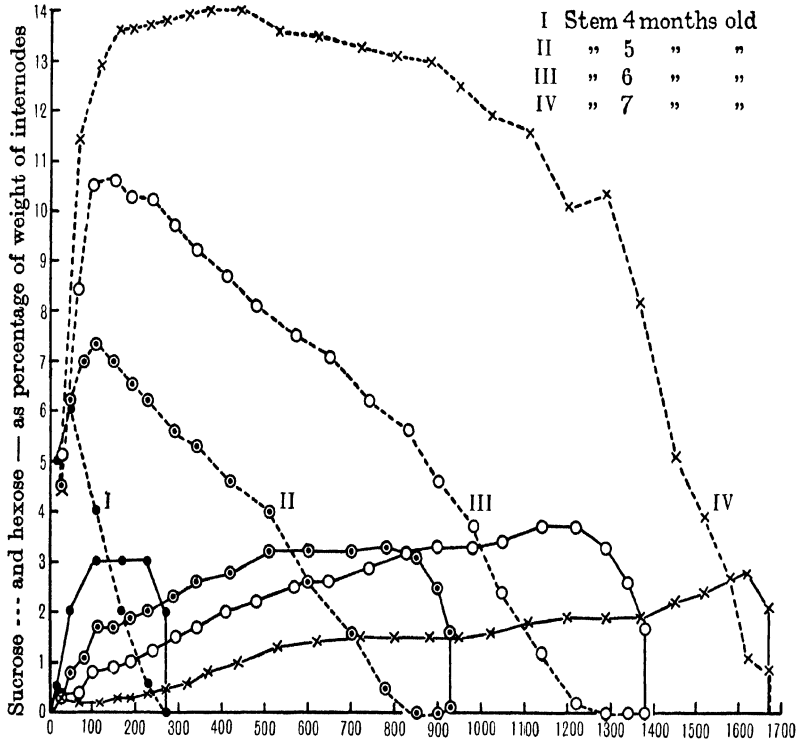


Fig. 1. Sucrose and hexose content of consecutive internodes of Sugar Cane (Went).

The main observations were as follows: The sucrose content increases with age of the internode (except close to the base of the stem) in any one stem. It also increases with the age of the plant during the vegetative period. In other words, the sucrose content is

very low near the growing-point and increases on passing down the stem until within a few internodes above the surface of the soil where the values fall. The sucrose value of any particular internode also increases with the age of the plant. The amount of hexose, as compared to sucrose, is small and varies inversely, that is, the greatest amount of hexose is found near the growing-point, and the values decrease on passing to the base of the stem. The amount of hexose, moreover, varies little with increasing age of the plant.

For carbohydrates in the leaf, qualitative tests only were made, and it was found to contain starch, sucrose and hexose. Starch accumulated by day and disappeared by night. There was a certain amount of starch in the younger internodes, but this disappeared from the older stem.

The phenomena are similar to those (to be described later) observed for the Mangold and Sugar Beet, though the storage organ in the Sugar Cane is the stem instead of the root. Sugar is synthesised in the leaf, and is translocated to the stem, where it is stored as sucrose, the hexoses diminishing in amount as storage proceeds.

Went forms the following picture of the changes taking place in any one internode from first development to maturity. Much starch is present at first. This gradually disappears, and is replaced by hexose, of which the quantity increases with growth in length. After growth ceases, the hexoses diminish, and sucrose accumulates, the maximum sucrose content coinciding with the minimum hexose content.

During growth, development of buds, roots, etc., the amount of hexose in the tissue increases; or when the supply of sugar from the leaf to stem ceases as in old ("over-ripe") stems. It would appear that a constant supply of hexoses is essential for the continuous formation of sucrose; if this supply falls off, that is, is diverted for growth, or the amount of synthesis falls, a reversal of the process takes place, and sucrose is hydrolysed to hexose.

Parkin (63) carried out estimations of glucose, fructose and sucrose, using leaves of the Snowdrop (*Galanthus nivalis*). This plant does not form starch in the leaf, except in very small quantities in the stomata, and Parkin was satisfied that maltose is absent. He compared normal leaves with insolated detached leaves, and, in addition, he compared leaves which had been kept in darkness with similar leaves after re-exposure to light. His observations are recorded in Table V.

The results may be summarised as follows: The proportion of sucrose to hexoses in the leaf decreases as the season advances (Table V, *a*). In a single leaf, the amount of sugar increases from above downwards, and, at the same time, the ratio of sucrose to hexose diminishes (Table V, *b*). Moreover, he states (Table V,

TABLE V. Sugars in Snowdrop leaf (Parkin)

(*a*) *Comparison during advance of season*

Date	% of dry weight		
	Sucrose	Hexose	Ratio
16. ii. 06	19·8	3·56	1 : 0·2
26. ii. 07	15·07	2·53	1 : 0·2
7. iii. 06	14·55	5·69	1 : 0·4
30. iii. 05	15·50	11·40	1 : 0·7
5. iv. 06	14·64	11·17	1 : 0·8
5. iv. 07	14·64	11·61	1 : 0·8
24. iv. 05	14·84	17·29	1 : 1·2
4. v. 05	10·30	12·78	1 : 1·2
11. iii. 10	12·74	5·67	1 : 0·4
30. iv. 10	10·42	12·38	1 : 1·19

(*b*) *Comparison of different portions of leaf*

Portion of leaf taken	Total sugars, % of dry weight	Ratio of sucrose to hexose
Upper $\frac{2}{3}$	25·95	1 : 0·6
Lower $\frac{2}{3}$	28·72	1 : 1·1
Upper $\frac{2}{3}$	21·98	1 : 1·05
Lower $\frac{2}{3}$	26·48	1 : 1·38
Upper $\frac{1}{2}$	22·48	1 : 1·39
Lower $\frac{1}{2}$	30·59	1 : 4·0
Upper $\frac{1}{2}$	22·93	1 : 0·57
Lower $\frac{1}{2}$	24·46	1 : 0·6
Upper $\frac{1}{2}$	19·26	1 : 0·5
Lower $\frac{1}{2}$	21·40	1 : 0·75
Upper $\frac{1}{2}$	24·03	1 : 0·65
Lower $\frac{1}{2}$	24·36	1 : 0·82

(*c*) *Comparison of leaves picked in the early morning with those gathered in late afternoon*

Time of estimation	% of dry weight			Remarks
	Sucrose	Hexose	Total	
9 a.m.	11·22	6·35	17·57	
3.30 p.m.	14·65	5·48	20·13	
	+3·43	−0·87	+2·56	
9 a.m.	9·80	12·38	22·18	
4.30 p.m.	14·65	11·61	26·26	
	+4·85	−0·77	+4·08	
8.15 a.m.	8·88	9·40	18·28	
4.15 p.m.	12·92	10·74	23·66	
	+4·04	+1·34	+5·38	
8.30 a.m.	9·74	11·61	21·35	Very cold
4.30 p.m.	10·10	11·94	22·04	day
	+0·36	+0·33	+0·69	
8.30 a.m.	5·09	2·77	7·86	After being
4.30 p.m.	12·55	3·61	16·16	in dark
	+7·46	+0·84	+8·30	4 days

(*d*) *Comparison of leaves picked in the evening with those gathered the following morning*

Time of estimation	% of dry weight			Remarks
	Sucrose	Hexose	Total	
4.30 p.m.	14·01	10·76	24·77	
8.0 a.m.	10·36	10·45	20·81	
	−3·65	−0·31	−3·96	
5.30 p.m.	15·46	11·41	26·87	
8.0 a.m.	10·84	12·64	23·48	
	−4·62	+1·23	−3·39	
4.0 p.m.	14·65	5·48	20·13	Plants
9.30 a.m.	9·64	5·67	15·31	covered
	−5·01	+0·19	−4·82	early morning
4.30 p.m.	14·65	13·66	28·31	Plants
12 noon	7·80	13·29	21·09	covered
	−6·85	−0·37	−7·22	till noon

TABLE V (*continued*)

(*e*) *Comparison of leaves detached and insolated with those left attached to the plant*

Time of estimation	% of dry weight			Remarks
	Sucrose	Hexose	Total	
4.30 p.m.	15·83	9·65	25·48	Attached
4.30 p.m.	19·02	8·85	27·87	Detached 9.30 a.m.
	+3·19	−0·80	+2·39	
4.30 p.m.	14·65	11·66	26·31	Attached
4.30 p.m.	20·42	10·50	30·92	Detached 9.30 a.m.
	+5·77	−1·16	+4·61	
4.30 p.m.	18·73	7·78	26·51	Attached
4.30 p.m.	20·66	7·41	28·07	Detached 9.30 a.m.
	+1·93	−0·37	+1·56	

(*f*) *Comparison of leaves detached from plants with those attached, both being kept in dark over night*

Time of estimation	% of dry weight			Remarks
	Sucrose	Hexose	Total	
4 p.m.	14·65	5·48	20·13	Control
9.30 a.m.	9·64	5·67	15·31	Attached
	−5·01	+0·19	−4·82	
9.30 a.m.	12·51	5·65	18·16	Detached
	−2·14	+0·17	−1·97	
6.30 p.m.	14·65	13·66	28·31	Control
Noon	7·80	13·29	21·09	Attached
	−6·85	−0·37	−7·22	
Noon	12·12	13·39	25·51	Detached; covered till noon
	−2·53	−0·27	−2·80	

(*g*) *Comparison after different periods of darkening*

	% of dry weight		
	Sucrose	Hexose	Total
Control	15·83	9·65	25·48
After 24 hrs.	8·13	9·83	17·96
After 48 hrs.	6·39	7·76	14·15
After 4 days	4·79	9·15	13·94
After 7 days	3·41	11·39	14·80
Control	14·65	11·66	26·31
After 11 days	2·27	9·54	11·81
After 14 days	5·05	6·54	11·59

c and *d*): "During any single day of the spring, the percentage of hexose sugars in the leaf remains fairly constant, no matter at what hour of the 24 the leaves may be examined. That of the sucrose, on the other hand, fluctuates greatly. It increases during the day and diminishes during the night. Further, leaves detached and insolated (Table V, *e*) contain decidedly more sucrose than their controls, but the quantity of hexose sugar remains much the same". In leaves which have a low concentration of sugars after darkening, on re-exposure to light (Table V, *c*) there is a rapid increase, of which the

chief source is sucrose. Leaves darkened for some days still contain a moderate quantity of sugar; the percentage falls rapidly during the first 48 hours of obscurity, and then remains fairly constant; the hexose may rise at the expense of sucrose and then may fall again (Table V, *g*). Fructose, as a rule, is in excess of the glucose, irrespective of the period or of time of day. Results also suggest that, as the total hexose sugar increases in proportion to the sucrose, so does the fructose rise in ratio to the glucose.

Parkin's deductions are essentially the same as those of Brown & Morris, namely, that sucrose is the first-formed sugar, and that fructose accumulates in relation to glucose.

Campbell (14) investigated the carbohydrates of the leaves of the Mangold. He estimated starch, total hexoses and sucrose at 2-hour periods during the day. By hydrolysing his extracts with hydrochloric acid he obtained values which he claimed to represent maltose. These values are only to be accepted with reservation; they may well represent glucoside sugars as he did not remove aromatic compounds with lead acetate. His values for sucrose have also been criticised by Davis, Daish & Sawyer (31) on the grounds that hydrolysis with citric acid under the conditions employed by him was incomplete. These authors also state that starch is absent from the leaves of the Mangold except in the young seedlings. Nevertheless, as will be mentioned later, Campbell's results fall into line with the general view of sugar interrelationships better than those of the other three investigators. The values (as % dry wt) found and the curves plotted are given in Table VI and Fig. 2.

TABLE VI. Carbohydrates in Mangold leaf. Percentages at 2-hour intervals (Campbell)

Time	6 p.m.	8 p.m.	10 p.m.	Mid-night	2 a.m.	4 a.m.	6 a.m.	8 a.m.	10 a.m.	Mid-day	2 p.m.	4 p.m.
Dry matter actual wt	4·04	3·56	3·62	3·3	3·78	3·63	3·70	4·35	4·12	4·26	4·52	4·84
Dextrose and levulose	4·67	3·58	3·98	3·92	3·81	2·64	4·70	4·43	3·44	4·33	3·57	4·65
Cane-sugar	2·20	1·46	1·23	1·47	0·68	0·45	0·79	0·63	1·72	1·76	1·92	2·20
Maltose	0·48	0·46	0·79	1·57	2·23	2·40	1·37	1·32	0·95	0·88	0·97	0·53
Starch	8·60	8·95	9·30	9·95	7·36	6·44	5·31	4·86	5·90	6·80	8·66	9·24

The chief results were as follows: the values for total hexoses were fairly constant, though the author considers the day value to be rather higher than the night value. Starch and sucrose increased during the day and diminished during the night.

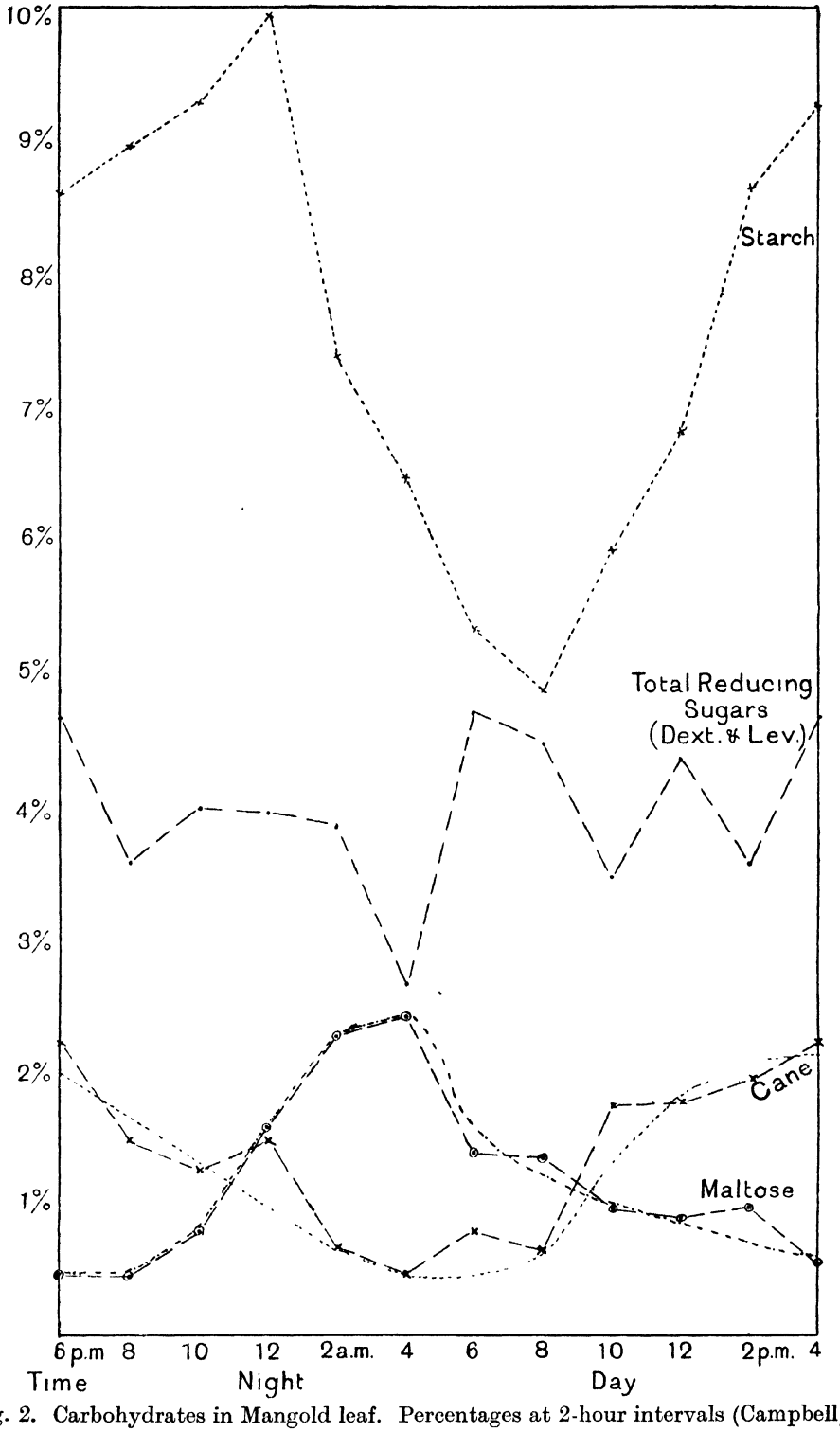


Fig. 2. Carbohydrates in Mangold leaf. Percentages at 2-hour intervals (Campbell).[1]

[1] Taken from *J. Agric. Sci.*, 1912, **4**, 252.

The author is inclined to the view that a hexose is the first product of photosynthesis.

Deleano (34) determined the amount of certain carbohydrates in the Vine leaf, namely, starch, total hexose and sucrose. The analyses were made in connection with an investigation on the respiratory activity of the leaves during varying periods of time, extending from 20 to 500 hours. Protein and certain soluble nitrogen compounds were also determined (see p. 211). In a series of experiments, two similar portions (*a*) and (*b*) were taken from a number of specially selected leaves. Portions (*a*) were dried, and estimations of starch, hexoses and sucrose were made. Of portions (*b*) the respiration was measured for varying periods. The portions (*b*) were dried at the end of each respiration period, and the carbohydrates estimated.

In brief, Deleano's results were as follows: after 100 hours' respiration there was a complete disappearance of sucrose and of the greater part of the starch from the leaf; at the same point, the values for hexoses also began to fall. A representative example of the initial values for carbohydrates in the leaf is the following:

	% dry weight	% fresh weight
Hexoses	11·59	2·85
Sucrose	1·08	0·26
Starch	9·65	2·37

It will be seen that the sucrose value is very low. This result is also commented upon by Davis, Daish & Sawyer (31). The Vine leaves were dried in Deleano's experiments in an oven at 100° C. Under these conditions there was possibly hydrolysis of cane-sugar, if not also of starch. It is therefore doubtful if reliance can be placed on the interrelationships of the various carbohydrates in this series of leaves.

The next work to be considered is that of Davis, Daish & Sawyer (31). These investigators used leaves of the Mangold. They estimated starch, glucose, fructose, maltose, sucrose, pentoses and pentosans in the leaves, mid-ribs and petioles at definite intervals of the day in certain periods during the season. They found the leaf only formed starch in the very young seedlings. Maltose, as already stated, was always absent. Their results for the three periods of the season are given in Tables VII, VIII and IX, and the graphs in Figs. 3, 4 and 5.[1]

[1] Tables VII, VIII and IX and Figs. 3, 4 and 5 are taken from *J. Agric. Sci.*, 1916, **7**, 255–326

Their observations were as follows: In the early stages of growth of the Mangold, the amount of sucrose in the leaves is greater than

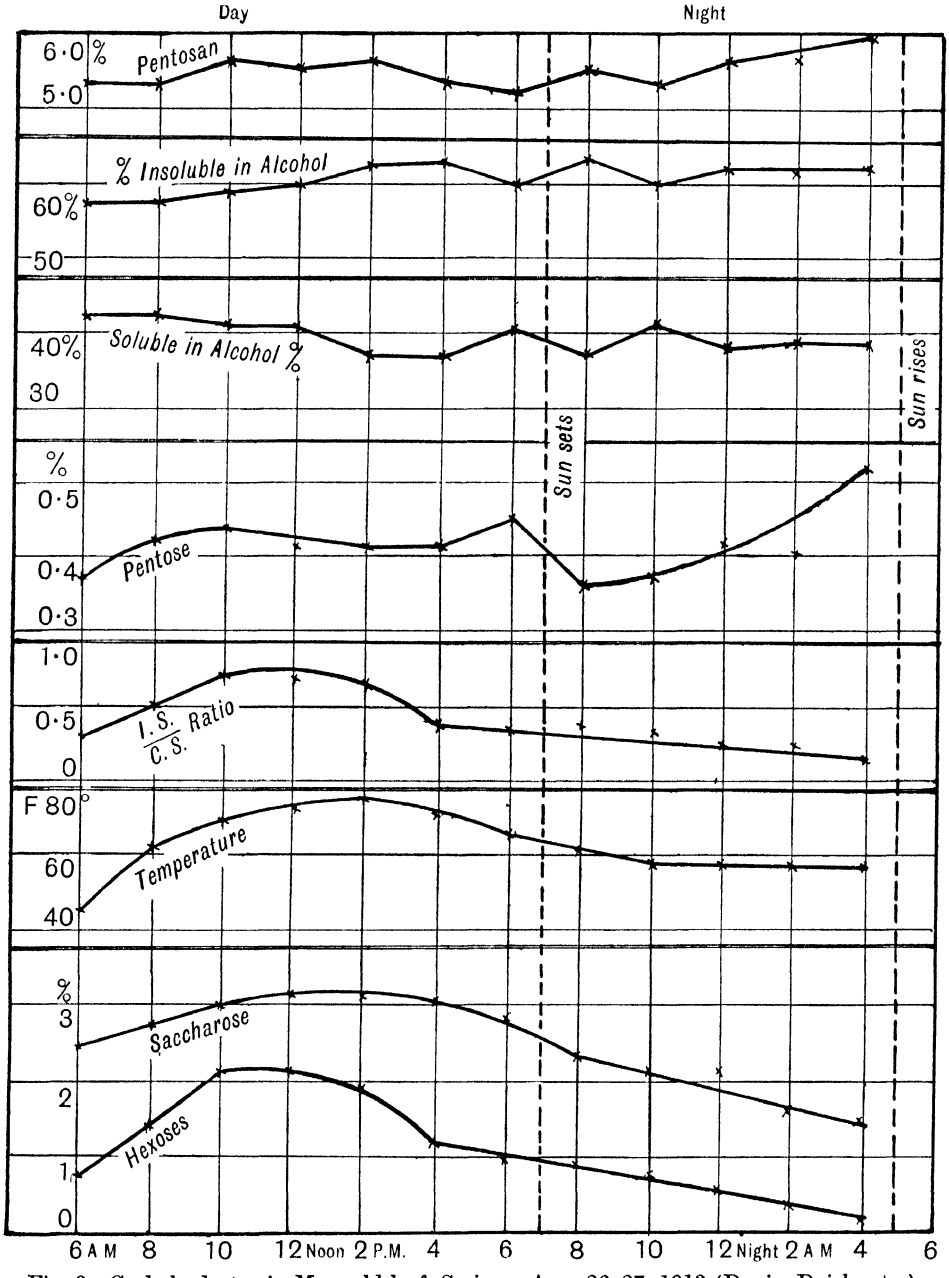


Fig. 3. Carbohydrates in Mangold leaf, Series I, Aug. 26–27, 1913 (Davis, Daish, etc.).

the hexoses. Later in the season, the hexoses preponderate. In the mid-ribs and petioles the hexoses always preponderate, increasing towards the bases. The authors consider the observations to be

explained best by regarding sucrose as the primary sugar of photo-synthesis. It is converted into hexoses in the veins, mid-ribs and petioles, and translocated as such to the root where it again becomes reconverted and stored as sucrose.

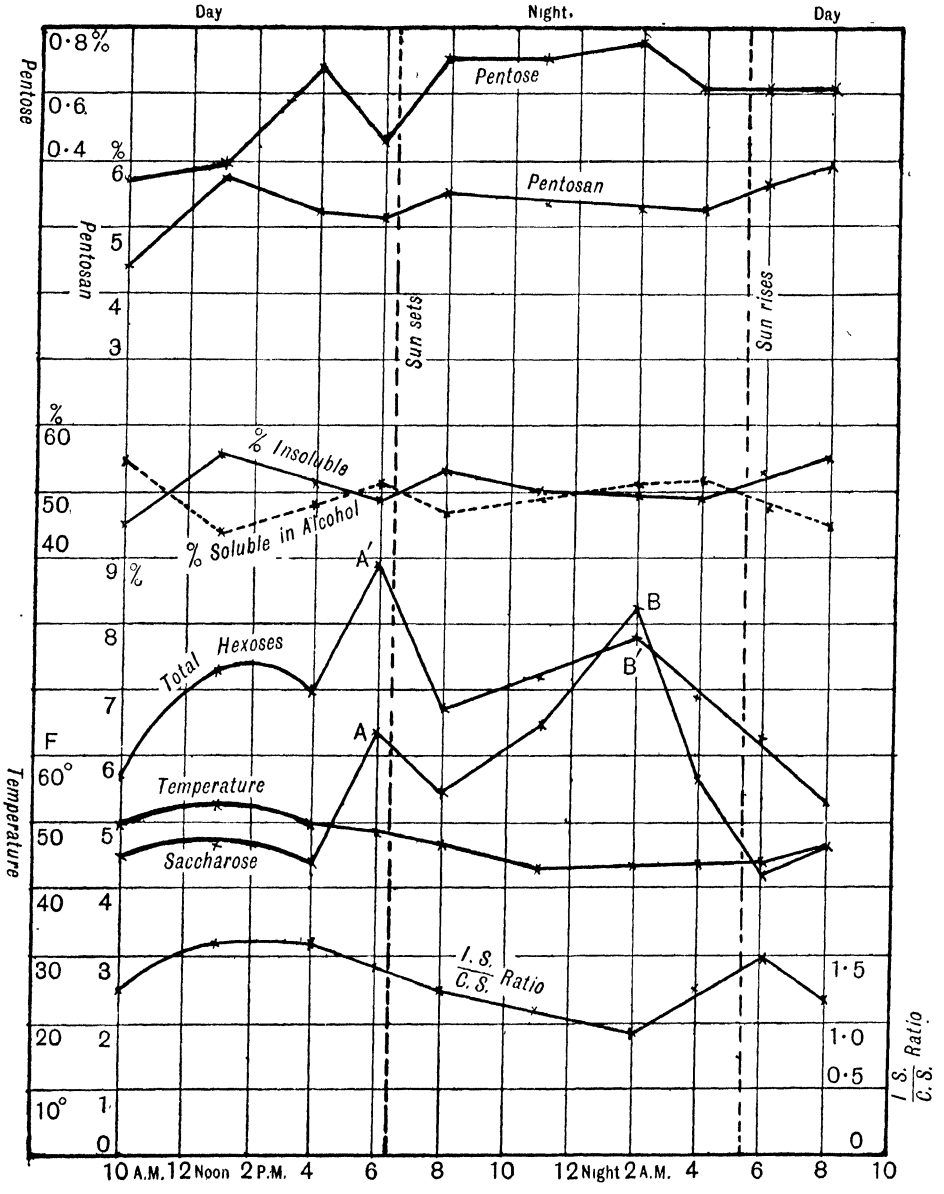


Fig. 4. Carbohydrates in Mangold leaf, Series II, Sept. 10–11, 1912 (Davis, Daish, etc.).

When, however, the data obtained by other investigators for sugars in the plant are examined, the impression gained is that the values

for the Mangold during the *later* periods, as shown graphically in Figs. 4 and 5, are not in accordance with the general interrelationships indicated between the various sugars. They are probably influenced by some variations in the standard to which they are related, and cannot be interpreted as the authors themselves believe.

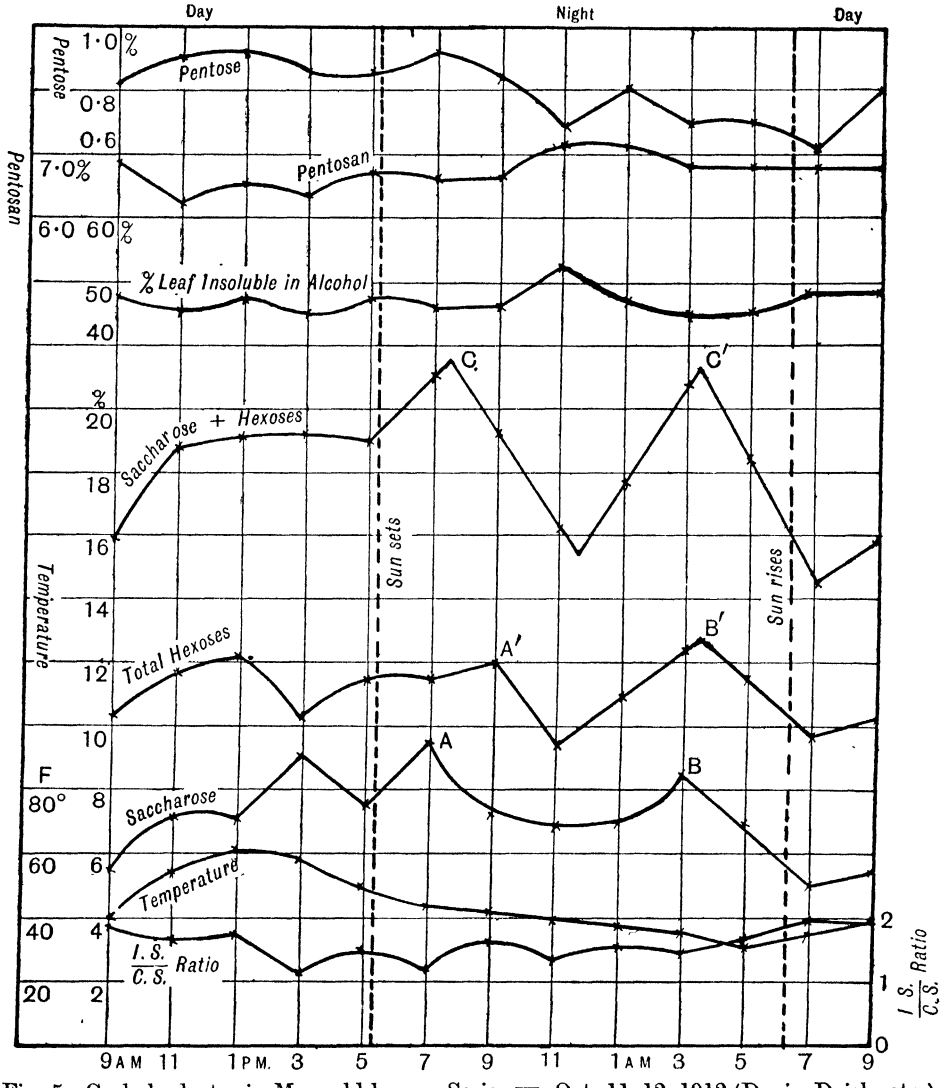


Fig. 5. Carbohydrates in Mangold leaves, Series III, Oct. 11–12, 1912 (Davis, Daish, etc.).

Further, Davis & Sawyer (33) carried out a similar investigation with leaves of the Potato. The results are given in Table X and Fig. 6.[1]

[1] Taken from *J. Agric. Sci.*, 1916, **7**, 352–384.

TABLE VII. Carbohydrates in Mangold leaves, Aug. 26–27, 1913 (Davis, Daish, etc.)

Aug. 26. Sun rises 5.4 a.m. Sun sets 7.0 p.m. Aug. 27. Sun rises 5.5 a.m. Sun sets 6.59 p.m.

Time	Temp.	% of leaf		Sacc. % in T.V.D.M.		Δ = C.A. − I.	Saccharose av. %	Hexoses % as I.S.	Saccharose + hexoses	Pentose %	Pentosan %	Maltose	Starch	I.S. / C.S. (av.)	Remarks
		Soluble in alcohol	Insoluble in alcohol	Citric acid	Invertase										
6 a.m.	45° F.	42·5	57·5	2·56	2·47	+0·09	2·51	0·77	3·28	0·37	5·38	0·00	0·00	0·307	Mists clearing away
8 a.m.	62°	42·5	57·5	2·76	2·75	+0·01	2·75	1·42	4·17	0·42	5·33	,,	,,	0·517	Brilliant sunshine
10 a.m.	70°	40·8	59·2	3·02	3·05	−0·03	3·04	2·16	5·20	0·44	5·65	,,	,,	0·710	,, ,,
12 noon	73°	40·6	59·4	3·17	3·04	+0·13	3·11	2·15	5·26	0·41	5·53	,,	,,	0·691	,, ,,
2 p.m.	75° (max.)	37·3	62·7	3·23	2·96	+0·27	3·09	1·94	5·03	0·41	5·61	,,	,,	0·628	Bright sun
4 p.m.	71°	37·2	62·8	3·09	3·01	+0·08	3·05	1·18	4·23	0·41	5·39	,,	,,	0·387	Still quite bright, but slightly hazy
6 p.m.	66°	40·1	59·9	2·96	2·68	+0·28	2·82	0·96	3·78	0·45	5·19	,,	,,	0·341	Bright sunshine
8 p.m.	61°	37·1	62·9	2·47	2·22	+0·25	2·35	0·90	3·25	0·36	5·50	,,	,,	0·383	Just dark
10 p.m.	58·5°	40·4	59·6	2·22	2·09	+0·13	2·15	0·74	2·89	0·37	5·31	,,	,,	0·344	Dry
12 night	58°	38·0	62·0	2·41	1·96	+0·45	2·18	0·57	2·75	0·42	5·65	,,	,,	0·261	Dry, but cloudy
2 a.m.	57°	38·6	61·4	1·70	1·46	+0·24	1·58	0·38	1·96	0·40	5·64	,,	,,	0·241	Cloudy
4 a.m.	56° (Min. 54° at 5 a.m.)	38·0	62·0	1·71	1·28	+0·43	1·50	0·20	1·70	0·52	5·96	,,	,,	0·133	Slight rainfall at 4 a.m.
6 a.m.	54·5°	—	—	—	—	—	—	—	—	—	—	—	—	—	First dawn = 4.45 a.m.
8 a.m.	57°	—	—	—	—	—	—	—	—	—	—	—	—	—	—

TABLE VIII. Carbohydrates in Mangold leaves, Sept. 10–11, 1912 (Davis, Daish, etc.)

Sept. 10. Sun rises 5.28 a.m. Sun sets 6.26 p.m. Sept. 11. Sun rises 5.30 a.m. Sun sets 6.24 p.m.

Time	Temp.	% of leaf*		Sacc. % in T.V.D.M.		Δ = C.A. − I.	Saccharose av. %	Hexoses %† as I.S.	Saccharose + hexoses	Pentose %	Pentosan %	Maltose	Starch	I.S./C.S. (av.)	Remarks
		Soluble in alcohol	Insoluble in alcohol	Citric acid	Invertase										
10 a.m.	50° F.	54·7	45·3	4·60	4·43	+0·17	4·51	5·72	10·23	0·34	4·42	0·00	0·00	1·27	Dull, cold wind
1 p.m.	53°	44·2	55·8	4·86	4·57	+0·29	4·62	7·50	12·12	0·39	5·74	,,	,,	1·59	,, ,,
4 p.m.	50°	48·5	51·5	4·41	4·35	+0·06	4·38	7·00	11·38	0·68	5·25	,,	,,	1·60	Dull, slight rain 3.30
6 p.m.	49°	51·0	49·0	6·46	6·32	+0·14	6·39	8·90	15·29	0·45	5·18	,,	,,	1·39	Dull, slight rain
Dark 7 p.m.															
8 p.m.	47°	47·2	52·8	5·61	5·27	+0·34	5·44	6·76	12·20	0·71	5·52	,,	,,	1·24	Clear, starlight
11 p.m.	43°	49·8	50·2	6·35	6·47	−0·12	6·41	7·10	13·51	0·71	5·31	,,	,,	1·11	,, ,,
2 a.m.	44°	50·7	49·3	8·28	8·26	+0·02	8·27	7·81	16·08	0·76	5·29	,,	,,	0·94	Cloudy
4 a.m.	44°	51·3	48·7	5·68	5·57	+0·11	5·62	6·91	12·53	0·62	5·26	,,	,,	1·23	,,
Light 5 a.m.															
6 a.m.	44°	47·5	52·5	4·23	4·24	−0·01	4·24	6·30	10·54	0·65	5·67	,,	,,	1·48	Overcast
8 a.m.	46°	45·7	54·3	4·79	4·42	+0·37	4·60	5·38	9·98	0·61	5·90	,,	,,	1·17	,,

* Calculated on total vacuum-dried matter of the leaf.

† After allowing for the pentoses present.

TABLE IX. Carbohydrates in Mangold leaves, Oct. 11–12, 1912 (Davis, Daish, etc.)

Oct. 11. Sun rises 6.19 a.m. Sun sets 5.15 p.m. Oct. 12. Sun rises 6.21 a.m. Sun sets 5.13 p.m.

Time	Temp.	% of leaf*		Sacc. % in T.V.D.M.		Δ = C.A. − I.	Saccharose av. %	Hexoses %† as I.S.	Saccharose + hexoses	Pentose %	Pentosan %	Maltose	Starch	I.S./C.S. (av.)	Remarks
		Soluble in alcohol	Insoluble in alcohol	Citric acid	Invertase										
9 a.m.	40° F.	52·2	47·8	5·67	5·38	+0·29	5·53	10·32	15·85	0·82	6·89	0·00	0·00	1·87	Foggy night, sun getting through at 9 a.m.
11 a.m.	55°	54·6	45·4	7·29	7·02	+0·27	7·16	11·62	18·78	0·91	6·21	,,	,,	1·62	Warm and sunny
1 p.m.	61°	52·5	47·5	7·19	6·83	+0·36	7·01	12·12	19·13	0·92	6·59	,,	,,	1·73	Very sunny
3 p.m.	58°	54·9	45·1	8·92	9·11	−0·19	9·02	10·24	19·26	0·86	6·35	,,	,,	1·14	Sunny
5 p.m.	50°	52·5	47·5	7·64	7·41	+0·23	7·52	11·46	18·98	0·86	6·68	,,	,,	1·52	Cooler, haze
Dark 6.30															
7 p.m.	44°	54·0	46·0	9·48	9·56	−0·08	9·52	11·47	20·99	0·92	6·60	,,	,,	1·20	Hazy
9 p.m.	42°	54·2	45·8	7·41	7·16	+0·25	7·28	11·98	19·26	0·84	6·65	,,	,,	1·65	,,
11 p.m.	39°	47·9	52·1	6·80	6·78	+0·02	6·79	9·39	16·18	0·68	7·15	,,	,,	1·38	Slight fog
1 a.m.	38°	52·6	47·4	7·15	6·68	+0·47	6·92	10·78	17·70	0·80	7·09	,,	,,	1·56	Cold and foggy
3 a.m.	36°	55·9	44·1	8·40	8·42	−0·02	8·41	12·41	20·82	0·70	6·78	,,	,,	1·48	Ice on leaves
5 a.m.	31°	54·7	45·3	6·93	6·88	+0·05	6·91	11·49	18·40	0·70	6·77	,,	,,	1·67	Ice thick on leaves
1st light 5.30 7 a.m.	35°	51·6	48·4	5·08	4·88	+0·20	4·98	9·62	14·50	0·61	6·77	,,	,,	1·93	Leaves frozen stiff

* Calculated on total vacuum-dried matter of the leaf.

† Allowance has been made for the pentoses present.

In general, though the Potato has a typical starch leaf, the observations were as with the Mangold leaf. Sucrose was found in excess of hexoses in the leaf, but the reverse was true for the petioles. During photosynthesis, starch and sucrose increase. The hexoses are present in small amount and fluctuate in value. The authors conclude that, again, sucrose is the first-formed sugar; this is hydrolysed in the veins, petioles, etc., and translocated to be stored as starch in the tuber.

Finally, a quotation may be made from one of the publications of the above authors (31): "In all plants of which a systematic examination has been made (mangold, sugar beet, potato, snowdrop, grape vine, dahlia, etc.) saccharose is formed directly in the mesophyll of the leaf, whence it passes into the veins, mid-ribs and stalks, undergoing more and more complete inversion in its passage".

Spoehr (81) made an examination of the carbohydrates of the succulent stem axes of the xerophyte *Opuntia phaeacantha*. He estimated pentoses, hexoses and sucrose, and, after hydrolysis, the total pentose polysaccharides and hexose polysaccharides (including starch which was not estimated separately). His investigations were on rather different lines from those of other workers, and covered a wider field. He determined, for instance, the effect of seasonal changes on carbohydrate metabolism; also the effect of change of temperature and artificially induced conditions of drought over long periods. Certain observations on variations in carbohydrates during day and night are given in Table XI.

The work of Weevers (89) constitutes a comparison of the sugars in the green and the white portions of the variegated leaves of a number of plants (*Acer Negundo, Ilex Aquifolium, Hedera Helix, Humulus Lupulus, Euonymus japonica, Aesculus Hippocastanum, Cornus sanguinea, Pelargonium zonale* and others). He claims to have shown that the green portions of the leaves contain both hexoses and sucrose; the white portions, sucrose only. He believes that a hexose is the first product of photosynthesis, and that sucrose only appears in transport. Of this, he considers, his results afford confirmation, for it has been shown by Sachs that starch is not found in white parts of variegated leaves; hence, they cannot form hexoses, and should contain only sucrose. The account of the methods and mode of representation of the data make it difficult to appraise the correctness of Weevers' values. He also maintains that in leaves of *Pelargonium*, depleted of starch and sugar by keeping in the dark,

on exposure to light, hexoses are formed first. The white portions also contain invertase.

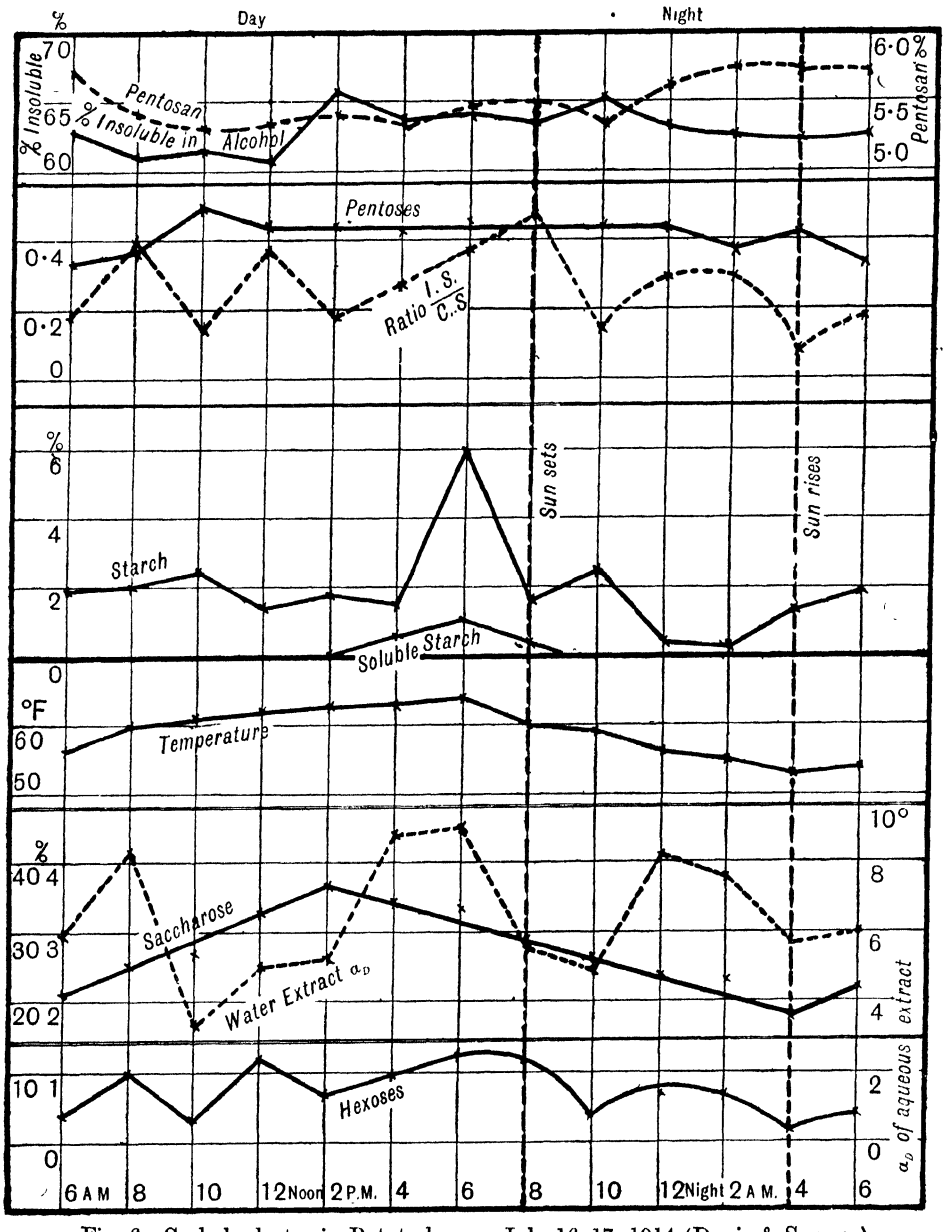


Fig. 6. Carbohydrates in Potato leaves, July 16–17, 1914 (Davis & Sawyer).

Miller (59) used, as material, leaves of varieties of Maize and Sorghum. In several series of estimations he determined the starch, total hexoses and sucrose at 2-hour intervals during the day and

TABLE X. Carbohydrates in Potato leaves, July 16–17, 1914 (Davis & Sawyer)

July 16. Sun rises 4.2 a.m. Sun sets 8.10 p.m. July 17. Sun rises 4.4 a.m.

Time	Temp. ° F.	% T.V.D.M. soluble in alcohol	Saccharose % on T.V.D.M.				Hexoses as invert sugar %	I.S./C.S.	Pentose %	Pentosan %	Maltose %	Aqueous extract		Soluble starch %	True starch %	Remarks
			Citric acid	Invertase	Δ = C.A. − I.	Average						α_D per 100 gm. T.V.D.M.	α_D calc. as sol. starch %			
6 a.m.	57	37·2	2·16	2·11	+0·05	2·14	0·40	0·19	0·35	5·72	0·00	6·02°	1·49	0·00	1·88	Sunny
8 a.m.	60	39·1	2·47	2·60	−0·13	2·53	1·00	0·39	0·37	5·37	,,	8·32	2·06	,,	2·00	Sunny
10 a.m.	61	38·5	2·81	2·65	+0·16	2·73	0·37	0·14	0·52	5·30	,,	3·36	0·83	,,	2·55	Slightly overcast
12 noon	62	39·3	3·39	3·19	+0·20	3·29	1·21	0·37	0·43	5·35	,,	5·06	1·24	,,	1·40	Shower 1.30
2 p.m.	63	34·1	3·81	3·50	+0·31	3·66	0·67	0·18	0·44	5·40	,,	5·30	1·31	,,	1·81	Shower 3.30
4 p.m.	63	36·2	3·56	3·34	+0·22	3·45	0·93	0·27	0·42	5·33	,,	8·88	2·20	0·58	1·56	Very bright
6 p.m.	64	35·8	3·46	3·22	+0·24	3·34	1·27	0·38	0·46	5·42	,,	8·96	2·22	1·00	5·95	Bright
8 p.m.	60	36·6	2·77	2·69	+0·08	2·73	1·22	0·45	0·42	5·51	,,	5·67	1·40	0·36	1·61	Dark 9.15 p.m.
10 p.m.	59	34·8	2·76	2·49	+0·27	2·63	0·40	0·15	0·45	5·35	,,	5·02	1·24	0·00	2·60	
12 night	56	36·6	2·48	2·30	+0·18	2·39	0·73	0·30	0·44	5·60	,,	8·32	2·05	,,	0·24	
2 a.m.	55	37·5	2·38	2·26	+0·12	2·32	0·68	0·29	0·37	5·74	,,	7·59	1·88	,,	0·28	1st light 3 a.m.
4 a.m.	53	37·7	2·09	1·44 (?)	+0·65 (?)	1·76	0·15	0·08	0·43	5·70	,,	5·71	1·42	,,	1·33	

night. The values obtained are expressed as percentages both of fresh weight of the leaf and of square metre of leaf surface; the latter figures are obtained by determination of the dry weight of a unit of leaf area for each period of analysis. Owing to the method of acid

TABLE XI. Variation in carbohydrate content of *Opuntia phaeacantha* during 24 hours. Results in percentages of dry weight (Spoehr)

	5 p.m.	7.30 a.m.	5 p.m.
Dry weight	17·58	18·80	17·85
Total sugars	16·18	19·40	20·45
Total polysaccharides	14·40	18·15	17·94
Disaccharides	0·58	0·34	1·18
Hexoses	1·06	0·83	1·27
Total pentose	8·60	8·33	9·78
Pentosan	8·34	8·22	8·55
Pentoses	0·26	0·20	0·23
Monosaccharides / Total polysaccharides	0·09	0·06	0·08

TABLE XII. Sugars in leaves of Maize and Sorghum (Miller)

Period	Percentage of fresh leaf									
	Dry matter		Water		Total sugars		Non-reducing sugars		Reducing sugars	
	Maize	Sorghum	Maize	Sorghum	Maize	Sorghum	Maize	Sorghum	Maize	Sorghum
6 a.m.	26·3	29·6	73·7	70·4	2·75	3·68	1·87	2·44	0·88	1·24
8 a.m.	27·3	30·6	72·7	69·4	4·68	4·64	3·44	3·19	1·24	1·45
10 a.m.	28·1	31·3	71·9	68·7	5·13	5·24	4·01	3·91	1·13	1·30
12 noon	28·7	32·4	71·3	67·6	4·89	5·50	4·25	4·80	0·64	0·70
2 p.m.	29·4	33·0	70·6	67·0	6·03	8·27	4·83	6·98	1·20	1·29
4 p.m.	28·9	33·6	71·1	66·4	5·13	—	4·44	—	0·69	—
6 p.m.	28·1	32·8	71·9	67·2	4·58	6·55	3·65	5·25	0·93	1·30
8 p.m.	26·8	31·5	73·2	68·5	3·31	4·22	2·54	3·28	0·77	0·94
10 p.m.	26·3	30·3	73·7	69·7	2·33	2·89	1·95	2·35	0·38	0·54
12 night	25·4	30·0	74·6	70·0	1·86	2·82	1·51	2·22	0·35	0·60
2 a.m.	24·3	28·4	75·7	71·6	2·19	3·16	1·60	2·20	0·59	0·96
4 a.m.	24·5	29·1	75·5	71·9	1·73	3·44	1·11	2·09	0·62	1·35
6 a.m.	26·4	29·3	73·6	70·7	2·61	3·75	2·02	2·52	0·59	1·23

hydrolysis employed for starch determination, the values obtained include other polysaccharides (pentosans, etc.), and are only comparative. Table XII and Fig. 7 represent, as a type, one series of estimations for Maize and Sorghum.

The facts observed are comparatively simple and are as follows: Both Maize and Sorghum form starch in the leaves and, at the same

time, they synthesise a not inconsiderable amount of sucrose, Sorghum producing more, relatively, than Maize. The approximate value for starch increases during the day and decreases again during the night. During 24 hours the amount of hexoses varies but little; there is, however, usually a definite, though small and irregular, increase during the day; this may be a real phenomenon or may be due to some undetected variation in the standard to which the values are related. In the case of sucrose there is undoubtedly, as compared

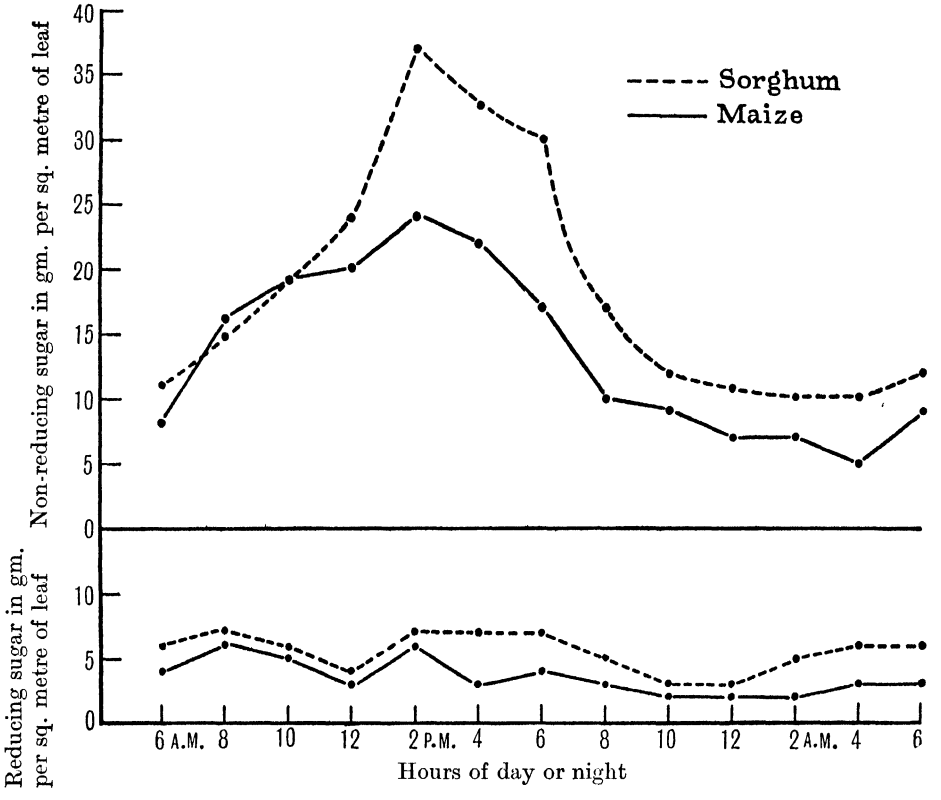


Fig. 7. Sugars of Maize and Sorghum leaves (Miller).

with the hexoses, a very considerable increase during the day, with a corresponding loss at night. Concordance in the results, as compared with those of some other investigators, is probably due to the successful choice of a standard for expression of the values and a relatively simple method of analysis.

The author concludes: "It would seem...that it cannot be determined definitely from chemical analyses whether the marked increase in the amount of the non-reducing sugars in the leaves during the day is due to the fact that they are the primary sugars of photo-

synthesis or to the fact that they are formed from the more simple sugars and accumulate in the leaves during the day as temporary storage products".

Evans (38), Archbold (4) and Haynes & Archbold (43), in a general investigation of the metabolism of the Apple, have made many determinations of the sugar content. Though the work is not strictly analogous to that on the leaves, etc., described in the previous pages, it is quoted here as it forms a comprehensive survey of sugars in the apple. They used, as material, different varieties of apples, and apples of the same variety grown on different soils. The sugars estimated were glucose, fructose and sucrose. The changes in sugar concentration taking place on storage at constant temperatures have also been investigated. Table XIII shows the orders of values in a typical case of storage.

TABLE XIII. Sugar content of apples stored at 12° C.
(Haynes & Archbold)

Date when analysed	Total sugar	Hexoses	Sucrose
2. xi. 27	11·62	9·97	1·64
15. xi. 27	12·13	10·80	1·30
29. xi. 27	11·25	10·36	0·88
13. xii. 27	11·01	10·33	0·67
4. i. 28	10·95	10·33	0·61
17. i. 28	10·82	10·26	0·55
7. ii. 28	10·33	9·85	0·48

From their analyses the authors draw the following conclusions concerning apples before picking: Hexoses, sucrose and starch are present in the young apple; starch increases at first, and the soluble carbohydrates steadily rise. When starch formation decreases, the soluble carbohydrate rapidly increases. In later stages, on ripening, starch rapidly disappears and the sucrose concentration rises. After picking, and on storage, the following changes gradually take place: the hexoses increase owing to hydrolysis of sucrose, but the value for total sugars falls owing to loss in respiration.

Evans (38) has determined, by the iodine oxidation method, the relative amounts of glucose and fructose. In mature apples the ratio is about 1 : 3. In storage, in the case of certain varieties, the ratio tends to decrease.

Mason & Maskell (57) have made an extensive examination of the carbohydrates of the vegetative organs of the Cotton Plant (*Gossypium*

herbaceum), chiefly with a view to finding out the path of translocation of carbohydrates and the mechanism of translocation.

The experimental method adopted differed in many respects from that of other workers. The tissues were frozen, the sap expressed, and the sugars in 5 c.c. of sap, after clearing with lead acetate, determined by the copper iodate method (Cole). Sucrose was estimated after inversion with invertase. From the values of dry weight determination of the tissue, concentration of sugars in the sap, weight of sap and total solids present in the sap, the weight of sugar associated with 100 gm. of water in the sap, and, finally, in the sample of tissue, was calculated. The absolute values of sugar were referred to the dry weight less total carbohydrates, this being considered more accurate than the usual methods. Polysaccharides were determined by hydrolysis of tissue with hydrochloric acid, and hence can only give comparative values.

The path of translocation of carbohydrates was explored by a series of physiological experiments, such as ringing, insertion of paper strips between bark and wood, etc., etc. After such treatment, the sugars were estimated in the various tissues affected, namely, bark (phloem and cortex), wood, leaves, etc. Conclusions are drawn from the correlations between concentrations in different organs and parts of organs.

The problem is treated more from the physiological than the biochemical aspect, and it seems doubtful whether the accuracy of the values for sucrose and hexoses is comparable with those obtained by the previous investigators we have been considering. On the whole they do not fall into good concordance with the general trend of results. The authors found, for instance, that the concentration of reducing sugars in the leaf is greater than that of sucrose, the latter being low; also that the concentration of both sugars rises during the day.

The results especially bearing on the problem of their investigation are as follows:

Diurnal variation of sugars in the leaf is, after a few hours, reproduced in the bark, much more than in the wood. The variation is chiefly in sucrose.

Ringing of the bark showed interruption of a downward transport of sugars. An accumulation of sugars occurred in bark and wood just above the ring, and in leaves some distance above; below the ring there was a loss of sugar. The greater part of the accumulation

and disappearance of sugar in bark and leaf is due to sucrose, change in reducing sugars being very small.

Complete separation of wood and bark by insertion of a paper strip had very little effect on the transport of sugar. It is therefore concluded that, in the main, the downward transport of carbohydrates is through the bark; that the bulk of carbohydrate transported through the bark is sucrose, and that, further, a difference of concentration determines the direction of movement. The process of migration resembles that of diffusion, but the rate is enormously greater.

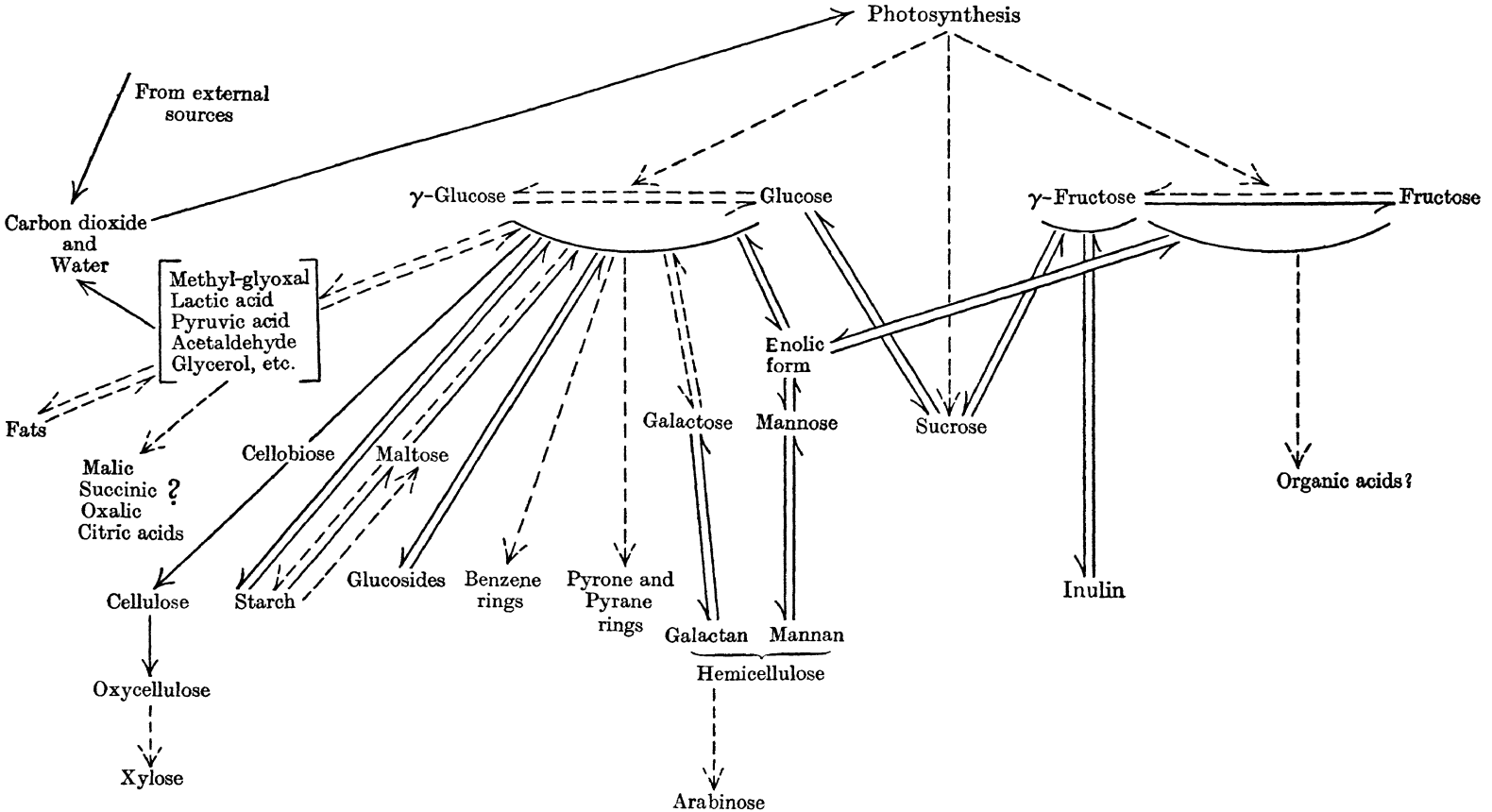
In a second communication (57) the authors explored more fully the analogy between the process of diffusion and the mechanism of transport. By experiments on ringing, removal of leaves, etc., they changed the direction of transport, and also restricted the channel of translocation.

From an examination of the radial distribution of sugars in the bark, and an estimation of the percentage of sieve tubes, they conclude that transport of sugar takes place mainly in the sieve tubes, and is determined by gradients of concentration; and, further, that it is analogous, except as regards absolute rate, to movement by diffusion.

Keulemans (47) has published a series of analyses of starch, hexoses, maltose and sucrose in the leaves of *Tropaeolum majus*. The half-leaf method of comparison was used (30–40 leaves), and the sugars were determined at intervals of 3, 6, 9 and 12 hours. The method of estimation was based on measurements of amounts of carbon dioxide produced by fermentation of the different sugars by selective strains of yeast. The values were referred to leaf area. The results are very irregular; in the main they are in accordance with those of earlier observers, but no new facts of fundamental significance are recorded. The impression is given that considerable errors may have been introduced owing to the complexity of the method, and the introduction of yeast preparations into the sugar extracts.

Iwanoff, Alexandrowa & Kudrjawzewa (96) have investigated the sugars of the Water Melon. In the very young fruit, glucose is present. Then small quantities of fructose appear, both sugars increase in quantity, but fructose definitely preponderates. At this point, saccharose appears, and increases in amount until the fruit is fully ripe.

Scheme of interrelationships between carbohydrates in the plant



------ indicates interrelationship uncertain.

The Interrelationships of the Sugars in the Plant

From the data available a survey of the interrelationships of the sugars will now be attempted. If the scheme on p. 45 is examined, it will be seen how complex the problem is, and how little, up to the present time, the various lines of investigation and the observed facts help us to find out the origin and relationships of the naturally occurring sugars.

Of the hexoses, glucose and fructose appear to be universally present, as is also the product of their condensation, sucrose. We do not know, however, whether both are formed in photosynthesis or whether they are readily interchangeable. Nor do we know whether the unstable (labile) γ-forms of glucose and fructose occur as such in the tissues, and, if so, what are their precise relationships to the stable (normal) forms in the plant.

Again, the position of maltose is quite uncertain. It is more reasonable to suppose that it is the direct product of hydrolysis of starch, but it is possible that it may not exist as such in starch and may, instead, be a condensation product of two hexose molecules when set free from the starch complex. It is doubtful whether it is present in the leaf and other assimilating organs.

Hence the case stands that, in such leaves as have been investigated, there are, in the main, only three sugars to be considered: glucose, fructose and sucrose.

Provisional suggestions

The outcome of a consideration of the various investigations on the carbohydrates shows that the published evidence does not cohere into a rational system. It has been suggested (Pringsheim;[1] Blackman, see p. 9) that active hexoses may play a part in sugar metabolism. The full significance, however, of this part may not yet have been brought to light. The possibility is here brought forward of developing this suggestion into certain propositions about the functions of active hexoses, which propositions, should they be substantiated, would serve as a key to the interrelations of the facts collected in this chapter.

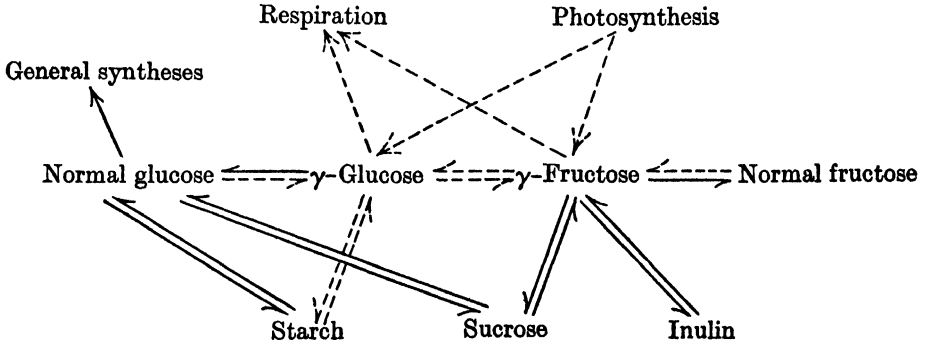
A preliminary statement of these propositions is made at this point, since it greatly facilitates the survey of certain aspects of the problem given in the following sections. Any application of these

[1] **Walton, R. P.** (editor). A Comprehensive Survey of Starch Chemistry. New York, 1928. This monograph contains an article: **Pringsheim, H.,** Bacterial Degradation and Constitution of Starch

views about active hexoses which can be made will be emphasised when dealing with the sections in question. The following are the suggestions to be considered:

1. Either γ-glucose or γ-fructose or both may be formed in photosynthesis.
2. γ-Glucose and γ-fructose may be interconvertible.
3. γ-Glucose may be a component of starch as γ-fructose is a component of sucrose and inulin.
4. γ-Glucose and γ-fructose are readily converted into stable glucose and fructose, but the reaction may not necessarily be reversible, unless there is some mechanism in the cell for the conversion of stable hexoses into γ-hexoses.
5. Sucrose may only be formed as an immediate product of photosynthesis and from the products of hydrolysis of starch.
6. Starch may only be formed as an immediate product of photosynthesis and from the products of hydrolysis of sucrose.
7. In the absence of photosynthesis, starch and sucrose are hydrolysed into their respective components (except in tissues where storage is taking place).
8. There may not necessarily be a direct equilibrium between sucrose and the stable glucose and fructose of the cell.
9. Either γ-glucose or γ-fructose or both may, in preference to the normal hexoses, form the substrate for respiration. Respiration, therefore, may be dependent either on the immediate products of photosynthesis or the presence of starch, sucrose or inulin (see p. 267).
10. Stable glucose (arising from the hydrolysis of starch and sucrose) may be the substrate for certain general syntheses, such as condensation to polysaccharides of the cell-wall, to glucosides and certain aromatic compounds.

The following scheme shows these suggested relationships:



The first sugar of photosynthesis

A question which has raised great interest and one which has given rise to much controversy is—Which sugar (or sugars) is first produced in photosynthesis? The alternative sugars suggested to play this *rôle* have been a hexose (glucose) and sucrose. As already seen, some investigators, namely Brown & Morris, Davis, Daish & Sawyer, Parkin and Went, have held the view that sucrose is the primary sugar of photosynthesis; others, namely Campbell, Priestley (65) and Weevers, that a hexose is the first-formed sugar. If sucrose is the first product, then it must either be condensed *en bloc* from smaller molecules, which are themselves the predecessors of sugar, or synthesised immediately from preformed glucose and fructose. In the latter case, these hexoses are constant components of the cell; so that additional production in photosynthesis should raise the concentration of hexoses already present, and lead to synthesis of sucrose. As we now know, however, that γ-fructose is a component of sucrose, we must assume that either this sugar is an immediate product of photosynthesis, or there is a mechanism in the cell for its formation from stable (normal) hexose.

With the exception of Davis, Daish & Sawyer, none of the advocates for sucrose as the primary sugar have made any definite suggestion as to the exact line of synthesis. In the discussion of this problem, Davis and his co-workers state: "It would seem, indeed, that plant leaves in general possess in *the chloroplasts a mechanism for elaborating cane sugar directly from the carbon dioxide of the air*". The evidence for regarding sucrose as the primary product has been, in the case of the majority of its partisans, the fact that the values for this sugar fluctuate much more than those for the hexoses, that is, it increases and decreases with varying external conditions, such as those, for instance, which control photosynthesis, whereas the hexoses remain more or less constant.

Though it should be borne in mind that erroneous deductions may be drawn where relative concentrations of metabolites are concerned, many of the facts recorded in the previous pages can be equally well interpreted by assuming a form of hexose as the primary product. There are then still alternatives: Is glucose the first product from which fructose may arise, and, subsequently, sucrose? Or are glucose and fructose formed simultaneously in photosynthesis? Whichever is true, the general trend of evidence suggests that the hexoses main-

tain a more or less uniform concentration. Increase in concentration of hexoses, on the one hand, should be expressed as an increase of sucrose concentration. A decrease in concentration of hexoses, on the other hand, should lead to hydrolysis of sucrose and a maintenance of equilibrium.

Schroeder & Horn (73) are opposed to the point of view of regarding sucrose as a temporary reserve product (see p. 56), since, in their investigations on *Tropaeolum* leaves, they found that the respective concentrations of sucrose and hexoses do not vary inversely. Further, it is true that, among the data of various observers, there is often apparently no direct connection between variations in sucrose and hexose concentration.

On the basis of the suggestions previously made on p. 47, a γ-hexose would be the product of photosynthesis. Sucrose is a stable compound in which the labile γ-fructose is in combination with normal glucose; it increases during photosynthesis, is temporarily stored and eventually translocated from the leaf. In the absence of photosynthesis, sucrose may be hydrolysed, as in a leaf or shoot in the dark. The γ-fructose is then available for respiration, and the residual stable glucose is available in the cell for general syntheses. There is no satisfactory evidence as yet that sucrose can be synthesised from stable glucose and fructose. Hence there may be no direct equilibrium.

À propos of this last point mentioned, the storage of sucrose in the root of the Beet from hexoses arriving from the leaves has, usually, been accepted as evidence for such a synthesis. Opinion, however, is divided as to which sugars really travel from leaf to root, and constitute the source for the storage of sucrose. An account of the various views held is given by Davis, Daish & Sawyer (31). Girard, Strohmer and Stephani may be quoted as authors maintaining that sucrose is formed in the leaf and travels as such to the root. Maquenne, Strakosch, Ruhland and Davis, Daish & Sawyer themselves, hold the opposite view, namely, that of the resynthesis from hexoses. Colin (19), however, is of the opinion that all three sugars can enter the root from the leaf.

The relationships in the plant between the synthesis of sucrose, which involves the presence of γ-fructose, and the balance of actions in inversion is still obscure.

The glucose-fructose ratio in the plant

Though we do not know whether glucose or fructose are both formed in photosynthesis, or whether glucose is directly converted into fructose, yet the question of the glucose-fructose ratio in various tissues has occupied the attention of several workers. From polarimetric data, Brown & Morris (12) found fructose in excess of glucose in the leaf of *Tropaeolum*. Parkin (63) also found this to be true for the Snowdrop leaf. Both authors considered that the glucose was used, in preference to fructose, in respiration. Lindet (see Davis, 29), on the other hand, found, on the whole, the opposite for the Sugar Beet. Davis (29), however, working on the Mangold leaf, points out that no real reliance can be placed on ratios obtained polarimetrically; for there are present, in the plant extract used, optically active substances, such as aspartic acid, asparagine, glutaminic acid and glutamine, which are not precipitated by basic lead acetate, and thus vitiate the estimations of the sugars. Davis is of the opinion that the fluctuations of the so-called glucose-fructose ratio observed in the leaves and leaf stalks are more likely to be due to fluctuations in the impurities than in the sugars, since they are seen also to be correlated with aberrant polarimetric values for sucrose as compared with the values obtained by reduction. A further error is introduced by the pentoses presumed to be present in the leaf, for there is no knowledge as to which of these are present and in what proportions. Arabinose and xylose, for instance, have very different specific rotatory powers, though their reducing powers are much the same. The above authors are of the opinion that such data as they obtained point to the presence of glucose and fructose approximately in the proportions of invert sugar; that, moreover, we have no idea of the metabolic significance of these sugars until the methods for estimation are improved. The same difficulty in estimating the glucose-fructose ratio was experienced for leaves of the Potato (Davis & Sawyer, 33).

Evans (38) used a chemical method for estimation of these two sugars in the Apple. He found that the fruit usually contains at least twice as much fructose as glucose, but the ratio varies considerably from variety to variety, those apples with a high sucrose content tending to have the higher fructose-glucose ratio. It is very difficult to gauge the significance of this ratio in the tissues, when it is obvious that each sugar may be involved in a number of different syntheses.

Concentration of hexoses

The amount of hexoses present in the tissues may vary in different plants and in different organs of the same plant. The general impression, from the data available, however, is that, over relatively long periods, the amount of total hexoses remains approximately the same. This is so in the case of leaves of the Mangold (Campbell), Potato (Davis & Daish), Maize and Sorghum (Miller), and Snowdrop (Parkin), as well as in apples (Haynes & Archbold). The hexoses show no rapid fluctuations as in the case of sucrose. The irregularities in the values shown by hexoses in the later estimations of Mangold leaves by Davis in September and October (pp. 32, 33) may, it would appear, be due to undetected alterations in the ultimate standard to which the values are referred.

The amount of hexoses in any organ may, however, gradually change through the various vegetative periods, as, again, for instance, in Mangold leaves, where the average amount found during the day is higher towards the end of the season. It is true that it is not possible in this case, as in many others, to calculate the actual concentrations, since the authors have not given values for the dry weights of the leaves, but it is obvious, from the actual amounts found, even allowing for a reasonable increase in the dry weight of the leaf with age, that the concentration of hexoses is greater at the end of the season. A similar seasonal rise in amount of hexose in the leaf was found for the Snowdrop by Parkin.

The values in Table XIV show the order of magnitude of hexoses as percentage of fresh weight (approximate concentration) in a few cases. Difficulty is usually experienced, as we have said, in ascertaining the concentration, because values for the dry weight are not given. The values of sugar per dry weight may, in extreme cases where the water content is either very small or very great, be misleading; for true comparison, concentration values are important.

Finally, we may consider how the suggestions made on p. 47 affect the question of hexose concentration. On the basis of these suppositions, one may regard the normal hexoses of the cell as residual, non-respirable and less active products than the γ-hexoses. They may, nevertheless, take part, in growing tissues, in certain syntheses, such as condensation to cellulose, aldol condensation to benzene rings and, especially, condensation with compounds of various natures to form glucosides. Their concentration would then depend to some

extent on the age and nature of the tissue. In mature organs, which have ceased to grow actively, or, in senescent organs, they may

TABLE XIV. Hexoses and sucrose (as percentage fresh weight) in leaves. Observations of various investigators

Observer	Plant	% fresh weight	
		Hexose	Sucrose
Kylin	*Tulipa sylvestris*	6·1	0·8
,,	*Narcissus poeticus*	2·5	0·8
,,	*Gentiana brevidens*	2·0	—
,,	*Hemerocallis fulva*	1·6	0·9
,,	*Fritillaria Imperialis*	1·2	0·4
,,	*Allium victoriale*	0·8	1·0
,,	*Veratrum nigrum*	0·8	0·8
,,	*Scilla sibirica*	0·6	0·2
,,	*Iris germanica*	0·4	0·4
,,	*Convallaria majalis*	0·1–2	—
,,	*Hosta siboldiana*	0·3	0·2
,,	*Tilia europaea*	0·3	0·8
,,	*Taraxacum officinale*	0·3	0·3
,,	*Bunias orientalis*	0·04	0·06
,,	*Acer platanoides*	0·02	0·2
Ahrns	*Tropaeolum majus*	0·3	1·0
,,	*Phaseolus vulgaris*	0·1	0·4
,,	*Helianthus annuus*	0·3	0·2
,,	*Pisum sativum*	0·3	1·0
,,	*Vitis vinifera*	0·4	0·7
Deleano	,,	2·9 (?)	0·3
Onslow	*Prunus Laurocerasus*	1·0	3·2
Spoehr	*Opuntia phaeacantha* (axis)	0·2	0·08
Campbell	*Beta vulgaris*	0·2	0·09
Haynes & Archbold	*Pyrus Malus* (fruit)	8·0 *ca.*	3·0 *ca.*
Miller	*Sorghum*	1·3	5·3
,,	*Zea Mays*	0·9	3·7

accumulate. Such might possibly be the explanation of the increased hexose values in mature leaves or the high values characteristic of certain ripe fruits.

Formation of starch and sucrose

If additional hexose is formed in photosynthesis, but the concentration in the leaves remains approximately the same, then the hexose must be condensed to other compounds; these are mainly starch and sucrose.

The case of starch is somewhat complicated by the observation that the leaves of all plants do not necessarily form starch to the same extent. In fact, five classes were differentiated by Meyer (58) (see also Parkin, 62) as a result of examination of a number of plants

in respect to the amount of starch formed in the leaves. These ranged from a class which forms abundant starch, through intermediate groups, to one from which starch was entirely absent. It was shown by various observers (Meyer, 58; Parkin, 62) that those tissues which form little or no starch can often be induced to do so by what is termed "sugar-feeding", that is, floating fragments of leaf in the dark on solutions (about 10–20 %) of various sugars, such as glucose, fructose, sucrose, invert sugar and galactose. With a few exceptions, the results show that sucrose is pre-eminently the best starch former; glucose, fructose and invert sugar rank about the same, whereas maltose and galactose have little effect. In a few cases, glycerine and mannite gave positive results.

As a consequence of these general observations, it has been concluded that a certain minimum concentration of sugar is necessary in any particular plant for starch formation. In the Dicotyledons this has been considered to be, on an average, rather low (0·2–0·5 %). For the Monocotyledons, on the other hand, it appears, as a rule, much higher, being from 15 to 20 %. In some Monocotyledons, namely the Onion (*Allium Cepa*), the Leek (*A. Porrum*) and the Snowdrop (*Galanthus nivalis*), no starch can be induced to form even on sugar-feeding. The outcome of these observations has led to a general use of the terms, starch leaves and sugar leaves. Of the leaves we have been especially considering in connection with sugars, the Potato is a typical starch leaf; the Mangold, according to Davis, is a sugar leaf, and only forms starch in the very young seedlings. The Snowdrop, another sugar leaf, was selected by Parkin, since, as it forms no starch, maltose would be expected to be absent.

On the whole, from such data as are available, it would appear that if sugar leaves do not form starch or other polysaccharides, they should have a higher concentration of total sugars than starch leaves (Gast, 40; Kylin, 48). From Tables XIV and XV this is seen to be the case, though there are exceptions, and some of the values, too, may not be very accurate. (It would be more satisfactory to express all values as concentrations, but in some cases no figures for dry weight are given.)

As a result of photosynthesis starch is formed in the leaf, presumably from glucose, and accumulates during the day. At night, hydrolysis exceeds synthesis, so that it diminishes in amount. There is good evidence, which will be discussed below, that, on hydrolysis, starch in the cell gives rise, under certain conditions, to sucrose It

has also been suggested (p. 47) that starch may contain a γ-hexose. If this were so, not only would the synthesis of sucrose from the products of hydrolysis of starch be significant, but also the fact mentioned earlier in this section that, conversely, sucrose far exceeds

TABLE XV. Analyses of carbohydrates in leaves and other organs (percentage of dry weight) by various investigators

Plant	Percentage of dry weight							
	Starch	Hexoses	Sucrose	Hexose and sucrose	Glucose	Fructose	Maltose	Other soluble carbohydrates
Tulipa (Kylin)	0·0	36·9	4·9	41·8	—	—	—	0·7
Narcissus ,,	0·0	16·1	5·4	21·5	—	—	—	4·2
Gentiana ,,	0·0	10·1	—	—	—	—	—	12·5
Hemerocallis ,,	0·0	9·9	5·1	15·0	—	—	—	4·3
Fritillaria ,,	0·0	11·1	3·4	14·5	—	—	—	4·5
Allium ,,	0·0	4·7	6·0	10·7	—	—	—	2·4
Veratrum ,,	A little	4·0	4·1	8·1	—	—	—	3·2
Scilla ,,	0·0	5·6	2·1	7·7	—	—	—	2·3
Iris ,,	0·0	1·6	1·6	3·2	—	—	—	3·6
Convallaria ,,	A little	0·5	—	—	—	—	—	—
Hosta ,,	Much	2·0	1·3	3·3	—	—	—	5·7
Tilia ,,	,,	0·8	2·6	3·4	—	—	—	5·0
Taraxacum ,,	Very much	1·4	1·7	3·1	—	—	—	2·4
Bunias ,,	,,	0·2	0·3	0·5	—	—	—	4·5
Acer ,,	,,	0·1	0·8	0·9	—	—	—	2·5
Tropaeolum (Brown & Morris)	4·6	0·4	3·9	4·3	0·0	0·4	5·3	—
,, (Ahrns)	6·3	2·0	6·9	8·9	—	—	0·8	—
,, (Gast)	6·4	3·8	4·4	8·2	0·5	3·3	1·1	—
Cucurbita (Gast)	6·1	0·9	2·6	3·5	0·0	0·9	0·6	—
Musa (Gast)	0·7	5·0	7·4	12·4	2·6	2·4	0·7	—
Canna (Gast)	1·3	0·3	5·9	6·2	0·0	0·3	0·4	—
Vitis (Gast)	5·3	2·7	3·1	5·8	1·0	1·7	0·5	—
,, (Deleano)	9·7	11·6	1·1	12·7	—	—	—	—
,, (Ahrns)	4·8	1·4	2·3	3·7	—	—	3·0	—
Phaseolus (Ahrns)	12·8	0·5	2·1	2·6	—	—	1·2	—
Helianthus (Ahrns)	4·1	1·6	1·5	3·1	—	—	2·0	—
Pisum (Ahrns)	4·1	1·9	5·8	7·7	—	—	4·3	—
Solanum (Davis & Sawyer)	6·0	1·3	3·5	4·8	—	—	0·0	—
Beta (Davis, etc.)	0·0	1·0	2·8	3·8	—	—	0·0	—
	0·0	8·9	6·4	15·3	—	—	0·0	—
	0·0	11·5	9·5	21·0	—	—	0·0	—
,, (Campbell)	8·6 (?)	4·7	2·2	6·9	—	—	0·5	—
Galanthus (Parkin)	0·0	3·6	12·6	16·2	—	—	0·0	—
Zea (Miller)	+	3·3	13·0	16·3	—	—	—	—
Sorghum (Miller)	+	4·0	16·0	20·0	—	—	—	—
Prunus Laurocerasus (Onslow)	+	3·0	9·7	12·7	—	—	—	—
Opuntia (Spoehr)	+	0·7	0·4	1·1	—	—	—	—
Pyrus Malus, fruit (Haynes & Archbold)	A little	*ca.* 57·0	21·0	78·0	—	—	—	—

stable hexoses in its power of producing starch artificially in leaves. That is, may not starch, apart from photosynthesis, only arise from the products of hydrolysis of sucrose and not from stable hexoses? Against this suggestion is the observed fact that stable glucose and fructose solutions possess the power of inducing starch formation to

a certain extent. This again would necessitate a mechanism for conversion of stable to γ-hexoses in the cell, though it is possible that, artificially, stable hexoses may induce starch formation in some indirect way. More concise experiments are needed, such as a balance-sheet of the carbohydrate interchanges during experiments on sugar-feeding.

Progress of photosynthesis, both in starch and sugar leaves, further results, as we know, in a rise of concentration of sucrose. This is shown from the values obtained for the Mangold (Campbell), Sorghum and Maize (Miller), Potato (Davis) and Snowdrop (Parkin). The question as to the channel through which sucrose may arise in photosynthesis has already been discussed. Though, as a rule, present in less amount than in sugar leaves, sucrose is nevertheless the chief sugar also for starch leaves.

Another source of sucrose in the plant arises, as already indicated, from the products of hydrolysis of starch. An illustration of this phenomenon is afforded by the formation of sucrose from an induced hydrolysis of starch brought about by desiccation. Starch formation can take place in the plant only, it would appear, within certain limits of sugar concentration. As already stated (p. 53) a minimal concentration, on the one hand, is necessary for starch formation. On the other hand, if the concentration of sugar is increased by desiccation, starch is hydrolysed, and the products of hydrolysis appear as sucrose.

A certain amount of research (Ahrns, 1; Schroeder & Horn, 73) has been devoted to the effect of desiccation on the carbohydrate balance in leaves. It is clear, from the results of the above investigators, that when a detached leaf containing considerable quantities of starch is allowed to dry, the starch disappears rapidly, whereas in a leaf kept supplied with water, there is little loss. Above a certain concentration of sugar, starch is not formed to replace hydrolysis. In such a leaf, also, provided starch is present, the sucrose content rises with loss of water and falls with gain of water. In no case, though, was starch re-formed on increasing the water content. In Table XVI are recorded some of the data from which Schroeder & Horn have drawn these conclusions.

Moreover, in leaves containing no starch, they found no increase, but a loss of sucrose, when subjected to drying; this loss, however, they claim to be less than in leaves which had been kept in water. Moreover, they point out from their results that the increase or

decrease of sucrose in their data is independent of the amount of hexose present. Hence, they do not consider that sucrose arises as a product due to increased concentration of hexoses. Unfortunately, in Schroeder & Horn's experiments, a complete balance-sheet for carbohydrates is not available, giving data for loss of starch in desiccation and loss of sugar through respiration, etc. Also some of their results are of doubtful value, owing to complications introduced by an attempted estimation of maltose by acid hydrolysis.

TABLE XVI. Effect of desiccation on carbohydrate content of *Tropaeolum* leaves (Schroeder & Horn)

Treatment of leaves	Water content	Percentage of dry weight				Starch
		Total sugar	Hexoses	Sucrose	Maltose	
A. Analysed at once	85·8	14·4	2·6	9·2	1·9	Much
B. Dried 24 hr., then analysed	67·9	26·4	10·2	13·9	1·6	None
		+12·0	+7·6	+4·7	−0·3	
A. Analysed at once	85·9	13·3	3·1	8·8	0·8	Moderate
B. On water 24 hr., then analysed	97·0	13·3	5·3	6·3	1·3	Scarcely less
		±0·0	+2·2	−2·5	+0·5	
A. Dried 5 hr., then analysed	81·1	19·6	5·4	11·6	2·0	Moderate
B. Dried 5 hr., 24 hr. on water, then analysed	97·3	16·0	8·3	6·4	0·9	Less
		−3·6	+2·9	−5·2	−1·1	
A. Analysed at once	88·3	14·4	0·2	9·8	3·5	Little
B. On water 24 hr., then analysed	95·5	9·2	2·8	4·8	1·3	Less?
		−5·2	+2·6	−5·0	−2·2	
A. Analysed at once	86·0	19·7	5·8	10·9	2·3	Moderate
B. Dried 4 hr., then analysed	83·2	20·5	5·5	11·9	1·9	Less
		+0·8	−0·3	+1·0	−0·4	

A more detailed series of analyses has been published by Ahrns (1) on the same lines for a number of different leaves. Though values are given for starch in this case, the results provide no more than a confirmation of those of Schroeder & Horn, the main difference being that in Ahrns' series, hexoses, in addition to sucrose, always increase on desiccation. Table XVII gives a selection of values from Ahrns' experiments.

A similar phenomenon has been investigated by de Wolff (93) for the Potato tuber. If slices of the tuber are artificially dried, the

TABLE XVII. Effect of desiccation on carbohydrate content of various leaves (Ahrns)

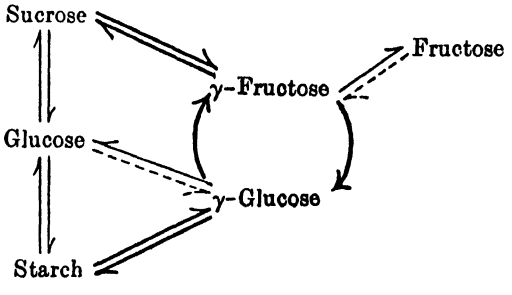
Plant and treatment	Water content, % fresh weight	Percentage of dry weight				
		Starch	Hexoses	Maltose	Sucrose	Total sugar
Tropaeolum majus:						
Analysed at once	85·92	6·29	1·97	0·81	6·94	10·11
24 hr. in dark and damp	87·20	4·07	3·85	1·82	5·70	11·74
		−2·22	+1·88	+1·01	−1·24	+1·63
Analysed at once	85·92	6·29	1·97	0·82	6·68	9·85
24 hr. in dark and dry	79·83	0·33	4·04	0·27	10·09	14·93
		−5·96	+2·07	−0·55	+3·41	+5·08
Vitis vinifera:						
Analysed at once	71·03	4·78	1·44	3·03	2·32	7·03
24 hr. in dark and damp	72·06	1·78	4·27	3·62	1·84	9·94
		−3·00	+2·83	+0·59	−0·48	+2·91
Analysed at once	71·03	4·52	1·44	3·09	2·28	7·03
24 hr. in dark and dry	69·75	0·75	4·52	1·92	4·37	11·07
		−3·77	+3·08	−1·17	+2·09	+4·04
Phaseolus vulgaris:						
Analysed at once	79·51	12·81	0·46	1·17	2·06	3·85
24 hr. in dark and damp	81·69	3·02	4·36	2·22	6·10	13·06
		−9·79	+3·90	+1·05	+4·04	+9·21
Analysed at once	79·51	12·81	0·61	1·13	1·98	3·85
24 hr. in dark and dry	70·36	1·28	1·76	1·82	7·53	11·59
		−11·53	+1·15	+0·69	+5·55	+7·74
Helianthus annuus:						
Analysed at once	84·77	4·13	1·64	1·99	1·46	5·25
24 hr. in dark and damp	86·01	1·62	3·02	1·13	1·26	5·49
		−2·51	+1·38	−0·86	−0·20	+0·24
Analysed at once	84·77	4·13	1·62	2·04	1·46	5·25
24 hr. in dark and dry	77·32	—	4·76	0·96	2·70	8·55
			+3·14	−1·08	+1·24	+3·30
Pisum sativum:						
Analysed at once	82·65	4·07	1·85	4·27	5·84	12·49
24 hr. in dark and damp	84·22	3·07	3·29	4·15	4·84	12·74
		−1·00	+1·44	−0·12	−1·00	+0·25
Analysed at once	82·65	4·03	1·95	5·28	6·72	14·36
24 hr. in dark and dry	77·69	1·31	4·33	3·79	8·31	17·04
		−2·72	+2·38	−1·49	+1·59	+2·68
Tropaeolum majus:						
Analysed at once	81·67	4·66	2·73	1·90	3·85	8·54
8 hr. in *light* and damp	82·45	1·68	4·83	2·06	5·50	12·77
		−2·98	+2·10	+0·16	+1·65	+4·23
Analysed at once	81·67	4·66	2·93	1·68	3·73	8·56
8 hr. in *light* and dry	76·86	—	3·62	3·00	6·03	13·08
			+0·69	+1·32	+2·30	+4·52

sugar content increases; this increase is definitely due to sucrose, the hexose content remaining approximately constant. The following table gives a typical set of data illustrating these relationships.

TABLE XVIII. Analyses of slices of Potato tuber subjected to drying (de Wolff)

Loss of weight during research %	Temp. of drying °C.	Time of drying (hours)	Sugar content		Increase of sucrose %
			Reducing sugar %	Sucrose %	
71·9	16–18	280	0·93	1·70	1·39
71·1	30·5	32	0·65	1·64	1·33
71·6	35·0	24	0·50	1·57	1·26
71·5	40·8	22	0·63	1·44	1·13
71·4	45·5	20	0·39	1·17	0·86
72·0	46·0	20	0·79	1·16	0·85
70·3	59·0	14½	0·68	0·86	0·55
69·4	98–100	6	0·80	0·52	0·21
Original potato	—	—	0·62	0·31	—

In addition, there are some experiments which provide evidence that, on adding water again to the dried material, there is a corresponding decrease in sucrose. From his extensive investigations, de Wolff concludes that the conversion of starch to sucrose is reversible, but takes place in several stages which he, however, is unable clearly to define. Though, on increase of water, the sucrose concentration falls, there is no actual evidence that starch itself is resynthesised, nor, from the data available, does there appear to be evidence that sucrose is synthesised from the stable glucose and fructose of the cell, even when the water content of the cell is decreased. It is therefore possible that the conversion of starch into sucrose and *vice versa* can only take place through the γ-hexoses, as is schematically represented below:



In conclusion, the following possible outline of metabolic interrelationships is suggested.[1] During photosynthesis, either γ-glucose and γ-fructose are both formed, or one only, which is readily converted into the other. They supply the substrate for respiration.

While photosynthesis is active, two types of temporary storage products are possible, starch and sucrose. γ-Glucose, stabilised by combination with normal glucose, is condensed to starch; γ-fructose, by combination with normal glucose, to sucrose. In some plants, possibly owing to water relationships, sugar concentration is too great (except in the young seedling) for starch to be formed, and the whole temporary storage consists of sucrose.

On cessation of photosynthesis, controlled hydrolysis of starch and sucrose takes place, supplying γ-hexoses for respiration. The products of hydrolysis of starch are probably converted into sucrose. Normal glucose is a by-product of starch and sucrose hydrolysis, but may take part in synthesis of polysaccharides of the cell-wall, glucosides and, possibly, some aromatic compounds. Upon the origin of stable fructose, light may be thrown when further investigations have been made as to its distribution and concentration in the tissues.

Sucrose is probably the chief sugar of translocation. The factors which determine the localisation of carbohydrates in storage organs are still obscure. Water relationships, apart from innate specific differences between plants, may possibly determine whether sucrose is deposited as starch, as for instance in most seeds, many bulbs, underground rhizomes and tubers; or whether sucrose itself forms the storage material, as in fleshy roots, stems and certain bulbs.

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[1] For recent researches which illustrate the catalysis of decomposition of hexose, of hydrolysis of sucrose, starch, etc., by plant tissues see Neuberg's work on leaves of *Nicotiana*, Bib. III.

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CHAPTER II

THE CELL-WALL

THE cell-wall is a non-living structure of very varied composition. It may be built up almost entirely of one constituent, or it may contain many different substances. Its constituents vary chiefly with the age of the tissue, that is, indirectly, with function, since, in individual cells and tissues, the cell-wall inevitably becomes adapted to the function of the tissue.

There appears to be some evidence that the primary membrane deposited between dividing nuclei contains nitrogen and fat, and is "protein-like" in nature. Later, as a rule, condensation products of sugars (polysaccharides) are deposited at the dividing membrane, and a cell-wall is developed. Of these polysaccharides, cellulose, a condensation product of glucose, is the chief representative. With cellulose are found, very frequently, pectic substances, i.e. condensation products of galactose, galacturonic acid and pentose. Universally distributed in the cell-wall, also, to a greater or less extent, are the hemicelluloses, so-called to distinguish them from true cellulose, since they are condensation products of other hexoses (galactose, mannose) and pentoses. The components of the pectic substances and hemicelluloses are, probably, derived initially from glucose by processes of transformation and oxidation. Cellulose itself may be modified later by oxidation and other changes. Further, in some unlignified tissues, extensive changes may result in "degradation products", such as gums and mucilages. In all these forms of polysaccharides, ions of potassium, calcium, magnesium and iron may play an important part.

Apart from the variations described above, there may be changes due to addition and association, as follows. In lignified tissues, lignin, a compound of unknown constitution, is deposited on the primary wall of pectic substance and cellulose. Similarly, in cuticularised tissues, a layer consisting chiefly of oxidised and condensed fatty acids is deposited upon the cellulose wall, or, at times, in the structure of the cellulose.

A more thorough study of the cell-wall might reveal many interesting points, for, as will be seen during the course of this chapter,

the wall is, to a certain extent, the non-living record of some of the metabolic activities of the protoplasm. The latter, on the one hand, is mainly composed of protein, that is, ultimately, of some twenty amino-acids linked together in various ways in very variable proportions. The cell-wall, on the other hand, may have, as components, at least six sugars of the pentose and hexose type, together with other derivatives, and these units, too, are condensed by different linkages in varying proportions. The process of condensation and consequent growth of the wall continues often throughout the life of the cell. Automatically, its chemical composition is an expression of biochemical processes taking place in the cell. The composition of the wall and the chemical reactions in the cell are mutually interdependent and progress together during tissue development. The cell-wall structure, therefore, might afford, if one could read it correctly, a record of some of the events taking place in the living contents which it encloses.

The various constituents and modifications of the cell-wall will be considered under the following sections:

Polysaccharides { Celluloses
Hemicelluloses
Pectic Substances
Gums and Mucilages
Cutin, Suberin
Lignin

Polysaccharides

In the previous chapter an account has been given of those units commonly present in the plant which represent the group of sugars of the carbohydrates. They can be enumerated as follows: *l*-arabinose, *d*-xylose, *d*-ribose, *l*-rhamnose, *d*-glucose, *d*-galactose, *d*-mannose and *d*-fructose. All may occur in the closed ring structure as well as in the straight chain. Of these ring structures there is evidence for the existence in the plant of two isomeric forms, depending upon which atoms are linked together, that is, to give a 5- or 6-membered ring. Moreover, of each of these ring structures, two stereo-isomeric forms are possible. The fact we are concerned with now is that any or all (except *d*-ribose) of these forms of sugar may be found condensed together by different linkages in various proportions to give rise to complexes greater than the di-, tri- and tetra-saccharides, that is, to the general group of the polysaccharides.

If we consider the polysaccharides from the aspect of what is usually termed "function", we can distinguish three types, though there is no rigid line of demarcation between them. First, those which form the structural cell-wall, i.e. celluloses, hemicelluloses and pectic substances. Second, those which form reserves of carbohydrates, i.e. starch and inulin. Though exceptional, there are cell-wall components which may subsequently, on hydrolysis, provide carbohydrates for metabolism. Third and last, there are "degradation products" of the cell-wall, the gums and mucilages.

In this chapter we are concerned only with the polysaccharides of the cell-wall. Celluloses, hemicelluloses, pectic substances, mucilages and gums will be considered in turn and, later, as far as possible, their origin and their biochemical significance in the whole system will be indicated.

Celluloses. (Bibliography II.)

The impression perhaps is still retained to some extent that the cell-wall of the higher plants is composed of cellulose. It is, as a matter of fact, exceptional for the cell-wall to consist of cellulose only, though it is usually the chief component. As a rule, there are many other components in the cell-wall, and these will be considered individually later.

Cellulose (or true cellulose, in contrast to hemicellulose) is a condensation product of glucose. In its purest form, which is known as α-cellulose, it is a white hygroscopic substance, insoluble in water and showing a certain amount of resistance to hydrolysis by mineral acids. On heating with 5 % sulphuric acid it is converted into glucose, the only product of hydrolysis. Two modifications, β- and γ-cellulose, commonly occur in varying proportions with α-cellulose. They differ from α-cellulose in being soluble in 17·5 % sodium hydroxide at ordinary temperatures, and from the alkaline solution, β-cellulose is precipitated on acidification with acetic acid, whilst γ-cellulose remains in solution. β- and γ-celluloses, moreover, are also less resistant to chemical reagents than α-cellulose. The β- and γ-modifications probably only differ from α-cellulose in degree of condensation or dehydration.

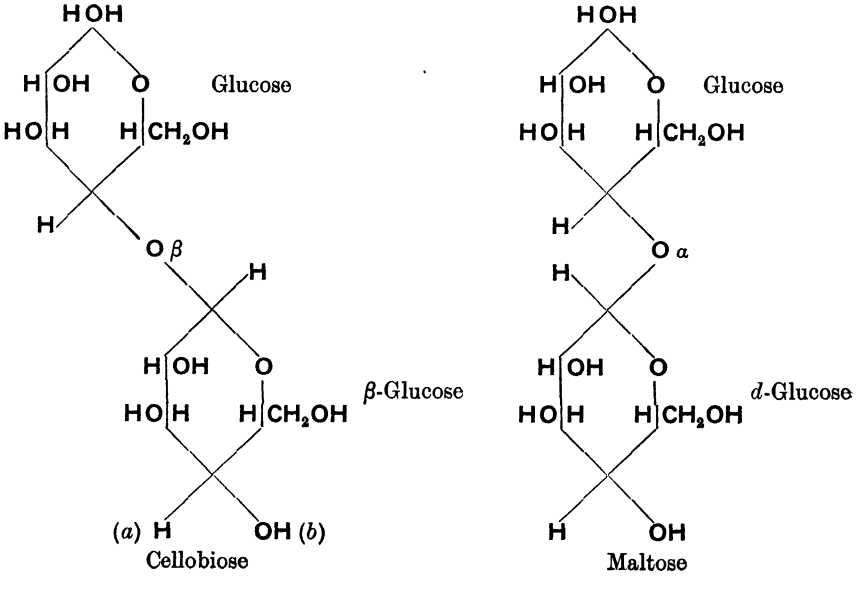
The cell-wall, as already stated, rarely consists of cellulose only. The best known example of such a case is the cell-wall of the hair-like outgrowths from the testa of the seed of the Cotton Plant. After a certain amount of purification, this product conforms to the

definition of α-cellulose. True cellulose, in the sense of yielding glucose only on hydrolysis, can, however, be obtained from practically all cell-walls after the removal of various other components, depending on the tissue used, which we shall consider later. Such cellulose frequently contains the modifications β- and γ-cellulose.

Much investigation has been expended in the effort to arrive at the constitution of cellulose, and many structural formulae have been suggested. As in the case of polysaccharides in general, two different points of view have been held, in the main, in connection with the constitution of the cellulose molecule: that, on the one hand, it is formed by the condensation, with elimination of water, of a number of glucose molecules, namely, by the ordinary type of linkage as in the di- or trisaccharides; or, on the other hand, that the cellulose molecule is derived from a more or less simple unit, such as an anhydride of two or three molecules of glucose, by polymerisation, residual valencies being involved.

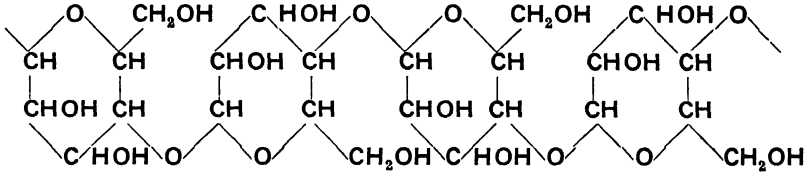
There is experimental evidence both for and against each point of view. In general, it appears certain that cellulose gives rise, on complete hydrolysis, to glucose only. It also seems clear, from the almost theoretical yield of 2 : 3 : 6-trimethyl glucose on methylation of cellulose, that the groups in positions 2, 3 and 6 of the hexose molecule are not involved in condensation.

Cellulose, moreover, on acetolysis, yields over 50 % of cellobiose, a disaccharide of similar structure to maltose, but a β-glucoside, instead of an α-glucoside:



Haworth (Bib. 1, 42) has suggested a formula for cellulose on the basis of the cellobiose structure (1 : 4, 1 : 4). Here, only glucosidic linkings are involved, and polymerisation does not enter into the question. It is further not impossible that the two ends of such a chain may be linked up.

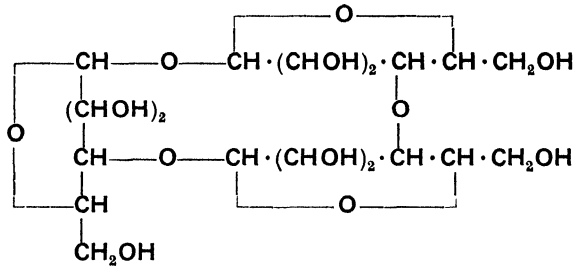
Arrangement of the condensed molecules on a certain pattern



enables Haworth's model to conform to the diagram recently demanded by an X-ray investigation of cellulose fibres.

Other investigators have regarded cellobiose as a reversion product, formed by the union of glucose molecules set free in decomposition of cellulose, their arrangement in the original molecule having been, of course, on different lines.

On the view of polymerisation, several types of basal unit have been suggested by various authors. One of the more recent formulae of this type is that, for instance, of Irvine & Robertson:

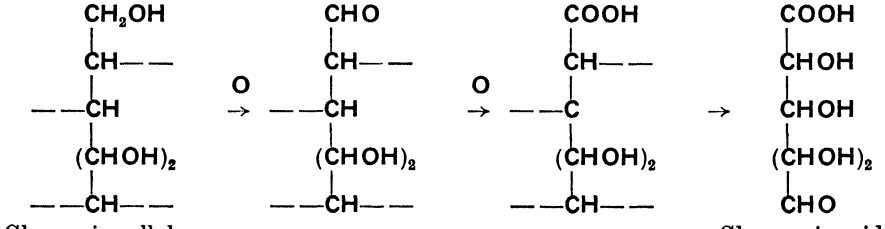


There are two further forms of cellulose, hydrocellulose and oxycellulose, both of which also occur commonly in the cell-wall:

Hydrocellulose is probably a stage in the hydrolysis of cellulose. It exhibits reducing properties, due possibly to the presence of carbonyl groups.

Oxycellulose. This derivative, though it occurs naturally, can be obtained artificially by the action of oxidising agents on cellulose. It includes, again, several modifications, α-, β- and γ-oxycelluloses, which are characterised by their behaviour towards dilute sodium hydroxide solution. Oxycellulose differs from cellulose in certain important points. It reduces Fehling's solution by virtue, probably,

of aldehyde groups; it has acid properties by virtue, probably, of carboxyl groups, and it gives furfuraldehyde on distilling with hydrochloric acid (the reason for this will be seen below). On the whole, the oxycelluloses are soluble in alkali. It has been suggested that the following reactions may explain the above properties:



Glucose in cellulose molecule

Glucuronic acid

This possible formation of carboxyl groups is of considerable interest as will be seen later.

To sum up: To find the cell-wall consisting only of true cellulose is rare. Nevertheless, cellulose forms a large part of the cell-wall together with other substances which we are about to consider. Cellulose in the wall is frequently present in varying modifications due to either partial hydration or to oxidation.

Hemicelluloses. (Bibliography II.)

It has been known for a long time that there are substances similar to cellulose in many cell-walls, and yet giving sugars other than glucose on hydrolysis. Evidence for their existence has been given by many investigators. Some of the most important pioneer work has been carried out by Schulze and his co-workers.

Schulze named these substances **hemicelluloses**, and he defined them, in general, as follows. They are insoluble in water, but are hydrolysed by weak acids (too weak to hydrolyse cellulose) into sugars, which are usually mixtures of both pentoses and hexoses, such as arabinose, xylose, galactose and mannose. The hemicelluloses can be extracted by sodium hydroxide (of concentrations about 4 %), from which extracts they are precipitated by neutralising with acid, especially if alcohol also is added.

Schulze and his co-workers investigated the hemicelluloses from the cell-walls of a large number of tissues, chiefly, however, the endosperm of ungerminated seeds. They first treated the material with ether, alcohol, very weak alkali (0·25–0·5 %), water, diastase (to remove starch), etc. The residue was finally hydrolysed with

weak sulphuric or hydrochloric acid (1–3 or 4 %); the sugars were isolated and carefully identified in each case. In some cases the hemicellulose was extracted with weak alkali and precipitated with acid.

They thus showed the presence of a number of complexes of various composition, galacto-mannans, galacto-arabans, mannoso-arabans, mannans, galactans, xylans, xylo-arabans, etc. Some occurred in one plant, others in another plant, as, for instance, in seeds of Pea, Soy Bean, Bean, Lupin, Coffee, Coconut, Date, in the bran of Wheat and Rye, in Pea and Bean pods, etc.

Thus the residue of the cell-wall material of Lupin seeds gave galactose and arabinose; of Coffee beans, galactose and mannose; of the Pumpkin seed, galactose only; of Wheat bran, arabinose and xylose; of Maize bran, xylose and galactose, etc. (see also Table XIX, where a number of examples have been selected from Schulze's publications).

In most of Schulze's investigations, the hemicelluloses were not extracted as such, but their constituents were obtained free from other substances and from cellulose by hydrolysing with very weak acid.

More recent work on the extraction of hemicelluloses has been carried out by Clayson, Norris & Schryver (Bib. III, 10), Clayson & Schryver (15), and Schryver & Thomas (52), sometimes, simultaneously, with that on pectic substances. Schryver and his co-workers isolated hemicelluloses, which they termed "cytopentans", from a number of sources, namely turnips, onions, pea-pods, cabbages, oranges and apples. The tissues were thoroughly ground, pressed, extracted with alcohol and washed. From the residue the hemicelluloses were extracted with 4 % caustic soda, precipitated with alcohol and subsequently dried. The products give a blue colour with iodine, do not reduce Fehling's solution, are not acted upon by taka-diastase, but are readily hydrolysed by 1 % hydrochloric acid, to give, presumably, reducing sugars. They contain large and varying proportions of pentoses, ranging from 40 % (onions, apples) to 85 % (pea-pods).

In addition, Norris & Schryver (Bib. III, 33) claim also to have obtained small quantities of hemicelluloses by treating preparations of pectin with alkali (when estimating the methoxyl groups, see p. 80). They therefore assume some form of hemicellulose to be associated with pectin.

Also, after extracting pectin with ammonium oxalate from the cell-wall, they again obtained hemicelluloses by treatment of the

TABLE XIX. Constituents of hemicelluloses
(Schulze and his co-workers)

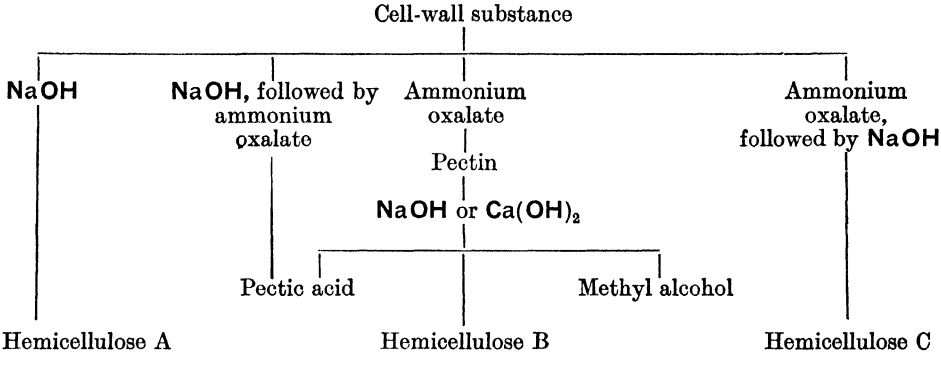
Material	Sugars
Pinus Cembra (seed coat)	Galactose, xylose (10)*
Zea Mays (seed and fruit coat)	,, ,, (56)
Triticum sativum (seed and fruit coat)	Arabinose, xylose (54)
Secale cereale (seed and fruit coat)	,, ,, (54)
Molinia caerulea (internodes)	Fructose, xylose (61)
Phoenix dactylifera (seed)	Galactose, mannose (67)
Elaeis guinensis (seed)	,, ,, (67)
Cocos nucifera (seed)	,, ,, (67)
Allium Porrum (root)	Galactose, arabinose (69)
Ruscus aculeatus (seed)	Mannose, arabinose (10)
Juglans regia (fruit shell)	Galactose, xylose (62)
Corylus avellana (fruit shell)	,, ,, (62)
Rumex acetosa (root)	Galactose, arabinose (69)
Rheum officinale (root)	,, ,, (69)
Anabasis aretioïdes (shoot)	,, ,, (69)
Paeonia officinalis (root)	,, ,, (69)
Cochlearia Armoracia (root)	,, ,, (69)
Prunus Amygdalus (seed)	,, ,, (62)
Alchemilla vulgaris (root)	,, ,, (69)
Lupinus luteus (seed)	,, ,, (67)
L. albus (seed coat)	,, ,, (10)
L. angustifolius (seed)	,, ,, (56)
L. angustifolius (seed coat)	,, ,, (10)
L. hirsutus (seed)	,, ,, (67)
Soja hispida (seed)	,, ,, (67)
Faba vulgaris (seed)	,, ,, (54),(67)
Pisum sativum (seed)	,, ,, (54),(67)
Vicia sativa (seed)	,, ,, (54),(67)
Phaseolus vulgaris (seed)	,, ,, (62)
Phaseolus vulgaris (seed coat)	,, ,, (62)
Medicago sativa (plant)	,, — (64)
Medicago sativa (root)	,, ,, (69)
Ricinus communis (seed)	,, ,, (62)
Sesamum indicum (seed)	Arabinose (56)
Heracleum Sphondylium (root)	Arabinose, galactose (69)
Daucus Carota (root)	,, ,, (69)
Lysimachia punctata (root)	,, ,, (69)
Coffea arabica (seed)	Mannose, galactose (67)
Cucurbita Pepo (seed)	Galactose (62)
Taraxacum officinale (root)	Arabinose, galactose (69)

residue with alkali. The various substances isolated are represented in Table XX. The estimation of pentose content, however, throws no light on the relationship, if any, between these various complexes.

* Numbers refer to Bibliography on Hemicelluloses.

Further, Schryver and his co-workers (15) have isolated a substance, which they claim to be a hemicellulose, from Wheat flour, and (52) from a number of starches, namely, those of Sago, Maize, Tapioca, Wheat and Rice. Since hemicelluloses are usually defined as condensation products of sugars other than glucose, and since the above product produces glucose only on hydrolysis, its claim to be included in this class may be said to rest on its solubility and the ease with which it is hydrolysed. It was extracted with 2–4 % caustic soda and precipitated by acid. It was not digested by taka-diastase. It was purified by precipitation with alkaline copper sulphate and subsequent decomposition with acetic acid. It is soluble in hot water, and, on hydrolysis with 1 % sulphuric acid, gives glucose only as a reducing sugar.

TABLE XX. Hemicelluloses from cell-wall (Norris & Schryver)



O'Dwyer (34) extracted a hemicellulose from the American White Oak, using dilute alkali, and precipitating with acetic acid and alcohol. It was purified by precipitation with Fehling's solution. Analyses gave 51·5 % of xylose, 18·5 % of arabinose and 30 % of mannan and galactan. It was hydrolysed with 1 % sulphuric acid.

Until recently hemicelluloses have been regarded as condensation products of sugars only. Later researches (O'Dwyer, 35) have claimed to show the presence of uronic acids (see p. 5) in hemicellulose complexes. O'Dwyer isolated two hemicelluloses (A and B) from Beech wood by extraction with 4 % sodium hydroxide solution. Hemicellulose A was directly precipitated by acidification, and yielded, on hydrolysis, xylose and carbon dioxide corresponding to 11 % glucuronic acid; hemicellulose B was precipitated only with acid and alcohol, and, on hydrolysis, gave arabinose and carbon dioxide corresponding to 63 % of galacturonic acid, together with

small amounts of galactose. It is important to note that the uronic acids were only obtained in small amounts, as they decompose in hydrolysis.

An additional point should be made in connection with the hemicelluloses: it appears that a complex which is a condensation product of one sugar only is often yielded to extraction with alkali. Such is the well-known xylan, a "wood-gum" obtained by extraction of the wood of forest trees. It may be, however, that uronic acids are commonly present also in these complexes, since, as O'Dwyer points out, they may be destroyed in hydrolysis. A similar well-known complex, araban, a condensation product of arabinose, can be isolated from cherry gum. This will be referred to later in the section on gums and mucilages.

It appears certain that the hemicelluloses, in addition to the part they play in the structure of the cell-wall, may also function as reserve material, more especially in the germination of seeds. Schulze, who demonstrated the presence of hemicelluloses in the cell-walls of the endosperm of many seeds, also showed by estimation that the hemicelluloses diminish during germination (Schulze, 57; Schulze & Castoro, 59). He stated, moreover, that true cellulose does not constitute a reserve. The same phenomenon is also obvious to the eye in the case of the endosperm of the seed of the Date, or of the *Phytelephas* Palm; the cells have greatly thickened walls containing a high percentage of hemicelluloses, which disappear on germination.

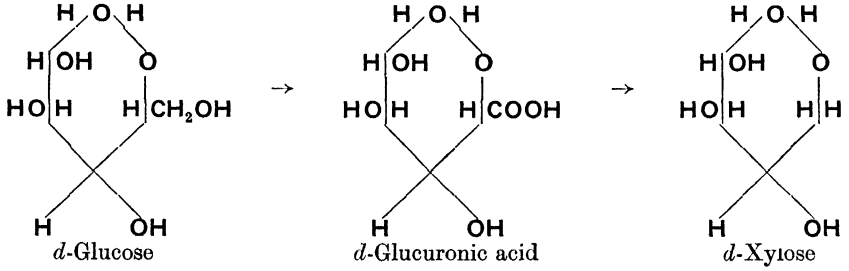
The question arises as to whether there are enzymes which catalyse the hydrolysis of hemicelluloses as in the case of other polysaccharides. Brown & Morris (8) claimed to have precipitated, from an extract of germinating Barley, an enzyme which would hydrolyse the cellulose walls of the endosperm of grasses, of tissues of the Potato, Carrot, Beet and Artichoke. It was thermolabile, and was termed by them "cytase". It did not affect the cellulose of the Coffee bean or of the seeds of the Date, Onion and Asparagus.

Bourquelot & Hérissey (3, 4) showed that, during the germination of the Carob Bean, an enzyme is produced which converts the polysaccharides of the bean into mannose and galactose. Later (5), it was found that enzymes (seminases), capable of hydrolysing mannogalactans and galactans into their derivative monosaccharides, occur in the fruits of Fenugreek, Lucerne, *Trifolium repens* and other plants: subsequently, they were also demonstrated in Orchid tubers (25).

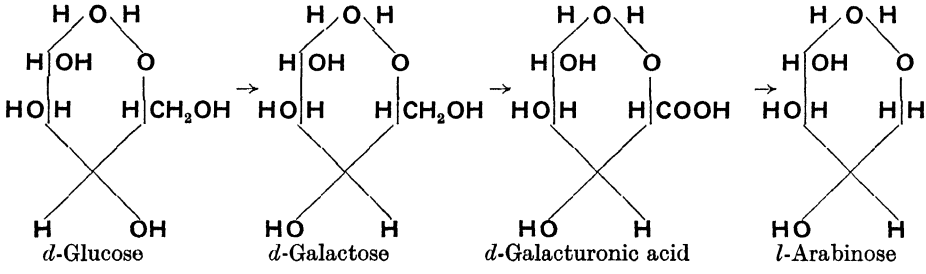
Grüss (20) has observed the presence of similar enzymes in germinating Date seeds.

Paton, Nanji & Ling (38) have given evidence for the presence of an enzyme in the Ivory Nut (*Phytelephas*) which hydrolyses the hemicelluloses of the cell-wall; 200 gm. of ivory-nut shavings in water kept at 45° C. for six days, gave 45 gm. of a reducing sugar; mannose was identified as a product of hydrolysis.

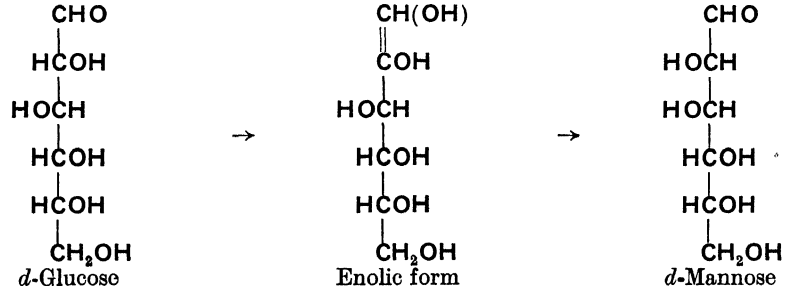
Now, the significance of the hemicelluloses in metabolism may be considered. We noted previously in Chapter I that various changes may be brought about in the glucose molecule. Thus, if the aldehyde group is fixed, formation of glucuronic acid may take place by oxidation, and subsequent loss of carbon dioxide will lead to the formation of *d*-xylose:



Or, glucose may be converted, by inversion, into galactose, from which, in a similar way, galacturonic acid and arabinose may arise:



Finally, glucose may be converted into mannose through the enolic form:



Hence it is possible that either, while the molecule of glucose is being incorporated into the cell-wall, or, after condensation has taken place, such changes as the following may occur:

Glucose	→ Glucuronic acid			
,,	→ ,,		→ Xylose	
,,	→ Galactose			
,,	→ ,,	→ Galacturonic acid		
,,	→ ,,	→ ,,		→ Arabinose
,,	→ Mannose			

If such changes take place to a considerable extent, and they may well do so owing to variations in metabolism of the living protoplasm, we have an explanation of the origin of alien components in the cell-wall, which would otherwise consist of true cellulose.

We can thus picture the following possibilities: a complex, formed from condensation of glucose alone, which is cellulose; of glucose and glucuronic acid, which is oxycellulose. From the decarboxylation of glucuronic acid a complex of xylose may arise, forming the pentosan, xylan. Or, by an extensive conversion of glucose into galactose, a galactan may be formed. Similarly, from galactose, by oxidation, galacturonic acid may be produced, the basal unit, as we shall see later, of the pectic substances. Further, by decarboxylation of galacturonic acid, an arabinose complex may arise, forming the pentosan, araban. Or, a different line of modification would result in mannose leading to the incorporation of a mannan. In the same cell-wall, almost all (if not all) these modifications may be present together. Evidence, however, points to a frequent association, on the one hand, of glucose and glucuronic acid (in, possibly, oxycellulose); of glucose, glucuronic acid and xylose; of glucose and xylose; and of glucuronic acid and xylose (O'Dwyer, 35). On the other hand, there is the association of galactose and galacturonic acid; of galactose, galacturonic acid and arabinose (see Pectic Substances); of galactose and arabinose; and of galacturonic acid and arabinose (O'Dwyer, 35).

Hence almost any cell-wall is a mixture of condensation products. How far the different complexes, the pentosans and hexosans, are actually combined or condensed together is not clear. It seems that the condensed products of the sugars, other than glucose, are, on the whole, characterised by their more ready solubility in alkaline solutions, and low resistance to hydrolysis by acids. Their solubility in alkali makes it possible to remove the whole complex from the cellulose it accompanies, but whether linkages are destroyed in the

process is not clear. The presence of carbonyl or carboxyl groups may render the complex more alkali-soluble, as, for instance in the α- and β-celluloses and oxycelluloses.

Pectic Substances. (Bibliography III.)

We next pass to other compounds which are very commonly components of the cell-wall, the pectic substances. These, again, can be extracted from accompanying cellulose and hemicellulose by differential solubility in certain solvents. In these compounds, the basal unit is pectic acid, a complex of galactose, galacturonic acid and arabinose. How this association may have come about is readily seen from the interrelationships set out above. The complex, pectic acid, has *in vitro* peculiar physical properties which have drawn attention to this particular class of cell-wall constituents.

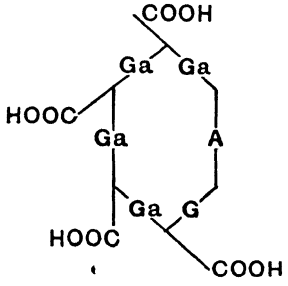
The pectic substances are present, in close connection with cellulose yet with none of its properties, in most, if not all, unlignified tissues of the higher plants. They are found, moreover, to the greatest extent, in fleshy and succulent tissues in, for example, currants, apples, pears, strawberries, oranges, onions, rhubarb, turnips, swedes, pea-pods, etc. The outstanding characteristic of these substances is that they form gels when in certain conditions and under certain circumstances. The literature on these compounds is very confusing, owing to the different methods employed in extraction, the impure state of the products obtained, and the changes brought about by the treatment during extraction.

A short account will be given first of the chief substances involved and their interrelationships: later, details will be given of the researches upon which more recent knowledge in this field is based. In the literature the following terms will be met with; they are set out here for assistance in reading the accounts of various investigations:

Substances	Enzymes (catalysing certain reactions connected with pectic compounds)
Pectic acid, synonymous with cyto-pectic acid (Pectinic acids)	Pectosinase
Pectin, synonymous with pectinogen (Neutral pectin)	Pectase
Protopectin or pectose, synonymous with insoluble pectin and pecto-cellulose	Pectinase

Possibly, the formula for **pectic acid**, first suggested by Nanji, Paton & Ling (25), but based largely on results of previous workers,

is the most satisfactory. It represents the molecule of pectic acid as a closed 6-membered ring, of which the members are one molecule of anhydro-arabinose, one of anhydro-galactose and four molecules of galacturonic anhydride, the four carboxyl groups of the latter being free:



G = Galactose

A = Arabinose

Ga = Galacturonic acid

Such a formula is in agreement with the values obtained, on analysis, for the calcium content of calcium pectate, the furfuraldehyde (from uronic acid and pentose), the carbon dioxide (from uronic acid), etc., of carefully purified preparations of pectic acid from various sources.

To F. Ehrlich (11) we owe the discovery that galacturonic acid is the keystone of the pectic substances. As material, he worked chiefly with Beet residues. In a recent communication, he describes, as the ultimate complex of pectic substances, a compound which he terms "pectic acid". It consists, on the basis of his analyses, of four molecules of galacturonic acid, one of galactose, one of arabinose and three of acetic acid; two molecules of galacturonic acid, are, moreover, esterified by methyl alcohol (see pp. 79, 89). As far as Ehrlich's products can be identified with those of other investigators, his terminology being somewhat different, the pectic acid he describes corresponds more closely to one of the pectin complexes to be described below. It differs from the pectic acid of other workers in being water-soluble and in containing methoxyl groups, as well as having acetic acid as a component.

Fellenberg (18–20), also using the Beet as material, isolated a pectic acid which consists, according to his analyses, of eight molecules of galacturonic acid, two of arabinose, one of galactose and one of a methyl pentose (see also p. 79):

$$(C_5H_8O_4)_2(C_6H_{10}O_5)(C_6H_{10}O_5)(C_5H_7O_4 \cdot COOH)_8 \cdot 2H_2O.$$

The extent to which pectic acid occurs free in the cell-sap or cell-wall is uncertain. It is insoluble in water: its salts with the alkali metals are soluble in water, from which they are precipitated by

TABLE XXI. Composition of pectic substances (various authors)

Schryver & Haynes (35).

"Pectinogen" ≡ Pectin.

"Pectin" ≡ Pectic acid ($C_{17}H_{24}O_{16}$).

Ehrlich (13).

"Rüben-Pektin" ≡ ? Pectose.

↓

"Hydropektin" ≡ Pectin.

Araban — Calcium-Magnesium salt of "Pektinsäure" ≡ ? Pectic acid + Pectin.

↓ — ↓

l-Arabinose — "Pektinsäure" ($C_{43}H_{62}O_{37}$).

↓

2 Methyl alcohol; 3 Acetic acid; 4 *d*-Galacturonic acid; *l*-Arabinose; *d*-Galactose

$$C_{43}H_{62}O_{37} + 10H_2O = 4C_6H_{10}O_7 + 2CH_3OH + 3CH_3\cdot COOH + C_5H_{10}O_5 + C_6H_{12}O_6$$

Beet "Pektinsäure" — Galacturonic acid — Methyl alcohol — Acetic acid — Arabinose — Galactose

Fellenberg (18, 19).

Pectose $(C_5H_8O_4)_2(C_6H_{10}O_5)(C_6H_{10}O_5)(C_5H_7O_4\cdot CO\text{-cellulose})_8$

Arabinose — Galactose — Methyl-pentose — Galacturonic acid

↓

Cellulose — Neutral pectin $(C_5H_8O_4)_2(C_6H_{10}O_5)(C_6H_{10}O_5)(C_5H_7O_4\cdot COOCH_3)_8\cdot 2H_2O$ or $C_{78}H_{120}O_{68}$

↓

Methyl alcohol — Pectinic acids $(C_5H_8O_4)_2(C_6H_{10}O_5)(C_6H_{10}O_5)(C_5H_7O_4\cdot COOCH_3)_n(COOH)_{8-n}\cdot 2H_2O$

↓

Methyl alcohol — Pectic acid $(C_5H_8O_4)_2(C_6H_{10}O_5)(C_6H_{10}O_5)(C_5H_7O_4\cdot COOH)_8\cdot 2H_2O$ or $C_{70}H_{104}O_{68}$

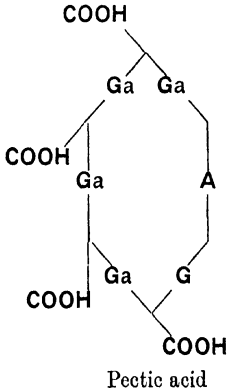
↓

Galactose-galacturonic acid — Arabinose — Methyl pentose

↓

Galacturonic acid + Galactose

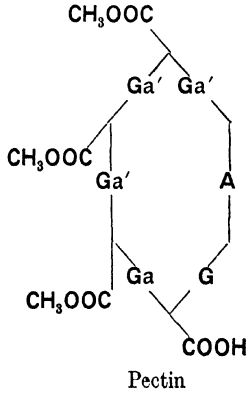
Nanji, Paton & Ling (25)



Pectic acid

(see p. 78)

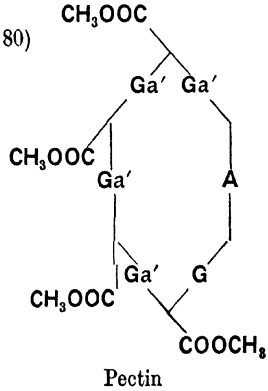
Norris & Schryver (33); **Norris** (31)



Pectin

(see p. 80)

Norman (30)



Pectin

alcohol as thick, gelatinous precipitates. On acidifying solutions of these salts, pectic acid separates out also as a gelatinous precipitate, or it may spontaneously set to a gel. Its salts with the alkaline earth metals are insoluble.

The next type of pectic substance we have to consider is known as **pectin**, or **soluble pectin**. It is present in the cell-sap of many tissues, and constitutes also, probably, a great part of the pectic substances of the cell-wall.

It includes derivatives of pectic acid in which some or all of the carboxyl groups are esterified by methyl alcohol. Evidence for the number of groups esterified is as follows:

Fellenberg, who first discovered the presence of methoxyl groups in pectic compounds, gives the name pectin to the completely esterified form of his pectic acid, that is the octomethoxy ester:

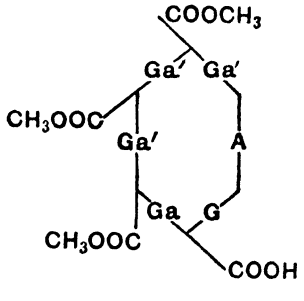
$$(C_5H_8O_4)_2(C_6H_{10}O_5)_2(C_5H_7O_4 \cdot COOCH_3)_8 \cdot 2H_2O = C_{78}H_{120}O_{68}.$$

He also claims to have split off, by hydrolysis with different hydrolytic agents, the methyl alcohol in stages, and thus obtained a series of pectic acids, with a decreasing number of methoxyl groups, termed by him "**pectinic acids**":

$$(C_5H_8O_4)_2(C_6H_{10}O_5)_2C_5H_7O_4 \cdot (COOCH_3)_n(COOH)_{8-n}.$$

Ehrlich's "hydropektin" ("pektinsäure" + araban) probably corresponds to pectin; it is obviously only partially esterified, and the author himself suggests that methyl alcohol may have been lost during the treatment of the commercial Beet residues he used as material. Ehrlich's pectin also contains calcium and magnesium.

Norris & Schryver (33), adopting the formula given above of Nanji, Paton & Ling, determined the methyl alcohol in pectin from turnips, onions, and pea-pods. Their values for methyl alcohol content were very variable, but were never greater than the value corresponding to the esterification of three carboxyl groups. They therefore incline to the view that in soluble pectin three carboxyl groups are esterified, and the fourth is free to react to form salts:



G = Galactose

A = Arabinose

Ga = Galacturonic acid

Ga' = Methylated galacturonic acid

A similar and more concordant value was found by Norris (31) for the pectin from both the juice and pulp of the Orange, signifying again an esterification of three carboxyl groups.

There is little doubt that, in the process of extraction of pectin, there may be a hydrolysis of the esterified groups to some extent. Moreover, when pectin preparations, even after careful reprecipitation, are purified again as an iron precipitate with subsequent removal of iron, they appear, on estimation of the calcium in the calcium salt, to lose accompanying substances, probably impurities. Thus Norman (30) found that the methoxyl values, recalculated after such a purification of pectin (prepared from lemon juice), were in accordance with a fully esterified pectic acid. So the question as to the constitution of soluble pectin is at present open to further investigation.

The pectins (i.e. more or less esterified pectic acids) are soluble in water, from which they are precipitated as gelatinous precipitates by alcohol. If free carboxyl groups are present, they can form salts with alkali and alkaline earth metals which are water-soluble, but are precipitated by alcohol as gelatinous precipitates.

Next to be considered is a third type of pectic substance, known as **insoluble pectin**, **pectose** or **protopectin**, which is insoluble in water and is present in the cell-wall. It is extracted only by special treatment, such as prolonged boiling with hot water or hydrolysis with acid. The condition in which this form exists in the wall has given rise to much controversy.

Fellenberg considers that the insolubility of pectose and its removal from the cell-wall only by hydrolysis are due to the combination of one or more of the carboxyl groups of pectin (others being methylated) with cellulose residues by condensation with elimination of water:

$$(C_5H_8O_4)_2(C_6H_{10}O_5)(C_6H_{10}O_5)(C_5H_7O_4 \cdot CO\text{-cellulose})_8.$$

Then, conversely, soluble pectin would be formed from pectose by prolonged hydrolysis with water or by acid. In support of this view, Sucharipa (36) claimed to have extracted complexes from the cell-wall by a series of solvents, which on analysis gave values for methoxyl and cellulose residues corresponding, to some extent, to the theoretical values based on Fellenberg's formulae for the pectin-cellulose series.

As far as Ehrlich is concerned, he states that the original pectin, which is present in an insoluble condition in the cell-wall of the Beet

root, is firmly united to the araban complex, but the combination is split by treatment with hot water.

This view of the union of pectin with cellulose has been upheld by Carré (7), who showed also that the degree of combination with cellulose was very variable in different products, some being readily hydrolysed by boiling water, others requiring more or less hydrolysis with acid, implying greater or less combination with cellulose groups.

Tutin (40), on the contrary, has cast doubt on the existence of pectose or protopectin, and has maintained that the difficulty of extraction with water is due to insufficient disintegration of the tissues. This point of view is not justified in the face of the experimental evidence of Fellenberg, Carré, etc.

Both Fellenberg and Carré have held the view that different preparations of pectin give methyl alcohol values which would correspond to the various esters suggested by Fellenberg. However, as we have seen, the methoxyl value may be very unreliable. There is therefore no real evidence, beyond the claim of Sucharipa to have isolated cellulose-pectin complexes, for this view of the constitution of pectose or protopectin.

A recent suggestion put forward by Nanji, Paton & Ling (25) and Nanji & Norman (24) is that several molecules of pectin may be associated together, in one or two of which there are some free carboxyl groups, these latter being replaced by iron or calcium. They point out that ferric chloride gives such an insoluble precipitate with pectin, which contains a certain amount of iron. This precipitate passes into solution on prolonged treatment with hot water, and at once is soluble in dilute acid.

Finally, there is a fourth type of pectic substance, that in which the pectic acid is combined as **pectate** and is insoluble. This seems to be specially true of the pectic substances of the middle lamella, the first cell-wall formed after cell-division, and eventually the outermost layer of the individual cell-walls. In the opinion of Nanji & Norman, the middle lamella cannot be the only source of combined pectic acid (pectate), as there is not sufficient of this layer. These authors also oppose the view that the combination of pectic acid in the middle lamella is with cellulose. The various pectic constituents of the cell-wall may be therefore summarised as follows:

Pectic acid	? in cell-sap, ? in cell-wall
Ca pectate	middle lamella
Soluble pectin	cell-sap and cell-wall
Insoluble pectin, pectose or protopectin ...	cell-wall

There are some points in connection with the solubilities of the various component complexes of the cell-wall which are tabulated here in order to be of assistance in reading the section. These complexes have been classified partially on their different solubilities in certain reagents; this has, perhaps, as will be mentioned later, rendered the classification to some extent an artificial one:

TABLE XXII. Solubilities of cell-wall constituents

Reagent	Cellulose	Hemi-celluloses	Pectose	Pectin	Pectic acid	Calcium pectate
Ammoniacal copper sulphate	Soluble	—	—	—	—	—
Caustic soda, 4 %	Insoluble	Soluble	—	—	—	—
Water	—	—	Insoluble (sol. on prolonged boiling)	Soluble	Insoluble	Insoluble
Oxalic acid, 0·5 %	—	—	Soluble	Soluble	Insoluble	Insoluble?
Ammonium oxalate, 0·5 %	—	—	Soluble	Soluble	Soluble	Soluble

Summing up the situation, we may say that pectic acid is probably an arabino-galacto-tetragalacturonic acid. If this formula is doubled, it differs from that of Fellenberg only by introducing a methyl pentose, instead of a galactose, molecule. From the researches of Ehrlich we conclude that he obtained a similar compound as his ultimate complex, differing only in the presence of methyl alcohol and acetic acid molecules (and in Flax "pectic acid" of xylose). Most investigators are agreed that soluble pectins are pectic acid in which an uncertain and possibly variable number of carboxyl groups are esterified by methyl alcohol. The explanation of the insolubility and fixation in the cell-wall of pectose or protopectin is still uncertain. Two suggestions are offered: either carboxyl groups of pectic acid are condensed with cellulose, or complexes of pectin molecules are rendered insoluble by loose combination with calcium or ferric ions. It seems certain that pectates of calcium or magnesium exist in the cell-wall, especially in the middle lamella.

Most of the investigators are probably dealing with the same substance, together with varying amounts of impurity depending upon methods of extraction and treatment. Thus, there is a definite re-

lationship between empirical formulae for the products of Fellenberg, Schryver & Haynes and Nanji, Paton & Ling:

Pectic acid

Nanji, Paton & Ling	$C_{35}H_{50}O_{33}$
Schryver & Haynes	$2(C_{17}H_{24}O_{16}) = C_{34}H_{48}O_{32}$
Fellenberg	$\frac{1}{2}(C_{70}H_{104}O_{68}) = C_{35}H_{52}O_{34}$

Pectin

Nanji, Paton & Ling	$C_{39}H_{58}O_{33}$
Fellenberg	$\frac{1}{2}(C_{78}H_{120}O_{68}) = C_{39}H_{60}O_{34}$

The good agreement between the analyses of many of the later workers is shown in Table XXIII.

We are then in a position to state that, widely distributed and in some way fixed in the unlignified cell-wall, sometimes in greater, sometimes in less quantity, we find the pectic complex, galacturonic acid plus galactose and arabinose. It is not extracted by dilute caustic alkali like the hemicelluloses, and the complex in the form of free pectic acid has the definite physical property of forming gels.

If we reconsider, for a moment, the hemicelluloses, in particular the example which was extracted by O'Dwyer from Beech wood, and which gave, on analysis, 63 % of galacturonic acid and 25 % of galactose and arabinose, the fact becomes evident that, though this product has the same components as pectic acid, it is regarded as a hemicellulose.

Hence it appears, looking at the matter in its widest aspect, as if the classification into hemicelluloses and pectic substances might be somewhat artificial. Rather, one would picture the cell-wall as consisting, primarily, of cellulose, which may give rise, however, to glucuronic acid and xylose, or, if galactose is incorporated, to galacturonic acid and arabinose. Then alkalies, possibly by destroying certain linkages, will extract some components, the hemicelluloses, from the whole and ammonium oxalate, by removing calcium and rendering them soluble, will extract other components, the pectic substances. Further, that the particular grouping, arabino-galacto-tetramethoxy-galacturonic acid (pectin), on account of some stability or affinity among its components, is an especially frequent unit, and is conspicuous, moreover, in having the peculiar physical property of forming gels.

Candlin & Schryver (5) have added certain observations which emphasise the wider point of view in regard to all these complexes. They point out that lignified tissues contain only traces (if any) of pectic substances, though considerable amounts of hemicelluloses;

unlignified tissues contain relatively large quantities of pectic substances, and smaller amounts of hemicelluloses. They suggest, therefore, that the galacturonic element of the pectins may undergo decarboxylation during lignification, and thus, from the pectic compounds, hemicelluloses would arise.

TABLE XXIII. Estimation of components of pectic substances

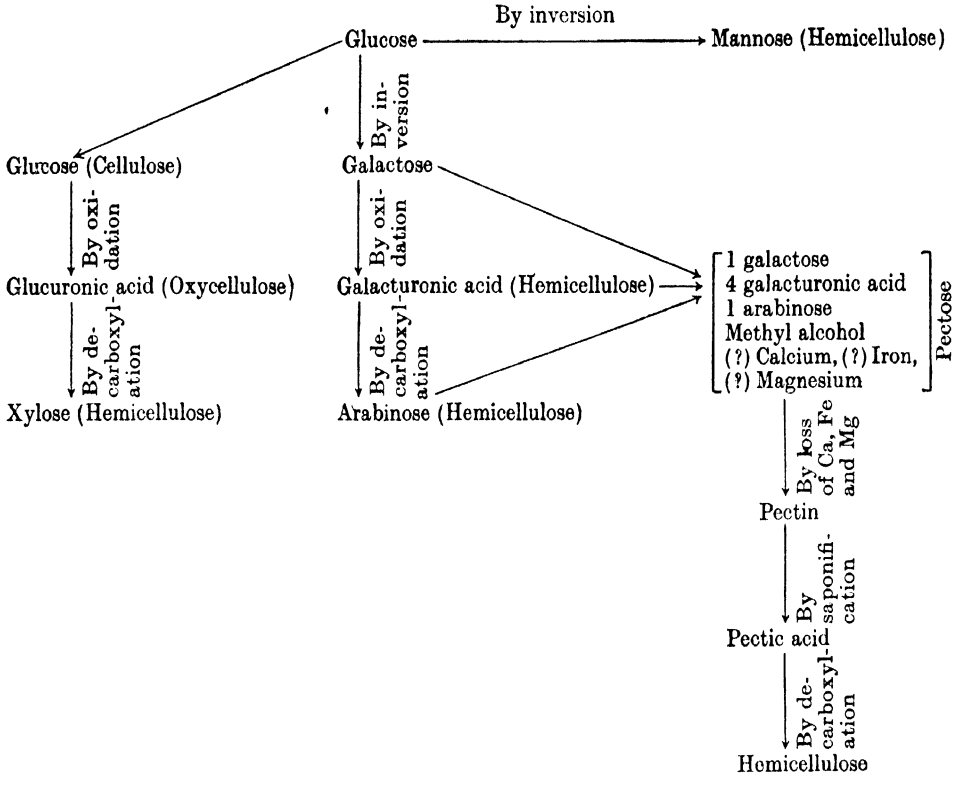
Substance	Source	Observer	Methyl alcohol %	Furfuraldehyde %	Carbon dioxide % from galacturonic acid	Calcium %
Pectic acid	Turnip	Schryver & Haynes (35)	—	20·4	—	—
,,	Rhubarb	,,	—	18·6	—	—
,,	Pea-pod	Clayson, Norris & Schryver (10)	—	20·7	—	—
,,	Turnip	,,	—	21·6	—	—
,,	Onion	—	—	20·7	—	—
,,	Orange	—	—	21·5	—	—
,,	Cabbage	—	—	20·3	—	—
,,	Apple	—	—	21·7	—	—
Pectin	From Turnip, Onion, Pea-pod	Norris & Schryver (33)	Not > 9·2	—	—	—
,, (crude)	Apple	Nanji, Paton & Ling (25)	—	—	13·79	—
,, (purified)	,,	,,	—	—	18·56	—
,, A	Beet	,,	—	—	18·08	—
,, B	,,	,,	—	—	19·81	—
Pectic acid	Orange	,,	—	—	18·09	—
,,	Onion	,,	—	—	18·00	—
Calcium pectate	—	,,	—	—	17·64	7·34
Pectic acid	Orange juice	Norris (31)	—	20·3	—	—
Pectin	,,	,,	8·92	19·2	—	
Pectic acid	Flax	Henderson (23)	—	—	20·90	
,,	,,	Norris (32)	—	20·16	17·80	—
Pectin	,,	,,	9·65	20·90	—	—
,,	Apple	Carré & Haynes (9)	—	—	—	7·62
,,	Lemon	Norman (30)	8·57	—	—	—
(Recalculated on basis of calcium pectate yield)	,,	,,	11·48	—	—	—
Pectin (calculated from formula of Nanji, Paton & Ling)	—	—	—	20·85	17·64	—
Tetramethyl ester (theoretical)	—	—	11·76	—	—	—
Trimethyl ester (theoretical)	—	—	8·94	—	—	—

In support of their view, they find that not only are the uronic acids of pectic substances decarboxylated by acids, but also by alkalies, and that if sodium pectate is heated with even weak alkali, 0·5 %, for some time, it is to a large extent decarboxylated, yielding hemicelluloses, very similar to those extracted from the natural wood.

Though this line of metabolism suggested by Schryver & Candlin is quite analogous to the origin of xylose from oxycellulose, or of arabinose from the galacturonic acid of hemicellulose, it does not

altogether carry conviction. Evidence, in general, is more in favour of an early lignification of tissues destined to become xylem, on the one hand, in contrast to an accumulation of pectic substances in the thick walls of fleshy unlignified tissues on the other hand. That is, all cell-walls contain cellulose and hemicellulose; in unlignified walls a preponderance of the special pectin complex is developed; in lignified walls other developments take place.

The various interrelationships of these substances we have been considering is indicated in the following scheme:



As all the various workers have differed greatly both as to their methods and the conclusions drawn from their results, it will be more satisfactory to deal with each one in chronological sequence. Previous to 1916, considerable investigation of pectic substances had taken place, both on macro- and micro-chemical lines. Much valuable information existed, though great confusion reigned, especially in regard to nomenclature, for the reasons already given at the outset of this account. In view of improved methods and increased knowledge of the carbohydrates in general, no details will be given of researches before 1916.

In 1916 Schryver & Haynes (35) published results on the isolation of certain pectic substances from turnips, strawberries, rhubarb stems and apples. They extracted the pectic compounds from the finely ground material (after expression of juice) with warm 0·5 % ammonium oxalate; these were precipitated by alcohol as gelatinous precipitates, which were purified by reprecipitation by alcohol from aqueous solution. The product in each case they termed "pectinogen". It is soluble in water, giving a viscous, opalescent, slightly acid solution. Its salts with alkalies and alkaline earths are also soluble. On allowing the solution to stand with alkali, it gives, on neutralisation, an insoluble gelatinous precipitate or gel. Also, if allowed to stand with lime water, a gelatinous precipitate separates out. The product formed, after action of alkali and neutralisation, was termed "pectin"; it was purified and analysed, giving an empirical formula of $C_{17}H_{24}O_{16}$. From the furfural value they concluded it contains one pentose group. We now know that the product isolated by Schryver & Haynes is probably a mixture of pectins, of varying methoxyl content, from the cell-wall, together with some pectic acid from the pectates of the middle lamella. (The ammonium salts of these would be extracted by ammonium oxalate, and subsequently would give the free compounds on addition of acid.) On treatment with alkali (saponification) and subsequent neutralisation, the pectin would give insoluble pectic acid, or on standing with lime water, calcium pectate is precipitated. The furfural value affords no clue to constitution, as it may arise from both galacturonic acid and pentose.

Fellenberg (18–20) in 1916–18, worked on pectic substances from Beet roots. He is of the opinion that pectose is probably a compound of pectin and cellulose formed by condensation of carboxyl groups with elimination of water. He concludes that it is not a calcium compound, since the greater part of this metal could be removed by acid without decomposing the pectose. He suggests that the conversion of pectose to pectin is a hydrolytic process, brought about artificially by acid, and catalysed in the plant by an enzyme. By removing all water and alcohol-soluble products from the material, he obtained a residue which he considers to be pectose and cellulose only. On treatment of this crude pectose with alkali, he detected, for the first time, methyl alcohol, as a component of pectin, pectic acid being produced simultaneously. In this process he concludes that pectose is first decomposed into cellulose and pectin, and the latter, subsequently, into methyl alcohol and pectic acid.

Fellenberg prepared pectin by hydrolysing pectose with water under pressure, the pectin formed being precipitated with alcohol. He confirmed the presence of galactose and arabinose in the pectic complex and, in addition, claimed to have shown the presence of methyl pentose. He proved that methyl alcohol and pectic acid are formed quantitatively by the action of alkali on pectin in the cold. By acid hydrolysis, he claimed to have produced a series of products, intermediate between octomethoxypectic acid (neutral pectin) and pectic acid, with a decreasing methoxyl content and an increasing acidity according to the strength of the hydrolytic treatment; these he termed "pectinic acids". Various natural pectins were found to give values for methyl alcohol conforming to those which would be derived theoretically from these intermediate derivatives: the values are here shown:

Substance	Source	Methyl alcohol %
Octomethoxy compound (neutral pectin):		
Theoretical value	—	11·94
Pectin I	Orange	11·60
Heptamethoxy compound:		
Theoretical value	—	10·50
Pectin	Apple	10·57
,,	Quince	10·26
Hexamethoxy compound:		
Theoretical value	—	9·07
Pectin	Currant	9·3
,,	Turnip	8·9
Pentamethoxy compound:		
Theoretical value	—	7·61
Pectin II	Orange	7·46

Elementary analyses of his preparations of neutral pectin, together with estimations of the barium and calcium content of barium and calcium pectates, also support his theoretical conclusions as to the formulae for these substances (see p. 79).

The researches of Ehrlich from 1917 to 1926 (11, 13) on the pectic substances of the Beet (and other plants) were very thorough, but his conclusions do not fall into line entirely with those of Fellenberg and other investigators. It is to Ehrlich we owe the discovery of galacturonic acid as the basal unit of all pectic compounds. He used, as material, residues of the Beet, from which soluble matter had been washed away by water. From these he extracted a crude pectin, "hydropektin", by boiling with water, or, more rapidly, by heating with water under pressure. This product could be readily separated,

further, by means of 70 % alcohol, into an araban, which was soluble, and a calcium-magnesium salt of "pectic acid" (in Ehrlich's terminology), which was insoluble. It seems probable that the latter, as it is soluble in water and contains methoxyl groups, is what most investigators are agreed to call soluble pectin (i.e. a mixture of more or less esterified products).

Free "pectic acid" was obtained from the calcium-magnesium salt by treatment with hydrochloric acid and precipitation with alcohol. By hydrolysis it was split up into its components which were carefully extracted, identified and estimated. The percentage composition was as follows:

Components of "pectic acid"	Calc.	Found
For 4 mols. galacturonic acid	66·3	64·8
„ 2 „ methyl alcohol	5·5	6·7
„ 3 „ acetic acid	15·4	12·8
„ 1 mol. arabinose	12·8	11·7
„ 1 „ galactose	15·4	13·1

The "pectic acid" then can be regarded as triacetyl-arabino-galacto-dimethoxy-tetragalacturonic acid (see also p. 79).

Ehrlich & Schubert (12) have also made an investigation of the pectic substances of the Flax. Again a "hydropektin" was extracted with hot water and was separated into a component (hexopentosan) soluble in 70 % alcohol, and a salt of "pectic acid". The hexopentosan, on hydrolysis, and identification and estimation of its sugars, was found to have the following composition:

$$\underset{}{C_{27}H_{46}O_{23}} + 4H_2O = \underset{\text{Pentose}}{3C_5H_{10}O_5} + \underset{d\text{-Galactose}}{C_6H_{12}O_6} + \underset{d\text{-Fructose}}{C_6H_{12}O_6}$$

The "pectic acid" molecule was hydrolysed, and gave on analyses:

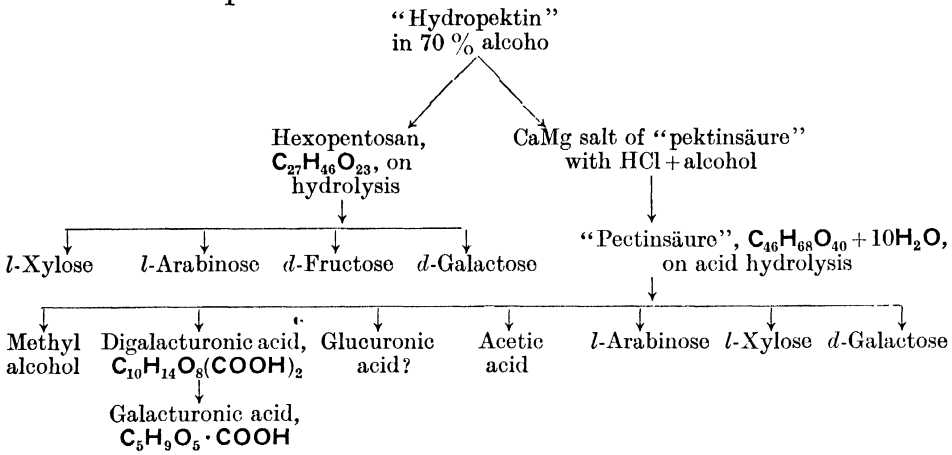
Components of "pectic acid"	Calc.	Found
For 4 mols. galacturonic acid	61·56	61·15
„ 2 „ methyl alcohol	5·08	4·13
„ 2 „ acetic acid	9·52	8·62
„ 1 mol. arabinose	11·90	10·90
„ 1 „ xylose	11·90	10·90
„ 1 „ galactose	14·28	13·60

from which is concluded that the hydrolysis took place as follows:

$$\underset{\substack{\text{Flax}\\\text{"pektinsäure"}}}{C_{46}H_{68}O_{40}} + 10H_2O = \underset{\substack{\text{Galacturo-}\\\text{nic acid}}}{4C_6H_{10}O_7} + \underset{\substack{\text{Methyl}\\\text{alcohol}}}{2CH_3OH} + \underset{\substack{\text{Acetic}\\\text{acid}}}{2CH_3COOH} + \underset{\text{Arabinose}}{C_5H_{10}O_5} + \underset{\text{Xylose}}{C_5H_{10}O_5} + \underset{\text{Galactose}}{C_6H_{12}O_6}$$

and that "pectic acid" may be regarded as a diacetyl-arabino-xylo-galacto-dimethoxy-tetragalacturonic acid.

The following scheme shows the composition and relationships of the various components:



In 1924, Sucharipa (36) made a series of extractions of pectic substances from the white portion of lemon peel, with special reference to pectose or protopectin, which he believes to be a compound of pectin with cellulose. He concludes, from his researches, that pectose is not homogeneous, but there may be a series of substances in which the methoxy groups are replaced by cellulose and *vice versa*. After a preliminary extraction of his material with water and alcohol, he prepared a protopectin by subsequent treatment with Schweitzer's reagent to remove cellulose. Then, by progressive decomposition of this protopectin with various reagents, such as water under pressure, 50 % sucrose solution and hot ammonium oxalate, he obtained pectin and an insoluble portion, which he identified as cellulose again by its solubility in Schweitzer's reagent. The various fractions of pectin from the protopectin gave a series of methoxyl values; the last and most stable fraction produced the smallest yield of methyl alcohol, and therefore, he concludes, has the greatest number of carboxyl groups replaced by cellulose. By the preliminary treatment with water, free or soluble pectin was removed which gave the maximum methoxyl value of 11·33 %. The results for the whole series are given in the table on p. 91.

The figures there given are comparable to those of Fellenberg for neutral pectin and pectinic acids (see p. 88).

In 1921, Clayson, Norris & Schryver (10) extracted and purified a number of pectins from various sources, such as apples, oranges,

cabbages, onions, pea-pods and turnips; these were then converted into pectic acid (termed by them "cytopectic acid"). They used, for extraction, salts, of which the anion gives an insoluble calcium salt, such as sodium carbonate, ammonium oxalate and tartrate. The analyses of the products were fairly concordant, and suggest again Schryver & Haynes' empirical formula, $C_{17}H_{24}O_{16}$. They also estimated the methyl alcohol formed during the conversion of pectin into pectic acid, and have found no definite ratio between the values for methoxyl and for pectic acid. This was not surprising, since, by using ammonium oxalate, etc., they probably extracted a certain amount of pectic acid; in addition to which, there may have been hydrolysis of the methoxyl groups during extraction. The resulting products, therefore, could scarcely be expected to give constant values for methyl alcohol.

Progressive decomposition of 100 *gm. of protopectin* (*Sucharipa*)

Product	% pectin	% methyl alcohol	% cellulose
Free pectin	7·63	11·33	—
Pectin (water under pressure)	3·31	10·20	16·30
,, (50 % sucrose)	0·99	8·03	16·74
,, (ammonium oxalate)	9·71	2·05	23·94

In 1922 Carré & Haynes (9) devised a method of estimating pectin (using apples as material) by conversion into pectic acid with alkali, and precipitation as calcium pectate in presence of acetic acid. The percentage of calcium in calcium pectate they found to be 7·66, and the empirical formula for pectic acid, $C_{17}H_{24}O_{16}$.

This was followed in 1922 by a paper by Carré (6) extending the method to the estimation of protopectin (pectose). This was hydrolysed to soluble pectin by heating in an autoclave with $N/20$ HCl to 110° C., and subsequent precipitation as a calcium salt. The changes undergone in the pectic constituents of apples in storage were followed by means of these estimations. The soluble pectin was found to increase rapidly from about October to March in apples kept at 1° C., and, afterwards, the increase was only very gradual. Conversely, the pectose, when allowances were made for certain complications, decreased steadily all through the year.

Carré supports Fellenberg's view that pectose consists of a combination of pectin and cellulose. She observes that pectose in different plant tissues varies in its behaviour to hydrolysing agents. In some

cases (ripe fruits) water alone will bring about considerable decomposition of pectose, whereas in unripe fruits, turnips, etc., acid hydrolysis was necessary. In regard to acid hydrolysis also, pectose is very variable, some samples being rapidly decomposed, others needing prolonged heating. She found also that different preparations of pectin gave different amounts of methyl alcohol. She concludes that both pectose and pectins may be mixtures of the various substituted pectic acids.

In 1923 Tutin (40), however, expressed the opinion that there is no chemical difference between insoluble and soluble pectin, and that the apparent insolubility of the former is only due to insufficient disintegration of the tissue. The whole, he maintains, can be extracted with water, if the tissues are very efficiently disintegrated, and sufficient time (40 days) is given for extraction. Tutin is probably incorrect in regard to insoluble and soluble pectin being identical, but correct, no doubt otherwise, since 40 days would enable the change to take place by slow hydrolysis. He also criticised Carré's method of estimation of pectic acid as calcium pectate, pointing out that other substances, insoluble in alcohol and acetic acid, might be precipitated under the same conditions.

In 1925, Carré (7) made a reinvestigation of the matter, and produced evidence showing that the conversion of pectose into pectin depends on the action of hydrolysing agents, and is not due to insufficient disintegration.

In 1925, Nanji, Paton & Ling (25) suggested the method of determination of galacturonic acid in pectic compounds by means of the estimation of carbon dioxide given off in decarboxylation on heating with acid. From calcium pectate they obtained the values given below based on carbon dioxide, furfural, estimation, etc. From these they deduct the ring structure for the pectic acid molecule mentioned at the beginning of this section (p. 78). The theoretical values are calculated on the basis of such a structure:

Components of pectic acid	As % of pectic acid	
	Found	Calc.
Galacturonic anhydride	70·56	69·7
Anhydroarabinose	14·27	14·25
Anhydrogalactose	15·17	16·55
Calcium	7·34	7·36
Total furfural	20·0	19·5

Nanji, Paton & Ling's formulae are therefore as follows:

Pectin (neutral): $C_5H_8O_4 \cdot C_6H_{10}O_5 \cdot C_5H_7O_4(COOCH_3)_4 = C_{39}H_{58}O_{33}$

Mono-, di-, tri-methoxy pectic acids ("Pectinic acids"):

$$C_5H_8O_4 \cdot C_6H_{10}O_5 \cdot C_5H_7O_4(COOH)_{4-n}(COOCH_3)_n$$

Pectic acid: $C_5H_8O_4 \cdot C_6H_{10}O_5 \cdot C_5H_7O_4(COOH)_4 = C_{35}H_{50}O_{33}$

The methoxyl values of the various esterified compounds are respectively (cp. Fellenberg, p. 88, and Sucharipa, p. 91):

Tetra-methoxy derivative (neutral pectin)			...	...	...	...	11·76 %
Tri-	,,	,,	...	...	...	...	8·94 %
Di-	,,	,,	...	...	...	...	6·04 %
Mono-	,,	,,	...	...	...	...	3·06 %

In 1925, Norris & Schryver (33) accepted the formula suggested by Nanji, Paton & Ling as the probable one for pectic acid. Estimations were made on preparations of pectin and pectic acid from various sources with a view to testing how far their products conform to this formula. Extraction was carried out with warm 0·5 % ammonium oxalate or oxalic acid solutions. They conclude, from the values obtained for methyl alcohol, that three carboxyl groups of pectic acid are usually esterified. There is evidence, also, of a stable form with one methoxyl group. By the action of alkali on pectin, they obtained the splitting off of a substance which they identified as a hemicellulose. They consider also that calcium enters into loose combination with the pectin molecule. It is probable that during their preparations, these authors may have extracted a certain amount of pectic acid by the use of oxalate; the pectins, also, may have been demethylated to some extent in extraction. These two causes would tend to give rise to mixed products.

In 1926, Emmett & Carré (16) introduced a modification into their method of estimation of pectic substances. It consists in precipitation with acid alcohol, and redissolving the precipitate before finally reprecipitating as calcium pectate.

O'Dwyer (34) in 1925 published a note on the extraction by means of ammonium oxalate of a substance claimed to be pectic acid from Beech wood. It is present to the extent of 0·3–0·5 % of the Beech wood sawdust.

Norris (31) in 1926 isolated pectin from the pulp of oranges by merely mincing and then pressing out the juice. From analyses he found that it closely resembles the products previously obtained by Clayson, Norris & Schryver by chemical means, i.e. it was a trimethylated ester of pectic acid. By allowing the enzyme (pectase) of the fruit to act on the pectin of the juice for some time, the pectin

was saponified, and an insoluble precipitate separated out, which, on purification and analysis, gave values identifying it with pectic acid.

In 1928, Nanji & Norman (24) published a method for routine estimation of pectic substances. Three solvents were employed, water, 0·5 % oxalic acid and 0·5 % ammonium oxalate. The water extracts free pectin; oxalic acid free pectin and pectose; ammonium oxalate free pectin, pectose and pectic acid (both free and combined as pectate). The determinations were made as calcium pectate.

Norman (30), further, in 1928 obtained a preparation of pectin from lemon juice, taking special precautions to avoid de-esterification. This gave a methoxyl content apparently corresponding to a trimethoxy derivative of pectic acid. A correction was made by referring the values to the calcium pectate yield of the preparation (this being the only known criterion of purity). The corrected figures then more nearly agreed with the value for a tetramethoxy derivative.

In 1928, Henderson (23), as a result of a research on the pectin of Flax (stems), suggested that, in place of the ring formula of Nanji, Paton & Ling, a simpler formula should be substituted, namely, galactose-tetragalacturonic acid:

$$(C_6H_{10}O_5 \cdot 4C_6H_8O_6 - H_2O)_n.$$

He further considers the arabinose component to be an impurity.

Later, in 1929, Norris (32) also made an investigation of Flax pectin by means of the ammonium oxalate extraction. The values obtained for furfuraldehyde, carbon dioxide and methoxyl indicate that this pectin also conforms to the constitutional formula of Nanji, Paton & Ling. He considers that there is no evidence for accepting Henderson's formula; nor are there grounds for the assumption of the presence of xylose as maintained by Ehrlich & Schubert.

From 1926 to 1929 a series of papers was published by Neuberg and his co-workers (26–29) concerning a research upon the volatile and other constituents of various samples of tobacco. The investigation also included the fresh leaf before drying. They detected (26) methyl alcohol as a product of decomposition of the pectic substances in the leaf. Furfuraldehyde and carbon dioxide from uronic acids were also estimated. Later (27, 29) they demonstrated the presence of the enzyme, pectase, which catalyses the saponification of the ester on autolysis.

Enzymes connected with pectic substances

The next point to consider is the question of enzyme action in connection with the changes described in the previous pages. There is a certain amount of evidence for the existence of an enzyme, **pectosinase** (propectase), which is responsible for the decomposition of pectose.

Carré (8) has maintained that, in the Apple, after soluble pectin has been washed away with cold water, the insoluble pectin is hydrolysed very slowly by cold water, more rapidly by hot water, and more rapidly still by hydrochloric acid ($N/100$ for 15 hours at 100° C. or $N/20$ in an autoclave for 1 hour at 110° C.). As evidence for enzyme action, however, she quotes the observation that the rate of development of soluble pectic substances by treatment with water at 45° C. is greater than when the tissues are treated with boiling water. The following experiment, moreover, points to the same conclusion. The soluble pectin was washed away from a quantity of ripe Apple pulp, and the residue left moist for 48 hours. On subsequent extraction with water a large amount of pectin was found to have developed, whereas a sample previously heated for an hour at 100° C. showed no marked increase of pectin.

The above observations, therefore, suggest the possibility that an enzyme plays a part in the natural decomposition of pectose in the plant.

On the other hand, there is now no doubt that the conversion of pectin (or of pectinic acids) into pectic acid, that is, the setting of pectin solution to a gel, is catalysed by an enzyme, **pectase**. Though not the discoverers of this enzyme, Bertrand & Mallèvre (1–3) have studied it extensively. They found it widely distributed in plants, being, in fact, identified by them in the juice of forty separate species. The time taken for mixtures of pectin solution and enzyme to set to a gel varied not only with the plant selected, but also in different parts of the same plant, as is shown in Tables XXIV and XXV on p. 96.

Very active preparations were obtained by Bertrand & Mallèvre from leaves of Clover and Lucerne by precipitation of the pressed-out juices with alcohol.

It would appear that the activity of the cell-sap is greatest in leaves of rapidly growing plants. Other factors, however, such as hydrogen ion concentration, may play a part. The retarding effect

of increased acidity was noted by Bertrand & Mallèvre; too acid saps must be neutralised before they will gel. Later, the influence of the presence of acids was studied by Euler & Svanberg (17). The former authors were also of the opinion that calcium plays a part in the pectase reaction. There appears to be no justification for this view. Possibly, the formation of a gelatinous insoluble calcium pectate may make the change from pectin to pectic acid more visible, especially in weak solutions; the calcium also may serve in some cases to decrease acidity.

TABLE XXIV

Plants	Time
Tomato (fruit)	48 hr.
Grape (fruit)	24 ,,
Currant (fruit)	15 ,,
Rhubarb (leaf)	12 ,,
Carrot (root, full-grown)	2 ,,
Delphinium (leaf)	1·5 ,,
Japanese Lilac (leaf)	20 min.
Carrot (root, very young)	15 ,,
Maize (leaf)	8 ,,
Florentine Iris (leaf)...	3 ,,
Potato, Clover, Lucerne, Plantain, Turnip	Less than 1 min.

TABLE XXV

Organs of Pumpkin	Time
Stem (base)	20 min.
,, (summit)	12 ,,
Petiole	8 ,,
Leaves (25 cm. across)	1 ,,
,, (7–9 cm. across)	3 ,,
,, (1–5 cm. across)	5 ,,
Corolla (male flowers)	45 ,,
Fruit (very young)	30 ,,

The reaction catalysed by the enzyme was shown by Fellenberg (18, 19) and later by Tutin (38) to be the same as that brought about by saponification with alkali; both authors found that methyl alcohol was produced during the gel formation. Tutin showed, by analysis, the pectic acid formed to be identical in each case.

Neuberg & Kobel (27) also have investigated the action of pectase from leaves of the Tobacco plant (*Nicotiana*). Both the pressed-out juice and an alcohol precipitate of the enzyme were used. The enzyme was found to produce a gel from solutions of pectin prepared from

the pericarp of *Citrus* fruit. The methyl alcohol produced was also estimated.

There is evidence for the existence of yet another enzyme termed **pectinase** which catalyses the hydrolysis of pectic substances with the production of reducing sugars. It was first detected by Bourquelot & Hérissey (4) in germinated Barley. A water extract from the grain, on precipitation with alcohol, gave a preparation of the enzyme. On adding the enzyme to a solution of pectin prepared from the root of Gentian reducing sugars were detected after 42 hours. Boiled solutions, as controls, gave no reducing sugar.

Gums and Mucilages. (Bibliography II.)

Less is known definitely of these two classes than of those which have preceded. Both gums and mucilages are abnormal products of the cell-wall, the former are frequently associated with injury, which may bring about hydrations and oxidations in the wall, thereby greatly altering the chemical composition of the normal complexes, and producing other complexes having the peculiar physical properties of this particular class. Cherry gum, which oozes from the various parts of the woody tissues of the tree, gives arabinose almost entirely on hydrolysis. Yet arabinose may be the chief component of other polysaccharides, which do not have the properties of a gum.

Norman (33) has recently made an investigation of gum arabic. This substance has been stated by previous workers to be a condensation product of arabinose, galactose and some unknown organic acid. The gum was purified by repeated precipitations with alcohol. It was suspected that the acid in gum arabic might be of the uronic type, since, on boiling with 12 % hydrochloric acid, carbon dioxide was evolved. Hence determinations were made of the pentosan and uronic acid contents by means of furfuraldehyde and carbon dioxide estimations respectively. On hydrolysis, arabinose and galactose were the only sugars detected. Different samples of gum arabic, however, gave different values for the components:

TABLE XXVI

Components	Sample I	Sample II
Uronic acid anhydride	12·56	17·56
Anhydro-arabinose	26·31	20·52
Anhydro-galactose	61·13	61·92
Yielding on hydrolysis:		
Arabinose	29·89	23·32
Galactose	67·92	68·80

This led the author to conclude that gum arabic is not a substance of definite empirical formula, though its general composition can be indicated, namely, a nucleus-acid consisting of galactose and a uronic acid, probably galacturonic acid, to which arabinose is linked by glucosidic linkages.

As a result of an investigation of the products formed after different periods of hydrolysis, the arabinose was found to be split off after a short treatment with acid, but the galactose does not seem to be split off except when the nucleus-acid is completely ruptured.

Investigations of other gums indicate a similar constitution, namely, a complex of hexose and pentose, together with uronic acid.

The following table, composed by Norman, gives the uronic acid content of various hemicelluloses and gums:

TABLE XXVII. Uronic acid anhydride content of some plant products

Substance	Source	Uronic content %	Sugars present	Authority
Pectic acid	Lemons	70·56	Galactose, arabinose	Nanji, Paton & Ling (III, 25)
Hemicellulose B	Beech wood	64·0	Galactose, arabinose	O'Dwyer (II, 35)
Mild oxidation products of pectin	Lemon pectin	60–55	Galactose, arabinose	Norman (unpublished)
Tragacanthin	Gum tragacanth	50·8	Arabinose	Norman (unpublished)
Hemicellulose by alkali treatment	Citrus pectin	37·3	Presumably galactose, arabinose	Candlin & Schryver (III, 5)
Hemicellulose B	Oat straw	31·8	? ?	Norman (unpublished)
Bassorin	Gum tragacanth	30·0	Arabinose, galactose, xylose?	Norman (unpublished)
Hemicellulose by alkali treatment	Onion pectin	21·5	Presumably galactose, arabinose	Candlin & Schryver
Acid gum arabic	(Sample II)	17·56	Galactose, arabinose	Norman
Hemicellulose B	Beech wood	18·8	? ?	Candlin & Schryver
Hemicellulose A	Turnip	15·4	? ?	Candlin & Schryver
Acid gum arabic	(Sample I)	12·56	Galactose, arabinose	Norman
Hemicellulose A	Beech wood	11·5	? ?	Candlin & Schryver
Hemicellulose A	Beech wood	11·0	Xylose (galactose?)	O'Dwyer
Hemicellulose A	Oat straw	10·8	Arabinose ?	Norman (unpublished)
Hemicellulose B	Turnip	10·7	? ?	Candlin & Schryver

Hence, as Norman points out, and as we have also emphasised on pp. 84–86, it is obvious that there is no essential difference, in terms of components, between the gums and hemicelluloses. It is rather that certain complexes, like the pectins, gums and mucilages, have been particularised on account of some special physical property peculiar to each group.

The mucilages present points of interest. These complexes swell up in water, giving thick slimy solutions. A characteristic example is the mucilage on the surface of the testa of linseed, the seed of the

Flax (*Linum*). When the dry seeds are placed in water, the outer portion of the seed swells, and finally gives a thick slimy solution. Various sugars, glucose, galactose, arabinose and xylose are obtained on hydrolysis, but this does not explain why this particular condensation product has its characteristic physical properties.

In connection with mucilages in certain other plants, members of the Cactaceae, some problems of interest arise. It has been pointed out by Spoehr that the pentosan content, by analysis, of *Opuntia phaeacantha*, one of the Cactaceae, is very high. Correlated with this is the abundant occurrence of mucilage in the tissues of the succulent stem structures of these plants, especially in certain cells.

Spoehr's view is as follows: owing to the restricted metabolic activity of these desert plants the concentration of hexoses is low. Hence oxidation may take place after the hexoses have condensed together by their reactive aldehyde groups being fixed. This oxidation takes place in the CH_2OH group attached to the sixth carbon atom with the production of glucuronic acid. From this acid, by decomposition, in sunlight probably, pentose arises, as we have already indicated. Spoehr's contention is that high pentosan content (and this is borne out by analysis in *Opuntia*, though the pentose content is not abnormally high) tends to produce polysaccharides of the mucilage type. Hence such are frequent in this species of *Opuntia*, and, being water-retaining, their production is correlated with the desert environment of these plants.

We may look upon this phenomenon, possibly, as an extreme case, where adverse conditions, such as drought, lead to increased condensation to polysaccharides and lack of formation of adequate material for respiration. In such a case, the cell-wall may itself be a source of material for oxidation with evolution of carbon dioxide. The glucose molecules, which ordinarily form a cellulose complex, are oxidised without hydrolysis. The resulting complex, mucilage, does not express itself as a structural cell-wall, but as a semi-solid mass dispersed through the cell, and thereby conferring on the tissues their water-retaining property. To a lesser extent, the formation of glucuronic acid groups in oxycellulose, in certain hemicelluloses and in pectic acid may be regarded as the same type of oxidation.

It thus appears that the components of the unlignified cell-wall may be of almost infinite variety, the changes being played upon a number of complexes, the condensation products of a definite specific number of sugars, together with acids which are themselves

oxidation products of the sugars. In struggling to classify these complexes as cellulose, hemicellulose, pectins, etc., we are probably trying to divide a greater complex of many parts which more or less grade into one another.

Cuticularisation and suberisation. (Bibliography IV.)

We now pass to another modification of the cell-wall, which is known generally as a cuticularised cell-wall. This type of change is chiefly to be found in the epidermis, the external layer of the higher plants, though the phenomenon is by no means restricted to this tissue. Suberisation of the wall occurs in various tissues, but, again, usually in the outer portions of stem and root.

As a result of many observations, largely, though not altogether, micro-chemical, the conclusion has been drawn that fatty substances are involved in these modifications which we are about to consider. More recently, investigations of Priestley and his co-workers have thrown considerably more light on this particular type of modification.

Cutin and **cuto-cellulose.** When we examine microscopically the cells of the epidermis of the leaves and shoots of vascular land plants, we find attached to the outer cellulose wall all over the plant a layer or skin of a substance impermeable to water. This layer varies in thickness, being generally, at one extreme, very thin in shade-loving plants growing in damp surroundings, and, at the other extreme, very thick in desert plants adapted to very dry situations. The substance, of which the cuticle consists, is known as cutin, which name must be taken as a collective term, not as a name for a chemical entity. Cutin is resistant to hydrolysis by acids, in fact to 70 % sulphuric acid and also to zinc chloride in hydrochloric acid, in which reagent cellulose is soluble. The greater portion of the cuticle is insoluble in fatty solvents. By treatment with alkali a certain amount of fatty acids can be detected and removed by formation of soaps. There is, however, a remainder which will not react in this way. No cellulose has been detected in the real cuticle. Below the cuticle, moreover, the cellulose walls of the epidermis may be cuticularised, that is cutin is deposited with the cellulose.

On the basis of experimental work, Priestley and his co-worker (2, 5) have formulated a theory as to the origin and formation of cuticle and cuticularised cellulose. Their view is outlined as follows. In the meristematic tissues of the growing point of the stem fat is

formed in the active metabolism of the tissues. Elsewhere, in dealing more especially with fats, it will be indicated how, possibly, by divergence from the main lines of respiratory metabolism, fats can be formed by successive condensation of molecules of pyruvic aldehyde and acetaldehyde formed in glycolysis and the first stages of respiration. Both saturated and unsaturated fatty acids may be produced in this way.

In the newly formed meristematic tissues, owing to lack of vascular tissue, there is a scarcity of metallic ions. Just behind the growing point, however, inorganic salts are brought by the vascular tissue, and the fats become saponified and converted into sodium, potassium, magnesium and calcium soaps. As soaps, the fatty substances then pass to the protoplasmic interfaces during differentiation and vacuolation of the cell contents, and also subsequently migrate, in solution, into the cell-wall. By evaporation and consequent movement of water, soaps are drawn to the surface of the epidermal walls and into contact with air, when those of the unsaturated fatty acids are oxidised with the formation of an insoluble varnish-like skin impermeable to water, analogous to the insoluble pellicle or skin which is produced when a thin layer of a drying oil, such as linseed oil, is spread on a surface. From an oily film the cuticle develops into a resistant varnish. In the movement of the soaps in the wall, Priestley suggests that the cell-wall may be chemically attractive, especially for unsaturated acids.

He regards cutin as a mixture of fatty acids with other substances which have undergone oxidation and condensation during exposure to air. Cuticularised layers, which may lie between the inner cellulose wall of the epidermis and cuticle, are of cellulose matrix, more or less heavily impregnated with deposits of fatty substances.

Evidence for their hypothesis has been gathered together by Priestley and his co-workers from various sources.

From estimations in Bean (*Vicia Faba*) seedlings they obtained evidence that in the apex of root and shoot the amount of fatty substance is relatively consistently higher in all stages of growth up to maturity. In the young plant, moreover, the fatty material is largely unsaturated, but becomes more saturated as the plant grows older.

The ratio of the bases in the soil may be expected to affect the thickness of the cuticle. Movement of fatty acids in the wall depends on the relative solubilities of the different soaps. Potassium, sodium

and magnesium are relatively soluble, and calcium, insoluble. The thickness of the cuticle depends on these ions in soil solution. Increase in the proportion of potassium is followed by greater mobility of fats and, therefore, increased thickness of cuticle, whilst increase in calcium diminishes the mobility and results in a thinner cuticle. The following figures show the relative thicknesses of cuticle of petioles of fruit trees grown on soils fertilised with different inorganic salts:

No potassium supplied	No calcium supplied
5·6μ	6·0μ
6·1	7·0
9·6	10·5
6·4	9·5
4·7	5·9

A certain amount of macro-chemical examination of the cuticle has been made by Lee (1). Cuticle was prepared from petals of *Chrysanthemum* flowers and from Rhubarb stalks. A large quantity of material was prepared and analysed with a view to finding out whether free fatty acid, glycerides (fats), soaps, etc., are present. The conclusion drawn was that cutin is a complex mixture of fatty acids, which have undergone condensation and oxidation. These occur both free and combined with alcohols. Soaps of fatty acids also occur, and, in addition, unsaponifiable matter, which probably contains higher alcohols. The relative quantities of these different constituents vary, but there is always a higher total quantity of oxy-fatty acids than of normal fatty acids. The chloroform-soluble fat, for instance, from the cuticle of out-door grown Rhubarb had a lower iodine value (40–43) than that of forced Rhubarb (90–91). In the damp sheds used for forcing, conditions appear to be less favourable for oxidation. In this change, moreover, light is considered by Priestley to play a part. He claims to have shown that fat, extracted from meristem, condenses into a varnish-like film on exposure to light, but not in darkness.

A very thin cuticle can be traced over the meristematic apex. As the shoot increases in volume, the cuticle will be stretched. As a matter of fact, it grows in thickness and may become very thick indeed (ordinarily, 10–12 times increase); therefore, there must be a continual addition of fatty acid to the cuticle. Priestley supposes that, up to a certain stage, the cuticle is permeable to fatty acids themselves. With increasing age it becomes oxidised and condensed and less soluble in fatty solvents; finally it loses its power of

dissolving additional fat. Then fats become deposited in the cuticularised layers below.

The cuticle, however, is not the only manifestation in the plant of this process of water-proofing of the cell-walls and rendering them impermeable both to water and solutes. There are also certain internal, more or less cuticularised, tissues. But to interpret the possible significance of these structures, it is necessary to summarise briefly some suggestions brought forward by Priestley. This author points out, as is well known, that the meristematic tissue of the growing point of the shoot of the higher plants is superficial. It is situated at the surface, and becomes raised up into folds, which eventually develop into the lateral organs, leaves, axillary shoots, etc. Moreover, directly behind the growing point is a mass of undifferentiated and vacuolated tissue lying between the meristem and the developing vascular tissue.

In the root the arrangement is different. The meristematic tissue is not superficial, but lies some distance behind the apex which consists of a mass of cells, the root-cap. From the meristem of the root the vascular tissues are developed inwardly and are in closer relationship with the meristematic layer. Moreover, as the tissues become differentiated, the central vascular cylinder becomes enclosed in a cylinder of cells, one layer thick, known as the endodermis. In the endodermis, round each of the transverse and radial longitudinal walls, is a band of cuticularised substances. So one may regard this layer as being really a cylinder of open mesh-work, where the mesh is a varnish-like cuticle impermeable to water and salts, and the meshes are filled in by living protoplasts which, though permeable to water, have a selective power on solutes which pass through. This cuticularised strip of the (primary) endodermis is known as the Casparian strip.

So much for the structure; as to chemical knowledge, Priestley and his co-worker (13) have made a chemical examination of the composition of the cell-wall of the apical meristem of both root and shoot. They have, in addition, with great labour, collected sufficient material of the actual Casparian strip from stems of *Potamogeton* to make a chemical analysis (9). Very briefly, we may say that they make the following observations and draw the following deductions.

In the cell-walls of the meristem of the shoot-apex (13), cellulose and pectic substances preponderate, as also in the tissues immediately behind the apex. The materials for synthesis at the growing-point

arrive by the vascular tissue chiefly, and must reach the meristem by diffusion. A cell-wall of cellulose and pectin has much the consistency of blotting-paper and, when saturated with water, offers little resistance to diffusion of soluble substances, whereas protoplasm may offer considerable resistance to diffusion of sugars, salts and organic acids.

The cell-walls of the meristem of the root, on the other hand, consist of cellulose but associated with less pectin and much more fat and also with protein. (They do not give the cellulose reaction with iodine and sulphuric acid except after boiling with 20 % caustic potash.) It is suggested that this meristem of the root, therefore, partaking more of the nature of protoplasm itself, offers more resistance to diffusion of water and solutes.

Hence, Priestley regards it as a suggestive fact that a predominant carbohydrate wall is found in the shoot-apex, associated with a free growth of meristem cells at the surface of the shoot, whereas in the root, the presence of protein and fats in the walls, which restrict free diffusion, is associated with an active meristematic growth in the inner layers of cells nearer the vascular supply. The cells at the outside of the meristem, indeed, fail to continue growth and are then added to the root-cap.

Whether this suggestion approximates to the truth or not, certainly these differences of distribution are connected with the different appearances of the two growing-points. The shoot-apex, growing freely at the surface, is thrown into folds of meristematic tissue, which are the initials of leaves and branches. The root tissues, however, continually added to from within the apex only increase the length of the root.

Further, it should be stressed that the fatty substances which, in the case of the root, are retained in the wall of the meristem cells, have also been present in the walls of the shoot meristem, as transitory products. Instead, however, of remaining there, they migrate immediately to the surface of the shoot, where they unite into the thin film of fatty substance, the cuticle.

In the root, these fatty substances never migrate freely; there is never a cuticle, but instead, at certain inner walls of the endodermis, fatty substances collect and are there oxidised on coming into contact with air, the cortex being well supplied with intercellular spaces. These oxidised and condensed fatty substances, localised in a definite region, constitute the Casparian strip. (There are further changes in

the old and more mature root, but it is neither necessary nor within the scope of this account to consider them.)

Evidence from micro-chemical examination and from analysis of a sample of the Casparian strip collected from *Potamogeton* stems led Priestley & North (8) to the following detailed conclusions. The walls of the endodermal cells, apart from the Casparian strip, are of cellulose and pectic substances. The strip consists of a basis impregnated with fatty substances. The latter consist of free fat and other fatty derivatives in some form of combination, yielding, on saponification, normal and oxidised fatty acids. The basis of the strip is in intimate connection with some substance containing nitrogen. It is suggested that it is similar to the meristematic cell-wall (protein-cellulose complex) of the growing point of the root, and that the laying down of the cuticularised strip commences very early in development, before the walls have lost their primitive character.

There is another set of phenomena observed by Priestley (6) of considerable interest in connection with the endodermis, namely those shown by the etiolated shoot. Now in either darkness or in light a root grows in much the same manner, and the structure is little altered. In view of the fact that the cells actively growing and dividing are sunk in the tissue of the root, this is perhaps natural. But the facts are different in regard to the superficial meristem of the shoot. When this is grown in darkness the phenomena of etiolation result. If a seedling of the Bean (*Vicia Faba*) or Pea is germinated in absolute darkness, i.e. never exposed to light, growth proceeds, but mainly consists in elongation of the stem by the formation of new cells, which vacuolate and separate from the basal rim of the meristem. There is practically no development of leaf, that is, it becomes an excessively elongated slender stem with a few rudimentary leaf initials.

When one examines the internal structure, several facts of interest are observed. First, there is in *Vicia* a well-defined functional endodermis with a Casparian strip as in the root. Such an endodermis is not found in the normal shoot in light. Second, the cell-walls of the growing-point of the etiolated shoot contain more fatty substances than those of the normal shoot. Third, the cuticle is thinner.

The results have been interpreted as follows. In the absence of light, the fatty substances released from the meristem cells stay on the walls of these cells instead of moving outwards and contributing

to the cuticle. The walls, therefore, interposed between the meristem and the vascular supply impede the diffusion of solutes to the meristem. The growth of the superficial layer of the meristem is thus diminished, no lateral folds are formed, and new leaf initials do not separate from the apex. The shoot in darkness resembles, then, to some extent, the root. Fatty substances do not readily travel to the surface, and are instead deposited as a Casparian strip, thus giving rise to a functional endodermis similar to that in the root.

When we consider, moreover, the structure of the endodermis, we see that organic solutes are unable to leak out from the central cylinder, because the endodermal protoplasts are relatively impermeable and permit very few solutes, and those mainly inorganic, to pass. The strip itself is impregnated with fatty substances, and is therefore impermeable to water and to substances soluble in water. In a root or stem with such an endodermis, the growth and structural development of the tissues outside the endodermis are severely restricted. The peculiar development of etiolated plants may be partly attributed to the restricting effect of this endodermis.

In connection with the etiolated shoot, there is yet another set of observations of interest made by Priestley (4), namely, the effect of illuminating gas or ethylene. Etiolated Bean seedlings were used. These were tall turgid stems, round in outline with a functional endodermis. The growth of such seedlings was continued in the dark, but over water in bell-jars into which a few c.c. of illuminating gas were bubbled. Within a few days, the stem of the "gassed plants" swelled in girth. In a few more days it was nearly square in outline, as in the normal plant, and subsequent examination showed that in the thicker portion grown in the coal-gas every trace of the Casparian strip had disappeared.

As previously mentioned, Priestley holds the view that unsaturated acids diffusing slowly through the cell-walls of the developing root are picked out by the basal layer in the endodermal cell-wall, and are thus concentrated in the walls of a layer of cells abutting upon intercellular spaces containing air, and are, subsequently, oxidised and condensed, forming the strip. The sharp localisation at the strip suggests that the diffusing unsaturated acid is picked up in this region by chemical means. In the presence of unsaturated hydrocarbons this strip fails to form, and hence the structural change in the stem. These hydrocarbons, diffusing into the root, appear to saturate the chemical linkages, which usually pick up the unsaturated

acids, and so these acids are no longer held up in the region of the future endodermis.

Priestley (6) obtained some remarkable results (which, to a large extent, await explanation) by exposing etiolated plants to two minutes' light only per day. This causes a considerable difference in the appearance of the plant, the apical leaves having opened out a little and leaves having appeared also at the second and third internodes.

There is yet another microscopical observation. The cells of the cortex of a completely etiolated seedling will not plasmolyse in strong glycerine or sugar solution. The protoplasm seems to be firmly attached or embedded in the cell-wall. This same phenomenon is true of the protoplasm attached to the Casparian strip of the endodermal cells. On plasmolysis, the protoplasm is withdrawn from the wall except in the region of the strip.

Priestley suggests that the walls of the cortex of the etiolated shoot and of the basal wall of the strip are of a primitive type, like that of the meristem of the root, which contains fat and protein, the latter being in continuity with the protoplasm. This primitive character is retained, that is, the wall is not really differentiated from the protoplasm, and the fatty substances of the strip are oxidised upon the basal substance and permanently embed the protoplasm, so that, on plasmolysis, the latter cannot leave the wall. As already pointed out, such a primitive wall, containing protein and fat, prevents adequate diffusion to the growing-point of the etiolated shoot (as well as in the root).

The remarkable fact observed is that the cortical cells of seedlings with two minutes' daily exposure to light will plasmolyse fully. Hence, such a short exposure accelerates the removal of these impregnating substances from the wall, there is greater movement of water and solutes through the walls, the distribution of the meristem is extended and there is a greater development of lateral organs.

Suberin. Finally, there is yet another change which may take place in the cell-wall, namely, the formation of "suberin". It is well known that, in various portions of the stem, petioles and root, sometimes superficially, sometimes so deep-seated as to be next to the vascular cylinder, certain cells may begin to divide tangentially. In the walls of the cells on the peripheral side a deposit is laid down which is collectively known as suberin. This layer of suberised cells is cork periderm, the meristem which gives rise to it, cork cambium or

phellogen. Large accumulations of cork periderm arise in such a tree as the Cork Oak and, when stripped off at intervals from the tree, constitute bottle cork.

Suberin, as a collective term, includes a number of substances similar to and analogous with "cutin". Suberin is also a mixture of fatty acids which have undergone oxidation and condensation.

If bottle cork is hydrolysed with alcoholic soda the salts of certain organic acids can be obtained. Two of the best known, which can be prepared in the crystalline state, are called phellonic ($C_{22}H_{43}O_3$) and phloionic ($C_{22}H_{40}O_7$) acids. These acids are soluble in the usual solvents for fatty acids. If, however, they are heated in sealed tubes substances are formed which are regarded as anhydrides or condensation products, and these are no longer soluble in fatty acid solvents.

Priestley (3) describes an experiment illustrating this point. He soaked a Soxhlet thimble in potassium phellonate, and then heated it in a sealed tube. After heating, the phellonate could no longer be removed from the thimble by the usual fat solvents. Moreover, the thimble was completely water-proofed and would hold water for any length of time.

Priestley, therefore, suggests that suberin of cork is also composed of mixtures of the anhydrides and oxidation products of the higher fatty acids which, like those of the cutin, migrate out as soaps.

Another case of interest is that of the Potato tuber cut across, and the surface left exposed to air (Priestley & Woffenden, 11). Various changes take place which are described by Priestley. The cells on the cut surface, which were previously crowded with starch grains, rapidly lose their starch (cp. the drying up of slices, p. 58), and the walls of the cells near the rupture become suberised. Within a few days a layer of cork cambium is formed, which proceeds to form periderm, that is, cells whose walls are suberised.

The whole process is of some complexity, but the interest of the case is that, after injury, there is a sudden conversion of a considerable amount of starch into sugar, and abnormal formation of fatty acids or substances of a fatty nature, which become oxidised and condensed in the cell-wall to suberin. The outcome is the formation of this impermeable layer over the cut surface.

Lignin. (Bibliography v.)

In addition to the changes already described in the cell-wall, there are yet others, due to deposition of what are termed "incrusting substances", the chief of which is known as lignin.

It is not in place here to describe the various types of lignified cells. Lignification is usually accompanied by considerable change in the form of the cell, as in the development of the vessels and fibres of the xylem; sometimes, however, as in xylem parenchyma, the shape undergoes relatively little modification. The lignification of the wall may be partial, as in the annular and spiral vessels of the protoxylem; or it may be almost complete, with small unlignified pores, as in stone cells of the flesh of the Pear, etc. Though formation of lignin is usually accompanied by death and disappearance of the protoplasm, this is not necessarily always the case, as, for instance, in certain types of xylem parenchyma.

Incrusting substances, such as lignin, naturally arise from the cell-contents. The question of the chemical composition of lignin, and the problem as to whether it is in true combination with cellulose or only in a state of physical adsorption, have occupied the attention of many workers.

Lignified tissues may contain tannins, colouring matters and other substances absorbed in some way. In addition, of course, there is the original cellulose basis with its various modifications. Thus, an analysis of wood may present the following components:

Cellulose

Hemicellulose { Arabinose, Xylose, Mannose, Galactose, Uronic acids

Lignin

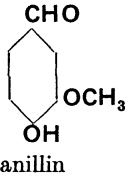
Tannins, colouring matters, etc.

The amount of hemicellulose content of any wood will depend on other changes which we have already considered. It is not directly connected with the amount of lignin formed, though it appears as if there may sometimes be a correlation between the extent of lignification and the amount of other components present.

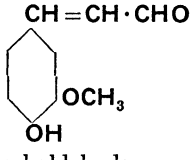
A number of colour reactions have been described for the detection of lignin. One, in particular, has been extensively used, and has a

certain interest. This is the bright magenta colour given with lignin by phloroglucinol in presence of strong hydrochloric acid and alcohol. If other phenols, resorcinol, pyrogallol, etc., are substituted for phloroglucinol, different and less brilliant colours are produced. Occasionally, woody tissues, as for instance, cherry wood, will give the colour with hydrochloric acid alone.

The question has been much debated as to whether the above colour reactions are due to aldehyde groups in lignin itself or to a substance of an aldehyde nature which accompanies lignin. Though the latter view is generally accepted, various observations are of interest in this connection. When wood is distilled with superheated steam the distillate will give the lignin reaction (Wichelhaus & Lange, 31). It has been suggested that such substances as vanillin and coniferyl aldehyde



Vanillin



Coniferyl aldehyde

may be responsible, since both give the lignin reaction with phloroglucinol and hydrochloric acid. Though both are found in the higher plants, yet they have not been shown to be universally distributed in lignified tissues. On the other hand, it is possible that aldehyde groups of lignin itself may be capable of producing the colour. The fact that wood, after treatment with sulphite, gives no phloroglucinol test, points to an aldehyde reaction; on the other hand, isolated lignin, it is said, does not give the reaction.

In reference to the lignin reaction, Czapek (2) isolated from wood a substance giving the phloroglucinol test, which he termed "hadromal". Later work (Hoffmeister, 16), employing a modification of Czapek's method, indicated that hadromal may be coniferyl aldehyde. Hoffmeister, moreover, considers it to be present in the form of a cellulose ester.

The study of lignin is rendered very difficult by the impossibility of isolating it unchanged from cellulose. Various methods have been employed by different investigators for this purpose. They may be enumerated as follows:

(1) By caustic soda solution. This extracts the lignin which is then precipitated by addition of acid.

(2) By calcium sulphite solution. This gives sulphonic acid derivatives of lignin.

(3) By concentrated acid. By this method the cellulose and hemicelluloses are converted into sugars, the lignin being relatively unchanged. Some workers have used concentrated sulphuric acid, others (Willstätter & Zechmeister, 32) concentrated hydrochloric acid. The action is carried out in the cold.

Lignin has been isolated by a number of workers employing various of these methods, and attempts have been made, on the basis of a determination of the reactive groups, to arrive at some idea of the constitution of the molecule. The following results of investigations have been tabulated by Dorée & Barton-Wright (3):

TABLE XXVIII.

Method of isolation*	Author	Empirical formula	No. of OH groups	No. of OMe groups	Corresponding basic "lignol"	Lignol	
						% C	% H
1. 17 % HCl cold	Friedrich & Diwald	$C_{39}H_{48}O_{14}$	1	5	$C_{34}H_{38}O_{14}$	60·8	5·6
2. 3 vol. HCl (*d.* 1·18) and 1 vol. H_3PO_4 (*d.* 1·7)	Urban	$C_{43}H_{48}O_{16}$	5	4	$C_{39}H_{40}O_{16}$	61·2	5·2
3. H_2SO_3 aq. at 110°	Dorée & Hall	$C_{26}H_{30}O_{12}S$	3 or 4	2	$C_{24}H_{24}O_9$	63·2	5·26
4. "	Klason (20, 21)	$C_{30}H_{32}O_{12}S$	3 (?)	2 (?)	$C_{28}H_{26}O_9$	66·1	5·1
5. Calcium bisulphite liquor—computed	Klason α-lignin (18)	$C_{22}H_{22}O_7$	1	2	$C_{18}H_{16}O_6$	65·85	4·84
6. 4 % NaOH at 10 atmos. pressure 1 hour	Dorée & Barton-Wright. Metalignin	$C_{20}H_{20}O_6$	1	2	$C_{18}H_{16}O_6$	65·85	4·84
7. 8–12 % NaOH at 140–160° for 6–10 hours	Powell & Whittaker (28)	$C_{45}H_{48}O_{16}$	5	4	$C_{41}H_{40}O_{16}$	62·4	5·07
8. 2 % alcoholic NaOH in cold on Rye straw	Beckmann, Liesche & Lehmann	$C_{40}H_{44}O_{15}$	4	4	$C_{36}H_{36}O_{15}$	61·01	5·08

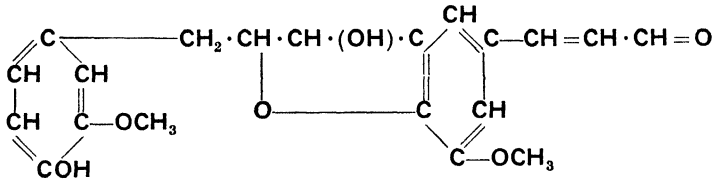
* Spruce wood or allied soft wood unless otherwise stated (Fuchs, 7, p. 281).

The general conclusions from these researches are that lignin contains hydroxyl, methoxyl and (?) acetyl groups. Also an aldehyde or ketone group. The methoxyl groups are probably of both the ester and the ether type. Unsaturated linkages are also present which unite with bromine or iodine. The empirical formula for the basic "lignol," a hypothetical substance free from sulphonic, acetyl or methyl groups, shows for all the preparations a certain similarity; the number of carbon atoms approximates to thirty-six (except in two cases, where it is half this value). There is little doubt that the product obtained is affected by experimental conditions, such as concentration of alkali used, time and temperature of extraction, etc. Methoxyl groups may be split off, oxidation may take place, or even more drastic changes may be brought about.

Some points may now be mentioned in connection with the lignin isolated by various workers.

Klason (17–21), using the sulphite method, isolated from the wood of the Spruce Fir the sulphonic derivatives of what he believed to be two components of lignin, namely, α- and β-lignin.

For α-lignin he assigned a formula containing the acrolein group —CH = CH.CHO:



and which may be considered to be derived from two molecules of coniferyl aldehyde. β-Lignin, on the other hand, is regarded as containing the corresponding acrylic acid group —CH = CH.COOH.

Dorée and Barton-Wright criticise the validity of this formula on the grounds that gentle oxidation of lignin yields no aromatic product, which fact could scarcely be possible if the above constitution were correct.

Beckmann, Liesche & Lehmann (1) used very gentle treatment for extraction of lignin from Rye straw, namely, a prolonged (48 hours) treatment with a cold 2 % aqueous-alcoholic caustic soda solution (the alcohol prevents solution of hemicelluloses). Their product, on analysis, was found to contain four hydroxyl and four methoxyl groups.

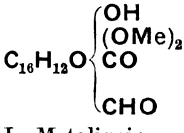
Powell & Whittaker (27, 28) investigated the lignins from Flax and also a number of woods (Pine, Spruce Fir, Ash, Birch, and Poplar). They used, for extraction, 8–12 % caustic soda solution at 140–160° C. for 6–10 hours. They consider the lignin to be little changed by this process. The products from different sources were of constant composition ($C_{45}H_{48}O_{16}$). They are of the opinion that lignin is derived from a basic substance, termed lignol, containing nine hydroxyl groups; the various lignins differ only in the extent to which the hydroxyl groups of lignol are methylated. The product, $C_{45}H_{48}O_{16}$, contains for instance, four methoxyl and five hydroxyl groups.

Dorée & Barton-Wright are of the opinion that prolonged action of caustic alkali may bring about removal of methoxyl groups from the natural product. Thus, during extraction, the latter may be entirely demethylated, or may be accompanied by demethylated

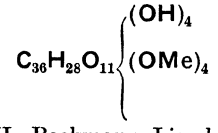
derivatives. With this idea in mind these authors, therefore, prepared lignin from Spruce Fir wood by adopting a method devised by Mehta (25), namely, extraction of previously purified sawdust with 4 % sodium hydroxide solution in an autoclave for an hour. The lignin was precipitated by acid, and purified by reprecipitation from glacial acetic acid. Owing to its rapidity, this method is supposed to cause the minimum change in the lignin, such as by demethylation, etc. The product obtained was a brown powder soluble in most organic solvents except ether, benzene and petrol ether. It has a m.p. of 186° C.

By means of the usual lines of investigation, they claim the presence of two methoxyl groups, one free hydroxyl group, one aldehyde and one ketone group. The iodine value corresponds to two unsaturated linkages in the molecule. The molecular weight was found to be—$C_{20}H_{20}O_6$—approximately half that obtained by most of the previous workers (except that for the α-lignin of Klason, with which it roughly agrees). Dorée & Barton-Wright term their product metalignin, and suggest that it is the unit from which natural plant lignins are derived by polymerisation. The usual form of lignin isolated has twice the molecular weight of metalignin.

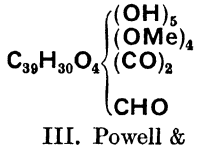
The schematic formulae representing the various alkali lignins are summed up by them as below:



I. Metalignin ($C_{20}H_{20}O_6$)

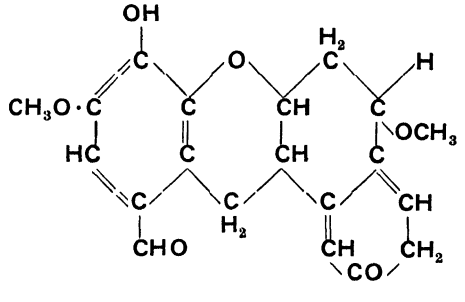


II. Beckmann, Liesche & Lehmann: alkali lignin from Rye straw ($C_{10}H_{44}O_{15}$)



III. Powell & Whittaker: lignin ($C_{45}H_{48}O_{16}$)

They suggest a constitutional formula differing from Klason's formula, and based on the existence in the molecule of hydroaromatic nuclei. In such a structure, they maintain, decomposition by oxidation would probably give rise to general disruption and production of oxalic acid and carbon dioxide:



As Dorée & Barton-Wright also point out, there is a connection between their product and the formula suggested most recently by Klason (20, 21) for the simplest acid obtainable by the action of sulphurous acid on wood, namely:

$$3C_{10}H_{10}O_3 \cdot H_2SO_3$$

Powell & Whittaker's results, which point to a similarity of products from different sources, suggest that there may be one unit upon which the natural lignins are based. From this unit, by different amounts of methylation, condensation or other changes, the various lignins in the vegetable kingdom are derived. There is evidence that varying amounts of methylation may even be present in the lignin of the same tree.

Below is given an analysis showing the proportions of the significant components of the wood of the Spruce Fir as determined by Dorée & Barton-Wright. The values are as percentages of the original dry sawdust:

	% of dry weight
Cellulose	55·0
Hemicellulose	7·8
Lignin	29·6

On the whole the value for lignin in woods of different species of trees varies from about 20 to 35 % of the dry weight of material.

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CHAPTER III

OXIDISING AND REDUCING SYSTEMS

FROM the tissues of the higher plants various enzymes have been extracted which will catalyse certain oxidations and reductions *in vitro*. It has generally been assumed that these enzymes catalyse such reactions in the living cells.

The enzymes described may, for the present, be classified under four main types, peroxidase, oxygenase, oxido-reductase and catalase.

Of these four types, peroxidase and oxygenase, though in reality independent catalysts, have been so much associated in the development of knowledge on plant oxidising enzymes, that it is not always possible to consider them entirely apart. Peroxidase may be said to be found in all the higher plants, but oxygenase is more restricted in its distribution. The distinguishing characteristic of peroxidase is the power of catalysing the decomposition of hydrogen peroxide (and, possibly, some organic peroxides) into water and nascent oxygen in the presence of certain oxidisable compounds; that of oxygenase of catalysing the autoxidation of aromatic compounds of a certain molecular structure. It has also long been known that, in general, crushed tissues, or tissue extracts, of plants containing oxygenase give a blue oxidation product (guaiacum blue) with guaiaconic acid, a constituent of guaiacum gum; tissues, or tissue extracts, on the other hand, of plants containing peroxidase alone, develop the blue colour only on addition of hydrogen peroxide. Both catalysts are thermolabile.

OXYGENASE-PEROXIDASE SYSTEM: GENERAL

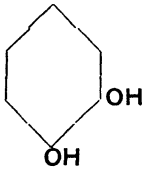
Since the reactions catalysed by oxygenase present considerable complexity, these will be discussed first in outline, leaving details for later consideration. In brief, it may be said that, according to the data available, rather over 60 % of the Phanerogams produce in their tissues compounds with the *ortho*-dihydroxy grouping characteristic of catechol. From these plants, also, a catalyst, oxygenase, can be extracted which catalyses the autoxidation of such compounds. It is with the mode of oxidation and the nature of the oxidation products that we are chiefly concerned.

The behaviour in solution of certain compounds with the *ortho*-dihydroxy grouping must first be mentioned. When solutions, in

ordinary distilled water, of catechol, neutralised protocatechuic and caffeic acids and other compounds with this grouping are allowed to stand in air at about p_H 7 for several days, such solutions gradually become brown, and the presence of a peroxide, namely hydrogen peroxide, as a product of autoxidation can be detected (other products formed will be discussed later). For the solutions will set free iodine from acidified potassium iodide, will give a blue colour with peroxidase and guaiacum and a yellow reaction with titanium sulphate, a reagent specific for hydrogen peroxide as contrasted with organic peroxides in general. Freshly prepared solutions of catechol, protocatechuic acid, etc., do not give the above tests.

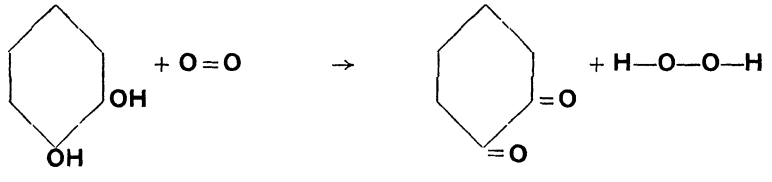
It is the autoxidation of these *ortho*-dihydroxy compounds which is catalysed by oxygenase. When a preparation of oxygenase is added to a freshly made solution of catechol, brown or reddish brown, oxidation products are rapidly formed, and, on addition of guaiacum, an intense blue colour appears.

Two suggestions have been made as to the reaction which is catalysed by the oxygenase; either that (1) by addition of molecular oxygen, an organic peroxide is formed which is subsequently decomposed in presence of water giving hydrogen peroxide

I.  + O=O → Catechol peroxide

II. Catechol peroxide + H_2O → Some oxidation product of catechol + H_2O_2

or that (2) by transference of hydrogen to molecular oxygen, an *ortho*-quinone is formed with simultaneous production of hydrogen peroxide. Thus, again, for catechol, the reaction would be

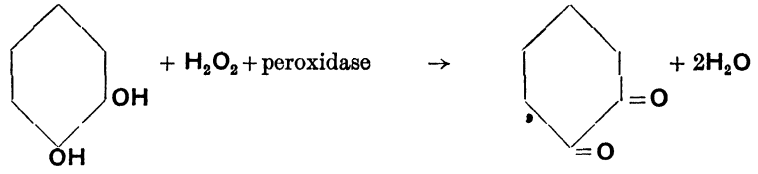
III. 

Ortho-benzoquinone is a powerful oxidising agent, and is capable of blueing guaiacum directly without the intervention of a catalyst.

As will be seen from details given later, there is experimental evidence that when a preparation of oxygenase is added to catechol, *ortho*-quinone is formed, and that an oxidation product (probably also the *ortho*-quinone) arises, which will blue guaiacum without the

intervention of an enzyme; there is also some indication that hydrogen peroxide is produced.

The evidence would seem then to point definitely to equation III as representing the reaction catalysed. But the possibility of accepting the simpler alternative is made difficult by the existence of two additional facts: first, that preparations of oxygenase have not so far been obtained free from peroxidase; secondly, that peroxidase and hydrogen peroxide have been shown experimentally to produce *ortho*-quinone from catechol:

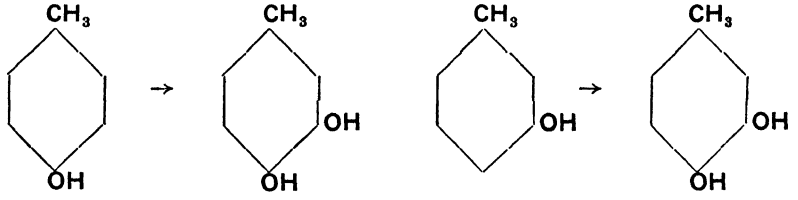
IV. 

Hence *ortho*-quinone may be formed in either or both of the above ways (III or IV). Should the preliminary oxidation of catechol by oxygenase be upon different lines as in I, then *ortho*-quinone would only be produced by the action of peroxidase and hydrogen peroxide on catechol.

Three additional points arise out of these reactions. First, the fact that secondary oxidations can be carried out by the products of oxidation of catechol, probably the *ortho*-quinone. Second, these secondary oxidations may be assisted by autocatalysis. Third, oxidation products (including *ortho*-quinone) formed can be adsorbed by colloidal surfaces present in the enzyme preparation.

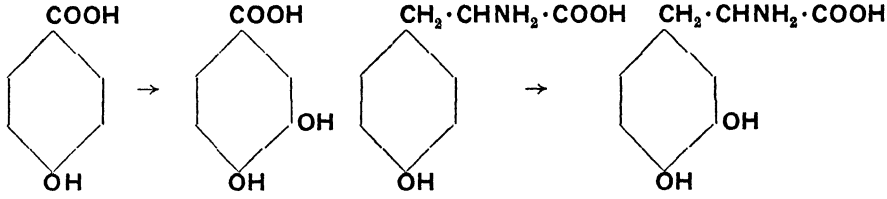
As *ortho*-quinone is the only product of oxidation so far identified, we have suggested it as the active agent for the secondary oxidations, and, in the following pages, it is provisionally assumed to be such. *Emphasis is here laid on the provisional nature of this assumption.*

Now let us deal with the above points separately. The additional secondary oxidation which concerns us first is that of certain monophenols to diphenols. The *ortho*-quinone from catechol appears, under certain conditions, to oxidise, for instance, phenol to catechol, and *p*-cresol and *m*-cresol to homocatechol:



p-Cresol Homocatechol *m*-Cresol Homocatechol

This power of oxidation may extend to other substances with monophenolic groupings, such as to *p*-hydroxybenzoic acid, which can be oxidised to protocatechuic acid, and, possibly, to tyrosine, which would give rise to dihydroxyphenylalanine:



p-Hydroxybenzoic acid — Protocatechuic acid — Tyrosine — Dihydroxyphenylalanine

The second point is that all the above reactions, except, perhaps, that on tyrosine, which will be dealt with later, are assisted by autocatalysis, that is, a trace of *ortho*-quinone would be in the first place sufficient to form a certain amount of the dihydroxy compound from the monohydroxy, and then the reaction can proceed with formation of further *ortho*-quinone and subsequent blueing of guaiacum.

The third point concerns the adsorption of the *ortho*-quinone. In addition to the catalyst, oxygenase, there is also present in oxygenase plants the substrate, that is, some substance with the *ortho*-dihydroxy grouping of catechol. During injury and exposure to air, which unavoidably accompanies the extraction of the enzyme, a certain amount of *ortho*-quinone is formed, and, in spite of ordinary precautions, is adsorbed by colloidal substances present in the enzyme preparation. Hence, such preparations will not only catalyse the oxidation of *ortho*-dihydroxy compounds, but may, by virtue presumably of adsorbed quinone and as a secondary oxidation, oxidise monophenolic compounds such as phenol, *p*-cresol, tyrosol, tyrosine, etc., to dihydroxy compounds. Moreover, a trace of quinone adsorbed to the preparation would be sufficient to initiate this secondary reaction.

This power of secondary oxidation of phenol, *p*-cresol, etc., can be removed by shaking the solution of the enzyme preparation with animal charcoal, which adsorbs and removes the *ortho*-quinone, or the portion of the colloidal complex with which it is united; it can be restored, however, by a trace of catechol, which, by providing *ortho*-quinone, initiates the reaction again.

Further details of the components of these systems will now be given.

Identity of oxygenase

The term oxygenase was originally used by Bach & Chodat (Chodat, 46) for enzyme-like substances, formed in both higher and lower plants, which are capable of uniting with the oxygen of the air to form peroxides. Later, when it was shown that in the higher plants Bach & Chodat's oxygenase could be resolved into an enzyme and a substrate, which, by interaction, form a peroxide, the term oxygenase was retained for the enzyme, in order to avoid the many complications which would arise by substituting a new terminology, since Bach & Chodat's conceptions, in the main, have been widely accepted.

From time to time, many investigators (Oppenheimer, 108) have questioned the existence of the catalyst here termed oxygenase, and have considered the phenomena recorded to be due to the presence of an autoxidisable substance only. It seems clear from observations made upon a considerable number of species of the Angiosperms that, if a plant contains a substance with the *ortho*-dihydroxy grouping (henceforth referred to as the catechol compound), then, in addition to the latter, a thermolabile catalyst can be extracted from the tissues, which, when added either to catechol itself or to the *ortho*-dihydroxy compound extracted from the plant, rapidly catalyses the oxidation of these substances. It is generally assumed that all the higher plants contain peroxidase, and observations, with a few exceptions, corroborate this assumption. On the other hand, only a certain percentage of this group contains a catechol compound and the accompanying oxygenase.

It must be admitted that the oxygenase of the higher plants has not yet been prepared free from peroxidase. A method has been recently described (Keilin, 83) whereby a peroxidase-free oxygenase can be obtained, but repeated trials of the method have failed to give the results claimed. The oxygenase can be precipitated by alcohol from water extracts. By fractional precipitation, precipitates are obtained containing, at first, oxygenase and peroxidase, and, finally, peroxidase only (Onslow, 93). Fractional precipitates by alcohol from the Basidiomycetes have been described by Chodat and other workers as representing, separately, different enzymes; but it should be emphasised that the oxidising systems of these Fungi, though analogous to those of the higher plants, are not identical with them, but show, in fact, various fundamental differences.

Distribution of oxygenase

The distribution of the oxygenase in the Angiosperms, by means of its action on catechol, has been explored (Onslow, 95). About 300 species were tested by the method described on p. 133, that is, the catechol compound was washed away from the tissues with alcohol, and the action of the tissue residue (containing the enzyme) on catechol tested. If oxygenase is present, catechol is oxidised and subsequently, on addition of guaiacum, a blue colour is developed.

The above 300 species represented 293 genera and 173 natural orders. Assuming the total number of natural orders of the Angiosperms to be 286, then 60 % were examined. Of these, the oxygenase reaction was detected in 63 %. The distribution was widely spread, and a consideration of the details indicates certain points of interest. It must be borne in mind, however, that the segregation into oxygenase and non-oxygenase orders in this investigation often rests on the evidence given by a few individual members. Subsequent research may modify the statements given here.

Of the total number of Monocotyledonous orders, 64 % were examined, and of these 79 % were found to give a positive reaction. The widely distributed grasses (Graminaceae) and allied orders contain oxygenase. Prominent exceptions are many bulb-producing plants of the orders Liliaceae and Amaryllidaceae, of which the Snowdrop (*Galanthus*) and Daffodil (*Narcissus*) are typical examples.

Of the total number of Dicotyledonous orders, 60 % were examined, and of these 60 % were found to contain oxygenase. The relative distribution in the two subsidiary groups, Archichlamydeae and Sympetalae, is different. Of the Archichlamydeae 57 % were examined, and of these 52 % were found to be oxygenase orders; of the Sympetalae 71 % were examined, and 84 % were found to be oxygenase orders.

Thus the Archichlamydeae, as far as examination has gone, contain the lowest percentage of oxygenase-containing orders; the Sympetalae, on the other hand, show the highest percentage. It may be said, therefore, that the more highly developed the phyla, the more universally distributed the system. The question as to whether this phenomenon has any biochemical significance will be discussed later. Among the Archichlamydeae, the Cruciferae have long been recognised as peroxidase plants only; other representative non-oxygenase orders are Crassulaceae, Geraniaceae, Tropaeolaceae, Malvaceae, and

those containing certain forest trees, Betulaceae, Fagaceae and Tiliaceae. The Sympetalae include almost entirely oxygenase orders, notable exceptions being the Primulaceae and Campanulaceae. All members of the highly developed and very widely distributed Compositae appear to be oxygenase plants.

A point to be emphasised is that oxygenase is usually found in most, if not all, the genera of an order, e.g. Compositae, Labiatae, Umbelliferae and Boraginaceae, but not infrequently both oxygenase and non-oxygenase plants may be represented in any one order; in the Leguminosae, for instance, the Pea (*Pisum*) and Lupin (*Lupinus*) are non-oxygenase, whereas the Broom (*Sarothamnus*) and Clover (*Trifolium*) are oxygenase plants. Similarly in Ranunculaceae, oxygenase is present in *Anemone*, but absent from the Winter Aconite (*Eranthis*); other orders with mixed genera are the Liliaceae and Saxifragaceae.

The distribution of oxygenase in the Angiosperms can be more concisely stated as follows:

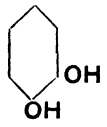
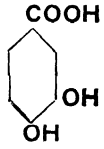
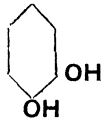
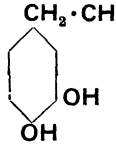
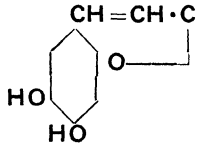
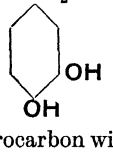
	% of orders examined containing oxygenase
Angiosperms. Of the total number of orders in the group 60 % were examined	63
Monocotyledons. 64 % examined	79
Dicotyledons. 60 % examined	60
Archichlamydeae. 57 % examined	52
Sympetalae. 71 % examined	84

The substrate for the oxygenase

As far as examination has gone, it appears that the oxygenase is accompanied in the plant by a catechol compound. In reality, the significant fact would appear to be the synthesis of this particular type of aromatic product. Provided it is formed, it is in some way intimately connected with a special colloidal surface in the tissues, which, after death at least, catalyses its oxidation.

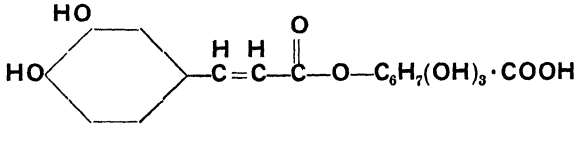
Various substances in different plants may serve as substrates; Reinke (122) was one of the first workers to demonstrate the presence of a catechol compound in the Potato tuber. Catechol tannins, that is, condensation products of protocatechuic acid, are widely distributed, and are probably accompanied by other derivatives with this same grouping. A selection of the compounds of this type, which have actually been isolated, are set out on p. 130. The species there

TABLE XXIX

Catechol compound	Plant in which found
Catechol 	*Populus, Salix* (Weevers, 142, 143) and other plants (Czapek[1])
Protocatechuic acid 	*Vitis vinifera* and other plants (Czapek[1])
Caffeic acid 	*Anthemis nobilis* (Power & Browning, 111); *Clematis vitalba* (Tutin & Clewer, 139)
Caffetannic acid, a derivative of caffeic acid	*Coffea arabica* and other plants (Czapek[1])
Dihydroxyhydrocinnamic acid (proteasäure) 	*Protea* (Czapek[1])
Aesculetin 	*Aesculus Hippocastanum* (Czapek[1])
Dihydroxyphenylalanine 	*Vicia Faba* (Guggenheim, 73) *Stizolobium* (Miller, 91)
Hydro-urushiol, complex hydrocarbon with *ortho*-dihydroxy grouping	*Rhus vernicifera* (Majima, 88)

recorded, moreover, are either known to contain oxygenase or to belong to orders of which such plants are typical.

In addition to the above-mentioned catechol compounds, there is the depside (simple tannin), chlorogenic acid, a condensation product of caffeic and quinic acids:



[1] **Czapek, F.** *Biochemie der Pflanzen.*

Gorter (70) has made an examination for this compound by means of a colour reaction with iron salts and weak alkali (to be described later), which, however, is unfortunately not specific for chlorogenic acid, but may be given by other catechol compounds. About 223 plants were examined, representing 220 genera and 65 orders; of these orders, 63 were among those examined by Onslow for oxygenase, though the genera selected were not always the same. With three exceptions, all the plants in which Gorter found a positive reaction have either been shown to contain the enzyme or are known to belong to oxygenase orders. In 14 oxygenase plants, however, no reaction was detected. It is not suggested here that chlorogenic acid, even if it were among the compounds giving the reaction in Gorter's series of plants, necessarily constitutes a substrate for the enzyme, but its presence would indicate that aromatic compounds of the catechol type are metabolic products of the plants in question.

The above facts also afford an explanation for the conclusions recorded by Wolff & Rouchelmann (155, 157) namely, that a substance resembling catechol is widely distributed in the higher plants. These authors showed that, on addition of an oxidising enzyme from a Basidiomycete to catechol solution, a peroxide is formed, which can be detected by the starch-iodide test. They subsequently observed that extracts of a large number of plants may be substituted for catechol with the same result. Hence their conclusion that catechol-like compounds are of wide occurrence.

The presence of a catechol compound in any plant can be readily demonstrated (Onslow, 92) without, necessarily, an accompanying isolation and identification. The tissues are extracted with hot alcohol, the alcohol removed by distillation, and the residue treated with aqueous lead acetate, whereby the catechol compound is precipitated. The lead is then removed with sulphuric acid. If, after neutralisation, an enzyme extract from the same plant (prepared as in the next section) is added to the catechol compound, oxidation takes place with subsequent blueing of guaiacum. The catechol compound will also give a green colour with ferric salts, turning pink, purple and blue on addition of very weak alkali, a reaction specific for the *ortho*-dihydroxy grouping.

By this method, about 30 species, mostly of different genera, which give the oxygenase reaction, have been found to contain substances giving the catechol reaction. On the other hand, about 15 species, also chiefly representatives of different genera, which give no oxy-

genase reaction, were found to be devoid of catechol compounds. No case has been noted yet in which oxygenase is present and no catechol compound, though the converse, absence of oxygenase and presence of catechol compound, may possibly be true for one or two plants examined.

The following is a list of plants in which presence or absence of *both* parts of the system has been determined (Robinson, 124; Onslow, 94, 96):

Oxygenase and catechol compound present

Banana (*Musa sapientum*), fruit
Mangold (*Beta vulgaris*), root
Fig (*Ficus Carica*), fruit
Apricot (*Prunus Armeniaca*), fruit
Peach (*P. Persica*), fruit
Cherry (*P. Cerasus*), fruit
Apple (*Pyrus Malus*), fruit
Pear (*P. communis*), fruit
Medlar (*Mespilus germanica*), fruit
Strawberry (*Fragaria elatior*), fruit
Grape (*Vitis vinifera*), fruit
Artichoke (*Helianthus tuberosus*), tuber
Groundsel (*Senecio vulgaris*), plant
Potato (*Solanum tuberosum*), tuber

Oxygenase and catechol compound absent

Horse-radish (*Cochlearia Armoracia*), root
Turnip (*Brassica campestris*), root
Red Currant (*Ribes rubrum*), fruit
Lemon (*Citrus Limonum*), fruit
Orange (*C. Aurantium*), fruit
Lime (*C. Limonum*, var. *acida*), fruit
Grape Fruit (*C. decumana*), fruit
Pineapple (*Ananas sativus*), fruit
Tomato (*Solanum Lycopersicum*), fruit
Melon (*Cucumis Melo*), fruit

Sometimes, in varieties of cultivated plants, e.g. Mangold, Potato, though the oxygenase is present, the catechol compounds may occur in much lower concentration than in the original type from which the variety has been derived (cp. the presence of emulsin, but absence of amygdalin, from the fruit of the Sweet Almond).

Preparation of oxygenase

Immediately the tissues of an oxygenase plant are injured in the presence of air, they discolour owing to the formation of oxidation products from the catechol compound. *These will be referred to as* ortho-*quinone, although they may also include organic peroxides, since we have, as yet, no definite knowledge of any other product.* A water extract will usually blue guaiacum, but as the reaction may be inhibited by excess of soluble carbohydrates, tannins, uronic acids, etc., which are frequently present, reliable information as to the presence of the oxygenase can often only be obtained by a different procedure.

One method of obtaining enzyme preparations has been to pound the tissues under alcohol, filter, and repeat the treatment several times, the whole process being carried out as rapidly as possible. The catechol compound is, as a rule, soluble in cold alcohol, and is thus removed together with sugars and, usually, tannins and organic acids. The residue, "tissue residue," in general, contains cellulose, proteins and enzymes. A water extract of the residue will give an enzyme solution, and if the preparation has been carefully carried out it should not blue guaiacum within a reasonable time, owing to the removal of the catechol compound and the prevention of formation of quinone. Traces of the latter will be present, however, even in the most carefully prepared product. When such a preparation is added to catechol, protocatechuic acid, caffeic acid, etc., oxidation is rapidly catalysed, quinones are formed and subsequently a blue colour is given with guaiacum. Such a preparation will also act on monophenolic compounds, such as phenol, *p*-cresol, etc., with formation of quinones and blueing of guaiacum. A somewhat purer and more concentrated preparation of enzyme can be obtained by precipitation of a water extract with strong alcohol, and again taking up in water.

The above method was originally adopted (Onslow, 93) to demonstrate the separation of the enzyme from the substrate, catechol compound. It also has the merit of providing a control for which the reaction is negative with guaiacum, should it be desired to test the reaction of the enzyme on various substrates.

If, however, in the above technique, the enzyme is allowed to react with the substrate in air in the injured tissue before extraction with alcohol, the residue will be brown owing to adsorption of quinone. The water extract will be discoloured, and will contain quinone, which will give a blue reaction with guaiacum.

Great difficulty is often experienced, especially with woody tissues, in obtaining a colourless residue by pounding and extracting with alcohol. Even with care, the quinone (or a condensation product of quinone) is adsorbed to the tissues and cannot be removed with alcohol. The water extract will therefore always give a blue reaction with guaiacum. The tubers of the Potato and Artichoke have been largely used for enzyme preparations, owing to the fact that the tissues are soft and free from lignified cells. In other cases the catechol compounds may not be readily soluble, or may even be insoluble, in alcohol, and thus are not removed by such treatment; it is possible

that this may sometimes be the case with the latex of laticiferous plants. Hence, all grades of preparations can be produced, ranging from those which have no action upon guaiacum to those which blue this reagent strongly. As will be seen later, this phenomenon of adsorption would appear to play an important part in these post-mortem oxidation systems.

In view of the varied nature not only of the catechol compounds serving as substrates, but also of the colloidal substances in the residue or precipitate, it is to be expected that adsorption will take place to a varying extent in different cases.

So also on extraction with water (elution), the amount of enzyme obtained in solution should depend upon the nature of the colloidal substance and of the quinone molecule, as well as upon other factors, and this is, as a matter of fact, borne out by experience. The extent to which the enzyme can be extracted from the residue (after alcohol) by water varies greatly with different tissues. When some substances, such as tannins, are present, which act as fixing agents, it may not be possible to extract the enzyme. Hence, to demonstrate the presence of oxygenase, the actual tissue residue must be placed in the catechol solution, when reaction takes place on the surface of the residue.

It should be realised that such a preparation as the above, even after precipitation, always contains peroxidase and also probably other enzymes, catalases, proteases, etc. It has not been possible to obtain the oxygenase entirely free from peroxidase by fractional precipitation with alcohol. Both enzymes are at first precipitated, but on repeated fractionation with alcohol the peroxidase alone is obtained in the final product.

Browning of tissues on injury

An additional phenomenon presented by plants which contain an oxygenase and a catechol compound is the readiness, on the whole, with which the plant turns brown, reddish brown or even black after death of the tissue brought about by mechanical injury, chloroform vapour, etc. This discoloration is the result of the post-mortem oxidation of the aromatic substrate by the oxygenase and, in addition, probably, the induced secondary oxidation of other aromatic substances in the plant. The browning of an apple and of many flower petals on injury is an example of this phenomenon. Specially striking cases of brilliantly coloured pigments have been described for

Dioscorea (Bartlett, 29), *Jacobinia* (Parkin, 109) and *Baptisia* (Clark, 48). Nevertheless, similar pigments do arise occasionally in plants without, apparently, the intervention of an oxidising enzyme. In these cases the chromogen of the pigment is usually in the tissues, in combination with sugar as a glucoside. On injury or autolysis the glucoside is hydrolysed and the free chromogen oxidises in air to a pigment. The production of indigo in the autolysed tissues of the Indigo plants (*Indigofera* spp., *Isatis tinctoria*) takes place on these lines. Similarly, blue pigments, hernidin[1] and dipsacotin[2] are produced after death in the vegetative parts respectively of the Annual Mercury (*Mercurialis annua*) and the Teasel (*Dipsacus*). According to the above investigators, no catalyst is involved in the oxidation. Bourquelot & Hérissey[3] likewise maintain that the pigment arising after death in leaves of the Aucuba Laurel (*A. japonica*) is produced apart from the action of an oxidising enzyme.

Products of the reaction catalysed by oxygenase

When a solution of catechol in ordinary distilled water at about p_H 7 is exposed to air, oxidation takes place, the solution gradually becomes brown, and a peroxide, hydrogen peroxide, can be detected. Solutions of other catechol compounds such as neutralised protocatechuic and caffeic acids, etc., behave in a similar way. If potassium iodide solution, acidified with acetic acid, is added to these autoxidised solutions, the elimination of free iodine by peroxide can be shown by the formation of a blue colour on addition of starch solution; or the addition to such a solution of a peroxidase extract (from Horseradish), for instance, followed by guaiacum solution, gives a blue colour; or, finally, the hydrogen peroxide can be detected with titanium sulphate, a reagent specific for this compound as contrasted with organic peroxides in general. Freshly prepared solutions of catechol, protocatechuic acids, etc., do not give the above results.

The reactions taking place in this autoxidation may follow either of the two courses which were suggested (equations I and III, p. 124) when the *catalysis* of this oxidation was considered. That is, either the formation of an unstable catechol peroxide which gives rise, on

[1] **Haas, P.**, and **Hill, T. G.** Mercurialis. II. The Occurrence of a Chromogen showing a remarkable Avidity for free Oxygen. *Biochem. J.*, 1925, **19**, 236–239.

[2] **Tammes, T.** Dipsacan und Dipsacotin, ein neues Chromogen und ein neuer Farbstoff der Dipsaceae. *Rec. Trav. Bot. Néerl.*, 1908, **5**, 51–90.

[3] **Bourquelot, Em.**, et **Hérissey, H.** Sur l'aucubine, glucoside de l'*Acuba japonica* L. *Ann. chim. phys.*, 1905 (8), **4**, 289–318.

decomposition, to hydrogen peroxide, or the simultaneous production of *ortho*-quinone and hydrogen peroxide.

The *ortho*-quinone from catechol has been prepared (Willstätter & Müller[1]) and shown to blue guaiacum directly. The autoxidised solutions of the catechol compounds do not blue guaiacum. Hence one might assume that *ortho*-quinone is not a product of autoxidation. It is possible, however, that at p_H 7 the *ortho*-quinone is unstable and forms condensation products. In more acid solutions, p_H 4·6, in which, as has been suggested (Szent-Györgyi, 133), the *ortho*-quinone may be more stable, autoxidation does not take place. On the other hand, the oxidation may proceed on different lines with preliminary formation of an unstable peroxide and subsequent decomposition, giving rise to condensation products, possibly, and hydrogen peroxide.

As already mentioned, the presence of hydrogen peroxide in such a solution is suggested by the fact that, on addition of peroxidase followed by guaiacum, a blue colour appears. The point has been verified, as far as qualitative tests are valid, by means of titanium sulphate (Onslow & Robinson, 100). After removal of unchanged catechol (which forms a colour with the reagent) with peroxide-free ether, an autoxidised solution will give a yellow colour with a solution of titanium sulphate. If a solution of catalase (from pig's liver) was added before testing with titanium no yellow colour was obtained.

In addition to the formation during autoxidation, it appears that hydrogen peroxide is produced when the oxidation is catalysed by oxygenase, for carefully controlled tests with titanium gave a positive result in a catechol solution which had been oxidised by an oxygenase preparation from the Potato. The reaction was again negative after addition of catalase.

Now, as to the question of the formation of *ortho*-quinone as a product of the action of oxygenase on catechol. It was first suggested by Szent-Györgyi (133) that the *ortho*-quinone is formed in this reaction, and that, when formed, it is most stable at p_H 4·6. Thus, if oxygenase from the Potato is allowed to oxidise catechol at this reaction, and if the enzymes and other colloidal substances are then precipitated from the oxidised solution by means of colloidal ferric hydroxide (or by picric acid solution), the filtrate, from which all

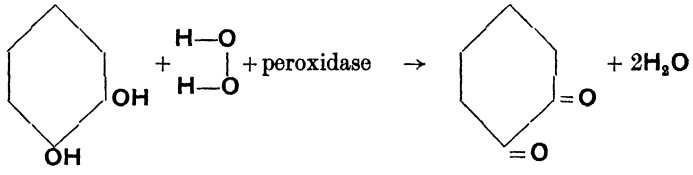
[1] **Willstätter, R.,** und **Müller, F.** Zwei Formen von Orthochinon. *Ber. d. D. chem. Ges.*, 1908 (2), **41**, 2580–2586.

enzymes are absent, and which contains the oxidation products, will be found to blue guaiacum. This blueing has been considered by Szent-Györgyi to be due to *ortho*-quinone.

The view that *ortho*-quinone can be formed under such circumstances was subsequently placed upon a firmer basis by the isolation of a crystalline anilino-*o*-quinone as the result of the action of an oxidising enzyme from the mealworm on catechol in the presence of aniline (see tyrosinase). In addition, it was shown, by means of the same crystalline aniline derivative, that the *ortho*-quinone can also be formed by the action of peroxidase (from Horse-radish) and hydrogen peroxide on catechol (Pugh & Raper, 114).

Their method has been repeated (Onslow, unpublished result) with the oxygenase preparation from the Potato. This was added to a catechol solution containing aniline and aerated for several hours, whereupon a red crystalline product with the characteristic properties of anilino-*o*-quinone was deposited.

Hence it would appear that *ortho*-quinone is certainly a final product in the action of the oxygenase preparation on catechol. But since the oxygenase preparations always contain peroxidase, and as hydrogen peroxide is formed during the oxidation, there is the possibility that the *ortho*-quinone does not arise from the direct action of oxygenase on catechol, but from the action of peroxidase and hydrogen peroxide on this substance:



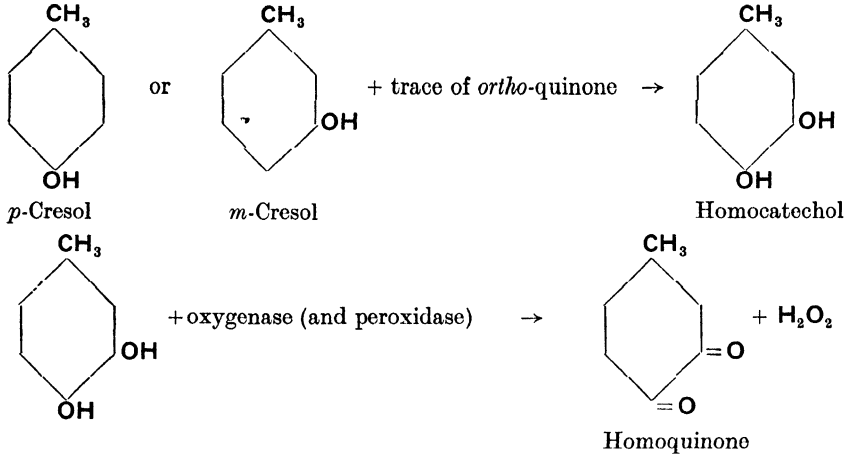
For the same reason, there is no direct proof that the guaiacum reaction may not also be given by peroxidase and hydrogen peroxide as well as by the *ortho*-quinone.

Secondary oxidation of monophenolic compounds

When a preparation of oxygenase as described on p. 133 is allowed to act upon *p*-cresol, an orange-red colour is developed and subsequently, with guaiacum, the oxidised solution gives a blue colour. If, moreover (Onslow & Robinson, 99), the *p*-cresol solution is tested, ½–3 hours after the addition of the enzyme, with dilute ferrous sulphate solution, a green colour is obtained, which changes to pink, purple and blue on addition of very weak sodium carbonate solution,

a reaction characteristic of the dihydroxy grouping of catechol. The original *p*-cresol does not give this reaction. A similar result is obtained when the enzyme is allowed to act upon phenol, *m*-cresol, *p*-hydroxybenzoic acid, *m*-hydroxybenzoic acid and tyrosol.

Thus, these monohydroxy compounds are oxidised to dihydroxy compounds and, since the position of the group is such (*para* or *meta* position) that an *ortho*-hydroxy compound arises, oxidation then proceeds in the normal way to *ortho*-quinones, and the whole reaction is assisted by autocatalysis:



Ortho-quinones have also been isolated as crystalline aniline derivatives from the action of the mealworm enzyme on *p*-cresol and phenol (see tyrosinase), a fact which gives greater certainty to the above conception.

As far as examination has gone, all oxygenase preparations from the higher plants have been found to act on *p*-cresol as well as on catechol.

Catalyst for secondary oxidations

From the evidence at hand the probability is in favour of regarding the trace of quinone, which is always present even in a carefully extracted oxygenase preparation, as the agent which initiates the secondary oxidation. The greater the amount of *ortho*-quinone adsorbed, the more rapid the oxidation of *p*-cresol by the enzyme preparation. If two preparations are made, one with great care and rapidity to avoid oxidation, and the other from tissue that has first been allowed to oxidise, there is no question that the former has a more rapid action upon *p*-cresol. The difference is best seen by adding the *p*-cresol solution to the tissue residue.

The following observation is also regarded as evidence for considering the *ortho*-quinone to be the agent in the secondary oxidation (Onslow & Robinson, 101). If a solution of the enzyme preparation is shaken once with Merck's animal charcoal, and filtered, the action of the filtrate on *p*-cresol is greatly diminished; after two or three additional treatments with charcoal it entirely disappears. If, then, to such a solution, a fraction of a milligram of catechol is added, the orange-red colour gradually develops. The deduction, therefore, can be fairly drawn that a trace of *ortho*-quinone will initiate the oxidation of *p*-cresol to homocatechol, and then the reaction proceeds assisted by autocatalysis.

Another alternative is that hydrogen peroxide is the agent which initiates the reaction, by converting mono- into diphenols (Onslow & Robinson, 99). That hydroxyl groups can be inserted into the benzene ring by oxidation with hydrogen peroxide *alone* has been shown by Dakin & Herter[1]. Thus, for instance, when ammonium benzoate was treated with hydrogen peroxide, *o*-hydroxybenzoic (salicylic), *m*-hydroxybenzoic and *p*-hydroxybenzoic acids were formed in approximately equal amounts. Moreover, when ammonium salicylate was treated with hydrogen peroxide, the 2 : 3-dihydroxy acid was produced, and when salts of *p*-hydroxybenzoic and *m*-hydroxybenzoic acids were oxidised, protocatechuic acid was the product, that is, the hydroxyl group is inserted in an *ortho*-position to the one already present.

If we postulate hydrogen peroxide as the active agent, then, in the case of well-dried preparations of oxygenase which act normally on *p*-cresol, the hydrogen peroxide must be assumed to arise when water is added to these preparations, that is, they must contain some compound of the nature of a catechol peroxide. Though, in the experiment with charcoal adsorption quoted above, hydrogen peroxide may certainly arise from autoxidation of the trace of catechol added, we are forced to assume, to make the explanation conclusive, that hydrogen peroxide can be removed by charcoal. Discussion of these points is continued in a following section.

Further, it is important to note that the oxidation of *p*-cresol to homocatechol is not brought about by peroxidase in the presence of hydrogen peroxide. Under these circumstances, a milky precipitate

[1] **Dakin, H. D.**, and **Herter, M. D.** On the Production of Phenolic Acids by the Oxidation with Hydrogen Peroxide of the Ammonium Salts of Benzoic Acid and its Derivatives, with some Remarks on the Mode of Formation of Phenolic Substances in the Organism. *J. Biol. Chem.*, 1907, **3**, 419–434.

is produced, a sensitive reaction which has long been in use for the detection of peroxidase (Chodat, 46).

The mechanism of oxygenase

It is difficult to postulate any conception for the mechanism of the system described in the previous pages. The following outline is purely suggestive, since very little experimental evidence exists at the moment. Obviously, plants which produce *ortho*-dihydroxy compounds of the catechol type also contain a colloidal surface which is associated in some way with the catalysis of the oxidation of these compounds. This colloidal surface has not been extracted free from traces of adsorbed products of oxidation. On rupture, by injury, of the cell of an oxygenase plant, it is suggested that the colloidal surface may fix and stabilise, for instance, a catechol peroxide formed by autoxidation of the catechol compound in air. Colloidal surface plus adsorbed peroxide then constitutes catalyst I, the oxygenase. In solution, hydrogen peroxide is formed from the adsorbed peroxide and, in the presence of peroxidase, will catalyse the oxidation of the catechol compound with formation of *ortho*-quinone. *Ortho*-quinone, in its turn, is adsorbed and stabilised by the colloidal surface, and surface plus adsorbed *ortho*-quinone constitutes catalyst II, which oxidises certain monophenols to diphenols. Or, we may assume that the hydrogen peroxide formed in the first instance (equation 2 below) from catechol peroxide is responsible for the oxidation of the monophenols. The various stages are represented thus:

(1) Catechol + O_2 → catechol peroxide.

Catechol peroxide + colloidal surface = catalyst I (oxygenase).

(2) Catechol peroxide + water → H_2O_2.

(3) Catechol + H_2O_2 + peroxidase → *ortho*-quinone.

Ortho-quinone + colloidal surface = catalyst II oxidising monophenols (or these may be oxidised by H_2O_2 from reaction (2)).

An alternative suggestion is that *ortho*-quinone may be the direct product of autoxidation of catechol, and that colloidal surface plus adsorbed *ortho*-quinone not only oxidises monophenols, but, in some way, catalyses the oxidation of catechol itself, though it is difficult to suggest how the latter process may be carried out.

Ortho-quinone is heat labile, as would be also, probably, organic peroxides of the type suggested above, so that, in this respect, they satisfy the definition of an enzyme. It seems clear that a special

colloidal surface is essential for the oxygenase; no surface is present in non-oxygenase plants which will serve in this way, for when their tissues are placed in catechol solution no catalysis occurs. Moreover, if a tissue residue, after alcohol extraction, of a peroxidase plant (Horse-radish) is placed in catechol solution and hydrogen peroxide is added, *ortho*-quinone is formed by the peroxidase in presence of hydrogen peroxide and the tissue turns brown, but the residue, after washing with alcohol, has no action on *p*-cresol.

Were some such conception of the nature of the enzyme correct as that set out above, then an oxygenase preparation (by alcohol), in the making of which the formation and adsorption of oxidation products has been avoided as far as possible, should be less active as a catalyst than a preparation made after oxidation of the tissues in autolysis has taken place. As frequently emphasised, no preparation, however carefully made, is entirely free from *ortho*-quinone. When, however, a preparation is made with great rapidity, and extreme precautions are taken to avoid oxidation as contrasted with another where the plant tissues have been allowed to oxidise before extraction, the action on *p*-cresol, as already mentioned, is more rapid in the latter case. The same result holds for catechol, though it is difficult to demonstrate, because the reactions here are very rapid, and differences in rate are less easily appreciated. For such tests, catechol solution should be poured on to the tissue residue spread out in a white basin, and the time noted for the first appearance of colour.

Again, some conclusions in regard to the nature of the enzyme might be drawn from the experiments with charcoal. On removal of the products of oxidation with charcoal, does the power to oxidise *p*-cresol disappear simultaneously with the action on catechol?

As a matter of fact, it has usually been found with preparations that the reaction on catechol persists, though much weakened, after that on *p*-cresol ceases. If, then, further adsorption by charcoal is carried out, the catechol reaction also disappears. But there is still left in solution a certain amount of peroxidase. This peroxidase, as we know, will oxidise catechol to *ortho*-quinone in the presence of hydrogen peroxide. Traces of hydrogen peroxide must also be assumed to be present in all solutions of catechol as a result of autoxidation. Hence, even after removal of all *ortho*-quinone by charcoal, there is yet the possibility of oxidation of catechol by means of peroxidase.

Additional Secondary Oxidations

We now see that the following represent some of the reactions possible in the oxygenase-catechol system:

Catechol + O_2 → unknown oxidation product + H_2O_2.

Catechol + O_2 + oxygenase → Catechol peroxide (or *ortho*-benzoquinone) + H_2O_2.

Homocatechol + O_2 + oxygenase → Homocatechol peroxide (or homoquinone) + H_2O_2.

Catechol + H_2O_2 + peroxidase → *Ortho*-benzoquinone + H_2O_2.

Phenol + *ortho*-benzoquinone → Catechol or homoquinone.

p-Cresol + *ortho*-benzoquinone → Homocatechol or homoquinone.

If a preparation of oxygenase (containing the usual traces of *ortho*-quinone) is added to solutions of a whole range of phenolic substances such as catechol, *p*-cresol, *o*-cresol, gallic acid, guaiacol, orcinol, phenol, phloroglucinol, protocatechuic acid, pyrogallol, quinol, quercetin, resorcinol, tannic acid and vanillin, there will be rapid oxidation only of those compounds which contain the *ortho*-dihydroxy grouping, less rapid oxidation of *p*-cresol, *m*-cresol and phenol, and relatively no oxidation of the other compounds. But if more than a trace of quinone be present, as may frequently be the case in oxygenase preparations, any of these additional substances may be oxidised to some extent. Hence a mixture of oxygenase with a supply of catechol compound, such as is present in the autolysed plant tissue, may oxidise, more or less extensively, a number of phenolic compounds of various structure. In addition, there is the ability of peroxidase and hydrogen peroxide to oxidise a wide range of phenolic compounds.

Another secondary oxidation due to *ortho*-quinone is that of deamination by oxidation of the amino-acids. This reaction, unlike the secondary oxidation of monophenols, is not assisted by autocatalysis. Hence, it proceeds only in the presence of a supply of *ortho*-quinone from either catechol or monophenols, such as phenol, *p*-cresol and tyrosol, which can be oxidised to *ortho*-diphenols. When oxygenase (from Potato tuber) is added to such amino-acids as glycine, alanine, leucine and phenylalanine, there is no oxidation.

But in the presence of the above-mentioned phenols, the amino-acids are oxidised, presumably, by the *ortho*-quinone, with removal of ammonia and carbon dioxide and formation of an aldehyde lower in the series:

$$CH_2(NH_2)COOH + O \rightarrow HCHO + NH_3 + CO_2.$$

Detailed observations of this reaction have been carried out in a modified form of the Barcroft differential manometer (Robinson & McCance, 125; Happold & Raper, 80) and the uptake of oxygen was measured, both for experiments with enzyme, phenols and amino-acids, and for control experiments with enzyme and phenols alone.

The conclusion to be drawn from the facts discussed in this section is that the nature and extent of secondary oxidation depends both on the amount of *ortho*-quinone present in the enzyme preparation and on the supply of catechol compound available.

Tyrosinase: Reactions and Distribution

This name was formerly given to what was believed to be a specific enzyme occurring in both higher and lower plants and animals. The characteristics of tyrosinase were defined thus: a power to catalyse the oxidation of tyrosine, producing, at first, a pink coloration which gradually deepens, through red, to black, with the ultimate formation of insoluble black compounds, known as melanins. In addition, it was said to oxidise *p*-cresol with the production of an orange-red colour.

It now seems certain that tyrosinase is intimately connected with oxygenase. When a preparation of oxygenase from the Potato tuber, for instance, made as described in the previous section, is added to tyrosine, the reaction characteristic of tyrosinase is given. The same is true for other oxygenase plants so that, in all probability, the so-called tyrosinase is widely distributed among the higher plants, and no plant, so far examined, gives tyrosinase reaction without also giving the oxygenase reactions described in the previous pages. The converse statement, that all oxygenase plants will act on tyrosine, does not hold completely; there are some exceptions which are given in the list below:

Plants giving oxygenase and tyrosinase reactions

Wheat (*Triticum vulgare*), seed
Banana (*Musa sapientum*), fruit
Fig (*Ficus Carica*), leaves
Beet (*Beta vulgaris*, var. *Rapa*), root
Mangold (*Beta vulgaris*), root
Oriental Poppy (*Papaver orientale*), leaves
Apple (*Pyrus Malus*), fruit
Pear (*P. communis*), leaves

Plants giving oxygenase and tyrosinase reactions (cont.)

Clover (*Trifolium pratense*), leaves
Broom (*Sarothamnus scoparius*), shoot
Laburnum (*Cytisus Laburnum*), leaves
Bean (*Vicia Faba*), leaves
Spurge (*Euphorbia Lathyrus*), leaves
Lac tree (*Rhus vernicifera*), leaves
Virginian Creeper (*Ampelopsis quinquefolia*), leaves
Chervil (*Chaerophyllum sylvestre*), leaves
"Indian Pipe" plant (*Monotropa uniflora*), plant
Potato (*Solanum tuberosum*), tuber
Tobacco plant (*Nicotiana Tabacum*), leaves
Artichoke (*Helianthus tuberosus*), tuber
Dahlia (*Dahlia variabilis*), tuber

Plants giving oxygenase but no tyrosinase reactions

Nettle (*Urtica dioica*), leaves
Lilac (*Syringa vulgaris*), leaves
Elder (*Sambucus nigra*), leaves
Groundsel (*Senecio vulgaris*), leaves

Plants giving neither oxygenase nor tyrosinase reactions

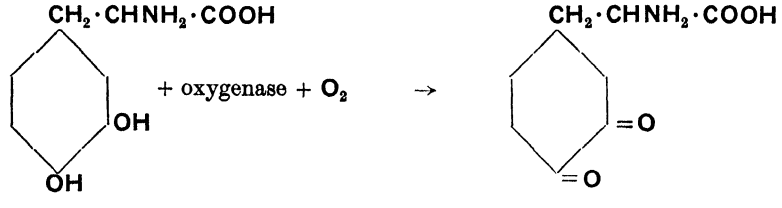
Sweet Alyssum (*Alyssum saxatile*), leaves
Wallflower (*Cheiranthus Cheiri*), leaves
Lupin (*Lupinus* sp.), leaves
False Acacia (*Robinia Pseud-acacia*), leaves
Lucerne (*Medicago sativa*), leaves
Medicago (*M. lupulina*), leaves
YellowVetchling (*Lathyrus pratensis*), leaves
Aucuba Laurel (*Aucuba japonica*), leaves

A list of plants containing tyrosinase is also given by Chodat & Evard (47).

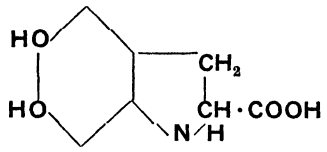
The course of the reactions brought about by an enzyme from the meal-worm having the characteristics ascribed to tyrosinase has been investigated by Raper (115, 116), and may be stated as follows.

A dihydroxy compound, dihydroxyphenylalanine, is first formed by oxidation of tyrosine, presumably by *ortho*-quinone (as in the case of *p*-cresol and other monophenolic compounds):

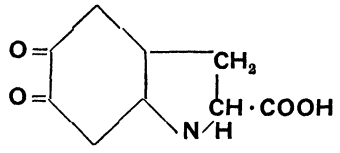
From this, as usual, an *ortho*-quinone arises:



This undergoes intramolecular change, and becomes converted into an indole compound:



of which the quinone is the red substance first visible in the action on tyrosine:



From this substance, by subsequent changes varying according to conditions, and for which the enzyme is not necessary, the black products (melanins) arise.

The mechanism of tyrosinase

It seems highly probable that, as far as the higher plants are concerned, the oxidation of tyrosine is initiated by the same agent and in the same way as in the case of *p*-cresol and other monophenols. Some of the evidence already stated for *p*-cresol in favour of *ortho*-quinone as the agent, also holds for tyrosine, namely, the greater the amount of oxidised products adsorbed to the oxygenase preparation, the more rapid the secondary oxidation, i.e. in this case the action on tyrosine.

How far, once initiated, the tyrosinase reaction is assisted by autocatalysis is not clear. It is obvious that eventually the *ortho*-quinone of dihydroxyphenylalanine is irreversibly converted into melanin if we accept the series of changes set out above.

Two courses, at least, would appear to be available according to circumstances.

Firstly, it must be admitted that an ordinary preparation of oxygenase (with the usual trace of *ortho*-quinone) from the Potato can bring about oxidation of tyrosine to the stage of melanin. This would suggest that a colloidal surface is present which stabilises a certain proportion of the *ortho*-quinone of dihydroxyphenylalanine, and enables it to catalyse the further oxidation of tyrosine; that is, the reaction is assisted by autocatalysis. This colloidal surface is possibly the same as that which serves for the catalysis of the oxidation of the monophenols, since the actions on *p*-cresol and tyrosine are almost always brought about by the same preparations.

Secondly, if pounded Potato tissue is left to autolyse, the tyrosinase reaction progresses very readily, and the mass becomes completely black in 24 hours. In this case, the catechol compound is present and forms a source of *ortho*-quinone apart from that which may arise from dihydroxyphenylalanine. Hence the conditions should be especially favourable for melanin formation.

Solutions of oxygenase treated with animal charcoal lose their power of acting on tyrosine (Onslow & Robinson, 101), and this, unlike the case of *p*-cresol, can only be restored to a slight extent by a trace of catechol. This would appear to be evidence against the presence of a stabilising colloidal surface in common to the two reactions, but the experimental work is not extensive and needs to be repeated.

Complications, as signified above, are no doubt responsible for some of the anomalies observed in connection with the behaviour of tyrosinase. Further, precisely the same set of factors may not always be required to bring about melanisation. It would appear that the reaction may be both initiated and catalysed by either *ortho*-benzoquinone or the quinone of dihydroxyphenylalanine, and these quinones may be stabilised by the same or by two different colloidal surfaces. This point of view is strengthened by the fact that a number of different preparations have been used, and considered as tyrosinase on account of their action on tyrosine. We have, for instance, as a source of enzyme, the ordinary preparation of oxygenase of the higher plants, Potato juice, many preparations from different Basidiomycetes (species of *Lactarius*, *Russula*, etc.), and, in addition, extracts from the mealworm and other insect tissues.

Though it is not within the scope of this book, a word may be said in regard to the Basidiomycete enzymes. The phenomenon observed in the Phanerogams appears to be unknown in these lower plants. It has not been possible to wash away a substance (the catechol compound) leaving a colloidal surface contaminated only by traces of oxidised products and having no action on guaiacum. If *Lactarius vellereus*, *Russula fragilis* and *Agaricus campestris* are thoroughly extracted with alcohol as for the Potato oxygenase, no readily identifiable aromatic compound is removed, and the residue, moreover, blues guaiacum in spite of repeated extraction (Robinson, 124). A possible explanation may be that on disorganisation of the cell, oxidised products (from aromatic and other compounds) are so readily adsorbed to the colloidal surfaces present that the latter are never

obtained relatively free (as is the Potato residue) from these substances. Therefore an enzyme preparation always blues guaiacum. (This constitutes a laccase; a similar phenomenon is demonstrated by the Potato residue if more than the normal amount of *ortho*-quinone is adsorbed, as will be explained in the next section.) Hence, there may be among the Basidiomycetes many possibilities of stabilisation, by various colloidal surfaces, of peroxides, quinones and other active molecules, some of which catalyse the oxidation of one type of aromatic compound, others another type. These systems may be the individual laccases, the tyrosinase free from both peroxidase and laccase, etc., which have been described by Chodat and other workers.

In regard to preparations from the mealworm and other insects, nothing is known of the aromatic compounds present, nor of the oxidation products which may be adsorbed. Dihydroxyphenylalanine is possibly present in insects, and its *ortho*-quinone, stabilised by a special colloid, might form a very active tyrosinase. In animals, as in the above-mentioned Fungi, there may also be the phenomenon of rapid adsorption, on disorganisation, of oxidation products to the tissue, so that the colloidal surface cannot be prepared relatively free from such products.

The outcome of these considerations suggests that in the higher plants the reactions brought about by tyrosinase are due to an initial oxidation of tyrosine to dihydroxyphenylalanine by *ortho*-benzoquinone, which arises from the catechol oxygenase system, and is stabilised by a colloidal surface. In presence of a source of catechol compound, the *ortho*-benzoquinone can take part in the further catalysis, or the reaction may be assisted by autocatalysis through the *ortho*-quinone of dihydroxyphenylalanine, if the latter is adsorbed and stabilised by a suitable colloidal surface.

The action of oxygenase and *ortho*-quinone on tyrosine is merely a special variation of the secondary oxidations previously described. In the case of phenylalanine, phenol, *ortho*-quinone and enzyme, we obtain the following result:

Phenol + (trace) *ortho*-quinone → Catechol.
Catechol + O_2 + oxygenase → *Ortho*-quinone + H_2O_2.
Ortho-quinone + phenylalanine → CO_2 + NH_3 + benzaldehyde.

In tyrosine the *ortho*-quinone and amino groupings form parts of the same molecule, and deamination does not follow the usual course.

It has been emphasised by Pugh (113) that from peroxidase, hydrogen peroxide and catechol, tyrosinase cannot be synthesised. It is evident from the above survey of the tyrosinase reaction that one would not expect such a synthesis for, though *ortho*-quinone is formed as a result of the action of peroxidase and hydrogen peroxide on catechol, the colloidal surface bringing about its stabilisation would be lacking. This has already been indicated by Onslow & Robinson (101), and the experimental observation quoted in regard to the failure of *ortho*-quinone to induce secondary oxidation of *p*-cresol in presence of tissues of a peroxidase plant (see p. 141) apparently points to the same conclusion.

There is no evidence that melanins are formed to any extent in the living plant, though they may be responsible for dark pigments in the testa of some seeds and the black spots on the flower of the Bean (*Vicia Faba*). At any rate, it is not clear that they have any definite biochemical significance. It is more probable that, in the case of the plant, the action of tyrosinase and the subsequent synthesis of the indole ring are post-mortem phenomena arising from fortuitous association of certain metabolites and catalytic surfaces.

Laccase

This is a term given by Bertrand to an enzyme first obtained from the Lac tree (*Rhus vernicifera*). The white latex of this tree rapidly darkens in air, producing a black, hard, shining surface, the lacquer of the Chinese and Japanese craftsmanship.

Bertrand obtained a precipitate from the latex which he called laccase. It possessed the properties of blueing guaiacum and of oxidising phenolic compounds of a certain constitution, namely, those with two hydroxyl groups in the *ortho* or *para* position. It had no action on tyrosine. Later, Bertrand applied the term laccase to a similar enzyme, from the Artichoke tuber and other plants, which we now know to be the oxygenase described in the preceding pages. The Lac tree is an oxygenase plant, the enzyme being present in the leaves, which also give the tyrosinase reaction. From the latex a hydrocarbon of high molecular weight and complex constitution has been isolated and shown to contain a side-chain with the *ortho*-dihydroxy grouping of catechol (Majima, 88).

It seems reasonable to conclude that, in the Phanerogams, laccase is oxygenase together with more or less adsorbed *ortho*-quinone. It

has been pointed out (p. 133) that in many preparations the tissue residue or enzyme preparation (by alcohol) cannot be obtained free from a considerable amount of adsorbed oxidation products and will therefore always blue guaiacum. Further, if Potato tissue is allowed to oxidise considerably before treatment with alcohol, and then the enzyme is extracted with water and reprecipitated with alcohol, the precipitate will again contain *ortho*-quinone, and, in solution, will blue guaiacum. Such a product is a laccase and, according to the suggestions outlined in the last section, should be synonymous with tyrosinase, provided the colloidal constituent can also stabilise the *ortho*-quinone of dihydroxyphenylalanine. But either this latter assumption, apparently, does not always hold, or the *ortho*-quinones of some particular catechol compounds are unable to initiate the reaction, for there are laccases, namely, that of Bertrand from the latex of *Rhus*, and the preparations from Nettle, Elder, Lilac and Groundsel (see p. 144), which will not act on tyrosine. On the other hand, it is more than probable that some additional unknown factor is concerned in the action of tyrosinase.

A laccase need not necessarily arise from the oxidation of an *ortho*-dihydroxy compound. Any reactive compound capable of blueing guaiacum and adsorbed to a colloidal surface might be termed a laccase. The laccases, moreover, of the Basidiomycetes, though similar to those of the Phanerogams, are not necessarily identical, and may also differ among themselves.

Peroxidase

The essential characteristics of this enzyme have been indicated in the previous pages. In the presence of hydrogen peroxide it will oxidise a number of phenolic substances of different constitution, such as pyrogallol, catechol, quinol, benzidine, guaiacum, guaiacol, *p*-phenylenediamine, *p*-cresol, etc. The last-mentioned reagent is useful for the detection of peroxidase, since this enzyme gives with it a reaction (milky precipitate) differing from that (orange colour) produced by laccase, i.e. oxygenase plus the oxidation products of catechol. The nature of the actual oxidations associated with peroxidase in the plant are unknown. *In vitro*, it will catalyse, in presence of hydrogen peroxide, the oxidation of nitrites to nitrates, but there is no evidence that this property is of biological significance.

The nature of the peroxidase is also unknown, though its isolation has been carried out to great perfection by Willstätter and his co-

workers (148–154). These investigators have isolated and purified a peroxidase preparation from the root of Horse-radish by a series of adsorptions (on protein, aluminium hydroxide, kaolin), elutions (by alkali) and precipitations (by alcohol, tannin). The final and most active product is free from protein and sugar. With purification, also, the iron content is decreased, though the activity is raised, so that there would appear to be no evidence for regarding iron as an essential factor for catalysis by the enzyme. Through his method of purification, Willstätter has succeeded in raising the pyrogallol number of the enzyme, i.e. the power of oxidation of pyrogallol in presence of peroxide under definite conditions, from about 0·25 in the original root to about 4900 in the purified preparation.

Oxido-reductases

The terminology used for the enzymes included under the above name depends on the particular view taken as to the ultimate mechanism of oxidation, a discussion which is not within the scope of the present chapter.

Oxido-reductase is here applied to the type of enzyme which catalyses the oxidation of one compound in the presence of another which is simultaneously reduced. The Schardinger enzyme of milk is a typical example. This catalyst will oxidise aldehyde to acetic acid in the presence of methylene blue, the latter being reduced at the same time to the leuco compound. In most cases, the oxidation-reduction process is more readily depicted if we assume that the process takes place through the elements of water:

$$CH_3 \cdot CHO + H_2O + MeB = CH_3 \cdot COOH + MeBH_2.$$

Bach (10) has actually considered that the catalysts involved are water-splitting enzymes.

A special form of this process is known as the Cannizzaro reaction, whereby two molecules of an aldehyde give rise to the corresponding acid and alcohol:

$$CH_3 \cdot CHO + CH_3 \cdot CHO + H_2O = CH_3COOH + CH_3 \cdot CH_2OH.$$

It is obvious that such reactions as the above can take place under anaerobic conditions, but, sometimes, they take place also in air, oxygen itself playing the rôle of the substance reduced, as for instance in the case of the xanthine oxidase of milk:

$$\underset{\text{Xanthine}}{C_5H_4N_4O_2} + H_2O + \underset{\text{or } O_2}{MeB} = \underset{\text{Uric acid}}{C_5H_4N_4O_3} + \underset{\text{or } H_2O_2}{MeBH_2}$$

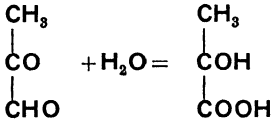
On the hypothesis of Wieland, the underlying conception of oxidation is the activation of hydrogen in the molecule of the compound to be oxidised (hydrogen donator) by a catalyst (dehydrase), and the transference of this hydrogen to the molecule of another compound which is reduced (hydrogen acceptor). In many cases the formation of a hydrate of the substance to be oxidised must be assumed before activation can take place. Hence, in effect, the result is the same as that arrived at by means of Bach's hypothesis.

The best known thermolabile catalyst of the oxido-reductase type in the plant is that which reduces nitrates in the presence of aldehydes. Though not the first to discover this form of enzyme, Bach (11) has made various investigations on the nitrate-reducing enzyme of the Potato tuber. He found that when Potato juice is added to a solution of sodium nitrate in the presence of a small amount of acetaldehyde, and the mixture kept at 60° C. for a short time, a positive test for nitrite can be obtained. The reaction does not take place in the absence of aldehyde.

Anderson (1) further investigated the enzyme, using the Griess-Ilosvay reagent. He detected it in some twenty-one plants (mostly shoots, though a few tubers and seedlings gave a positive reaction). These included the Sunflower (*Helianthus annuus*), Artichoke (*H. tuberosus*), Flax (*Linum usitatissimum*), Pea (*Pisum sativum*), Radish (*Raphanus sativus*), Wheat (*Triticum vulgare*), Clover (*Trifolium pratense*), etc. The activity of the reducing mechanism was variable. In several plants, aldehyde was unnecessary, and in a few it actually retarded the reduction. The expressed sap of about 100 species was also examined for nitrite itself with the Griess-Ilosvay reagent, and about 25 % showed a positive reaction. Anderson points out that the activity is considerable only at high temperatures and in presence of aldehyde; it is, moreover, of comparatively rare occurrence. These facts appear to militate against its playing a part in nitrogen metabolism.

The nitrate-reducing enzyme from the Potato tuber has been extracted and more extensively investigated by Michlin (89), who has shown that the place of acetaldehyde, as a hydrogen donator, can be taken by other aldehydes. This was confirmed by Bernheim (31), who also claimed that nitrate, as a hydrogen acceptor, can be replaced by both quinone and methylene blue. Michlin (90) has pointed out, however, that the latter reduction takes place only to a small extent over a narrow range of p_{H}.

Other enzymes of the oxide-reductase type are doubtless involved in glycolysis, the primary anaerobic stage of respiration. There is reason to believe that this process in the higher plants takes a course similar in outline to that known to be followed by Yeast. A "zymase" has been extracted from Beet roots and potatoes, which will ferment sugar, and hence must contain the various catalysts responsible for this process. A specific catalyst, which carries out the oxidation of pyruvic aldehyde (methyl-glyoxal) to lactic acid,



has also been detected in the higher plants. These catalysts, however, are more suitably considered in connection with respiration (Chap. VI).

Wieland's conception of hydrogen transport has been applied to catalysts of the tissues. Thunberg, in particular, has claimed to support the hypothesis by showing that washed tissues contain catalysts which will oxidise various metabolites, if methylene blue is present as a hydrogen acceptor. From the plant also he has obtained similar results; he found (136), for instance, that the endosperm of the Bean (*Phaseolus vulgaris*) decolorised methylene blue in presence of malic, formic and succinic acids and other substances. His researches have been extended to show the existence of further dehydrases: one, from seeds of species of Mallow (*Malva*), the Orange (*Citrus Aurantium*) and the Plum (*Prunus communis*) oxidises oxalic acid (137), and another from the seeds of the Cucumber (*Cucumis sativa*) oxidises citric acid (138). In both cases methylene blue was used as a hydrogen acceptor.

In an endeavour to introduce uniformity among oxidising systems, it has been suggested that there is activation and transference of hydrogen in the process of oxidation of catechol by the oxygenase. Experiments have been carried out (Onslow & Robinson, 100) with a view to oxidising catechol, anaerobically, by means of the oxygenase in presence of methylene blue and other more readily reducible indicators of Mansfield Clark. No evidence was obtained that the oxygenase can act in this capacity.

Additional oxidising systems

Here must be included a few oxidising and reducing mechanisms which have been described, but which cannot be conveniently classified among those already defined.

The statement that a thermostable laccase has been obtained by Euler & Bolin (52–54) from the Lucerne (*Medicago sativa*) has been much quoted in literature upon the subject. *Medicago* is not an oxygenase plant; so that the phenomena observed must be of a different type. The authors prepared the enzyme by precipitating the juice of the Lucerne with alcohol, and they claim it to be an impure mixture of the calcium salts of glycollic, citric, malic and mesoxalic acids. The precipitate catalyses the absorption of oxygen by quinol, especially in the presence of manganese salts.

Bunzel (45), on repeating Euler & Bolin's work, states that no oxidase (laccase) is present, and that the catalytic effects on the oxidation of quinol observed by the latter authors were due to the alkalinity of the solutions of the salts obtained in their preparations.

A substance, which is of considerable significance in oxidative metabolism of animal tissues and Yeast, namely, the autoxidisable peptide, glutathione (Hopkins, 81), may also prove to be of equal interest in the plant. Qualitative reactions indicate that it is widely distributed in the Phanerogams, but the evidence by isolation is not yet conclusive (Kozlowski, 84).

Recently, Szent-Györgyi (135) has isolated from the animal (adrenal gland) and from certain peroxidase plants a crystalline substance, termed by him "hexuronic acid". He claims it to be an active and unstable isomer of glucuronic acid, probably fructuronic acid, and he considers it to be of importance in oxidation-reduction processes of the cell. The plant material used was as follows: fruits of Orange, Lemon, Grape Fruit, Tomato and Pineapple; roots of Radish (*Raphanus sat. niger*) and Turnip; bulbs of Onion and Leek; and leaves of Cabbage. He showed that a strong reducing agent is present in the expressed juice which reduces hydrogen peroxide, so that excess of this reagent must be added in order to obtain the blueing of guaiacum with the peroxidase also present in the juice. Iodine and phosphotungstic acid are also reduced.

From the fruit of the Orange and the leaves of the Cabbage he has extracted a crystalline substance (m.p. 175–188° C.; rotation $[\alpha]_D^{20°} + 24°$) responsible, in his opinion, for the reducing power of the expressed juices, and which he claims to be identical with the product isolated by him from the suprarenal gland. This so-called "hexuronic acid", when isolated from the plant, was found to be oxidised, by peroxidase and hydrogen peroxide, only through the medium of a phenolic substance. Szent-Györgyi also considers that

the oxidation of hexuronic acid is reversible under anaerobic conditions. The system is thus expressed:

$$H_2O_2 + \text{peroxidase} \rightarrow \text{phenol} \rightarrow \text{hexuronic acid}$$
$$\rightarrow \text{other oxidation systems.}$$

It may be pointed out that the plant tissues used by Szent-Györgyi are such as contain exceptionally large amounts of pectic substances, and in the cell-walls changes are taking place which might result in the presence of free galacturonic acid in the cell. Galacturonic acid reduces Fehling's solution in the cold, and a sample derived from the pectic acid extracted from Beet and Flax (Ehrlich, see p. 89) had a m.p. of 159° C., and showed a final rotation $[\alpha]_D^{20^\circ} + 53^\circ$. There does not seem to be conclusive evidence that Szent-Györgyi's product from the plant has properties which might not also be characteristic of galacturonic acid; or, at any rate, that the latter was absent from his preparation.

From Szent-Györgyi's results one can conclude that the press-juice of certain peroxidase plants has a definite reducing power; this is presumed to be due to a uronic acid, since crystals of a product conforming in composition to such a substance have been obtained from juice of the Orange and from leaves of the Cabbage. The uronic acid is oxidised by the *ortho*-quinone formed by the action of hydrogen peroxide and peroxidase on a polyphenol, and can be reduced again by the plant juice. The origin of hydrogen peroxide in peroxidase plants is left undefined. It is obvious that the reducing action of the uronic acid would not be apparent in plant juices from oxygenase plants, since it would be oxidised by the *ortho*-quinone formed from the catechol compounds present.

As will be mentioned later in the section on oxidising enzymes and respiration, it is difficult to deduce whether the interactions *in vitro* of the fortuitous selection of metabolites occurring in an expressed plant juice have necessarily any corresponding significance in the living cell.

The oxidising mechanism connected with certain haematin compounds probably universally distributed in cells (Keilin, 83) will be referred to in the next section.

Oxidising systems and respiration

It is difficult to appraise the evidence for and against the view that the catechol-oxygenase peroxidase system plays a part in respira-

tion. Though other oxidising mechanisms no doubt exist in the plant, this particular system has been extensively studied, and constitutes a typical and characteristic oxidase. Were the answer to the question known, as to whether this type of catalyst does or does not form an essential component of the respiration process, it might be decisive for all similar mechanisms.

Palladin's hypothesis of respiration pigments is based on the phenomena brought about by the catechol oxygenase. Palladin noted that many plants, on autolysis, produce red, brown, blue or black pigments, which are reduced again by the plant tissue on exclusion of air. That is, *ortho*-quinones, and all secondary oxidation products formed in oxygenase plants during autolysis, are deemed to serve in the living plant, their production being suitably controlled, as hydrogen acceptors in reactions taking place in the first anaerobic stages of respiration, and are thereby reduced to the original unoxidised state. Subsequently, they are re-oxidised, and, again, reduced and so forth. Palladin's hypothesis has lately been revived with new observations by Oparin (102, 104).

The catechol-oxygenase-peroxidase system theoretically fulfils the rôle as an agent for supplying oxygen in a suitable form for oxidation of metabolites produced in the first, anaerobic, stages of respiration, for, not only can *ortho*-quinone serve as a hydrogen acceptor in oxidation-reduction processes, but hydrogen peroxide is also produced simultaneously; peroxidase, in addition, is present, and hence, in the presence of hydrogen peroxide, can catalyse other secondary oxidation processes.

The difficulty in accepting this scheme lies in the fact that the catechol-oxygenase system is absent from rather under 50 % of the total number of natural orders of the Angiosperms. Yet the impression gained from the study of the phenomena of respiration is that the reactions underlying this process are fundamentally the same for all green plants, and that the transference of molecular oxygen to oxidisable metabolites does not depend on the fortuitous occurrence of some particular type of aromatic compound. It is immaterial to the growth and development of a plant as to whether it is of the catechol type or not. So far, no autoxidisable mechanism, in any way comparable to the catechol-oxygenase system, has been detected in peroxidase plants, and, though not conclusively shown by experiment, it is probable that, on autolysis, the tissues of the latter do not absorb oxygen to any marked extent. Such, in fact,

is indicated in some studies made by Thoday[1] on the effect of small amounts of chloroform on respiration; for these experiments, leaves of the Cherry Laurel (*Prunus Laurocerasus*), the Portugal Laurel (*P. lusitanica*), the Artichoke (*Helianthus tuberosus*), all oxygenase plants, and of the Garden Nasturtium (*Tropaeolum majus*), a peroxidase plant, were used. A considerable difference of behaviour was noted between the oxygenase and peroxidase plants when the amount of chloroform administered was large enough to initiate visible disorganisation of the tissues (death point); a large absorption of oxygen was observed in the oxygenase plants accompanied by browning of the leaf, but in *Tropaeolum* no comparable phenomenon took place.

If we therefore consider that the catechol-oxygenase system is continually oxidised and reduced in the living cell for one type of plant, no comparable system has yet been detected in the other type, though we do not wish to deny the possibility of its existence. There is, moreover, an alternative, in that some other, fundamentally different, mechanism is supplying oxygen to metabolites in the living cell. Then, when the cell-structure is destroyed by narcotics or mechanical injury, oxygen gains free access, and uncontrolled oxidation follows. At this stage, the oxidation of any autoxidisable substances which may be present is catalysed by a surface inherent to the structure of the cell, and the phenomenon of an oxidase is exhibited. From this point of view the catechol compounds would act as indicators of the condition when oxygen is available for their oxidation, and, though the latter may be dependent upon the main oxidation mechanism in the cell, it is a subsidiary process, and the catechol substances and their oxidation products are not participants in the metabolic chain of the respiration processes.

Though the *prima facie* evidence for regarding oxidising enzymes of the catechol oxygenase type as integral components of respiration in the higher plants is not convincing, the evidence in the case of Yeast and animal tissues has been taken as conclusive by Keilin (83). A brief outline of the facts in connection with Yeast is given here, since a comparison has been made by the above author between the oxidase and respiratory mechanism of this Ascomycete and those of the higher plants.

Keilin has carried out the detection of the oxidase of Yeast by means of the indophenol reagent. This is an alkaline mixture of

[1] **Thoday, D.** On the Effect of Chloroform on the Respiratory Exchanges of Leaves. *Ann. Bot.*, 1913, **27**, 697–717.

p-phenylenediamine and *α*-naphthol, giving rise, on oxidation, to a product, indophenol blue, of which the reduction product is leuco-indophenol. The reagent penetrates the cell, and the indophenol blue is deposited within the cell. Yeast oxidase, like the oxidase of animal tissue, will not blue guaiacum. A possible reason for this may be that the oxidases of animal tissues and of Yeast are attached to the protoplasm, and cannot be extracted and obtained in solution. Such oxidases, or the products of their activity, will not transfer oxygen to guaiacum, as in the case of plant enzymes which are soluble, but will only act on the indophenol reagent which is itself adsorbed to the protoplasm. In regard to the catechol-oxygenase system, the oxygenase preparation alone will not act on indophenol, but in presence of catechol, or adsorbed *ortho*-quinone, a positive reaction is given with indophenol as well as with guaiacum; peroxidase plants do not give the indophenol reaction within the time allotted for a positive test, though granules of indophenol blue may eventually be deposited in the tissues (Reed, 121). Hypothetical suggestions for the comparison of the various enzymes are set out below:

Possible nature of enzyme				Reactions		
Lactarius	Yeast	Higher plants	Higher animals	Guaiacum	Indophenol	Catechol
—	—	Oxygenase = colloidal surface + *trace* of adsorbed oxidised products (*ortho*-quinone, etc.)	—	Negative	Negative	Positive
Soluble laccase = colloidal surface + adsorbed oxidation products	—	Soluble laccase = oxygenase + adsorbed oxidation products (*ortho*-quinone, etc.)	—	Positive	Positive	Positive
—	Insoluble oxidase	—	Insoluble oxidase	Negative	Positive	Negative

The phenomena shown by Yeast may be stated in outline as follows: the reagent will penetrate the yeast cells suspended in buffer solution, but in the normal untreated cell no reaction takes place. On addition of the reagent, however, after certain treatments, other effects are obtained, which will now be described:

1. If a suspension of Yeast cells is heated to 52° C. for 1½ hours, then, on adding the reagent, indophenol blue is formed in the cells

(that is, a positive oxidase reaction is given). Keilin considers this to be due to the destruction of a reductase enzyme in the cell by the above treatment. Hence there is no catalysis of reduction and oxidation proceeds.

2. If the suspension of Yeast cells is narcotised with phenylurethane, there is a positive oxidase reaction. Keilin considers this to be due to inhibition of reductase enzymes.

3. If the suspension of Yeast cells is cooled from $-2°$ to $-4°$ C., there is a positive oxidase reaction. Keilin considers this to be due to different temperature coefficients for oxidation and reduction.

More recently (163) Harrison claims to have shown that some of Keilin's observations and deductions in connection with the oxidising and reducing systems of Yeast are erroneous. Harrison suggests another scheme of reactions. It is not within the scope of this chapter, however, to discuss their respective merits. For the time being, Keilin's results are accepted.

Other observations were made with plasmolysed Yeast, dried Yeast, acetone Yeast and Yeast treated with alcohol, but as it is difficult to know, without experimental details, what takes place under these treatments, the account here is confined to the experiments which are more precisely described.

Now let us compare these results with any similar phenomena shown by the higher plants. Experiments (1) and (3) are difficult to demonstrate in a multicellular organism unable to live under the same conditions as Yeast. With respect to experiment (2), chloroform is usually employed in connection with the higher plants for experiments on narcosis. It increases the permeability of the cell, and at the death point, in catechol-oxygenase plants, browning occurs, and subsequent blueing of guaiacum or indophenol. This would be regarded as due to access of oxygen rather than to inhibition of reductases. Similarly, when a suspension of Yeast cells is treated with chloroform, with subsequent addition of the reagent, indophenol blue is formed.

If, moreover, the cells of the higher plants are rendered permeable to air by rupture due to mechanical injury, again there is browning, and a positive reaction will be given with guaiacum and indophenol. Here access of oxygen would be postulated as the cause of the phenomenon, rather than the destruction of reductases.

If Yeast cells are ground up with powdered glass (a method not adopted by Keilin) and then treated, in suspension, with the indophenol reagent, indophenol blue is formed; a control experiment

carried out with Yeast cells mixed with powdered glass gives a negative reaction. This effect, however, may be due to the washing out or dilution of the reducing metabolites, and may not be concerned with the reductase.

It appears difficult to explain the results described for the higher plants solely on the hypothesis of inhibition or destruction of reductases. If, however, one assumes that injury leads to increased permeability to oxygen, then reducing metabolites are oxidised, and autoxidation occurs with subsequent transference of oxygen to the indophenol reagent. Or, we may postulate that the respiration capacity of tissues or cells is decreased or destroyed by the treatments described, in which case, the supply of reducing metabolites produced in the anaerobic stage is lowered, with the resultant final oxidation of autoxidisable substances in the cell.

Keilin further proceeds to demonstrate that the oxidase of Yeast is inhibited by potassium cyanide and sulphuretted hydrogen. The catechol-oxygenase system was already known to be inhibited by cyanide; this was confirmed by Keilin, and also the same effect was shown to be produced by sulphuretted hydrogen.

Finally, Keilin showed experimentally that carbon monoxide will inhibit the oxidase reaction of Yeast in the dark, but, on exposure to light, the unstable compound with carbon monoxide is decomposed, and the oxidase reaction appears. The same inhibition is produced on the oxygenase in regard to the oxidation of catechol, but in this case the effect is not removed in the light.

Entirely similar results to those obtained with Yeast were found by Keilin to be given by the oxidase of heart muscle.

In connection with observations made by Warburg and others on the effect of inhibitors on respiration of cells, Keilin states "...that the factors such as KCN, H_2S, CO in dark and CO in light...inhibit the indophenol oxidase activity to about the same degree as they inhibit the normal respiration of the cells. This clearly shows that the indophenol or paraphenylenediamine oxidase takes an essential part in the normal respiratory activity of the cells, and that this oxidase is identical with the 'respiratory ferment' of Warburg".

In Keilin's system the oxidase is regarded as functioning in connection with certain intracellular haematin compounds (cytochrome) which occur in Yeast and muscle. Owing to lack of investigation, the presence of these haematin compounds in the higher plants has been confirmed by a few observations only.

In Yeast and muscle, cytochrome is considered by Keilin to be oxidised by oxygen activated through the oxidase, and reduced by reducing metabolites activated through the reductase. It therefore acts as a carrier between the two activating systems. This conclusion is based on the fact that the oxidation and reduction of cytochrome in the cell is inhibited or abolished by the same factors (KCN, H_2S, CO, urethane, heat, etc.) which respectively inhibit or abolish the activity of the oxidase and reductase.

Keilin's evidence appears conclusive until we consider the case of the peroxidase plants, where respiration takes place without any surface which will directly catalyse the oxidation of catechol, or, indirectly, that of leucoindophenol, at any rate to the extent demanded for a catalyst. Nor in these plants is there an indication of any other system playing a similar rôle. That is, in peroxidase plants, autoxidisable substances are either absent or present to a small extent. Hence all the post-mortem phenomena described for the oxygenase plant, or for the Yeast cell under the abnormal conditions described above, are absent. It is possible that we should regard the oxidase and reductase effects as indicators merely of oxidation and reduction capacity of a complex mixture of metabolites and surfaces under certain conditions, rather than essential participants in the actual respiration processes of the cell. (See also p. 304.)

Historical sequence of investigations[1]

The literature on the oxidising enzymes of plants is very considerable. Conflicting results have often been obtained owing to various causes. Material, for instance, from both lower and higher plants has been used, and conclusions drawn indiscriminately from both sources. The enzymes of the Basidiomycetes resemble those of the higher plants, but strict comparison is not possible until more is known of the former. Again, tissues containing oxidising enzymes have given negative results when tested with reagents through failure to remove inhibiting sugars and tannins; or, preparations have been made in many different ways, and have contained varying amounts of *ortho*-quinone, a fact which, as we have seen, may confer different oxidative powers on the preparations.

First will be mentioned investigations on the oxidase (catechol-

[1] The names oxygenase, oxidase, tyrosinase, etc., mentioned in this section are those actually used by the authors in question. Attempts to rectify discordance in nomenclature are limited to previous sections.

oxygenase-peroxidase) system. The greater part of the pioneer work on plant oxidising enzymes is due to Bach & Chodat, who made a long series of classical researches (2–28, 46). The system in the higher as well as the lower plants, which causes the direct blueing of guaiacum, was termed by Bach & Chodat an oxidase. These authors considered such a system to consist of two components, an oxygenase and a peroxidase. Chodat (46) has defined an oxygenase as an enzyme-like substance, which can combine with atmospheric oxygen to form a peroxide, but neither author has, at any time, differentiated between the enzyme (oxygenase) and the substrate (catechol compound). Since practically all the higher plants contain peroxidase, then, according to Bach & Chodat, those which contain oxygenase in addition will blue guaiacum directly; on the other hand, plants which contain only peroxidase can only blue guaiacum indirectly, that is, after the addition of hydrogen peroxide.

Bach & Chodat's conception of the mechanism of the oxidase was as follows: The oxygenase (which is an enzyme-like substance) is capable of forming an organic peroxide in air. They had previously shown that peroxidase catalyses the decomposition of hydrogen peroxide and organic peroxides (such as ethyl peroxide) with the formation of active oxygen. Hence the peroxidase, which is always present, would catalyse the decomposition of the peroxide formed by the oxygenase, the active oxygen being accepted by guaiacum, etc.

In 1911, Wheldale (Onslow) (144) first pointed out that extracts of plants which show the direct oxidase reaction contain a substance giving the catechol reaction with iron salts and dilute alkali, whereas no such substance is to be found in peroxidase plants. She also emphasised the connection between the direct oxidase reaction and browning of the tissues on injury. An additional observation was made, namely, that catechol solution itself autoxidises in air with production of peroxide. Later (Onslow, 93) it was demonstrated that, of a number of commonly occurring aromatic compounds, only those with the *ortho*-dihydroxy grouping autoxidise in air with formation of peroxide, and that such compounds are present in plants showing the direct oxidase reaction; further, that they are accompanied by an enzyme, oxygenase, which catalyses this autoxidation with production of brown pigments and subsequent blueing of guaiacum. The system was found to be absent from peroxidase (indirect oxidase) plants. The separation of enzyme from substrate was also accomplished by extraction with alcohol; the catechol compound can then

be isolated from the extract, and the system again united as it formerly existed in the tissues.

In 1925, Onslow & Robinson (99) showed that a preparation of oxygenase not only oxidises *ortho*-dihydroxy compounds, but also compounds with a monophenolic grouping, such as *p*-cresol, *m*-cresol, tyrosol, *m*- and *p*-hydroxybenzoic acids, the corresponding dihydroxy compounds being produced.

In 1925, Szent-Györgyi (133) suggested that *ortho*-benzoquinone is the product of oxidation of catechol by oxygenase, and is solely responsible for the blueing of guaiacum. He allowed oxygenase from the Potato tuber to act on catechol solution, removed, as he believed, the enzymes by precipitation with alcohol, and showed that the filtrate blued guaiacum by virtue of the presence of an oxidised product, *ortho*-quinone, apart from any enzyme action. He gave, however, no proof of the identity of the quinone.

In 1926, Onslow & Robinson (100) confirmed the formation of an oxidation product, from the action of Potato oxygenase on catechol, which blues guaiacum in the absence of enzymes. They found, however, that enzymes cannot be removed completely from the reaction mixture, as maintained by Szent-Györgyi, by precipitation with alcohol. They can be eliminated, however, by means of colloidal ferric hydroxide or by picric acid solution. These authors also demonstrated, with titanium sulphate, the presence of hydrogen peroxide, both in autoxidised solutions of catechol and as a product of the action of oxygenase on catechol. This was corroborated by Platt & Wormall (110), using Potato enzyme.

In 1927, Pugh & Raper (114), through the isolation of crystalline aniline derivatives, confirmed the formation of *ortho*-quinones from phenol, *p*-cresol and catechol by the action of an oxidising enzyme (termed by them tyrosinase) from the mealworm; also of the formation of *ortho*-quinone from catechol by the action of peroxidase (from the Horse-radish) and hydrogen peroxide.

Finally, Onslow & Robinson (101) provided evidence that the power of oxygenase preparations to oxidise monophenols can be removed by treatment with animal charcoal, and, further, that it can be restored again by the addition of a trace of catechol. The deduction was drawn that the secondary oxidation of monophenols is due to a trace of *ortho*-quinone adsorbed to a colloidal surface, which complex is removed by treatment with charcoal.

Now let us turn to the sequence of investigation in connection

with tyrosinase. Considerable confusion has probably arisen in the matter of tyrosinase by, again, the indiscriminate use of both the lower (Basidiomycetes) and higher plants as materials for the study of this enzyme. Analogous systems doubtless exist in the two cases, but there is no close parallelism. Though Chodat & Bach[1] and Bach (20) claim to have separated, to some extent, the oxygenase (enzyme-like peroxide) component from the peroxidase component of the Basidiomycete oxidase (laccase), no resolution of the oxygenase into enzyme and substrate has yet been brought about. For, in the case of *Lactarius vellereus*, *Lepiota Prossera*, *Agaricus campestris* and *Russula fragilis*, even after prolonged washing with alcohol, no substance analogous to the catechol derivative is removed, and the mycelium residue still blues guaiacum directly (Robinson, 124). It appears that tyrosinase, free from both oxidase (laccase) and peroxidase, has been prepared from *Lactarius vellereus* (Chodat, 46) and from *Russula* sp. (Bertrand[2]). The freedom of tyrosinase from laccase should be more readily capable of demonstration in the Basidiomycetes than in the higher plants, since the action of the oxidase (laccase) of the former on guaiacum is not affected by washing away any component by alcohol as in the case of the latter.

Later, additional confusion arose in connection with the researches of Raper and his co-workers. These authors at first (80, 110, 119, 120) used a preparation of tyrosinase from the Potato tuber, and later (114, 115, 116) one from the mealworm. As pointed out in the previous pages, we do not know how far, in detail, these systems from different sources resemble each other. Moreover, Raper employed the term, tyrosinase, for the catalyst, which has been designated oxygenase by Bach & Chodat and by Onslow. As stated in the foregoing sections, evidence, on the whole, suggests that tyrosinase (using the term only in respect of an enzyme acting on tyrosine) is oxygenase, together with some oxidation product (*ortho*-quinone), adsorbed to a specific colloidal surface. It is, therefore, not surprising that, without some agreement both in outlook and terminology, it is difficult to write a clear account of the various lines of investigation.

Chodat, who from his methods of preparation and purification believed tyrosinase, both of the higher and lower plants, to be a

[1] **Chodat, R.**, und **Bach, A.** Untersuchungen über die Rolle der Peroxyde in der Chemie der lebenden Zelle. V. Zerlegung der sogenannten Oxydasen in Oxygenasen und Peroxydasen. *Ber. d. D. chem. Ges.*, 1903, **36**, 606–608.

[2] **Bertrand, G.** Sur la présence simultanée de la laccase et de la tyrosinase dans le suc de quelques champignons. *C.R. Acad. sci.*, 1896, **123**, 463–465.

single and separate enzyme, first suggested (46) that it oxidises amino-acids (including tyrosine) with evolution of carbon dioxide and ammonia and the formation of the aldehyde of the acid next lower in the series, that is, it constitutes an oxido-deaminase (also Schweizer, 129). Some of Chodat's evidence is based on results obtained with the Basidiomycete enzyme; he claims, however, to have obtained tyrosinase from the Potato also free from oxidase (phenolase, laccase), though peroxidase remained. Chodat and Schweizer maintained that a tyrosinase preparation from the Potato would deaminise glycine with production of formaldehyde, carbon dioxide and ammonia; phenylglycine, also, with production of carbon dioxide and benzaldehyde, the latter being detected by its odour. They considered this deamination to take place, either in the presence or absence of *p*-cresol. [Tyrosinase, in the presence of *p*-cresol and glycine, gives a deep red colour changing to blue, "cresol-blue". Similar colours are obtained with other amino-acids (Chodat, 46); *p*-cresol may be replaced by catechol or phenol.]

Bach (17) maintained, for the higher plants, that he was not able at any time to prepare tyrosinase free from oxidase (laccase, phenolase). He believed tyrosine to be first deaminised by an oxy-reductase component of tyrosinase, and subsequently the products were oxidised by the phenolase component. Potato tuber was used as a source of enzyme.

It was first shown by Robinson & McCance (125), and, later, confirmed by Happold & Raper (80), that deamination of aliphatic amino-acids by tyrosinase will not take place unless catechol or *p*-cresol is present. Robinson & McCance used *Lactarius* enzyme, and Robinson, separately, recorded the same fact for the Potato enzyme (prepared according to Onslow) with catechol, *p*-cresol, and tyrosol. It was made clear by these authors that either an *ortho* or *para* compound was necessary for deamination. The slight deamination observed by Chodat, in the absence of *p*-cresol, may have been due to the precipitation of a relatively large amount of *ortho*-quinone with the enzyme. Happold & Raper employed dialysed Potato juice as a source of enzyme, and the term used by them for the catalyst concerned was tyrosinase.

In 1923, it was suggested by Onslow (97) that the initial stage of the action of tyrosinase (from Potato) on tyrosine is the formation of dihydroxyphenylalanine, and that traces of peroxide in oxygenase preparations might oxidise tyrosine to the above compound. From

this, active oxygen (now known to be *ortho*-quinone) would arise which would deaminise the side-chain. Onslow & Robinson (99) also found that, when tyrosinase from Potato was allowed to act on tyrosine for a short time, a green coloration was obtained with ferrous sulphate, though no characteristic purple appeared on addition of sodium carbonate.

Later, Happold & Raper (80) suggested that *ortho*-quinone is formed by the action of *tyrosinase* (cp. Szent-Györgyi) on *p*-cresol and catechol, and that the quinone is responsible for deamination of amino-acids. They also claimed to have demonstrated the deamination of glycine by *ortho*-quinone prepared according to Willstätter & Müller.

In 1926, Raper (115), acting on previous suggestions, isolated dihydroxyphenylalanine as the first product of the action of tyrosinase on tyrosine. He used tyrosinase from the mealworm, and, after allowing the action on tyrosine to proceed to the red stage, the reaction was stopped. Dihydroxyphenylalanine was isolated from the products of the reaction by precipitation with lead acetate in alkaline solution. It was obtained crystalline and identified.

It is further due to Raper & Wormall (120) and Happold & Raper (80) to have shown that there is no evidence for the liberation of ammonia during the action of tyrosinase on tyrosine. Dialysed Potato juice was employed as a source of enzyme.

Finally, Raper (116) made a series of investigations of the intermediate products of the action of tyrosinase (from mealworm) on tyrosine, with a view to determining the course of the reaction in the formation of melanin. Several intermediate products with the indole structure were isolated, and the enzyme is regarded as responsible only for the first stage of oxidation, namely until the appearance of a red coloration.

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OXIDISING AND REDUCING SYSTEMS

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CHAPTER IV

THE PLANT PROTEINS

In the plant, proteins form a considerable portion of the protoplasm and they also occur in the cell-sap. A high percentage of protein is found in reserve tissues of seeds, tubers, bulbs, etc., often in the form of special bodies, termed aleurone, crystalloids, etc. Seeds have, so far, constituted the chief material for the investigation of proteins. As a result of these investigations, certain well-known types have been established, depending upon their solubility in various solvents: (1) Albumins; (2) Globulins; (3) Prolamins; (4) Glutelins.

Albumins

These proteins have the following characteristics. They are soluble in water and are coagulated by heat. Unlike animal albumins, they may be precipitated by saturation with sodium chloride or magnesium sulphate. They occur in relatively small amounts, but, nevertheless, a certain number of well-defined albumins have now been isolated and named as set out in Table XXX.

Globulins

The greater part of the reserve proteins of seeds are globulins. They are characterised, broadly speaking, by their insolubility in water and solubility in salt solutions. They are not precipitated in general by saturating with magnesium sulphate (as are animal globulins), but saturation with sodium sulphate at 35° C. precipitates all so far tested. The degree of saturation with ammonium sulphate required for precipitation varies with different proteins. They are only imperfectly coagulated by heating. One of the most striking characteristics is that they can, in many cases, be obtained in well-defined crystals, usually octahedra, either by dialysis or dilution of salt solutions. A large number of well-defined globulins have now been isolated and named as set out in Table XXXI.

Prolamins

The proteins of this group are soluble in 70–90 % alcohol, nearly or entirely insoluble in water and salt solutions, but soluble in acid or alkali. They have been found in the seeds of all cereals examined,

TABLE XXX. Plant albumins

Albumin	Plant in which found	Protein content, % of dry weight	% nitrogen in protein
	In seed		
Leucosin	Barley (*Hordeum vulgare*), 82[1]	0·3	16·6
,,	Rye (*Secale cereale*), 81	0·4	16·7
,,	Wheat (*Triticum vulgare*), 72, 85, 105, 124, 126, 137, 138	0·3	16·8
,,	Wheat, embryo, 104	—	16·4
Albumin	Wheat, bran, 55, 56	2·9	15·4
,,	Jack Bean (*Canavalia ensiformis*), 62	1·5	16·4
Legumelin	Soy Bean (*Glycine hispida*), 103	—	16·1
,,	Lentil (*Ervum lens*), 99	1–1·2	16·2
,,	Adzuki Bean (*Phaseolus radiatus*[2]), 97, 119	—	16·1
,,	Pea (*Pisum sativum*), 98, 129	2·0	16·3
,,	Broad or Horse Bean (*Vicia Faba*), 100	1–1·2	16·2
,,	Vetch (*V. sativa*), 101	1–1·5	16·2
,,	Cow-pea (*Vigna sinensis*), 96	—	—
Albumin	Adsuki Bean (*Phaseolus angularis*), 53	0·05	—
,,	Mung Bean (*P. aureus*), 49	0·05	14·8
,,	Lima Bean (*P. lunatus*), 59	1·8	14·2
Phaselin	Kidney or Navy Bean (*P. vulgaris*), 80	2·0	14·6
Albumin	Georgia Velvet Bean (*Stizolobium deeringianum*), 48	0·6–0·8	15·9
Ricin	Castor Bean (*Ricinus communis*), 69, 134	1·3	18–19
	In other organs		
Albumin	False Acacia (*Robinia Pseud-acacia*), bark, 61, 66	2·5	14·8

[1] Numbers refer to the bibliography.
[2] Incorrectly identified by the authors; should be *P. angularis*.

TABLE XXXI. Plant globulins

Globulin	Plant in which found	Protein content, % of dry weight	% nitrogen in protein
	In seed or fruit		
Globulin	Coconut (*Cocos nucifera*), 40, 47, 65	10·0	18·5
,,	Cohune-nut (*Attalea Cohune*), 41	—	17·8
Avenalin	Oat (*Avena sativa*), 75, 76, 79	1·5	17·9
Globulin	Barley (*Hordeum vulgare*), 82	—	18·1
,, I	Rice (*Oryza sativa*), 58, 68, 144	0·09	16·3
,, II	,, ,,	0·07	17·9
,,	Rye (*Secale cereale*), 81	—	18·4
,,	Wheat (*Triticum vulgare*), 105, 124, 126, 137	0·6	18·4
,,	Wheat, embryo, 104	—	18·6
,,	Wheat, bran, 55, 56	2·3	17·8
,,	Maize (*Zea Mays*), 24, 84	—	—
Castanin	Chestnut (*Castanea vulgaris*), 10	—	—
Corylin	Hazel or Filbert (*Corylus tubulosa*), 92	—	19·2

TABLE XXXI (*continued*)

Globulin	Plant in which found	Protein content, % of dry weight	% nitrogen in protein
	In seed or fruit		
Juglansin	European Walnut (*Juglans regia*), 92	—	19·1
,,	American Black Walnut (*J. nigra*), 122	—	18·9
,,	American Butter-nut (*J. cinerea*), 122	—	18·6
Edestin	Hemp-seed (*Cannabis sativa*), 1, 2, 66, 78, 133	—	18·7
Globulin	Buckwheat (*Fagopyrum fagopyrum*), 37	7·0	17·4
Amandin	Almond (*Prunus Amygdalus*), 92, 113	—	19·3
,,	Peach (*Prunus Persica*), 92	—	19·3
Arachin	Peanut (*Arachis hypogaea*), 43, 44, 45, 46, 66	—	18·3
Conarachin		27·0	18·3
Canavalin	Jack Bean (*Canavalia ensiformis*), 62, 147, 148	—	16·7
Concanavalin A and B		20·5	16·4
Glycinin	Soy Bean (*Glycine hispida*), 66, 67, 103, 111	—	16·9
Vicilin*	Lentil (*Ervum Lens*), 99	—	17·4
Legumin†	,, ,,	—	18·1
Conglutin A	Lupin (*Lupinus*), 6, 93	—	17·6
,, B	,, ,,	—	18·4
Globulin α	Adsuki Bean (*Phaseolus angularis*), 53	0·4	15·6
,, β	,, ,,	2·8	16·5
Globulin α	Mung Bean (*P. aureus*), 49	0·4	15·7
,, β	,, ,,	5·8	16·8
Globulin α	Lima Bean (*P. lunatus*), 59	0·6	15·6
,, β	,, ,,	1·6	14·8
Globulin	Adzuki Bean (*P. radiatus*[1]), 97	—	16·4
Phaseolin	Kidney or Navy Bean (*P. vulgaris*), 30, 80, 107, 150	16–20	16·0
Conphaseolin		0·4	15·9
Legumin†	Pea (*Pisum sativum*), 91, 98, 108, 127, 128	10·0	17·7
Vicilin*	,, ,,	—	17·1
Stizolobin	Chinese Velvet Bean (*Stizolobium niveum*), 38, 64, 66	—	16·1
Globulin α	Georgia Velvet Bean (*S. deeringianum*), 48	2·8–3·0	16·6
,, β	,, ,,	1·3	17·3
Legumin†	Broad or Horse Bean (*Vicia Faba*), 3, 100	—	18·1
Vicilin*	,, ,,	—	17·5
Legumin†	Vetch (*V. sativa*), 91, 101, 131	—	18·0
Vignin	Cow-pea (*Vigna sinensis, V. Catjang*), 96, 130	—	17·2
Acerin	Maple (*Acer saccharinum*), 9	—	18·3
Globulin	Flax (*Linum usitatissimum*), 77, 78	—	18·6
Globulin α	Cotton (*Gossypium herbaceum*), 50, 139	2·6	18·2
,, β	,, ,,	16·0	17·8
Excelsin	Brazil-nut (*Bertholletia excelsa*), 78, 109	—	18·3
Globulin	Castor Bean (*Ricinus communis*), 78	—	18·7
Globulin α	Tomato (*Lycopersicum esculentum*), 42	—	18·3
,, β	,, ,,	—	16·0
Globulin α	Sesame (*Sesamum indicum*), 57	32·0	18·4
,, β	,, ,,	7·5	17·6
Globulin	Cantaloupe (*Cucumis Melo*), 54	28·2	18·4
,,	Squash (*Cucurbita maxima*), 78, 112	—	18·5
,,	Sunflower (*Helianthus annuus*), 95	—	18·6
	Organs other than seed or fruit		
Globulin	False Acacia (*Robinia Pseud-acacia*), bark, 61	1·4	—
,,	Potato (*Solanum tuberosum*), tuber, 90, 145	—	16·2

but not in any other natural order. The chief prolamins are set out in Table XXXII.

TABLE XXXII. Plant prolamins

Prolamin	Plant in which found	Protein content, % of dry weight	% nitrogen in protein
	From seed or fruit		
Kafirin	Sorghum (*Andropogon*), 36, 63, 66	7·9	16·4
Hordein	Barley (*Hordeum vulgare*), 39, 71, 82, 110	4·0	17·2
Prolamine	Rice (*Oryza sativa*), 35, 136	0·1	16·2
Gliadin	Rye (*Secale cereale*), 81, 115	4·0	17·7
,,	Wheat (*Triticum vulgare*), 66, 105, 117, 124, 132, 136, 137	4·2	17·7
Prolamine	Wheat, bran, 55, 56	5·3	15·4
Zein	Maize (*Zea Mays*), 24, 27, 28, 66, 84, 114, 132, 133	5·0	16·1

GLUTELINS

The proteins of this group are characterised by their insolubility in water, salt solutions and alcohol, but they are, in the widest definition of the group, soluble in weak alkali and acid.

The first well-defined member to be described was the glutenin of the Wheat grain. It was originally extracted by very weak alkali, from which solution it was precipitated on neutralisation with acid. Similar, less well-defined, proteins were later extracted by this method from other cereals.

More recently, Csonka & Jones (25, 26, 51, 52) have modified the method of extraction and purification of glutelins. It was found by these authors that glutelins can be precipitated from alkaline solutions by, as a rule, small amounts of ammonium sulphate, too small to precipitate globulins; these amounts also lie within rather narrow limits, so that the glutelins desired can be obtained free from significant amounts of other proteins.

By means of this technique, other well-defined glutelins have been obtained from the grain of Oat, Rice and Maize, in addition to that of Wheat. Further, by fractional precipitation with different amounts of ammonium sulphate, two glutelins (α and β) have been isolated, in place of the original glutenin, from the Wheat grain; these are precipitated from alkaline solution by addition of ammonium sulphate to, respectively, 0·02 and 0·16 % of saturation. Similarly,

from the Maize kernel, α- and β-glutelins are precipitated by addition of ammonium sulphate to 3 and 16 % of saturation.

Proteins, which may, by definition, be also included in this group are those, to be described later (p. 178), derived from the cytoplasm of the vegetative tissues of the higher plants.

The chief glutelins are set out in Table XXXIII.

TABLE XXXIII. Plant glutelins

Glutelin	Plant in which found	Protein content, % of dry weight	% nitrogen in protein
	From seed or fruit		
Glutelin	Oat (*Avena sativa*), 5, 25, 73	1·9	17·5
Oryzenin	Rice (*Oryza sativa*), 51, 136, 144	1·5	17·6
α-Glutelin	Wheat (*Triticum vulgare*), 7, 26, 66, 105, 126, 137	1·0	17·1
β- ,,	,, ,,	0·1	16·1
α- ,,	Maize (*Zea Mays*), 52, 84, 114	0·7	16·1
β- ,,	,, ,,	—	—
Glutelin	Cotton (*Gossypium herbaceum*), 50	0·7	15·3
,,	Cantaloupe (*Cucumis Melo*), 54	5·8	16·3
Glutelin?	Buckwheat (*Fagopyrum fagopyrum*), 70	—	17·4
	From inflorescence		
Glutelin?	Cauliflower (*Brassica oleracea* var.), 74	1·7	13·4
	From leaves		
Glutelins?			
Cytoplasmic	Maize (*Zea Mays*), 22	2·3	14·4
Vacuole	Spinach (*Spinacia oleracea*), 18	0·7	14·0
Cytoplasmic (Spinacin)	,, ,,	4·9	16·3
Vacuole	Alfalfa (*Medicago sativa*), 21	0·25	13·2
Cytoplasmic	,, ,,	3·2	15·8
Cytoplasmic	Broad Bean (*Vicia Faba*), 20	—	16·8
,,	Runner Bean (*Phaseolus multiflorus*), 20	—	15·8
,,	Cow-parsnip (*Heracleum Sphondylium*), 20	—	15·9
,,	(*Crambe cordifolia*), 20	—	15·7
,,	Cabbage (*Brassica oleracea*), 20	—	14·6
,,	Horse-radish (*Cochlearia Amoracia*), 20	—	14·4
,,	New Zealand Spinach (*Tetragonia expansa*), 20	—	14·5
,,	Sunflower (*Helianthus annuus*), 20	—	13·7
,,	Fig (*Ficus Carica*), 20	—	13·4

In regard to the proteins from other plant tissues, less is known. Only in a few cases have well-defined products been isolated. Tuberin, a globulin, has been obtained from the Potato tuber, and a globulin has also been extracted from the inner bark of the Locust Tree, or False Acacia.

The proteins of the cell-sap and cytoplasm of vegetative organs

(mainly leaves) have been studied by Chibnall (11–19), Chibnall & Grover (20), Chibnall & Nolan (21, 22), Chibnall & Schryver (23) and Osborne, Wakeman & Leavenworth (140, 141).

Chibnall and his co-workers (14, 20) have devised a method for extracting the proteins of the cell-sap and cytoplasm separately. By treating the tissues with ether, the cells are plasmolysed, but not ruptured; the cell-sap was then pressed out in a Buchner press, and washed free from the cytoplasm. The protein was obtained free from the extract by coagulation. In this way vacuole proteins have been prepared from Spinach and Alfalfa (see Table XXXIII).

An extract of the cytoplasmic proteins was obtained by grinding and washing out the residue with water. The protein was subsequently precipitated by the addition of dilute acid. Following this procedure, cytoplasmic proteins have been isolated from a number of plants. They are insoluble in water and salt solutions, but are soluble in a very small excess of either acid or alkali; hence, they may be classed as glutelins. Though they may be mixtures, their chemical properties appear to be similar as far as determined by analysis (see Table XXXIV below).

In connection with a series of experiments on the nitrogen metabolism of the leaf of the Runner Bean (*Phaseolus multiflorus*), Chibnall & Schryver (23) extracted and estimated the protein. In this case, however, vacuole and cytoplasmic proteins were pressed out together, and subsequently coagulated by heat.

An investigation of the proteins of the inflorescence buds (head) of the Cauliflower (*Brassica oleracea* var.) has been attempted (McKee & Smith, 74). A cytoplasmic protein was extracted by washing out, and then coagulating by heat. A second portion was obtained by further extraction of the tissues with weak alkali, and a subsequent precipitation with acid.

A certain interest has centred on the relationship between the isoelectric points of plant proteins and the hydrogen ion concentration of plant tissues.

The isoelectric points of a representative selection of plant proteins have been determined by Pearsall & Ewing (142) as below. The range of p_H of the isoelectric points was, with a few exceptions, from 4·5 to 3·3. Since the cell-sap of plant tissues is usually of the reaction p_H 5·5–6·5, they conclude that the principal proteins are normally on the alkaline side of their isoelectric points, and hence behave as anions.

Edestin	5·5–6·0	Gliadin	3·5–5·5
Vicilin	3·4	Leucosin	4·5
Legumin	4·4–4·6	Globulin (Potato)	4·4
Legumelin	4·2	Globulin (Carrot)	4·1–4·4
Glutenin	4·4–4·5		

Chibnall & Grover (20) have further tested this relationship during their investigation of the proteins of leaf cell cytoplasm mentioned above. Determinations of the p_H of the tissues of the plants used were made:

Ficus	6·9	*Vicia*	5·7
Spinacia	6·6	*Medicago*	5·7
Helianthus	6·4	*Zea*	5·7
Heracleum	6·2	*Brassica*	5·6
Tetragonia	6·1	*Crambe*	5·6
Phaseolus	6·0	*Cochlearia*	5·4

Determination of the isoelectric points of the proteins was attempted by the method of maximum precipitation. The range lay approximately between p_H 4·0 and 5·0. Therefore the proteins must be present in the cytolysed cells as anions, but at a p_H not far removed from their isoelectric point.

The proteins could not be extracted from certain other plants, namely, *Parthenocissus* spp., *Rumex acetosella* and *Vitis vinifera*, which have a tissue p_H of 3·7–3·1. It was only possible to extract proteins from leaves of which the sap is alkaline with respect to the isoelectric point.

From Table XXXIV it will be seen that vegetable proteins can be resolved into the same amino-acids as animal proteins, though the relative proportions of the various components are, as would be expected, often very different. There is, however, no fundamental chemical difference.

Of the distribution of the amino-acids, by comparison with animals, it may be said that plants show:

1. Greater percentages of glutaminic acid and ammonia.
2. Less lysine in many cases; it is sometimes absent.
3. Relatively large amounts of proline.
4. Arginine in larger proportions.
5. Often very low values for glycine and alanine; the former may be absent.
6. Sometimes absence of tryptophane.

TABLE XXXIV. Products of hydrolysis of plant proteins. (From various authors)

	Glycine	Alanine	Valine	Leucine	Proline	Phenylalanine	Aspartic acid	Glutaminic acid	Serine	Tyrosine	Cystine	Lysine	Histidine	Arginine	Tryptophane	Ammonia
From seed or fruit																
Cocos (Coconut). Globulin ...	Trace	4·1	3·6	6·0	5·5	2·1	5·1	19·1	1·8	3·2	1·5	5·8	2·4	15·9	1·3	1·6
Attalea (Cohune-nut). Globulin	.	.	.	.	.	.	.	.	.	.	2·2	7·4	1·7	17·2	0·7	.
Andropogon (Sorghum). Kafirin	0·0	8·1	4·3	15·4	7·8	2·3	2·3	21·2	.	5·5	0·6	1·0	1·1	1·6	0·9	3·5
Avena (Oat). Glutenin	1·0	2·5	1·8	15·0	5·4	3·2	4·0	18·4	.	1·5	3·0	5·0	2·3	8·3	.	.
Hordeum (Barley). Hordein ...	0·0	1·3	1·4	7·0	5·9	5·5	1·3	41·3	0·1	4·0	1·6	0·9	2·3	2·8	0·8	4·3
Oryza (Rice). Globulin I ...	.	.	.	.	.	.	.	.	.	5·6	2·1	7·1	2·4	7·9	2·7	.
Globulin II	.	.	.	.	.	.	.	.	.	7·5	2·9	3·6	3·0	15·2	2·3	.
Oryzenin (glutelin)	.	.	.	.	.	.	.	.	.	5·3	2·4	4·7	2·4	11·1	2·5	2·2
Secale (Rye). Gliadin	0·1	1·3	.	6·3	9·8	2·7	0·3	33·8	0·1	1·2	2·6	0·0	0·4	2·2	0·6	5·1
Triticum (Wheat). Leucosin ...	0·9	4·5	0·2	11·3	3·2	3·8	3·4	6·7	.	3·3	.	2·8	2·8	5·9	.	1·4
Gliadin	0·0	2·0	3·3	6·6	13·2	2·3	0·8	43·0	0·1	3·3	1·6	1·2	2·2	3·0	1·4	5·2
Glutenin (original)	0·9	4·7	0·2	6·0	4·2	2·0	2·0	25·7	0·7	4·3	1·6	1·9	1·8	4·7	1·7	4·0
α-Glutelin	.	.	.	.	.	.	.	.	.	.	3·3	2·8	3·5	5·8	.	.
β-Glutelin	.	.	.	.	.	.	.	.	.	.	7·5	5·8	3·7	3·1	.	.
Wheat bran. Albumin ...	.	.	.	.	.	.	.	.	.	4·2	3·3	4·5	2·6	10·0	4·8	.
Prolamine	.	.	.	.	.	.	.	.	.	3·4	2·3	2·5	0·8	4·4	1·4	.
Globulin	.	.	.	.	.	.	.	.	.	3·7	1·5	11·8	2·8	14·1	2·9	.
Zea (Maize). Zein	0·0	9·8	1·9	25·0	8·9	7·6	1·8	31·3	1·0	5·2	0·9	0·0	0·8	1·8	0·0	3·6
Glutenin (original)	0·3	.	.	6·2	5·0	1·7	0·6	12·7	.	3·8	.	2·9	3·0	7·1	+	2·1
α-Glutelin	.	.	.	.	.	.	.	.	.	.	2·8	6·7	1·7	7·6	.	.
Corylus (Hazel-nut). Corylin ...	.	.	.	.	.	.	.	17·9	.	.	.	.	.	.	.	2·2
Juglans (Walnut). Juglansin ...	.	.	.	.	.	.	.	.	.	.	2·2	.	.	.	2·8	.
Cannabis (Hemp-seed). Edestin	3·8	3·6	0·0	20·9	1·7	2·4	10·2	19·2	0·3	2·1	1·0	2·2	2·1	15·8	2·5	1·9
Fagopyrum (Buckwheat).																
Glutelin (glutenin)?	.	.	.	.	.	.	.	.	.	.	.	1·5	0·8	7·0	.	.
Globulin	.	.	.	.	.	.	.	.	.	.	2·5	7·9	0·6	13·0	2·7	.
Prunus (Almond). Amandin ...	0·5	1·4	0·2	4·5	2·4	2·5	5·4	23·1	.	1·1	0·9	0·7	1·6	11·9	1·4	3·7

Arachis (Peanut). Albumin ...	.	.	.	.	.	.	.	.	.	.	1·5	.	.	.	2·3	.
Arachin...	0·0	4·1	1·1	3·9	1·4	2·6	5·6	19·5	.	5·5	1·1	5·0	1·9	13·5	0·9	2·0
Conarachin	.	.	.	.	.	.	.	.	.	.	3·0	6·0	1·8	14·6	2·1	.
Canavalia (Jack Bean). Albumin	.	.	.	.	.	.	.	.	.	.	1·5	.	.	.	4·3	.
Canavalin	.	.	.	.	.	.	.	.	.	5·5	1·0	.	.	.	0·2	.
Concanavalin A	.	.	.	.	.	.	.	.	.	5·2	0·4	.	.	.	2·2	.
,, B	.	.	.	.	.	.	.	.	.	9·4	3·2	.	.	.	2·3	.
Glycine (Soy Bean). Glycinin ...	1·0	.	0·7	8·5	3·8	3·9	9·4	18·5	.	1·9	1·2	9·1	1·4	8·1	1·7	2·3
Lens (Lentil). Legumin† ...	.	.	.	.	.	.	.	.	.	.	0·7	.	.	.	0·9	1·7
Vicilin*...	.	.	.	.	.	.	.	.	.	.	0·4	.	.	.	0·7	1·8
Lupinus (Lupin). Conglutin ...	0·8	2·5	1·1	6·8	2·6	3·1	3·0	6·5	.	.	.	2·7	2·5	10·9	.	.
Phaseolus angularis (Adsuki Bean)																
α-Globulin	.	.	.	.	.	.	.	.	.	.	1·6	8·3	2·3	5·5	1·7	.
β-Globulin	.	.	.	.	.	.	.	.	.	.	0·9	8·4	2·5	7·0	1·0	.
P. aureus (Mung Bean). Albumin	.	.	.	.	.	.	.	.	.	.	1·3	.	.	.	2·3	.
α-Globulin	.	.	.	.	.	.	.	.	.	.	1·7	6·1	3·3	5·1	2·0	.
β-Globulin	.	.	.	.	.	.	.	.	.	.	0·4	9·3	2·0	7·5	1·2	.
P. lunatus (Lima Bean). Albumin	.	.	.	.	.	.	.	.	.	.	1·1	6·0	2·5	5·7	1·4	.
α-Globulin	.	.	.	.	.	.	.	.	.	.	1·6	7·8	3·7	5·7	1·9	.
β-Globulin	.	.	.	.	.	.	.	.	.	.	0·8	8·5	2·6	5·1	2·2	.
P. vulgaris (Kidney or Navy Bean). Phaseolin	0·6	1·8	1·0	9·7	2·8	3·3	5·2	14·5	0·4	2·2	0·6	7·9	3·3	6·1	0·9	2·1
Conphaseolin	.	.	.	.	.	.	.	.	.	.	1·5	10·7	0·9	6·9	2·8	.
Pisum (Pea). Legumelin ...	0·5	0·9	0·7	9·6	4·0	4·8	4·1	13·0	.	1·6	.	3·0	2·3	5·5	.	1·3
Legumin†	0·4	2·1	.	8·0	3·2	3·8	5·3	17·0	0·5	1·6	0·8	5·0	1·7	11·7	1·8	2·0
Vicilin*...	0·0	0·5	0·2	9·4	4·1	3·8	5·3	21·3	.	2·4	0·6	5·4	2·2	8·9	0·2	2·0
Stizolobium niveum (Chinese Velvet Bean). Stizolobin ...	1·7	2·4	2·9	9·0	4·0	3·1	9·2	14·6	0·7	6·2	1·6	8·5	2·3	7·1	1·4	1·6
S. deeringianum (Georgia Velvet Bean). Albumin	.	.	.	.	.	.	.	.	.	.	2·7	8·2	0·8	6·1	2·5	.
α-Globulin	.	.	.	.	.	.	.	.	.	.	3·3	8·3	1·2	7·2	2·3	.
β-Globulin	.	.	.	.	.	.	.	.	.	.	0·7	8·5	3·4	8·2	.	.
Vicia Faba (Horse Bean).																
Legumin†	1·0	2·8	1·0	8·2	2·3	2·0	4·0	16·3	.	2·8	.	.	.	.	.	1·6
Vicilin*...	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1·9
V. sativa (Vetch). Legumin† ...	0·4	1·2	1·4	8·8	4·0	2·9	3·2	18·3	.	2·4	.	4·0	2·9	11·1	.	2·1
Vigna (Cow-pea). Vignin ...	0·0	1·0	0·3	7·8	5·3	5·3	4·0	16·9	.	2·3	0·5	4·3	3·1	7·2	1·7	2·3
Acer (Maple). Acerin	.	.	.	.	.	.	.	.	.	.	0·6	6·1	1·4	10·1	.	.

* and † denote respective globulins identical.

TABLE XXXIV (*continued*)

	Glycine	Alanine	Valine	Leucine	Proline	Phenylalanine	Aspartic acid	Glutaminic acid	Serine	Tyrosine	Cystine	Lysine	Histidine	Arginine	Tryptophane	Ammonia
From seed or fruit																
Linum (Flax). Globulin... ...	.	.	.	.	.	.	.	.	.	.	1·2	.	.	.	4·0	.
Gossypium (Cotton). Globulin ...	.	.	.	.	.	.	.	17·6	.	.	1·1	2·1	3·5	13·5	2·6	1·9
Bertholletia (Brazil-nut). Excelsin	0·6	2·3	1·5	8·7	3·7	3·6	3·9	12·9	.	3·0	1·8	1·6	1·5	16·0	2·6	1·8
Ricinus (Castor Bean). Ricin ...	0·0	1·0	2·0	16·0	4·6	0·4	2·0	20·0	.	2·7	1·0	6·3	0·0	11·7	0·4	2·8
Globulin	.	.	.	.	.	.	.	14·5	.	.	.	1·5	2·7	13·2	.	2·0
Lycopersicum (Tomato).																
α-Globulin	.	.	.	.	.	.	.	.	.	.	1·2	4·9	1·2	14·0	1·2	.
β-Globulin	.	.	.	.	.	.	.	.	.	.	1·1	6·4	3·8	10·7	1·5	.
Sesamum (Sesame). α-Globulin	.	.	.	.	.	.	.	.	.	4·7	1·9	5·4	2·7	15·1	2·8	.
β-Globulin	.	.	.	.	.	.	.	.	.	4·5	1·5	4·0	3·5	15·6	2·7	.
Cucumis (Cantaloupe). Globulin	.	.	.	.	.	.	.	.	.	.	1·3	3·3	4·2	16·3	2·8	.
Glutelin...	.	.	.	.	.	.	.	.	.	.	1·1	4·6	2·7	12·4	3·2	.
Cucurbita (Squash). Globulin ...	0·6	1·9	0·3	7·3	2·8	3·3	3·3	12·4	.	3·1	1·4	3·7	4·5	15·7	3·0	1·6
Attalea (Cohune-nut). Globulin	.	.	.	.	.	.	.	.	.	.	2·2	.	.	.	0·7	.
Helianthus (Sunflower). Globulin	.	.	.	.	.	.	.	21·8	.	.	1·6	.	.	.	2·5	2·6
From bark																
Robinia (Acacia). Bark. Albumin	.	.	.	.	.	.	7·7	4·5	.	6·3	1·4	5·5	1·7	4·4	4·2	.
Globulin	.	.	.	.	.	.	.	.	.	.	1·3	.	.	.	3·2	.
From tuber																
Solanum (Potato). Tuber. Globulin	.	4·9	1·1	12·2	3·0	3·9	.	4·6	.	4·3	4·4	3·3	2·3	4·2	.	1·8
From leaves																
Spinacia (Spinach). Spinacin ...	.	.	.	.	.	.	.	.	.	.	.	8·2	2·3	6·9	.	.
Medicago (Alfalfa). Cytoplasmic glutelin	.	.	.	.	.	.	.	.	.	.	.	8·2	1·8	7·5	.	.
From inflorescence																
Brassica oleracea var. (i) ...	.	.	.	.	.	.	.	.	.	.	.	7·5	2·6	5·4	.	.
„ „ (ii)	.	.	.	.	.	.	.	.	.	.	.	7·1	1·3	4·9	.	.

All the proteins isolated have been arranged in the preceding tables according to the natural orders of the plants. Though we see that proteins of allied genera are often very similar, the data are too scanty to allow any general conclusions to be drawn. The range of variation of the basic amino-acids is great; the arginine, histidine and lysine content for the cereals is low, lysine being absent from zein, and from the gliadin of Rye. On the other hand, it is high for some wheat-bran proteins, and also for many others, as can be seen on reference to Table XXXIV. The glutaminic acid content is high for all plant proteins, but especially for the cereals. The cystine content is low and especially small for some of the Leguminosae. The cystine and tryptophane estimations are by colorimetric methods.

From a comparison of the values obtained for ammonia nitrogen and for the glutaminic and aspartic acid content of various proteins, there is little doubt that the ammonia nitrogen is derived from ammonia in amide combination with one of the carboxyl groups of these acids.

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CHAPTER V

NITROGEN METABOLISM

General Nitrogen Metabolism. (Bibliography VIII)

Nitrogen metabolism denotes the series of chemical reactions which leads to the formation of the complex organic nitrogen compounds of the plant from such simple substances as inorganic nitrates, ammonium salts, carbon dioxide and water. Plants, unlike animals, do not eliminate nitrogen; all nitrogen is retained, none being lost, except, perhaps, a small amount during leaf-fall.

The plant nitrogen compounds can be, more or less artificially, classified as:

1. Amino-acids and their condensation products, the polypeptides, peptones, proteoses and proteins. Also among the amino-acids are included substances from 2, 3 and 4.
2. Substances containing the pyrrole ring, such as proline and chlorophyll.
3. Substances containing the indole ring, such as tryptophane and indoxyl.
4. Substances containing the iminazole ring, such as histidine.
5. Purine and pyrimidine bases. Adenine, guanine, uracil and cytosine are components of plant nucleic acids.
6. Cyanogen compounds, in which prussic acid, HCN, is a component of the molecule.
7. Simple bases, amines and betaines, such as methylamine, choline and betaine; the latter are widely distributed and important because of their connection with the lecithins.
8. A special and complex group of bases, the alkaloids.
9. Urea, which has been detected in small quantities in some of the higher plants.
10. Amides.

All the above groups must be ultimately derived from inorganic compounds. In the case of parasites, saprophytes and symbiotic plants, organic nitrogenous substances are directly absorbed; these, however, have been previously synthesised by the host plant, so that the initial process is only set back a stage.

General considerations

Of nitrogenous substances, it is natural that the synthesis of proteins should be the problem to which most attention has been paid. Two possible modes of synthesis in the plant will be outlined and criticised here, namely:

Hypothesis A. This suggests the separate synthesis of each amino-acid. These are then later by a series of stages, the converse of the hydrolytic process, condensed to form peptides, peptones, proteoses and proteins.

Hypothesis B. The amino-acids are not synthesised separately, but large constituent molecules of the protein are condensed *en bloc* from compounds of greater simplicity than amino-acids.

To consider first hypothesis A. This can obviously be regarded as involving two stages:

I. The synthesis of the amino-acids. Here again, as far as nitrogen is concerned, two problems are involved:

(*a*) The introduction of the NH_2-grouping.

(*b*) The synthesis of the heterocyclic (pyrrole, indoxyl and imina-zole) rings.

II. The condensation of these substances in ascending series of complexity, through polypeptides, peptones, proteoses to proteins.

It is stage I which presents the difficult problem. For stage II there is ample confirmation of the existence of proteolytic enzymes, which, presumably, may catalyse the synthesis of proteins from amino-acids. The consideration of stage I (*b*) is left till later (see p. 216).

First, to consider stage I (*a*). It has naturally been supposed that ammonia is the fundamental nitrogen compound essential to the formation of amino-acids. Ammonium salts are absorbed by many plants, and though the exact reactions are not known, the method by which the ammonia of these salts is made available in metabolism would not appear to present a difficult problem. In fact, it will be indicated later how ammonia from ammonium salts may be readily synthesised to organic nitrogen compounds.

In the case of supply of nitrogen from nitrates, it has usually been assumed that they are first reduced to nitrites. A nitrate-reducing enzyme of the water-splitting type has been detected in plants, whereby nitrate is reduced by hydrogen to nitrite, if a suitable oxygen acceptor, such as aldehyde, is present (Bach, Bib. VI, 11). However, neither the distribution of this enzyme nor that of nitrites in the

plant has been shown to be wide (Anderson, Bib. VI, 1). Whether such nitrate-reducing enzymes have a real significance in the nitrate-protein metabolism is therefore rather uncertain.

How nitrites may directly enter into amino-acid and protein synthesis has been suggested by Baly (3, 4) on more or less theoretical grounds which will be referred to later. On the other hand, it has again generally been assumed that nitrites may afford a source of ammonia by further reduction, though the exact mechanism has not been formulated. Given a source of ammonia, the question next to be considered is how the amino-acids may be formed.

It is probably best to approach the problem first from a theoretical and chemical standpoint. If we examine the list of the best known amino-acids we see that they might arise by amination with ammonia of a corresponding series of non-nitrogenous acids:

TABLE XXXV

Aliphatic compounds

Mono-carboxylic mono-amino acids:

Glycine or α-amino-acetic acid

$$CH_2(NH_2)\cdot COOH$$

Alanine or α-amino-propionic acid

$$CH_3\cdot CH(NH_2)\cdot COOH$$

Valine or α-amino-iso-valeric acid

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!\rangle CH\cdot CH(NH_2)\cdot COOH$$

Leucine or α-amino-iso-caproic acid

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!\rangle CH\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

Iso-leucine or α-amino-β-methyl-β-ethyl-propionic acid

$$\begin{matrix} CH_3 \\ C_2H_5 \end{matrix}\!\!\rangle CH\cdot CH(NH_2)\cdot COOH$$

Serine or α-amino-β-hydroxy-propionic acid

$$CH_2OH\cdot CH(NH_2)\cdot COOH$$

Dicarboxylic mono-amino acids:

Aspartic acid or α-amino-succinic acid

$$COOH\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

Glutaminic acid or α-amino-glutaric acid

$$COOH\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

Mono-carboxylic di-amino acids:

Arginine or δ-guanidine-α-amino-valeric acid

$$HN{=}C\begin{matrix}\diagup NH_2 \\ \diagdown\end{matrix}\!\!-NH\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

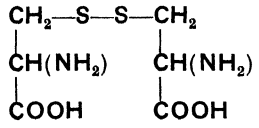
Lysine or α-ε-di-amino-caproic acid

$$CH_2(NH_2)\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

TABLE XXXV (*continued*)

Dicarboxylic di-amino acid:

Cystine (dicystëine) or di-β-thio-α-amino-propionic acid



Aromatic compounds

Mono-carboxylic mono-amino acids:

Phenyl-alanine or β-phenyl-α-amino-propionic acid

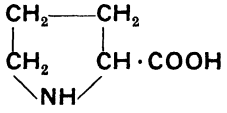
$$C_6H_5 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$$

Tyrosine or *p*-hydroxy-phenyl-alanine

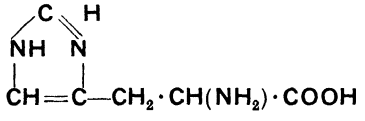
$$OH \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$$

Heterocyclic compounds

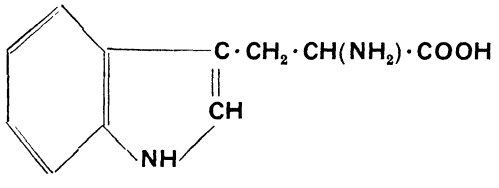
Proline or α-pyrrolidine-carboxylic acid



Histidine or β-iminazole-alanine



Tryptophane or β-indole-alanine $C_8H_6N \cdot CH_2 \cdot CH(NH_2) \cdot COOH$,



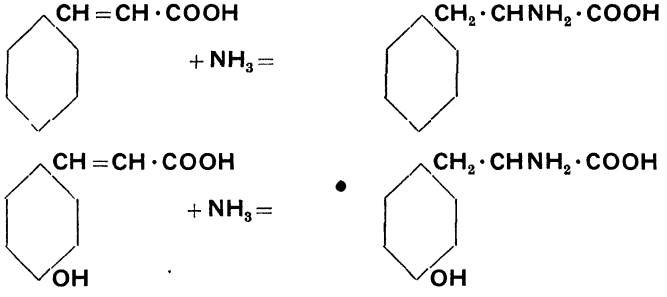
The whole subject is of considerable interest. Why this curious assortment of compounds? Why are these, and these only, united together to form protein? The fact, also, that the primary synthesis of amino-acids lies with the plant, the animal being incapable of synthesising these substances, is of great significance.

We have very little evidence for the process of amination. It is possible that ammonia may combine with a corresponding series of unsaturated acids. Diaminocaproic acid, for instance, has been obtained by the action of ammonia on sorbic acid:

$$CH_3CH=CH \cdot CH=CH \cdot COOH + 2NH_3 = CH_3 \cdot CH_2 \cdot CHNH_2 \cdot CH_2 \cdot CHNH_2 \cdot COOH.$$

So also might aspartic acid, phenylalanine and tyrosine arise from fumaric acid, cinnamic and *p*-coumaric acids respectively:

$$COOH \cdot CH = CH \cdot COOH + NH_3 = COOH \cdot CH_2 \cdot CHNH_2 \cdot COOH,$$



Fumaric acid is frequently present in plants; it is very probable that cinnamic and *p*-coumaric acids are also to be found.

It is always possible that any biochemical process in the plant may be reversible, that is, it may take place on the same lines, but in opposite directions; hence, it may be worth while to consider how the process of deamination may be brought about in the living organism, with a view to considering its reversal as a means to synthesis. Three ways may be enumerated:

(*a*) By reduction:

$$R \cdot CH \cdot NH_2 \cdot COOH + H_2 = R \cdot CH_2 \cdot COOH + NH_3$$

with the formation of the corresponding fatty acid series. Thus from phenylalanine, tyrosine and leucine, would arise phenylpropionic, oxyphenylpropionic and caproic acids. Of this series of acids, iso-valeric, succinic and glutaric acids are known to be distributed in plants, the two latter widely so. Acetic, propionic, iso-caproic, β-phenylpropionic and its *p*-oxy-derivatives are probably to be found.

(*b*) By hydration with formation of α-hydroxy-acids:

$$R \cdot CH \cdot NH_2 \cdot COOH + H_2O = R \cdot CHOH \cdot COOH + NH_3.$$

Of such acids, glycollic (hydroxy-acetic) and malic are present in plants.

(*c*) By oxidation with formation of α-keto-acids:

(i) $$R \cdot CH \cdot NH_2 \cdot COOH + O = R \cdot CO \cdot COOH + NH_3.$$

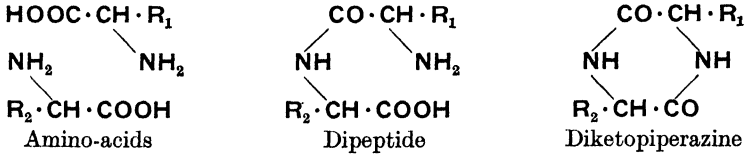
In presence of carboxylase, the reaction goes further:

(ii) $$R \cdot CO \cdot COOH = R \cdot CHO + CO_2.$$

The last process, deamination by oxidation, is catalysed *in vitro* by the catechol oxidase system of the higher plants (see p. 42). There is, however, no evidence that the reaction takes place *in vivo*. Except in the case, (*c*, ii), of decarboxylation, which has not been shown to be reversible, it is possible that the converse of any of the above processes might take place with the formation of amino-acids.

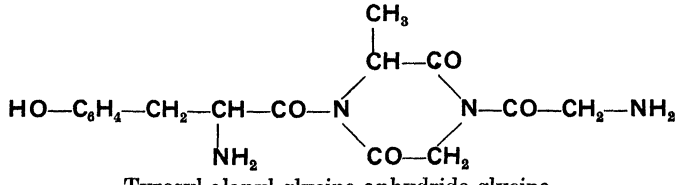
It has been suggested by Smirnow (147) that the keto acids are most suitable for amination. The existence in the plant, however, of a series of commonly occurring acids from which the amino-acids are derived by amination is not in any way obvious, though it is not essential that all should be synthesised on one plan.

Now, we turn to hypothesis B. This is the outcome of the consideration of the possible part played by asparagine in protein synthesis, for which the evidence will be given in detail later. It has been suggested that asparagine is the primary stable product of nitrogenous metabolism, and is supplied, together with carbohydrates, to tissues where protein synthesis is taking place. By oxidation in respiration, asparagine might give rise to 2-carbon residues and ammonia. These, together with methyl-glyoxal, urea, acetaldehyde, pyruvic aldehyde, and other active molecules (in actively respiring tissue) may condense *en bloc* to form large polypeptide complexes. These would include heterocyclic rings (from methyl-glyoxal, urea, etc., see p. 216) and aliphatic amino groupings from simple carbon compounds. The asparagine residues and ammonia might take part in the peptide and diketopiperazine linkages, if we consider the latter to form part of the protein molecule, according to the recent ideas of Abderhalden. This author, as is now well known, suggests that "the protein molecule is built up of a number of diketopiperazine-containing complexes which are associated or held together by means of secondary valencies" (Vickery & Osborne, 168). If we wish to regard protein synthesis as the laying down of material according to a pattern peculiar to an individual species, Abderhalden's conception (or in fact any of the recent views which postulate some formation of ring complexes) is possibly more helpful than an unvaried linking together of chains of amino-acids by the peptide linkage:



Amino-acids Dipeptide Diketopiperazine

An example of greater complexity may be represented as:



Tyrosyl-alanyl-glycine-anhydride-glycine

In the event of protein formation taking place by condensation *en bloc*, the amino-acids would be regarded as secondary products formed only from hydrolysis of protein, the cyclic and heterocyclic rings of phenylalanine, tyrosine, proline, histidine and tryptophane being stable, in contrast to the diketopiperazine rings.

We have now considered the possibilities as to *how* the synthesis of amino-acids may take place. Next to be considered is *where* the synthesis takes place.

Since amino-acids in the plant *may* arise *de novo* from inorganic salts and products of photosynthesis, as well as indirectly from hydrolysis of pre-existing protein, it has been usual to speak of primary (*de novo*) and secondary (from hydrolysis) formation.

At the growing-points and in the cambial regions of stem and root there is a continuous synthesis of protein; also in reserve tissues, such as seeds, tubers, etc. There must in these regions, therefore, be a constant supply of either the whole range of the amino-acids or the substances from which they arise. If of amino-acids themselves, these must be *somewhere* synthesised from carbohydrate and ammonium salts or nitrates, thus involving not only the production of the amino grouping, but also the synthesis of the pyrrole, iminazole and indole rings. It is known that sugars are translocated from the leaves (the seat of synthesis) to the growing-points, developing tissues, etc. Ammonium salts or nitrates are also supplied in the transpiration current to all tissues. There is evidence, to be considered later, which indicates that a primary synthesis of nitrogen compounds may take place in the green leaf. The questions at issue are: Does the whole protein synthesis (including if necessary amino-acid synthesis) take place at the growing-points and in developing tissues? If not, is the whole series of the amino-acids first synthesised in the leaves and then translocated to these tissues? Or is there some more simple form of nitrogenous translocatory product?

The experimental evidence has developed on several lines, though the most important is doubtless derived from the plant itself, and, for this, there are various sources, namely, the germinating seedling, the ripening seed, the leafy shoot, and the green leaf. In the green leaf it seems probable, as already stated, that there is primary synthesis of nitrogen compounds; it appears certain, as we shall see later, that there is also protein hydrolysis. Thus, translocation products may be either of primary or secondary origin, and this renders the problem more complex in the case of the leaf. The case

of the seedling is more simple, for here we are, in the early stages at least, only dealing with secondary products from hydrolysis of reserve material, and the synthesis of these again in the very young plant. Thus, it may be said that the break-down products are formed from proteins, and, again, re-form proteins in another place. If light is excluded and normal growth inhibited, these products may accumulate and are more readily identified.

Nitrogen metabolism of the seedling. Work of Schulze

Let us first consider the case of the seedling. The reserve proteins of the tissues of the endosperm or embryo, as the case may be, are hydrolysed by proteolytic enzymes to amino-acids, and these, or secondary break-down products, are translocated to the growing-points of the seedling.

In many seedlings, especially among the Leguminosae, there is a high percentage of asparagine. There is no doubt that more asparagine is produced than could arise from the reserve protein of the seed. In other seedlings it may similarly be glutamine. Hence, it has long been considered probable that asparagine (or glutamine) is of considerable significance in the synthesis of nitrogen compounds.

Most of the evidence is based on careful analyses and estimations of amino-acids in seedlings by Schulze (90–145). This author came to various conclusions, as follows:

From young 6- to 7-day old seedlings a whole series of amino-acids could be isolated, such as leucine, tyrosine, arginine, histidine, lysine, etc., but, in older seedlings, many of these disappear, and there is instead an accumulation of asparagine, which substance can be readily crystallised from extracts.

This accumulation of asparagine is far greater than could arise from the aspartic acid present in the reserve protein of the seed, which would be split off as amide by enzyme hydrolysis. Thus Schulze & Castoro (132) found, during germination, of etiolated seedlings of *Lupinus albus* the following amounts:

4 days	3·11 %	of dry weight
7 ,,	12·78	,,
18 ,,	25·7	,,

that is, about half of the total nitrogen of the seedling, or 73 % of the hydrolysis products from the protein, whereas the amount which could arise from protein hydrolysis alone is from 2 to 3 %. From

seedlings of *L. luteus* even greater quantities, as much as 28·7 % of the dry weight, were obtained.

In normal green seedlings there is less accumulation, but considerable amounts are still present. *Lupinus albus*, for instance, gave:

	Stem	Cotyledon
After 14 days	21·1	17·6 % dry weight

Though an accumulation of asparagine is especially great in the Leguminosae (*Lupinus*, *Vicia*, *Soja*), Schulze detected the same phenomenon, to a lesser extent, in seedlings of the Graminaceae, *Papaver somniferum*, *Tropaeolum majus*, *Helianthus annuus*, *Picea excelsa* and *Pinus sylvestris*.

Schulze found an accumulation also of glutamine instead of asparagine in some other plants, for instance in seedlings of *Ricinus*, *Cucurbita*, and many Cruciferae. The accumulation of glutamine, however, is less in quantity, 2·5 % dry weight from etiolated *Ricinus* seedlings being the greatest obtained (Schulze, 123).

Schulze showed, from comparative determinations of protein, amino-acids, and amide nitrogen, that, after all protein is hydrolysed, there is an increase of amide nitrogen at the expense of the amino-acid nitrogen. This is also obvious from the fact that, although the amino-acids disappear in etiolated seedlings, yet there is, at the same time, no synthesis, but a loss, of protein, and an accumulation of asparagine (or glutamine) only. A characteristic analysis of *Lupinus luteus* gave as follows:

TABLE XXXVI

	Per 100 gm. ungerminated seeds without testas		
	6-day seedlings	15-day seedlings	24-day seedlings
Protein nitrogen	5·49	1·71	1·78
Asparagine nitrogen	1·16	4·02	5·09
Diamino nitrogen	0·97	1·22	1·03
Other nitrogen (including monoamino)	1·72	2·39	1·40
	9·34	9·34	9·30

When photosynthesis commences in a normal seedling, or when etiolated seedlings are exposed to light, the asparagine (or glutamine) gradually disappears, being used for protein synthesis. That asparagine is found in green seedlings, and is even found to increase after photosynthesis has begun, is considered by Schulze to be in favour

of his hypothesis, i.e. that asparagine is a more favourable material for protein synthesis than amino-acids, and hence it continues to be formed, to a greater or less degree, from the latter. This is shown in the analysis from *Lupinus* seedlings:

TABLE XXXVII

	In percentage of total nitrogen	
	Kept 10 days in dark	Kept 10 days in dark, and then 21 days in light
Protein nitrogen	25·2	33·2
Asparagine nitrogen	34·2	42·0
Other nitrogen (including amino-acid)	40·6	24·8

Analysis of different portions of seedlings (Schulze & Castoro, 132) also brings out another point, namely, that the accumulation of asparagine is less in the leaves than in the petioles, and thus affords evidence, according to Schulze, of its participation in protein synthesis. This was shown in 14-day-old normal green seedlings of *Lupinus albus*:

TABLE XXXVIII

	In percentage of dry weight			
	Cotyledons	Plumule	Stem	Root
Protein	14·64	24·66	9·56	11·22
Asparagine	17·59	6·65	21·12	10·23

and was interpreted by Schulze as being due to the active formation of protein in the leaves at the expense of asparagine arriving in large quantities through the petioles.

In some cases Schulze (121) found that arginine did not disappear to the same extent as the other amino-acids in the development of the seedling. Its production, however, decreased with decrease of reserve protein and presented a totally different phenomenon from the accumulation of asparagine. (See also p. 231.)

Hence Schulze concludes that the amino-acids undergo a secondary decomposition, possibly by oxidation, with production of ammonia, and subsequently asparagine is formed, which is therefore of secondary origin. He does not definitely state the origin of the non-nitrogenous portion of asparagine, though he points out the

relationship of asparagine to succinic acid (detected by him in seedlings of *Lupinus* and *Cucurbita*) and suggests that asparagine may arise by amination of succinic acid, and the formation and subsequent decomposition of ammonium aspartate:

$$R \cdot COONH_4 - H_2O = R \cdot CONH_2 + H_2O.$$

Schulze regards the work of Butkewitsch (6–9) as a confirmation of the above view, namely, that ammonia from decomposition of amino-acids gives rise to asparagine (or glutamine). Butkewitsch showed that, in seedlings which were anaesthetised by toluol, there was an accumulation of ammonia, but no asparagine was formed. This is to be explained by the inhibition, in anaesthesia, of synthetic processes, though catalytic processes remain unaffected. Oxygen, moreover, was essential for this accumulation, which fact points to the origin of ammonia by oxidation of amino-acids. Suzuki (156) and Palladin (58) have also claimed that formation of asparagine in seedlings only takes place in the presence of oxygen. To a fuller discussion of this point we shall return later (see p. 213).

That oxidation of amino-acids might take place owing to a fall in the carbohydrate supply is a deduction which may be drawn from further observations of Schulze, though he himself does not put forward this interpretation. The series of observations referred to was made by Schulze (111) in connection with the relationship between the breakdown of reserve protein of seedlings and their non-nitrogenous reserve products. He makes the following comparisons between loss of reserve protein in 15-day-old seedlings and their original carbohydrate or fat content:

TABLE XXXIX

	Percentage of original protein	Percentage of seed weight
Lupinus luteus	80·4	18 (carbohydrate)
L. angustifolius	75·7	39 (carbohydrate)
Cucurbita	27·9	52 (fat)
Zea Mays	27·4	Rich in starch

From this we see that the loss of protein is proportional to the non-nitrogenous reserve. Schulze's line of argument is as follows: The protein, on hydrolysis, produces a mixture of nitrogenous substances, which are later, to a great extent, converted into asparagine (or glutamine). The non-nitrogenous reserves are hydrolysed into

glucose. Glucose (or possibly other carbohydrates) together with the amides (and conceivably other soluble nitrogenous substances) are regenerated into protein at the growing-points. The richer the seedling in carbohydrate, the more amide is regenerated to protein. In etiolated seedlings, therefore, the loss of protein is less the greater the proportion of non-nitrogenous reserve.

Nitrogen metabolism of the seedling. Work of Prianischnikow

In addition to Schulze, Prianischnikow has published a series of papers (66–82) extending over many years on the subject of nitrogen metabolism. He confirmed, from experiments on seedlings, the fact that there is an accumulation of asparagine on germination, and that it is of secondary origin from the amino-acids. He does not, however, regard with favour the view that asparagine is a more suitable source for protein synthesis than amino-acids. He points out that asparagine often accumulates rather than disappears in the presence of considerable quantities of carbohydrate. Its accumulation, in his opinion, and contrary to that of Schulze, may point to the fact that it is less readily used for protein synthesis than amino-acids. Prianischnikow prefers to consider asparagine as analogous to urea, that is, as a safe combination for free ammonia, which would, if it accumulated, be injurious to the tissues. Asparagine, however, unlike urea, is not excreted, but constitutes a temporary reserve for nitrogen until it is used again in protein synthesis.

Much of Prianischnikow's evidence is based upon experiments on seedlings, but from another aspect, that of water culture. He grew seedlings on solutions of ammonium salts, chiefly chloride and sulphate, and subsequently estimated the total amide and ammonia nitrogen in these and in control plants. His experiments were numerous, and the results are embodied in many publications. Concisely stated, however, they are as follows: The various seedlings could be divided into three groups, according to their behaviour (82):

(1) Seedlings of Barley. On solutions of ammonium chloride or sulphate these grew and yielded increased amounts of asparagine and ammonia nitrogen. The culture solution also became acid.

(2) Seedlings of Pea. On ammonium chloride or sulphate no increase of nitrogen content was obtained. If calcium carbonate or sulphate was added, there was an increase of asparagine and ammonia nitrogen.

(3) Seedlings of *Lupinus*. No increase of nitrogen compounds was obtained even after addition of calcium salts.

Later Prianischnikow (75) made further experiments. By using older Barley seedlings without carbohydrate reserve he showed that they behave similarly to Lupin seedlings. Conversely, by supplying Lupin seedlings with soluble carbohydrate, i.e. sugar, they will behave as Barley seedlings. Prianischnikow explains the difference of behaviour on the ground of the presence or absence of carbohydrate reserve. He also maintains that asparagine serves as an innocuous and safe storage product for nitrogen, since excess of ammonia has been absorbed by the seedlings under the conditions of the experiment.

The phenomena, however, might indicate rather more complexity in the situation, such as follows. The Graminaceae are known to be able to tolerate an acid p_H in their medium for growth. Therefore they can absorb ammonia, and they have, in addition, a good carbohydrate reserve for asparagine synthesis. The Pea cannot tolerate such an acid p_H, but needs calcium for, presumably, neutralisation. Given this, it has a good carbohydrate reserve for asparagine synthesis. The Lupin can neither tolerate an acid p_H nor has it probably quickly available carbohydrate reserve, since little starch is present and it is dependent on hemicellulose.

Similar, though more elaborate, investigations have been made by Smirnow (147). He showed, on the same lines, that seedlings of Lupin supplied with ammonia nitrogen and glucose had more asparagine and less ammonia nitrogen in their tissues than seedlings of the same age which had been supplied with ammonia nitrogen alone and no glucose.

Still more recent work on very specialised lines on absorption of ammonium salts has been carried out by Mevius & Engel (191, 192).

To sum up: Schulze and Prianischnikow both agree that in germinating seedlings much asparagine is formed, and it is of secondary origin from amino-acids. Ammonia is formed by deamination, probably by oxidation, of amino-acids. The origin of the succinic acid basis is somewhat obscure, as there is not always abundant carbohydrate. Nor is the fate of this residue obvious when relatively large quantities of asparagine are again used in protein synthesis.

Except as to the origin and fate of asparagine, Schulze and Prianischnikow are not in very close agreement. Schulze is inclined to think that the amino-acid to ammonia breakdown with subsequent formation of asparagine is, to a large extent, an essential process,

and that asparagine is the chief source of nitrogen for protein synthesis. Prianischnikow (78, 79) regards asparagine chiefly as a by-product, in which form surplus and harmful, though valuable, ammonia may be stored for future use.

Nitrogen metabolism of ripening seed

Next, we turn to evidence from the ripening seed. In a sense this process is the converse of that which we have just been considering, that is a synthesis, in contrast to a hydrolysis, of reserve protein, and, again, it is taking place in one direction only, but is resulting in a stable condition, that of the reserve protein of the seed. Many observers (Woodman & Engledow (172) and Zaleski (177, 178)) have found evidence for the statement that, in ripening seeds, soluble nitrogen compounds pass from the leaves to the fruits, and disappear there with formation of protein. In the unripe seed, there is relatively little protein in comparison with soluble nitrogenous substances; in the ripe seed, there is a high percentage of protein and a very low percentage of soluble nitrogenous substances.

As in the case of seedlings, so with ripening seeds, the chief investigations of value are due to Schulze and his co-workers (127, 145). Schulze worked chiefly with developing pods and seeds of *Pisum sativum*. He found that, during ripening, there is a breakdown of protein in the pod, and soluble nitrogen compounds pass from pod to seed, that is, the pod serves to some extent as a reserve organ for the seeds. From the pods he identified various amino-acids in small quantities, such as arginine, leucine, histidine, tryptophane, but about half of the total soluble nitrogen was found to be due to asparagine. From the seeds also he identified a number of amino-acids, such as arginine, lysine and tyrosine, but only small quantities of asparagine and glutamine. He is of the opinion that probably all the amino-acids are qualitatively present, but it is difficult to effect an isolation and identification in each case. The outcome of his observations appears to be that there is far more asparagine in the pods than in the seeds, and more arginine in the seeds than in the pods. In addition Schulze is of the opinion that arginine may be actually synthesised in the seed. It was found that practically the same distribution is true for ripening fruits of *Phaseolus vulgaris* and *Vicia sativa*. He points out that soluble nitrogen compounds may also pass direct from the leaves to the ripening seeds without necessarily accumulating in the pods. To obtain some idea of the

amino-acid content of the whole plant, he examined those of *Vicia*, *Trifolium* and *Medicago*, and found it to be very low indeed, so that the various compounds were not detected with any certainty, except asparagine, which occurred to the extent of about 2 % of the dry weight and may represent nearly 40 % of the non-protein nitrogen compounds. Hence Schulze again concludes that asparagine is used in protein synthesis. This would explain the facts observed, namely that the seeds have very little asparagine, the pods much; of plants grown in the light, the leaves, where protein synthesis is active, have less asparagine than the stems, but, in the dark, the asparagine accumulates in the leaf. These results, however, leave us again in the same position of uncertainty as to whether asparagine is the chief, if not sole, translocation product of nitrogen. Do all or any of the amino-acids break down into asparagine before being re-synthesised into protein?

Schulze's examination of the vegetative parts of the mature plant and the high values obtained for asparagine content forms, as a matter of fact, part of the evidence to be considered next, namely, that from a study of the green leaf or vegetative shoot.

Nitrogen metabolism of the starved leaf and shoot

It has been observed by various workers that, when green leaves and shoots have been kept for some days with their stalks in water in the dark or in diffused light, there is a considerable increase of asparagine.

Schulze (91) placed young shoots of the Birch in water for 10–12 days, and found an accumulation of asparagine in the young leaves; Schulze & Bosshard (129) found asparagine to be formed in shoots and young leaves of twigs of forest trees kept in water. Schulze, Bosshard & Kisser (129, 138) found that plants of *Trifolium pratense* and *Avena sativa* when kept in the dark showed a loss of protein, and a formation of asparagine. Schulze (100) also isolated glutamine from plants of *Saponaria officinalis*, and leaves of Beet which had been kept for 5 days in a dark room. Amino-acids were found after a short interval in the dark, and, later, these were replaced by asparagine or glutamine. Plants of *Medicago sativa* (see above) gave no histidine or arginine in the fresh state, but, after being in water 2 days, asparagine could be detected. Schulze & Bosshard (129) kept young Oat plants (without roots) in water in a dark room for 6–7 days, and

found, on analysis of these and a control set of plants extracted at once, the following values:

TABLE XL

	Control	Plants in dark
		% of dry weight
Total nitrogen	4·12	4·50
Protein nitrogen	3·51	1·46
Asparagine nitrogen	0·15	1·69
Other nitrogen	0·46	1·35

Such results were confirmed by Butkewitsch (6), who kept plants of *Avena sativa* and *Vicia Faba* in the dark, and showed by isolation and weighing that asparagine was formed at the expense of protein nitrogen.

Later work in this direction has been carried out by Chibnall (15), who kept leaves of the Runner Bean (*Phaseolus multiflorus*) in water in the dark or in diffused light for 4–5 days, a control being analysed at the beginning of the experiment. Several series of leaves were analysed with concordant results. The values for one out of the several experiments are as follows:

TABLE XLI

No. of days in water	In percentages of total leaf nitrogen				In percentages of total non-protein nitrogen			
	Total non-protein N	Ammonia N	Amide N (Sachsse)	Free amino N	Total non-protein N	Ammonia N	Amide N (Sachsse)	Free amino N
0	16·50	0·34	0·89	6·81	16·50	2·00	5·98	41·30
5 in dark	35·14	0·57	5·85	15·82	35·14	1·62	16·65	45·00
Increase	18·64	0·23	4·96	9·01	18·64	−0·38	10·67	3·70

Since the amide nitrogen (as estimated by distillation with magnesium oxide) may arise from any amide, and is not a direct proof of the presence of asparagine, the author succeeded in isolating, by precipitation and crystallisation, 48·8 % of the nitrogen indicated by Sachsse's method. The true percentage is undoubtedly higher, as all the asparagine would not be isolated by Chibnall's method, but the nature of the remaining amide compound is uncertain, since glutamine was shown to be absent. It may be derived from polypeptides of asparagine.

Chibnall's results show that in starved leaves, in terms of total nitrogen, there is an increase of non-protein nitrogen (chiefly amide and amino), with a corresponding decrease in protein nitrogen. In terms of water-soluble nitrogen, the only increase is in amide nitrogen, the amino nitrogen remaining about the same.

From the preceding account we see that most investigators agree that, under certain circumstances, of which the chief is lack of carbohydrate, ammonia and asparagine arise through amino-acids from protein, and that again, later, asparagine is resynthesised to protein. But no precise course has yet been framed for either protein synthesis or protein decomposition.

Nature of primary compounds for protein synthesis

First, to consider protein synthesis in more detail. The evidence from germinating seedlings gives no clue as to whether protein synthesis at the growing-point takes place by recondensation of the amino-acids formed from hydrolysis of reserve protein, asparagine then being merely a temporary reserve product for ammonia formed in unavoidable oxidation of amino-acids, or whether all amino-acids are converted into asparagine, which is the only source of nitrogen and an essential step in protein synthesis, this then taking place through the condensation of smaller molecules as indicated previously (see p. 196). It may be pointed out that if the nature of the translocation products to the growing-point were known, then the actual mode of protein synthesis might be more readily formulated. Accumulation or disappearance of the respective products will serve equally as evidence for either hypothesis. As an example of this dilemma, we may quote the figures given in Table XXXVII. Prianischnikow, on the basis of the fall in amino-acid content in the second set of seedlings, draws the conclusion that amino-acids, rather than asparagine, are used in protein synthesis; this point of view, of course, is opposed to that of Schulze.

When we turn to the leaf, the foremost question is whether this organ can itself synthesise protein. It would be assumed, *a priori*, to be inevitably the case, otherwise the protein content of the leaf cannot increase after first development. From experimental evidence, it would appear that the protein content of the leaf increases by day and diminishes by night during the vegetative period. This was shown by Schulze & Schütz (89) for leaves of *Acer Negundo* and by Suzuki (see Chibnall, 14) for a number of different plants;

these results were also confirmed by Chibnall (14) for *Phaseolus multiflorus*. The latter considers the values of protein nitrogen expressed as percentage of fresh weight of leaf to be the most correct, and gives the diurnal variation as percentage of the day value. For *Phaseolus* the loss by night is 1·8 %, and for *Acer* (recalculated on the above basis) about 6·5 %. From the evidence at hand, therefore, we must assume that there is a hydrolysis of protein by night and a resynthesis by day.

Investigations, on other lines, have been made to determine whether there is an increase of protein in detached leaves supplied with inorganic nitrogen and carbohydrate. Sapoznikow (86), for instance, estimated the increase of protein in detached leaves of the Vine kept with their petioles in nitrate solution in the light. The increase of protein amounted in one experiment to 1·782 gm. per sq. metre in 2½ days. Zaleski (173, 175) employed leaves of *Helianthus* floating on various solutions, and compared them with controls, both being kept in the dark. His results were as follows:

Increase in mgm. of nitrogen per sq. metre as compared with control		
Nitrate + sugar	Sugar, but no nitrate	Nitrate, but no sugar
+232	−4	−393
+227	−1	−104
+214	+7	−246
+194	+6	—

The above results may serve as evidence that protein synthesis can take place in the mature leaf, but do little to advance our knowledge as to the actual line of synthesis.

On the one hand, it is possible that all the amino-acids are separately synthesised *de novo* in the leaf (including, of course, the heterocyclic rings, see p. 216), and are translocated to the growing-points, etc. We have, at present, no evidnce for this. [A side issue bearing on this point, however, and one which has often been raised, is whether the synthesis of protein can take place in the dark. It seems clear that such is the case in bulbs and tubers germinating in the dark. But this may be regarded as regeneration from pre-existing protein, and may involve only a breakdown and resynthesis of amino-acids. If, however, we should assume that heterocyclic rings can only be formed in green organs in the presence of light, then the leaf *must* be the seat of production of amino-acids which are trans-

located as such to the growing-points. If we accept Zaleski's results as evidence on this point, then light is not essential; but his experiments, like those of various other workers in connection with the same problem, are scarcely sufficient to warrant this assumption. Evidence from etiolated seedlings is not satisfactory, since it is not, as a rule, possible to determine from the data whether there has actually been an increase of total protein and not merely regeneration. Results obtained with Bacteria and Fungi on protein synthesis are not of critical value as a guide to the metabolism of the higher plants.]

On the other hand there is the following aspect of the problem in contradiction to the point of view of separate synthesis of the whole range of amino-acids. Chibnall (14, 15) has shown that there is not only, as we have mentioned, a fall of protein nitrogen during the night, but also a decrease of non-protein nitrogen to the extent of 9 % per day value. From this he concludes that there is a translocation of soluble products from the leaf. That the chief of these is asparagine he deduces from his observations on the starved leaf, in which the protein is gradually converted, through amino-acids, into asparagine. Finally, his view is that there is a continuous decomposition of the protein of the normal leaf, by day and by night, on the lines of the starved leaf; but, by day this is masked, as the rate of protein synthesis exceeds that of protein degradation. The asparagine content is always found to be low in normal leaves (Chibnall, 15).

From evidence presented a reasonable point of view would be that asparagine is synthesised *de novo* in the leaf by way of succinic or malic acid (from products of respiration) and ammonia, and that it forms the only nitrogen translocation product. As long as carbohydrate concentration is high, the leaf protein remains unchanged; if there is a fall in concentration, protein is hydrolysed to some extent (as by night). On continued fall of carbohydrate concentration (starved leaf in the dark) the protein is gradually converted into asparagine *via* amino-acids, and is translocated away. On this hypothesis, however, asparagine must serve as the chief, if not the only, source for protein synthesis both in the leaf and elsewhere.

Prianischnikow's results, obtained from growing seedlings on ammonium salts, might be taken as evidence that asparagine was the first product of nitrogen synthesis. Both Prianischnikow and Smirnow lay stress on the fact that carbohydrates are essential for this formation; in their absence, there is only accumulation of ammonia. One must assume, therefore, as before, that succinic (or

malic) acid is formed as a by-product in respiration, and is subsequently aminated (see p. 220).

It should be noted, though, that Prianischnikow's seedlings did not form protein at all (? or rarely). It may be, therefore, that, owing either to absence of light or unfavourable conditions, protein cannot be synthesised. Asparagine, then, would merely rank as a temporary storage product, and we cannot conclude that it is an essential step in synthesis.

Whichever of these alternative *rôles* asparagine may play, one must assume that the nitrogen of the molecule is readily available. The amino nitrogen may be removed by oxidation, as in the case of any amino-acid. The amide nitrogen should also be available, that is the reaction

$$R \cdot CONH_2 + H_2O \rightarrow RCOOH + NH_3$$

should take place readily in the plant.

There has, in the past, been much confusion between the terms deamination and deamidation, that is, the origin of ammonia from the amino-acid and the amide groups respectively. An enzyme, asparaginase, has been postulated for the deamidation of asparagine, and its activity has been more or less successfully demonstrated by various observers. Recently, Grover & Chibnall (28) claim to have shown the presence of such an enzyme in the roots of Barley. For convenience they used what was practically malt, roots of dried germinated Barley. They extracted these with water, and precipitated the enzyme with alcohol.

After incubation of asparagine with the enzyme for 27 days, they isolated and identified the free aspartic acid. The enzyme acted on *l*-asparagine, but not on the dextro-form. It also acted on *d*-glutamine, but not on the simple amides of acetic and propionic acids.

Grover & Chibnall also found that asparagine was deamidated by erepsin from pig's intestine. Hence, it appeared possible that asparaginase, the enzyme postulated, might be merely a plant erepsin, as it is well known that all plants contain erepsins capable of splitting peptides. The action of the Barley root enzyme was then tried upon a peptide, glycyl-glycine, with the result that the peptide was hydrolysed.

Hence, Grover & Chibnall consider it unnecessary to postulate a specific asparaginase for asparagine and glutamine, but assume that the general type of erepsin (or, to adopt the more modern nomenclature, peptidase) is responsible for splitting, not only the peptide

linkage, but also the amide linkage. Asparagine, they suggest, need no longer be considered as a substance apart, but merely a dipeptide, of which class it is the simplest example.

Decomposition of protein in relation to carbohydrate concentration

Next, let us consider the decomposition of protein in greater detail. The facts of observation are that asparagine (or glutamine) is formed from protein *via* amino-acids in germinating seedlings and in detached leaves and shoots kept in darkness. One of the conditions incident to this change is apparently a low concentration of carbohydrate; another, the presence of oxygen. In the starved leaf and shoot, it is clear that concentration of carbohydrates is rapidly diminishing owing to respiration. The relationship between carbohydrate and nitrogen metabolism and respiration in a starved leaf has been investigated by Deleano (20). The latter divided Vine leaves into two halves; in one series of half-leaves, he estimated the carbohydrates (reducing sugars, cane-sugar, starch, etc.) and nitrogen compounds (total, soluble and ammonia nitrogen); of the other half-leaves, the respiration was measured for definite periods, and then analyses were made as in the case of the controls. A number of experiments were conducted so as to measure the respiration for periods from 22 to 500 hours. Deleano's experiments show, broadly speaking, that at the end of 100 hours most of the starch has disappeared, the cane-sugar entirely; at this point, too, the reducing sugar concentration begins to fall. In the case of the nitrogen compounds, at the same point, hydrolysis of protein commences and continues; also soluble nitrogen and ammonia appear, and increase continuously. This suggests that, as soon as the reserve carbohydrates are used up, the concentration of reducing sugars begins to fall, and, simultaneously, hydrolysis of protein takes place with the production of amino-acids and amide. Another point brought out by Deleano is the fact that more carbon dioxide was formed in respiration of the leaf than could be obtained theoretically from the amount of carbohydrate and organic acids estimated in the leaf. From graphs it is clear that the divergence between observed and calculated amounts begins a little later than the 100-hour period.

Now, to turn to the germinating seedling. The cotyledons (or indirectly the cotyledons embedded in the endosperm) are in a sense analogous to the starved leaf. It has been shown by Schulze (111) that the amount of asparagine formed in the early stages of germination

is proportional to the reserve of carbohydrates or fat. (This point should be clearly differentiated from the later accumulation of asparagine due to the fact that carbohydrate is needed also for its synthesis to protein.) It is possible that a series of events similar to those pictured in the starved leaf takes place in the germinating seedling. When the sugar content falls to a certain concentration, protein hydrolysis and amino-acid decomposition commence. Though more precise data are needed for carbohydrate metabolism of the seedling, it is clear that the mobilisation of carbohydrate and protein must be rapid in albuminous seeds (i.e. with endosperm), for the cotyledons must cease to form contact with this tissue before they can be withdrawn from the testa.

The converse of the state of affairs we have been considering is the prevention of the formation of asparagine when shoots or seedlings are provided with sugar solution. Monteverde (see Iwanoff, 34), for instance, kept twigs of *Syringa* and shoots of *Pisum* in glucose solutions (6·8 %), controls being placed in water. Asparagine was formed in the controls only. Prianischnikow also demonstrated the same result with etiolated seedlings of *Vicia Faba*, as follows:

% of total N	10 days in water	10 days in water +10 in sugar	20 days in water
Protein N	55·6	53·3	46·4
Asparagine N	20·5	22·0	29·4

Decomposition of amino-acids in relation to presence of oxygen

Palladin (58) claims to have shown that, in seedlings of *Triticum vulgare* kept in the dark in air, asparagine is almost the only decomposition product of protein. When kept in the absence of oxygen, asparagine was produced in very small quantity, the chief decomposition products being leucine and tyrosine. Suzuki (156) also showed that in etiolated seedlings of *Hordeum* and *Soja*, in the absence of oxygen, there was no increase in asparagine as compared with controls, but a striking increase in the presence of oxygen, accompanied by a decrease in amino-acids.

Decomposition of amino-acids by oxidation

The observations recorded in the previous paragraphs suggest that asparagine may be formed by oxidation of the amino-acids, and that this oxidation only takes place below a certain concentration of soluble carbohydrate.

A mechanism in the higher plants which will bring about a deamination of amino-acids is that of the catechol-oxidase system (see p. 142). It has been shown that the oxidase of the Potato tuber will, *in vitro*, deaminate amino-acids in the presence of oxygen as follows:

$$\begin{aligned} R\cdot CHNH_2\cdot COOH + O &= R\cdot CHNHOH\cdot COOH \\ &= RCO\cdot COOH + NH_3 \\ &= RCHO + CO_2 \end{aligned}$$

This might readily explain the accumulation of ammonia on autolysis of tissues or juice of oxidase plants, as observed, for instance, by Kiesel (38) for *Vicia Faba*, but whether the mode of oxidation of amino-acids in the living plant is on this plan is an open question. In any case, it is assumed that the succinic (or malic) acid residue arises from respiration of carbohydrate, and, whether the concentration of carbohydrate is low, as in a starved leaf, or high, as one would suppose in the case of Prianischnikow's seedlings supplied artificially with sugar, this residue is the same.

There is an alternative suggestion for the formation of asparagine from amino-acids. It is possible that the grouping

$$-CH\cdot CHNH_2\cdot COOH,$$

which is common to practically all members of the series, may be oxidised, with the removal of a 2-carbon residue. The residues, from two molecules of amino-acids, may then condense to form asparagine, two molecules of ammonia being simultaneously available. Further, that the whole asparagine molecule may arise from oxidation of arginine, has been suggested by Schulze & Castoro (132):

$$\text{Arginine} + O_2 = NH_2C(NH)NH(CH_2)_3\cdot COOH + CO_2 + NH_3$$

$$NH_2C(NH)NH(CH_2)_3\cdot COOH + O_2 = \underset{\text{Guanidine}}{NH_2C(NH)\cdot NH_2} + \underset{\text{Succinic acid}}{COOH\cdot CH_2\cdot CH_2\cdot COOH}$$

Kostytschew (46) also suggests that aspartic acid may arise from the oxidation of glutaminic acid and leucine:

$$(CH_3)_2\cdot CH\cdot CH_2\cdot CH(NH_2)\cdot COOH \rightarrow COOH\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

$$COOH\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH \rightarrow COOH\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

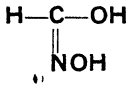
These three amino-acids, moreover, are those occurring, as a rule, in greatest quantity in plant proteins.

Synthesis of amino-acids in vitro. *Work of Baly*

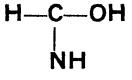
Some evidence of quite a different nature is expressed in the researches of Baly, Heilbron & Hudson (3). These investigators have attempted to carry out the synthesis of amino-acids by means of the effect of ultra-violet light on mixtures of certain inorganic compounds. In their experiments carbon dioxide was passed through

aqueous solutions of nitrite, and exposed to ultra-violet light. In this way, they assume that formaldehyde is produced in a specially activated form, which is expressed by the formula H—C—OH, activity being due to divalent carbon.

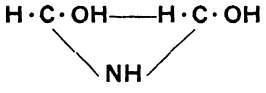
This activated formaldehyde is then assumed to react with nitrous acid to give formhydroxamic acid:



and this, by loss of oxygen, becomes



The above compound may be regarded as a hydrate of prussic acid, and therefore may be of interest in the plant. It is further assumed to condense with activated formaldehyde to give a labile ring



which, by intramolecular rearrangement, produces glycine. Glycine reacts further with active formaldehyde to form homologues of glycine. In support of these views, Baly obtained in his mixtures, after exposure to the ultra-violet rays, substances which gave the ninhydrin reaction for amino-acids, and, later, from similar experiments, the diazobenzene reaction for histidine. Further work on the synthesis of alkaloid-like compounds *in vitro* has been severely criticised.

In reviewing the discussions of the previous pages, a final emphasis should be laid on the fact that asparagine arises from three sources:

(1) From aspartic acid, which is a component of the plant protein.

(2) From oxidation of amino-acids.

(3) From ammonium salts in the presence of sufficient concentration of sugar.

The observation that asparagine is found as a result of lack of carbohydrate, and yet may sometimes be present with relatively high concentrations of the same substances, has been a matter of controversy among the various workers on nitrogen metabolism. It is obvious that this may well be the case, when asparagine is supposed to arise by a down-grade process as in (2) and an up-grade process as in (3). If, for certain reasons, asparagine formation does not proceed to protein synthesis, it may accumulate, together with carbohydrate (Prianischnikow's seedlings, see p. 202). On the other

hand, without a certain concentration of carbohydrate, protein formation will not take place from asparagine (etiolated seedlings).

Kostytschew (46) has also considered the reverse problem. When, as in etiolated seedlings after exposure to light, very large quantities of asparagine are used for protein synthesis, what becomes of the succinic or malic acid residue? "What", Kostytschew writes, "happens to the carbohydrate residue of asparagine after splitting off of ammonia? Aspartic acid is not found. As much as 70 % of the nitrogen of protein breakdown products is sometimes due to asparagine; one would expect this residue to appear in large amounts. As a matter of fact, this residue is never found." He suggests that it may be oxalacetic acid

$$COOH \cdot CH_2 \cdot CO \cdot COOH = CH_3CHO + 2CO_2$$

which would be split by carboxylase into acetaldehyde and carbon dioxide. Kostytschew is apparently of the opinion that no portion of the aspartic acid molecule except the ammonia can be used in protein formation. Loew (50), on the other hand, suggests that aspartic aldehyde may take part in protein synthesis.

Recent investigations on nitrogen metabolism. Work of Ruhland & Wetzel, Mothes, Engel and Grüntuch

Some new conceptions, which have thrown fresh light on the whole problem of nitrogen metabolism, have recently been introduced by Ruhland & Wetzel in three preliminary communications (196–198). The chief of these conceptions is the recognition, in respect of nitrogen metabolism, of two types of plants, the "amide" plants and the "ammonia" or "acid" plants. The former we are familiar with from the preceding pages. In these, asparagine (or glutamine) arises from oxidative deamination of amino-acids. It is formed, it has been suggested by previous workers, either from the amination by ammonia of succinic or malic acid (produced in respiration), or the whole molecule of asparagine might arise from such amino-acids as glutaminic acid or leucine by oxidation (see p. 213).

In the case of the "acid" plants, distinguished on this new basis by Ruhland & Wetzel, deamination results in the simultaneous formation of organic acids, such as malic and oxalic acids, and ammonia. High percentages of acids and ammonia are found under conditions, which, in the other class of plants, would give rise to accumulation of amides. The non-nitrogenous residues produced on deamination of amino-acids are, in the opinion of the above authors, the precursors of such

acids as malic and oxalic acids. Since, further, both acid and ammonia are the products of one and the same line of metabolism, the injurious effect of ammonia is automatically cancelled to a large extent by the production of acid. Ruhland & Wetzel are of the opinion that such acids as malic and oxalic acids are not products of the respiratory mechanism, since they can find no correlation between the amount of their formation and the degree of respiratory activity.

Their first investigations (196) were carried out on *Begonia semperflorens*. The leaves of this plant they found to contain (per dry weight) on an average 20 % oxalic acid, 0·5 % malic acid and 0·3 % succinic acid. The leaves, moreover, contain in the morning 5–10 times as much ammonia (per total nitrogen) as plants which have no significant amount of organic acids. On the other hand, they found that the amino-acid content is relatively low, so that in *Begonia* the value for ammonia-nitrogen is 50 % of that of the amino-acid nitrogen[1], instead of 2–3 % as in an "amide" plant. Amides, moreover, under normal conditions are practically absent.

In abundant supply of nitrogen, as in cultures on nitrogen-containing salts, the value of the ammonia nitrogen may rise to 75 % of the amino nitrogen.

A further, quite enormous, increase of ammonia content can be brought about experimentally, if lack of carbohydrate is ensured by prolonged darkening at a high temperature (28–35° C.). Such conditions force protein breakdown. Ammonia then increases as compared with amino-acids; the latter increase also, but as they are rapidly deaminised, the ammonia nitrogen, after 11 hours, oversteps the amino nitrogen in amount, and its final value, after 48 hours, may reach 171 % of the amino value (or after 106 hours, almost 30 % of the total nitrogen). Amides, again, are quite insignificant.

In correlation with the accumulation of ammonia in "acid" plants, there is a corresponding accumulation of organic acid. Ruhland & Wetzel conclude that, in deamination of amino-acids, oxy- or α-keto-acids are formed, and are the precursors of oxalic acid.

In *Begonia* plants on nitrogen-free cultures, that is, with a shortage of nitrogen, there is little deamination and formation of ammonia, but relatively high amino-acid content (cp. Engel, p. 232). With this is correlated a low oxalic acid production, in spite of abundant carbohydrate and high respiration value. Hence the authors regard this as

[1] For simplicity we use amino-acid-N for Ruhland & Wetzel's and Mothes' expression, *Rest*-N (that of amino-acids and organic bases).

evidence against the view that oxalic acid is a product of carbohydrate metabolism.

Ruhland & Wetzel make further general statements on nitrogen metabolism of *Begonia*, and these we shall summarise in the following paragraphs.

There is an increase of oxalic acid during the night, especially in young leaves. Simultaneous nitrogen estimations have shown that there is an active deamination of amino-acids in young leaves by night, so that their content of amino-acids may fall about 50 % if translocation to them from other organs is prevented; if, on the other hand, they receive amino-acids, the content may rise about 37 %.

In old leaves, hydrolysis of protein dominates by night, but, in contrast to young leaves, there is no active deamination. By preventing translocation from the old leaf by night, the content of amino-acids may rise more than 200 %; with free translocation, it may fall 33 %. These results lead to the conclusion that, during the night, considerable quantities of amino-acids are translocated from the old leaves, and conducted to the young leaves. The ammonia content falls by night in both old and young leaves. While ammonia is used in young leaves for protein synthesis, it is translocated from old leaves, or, should translocation be prevented, it is converted into amide. In young leaves by night, there is active deamination both of their own amino-acids and of those received by translocation, and considerable protein synthesis takes place (cp. Mothes' results given below).

Thus, in old leaves by night hydrolysis of protein predominates, of which the products are conducted, for the most part, to the young leaves, and are there used up. Consequently, by prevention of translocation, the soluble nitrogen content may increase $2\frac{1}{2}$ times the evening value, while in young leaves, under the same conditions, it falls about 30 %.

Finally, the authors remark that the degree of deamination of amino-acids and of protein synthesis, on the one hand, and the increase in free oxalic acid on the other hand, run parallel throughout in both old and young leaves. The same correlation also holds between the daily variations of oxalic acid in old and young leaves and the change in the ratio $\frac{\text{soluble N}}{\text{protein N}}$.

In a second communication (197) Ruhland & Wetzel give a preliminary account of investigations with another "acid" plant, namely, the Rhubarb (*Rheum hybridum hort.*). Since, in amide plants, the young developing stage is especially suitable for demonstrating amide

production, the above authors have selected the Rhubarb as an acid plant which is easily grown, and of which the developing stages can be readily investigated.

In regard to the relationship between nitrogen and acid metabolism in the developing petioles, the authors state that an active deamination, with formation of ammonia, takes place, so that the toxic effect of the latter must be counteracted. This is brought about by the production of acid, which is practically sufficient in quantity to neutralise the ammonia.

In active deamination, the danger from toxic ammonia is thus automatically removed, if ammonia and acids are final products of one and the same chemical process, that is, if the latter are themselves nitrogen-free deamination products, a conclusion which the authors' results vindicate.

Ruhland & Wetzel point out, moreover, that the former conception of the origin of organic acids in respiration appeared plausible because no other sufficiently active line of metabolism suggested itself as a source of acid. In the Rhubarb there is a nitrogen metabolism of great intensity, directly from the beginning of the petiole development, in which deamination and acid formation result simultaneously, and also correspond in output. In older organs which, in contrast to an active carbohydrate metabolism, show only very limited nitrogen metabolism, there is scarcely any appreciable fresh production of acid.

They further remark that succinic and malic acids are the first deamination products found in Rhubarb petioles which suggests a prior formation of asparagine or glutamine. Oxalic acid appears later, and is mostly accompanied by a corresponding and simultaneous decrease of malic and succinic acids. They do not consider oxalic acid to be a primary deamination product, which point of view is supported by the facts that it increases continuously in old petioles in spite of decreasing deamination, and this increase is accompanied, moreover, by loss of succinic and malic acids.

Yet other facts in connection with the metabolism in the Rhubarb have been collected by Ruhland & Wetzel (198) in support of their theory. They observed that when the rhizome sprouts, the amino-acids which are translocated to the developing petioles are actively deaminated. There thus arises, simultaneously, and in the same region, so much organic acid (especially malic acid) that a close chemical connection must exist between the two processes.

They observed, moreover, that when detached laminae are exposed

to prolonged darkness in warm weather, there is first loss of carbohydrate and active hydrolysis of protein. Then there sets in an intense deamination, and the acid suddenly increases 26 %; there is at the same time ammonia production. The process leads eventually to formation of considerable quantities of acid, of which the molecular concentration corresponds well with the increase of ammonia.

They were further able to show the presence in the petioles of two forms of malic acid, *i*- and *l*-malic acids. They maintain, moreover, that the optically active acid is the newly-formed substance, whereas the inactive acid is translocated from the rhizome. This conclusion is based on estimations carried out during the whole development from the bud to the autumnal leaf. At the beginning, in the rhizome and youngest bud, only the inactive malic acid is found. With growth of the petiole and consequent continuous deamination, *l*-malic acid appears in steadily increasing amount, accounting in summer for nearly two-thirds of the total acid content. In this, and, further, in material kept in darkness, in which the metabolism of both ammonia and acid is stationary, it was found that the relationship of molecular concentration of ammonia and *l*-malic acid is practically constant and equal to unity. The newly-formed acid, they conclude, is *l*-malic acid only, and is derived from the amino-acids translocated from the rhizome. Amides are only represented in quite subordinate amount.

Acids, therefore, are only found in the Rhubarb in connection with deamination. Also in the petioles only so long as they are growing; after growth ceases, until autumn, they are no longer produced. Ruhland & Wetzel conclude again from these facts that the metabolism of respiration has nothing to do with acid formation.

Further they consider that malic acid is not subsequently oxidised completely, but only partially to oxalic acid. Nor is the whole of the acid so oxidised, but, at the end of the vegetative season, both acids are found together.

Thus the picture drawn by Ruhland & Wetzel indicates a definite line for nitrogen metabolism in the "acid" plants. When amino-acids from protein hydrolysis are the source of nitrogen, they are deaminised, and the ammonia is used for protein synthesis, the non-nitrogenous remainder being represented at first by malic (or succinic) acid, which is, subsequently, oxidised to varying extent to oxalic acid.

If this conception of Ruhland & Wetzel is applied to the case of "amide" plants, it may modify the aspect outlined in the previous pages. It is reasonable to suppose that the amides of aspartic and

glutaminic acids fulfil a similar *rôle* to that of the ammonium salts of malic and oxalic acids. It would appear, moreover, that in amide plants also, as far as the source of nitrogen from protein, *via* amino-acids, is concerned, deamination and amide formation are essential processes in resynthesis to protein. It would likewise appear probable that the non-nitrogenous residue of the asparagine (or glutamine) molecule may arise from amino-acids, rather than as a product of respiration metabolism.

We now pass to the recent researches of Mothes (194) on the nitrogen metabolism of leaves. The work carried out by this author so thoroughly extends and amplifies much of that of the earlier investigators that his data are reproduced here somewhat extensively.

Mothes has obtained results bearing upon several of the debated questions, such as metabolism of nitrogen compounds of the leaf during the night, during carbohydrate starvation, on carbohydrate feeding, etc. These points will now be considered in detail. In addition, fresh data are supplied from young, as contrasted with old, leaves.

The investigation of change in nitrogen compounds of the leaf during the night was first carried out on leaves attached to the plant. The results obtained are given in Table XLII.

The conclusions drawn by Mothes are that, with the exception of ammonia nitrogen, there is a loss of all forms of nitrogen during the night. Further, the increase in ammonia nitrogen (which has been observed by other authors) points to a slower using up of this product While the decrease of amide nitrogen is significant, that of protein is insignificant. Young leaves show the changes either not at all or to a less degree.

With a view to throwing additional light on the processes taking place, Mothes next made observations on the changes, by night, in detached leaves. Portions of leaves were floated with the upper side on water during the night, control portions being analysed at once. The results are given in Table XLIII.

From Table XLIII it is concluded that protein and ammonia nitrogen decrease. The total soluble nitrogen increases, and of this, the amide more than the amino nitrogen. Whether the increase in amide nitrogen arises from the disappearance of ammonia nitrogen, or from some other source, it was not possible to decide.

The effect of temperature on the nitrogen partition in leaves, both attached and detached, was also investigated. Though leading, in general, to an increase of soluble nitrogen, it was found that increase

TABLE XLII. Nitrogen distribution in leaves during the night (Mothes)

Material	Date	Time of gathering	Nitrogen as percentage of fresh weight					
			Ammonia	2-amide	Amino* (*Rest*)	Soluble	Protein	Total
(1) *Vicia Faba*	23/24. ix. 24	5·00	0·04	0·11	0·43	0·58	4·49	5·07
		7·00	0·07	0·06	0·21	0·34	4·21	4·55
		Difference†	+75	−45	−50	−40	−6	−10
(2) Ditto	24/25. iii. 25	5·00	0·05	0·65	0·23	0·93	4·30	5·23
		7·00	0·06	0·44	0·19	0·69	4·17	4·86
		Difference	+20	−32	−17	−26	−3	−7
(3*a*) Ditto Mature leaves	24/25. iv. 25	6·00	0·04	0·12	0·50	0·66	4·90	5·56
		11·00	0·05	0·10	0·48	0·63	4·70	5·33
		Difference	+25	−17	−4	−5	−4	−4
(3*b*) Ditto Young leaves	24/25. iv. 25	6·00	0·06	0·50	0·93	1·49	7·57	9·06
		11·00	0·07	0·51	0·87	1·45	7·45	8·90
		Difference	+16	+2	−7	−3	−2	−2
(4) *Lupinus luteus*	16/17. ix. 24	5·00	0·03	0·07	0·18	0·28	6·05	6·33
		7·00	0·03	0·04	0·13	0·20	5·80	6·00
		Difference	—	−43	−28	−29	−4	−5
	16/17. ix. 24	5·00	0·03	0·06	0·17	0·26	6·23	6·49
		7·00	0·03	0·03	0·14	0·20	5·60	5·80
		Difference	—	−50	−18	−23	−10	−11
(5) *Phaseolus multiflorus*	24/25. ii. 25	5·15	0·01	0·25	0·79	1·05	3·83	4·88
		9·00	0·01	0·16	0·72	0·89	3·75	4·64
		Difference	—	−36	−9	−15	−2	−5

* Amino-acids and organic bases. † Expressed as percentages of evening values.

TABLE XLIII. Nitrogen distribution in detached leaves during the night (Mothes)

Material		Nitrogen as % fresh weight Total	As percentage of total nitrogen				
			Ammonia-N	2-amide-N	Amino-N (*Rest*)	Soluble-N	Protein-N
Phaseolus multiflorus	Evening	4·82	2·02	4·60	17·58	24·20	75·80
	Morning	4·77	1·14	5·23	20·45	26·82	73·18
Vicia Faba	Evening	5·48	1·32	1·96	8·57	11·85	88·15
	Morning	5·57	0·94	3·23	9·24	13·41	86·59

of temperature might reverse some of the changes observed above. In Mothes' opinion the results, on the whole, indicate that three processes are being carried out side by side in the leaves. Synthesis of protein at the expense of asparagine, hydrolysis of protein, with subsequent formation of asparagine as a secondary oxidation product, and a translocation away of soluble compounds. The rates of the different processes vary with temperature, so that sometimes one, sometimes another, effect predominates.

The problem of the effect of carbohydrate starvation on nitrogen metabolism was also investigated by Mothes. Though the conclusions drawn from his results confirm those of Schulze, Prianischnikow, Chibnall, etc., considered by us in previous pages, a selection of Mothes' data are included here since they give a good representation of the phenomena.

Mothes first made analyses of the various forms of nitrogen in the leaves of plants which had been kept in the dark from 4 to 18 days as compared with control plants in the light. The conclusions are as follows: Whereas the amounts of various nitrogen compounds in the latter remain fairly constant, the former show a decrease of total nitrogen and of protein nitrogen; the soluble nitrogen on the other hand increases, and of this, the amide more than the amino nitrogen. If, of a plant kept in the light, a certain number of leaves only were darkened, we find the loss of total and protein nitrogen to be greater than in the case when the whole plant was darkened.

Again, in order to throw light, if possible, on the process, Mothes repeated the investigation of the effect of carbohydrate starvation using detached leaves, either with their petioles in water, or floating on their upper surfaces. The results are given in Table XLIV and Fig. 8.

The observations drawn from Table XLIV are that, in regard to detached and darkened leaves, the protein nitrogen undergoes considerable loss, the soluble nitrogen, conversely, increasing; the amide nitrogen increases more than the amino nitrogen, and may reach a value of 50 % of the total soluble nitrogen. The ammonia nitrogen, moreover, increases at the expense of the amino nitrogen. The main conclusion, already arrived at by previous workers, is that in carbohydrate starvation there is an oxidative decomposition of the products of hydrolysis of the proteins. Mothes also investigated the effect of change of temperature on the nitrogen partition of leaves kept in the dark, and found that the various processes were affected to a different

degree. The question further arises as to whether the occurrence of amides normally in the leaf denotes that this process of deamination is always going on, the carbohydrates present preventing accumulation of products.

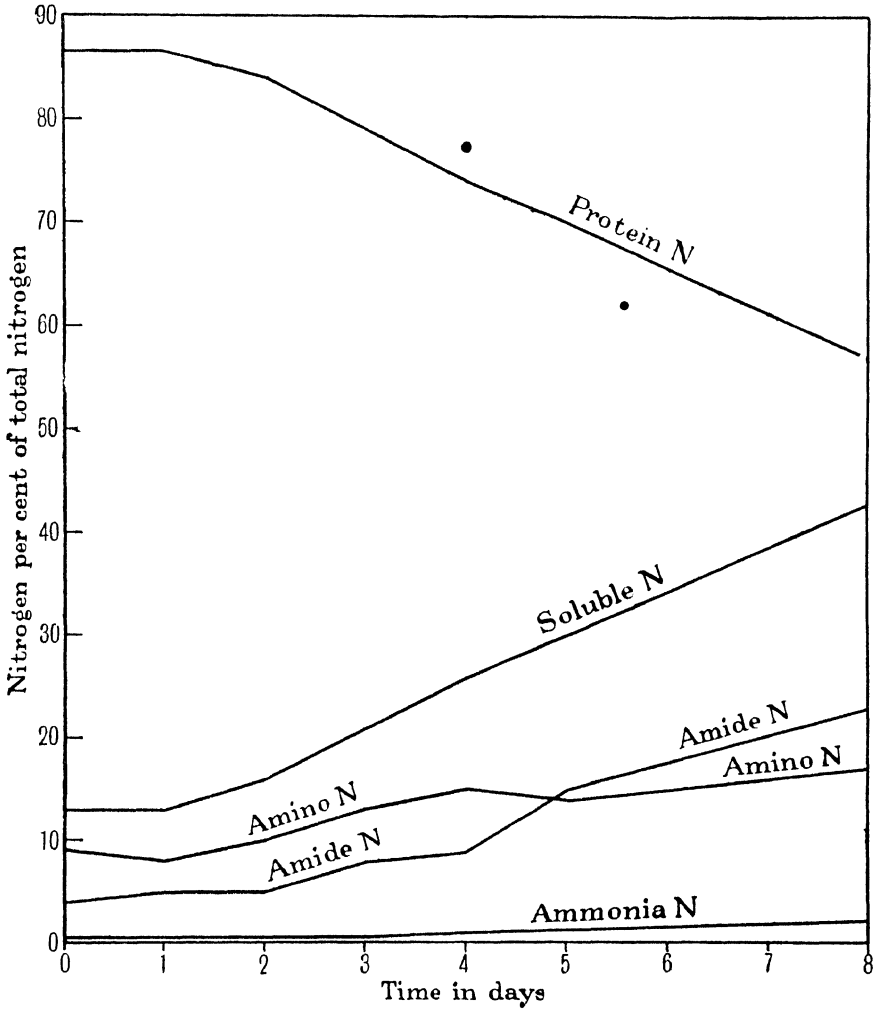


Fig. 8. Nitrogen distribution in leaves of *Vicia Faba* 8 days in the dark (Mothes).

Experiments were next carried out on leaves supplied with carbohydrate either by exposure to light or by sugar-feeding under sterile conditions. The results are given in Table XLV.

From Table XLV, Mothes concludes that there may be considerable variation in behaviour in regard to the illuminated leaves. In general, there is protein hydrolysis with increase of amide and amino nitrogen. In *Lupinus*, protein hydrolysis is as great as in absence of light, and,

obviously, the intensity of light plays a great part. Since, however, all the leaves after illumination (except in Expt. 1 *c*) were found to contain starch, one cannot speak of lack of carbohydrate. In one experiment (4) there is, apparently, synthesis of protein at the expense

TABLE XLIV. Effect of carbohydrate starvation on nitrogen distribution in leaves (Mothes)

Material	Period	As percentage of total nitrogen					As percentage of soluble nitrogen		
		Ammonia N	2-amide N	Amino (*Rest*) N	Soluble N	Protein N	Ammonia N	2-amide N	Amino N
1. *Vicia Faba*	16. vii	0·25 0·26	2·33 2·33	6·33 6·11	8·91 8·70	91·09 91·30	2·87 2·98	26·15 26·78	70·89 70·24
	After 1 day	0·25 0·25	3·78 3·80	12·98 12·96	17·01 17·01	82·99 82·99	1·47 1·47	22·22 22·34	76·31 76·19
	After 4¾ days	0·94 0·88	12·49 12·25	16·79 19·27	30·22 32·40	69·78 67·60	3·11 2·71	41·33 37·89	55·56 59·40
	After 6 days	2·60	13·07	17·38	33·05	66·95	7·81	39·54	52·65
2. *Lupinus luteus*	Average initial value	—	1·00	3·00	4·00	96·00	—	25	75
	After 3 days	0·38 0·50	5·06 4·85	11·54 11·39	16·98 16·74	83·02 83·26	2	29	69
	After 5 days	1·26 1·34	13·11 12·55	16·29 19·11	30·66 33·30	69·34 66·70	4	41	55
3. *Linosyris vulgaris*	Initial value After 5 days After 7 days	1·2 1·1 1·1	0 8 4·2 5·9	7·4 13·4 16·9	9·4 18·7 23·9	90·6 81·3 76·1	13 6 5	9 23 25	78 71 70
4. *Vicia Faba*	Initial value After 1 day After 2 days After 3 days After 4 days After 5 days After 8 days	0·46 0·51 0·62 0·70 1·20 1·30 2·40	4·14 4·78 5·42 7·60 9·40 14·80 22·90	8·60 8·11 9·61 12·80 15·30 13·70 17·40	13·20 13·40 15·65 21·10 25·90 29·80 42·70	86·80 86·60 84·35 78·90 74·10 70·20 57·30	4 4 4 3 5 4 6	31 36 35 36 36 50 53	66 60 61 61 59 46 41
5. *Phaseolus multiflorus*	Initial value After 8 days	1·5 3·0	1·8 21·2	6·1 14·9	9·4 39·1	90·6 60·9	16 8	19 54	65 38
6. *Phaseolus multiflorus*	Initial value After 8 days	1·2 1·6	1·3 10·7	5·2 25·0	7·7 37·3	92·3 62·7	16 4	17 29	67 67

of amino nitrogen. In contrast to the results obtained in absence of light, there is no increase of ammonia parallel to the increase of amide nitrogen. In regard to sugar feeding, there is a relationship to temperature; the higher the temperature, the greater must be the con-

TABLE XLV. Effect of increased carbohydrate on nitrogen distribution in leaves (Mothes)

Material	Conditions	Remarks	Nitrogen as % fresh weight. Total	As percentage of total nitrogen				
				Ammonia N	2-amide N	Amino N (*Rest*)	Soluble N	Protein N
1. *Lupinus luteus*	Initial value	—	5·2	—	1	3	4	96
	3 days in light	Cloudy	—	0·35	6·89	9·23	16·47	83·53
			—	0·38	5·51	11·83	17·72	82·28
	5 days in light	Cloudy	—	0·25	15·92	16·24	32·40	67·60
2. *Phaseolus multiflorus*	1. iii. 25	—	4·33	0·1	8·8	11·9	20·8	79·2
	2¼ days in light	Sunny	—	0·1	13·0	17·2	30·3	69·7
3. *Phaseolus multiflorus*	25. iii. 25	—	4·05	0·06	4·41	13·05	17·52	82·48
	3 days in light	7° C. mist	—	0·07	6·60	15·11	21·78	78·22
	3 days in dark	14° C. „	—	0·60	10·23	19·21	30·04	69·96
	6 days 1 % glucose	14° C. „	—	0·07	11·23	18·15	29·45	70·55
4. *Phas. multifl.* young plant	3. iv. 25	—	5·03	0·1	7·1	11·1	18·3	81·7
	4 days in light	Very sunny	—	0·1	6·3	3·1	9·5	90·5
5. *Phas. multifl.*	24. iii. 25	—	4·42	0·07	4·92	8·30	13·29	86·71
	6 days 2 % glucose	13·5° C.	—	0·07	7·08	13·57	20·72	79·28
6. *Phas. multifl.*	16. iii. 25	—	5·02	0·1	8·8	13·9	22·8	77·2
	5 days 2·5 % glucose	21° C.	—	0·1	12·6	14·1	26·8	73·2
7. *Phas. multifl.* young plant	14. ii. 25	—	5·37	0·18	4·98	19·89	25·05	74·95
	4 days 2·5 % glucose	17° C.	—	0·20	4·95	16·61	22·06	77·94
	2 days H_2O	24° C.	—	1·53	7·83	31·11	40·47	59·53
	2 days 2·5 % glucose	24° C.	—	0·23	5·73	21·35	27·31	72·69
8. *Phas. multifl.* A	3. iv. 25	—	4·65	0·1	7·0	12·0	19·1	80·9
	4 days 4 % glucose	17° C.	—	0·1	7·3	12·1	19·5	80·5
B	3. iv. 25	—	—	0·1	6·5	11·7	18·3	81·7
	4 days H_2O	17° C.	—	0·7	22·2	12·4	35·3	64·7
9. *Phas. multifl.*	1. iv. 25	—	4·0	0·1	8·5	9·8	18·4	81·6
	5 days 5–7 % glucose	14° C.	—	0·1	7·8	8·7	16·6	83·4
10. *Vicia Faba*	30. i. 25	—	4·98	0·17	12·79	20·69	33·65	66·35
	3 days H_2O+$CaSO_4$	12° C.	—	0·65	14·34	25·58	40·67	59·33
	3 days 2 % glucose + $CaSO_4$	12° C.	—	0·25	11·78	22·47	34·51	65·49
11. *Vicia Faba* poor in starch	24. iii. 25	—	5·15	0·13	9·77	5·62	15·72	84·28
	3 days 1 % glucose	23° C.	—	0·13	24·37	19·15	43·65	56·35
12. *Lupinus albus* in flower	20. iii. 25	—	6·1	0·1	3·0	9·3	12·4	87·6
	3 days H_2O	—	—	0·4	14·9	14·1	29·4	70·6
	1 day 1·25 % glucose	20° C.	—	—	—	—	—	—
	3 days H_2O	—	—	0·1	7·3	12·2	19·6	80·4
	3 days 2·5 % glucose	—	—	0·1	3·6	11·6	15·3	84·7

centration of sugar to prevent protein hydrolysis. Though in some experiments the concentration of sugar keeps the amide value constant, yet protein synthesis at the expense of amide nitrogen is never attained, and only in Expts. 7 *a* and 9 is there a decrease of amino nitrogen. Again, as in light cultures (but in contrast to cultures in darkness) in spite of a great increase in amide, there is no increase in ammonia nitrogen, which is regarded as a further support to the view that in presence of carbohydrate, ammonia appears in the form of

TABLE XLVI. Nitrogen distribution in leaves in narcosis (Mothes)

Experiment	Remarks	$CH_3.Cl_3$ per 8 ltr.	Temp.	Nitrogen as % fresh weight. Total	As percentage of total nitrogen				
					Ammonia N	2-amide N	Amino N (*Rest*)	Soluble N	Protein N
(1) 6. v. 25	—	—	—	4·26	1·3	2·9	8·4	12·6	87·4
A. 2 days' narcosis	Much injured	2·4	22°	—	3·5	2·5	23·3	29·1	70·9
6. v. 25	—	—	—	—	1·3	2·6	6·1	10·0	90·0
B. 3 days in the dark	—	—	22°	—	1·6	6·8	11·8	20·0	79·8
(2) 18. v. 25	—	—	—	4·6	1·1	3·5	10·3	14·9	85·1
2 days' narcosis	Uninjured	1·0	30°	—	3·8	3·0	18·5	25·3	74·7
(3) 23. v. 25	2 days darkened	—	—	5·38	1·0	7·1	18·5	26·6	73·4
5 days in the dark	Leaves yellow –yellow green	—	21°	—	2·9	13·2	39·6	55·7	44·3
5 days' narcosis	Leaves good green	0·5	21°	—	14·7	1·8	22·6	39·1	60·9
(4) 15. vi. 25	5 days darkened	—	—	—	1·6	4·8	10·1	16·5	83·5
5 days' narcosis	Leaves green	0·5	17°	—	12·8	2·0	15·8	30·6	69·4
(5) 22. vi. 25	Young plant	—	—	—	1·0	9·5	7·2	17·7	82·3
4 days' narcosis	—	0·5	18°	—	13·6	4·1	19·5	37·2	62·8
4 days' narcosis	8 hrs. in light, 40 hrs. darkened	0·5	18°	—	3·1	19·9	17·5	40·3	59·5

asparagine. In all sugar cultures, protein hydrolysis is less than in water cultures. Finally, there is an obvious difference in behaviour between old and young leaves (Expts. 5 and 7).

The next series of experiments was concerned with narcosis on the lines of those of Butkewitsch mentioned in previous pages. Detached leaves of *Phaseolus* were kept in water in a closed space in the dark and treated with chloroform vapour. Comparisons were also made with leaves kept in the dark only. The results are given in TableXLVI.

These results confirm those of the older workers, and Mothes draws the same conclusions, namely, that all synthetic processes are inhibited in narcosis; hence, amides (asparagine) cannot be formed from the ammonia produced in oxidative decomposition of the products of protein hydrolysis, and, consequently, ammonia accumulates. In all cases there is considerable protein hydrolysis, less, however, than in darkened leaves, owing, possibly, to accumulation of products of hydrolysis. Increase of amide nitrogen is never observed; for the loss of amide nitrogen shown in some instances, the explanation is doubtful. All experiments show considerable increase of ammonia nitrogen derived from oxidation of amino-acids. Expt. 5 indicates that such ammonia nitrogen can be converted into asparagine if the plant is not injured by the chloroform, and photosynthesis is rendered possible.

The next series of experiments was connected with nitrogen metabolism under anaerobic conditions. Detached leaves in water kept in an oxygen-free atmosphere in the dark were used, a control experiment being carried out under aerobic conditions. The results are given in Table XLVII.

TABLE XLVII. Nitrogen distribution in leaves in anaerobiosis (Mothes)

Plant	Conditions	As percentage of total nitrogen				
		Ammonia N	2-amide N	Amino N (*Rest*)	Soluble N	Protein N
1. *Phaseolus multiflorus*	Initial value	1·4	2·4	6·7	10·5	89·5
	3 days' anaerobiosis	1·8	3·1	22·4	27·3	72·7
	Initial value	1·3	2·6	6·1	10·0	90·0
	3 days' aerobiosis	1·6	6·8	11·8	20·2	79·8
2. Ditto	Initial value	1·15	1·94	6·05	9·14	90·86
	3 days' narcosis + anaerobiosis	1·63	1·91	19·55	23·09	76·91

From Table XLVII it will be seen that there is only a slight increase of amide nitrogen as compared with the control under aerobic conditions. Mothes concludes that, on the whole, as with seedlings, so also with mature leaves, no formation of amide and ammonia takes place in absence of oxygen. Expt. 2 shows that the ammonia formed in narcosis must arise by oxidation.

A number of experiments with leaves supplied with solutions containing nitrogen were also carried out by Mothes. The substances selected were ammonium salts and asparagine. Mature, but not too old, leaves were used, and sterilised solutions of ammonium salts and asparagine, both with and without sugar solutions of various concentrations. The experiments were carried out in the dark unless otherwise mentioned. The results are given in Table XLVIII.

Table XLVIII. Nitrogen distribution in leaves supplied with ammonium salts (Mothes)

Plant	Conditions	Nitrogen as percentage of fresh weight					
		Ammonia	2-amide	Amino (*Rest*)	Soluble	Protein	Total
1*a*. *Phas. multifl.* younger	NH_4Cl	1·16	1·07	2·40	4·63	4·60	9·23
	NH_4Cl + glucose	0·69	0·95	2·12	3·76	6·30	10·06
1*b*. Ditto older	NH_4Cl	1·05	0·80	1·65	3·50	4·51	8·01
	NH_4Cl + glucose	0·56	0·67	1·60	2·83	5·66	8·49
2. *Vicia Faba*	A. Analysed at once	0·07	0·39	1·03	1·49	3·53	5·02
	B. 4 days NH_4Cl	1·09	0·74	1·74	3·57	2·46	6·03
	C. 4 days NH_4Cl + 1 % glucose	0·55	1·28	1·22	3·05	3·34	6·39
	D. 4 days NH_4Cl + 5 % glucose	0·38	0·53	1·30	2·21	5·12	7·33
	Difference in % of initial value B	+1460	+ 90	+69	+140	−30	+20
	C	+ 690	+230	+18	+105	− 5	+26
	D	+ 440	+ 36	+26	+ 48	+45	+45
3. *Phas. multifl.*	Analysed at once	0·06	0·29	0·66	1·01	5·52	6·53
	4 days in light	0·04	0·27	0·61	0·92	5·32	6·24
	1 day NH_4Cl	0·87	0·48	0·94	2·29	6·48	8·77
	1 day NH_4Cl + 3 days 7 % glucose	0·16	0·22	1·16	1·54	7·19	8·73
	1 day NH_4Cl + 3 days 2 % glucose	0·32	0·36	0·99	1·67	6·88	8·55
	1 day NH_4Cl + 3 days' light	0·39	0·40	1·27	2·06	6·97	9·03
4. *Phas. multifl.*	Analysed at once	0·06	0·12	0·30	0·48	2·87	3·35
	NH_4Cl (rich in starch)	1·71	0·80	0·65	3·16	2·53	5·69

In general, Mothes concludes, the results in Table XLVIII show that solutions of ammonium salts are readily used by mature leaves for protein synthesis if sufficient nitrogen-free material is present for such synthesis to take place. If there is lack of carbohydrate, there is amide formation at the expense of ammonia; if there is still greater shortage, then amide is formed at the expense of protein. The absorption of ammonia in both cases is considerably less. It is immaterial whether the necessary nitrogen-free material is derived from photosynthesis or from sugar-feeding. The experiments on feeding with asparagine are given in Table XLIX.

With asparagine alone, as is seen from Table XLIX, there is protein hydrolysis. In Expts. 5 and 6, the addition of sugar only prevents this hydrolysis. Expts. 7, 8 and 9 show that a high concentration of sugar is necessary for protein synthesis from amide, and that the concentration necessary varies according to that of asparagine. A further point, which is very interesting, is that amino (*Rest*) nitrogen can increase considerably at the cost of amide nitrogen, protein nitrogen being stationary.

TABLE XLIX. Nitrogen distribution in leaves supplied with asparagine (Mothes)

Material		Percentage of fresh weight					
		Ammonia	2-amide	Amino (*Rest*)	Soluble	Protein	Total
5. *Vicia Faba*	Initial value	0·01	0·14	0·43	0·59	4·95	5·53
	2 days asparagine	0·08	3·23	0·59	3·90	4·27	8·17
	2 days asparagine +2 days glucose	(0·10)	2·72	1·11	3·93	4·04	7·97
	2 days asparagine +4 days glucose	0·04	2·23	1·71	3·98	3·97	7·95
6. *Phas. multifl.*	Initial value	0·01	0·52	0·92	1·50	5·24	6·74
	3 days asparagine +glucose	0·02	2·86	1·15	4·03	5·30	9·33
		Percentage of total nitrogen					
7. *Phas. multifl.*	1 day 1 % asparagine	0·4	41·8	4·5	46·7	53·3	
	Afterwards 5 days 2 % glucose	0·1	32·3	14·0	46·4	53·6	
8. *Phas. multifl.*	1 day 0·5 % asparagine	0·6	40·4	8·5	49·5	50·5	
	Afterwards 4 days 7 % glucose	0·2	27·1	11·4	38·7	61·3	
9. *Phas. multifl.*	(*a*) 1 day 0·5 % asparagine	0·4	39·7	9·1	49·2	50·8	
	Then 3 days 5 % glucose	0·1	44·0	10·7	54·8	45·2	
	(*b*) 1 day 0·1 % asparagine	0·4	21·8	11·3	33·5	66·5	
	Then 3 days 5 % glucose	—	19·0	12·7	31·7	68·3	

In some of the experiments in Tables XLV and XLIX, as Mothes points out, there may be either hydrolysis of protein or failure to synthesise protein in spite of the presence of abundant carbohydrate. These anomalies have been found to be due to varying behaviour according to the age of the leaf. A considerable amount of additional research has been devoted by Mothes to this question, but it is too extensive to be quoted here. The results, however, may be summarised as follows:

In presence of sufficient carbohydrate, protein synthesis is always brought about in young leaves, if ammonia or asparagine is presented. It is immaterial whether the source of nitrogen is from protein of the

leaf itself (through exposure to darkness) or is derived from without. Excess of ammonia is first deposited as amide. There is also considerable increase of amino nitrogen, but it is questionable whether the latter can be regarded as material for protein synthesis. There is no protein breakdown in young leaves in presence of carbohydrate; nor is there such in narcosis.

Old leaves maintain their protein content either with difficulty or not at all, even with supply of carbohydrate. There is protein breakdown not only when carbohydrate is increased by photosynthesis, but also when glucose is supplied artificially. This protein breakdown is hindered, but not prevented, by addition of nitrogen in the form of ammonia or asparagine.

Some of Mothes' main conclusions are enumerated as follows:

1. Amides are formed in leaves of darkened plants and in detached and darkened leaves (Table XLIV).

2. This amide formation is quickened by raising the temperature.

3. Leaves which have become very poor in carbohydrate, owing to prolonged darkening, show a formation of ammonia, instead of a further increase of amide (Table XLIV).

4. If detached leaves, which are not too old, are exposed to daylight or fed with glucose in the dark, amides do not arise; those present disappear, and are used for protein synthesis (Table XLV).

5. Numerous considerations prove that amides and ammonia arise directly from proteins, or their decomposition products.

6. Oxygen is a limiting factor for amide formation. Its complete absence prevents the formation of amides in leaves poor in carbohydrate. There is also no increase of ammonia, but, instead, an increase of amino-acids and organic bases (Table XLVII).

7. In narcotised leaves poor in carbohydrate, in which synthetic processes are prevented, no amides appear, but we observe abundant ammonia and amino nitrogen (Table XLVI).

8. In leaves which are not too old, and have abundant carbohydrate, neither ammonia nor amide appear on narcosis. The production of amino nitrogen is unaffected.

9. In autolysis, no amides appear, but abundant amino nitrogen and ammonia.

10. In leaves which are not too old, and have abundant carbohydrate, ammonia supplied from without is rapidly synthesised to protein; in shortage of carbohydrate it is stored as asparagine. In

complete lack of carbohydrate it is deposited unaltered in the cells until the leaf dies from ammonia poisoning (Table XLVIII).

11. In similar material as 10, asparagine supplied from without is converted into protein in presence of abundant carbohydrate; in absence of carbohydrate it is stored as such (Table XLIX).

Though no conclusive evidence is given as to whether amide formation is an essential step for the transference of nitrogen and the synthesis of protein, Mothes' final conclusion may be quoted as follows: "As a result of my researches, I am of the opinion that the significance of amide cannot be, primarily, that of a medium of translocation. There is translocation of amides only if they, through accumulation, disturb equilibrium in the region of their formation, or if they are needed in young leaves for protein synthesis. In both the above cases, there is no ground for regarding the amides as other than substances in the form of which ammonia is rendered innocuous."

A second investigation by Mothes (195) deals with the nitrogen metabolism of seedlings of the Coniferae; it is, however, chiefly limited to the part played by arginine—of which there is a high percentage in such plants—in the interchange of products. The various forms of nitrogen (protein, basic, amide, amino and ammonia) were determined in seedlings of *Pinus nigra*, *P. Thunbergii* and *Picea excelsa*, grown both in light and in darkness.

From relative amounts of amide and bases (arginine), formed in light and darkness during various periods of the seedlings' existence, Mothes arrives at certain conclusions in regard to the part played by arginine in nitrogen metabolism.

The seedlings show the characteristics of amide plants, both in light and in darkness, the amide nitrogen increasing at the expense of the amino nitrogen, and falling again in the normal (light) cultures. The arginine nitrogen also increases, and, in time, slowly falls (more rapidly in *Pinus Thunbergii* and *Picea excelsa*). Among other reasons, the fact that conditions which favour oxidative deamination affect asparagine, more than arginine, production led Mothes to conclude that arginine is not, like asparagine, an unspecific reserve product of nitrogen. He is inclined to regard it as a (to the plant) valuable substance, which is not readily deaminated, and which accumulates, owing to the fact that all proteins have not the same arginine content, and so in protein synthesis it may only be slowly assimilated. Such a view is contrary to the conception that all protein hydrolysis products are broken down to ammonia, and that ammonia, again, is the first essential to

protein synthesis. This outlook, apparently, Mothes accepts, even so far as to suggest that deamination may be characteristic only of seedlings, cultures in darkness, and ageing organs, "ammonia" plants, perhaps, forming an exception. Any amino-acid, he further points out, may be considered to serve as a specific reserve of nitrogen, should its participation in protein synthesis be delayed.

The researches of Engel (188) are concerned with a different aspect of the problem. This author set out to investigate the processes which take place in a growing plant to which no nitrogen is supplied from without. He points out that in this case nitrogen for growth must be obtained from the older organs, and, consequently, there is an active protein breakdown. Since this takes place in the presence of abundant carbohydrate, there can be no increase of ammonia or amide nitrogen, and these must be reduced to a minimum, whereas, in consequence of protein hydrolysis, the amino-acid nitrogen must approach a maximum.

Shoots of *Callisia martensiana*, on which were a few leaves, were grown in water containing 0·002 % calcium sulphate for about three months. At the end of the period, they had produced a certain number of new leaves. The latter were analysed, and compared with those of normal plants grown in pots. The total nitrogen was found to have decreased, as also the protein nitrogen. The changes in the various forms of soluble nitrogen content in percentages of total soluble nitrogen are shown as follows:

	Soluble N	Ammonia N	2-amide N	Amino N
Normal leaves	100	8	40	52
Nitrogen starved leaves	100	9	20	71

Additional analyses show that the youngest leaves, formed during experiments conducted on the above lines, contain most nitrogen, and, of this, the greater quantity is ammonia and amide nitrogen. The amino nitrogen remains almost constant.

Engel suggests that amino-acids are translocated to growing regions, and are there deaminised, and the ammonia is used for protein synthesis.

A similar series of experiments were made, using Maize seedlings in water-culture. Of these seedlings, the oldest leaves were removed at intervals and analysed. The relationships between the various forms of soluble nitrogen are shown as follows:

The oldest leaves	Soluble N	Ammonia N	2-amide N	Amino N
On 4 July, 1928	100	9	35	56
On 9 July, 1928	100	9	8	81
On 11 July, 1928	100	5	9	86

The ammonia and amide nitrogen have decreased, and there is a considerable increase in amino-acid nitrogen. In spite of ample starch in the leaves, a compulsory breakdown of protein is brought about, with subsequent translocation to the regions where growth takes place.

It is obvious, in Engel's opinion, that the protein breakdown is arrested at the amino-acid stage, and it is these substances which are translocated from the leaf. Nitrogen shortage does not lead to deamination. He further points out that in carbohydrate shortage, protein is hydrolysed, and the amino-acids are oxidised, thereby providing energy, and ammonia finally remains. In nitrogen shortage, on the other hand, protein is hydrolysed to obtain nitrogen at disposal for protein regeneration at the growing-points; owing to excess of carbohydrate, no amide or ammonia is formed.

In addition, Engel mentions that similar phenomena, noted by Ruhland (see p. 216), occur in *Begonia*. Engel concludes therefore that there is a far-reaching correspondence between "ammonia" (*Begonia*) and "amide" (Maize) plants. In nitrogen shortage, the breakdown of protein leads to accumulation of amino-acids only; both types of plants avoid the necessity for neutralising the toxic effect of ammonia, which, under ordinary circumstances, results in the formation of ammonium oxalate or asparagine—the final products of deamination of amino-acids. In the roots, on the contrary, owing to shortage of carbohydrate, the amounts of ammonia and amide nitrogen are greater than in the leaves.

Engel suggests that in protein synthesis, oxy- and keto-acids (arising from carbohydrates) are neutralised by ammonia, and from these ammonium salts amino-acids ultimately arise (see Fig. 9). He also considers that the non-nitrogenous remainder of asparagine can recombine with ammonia derived from any source.

Finally, the investigations of Grüntuch (189) may be mentioned. This author investigated the nitrogen metabolism of a number of underground reserve organs, such as rhizomes, roots, tubers, etc. He found that though such organs have a low total nitrogen content as compared with seeds, yet they have a characteristically high soluble

nitrogen content, even containing as much as 88 % of the total nitrogen (Table L).

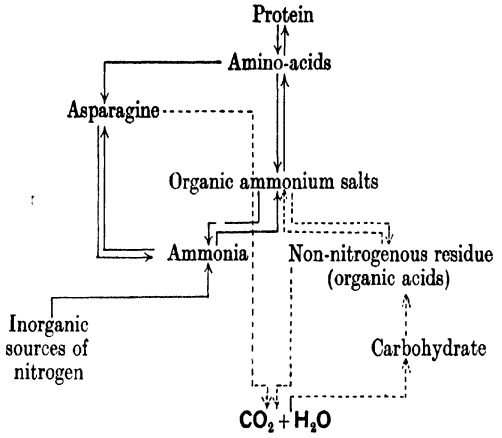


Fig. 9. Scheme for protein synthesis and breakdown (Engel).

TABLE L. Nitrogen content of various underground storage organs (Grüntuch)

Plant	Organ	Date	Sol. N ‰	In % of total N	Protein N ‰	In % of total N	Amino N (*Rest*) ‰	In % of total N	2-amide N ‰	In % of total N	NH_3N ‰	In % of total N	Total N
Oxalis tetraph.	Bulb	1. vi. 27	6·61	56·7	5·02	43·3	6·24	53·5	0·26	2·7	0·11	0·9	11·63
Allium Cepa	Bulb	12. vi. 27	2·07	67·4	1·00	32·6	1·28	41·6	0·66	21·6	0·13	4·10	3·08
Asparagus offic.	Rhizome	27. vi. 27	1·87	36·7	3·23	63·3	1·41	29·5	0·31	6·2	0·05	0·97	5·1
Iris florentina	Rhizome	1. vii. 27	1·51	64·8	0·84	35·8	1·26	53·4	0·14	7·1	0·1	4·4	2·36
Iris florentina	Root	13. ii. 27	1·80	34·0	3·50	66·0	—	—	—	—	—	—	5·30
Asparagus offic.	Root	18. iv. 27	3·45	70·0	1·38	30·0	1·45	42·0	1·28	26·5	0·76	1·5	4·83
Dahlia var.	Tuberous root	18. iv. 27	0·84	72·0	0·35	28·0	—	—	—	—	—	—	1·19
Canna indica	Tuberous root	25. i. 28	1·92	64·0	1·09	36·0	—	—	—	—	—	—	3·01
Daucus Car.	Tuberous root	25. i. 28	1·24	54·5	1·04	45·5	—	—	—	—	—	—	2·28
Beta vulg.	Tuberous root	13. ii. 28	4·20	47·5	4·65	52·5	—	—	—	—	—	—	8·85
Oxalis tetraph.	Tuberous root	8. ix. 27	1·71	47·6	1·84	52·4	1·30	36·1	0·24	6·6	0·17	4·7	3·55
Helianthus tub.	Tuber	18. iv. 28	1·18	42·5	1·60	57·5	—	—	—	—	—	—	2·78
Solanum tub.	Tuber	26. ix. 27	1·27	34·0	2·06	66·0	0·25	9·0	0·75	24·5	0·03	1·3	3·33

GENERAL CONCLUSIONS (see Fig. 10)

It is not easy to make concise generalisations from the results of the earlier workers and those outlined in the previous section.

On the whole it seems probable that protein may be synthesised, in the meristematic tissues of growing-points and other regions, by the laying down of a pattern, the result of condensation of small active molecules formed in glycolysis, nitrogen being supplied as ammonia, and sugar as sucrose (γ-fructose + normal glucose).

It would appear that protein can be both synthesised and hydrolysed in other organs also, such as leaves. The predominating effect, i.e. synthesis or hydrolysis, depends both on the age of the organ and on the amount of carbohydrate present. Synthesis predominates in the youngest members, hydrolysis in the oldest. Abundance of carbohydrate is unfavourable to protein hydrolysis. With continuous fall in carbohydrate, hydrolysis proceeds to amino-acids, followed by deamination to amides (or ammonium salts of organic acids) and ammonia.

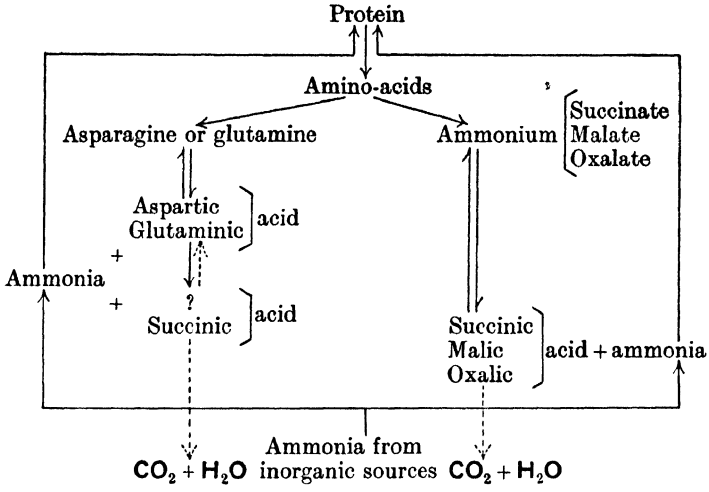


Fig. 10. Tentative scheme for protein synthesis and hydrolysis.

Amino-acids may be products of hydrolysis only, and, as such, incapable of resynthesis to protein. In the presence of decreasing carbohydrate, they undergo deamination, with the formation of amides (asparagine and glutamine) in "amide" plants, and ammonium salts of organic (malic, succinic and oxalic) acids in "acid" plants. The non-nitrogenous part, either of the amide or salt, arises, in all probability, from some of the amino-acids deaminated, such as glutaminic acid or leucine, and not as a by-product of respiration.

Nitrogen may be translocated as amino-acids, amides (or ammonium salts of organic acids) or both.

At the growing-points sucrose, the sugar of translocation, may be rapidly hydrolysed, the γ-fructose being used in respiration, the inactive glucose in cell-wall formation. Since there may be relatively little carbohydrate present at the growing-point, amino-acids would

be converted into amides (or ammonium salts of organic acids), and these further yield ammonia.

Since nitrogen is difficult to attain, it is withdrawn from proteins of older organs to supply (*via* amino-acids and amides) the growing-points and younger organs. Ammonia from inorganic sources may recombine with deamidated asparagine and glutamine, or with organic acids. The non-nitrogenous remainders of the above may eventually be oxidised to carbon dioxide.

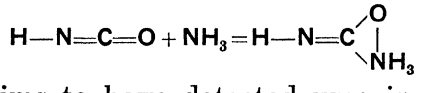
Many other cycles of nitrogen metabolism must take place in the plant; these involve nitrates, cyanogen compounds, purine bases, amines and alkaloids.

One of the most striking objections in regard to the above summary is the case of Prianischnikow's seedlings (p. 202). If these contain ample carbohydrate, they yield increased amounts of asparagine on solutions of ammonium salts, the non-nitrogenous part of asparagine being assumed to arise from carbohydrate. It is possible, however, to suppose that the asparagine is a down-grade product accumulating, because, in presence of carbohydrate, it cannot be further broken down (see Mothes, p. 226).

PURINE METABOLISM. (Bibliography IX)

We now turn our attention from ammonia in its *rôle* as a source of amino-acid and amide synthesis to its connection with purine metabolism.

The basal substance in this connection is probably urea, which can be regarded as a condensation product of cyanic acid and ammonia:



Fosse (3, 4) claims to have detected urea in small quantities in various plants, such as Spinach, Cabbage, Carrot, Potato and Chicory. It is more than probable that it is widely distributed, if not universally present, in the plant. Its absence, or presence in very small quantities only, may very well be due to the fact that it is rapidly synthesised into other substances.

There is also widely distributed in plants an enzyme, urease, which catalyses the decomposition of urea into ammonia and carbon dioxide (ammonium carbonate). Urease has also been shown to catalyse the reverse reaction[1] *in vitro*, and it is justifiable to assume that it does

[1] **Kay, H. D.** *Biochem. J.*, 1923, **17**, 277.

so in the plant. Thus we may suppose ammonia and carbon dioxide to combine in the plant with formation of urea:

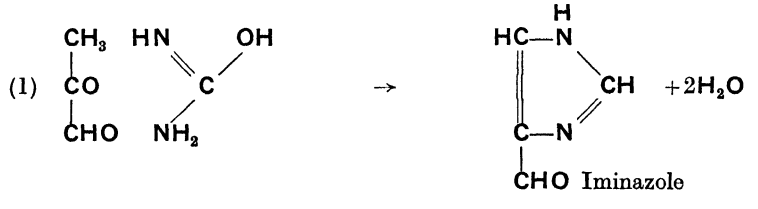
$$NH_3 + CO_2 = H{-}N{=}C{=}O + H_2O$$
$$H{-}N{=}C{=}O + NH_3 = HNCONH_3$$

The distribution of urease has been investigated by various authors. Zemplén (31) found it to be present in *Amorpha fruticosa*, *Robinia Pseud-acacia*, *Caragana arborescens*, *Lupinus albus*, *L. luteus*, *Cytisus Laburnum*, *Anthyllis vulneraria*, *Onobrychis sativa*, *Morus alba*, *Cannabis sativa*, *Vicia Faba*, *Trifolium incarnatum*, *Medicago lupulina*, *Paliurus aculeatus*, *Glycyrrhiza glabra*, *Colutea arborescens*, *Lotus corniculatus*, *Ulex europaeus*, *Pinus maritima*, *Ornithopus sativus*, *Medicago sativa*, *Spartium scoparium*, *Galega officinalis*, *Melilotus coeruleus*, *Pisum sativum*, *Taxus baccata*, *Capparis spinosa*, *Polygonum Fagopyrum*. [It was absent from summer Wheat, Barley, Rye and Oats, *Cercis Siliquastrum*, *Lathyrus odoratus*, stubble Beet, Poppy, *Sinapis alba*, *Helianthus annuus*, Flax, *Ervum Lens*, *Panicum album*, *Vicia sativa*, and field Maize.]

Kiesel & Troitzki (13) showed it to be present in *Vicia sativa*, *Phaseolus vulgaris*, *Pisum sativum*, *Angelica sylvestris*, *Helianthus annuus*, *Trifolium pratense*, *Lupinus albus* and *Secale cornutum*. [Absent from *Pyrus Malus* (flesh of fruit), *Citrus Limonum* (seeds), *Allium Cepa* (bulb scales), *Beta vulgaris* (root), *Daucus Carota* (root), Potato (tuber), *Pelargonium zonale* (leaves and stems).]

Given urea, we can next proceed to possible suggestions for the synthesis of some of the heterocyclic rings. They are, as previously stated, (1) the iminazole, (2) the pyrimidine, and (3) the purine.

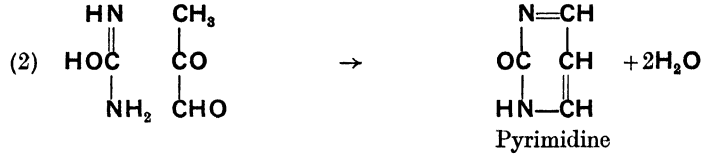
The iminazole ring can be considered to be formed from one molecule of urea and one molecule of methyl-glyoxal with elimination of water:



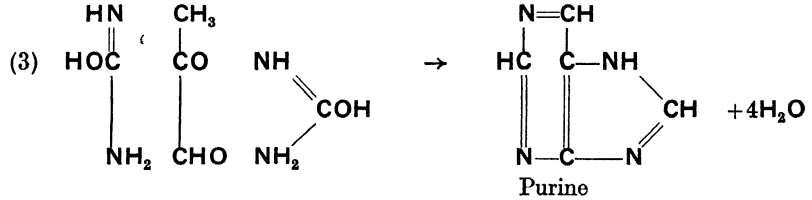
The iminazole ring occurs in histidine. Since methyl-glyoxal, as we have seen, is probably a product formed in respiration, it would be universally present, and we can see how this ring might be readily synthesised at any moment in the cell.

Similarly, the pyrimidine ring can be synthesised from one molecule

of urea and one molecule of methyl-glyoxal, and the purine ring from two molecules of urea and one molecule of methyl-glyoxal:

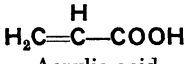


Pyrimidine

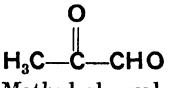


Purine

Thus the materials for these two rings are also readily available. Sometimes it is suggested that an unsaturated acid, such as acrylic acid, combines with urea to form the purine and pyrimidine rings, but the relationship is, in any case, very close between acrylic acid and methyl-glyoxal:

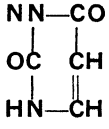


Acrylic acid

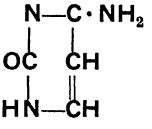


Methyl-glyoxal

The two chief pyrimidine bases which occur in the plant are **uracil** and **cytosine**. They are known only as components of nucleic acid (see below):

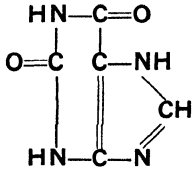


Uracil

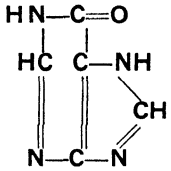


Cytosine

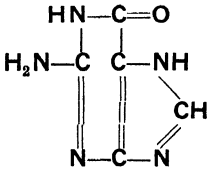
The purines occurring in plant tissues are **xanthine**, **hypoxanthine**, **guanine** and **adenine**. They are widely distributed, and have been isolated by Schulze from many plants. All four were also identified by Kiesel (Bib. VIII, 45) from ears of Rye. Schittenhelm (19) has identified an enzyme in Lupin seedlings which converts guanine into xanthine.



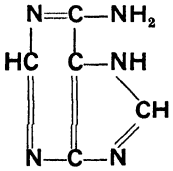
Xanthine



Hypoxanthine



Guanine



Adenine

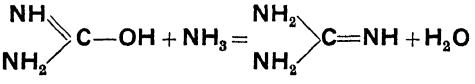
Caffeine and **theobromine** are methylated derivatives of xanthine. Methylation is common in the plant, formaldehyde or methyl alcohol being probably always available.

The interest of the purines centres largely in the fact that they form part of nucleic acid:

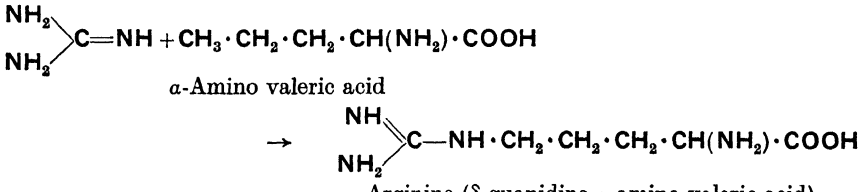
phosphoric acid—pentose—guanine
|
phosphoric acid—pentose—adenine
|
phosphoric acid—pentose—cytosine
|
phosphoric acid—pentose—uracil

The only nucleic acid investigated in the higher plants is that from Wheat embryos.

The next compound of interest in connection with urea is **guanidine**. Guanidine is urea in which the oxygen has been replaced by the imino group:



It has been found by Schulze in seeds of Vetch and in Beet root (?) residues. It is curious that guanidine combines with α-amino valeric acid to form the diamino-acid, arginine, which is always found, generally in large quantities, in plant proteins:



α-Amino valeric acid

Arginine (δ-guanidine-α-amino valeric acid)

There are two ways in which arginine may be split into simpler molecules in the plant, and, conversely, possibly, synthesised. One way has already been considered in connection with amino-acid synthesis, namely, deamination by oxidation:

(i) $NH_2 \cdot C(NH) \cdot NH(CH_2)_3 \cdot CH(NH_2) \cdot COOH + O_2$
$= NH_2 \cdot C(NH) \cdot NH(CH_2)_3 \cdot COOH + CO_2 + NH_3$

(ii) $NH_2 \cdot C(NH) \cdot NH(CH_2)_3 \cdot COOH + O_2$
$= NH_2 \cdot C(NH) \cdot NH_2 + COOH \cdot CH_2 \cdot CH_2 \cdot COOH$
Guanidine Succinic acid

There is no special experimental evidence in support of this sequence of events in plants, except the occurrence of guanidine, and the presence of asparagine as a derivative of succinic acid, and therefore a breakdown product of arginine.

The second way in which arginine may be split in the plant is by the enzyme, arginase, which catalyses the decomposition into urea and ornithine (α-, δ-diamino valerianic acid):

$$NH_2 \cdot C(NH) \cdot NH(CH_2)_3 \cdot CH(NH_2) \cdot COOH + H_2O = \underset{\text{Urea}}{NH_2 \cdot C \cdot NH \cdot OH} + \underset{\text{Ornithine}}{NH_2 \cdot (CH_2)_3 \cdot CHNH_2 \cdot COOH}$$

Ornithine has not yet been found in the plant, but the decomposition of arginine has been demonstrated by Kiesel (9) *in vitro*. He found that the expressed juice of *Lupinus luteus* would split arginine; later (10), he again demonstrated the same action with ground (etiolated) seedlings of white and blue *Lupinus*, but in no case were the products detected. With Wheat seedlings, however, ornithine was detected. The urea formed was decomposed by urease, which was shown to be present in both Wheat and Lupin (etiolated) seedlings. None of the preparations affected guanidine. Still later (12) ornithine was obtained from arginine in *Vicia sativa* seedlings, and both arginase and urease were shown to be present.

Proteolytic Enzymes. (Bibliography x)

In the plant tissues, the process of hydrolysis of proteins and, presumably, that also of synthesis is catalysed by proteolytic enzymes. The same ultimate units, the amino-acids, are produced as in acid hydrolysis. Both these and the intermediate products, proteoses, peptones and polypeptides, are undoubtedly present in all living tissues.

The presence of proteolytic enzymes has been demonstrated by their action in autolysis of tissues. Butkewitsch (12, 13) found that the amino nitrogen increased in autolysis of seedlings of Lupin, Castor-oil plant and Bean. A crude alcoholic precipitate of the enzyme from a glycerol extract also acted upon conglutin, the protein of Lupin. From the digestion mixtures, in both cases, leucine and tyrosine were identified. Schulze & Castoro (Bib. viii, 134) identified arginine, leucine and tyrosine from the autolysis of Lupin seedlings. An increase of soluble nitrogenous substances and a loss of protein was demonstrated also by Zaleski (Bib. viii, 178) on the autolysis of a preparation, by acetone, from unripe seeds.

A new conception was introduced by Vines (21–31), namely, that the proteolytic enzymes of the higher plants are of two types: (1) enzymes of the pepsin type which hydrolyse proteins, through proteoses to peptones; (2) enzymes of the erepsin type which

hydrolyse peptones to amino-acids. Vines was indeed able to detect the formation of tryptophane on autolysis of a number of different kinds of tissues—from resting and germinating seeds, leaves, fruits, bulbs and roots. Separately, the erepsin can be much more readily detected than the pepsin; Vines demonstrated that many plant tissues will form tryptophane when added to peptone. It was less easy to demonstrate the presence of the pepsin by action on fibrin, especially in leaves, though he maintains it can be done in the case of seedlings of Bean, Pea, Lupin, Maize and others; in fruits of Cucumber and Melon and in bulbs of Tulip and Hyacinth.

Vines claims to have separated the erepsin from the pepsin, in the seed of Hemp, by fractional precipitation of a 10 % sodium chloride extract with dilute acetic acid.

An erepsin, rather more carefully prepared by precipitation with ammonium sulphate from Cabbage (Blood, 11), was found to produce tryptophane from peptone, but it did not act, apparently, upon either animal or vegetable proteins, nor did it produce tryptophane on autolysis, i.e. it could not act upon the proteins of the Cabbage precipitated with it.

The presence of proteolytic enzymes in the vegetative parts of plants was shown by Fisher (18). He used the whole plant, dried and very finely ground, of Barley, Oats, Maize, Rye, Clover, Lucerne, Vetch, Pea, Bean, Buckwheat and Mustard. This material was added both to Witte peptone and to a plant protein, legumin, and the increase in amino-acid nitrogen estimated quantitatively. Evidence was obtained of the existence of both types of enzyme in all the plants, though the amount of hydrolysis varied. The activity of the enzyme increases with increasing maturity of the leaves, and, in seedlings, with the progressive germination of the seed.

Though not noted by the author himself, a fact of further interest emerges from Fisher's work, namely, that the enzymes of the Pea plant hydrolyse the legumin employed much more readily than the proteins of the other plants. This fact was later discovered by Blagoveschenski (8). This author made a series of experiments with proteins extracted from the seeds of a number of plants and the enzymes from the corresponding germinated seedlings. The seeds used were of Hemp, Rape, Lupin, *Phaseolus* sp., *Hibiscus*, *Dolichos* and *Lathyrus*. The protein (globulin) was extracted from the seeds, and the juice of the seedling was used as a source of enzyme. Many different combinations of enzyme and protein were made, and the

amino nitrogen was determined quantitatively both before and after incubation.

Assuming that the maximal action for each enzyme is equal to 100, the values of the others are as follows:

Enzymes from	Globulins from			
	Phaseolus	*Lupinus*	*Brassica*	*Cannabis*
Phaseolus	100	38	18	71
Lupinus	72	100	—	38
Brassica	0	73	100	—
Cannabis	0	0	0	100

Enzymes from	Globulins from				
	Phaseolus	*Dolichos*	*Cannabis*	*Hibiscus*	*Lathyrus*
Phaseolus	100	99	20	59	4
Dolichos	67	100	—	—	53
Cannabis	—	—	100	44	0
Hibiscus	—	84	—	100	26

that is, the enzyme from every plant hydrolyses its own globulin more readily than the globulins of other plants.

Blagoveschenski & Bielozerski (10) have also demonstrated another point with regard to the enzymes acting upon peptone (called by them, peptase), namely, that the p_H optimum varies and is specific for each plant. The powdered dried leaves of the Apple, Pear, Vine, Walnut, Cotton Plant, Lilac, Ash, Plane and Oak were used, and the increase in amino-acid estimated. The p_H optimum varied from 4·5 to 7·7.

Finally Blagoveschenski (9) claims to have shown that the proteolytic enzymes of seeds of *Phaseolus Mungo* can synthesise polypeptides from leucine and glycine. A mixture of these two amino-acids with the enzyme in strong glycerol shows a decrease of amino nitrogen.

Proteoses, peptones and polypeptides

The presence of these compounds in plant tissues is not readily demonstrated, since they are transitional substances, and only small quantities are present at any moment. It appears probable that among other polypeptides, glutathione is present in many tissues. Kozlowski (Bib. VI, 84) has shown that a compound with the properties and reactions of glutathione is present in the seeds of the Pea.

Its actual components, however, have not been satisfactorily demonstrated.

Amino-acids. (Bibliography VIII)

Almost all the amino-acids have now been isolated, often in considerable quantities, from plant tissues. The researches of most value in this direction are those of Schulze and his co-workers. Schulze largely employed germinating seedlings of many plants as his material. By very careful methods of isolation, he not only discovered the two amino-acids, arginine and phenylalanine, but he also isolated most of the other known amino-acids, frequently from a number of different plants; only leucine and iso-leucine had previously been extracted from plants. Schulze's researches are published in a series of papers extending from about 1880 to about 1911 (see also Table LI).

Now let us consider the individual amino-acids. Schulze did not isolate glycine, alanine, serine or cystine from plant tissues. **Glycine** and **cystine**, as we have seen, occur in very small amounts in plant proteins. Quite recently, Vickery (167) claims to have isolated **alanine** and **serine** for the first time from plant tissues. He used the juice from about 24 kilos of the Lucerne plant or Alfalfa (*Medicago sativa*), and obtained these two acids by the ester distillation method.

Valine was isolated by Schulze from seedlings of Lupin and Vetch.

Leucine was also isolated by Schulze from the same seedlings, and also from buds of Horse Chestnut.

Aspartic acid. This acid, in its free state, does not, as a rule, appear as a product in proteolysis, but as asparagine. Kiesel (45) claims to have isolated considerable quantities of free aspartic acid from ripening ears of Rye.

Asparagine, the amide of aspartic acid, is found widely and abundantly distributed in plant tissues. It has possibly an important function in protein synthesis, to which reference has already been made. It has been isolated from many plants by various workers, though chiefly by Schulze. He isolated it from seedlings of Vetch, Lupin, Fir, Pine, Wheat, Maize, Poppy, Nasturtium, Sunflower, Vegetable Marrow; from buds of the Plane, Sycamore, Horse Chestnut, Lime, Beech, Poplar, Birch, Alder and Vine; from tubers of Potato, Dahlia and Artichoke. He found asparagine in all Leguminosae and Graminaceae examined.

Glutaminic acid, in the same way as aspartic acid, does not

occur in the free state, but as the amide glutamine in proteolytic products.

Glutamine is widely distributed. It is less easily detected than asparagine as it does not crystallise so readily. It was also discovered by Schulze, and has been isolated by him from seedlings of Vetch, Gourd, Rape, Soapwort, Beet, Fir, Castor Bean, Sunflower, Radish, Cress, Spinach; roots of Carrot, Beet, Radish, Celery. Schulze maintains that glutamine, and not asparagine, is common to the Cruciferae and Caryophyllaceae.

Arginine was first discovered by Schulze and isolated from seedlings of Lupin, Vegetable Marrow, Castor Bean, Vetch and Pea. It is especially abundant in seedlings of Fir and Pine, and has been extracted from roots of Chicory and Artichoke. It has also been found by Kiesel (37) in Clover leaves.

Lysine was isolated by Schulze from seedlings of Vetch, Pea and Lupin.

Phenylalanine was first discovered by Schulze, and isolated by him from seedlings of Soy Bean, Vetch, Lupin and Pea.

Tyrosine is rather readily detected, and has been isolated from many plants, by Schulze from seedlings of Vegetable Marrow, Lupin, Castor Bean, Vetch, Pea; also from tubers of Potato and Dahlia, and roots of Beet.

Proline has been isolated from Lupin seedlings by Schulze & Winterstein.

Histidine has been isolated by Schulze from seedlings of Lupin, Pea, Soy Bean and Vetch.

Tryptophane has been isolated by Schulze from seedlings of Lupin and Vetch.

Dihydroxyphenylalanine is an amino-acid which does not occur in proteins, as far as is known. It is present in considerable amounts in the Broad Bean (*Vicia Faba*) (Guggenheim, Bib. VI, 73) and in the Velvet Bean (Miller, Bib. VI, 91).

Schulze, in his long series of papers, notes many subsidiary phenomena. The amount of different amino-acids varies considerably at different ages of the seedling, so that some observers have declared some amino-acids, as, for example, tyrosine, to be absent, while others have found it to be present. Also the amino-acids varied in the same

TABLE LI. (Bibliography VIII.) Distribution of amino-acids. (Results based on researches of Schulze and his co-workers, except when otherwise stated)

Amino-acid	Plant
Glycine	—
Alanine	*Medicago sativa* (Vickery, 167)
Valine	Seedlings of *Lupinus albus, L. angustifolius, L. luteus, Vicia sativa*; plant of *Medicago sativa* (Vickery, 167)
Leucine	Seedlings of *Cucurbita Pepo, Lupinus albus, L. angustifolius, L. luteus, Pisum sativum, Vicia sativa*; root or tuber of *Beta vulgaris, Solanum tuberosum*; pods of *Pisum sativum*; plant of *Medicago sativa* (Vickery, 167)
Iso-leucine	Seedlings of *Lupinus albus, Vicia sativa*
Serine	Plant of *Medicago sativa* (Vickery, 167)
Aspartic acid	Ears of *Secale cereale* (Kiesel, 45)
Asparagine	Seedlings of *Arrhenatherum elatius, Cucurbita Pepo, Helianthus annuus, Lupinus albus, L. angustifolius, L. luteus, Lolium perenne, Phleum pratense, Papaver somniferum, Picea excelsa, Pinus sylvestris, Soja hispida, Triticum vulgare, Tropaeolum majus, Vicia sativa, Zea Mays*; buds of *Acer campestre, A. pseudoplatanus, Aesculus Hippocastanum, Alnus* sp., *Betula alba, Fagus sylvatica, Platanus orientalis, Populus nigra, Tilia parviflora, Vitis vinifera*; roots or tubers of *Althaea officinalis, Apium graveolens, Beta vulgaris, Brassica oleracea, B. Napus, Dahlia variabilis, Helianthus tuberosus, Scorzonera hispanica, Solanum tuberosum*; plants of *Avena sativa, Medicago sativa, Trifolium pratense, Vicia Faba* (Prianischnikow, 72), *V. sativa*; pods and unripe seeds of *Pisum sativum, Phaseolus vulgaris, Vicia sativa*; embryo of *Triticum*
Glutamine	Seedlings of *Brassica Napus, Camelina sativa, Cucurbita Pepo, Helianthus annuus, Lepidium sativum, Picea excelsa, Raphanus sativus, Ricinus communis, Sinapis alba, Spergula arvensis, Spinacia glabra*; leaves of *Beta vulgaris, Brassica oleracea, B. Napus, Saponaria officinalis*; roots of *Apium graveolens, Beta vulgaris, Brassica oleracea, Daucus Carota, Raphanus sativus, Stachys tubifera*; young seeds of *Pisum sativum*
Arginine	Seeds of *Helianthus annuus, Lupinus albus, L. luteus, Soja hispida, Pinus Cembra, Pisum sativum*; embryo of *Arachis hypogaea, Triticum*; seedlings of *Abies pectinata, Cucurbita Pepo, Lupinus albus, L. luteus, Picea excelsa, Pinus sylvestris, Pisum sativum, Ricinus communis, Soja hispida, Vicia sativa*; roots of *Brassica Napus, Cichorium Intybus, Helianthus tuberosus, Ptelea trifoliata*; young plant of *Medicago sativa*; leaves of *Trifolium pratense* (Kiesel, 37); pods and young seeds of *Pisum sativum, Phaseolus vulgaris* and *Vicia sativa*
Lysine	Seedlings of *Lupinus albus, L. luteus, Pisum sativum, Vicia sativa*; plant of *Medicago sativa* (Vickery, 167); young seeds of *Pisum*
Cystine	—
Phenylalanine	Seedlings of *Lupinus albus, L. luteus, Pisum sativum, Soja hispida, Vicia sativa*; plant of *Medicago sativa* (Vickery, 167); ears of *Secale cereale* (Kiesel, 45)
Tyrosine	Seedlings of *Arachis hypogaea, Cucurbita Pepo, Lupinus albus, L. angustifolius, L. luteus, Pisum sativum, Ricinus communis, Tropaeolum majus, Vicia sativa*; roots or tubers of *Beta vulgaris, Brassica oleracea, Dahlia variabilis, Solanum tuberosum*; plant of *Medicago sativa* (Vickery, 167); young seeds of *Pisum*; pods of *Phaseolus vulgaris*
Proline	Seedlings of *Lupinus albus*
Histidine	Seedlings of *Lupinus albus, L. luteus, Pisum sativum, Soja hispida, Vicia sativa*; pods and unripe seeds of *Pisum*
Tryptophane	Seedlings of *Lupinus albus, Vicia sativa*; pods of *Pisum sativum, Phaseolus vulgaris*

species, according to whether it was germinated in light or darkness. For instance, green seedlings of *Vicia sativa* had only leucine; etiolated seedlings, leucine, valine and phenylalanine; green seedlings of *Lupinus albus* had leucine; etiolated seedlings, phenylalanine and valine. Different species of the same genus, also, may contain different amino-acids. *Lupinus albus* and *L. angustifolius* had no arginine, though it was present in *Lupinus luteus*.

Schulze suggests that probably all the amino-acids are present qualitatively, but in quantitatively different amounts, since the metabolism of different seedlings progresses at different rates during germination. One special point of interest was noted by Schulze in regard to glutamine and asparagine. Though, apparently, each of these amides is characteristic, on the whole, of various genera, if not orders, it is curious that certain seedlings, as, for instance, those of the *Cucurbita Pepo* and *Picea excelsa*, are rich in glutamine and poor in asparagine in one culture, whereas in other cultures they have no glutamine, but much asparagine. In Sunflower seedlings the same phenomenon may occur, and also in roots of Beet and Kohl-rabi, sometimes one amide preponderating, sometimes another.

It is interesting to note the results at this stage obtained by Vickery (167) in his estimation of the free amino-acids in Lucerne. He isolated a number of amino-acids from Lucerne, using the expressed juice from about 24 kilos of material. He employed various precipitants, lead acetate, phosphotungstic acid, mercuric salts, etc.; the point of interest, especially, is the idea given of the amounts of free amino-acids in 100 gm. of a fresh plant, such as Lucerne:

Alanine	·005 gm.	Arginine	·008 gm.
Valine	·012 ,,	Lysine	·001 ,,
Leucine	·009 ,,	Phenylalanine	·003 ,,
Serine	·010 ,,	Tyrosine	·0005 ,,
Asparagine	·101 ,,		

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CHAPTER VI

RESPIRATION

SUCH facts as we know in connection with the metabolism of respiration of the higher plants definitely point to a similarity between certain stages of this process and those of fermentation by Yeast. In the case of the latter organism there is a great accumulation of knowledge, and so rapid is the progress of experimental research that it is scarcely possible to give an up-to-date account of the subject. Since knowledge of the metabolism of respiration is scanty in regard to the higher plants, the simplest representation of the suggested lines of metabolism of fermentation by Yeast will suffice for the present as a possible model for the higher plants.

METABOLISM OF FERMENTATION BY YEAST

Extensive researches on fermentation, respiration and general metabolism of Yeast have been carried out by Euler, Harden, Neuberg, Meyerhof, and other workers. As an outcome of investigation, some of the metabolic reactions involved in fermentation have been determined, and are very briefly summarised in the following pages.

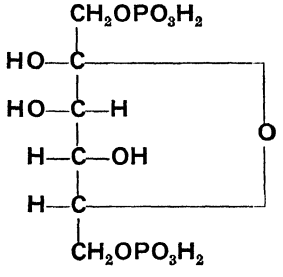
It is known that phosphate (R_2HPO_4) accelerates the fermentation of sugar solutions by Yeast juice and dried preparations of Yeast. On living Yeast, phosphate has no effect. Recently, however, Harden[1] has expressed his views as follows: "Neither arsenate nor phosphate has an accelerating action on the rate of fermentation by living Yeast. This may be due to the fact that the supply of inorganic phosphate in the interior of the Yeast cell is already optimal, but some doubt exists as to whether or not these salts freely penetrate the cell.... I have assumed up to now that the processes in the living cell are essentially of the same kind as those which occur in the various preparations made from the dead cell, but differ from these mainly in the relative intensity of some of the reactions, and I know no valid argument against this assumption.

"The cycle undergone by the phosphate in the series of changes which constitutes ordinary fermentation clearly consists in the alternate formation of a phosphoric ester and the hydrolysis of this to free phosphoric acid. A simple calculation based on the phosphorus content of living Yeast shows that the whole of this phosphate must pass

[1] **Harden, A.** *Nature*, 1930, **125**, 277; **Harden, A., and Macfarlane, M. G.** *Biochem. J.*, 1930, **24**, 343.

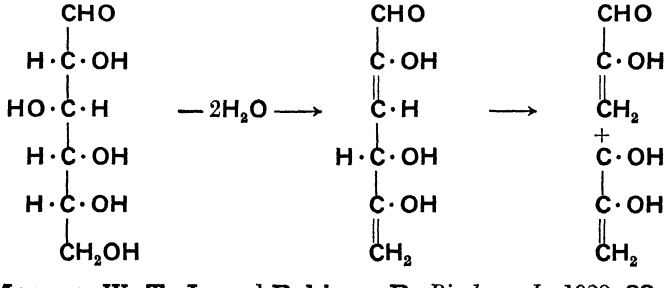
through the stage of phosphoric ester every five or six minutes in order to maintain the normal rate of fermentation, whereas in an average sample of Yeast juice the cycle, calculated in the same way, would last nearly two hours."

As outlined above, the processes which underlie this acceleration are considered to be the formation, by esterification of phosphoric acid, of hexose-phosphates, thereby affecting a change in the hexose molecule, and a subsequent hydrolysis again of the hexose-phosphate. The reactions are catalysed by an enzyme, hexosephosphatase. Glucose, fructose and mannose, which are all fermented with ease by Yeast, form an ester with phosphoric acid, hexose-diphosphate. At the same time, other mono-phosphates are formed; the exact relationship of mono- to di-phosphates is not yet defined, and does not seriously concern us at the moment. What is of importance is the fact that, whichever sugar is fermented by Yeast juice, the diphosphate formed during esterification is a fructose diphosphate. Further, that the fructose in the phosphate molecule is probably present in the active, or so-called γ-form[1]:



General considerations of the splitting of the hexose molecule lead to the view that the active form of hexose is more readily resolved into 3-carbon compounds than the inactive.

The first stage of splitting of hexose is considered to lead to the formation (after enolisation) of two molecules of pyruvic aldehyde (methyl-glyoxal):



[1] **Morgan, W. T. J.**, and **Robison, R.** *Biochem. J.*, 1928, **22**, 1270.

Then a Cannizzaro reaction takes place, catalysed by an oxido-reductase, with the formation of an alcohol (glycerol) and an acid (pyruvic acid) respectively:

$$\begin{array}{l} CH_2{=}C(OH)\cdot CHO + H_2O \\ CH_2{=}C(OH)\cdot CHO \end{array} + \begin{array}{c} H_2 \\ | \\ O \end{array} = \begin{array}{l} CH_2(OH)\cdot CH(OH)\cdot CH_2(OH) \\ + \\ CH_2{=}C(OH)\cdot COOH \end{array}$$

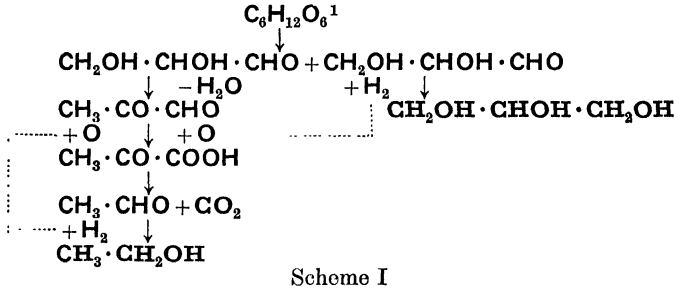
Then the decomposition of pyruvic acid, a keto-acid, is catalysed by the enzyme, carboxylase, with the evolution of carbon dioxide and formation of acetaldehyde:

$$CH_3\cdot CO\cdot COOH = CH_3\cdot CHO + CO_2.$$

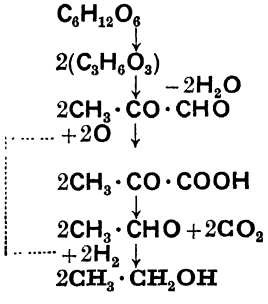
Further, two different aldehydes, acetaldehyde and pyruvic aldehyde, by a Cannizzaro reaction, give rise to pyruvic acid and alcohol:

$$\begin{array}{l} CH_3\cdot CO\cdot CHO \\ CH_3\cdot CHO \end{array} + \begin{array}{c} O \\ | \\ H_2 \end{array} = \begin{array}{l} CH_3\cdot CO\cdot COOH \\ + \\ CH_3\cdot CH_2OH \end{array}$$

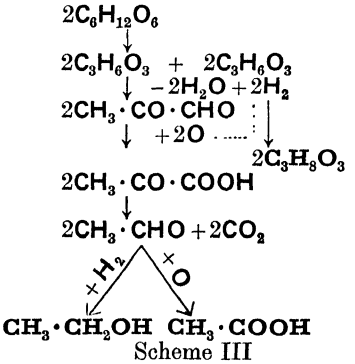
Then pyruvic acid is again converted into acetaldehyde and carbon dioxide. The whole series of the above reactions is summarised in Scheme I (see below).



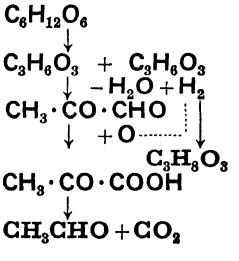
Scheme I



Scheme II
(Neuberg's 1st form)



Scheme III
(Neuberg's 3rd form)



Scheme IV
(Neuberg's 2nd form)

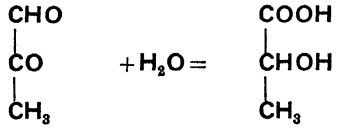
If the four schemes arranged above be examined, it will be seen that, in regard to the Cannizzaro reaction, there are various possibilities. It may take place between two molecules of methyl-glyoxal, or between one molecule of methyl-glyoxal and one molecule of

[1] Absolute number of molecules not accounted for.

acetaldehyde (Schemes I and II). There is also the possibility of its taking place between two molecules of acetaldehyde (Scheme III). As a matter of fact Neuberg has shown that various conditions of the medium will affect the preponderance of one reaction over another. Under some conditions, acetaldehyde alone may act as hydrogen acceptor in the oxidation of pyruvic acid. When alkaline carbonates or phosphates are added after fermentation has started, methyl-glyoxal also acts as an acceptor, a Cannizzaro reaction taking place at the same time between molecules of acetaldehyde. This type of fermentation is known as Neuberg's third form of fermentation.

Another variation of the original scheme of fermentation may be brought about by introducing sodium or calcium sulphite into the fermentation mixture. Acetaldehyde is fixed by the sulphite and leads to an increased formation of this product. At the same time there is a corresponding increase in glycerol formed. Neuberg proposes the course of events shown in Scheme IV and known as his second form of fermentation.

Zymase is the name given to the catalyst, expressed out in Yeast juice, first associated with the fermentation of hexoses with formation of carbon dioxide and alcohol. It is clear that zymase must contain carboxylase. How many additional enzymes are concerned is not yet defined. Neuberg has shown the presence in Yeast of a catalyst, glycolase, which produces methyl-glyoxal from hexose-phosphate, and yet another called by him ketonaldehydemutase, which will, by an internal Cannizzaro reaction, form lactic acid from pyruvic aldehyde (methyl-glyoxal):



There is further the evidence for an aldehydemutase, which will give rise either to acetic acid and ethyl alcohol or to pyruvic acid and ethyl alcohol according to the conditions of fermentation.

From zymase, a thermo-labile co-enzyme, co-zymase, can be removed by dialysis and other treatments. The inactive residue, apo-zymase, is incapable of bringing about fermentation, but can be reactivated by addition of the co-enzyme. The question as to whether the co-enzyme activates the component catalyst of zymase which splits the sugar molecule or the mutase or both does not seriously concern us at present.

Another observation of far-reaching importance is that of Meyerhof[1]. This author has shown that the presence of oxygen affects the fermentation of sugar in a quantitative relationship. One molecule of respired oxygen suppresses a fermentation equivalent to $1\frac{1}{2}$–2 molecules of carbon dioxide, or, in other words, through the oxidation of one molecule of sugar, 4–6 other sugar molecules are protected from fermentation. The importance of this relationship lies in the fact that the respiratory quotient is completely independent of the connection between amount of fermentation and respiration, which in different strains of yeast can fluctuate more than 30 times in amount.

Metabolism of respiration in the higher plants: General considerations

It is proposed first to enumerate shortly the points on which there may be resemblance between the metabolic processes of respiration of the higher plants and of fermentation by Yeast. Later, these points will be considered in detail.

There is very definite evidence from sugar analyses of respiring plant organs that a hexose sugar is the substrate for respiration. Further, the carbohydrate balance sheet of an organ which is respiring, though not synthesising, sugar, indicates the possibility that the γ-fructose formed from hydrolysis of sucrose is preferentially respired.

A number of early workers—L. Iwanoff, N. Iwanoff, Kostytschew and Zalcski—sought to ascertain whether, on analogy with Yeast preparations, phosphates had an accelerating effect on the respiration of seeds and vegetative organs; they used, as material, plants and seeds which had been killed by freezing or drying, but in which, it was hoped, the enzymes were intact, so that the research may be termed one upon post-mortem respiration rather than respiration of the living plant. Though most workers were in agreement that phosphates, both inorganic and organic (from Yeast-fermented solutions), increased the output of carbon dioxide, opinion was not unanimous as to the cause of the increase.

Later, Bodnár & Hoffner (6), by experiments on similar lines, showed that when a meal from dried germinated peas was moistened with phosphate (K_2HPO_4) solution, the output of carbon dioxide and alcohol in anaerobic respiration was nearly double that produced when

[1] **Meyerhof, O.** *Biochem. Zs.*, 1925, **162**, 43.

the meal was moistened with water alone. From this they deduce that the phosphate increases not only the output of carbon dioxide, but also that of alcohol. The formation of alcohol in anaerobic respiration of the higher plants will be considered later (see p. 295).

In connection with the question as to the existence of a hexose-phosphatase in the higher plants, Bodnár (4) also claims to have shown that ground pea meal will convert inorganic into organic phosphate, probably by esterification. Neuberg & Kobel (61), moreover, have demonstrated the presence of hexosephosphatase in *Nicotiana Tabacum* through the hydrolysis, by means of leaves pounded in water, of sodium and magnesium hexose-diphosphates. Such a catalyst is probably widely distributed.

The possibility of obtaining a crude preparation of zymase from the higher plants was long ago demonstrated by Stoklasa & Czerny (104). These workers obtained a precipitate, by alcohol, from the juice of potatoes, beet and other tissues, which fermented sugars actively with the production of carbon dioxide and alcohol in theoretical proportions. Stoklasa's preparation of zymase was later confirmed by Bodnár (3).

Assuming, on the above grounds, that the zymase enzymes of higher plants and Yeast are similar, in that they bring about the fermentation of sugar to carbon dioxide and alcohol, we may briefly consider the other catalysts known to be associated with the various stages of decomposition of sugar. How far these additional catalysts are to be regarded as components or not of the zymase complex does not seriously affect us at present.

The actual splitting of the glucose molecule into methyl-glyoxal (pyruvic aldehyde) is, presumably, the function of glycolase. Methyl-glyoxal has been detected in Tobacco leaves (Kobel & Scheuer, 134). The dismutation of methyl-glyoxal to pyruvic acid and glycerol is assumed to be catalysed by an oxido-reductase (dehydrase, dehydrogenase). Such a catalyst, methylglyoxalase or ketonaldehydemutase, which, as already mentioned in the preceding section, converts methyl-glyoxal, by an inner mutation, into lactic acid, has been shown to be present in Yeast. Neuberg & Gorr (57) also demonstrated its presence in peas, beans (*Vicia Faba*) and Lupin (*Lupinus albus*) seeds, and Neuberg & Kobel (61) in Tobacco leaves.

For the presence of carboxylase in the higher plants, there is ample evidence. It was shown by Zaleski & Marx (122) that peas, beans and Lupin seeds would form acetaldehyde and carbon dioxide from pyruvic

acid. Bodnár (3), moreover, obtained a crude precipitate of carboxylase from Potato and Beet juice pressed out in a Buchner press.

The formation of acetaldehyde, as, apparently, an intermediate product of respiration in Pea meal, was first demonstrated by Neuberg using his *Abfangmethode* of fixing the aldehyde with sulphite. Neuberg's experiments were carried out anaerobically, but Klein & Pirschle (30) claim to have shown fixation of acetaldehyde in normal respiration of a number of different vegetative organs.

The exact products which arise in the final stage of respiration in the higher plants depend upon the presence or absence of oxygen. Many investigators have provided ample evidence that, in absence of oxygen, both living and dead tissues form alcohol as the result of reduction of acetaldehyde, the ratio of carbon dioxide to alcohol often agreeing with the theoretical value. In other cases, very little or no alcohol is produced, some other substance than aldehyde acting, probably, as hydrogen acceptor in anaerobic oxidation. In normal respiration, of course, alcohol is not formed.

In regard to the dismutation of acetaldehyde, there has been no satisfactory demonstration that this process takes place in the higher plants. Zaleski & Schatalowa-Zaleskaja (125) and Bodnár & Bernauer (5) have obtained a disappearance of acetaldehyde added to ground up plant tissues, but there is no real evidence, from estimation of acid and alcohol, that a Cannizzaro reaction has taken place. Since Zaleski & Marx (122), however, claim to have obtained alcohol from pyruvic acid added to Pea meal, possibly acetaldehyde in a nascent condition (from pyruvic acid) is essential for dismutation.

It is appropriate before turning to the aerobic stage of respiration to mention that research on the question of a co-enzyme in the higher plants is not lacking. Bodnár & Hoffner (6) maintain that dialysed Pea meal loses its power of producing carbon dioxide and alcohol, and that this can be restored by addition of boiled extract of the meal. The question as to whether the co-enzyme activates, in addition to apo-zymase, the catalysis of esterification of phosphate and of dismutation of acetaldehyde has been investigated by Zaleski & Pissarjewsky (124) and by Zaleski & Schatalowa-Zaleskaja (125).

The products of the final stage of normal respiration are carbon dioxide and water, and it would appear that acetaldehyde must be oxidised to these compounds, since it neither accumulates nor is reduced to alcohol. We have at present, however, no definite knowledge of the process of oxidation of aldehyde in the plant.

There is yet another line of metabolism in which acetaldehyde may be concerned, namely a condensation with pyruvic acid. Such a combination forms the starting point of a series of reactions, in which repeated condensations of aldehydes, of ever-increasing length of carbon chain, with pyruvic acid may lead eventually to the formation of fats.

Finally, there is the question of participation of molecular oxygen in respiration, i.e. the mechanism by which the hydrogen received by hydrogen acceptors is finally oxidised in air, and by which also acetaldehyde is oxidised. The theory of Palladin of a system of aromatic respiration pigments and oxidases is not satisfactory, since such a system is not universally distributed. Other systems, suggested by animal biochemists, have not yet found acceptance in the case of the plant.

Another complexity is introduced into the study of the metabolism of aerobic respiration by the discovery (Blackman, 2) that about three times as much sugar is decomposed in glycolysis as is finally oxidised to carbon dioxide and water. These facts imply an oxidative anabolism at some stage, but no suggestions have been made as to how it may be carried out.

THE SUBSTRATE FOR RESPIRATION

It is clear that, in the case of Yeast, any one of the three hexoses, glucose, fructose or mannose, can be regarded, as far as is here significant, as equally readily fermented. Sucrose or maltose, moreover, are hydrolysed, and the resultant sugars decomposed. From the previous section, we conclude that an esterification of phosphoric acid by hexose occurs, and that di- and mono-phosphates are formed. It has been shown that the diphosphate is probably a compound of γ-fructose and phosphoric acid (Morgan & Robison) and the identification of a monophosphate of γ-glucose has been claimed by Levene & Raymond.[1]

The conclusion obviously to be drawn is that, as far as Yeast is concerned, on esterification or phosphorylation, hexose is probably "activated" to the γ-form, this being, presumably, more readily split into the intermediate 3-carbon compounds of the first stage of respiration. In the case of sucrose, though one half of the molecule is already present as γ-fructose, this apparently does not result in any selective fermentation.

[1] **Levene, P. A.,** and **Raymond, A. L.** *J. Biol. Chem.*, 1929, **81**, 279.

When we turn to the higher plants, there is definite evidence that it is, in all probability, a hexose which constitutes the substrate for respiration; what additional deductions can be made as to the precise nature of the hexose are set out in the following pages.

In order to find out such clues as are available, it is necessary, as we shall see later, to have a complete balance-sheet of carbohydrate interchange during metabolism. At the outset it may be suggested that there are various possibilities in the higher plants as follows:

(1) That the sole source of substrate for respiration is γ-fructose provided by the hydrolysis of sucrose.

(2) That, in addition to this γ-fructose, normal stable glucose, mannose or fructose from any source, can be readily converted into the γ-form, and can thus also serve as a substrate; then, as in the case of yeast, it would be immaterial which of these sugars is available in the plant.

(3) That γ-fructose (from sucrose) is preferentially respired. When the supply of this sugar fails, then normal stable hexose serves as a last resource. The latter is either converted into the γ-form and split as usual, or is oxidised in some other way.

Let us now examine what evidence there may be in favour of any of these suggestions given above. First, it should be recalled that in Chapter I (p. 47) a hypothetical case has been made out in favour of γ-fructose and γ-glucose as all-important sugars of metabolism of the higher plants. It was there suggested that a γ-hexose may be a product of photosynthesis. We know that in photosynthesis sucrose is always a product, and, usually, but not always, starch too is formed to a greater or less extent. In the most actively growing and respiring organs there is relatively less sucrose to hexose; in older and more mature organs, the sucrose may increase considerably. In the same way, the amount of starch increases during active photosynthesis as well as in storage organs which have ceased to grow and do not respire actively. When growth takes place in absence of photosynthesis, both sucrose and starch are rapidly hydrolysed. It is clear, moreover, from observations on interchange of carbohydrates during storage of fruit[1] (see Table LIV), that, as starch disappears, sucrose, though constantly undergoing hydrolysis, may even accumulate. The similar replacement of starch by sucrose in leaves and Potato tubers on desiccation has been considered in detail in Chapter I (pp. 55–58).

[1] **Bridel, M., et Bourdouil, C.** Sur la transformation des glucides au cours du mûrissement des bananes. *C.R. Acad. sci.*, 1929, **189**, 543–545.

We can assume therefore that sucrose may be formed directly from the products of hydrolysis of starch. Conversely, though starch may be formed, on sugar-feeding, from several of the sugars found in the plant, there is evidence that, of these, it is most readily produced from sucrose (see p. 53).

There is a certain amount of experimental evidence in favour of the view that γ-fructose arising from sucrose is either preferentially respired or may form the sole substrate for respiration. On the basis of either assumption, the following relationship should hold in respiring organs (when there is a supply of sucrose or starch and yet no increase of carbohydrate due to photosynthesis) namely, the sugar lost in respiration should, during any period, be equivalent in amount to half the sucrose hydrolysed during that period. Also there should be a gain of hexose equivalent to the unrespired glucose derived from sucrose hydrolysed.

The task of determining, by means of a balance-sheet of carbohydrate interchange, if such a relationship exists, is rendered possible only by using organs detached from the plant and prevented from synthesising additional carbohydrate. Fruits in storage, such as apples, pears and bananas, are useful for this purpose.

The following data are reproduced from analyses made by Evans (Bib. I, 38) of sugars in samples of apples taken at certain intervals from storage at definite temperatures. During storage, sucrose is hydrolysed, and hexose increases, but, at the same time, it is found that the total sugar [sucrose (as invert sugar) + hexose] diminishes owing to loss in respiration. In Table LII are given a number of values for the total sugar lost and the amount of sucrose hydrolysed during definite periods of storage of different varieties. It will be seen that, by adding all values together, the total sugar lost (respired), i.e. 11·3 gm., and half the total sucrose hydrolysed, i.e. 9·8 gm., approach equality; the total gain of hexose is, however, somewhat lower.

The values on p. 269 are taken similarly from data for change in metabolites during storage of Conference pears (Emmett, Bib. III, 15).

In this case, there is practically equality between the total sugar lost, half the total sucrose hydrolysed and the total hexose gained.

The data of Bridel & Bourdouil (see p. 267) on the analysis of bananas provide similar evidence. In this case the fruit contained starch in addition to sucrose and hexose. Analyses were made, at intervals, of all three components. From Table LIV it will be seen that sucrose values increase at the expense of the starch, and that if

both sucrose and starch are expressed as invert sugar, there is a loss of total sugar. It will be seen also that there is quite a good agreement between the total loss of sugar, half the total sucrose hydrolysed and the total hexose gained.

TABLE LII. Sugars of apples in storage (Evans)

Period in days	% of fresh wt. of apple		
	Gain of hexose	Loss (by hydrolysis) of sucrose (as hexose)	Loss of total sugar (as hexose)
104	0·36	1·40	1·04
84	1·62	2·34	0·72
58	0·90	1·44	0·54
56	0·40	1·16	0·76
,,	0·37	0·39	0·02
?120	0·47	0·69	0·22
,,	0·31	0·95	0·54
,,	0·24	0·71	0·47
,,	0·25	0·75	0·50
,,	[−0·09]	0·77	0·86
,,	[−0·21]	0·41	0·62
,,	0·23	0·87	0·64
,,	0·29	0·65	0·36
,,	0·19	0·81	0·62
76	0·60	1·24	0·64
,,	0·94	1·31	0·37
,,	0·11	1·08	0·97
,,	0·50	0·86	0·36
,,	0·80	1·80	1·00
Totals	**8·28**	19·63 $\frac{\text{Total}}{2}$ = **9·81**	**11·25**

TABLE LIII. Sugars of pears in storage (Emmett)

Period in days	% of fresh wt.		
	Gain of hexose	Loss (by hydrolysis) of sucrose (as hexose)	Loss of total sugar (as hexose)
113	0·45	1·35	0·90
76	0·88	1·23	0·35
194	0·43	0·99	0·56
Totals	**1·76**	3·57 $\frac{\text{Total}}{2}$ = **1·78**	**1·81**

The evidence so far given for a preferential respiration of the γ-fructose component of sucrose has related to senescent organs such as ripening fruits. The question arises as to whether such a phenomenon

is characteristic of ordinary vegetative tissues. On consideration, it is obvious that such a relationship is difficult to demonstrate. The organ selected for experiment must of course be kept in the dark to avoid synthesis of sugar, and must be severed from the parent plant to avoid loss due to translocation. Temperature and other conditions should be such that normal respiration may take place, otherwise the products of hydrolysis of sucrose may merely accumulate. If we assume, moreover, that the glucose component of sucrose is not

TABLE LIV. Carbohydrates of bananas in storage (Bridel & Bourdouil)

Time in days	Hexose % fresh wt.	Sucrose % fresh wt.	Sucrose (as hexose)	Starch % fresh wt.	Starch (as hexose)	Sucrose +starch (both as hexose)	Loss of sucrose (as hexose)	Total sugar	Loss of total sugar
(March 21)	0·06	0·75	0·79	22·94	25·23	26·02	—	26·08	—
2	0·20	2·00	2·10	21·55	23·71	25·81	0·21	26·01	0·07
4	1·27	6·35	6·67	13·40	14·74	21·41	4·61	22·68	3·40
6	2·15	10·93	11·48	8·75	9·63	21·11	4·91	23·26	2·82
7	3·12	12·41	13·03	6·00	6·60	19·63	6·39	22·75	3·33
8	3·83	13·03	13·68	3·63	3·99	17·67	8·35	21·50	4·58
9	4·04	13·62	14·30	3·28	3·61	17·91	8·11	21·95	4·13
(April 1)									
10	4·33	13·73	14·41	2·18	2·40	16·81	9·21	21·14	4·94
12	4·79	14·38	15·10	2·03	2·23	17·33	8·69	22·12	3·96
14	5·32	13·11	13·77	0·94	1·03	14·80	11·22	20·12	5·96
15	5·93	12·44	13·06	0·87	0·96	14·02	12·00	19·95	6·13
17	6·52	10·42	10·94	0·76	0·84	11·78	14·24	18·30	7·78
19	7·18	9·01	9·46	0·83	0·91	10·37	15·65	17·55	8·53
21	8·26	7·87	8·26	—	—	8·26	17·76	16·52	9·56
23	7·79	7·66	8·04	—	—	8·04	17·98	15·83	10·25
25	**8·64**	6·24	6·55	—	—	6·55	19·47	15·19	**10·89**
							Total / 2 = **9·73**		

normally respired, but is used in syntheses, as, for instance, in cell-wall formation, then it is also important that the organ should not be actively growing.

Such a state of affairs would on the whole be realised in a mature green leaf kept in darkness and under such conditions of temperature, humidity, etc. that it is able to respire. Analyses of total carbohydrates (starch and sugars) should be made on control material at the time when the leaves are detached from the plant, and on the experimental leaves after an interval of 24–48 hours (depending on the temperature) in darkness.

A few data exist which give such a carbohydrate balance-sheet. Though *all* individual experiments do not show very clearly the sug-

gested relationship, namely, that the loss of sugar in respiration is equivalent to half the sucrose hydrolysed, yet such an agreement is closely realised when the total of a number of experiments is taken as set out in Table LV. It will be noted that the total gain of hexose also approaches the theoretical value.

These experiments were not designed to demonstrate the point in question. Hence it is obvious that essential conditions, such as

TABLE LV. Carbohydrates of detached leaves kept in darkness (Expts. 1–5, Ahrns, see p. 57; Expts. 6–7, Brown & Morris, see p. 23)

Expt.	Hexose	Gain of hexose	Sucrose (as hexose)	Starch (as hexose)	Sucrose +starch (both as hexose)	Loss of sucrose (as hexose)	Total sugar	Loss of total sugar
1. *Tropaeolum majus*								
Control	1·97	—	7·29	6·92	14·21	—	16·18	—
In dark 24 hrs.	3·85	1·88	5·99	4·48	10·47	3·74	14·32	1·86
2. *Vitis vinifera*								
Control	1·44	—	2·44	5·26	7·70	—	9·14	—
In dark 24 hrs.	4·27	2·83	1·93	1·96	3·89	3·81	8·16	0·98
3. *Phaseolus vulgaris*								
Control	0·46	—	2·16	14·09	16·25	—	16·71	—
In dark 24 hrs.	4·36	3·90	6·41	3·32	9·73	6·52	11·09	2·62
4. *Helianthus annuus*								
Control	1·64	—	1·53	4·54	6·07	—	7·71	—
In dark 24 hrs.	3·02	1·38	1·32	1·78	3·10	2·97	6·12	1·59
5. *Pisum sativum*								
Control	1·85	—	6·13	4·48	10·61	—	12·46	—
In dark 24 hrs.	3·29	1·44	5·08	3·38	8·46	2·15	11·75	0·71
6. *Tropaeolum majus*								
Control	1·41	—	10·48	4·06	14·54	—	15·95	—
In dark 24 hrs.	4·04	2·63	3·66	3·28	6·94	7·60	10·98	4·97
7. ditto. Control	2·11	—	7·70	5·97	13·67	—	15·78	—
In dark 24 hrs.	5·10	2·99	3·52	1·00	4·52	9·15	9·62	6·16
		17·05				35·94 Total / 2 **=17·97**		**18·89**

maturity of leaf, activity of respiration, etc., may have failed, some in one experiment, others in another.

If reference is made to the original analyses of leaves by Ahrns (see p. 57) and Brown & Morris (see p. 23), it will be noted that no allowance has been made in Table LV for the maltose claimed to have been estimated by the above authors in the leaf material. These

values for maltose rest on the increased reducing power of extracts after hydrolysing with acid. We have already quoted evidence and reasons (see p. 17) for the belief that such procedure is not valid. In fact, there is no satisfactory evidence that starch is hydrolysed to maltose in the living leaf, and we have consistently postulated, for reasons already stated, the conversion of products of hydrolysis of starch into sucrose.

TABLE LVI. Carbohydrates of detached leaves kept in darkness

Material	As percentage of fresh wt.							
	Hexose	Gain of hexose	Sucrose (as hexose)	Starch (as hexose)	Sucrose +starch (both as hexose)	Loss of sucrose	Total sugar	Loss of total sugar
1. *Vitis*, 2 combined Expts. in May and in June.								
Controls	1·31	—	2·92	5·36	8·28	—	9·59	—
After 72 and 144 hrs.	2·47	**1·16**	2·55	0·74	3·29	4·99	5·76	**3·83**
						Total / 2 = **2·49**		
2. *Vitis*, 2 combined Expts. in July and August.								
Controls	1·09	—	2·89	1·15	4·04	—	5·13	—
After 36 and 48 hrs.	1·90	**0·81**	1·58	0·31	1·89	2·15	3·79	**1·34**
						Total / 2 = **1·07**		
3. *Vitis*								
Ahrns (see Table LV)	0·42	—	0·71	1·52	2·23	—	2·65	—
	1·19	**0·77**	0·54	0·55	1·09	1·14	2·28	**0·37**
						Total / 2 = **0·57**		
Total (1, 2, 3)	—	**2·74**	—	—	—	**4·13**	—	**5·54**
Total (2 and 3)	—	**1·58**	—	—	—	**1·64**	—	**1·71**

A few preliminary data (unpublished) are given in Table LVI for analyses[1] of Vine leaves (using the half-leaf method). It would appear that these results illustrate the point made at the outset in connection with stable glucose. In active metabolism, there are many ways in which this sugar may be used, even in the detached leaf, such as, formation of cell-wall, of glucosides or, possibly, of aromatic compounds.

[1] I am indebted to Miss D. G. Griffiths for these analyses.

Hence we can scarcely expect to find the stable ratios until comparatively late in the season. Though the experiments in Table LVI are scanty, they indicate such a shortage of hexose during May and June. Later, in July and August, the values are nearer equality.

If the values calculated in Tables LII to LV are summarised, it will be seen that equality between the loss of total sugar and half the sucrose hydrolysed is maintained to within 9 per cent.

	$\frac{\text{Loss of sucrose}}{2}$	Loss of total sugar
Apples (Evans)	9·81	11·25
Pears (Emmett)	1·78	1·81
Bananas (Bridel & Bourdouil)	9·73	10·99
Leaves (Ahrns, Brown & Morris)	17·97	18·89
Totals	39·29	42·94

We can conclude that for tissues in general, when sucrose is available, the amount of sugar respired is approximately equal to half the sucrose hydrolysed. Whether this relationship constitutes a proof that the γ-fructose of sucrose is either preferentially respired or forms the sole substrate for respiration, future investigation will decide.

On the assumption that γ-fructose is respired, the hexose gained should be glucose. From Evans' data (Bib. I, 38), 80 % of the total hexose accumulated in storage of apples is glucose.

On the same assumption too, there is at present little evidence in regard to the respiration of normal stable hexose. From general considerations it would appear that, should the supply of invert sugar from sucrose in tissues be exhausted, the stable hexoses will diminish. As yet we have no information to show whether this is normal respiration of sugar or some form of abnormal breakdown.

THE PART PLAYED BY PHOSPHATE IN THE RESPIRATION OF THE HIGHER PLANTS

(1) *Earlier researches* (*before* 1914)

Though in the higher plants there is some evidence in favour of regarding γ-fructose (from sucrose) as the substrate for respiration, yet it is possible that stable hexoses may also serve this function. Should then stable hexose in this event be converted into the instable γ-form as a preliminary to respiration, the obvious assumption to make is that phosphate, as in the case of yeast, may be concerned in this change. The effect of phosphates on respiration of the higher

plants has been much debated, and forms a part of an experimental investigation, chiefly on post-mortem respiration, ranging over twenty years or more.

Some of the earliest investigations were carried out by the Russian school of biochemists working in St Petersburg from about 1900 to 1914. For the most part, we may consider the material employed by them to have been "dead." Sometimes either soaked or germinated seeds were used, but frequently they made use of ground seeds (a mixture of dead and living cells), the grinding, presumably, enabling solutions to penetrate the tissues. Occasionally, frozen (and subsequently thawed) material was used. In many cases, toluol, which would destroy the normal cell structure, was added as an antiseptic.

On the whole, the view of these authors was that respiration takes place in two stages, an anaerobic stage in which zymase is responsible for the output of carbon dioxide, with or without formation of alcohol, and subsequently an aerobic stage in which intermediate products of respiration are oxidised. The fact that "dead" tissues continue to evolve carbon dioxide and absorb oxygen, and, in some cases, to produce alcohol would be a proof that respiration is, to some extent, an enzymatic process.

Though all the above-mentioned group of workers largely employed such material as described above, and showed that it had a definite gaseous exchange, Zaleski & Reinhard (127) have especially considered the effect of freezing and grinding on the gaseous exchange, i.e. the so-called post-mortem respiration of their material. They estimated the CO_2-output of peas, rape-seed and maize grains, after freezing, as compared with controls, and found that, though all produced carbon dioxide, only peas (with testa) showed a marked increase in CO_2-output. Seedlings of Lupin and Maize, on freezing, showed a much decreased output. Of peas, lupin seeds and wheat grains, on pounding, only the peas again showed marked increase of CO_2-output. Dried and ground seedlings of the Pea and Wheat also gave off carbon dioxide, and the same was true for frozen and pounded peas in hydrogen. The authors consider destruction of cell-structure to diminish aerobic respiration more than anaerobic.

In a later communication (129) Zaleski & Reinhard extended their range of material and somewhat modified their conclusions. They give the following table of results as to the effect of disintegration on CO_2-output. Again they found the phenomena to be also exhibited in hydrogen.

TABLE LVII. Effect of pounding on CO_2-output (in mg.) from tissues (Zaleski & Reinhard)

Material	Uninjured	Pounded	Difference
100 soaked peas	17·5	33·8	+ 93
Lupinus angustifolius, 100 soaked seeds	7·5	12	+ 60
Wheat grains, soaked (50 gm.)	6	12	+100
Wheat blades, etiolated (20 gm.)	15	0	—
Vicia Faba, leaves, etiolated (15 gm.)	11	10·5	—
Vicia Faba, leaves, etiolated (10 gm.)	7	5	- 40
Begonia leaves (12 gm.)	12	5	-140
100 Wheat (5 day) seedlings	8	2·5	-220
15 Pea (15 day) ,,	5	7·5	+ 50
30 Pea (10 day) ,,	10	15	+ 50
25 Pea (15 day) ,,	8	14·5	+ 81
Pea seedlings, cotyledons	4	11	+175
Pea seedlings, axes	7·5	3·5	-113
Wheat blades, green (5·2 gm.)	7	0·7	—
Cucurbita (10 day) seedlings	24	17·5	- 37
Populus, buds (14·7 gm.)	8	5	- 60

Temperature for all experiments 15–17° C.

It will be seen that the result of disintegration is variable. Additional observations, however, were made which are embodied in Table LVIII. Here, a portion of the material was frozen at − 15° to − 20° C. Other portions were dried at 36° C. and subsequently soaked; or dried and ground, and then moistened with water.

It is to be gathered from Table LVIII that actively respiring organs, such as shoot tips (Expt. 3) and leaves (Expts. 4, 7, 8 and 9) show the same CO_2-output after freezing (column C) as after drying with subsequent moistening (column E), so that we can correctly regard the dried material as being dead. Further pounding of dead, in contrast to living, plant material, causes either no effect on the CO_2-output or, more usually, an increase. Pounding does not increase the CO_2-output from dead Wheat germs (Expt. 16), whereas pounded and dried shoot tips and leaves of *Vicia Faba* (Expts. 3 and 5) give out more CO_2 (column F) than the same after freezing (column C). In leaves of Begonia (Expts. 9 and 10) the CO_2-output, after drying and pounding, is in excess of that produced by the living leaves.

We shall frequently see later, from a study of the work of the Russian school, that both frozen and ground material will absorb oxygen and evolve carbon dioxide, but what relationship this phenomenon bears to respiration of living plants remains to be considered.

It should be realised that injured (pounded) tissues of oxidase plants, as, for example, Bean (*Vicia Faba*) and Wheat (*Triticum*

vulgare), will absorb oxygen, and may evolve carbon dioxide as a result of the deamination of amino-acids by *ortho*-quinone (see p. 142).

TABLE LVIII. CO_2-output (in mg.) of tissues after various treatments (Zaleski & Reinhard)

Material	No. of expt.	Time in hrs.	A Living	B Pounded	C Frozen	D Frozen and pounded	E Dried and soaked	F Dried, ground and moistened
Vicia Faba, etiolated shoot tips (6 gm.)	3	4	12	—	5	—	—	—
Ditto	,,	,,	12	—	—	—	5	—
Ditto	,,	,,	14	—	—	—	—	7
Vicia Faba, etiolated leaves (15 gm.)	+	1	10	—	4·5	—	4·5	—
Ditto	—	,,	9·5	—	—	—	—	4·5
Ditto (9·2 gm.)	5	,,	7	—	—	—	—	5
Wheat, leaves, etiolated (30 gm.)	7	1	19·5	—	Trace	—	Trace	Trace
Ditto (20 gm.)	8	,,	14·5	—	,,	—	,,	,,
Begonia, leaves (46 gm.)	9	2	6·5	—	2·5	—	2	—
Ditto	—	,,	6·5	—	—	—	—	10
Begonia, leaves (8·5 gm.)	10	½	—	—	—	—	—	25
	—	3	—	—	—	—	—	45
	—	2	—	—	—	—	—	15
	—	5	—	—	—	—	—	23
	—	13½	—	—	—	—	—	8
Ficus elastica, leaves (25 gm.)	11	1	2·5	—	—	—	—	4·5
Ditto (8 gm.)	12	,,	—	—	—	—	—	7·5
Pisum, 100 soaked seeds	13	1	—	—	16·5	29·6	—	—
Zea Mays, 100 (4 day) seedlings	14	1	3·4	—	3·4	—	—	—
Ditto 100 (10 day) seedlings	,,	,,	25·8	—	6·6	—	—	—
Pisum, 40 (10 day) seedlings without cotyledons	15	1	6·5	—	1	—	—	—
Ditto, 100 (9 day) seedlings	17	,,	35	20	—	—	—	—
Ditto, 100 (14 day) seedlings	,,	,,	45	12·5	—	—	—	—
Ditto, 100 (16 day) seedlings	,,	,,	28	25	—	—	—	—
Dead Wheat germs	16	—	—	14·5 (Control, uninjured =15·0)	—	—	—	—

Temperature 15–16° C. for most of the above experiments.

As the supply of amino-acids may be maintained by the action of proteases on autolysis, the output of carbon dioxide from this source might be maintained for an appreciable time.

It is, however, chiefly on such material as described above that the effect of phosphate has been determined. Stimulation of CO_2-output by phosphate was first indicated by (Nicolaus) Iwanoff. Later, a careful series of experiments was carried out by (Leonid) Iwanoff (22) on the effect of phosphate on the CO_2-output of tissues of seeds. He used, as material, the germ (embryo) of Wheat, seeds of Pea, Bean (*Vicia Faba*), Vetch (*V. sativa*), Sunflower (*Helianthus annuus*) and Maize grains. Though sometimes whole seeds were used, usually finely or coarsely ground seeds were employed, moistened with water or other solutions, toluol being used as an antiseptic. An additional preparation was made by treating seeds with acetone.

The acetone-treated material can naturally be regarded as "dead", the remaining material as a mixture of living and dead cells, the former gradually dying as toluol penetrates the tissues. Reactions underlying gaseous interchange in such cases can therefore be regarded as enzymatic.

L. Iwanoff's results show that disodium phosphate (Na_2HPO_4) increases CO_2-output to the extent of 20–30 % (even sometimes 100 %) in all material, living, ground, with or without toluol and acetone-treated, and also in absence of air (in hydrogen). Table LIX shows some of the results for peas, and similar tables are given by L. Iwanoff for the Wheat germ (embryo). Table LXI shows the effect of phosphate on seeds of various other plants.

The stimulation in an atmosphere of hydrogen can be observed by a manometer; hence it follows that the phosphate raises the respiratory quotient, that is, increases the CO_2-output which is not connected with oxygen absorption. This fact suggests that we are dealing with increase of carbon dioxide arising from alcoholic fermentation (anaerobic respiration).

Zaleski & Reinhard (127) also obtained increase of CO_2-output with phosphate, using ground peas, Maize grains, Wheat germs and ground seeds of Lupin as shown in Table LXII. As will be seen from the latter table, ground, acetone-treated and also frozen material was used. The authors believe that, in the case of Wheat grains, the duration of the experiment was too short to show stimulation by phosphate. Experiments in which acid phosphate (NaH_2PO_4) was used showed that this lowers the CO_2-output.

The above authors found that phosphate also increased the CO_2-output in hydrogen; so they conclude that phosphate affects not only aerobic, but also anaerobic respiration. They suggest that the aerobic

results may be due to stimulation of processes of oxidation, and they quote data showing such an effect of phosphate on oxidase, catalase and reductase.

TABLE LIX. Effect of phosphate on the CO_2-output (in mg.) of Peas (L. Iwanoff)

No. of expt.	I. Seeds with toluol	CO_2-output in mg.; time in hrs.			Total	Difference mg.	Difference %
23	10 gm. seeds pour. led	19 h. 30 m.	9 h.	—	28 h. 30 m.		
	20 c.c. water	57	44	—	101	47	40
	„ phosphate*	100	48	—	148		
24	10 gm. seeds	23 h. 30 m.	18 h.	—	41 h. 30 m.		
	Whole: 20 c.c. water	32	50	—	82	19	23
	Whole: „ phosphate	37	64	—	121		
	Pounded: 20 c.c. water	104	94	—	198	78	39
	Pounded: „ phosphate	155	121	—	276		
25	10 gm. seeds pounded	24 h.	—	—	24 h.		
	Air: 20 c.c. water	95	—	—	95	63	66
	Air: „ phosphate	158	—	—	158		
	Hydrogen: 20 c.c. water	60	—	—	60	49	81
	Hydrogen: „ phosphate	109	—	—	109		
26	10 gm. seeds pounded	20 h.	—	—	20 h.		
	Air: 20 c.c. water	53	—	—	53	57	108
	Air: „ phosphate	110	—	—	110		
	Hydrogen: 20 c.c. water	23	—	—	23	43	187
	Hydrogen: „ phosphate	66	—	—	66		
27	10 gm. seeds pounded in hydrogen	10 h.	—	—	10 h.		
	20 c.c. water	28	—	—	28	20	71
	„ phosphate	48	—	—	48		

	II. Living seeds, without toluol									
28	10 gm. seeds†	12 h.	9 h.	24 h.	14 h.	9 h.	24 h.	92 h.	76	25
	10 c.c. water	48	30	67	40	29	93	307		
	„ phosphate	46	35	66	70	46	120	383		

	In manometer	mm. pressure				
29	10 gm. seeds pounded	10 h.	—	—	—	
	Without toluol: 20 c.c. water	33	27	—	—	
	Without toluol: „ phosphate	60				
	With 0·2 c.c. toluol: 20 c.c. water	24	21	—	—	
	With 0·2 c.c. toluol: „ phosphate	45				
30	10 gm. seeds pounded +0·2 c.c. toluol	6 h.	20 h.	27 h.	45 h.	
	20 c.c. water	18	61	85	125	52
	10 c.c. phosphate	27	93	125	177	

* 1 % Na_2HPO_4 solution used throughout. † Previously soaked 6 hrs. at 15° C.

Kostytschew, on the other hand, as the result of a series of researches (35) states that the CO_2-output from Wheat germs is not

increased by phosphate alone. L. Iwanoff (22) suggests that with the concentration (3 to 3·5 %) of phosphate used by Kostytschew, stimulation is delayed, and, in addition, the first-formed CO_2 may be absorbed by alkaline phosphate.

Later, N. Iwanoff (27) published results confirming his first attempts, and those we have given of L. Iwanoff and of Zaleski & Reinhard. N. Iwanoff showed that phosphate increased the CO_2-output from Wheat germs (incapable of germination), and for those treated with acetone and also for dried material. In addition, he used etiolated tips of 10–12 day-old seedlings of *Vicia Faba*. These were

TABLE LX. Effect of organic phosphoric acid[1] on the CO_2-output of Peas (L. Iwanoff)

No. of expt.	In flask about 10 gm. pounded seeds with toluol	CO_2-output in mg.; time in hrs.			Total	Difference mg.	Difference %
		5 h.	18 h.	—	23 h.		
31	Current of air { 20 c.c. water	12	85	—	97	77	79
	„ organic phosphoric acid	13	161	—	174		
		19 h.	9 h. 30m.	18 h.	46 h. 30 m.		
32	Current of air { 20 c.c. water	61	32	74	167	87	52
	„ organic phosphoric acid	87	50	117	254		
		42 h.	—	—	42 h.		
33	Current of hydrogen { 20 c.c. water	104	—	—	104	31	30
	„ organic phosphoric acid	135	—	—	135		
		20 h.	—	—	20 h.		
34	Current of hydrogen { 20 c.c. water	27	—	—	27	38	140
	„ organic phosphoric acid	65	—	—	65		

unaffected by phosphate, but, after freezing, there was an increased CO_2-output with 1–2 % Na_2HPO_4. He is of the opinion that in dead material phosphate stimulates the anaerobic CO_2-output only.

Another method of demonstrating the stimulating effect of phosphate consisted in using sugar solutions which had been fermented by zymin. Such solutions are known to contain organic phosphate. It was shown by Kostytschew (35, 38) that these fermented solutions caused increased CO_2-output from Wheat germs. This was confirmed by L. Iwanoff (22) for Wheat germs and peas (Table LX). Also by Zaleski & Reinhard (129) for Wheat germs (sometimes pounded and in presence of toluol) and for ground Wheat and Pea meal (both with and without toluol).

[1] Filtrate from zymin fermentation.

TABLE LXI. Effect of phosphate on CO_2-output of seeds of various plants (L. Iwanoff)

No. of expt.		Manometer reading in cm. mercury Time in hours												Total	Difference
	Helianthus annuus	5	3	*circ.* 0·5 c.c. toluol added	13	24	24	24	24	24	24	24	24	205	
35	10 gm. (*circ.*) split and shelled seeds — 10 c.c. 10 % sucrose	−6	−7	—	3	1	25	36	33	30	12	12	12	164	123
	10 gm. (*circ.*) split and shelled seeds — Ditto + phosphate*	−6·5	−8	—	6	4	24	69	75	54	23	17	15	287	
		Time in days													
		2	1	1	1	2	1	—	—	—	—	—	—	8	
36	Ditto + 0·5 cc. toluol — 15 c.c. 10 % glucose	8	29	13	6	5	5	—	—	—	—	—	—	66	62·5
	Ditto + 0·5 cc. toluol — Ditto + phosphate	12	30·5	32	28	16	10	—	—	—	—	—	—	128·5	
	Vicia sativa	1	1	1	1	1	1	1	1	—	—	—	—	8	
37	10 gm. (*circ.*) coarsely ground seeds with testas + 0·2 c.c. toluol — 15 c.c. water	8	15	32	19	12	11	9	0	—	—	—	—	106	132
	10 gm. (*circ.*) coarsely ground seeds with testas + 0·2 c.c. toluol — „ phosphate	5	23	45	40	35	30	30	30	—	—	—	—	236	
	Vicia Faba	1	1	1	1	1	1	1	1	—	—	—	—	8	
38	10 gm. (*circ.*) coarsely ground seeds with testas + 0·4 c.c. toluol — 20 c.c. water	16	3	4	3	0	5	4	4	—	—	—	—	39	137
	10 gm. (*circ.*) coarsely ground seeds with testas + 0·4 c.c. toluol — „ phosphate	25	28	28	30	26	20	12	7	—	—	—	—	176	
	Zea Mays	1	1	1	1	1	—	—	—	—	—	—	—	5	
39	10 gm. (*circ.*) coarsely ground seeds + 0·3 c.c. toluol — 20 c.c. 10 % glucose	5	11	4	—	—	—	—	—	—	—	—	—	20	17
	10 gm. (*circ.*) coarsely ground seeds + 0·3 c.c. toluol — Ditto + phosphate	5	17	15	—	—	—	—	—	—	—	—	—	37	
40	Ditto + 0·5 c.c. toluol — 20 c.c. 10 % glucose	−1	12	−2	−3	−8	—	—	—	—	—	—	—	−2	55
	Ditto + 0·5 c.c. toluol — Ditto + phosphate	7	16	14	9	7	—	—	—	—	—	—	—	53	

* 1 % Na_2HPO_4 solution used throughout.

Yet a third method was to use an extract of zymin. This was found by Zaleski & Reinhard (129) to cause an increased CO_2-output from ground Wheat germs (with toluol) and ground Wheat grains and peas. This result was further confirmed by L. Iwanoff (24) for ground peas, using a boiled extract of zymin or, sometimes, hefanol (Yeast dried by heat) as shown in Table LXIII.

TABLE LXII. Effect of phosphate on CO_2-output of ground seeds (Zaleski & Reinhard)

No. of expt.	Temp. ° C.	Material		CO_2 per hr. in mg.	Difference in %
		Pisum sativum			
34	17	Meal from living seeds (50 gm.)	Water	29·5	
			1 % Na_2HPO_4	52·0	+76
36	16	Seeds, first soaked in water, then dried and ground (50 gm.)	Water	31·5	
			1 % Na_2HPO_4	49	+55
37	17·5	Acetone meal (50 gm.)	Water	29·5	
			1 % Na_2HPO_4	42	+42
38	16·5	Meal from living seeds (25 gm.)	Water	12·5	—
			0·4 % NaH_2PO_4	11·5	− 8
			0·5 % K_2HPO_4	19	+52
			1·2 % K_2HPO_4	19	+52
			1 % Na_2HPO_4	21	+68
39	15	Frozen meal (50 gm.)	Water	24	
			1 % Na_2HPO_4	42	+75
40	17	Meal (50 gm.)	Water	34	
			10 % Na_2HPO_4	19	−44
		Lupinus angustifolius			
41	15	Meal from seeds (25 gm.)	Water	5·5	
			1 % Na_2HPO_4	11·5	+109
		Zea Mays		CO_2 per 2 hrs.	
42	16	Meal from grains (50 gm.)	Water	6·5	
			1 % Na_2HPO_4	11·5	+76
		Wheat			
43	15·5	Meal from grains (25 gm.)	Water	7·5	
			1 % Na_2HPO_4	5·5	−26
				CO_2 per hr.	
45	17	Grains first soaked in water, dried and ground (25 gm.)	Water	8	
			1 % Na_2HPO_4	7	−12
46	15	Meal from germs (25 gm.)	Water	22	
			1 % Na_2HPO_4	35·5	+61

There was, moreover, in the presence of the same extract, an increased formation of alcohol (see Table LXIV).

Zaleski (116) has also shown that a boiled extract of hefanol increases the CO_2-output of peas, seeds of *Lupinus luteus*, Wheat grains (both ungerminated and germinated 3 days), etiolated (25 days) Wheat seedlings and axes of seedlings (20 days) of *Lupinus luteus*, but not of the stem-tips of etiolated seedlings of *Vicia Faba*. All

material was considered to be dead, as it was dried, and subsequently ground, and toluol was employed. In most cases stimulation took place not only in air, but also in a vacuum. He regards it as obvious that hefanol extracts only stimulate the anaerobic CO_2-output.

Zaleski & Reinhard (127) in their first investigations came to the conclusion that phosphate increases aerobic as well as anaerobic respi-

TABLE LXIII. Effect of zymin extract on CO_2-output of ground peas (L. Iwanoff)

No. of expt.	Material		CO_2 (mg.) evolved in time (hrs.)	
			31 hrs.	17 hrs.
3	Pea meal (5 gm.)	10 c.c. water	37·8	26·8
		„ zymin ext.	80·8	49·3
		„ 4 % glucose	37·1	24·5
			24 hrs.	
4	Ditto	10 c.c. water	30·4	
		„ zymin ext.	75·7	
		„ 1 % Na_2HPO_4	47·1	
			17 hrs.	
5	Ditto	10 c.c. water	19·0	
		„ hefanol ext.	35·8	
		„ zymin ext.	49·8	
		„ 1 % Na_2HPO_4	27·0	

TABLE LXIV. Effect of zymin extract on formation of alcohol from ground peas (L. Iwanoff)

No. of expt.	Material		CO_2 (mg.) evolved	Alcohol (mg.) formed	Alcohol per $CO_2=100$
9	Pea meal (5 gm.)	10 c.c. zymin ext.	142·1	130	91·5
10	Pea meal (7 gm.)	15 c.c. zymin ext.	134·0	110	82·0
11	Pea meal	15 c.c. zymin ext.	154·7	140	96·8

ration (alcohol fermentation). In later publications, Zaleski and his co-workers (120, 129) maintain agreement with L. Iwanoff that only the alcoholic fermentation of Wheat germs is raised by secondary phosphate and by fermented sugar solutions.

We thus see from all that has preceded that there is evidence of an increased CO_2-output from the tissues of dead seeds and seedlings, etc., on treatment with inorganic phosphate and fermented sugar

solutions (possibly owing to organic phosphate produced) and also by zymin extracts.

Nevertheless, though N. and L. Iwanoff and Zaleski & Reinhard have accepted the view that phosphate causes increased CO_2-output chiefly as a result of the stimulation of anaerobic respiration (fermentation), and that the increase brought about by fermented sugar solutions is due to organic phosphate, Kostytschew and his co-workers are of a different opinion. These latter authors state:

(1) That fermented sugar solutions increase the CO_2-output not by virtue of inorganic or organic phosphate content, but because they contain intermediate products of respiration, which are finally oxidised to carbon dioxide by the Wheat germs, peas, etc., that is, aerobic respiration is stimulated. An additional demonstration, he claims, is the evolution of carbon dioxide which occurs when peroxidase and hydrogen peroxide are added to a solution of glucose after fermentation by zymin.

Again, as evidence, Kostytschew (38, 39) states that, using Wheat germs as material, fermented sugar solutions do not cause an increase of alcohol formation, which should be the case if the increased CO_2-output (79–142 %) arises from anaerobic respiration (fermentation). He maintains that in L. Iwanoff's researches, the germs were usually dead and covered with water, and thus fermentation was induced. His (Kostytschew) researches were carried out with good aeration and with living germs.

(2) That phosphates only increase the CO_2-output by virtue of their alkalinity (Kostytschew & Scheloumoff, 44). This statement is based on the effect of solutions of neutralised phosphate and other alkaline salts on the CO_2-output of Wheat germs (incapable of germination).

On the other hand N. Iwanoff, L. Iwanoff, Zaleski & Reinhard and Zaleski & Marx bring forward various criticisms and statements as follows:

(1) The increased CO_2-output with phosphate from Wheat germs and frozen shoot tips of *Vicia Faba* seedlings arises from the primary (anaerobic) and not secondary (aerobic) respiration, as it also takes place in absence of air or in an atmosphere of hydrogen; the respiratory quotient, moreover, increases (N. Iwanoff, 27). The same point of view is held by L. Iwanoff (22) in regard to the similar behaviour observed for ground peas (in presence of toluol). He found, moreover, that Pea meal may form considerable quantities of alcohol even in a

current of air, owing to lack of oxidation (24) [Palladin had likewise found peas, after freezing, to form alcohol in a current of air]. L. Iwanoff is further of the opinion that the question as to whether alcohol happens to be formed or not in connection with anaerobic CO_2-output is not significant in regard to the point at issue (23).

Zaleski & Reinhard (129) have also shown that sugar solutions fermented by zymin and extracts of zymin and hefanol have a stimulating effect on the respiration of whole and ground Wheat germs, as well as ground Wheat grains and peas. Since the material produces an equal CO_2-output in both air and hydrogen, they conclude that fermented sugar solution and zymin and hefanol extracts only stimulate anaerobic respiration.

(2) Zaleski & Reinhard (129) and L. Iwanoff (24) consider that death of the plant decreases the aerobic (oxidation) processes, but anaerobic respiration is little injured. The oxidation mechanism comes to an end before the CO_2-output ceases; therefore the post-mortem exchange of gases in pea-meal can scarcely be regarded as respiration. Zaleski & Reinhard and L. Iwanoff are of the opinion that the germs used by Kostytschew were probably dead.

(3) The increased CO_2-output brought about by organic phosphate using Wheat germs (with toluol) is not accompanied by any definite increase of oxygen absorption (L. Iwanoff, 24), which would be the case if oxidation of intermediate products were taking place.

(4) Zaleski & Marx (120), though they find alkali to increase CO_2-output from ground peas and Wheat germs (in presence of toluol), consider that Kostytschew has not shown that alkalinity increases the CO_2-output from *aerobic* respiration, nor that it does so in the absence of phosphate. They suggest that the effect of alkalinity may be superposed on that of phosphate.

(5) L. Iwanoff states (24) that he has, as a matter of fact, found heated zymin extract to increase alcohol formation in Pea meal and Wheat germs in air.

(6) Zaleski (116) claims to have removed the activating substance from hefanol extract with acetone, in which solvent hexose-phosphate is insoluble. Hence he assumes the existence of some additional unknown substance which stimulates the anaerobic CO_2-output.

Kostytschew (40), Kostytschew & Scheloumoff (45) and Kostytschew, Brilliant & Scheloumoff (42) make, on the other hand, the following statements:

TABLE LXV. Respiration of wheat germs (Kostytschew & Scheloumoff)

No. of expt.	Material	Solution	CO_2 evolved (mg.)	Alcohol formed (mg.)	Time (hrs.)	Atmosphere (current)
		Complete aeration				
1	*Living Wheat germs* (20 gm.)	100 c.c. water	256	0	6	Air
		,, 1 % Na_2HPO_4	278	0	,,	,,
2	Ditto	,, water	204	203	,,	Hydrogen
			Ratio 100 : 100			
3	Ditto	,, 1 % Na_2HPO_4	265	15	,,	Air
		,, ,,	229	213	,,	Hydrogen
			100 : 93			
4	Ditto (15 gm.)	2 % glucose solution fermented with zymin	140	16	3	Air
	,,	,, ,,	136	8	,,	,,
	,,	,, ,,	274	3	6	,,
5	Ditto (20 gm.)	100 c.c. 5 % glucose solution fermented with zymin	298	5	,,	,,
6	Ditto	Ditto	304	299	,,	Hydrogen
			100 : 98			
	,,	,,	368	—	,,	Air
		Incomplete aeration				
7	Ditto	40 c.c. water	241	105	,,	Air
			100 : 43			
	,,	,, 1 % Na_2HPO_4	234	122	,,	,,
			100 : 52			
8	,,	40 c.c. solution fermented as in 4	367	198	,,	,,
			100 : 54			
9	,,	40 c.c. 2 % glucose solution	239	124	,,	,,
			100 : 52			
	,,	,, solution fermented as in 4	324	193	,,	,,
			100 : 59			
		Complete aeration				
10	Wheat germs *incapable of germination* (100 gm.)	500 c.c. water	84	23	5	Air
			100 : 27			
11	Ditto	500 c.c. 5 % glucose solution + Na_2HPO_4	165	93	,,	,,
			100 : 55			
12	Ditto	500 c.c. solution fermented as in 5	337	138	,,	,,
			100 : 41			
13	Ditto	200 c.c. solution fermented as in 5	358	348	,,	Hydrogen
			100 : 97			

They (45) claim to have shown (see Table LXV):

(1) That living Wheat germs (capable of germination) (*a*) produce no alcohol if *fully aerated*, (*b*) develop a true alcohol fermentation in absence of oxygen, (*c*) have a greater CO_2-output in air than in hydrogen.

(2) That even the respiration of Wheat germs treated with toluol (after which they cannot germinate) is almost entirely aerobic.

(3) That if aeration of living germs is incomplete (i.e. more liquid is added to the material as in the case of L. Iwanoff's experiments) at least 50 % of the CO_2-output is anaerobic.

(4) That the CO_2-output of Wheat germs (incapable of germination) if fully aerated, is weaker, but is about 50 % anaerobic and 50 % aerobic.

(5) That Zaleski's Wheat germs were dead, but Kostytschew had previously used living germs. Capability of germination is not necessarily a criterion of whether germs are living or not. Germs incapable of germination may yet respire normally.

Finally, they point out that Iwanoff is obliged to postulate a co-ferment (in addition to phosphate) to explain the stimulating effect of zymin extract.

Much emphasis is laid by Kostytschew and his co-workers on the fact that respiration of Wheat germs cannot be considered as wholly anaerobic. Even in the case of weakly respiring germs incapable of germination, 50 % of the respiration is *aerobic*. They regard the amount of alcohol formed as a measure of anaerobic respiration. Hence they consider that Iwanoff's statements, without any corroborative estimation of alcohol, that the respiration of Wheat germs is anaerobic, to be reprehensible. In the case of the weakly respiring germs (incapable of germination) they subtract the CO_2-equivalent, of alcohol formed, from the total CO_2-output, and regard the remainder (about 50 %) as the carbon dioxide of aerobic origin. It had previously been shown that the germs do not oxidise alcohol.

Later, Kostytschew, Brilliant & Scheloumoff (42) again define their position as follows: The respiration of germs (*capable of fermentation*) is normal. The CO_2-output is not increased by inorganic phosphate. Fermented sugar solutions increase the CO_2-output, and this can be referred back to normal respiration.

Further, that Iwanoff and Zaleski's experiments were carried out with germs incapable of germination and in incomplete aeration. They, moreover, define once more these latter authors' views as

follows: Wheat germs show only anaerobic respiration (alcoholic fermentation). The CO_2-output is raised both by inorganic phosphate and by fermented sugar solutions. This increased CO_2-output is not accompanied by increased O_2-uptake. Products of fermentation effect an increased CO_2-output and also increase alcohol formation.

Kostytschew, Brilliant & Scheloumoff then describe a series of observations on the O_2-uptake and CO_2-output, i.e. the respiratory quotient $\frac{CO_2}{O_2}$ for Wheat germs, using two types, namely one *capable* and the other *incapable* of *germination*, each was investigated in *complete* and in *incomplete aeration*. It was found that in *incomplete* aeration of both types of germ, the O_2-uptake is greatly diminished.

The authors further investigated the effect of inorganic phosphate on O_2-uptake and the respiratory quotient of *living* germs, and showed that this is negative. Finally, they investigated the effect of sugar solutions fermented with zymin on the two types of germs. In the living germs, there was an increased O_2-uptake and CO_2-output, and the respiratory quotient remained unaltered. In the other type, even in perfect aeration, there was an increased CO_2-output only, so that the respiratory quotient increased.

Zaleski later (117), answering Kostytschew's criticisms, explains that his method of aeration differed very little from that of Kostytschew. As to his (Zaleski) statement that fermented sugar solution affects only the CO_2-output of "dead" germs, he points out that Kostytschew himself has obtained the same result. In regard to his (Zaleski) further statement (to which Kostytschew had taken exception) that the CO_2-output of "dead" germs is of anaerobic origin only (Kostytschew, himself, maintaining that 50 % is of aerobic origin as measured by alcohol formation), Zaleski makes the following reply: That there is a possibility of the dead germs forming less alcohol in air than would be expected from the CO_2-output. He further points out that the alcohol is derived from acetaldehyde (from pyruvic acid), and changes may take place on other lines than reduction to alcohol.

L. Iwanoff (26) also, in answer to Kostytschew's criticisms, points out how different samples of Wheat germs may vary, the difficulty, moreover, of defining capability of germination, which may range from 1 to 100 %. From a complicated controversial argument, the following additional points made by L. Iwanoff may perhaps be emphasised. (1) That an increased CO_2-output was obtained from

"dead" Wheat germs and peas, by addition of boiled zymin extract, and, in this case, there can be no question of intermediate organic products of respiration, though, in all probability, inorganic and organic phosphates are present. (2) That he (L. Iwanoff) in the case of experiments with Wheat germs, is only measuring anaerobic respiration. Hence, whether aeration is complete or incomplete makes no difference to the CO_2-output.

To sum up the position we have been considering in this section, all observers agree in obtaining increased CO_2-output from "dead" tissues by means of solutions of inorganic phosphate, fermented sugar solutions and zymin extracts. The increase, due to inorganic phosphate, is interpreted on the hexose-phosphate hypothesis by Iwanoff, Zaleski and others, but by Kostytschew as being due to alkalinity. In the case of fermented sugar solutions, the increased CO_2-output is considered by Iwanoff, Zaleski and their co-workers to be again due to stimulation of the anaerobic process by phosphate, but by Kostytschew to oxidation, in aerobic respiration, of intermediate products. Further, Zaleski and Iwanoff maintain that the respiration of the dead germs is anaerobic because the oxidising mechanism is destroyed, and Kostytschew because the aeration is imperfect. The matter thus finally becomes a question as to whether the respiration of dead Wheat germs is wholly anaerobic or partly aerobic as well. The method of using alcohol estimation as a measure of anaerobic respiration is unsatisfactory, as, apparently, the amount formed depends on the aeration and other conditions (see p. 295), and may not be a true measure of the anaerobic respiration. As Kostytschew bases his calculation of aerobic CO_2-output on the estimation of alcohol, he is therefore not necessarily correct.

At this date (1914) the controversy, which had become very complex, ceases. The observations of these earlier workers are doubtless, in the main, correct, and they will be very helpful to others (as shown in the following section) using similar material and working in the light of more modern knowledge.

(2) *Later researches* (*since* 1914)

These have been, in general, conducted in much the same way as those devised by the earlier workers. New facts in connection with the biochemistry of fermentation of Yeast have suggested some additional lines of investigation.

One of the more important of these is the question as to whether there is an enzyme in the higher plants which catalyses the synthesis

and hydrolysis of hexose-phosphates. This point had already been considered by L. Iwanoff (24, 25) and Zaleski & Marx (120) but with negative results.

Later, Bodnár (4) reinvestigated the question. He used, as material, peas which had been soaked in water for 2–3 hours, then dried at 30° C. after removing the testas, and finally ground. To this meal, phosphate (Na_2HPO_4) solution was added. The loss of inorganic phosphate was estimated after definite intervals. The figures in Table LXVI indicate some of the values obtained.

TABLE LXVI. Conversion of inorganic into organic phosphate (Bodnár)

Material	Temp. (° C.)	Time (hrs.)	Loss of phosphate (mg.)
5 gm. Pea meal + 5 c.c. phosphate solution (≡0·0528 gm. P_2O_5)	20–21	3	5·9
Ditto	20–21	6	13·9
Ditto	20–21	24	50·2
Ditto	30	6	24·9

No loss of phosphate was observed with Pea meal which had been heated. Organic phosphate was detected in the filtrate after precipitation of inorganic phosphates for estimation. Bodnár inclines to the view that esterification of the phosphoric acid by sugar has taken place. On the other hand, Zaleski & Pissarjewsky (124) maintain that they could not repeat Bodnár's phosphate conversion in unripe peas and seeds of other plants.

An enzyme, **hexosephosphatase**, catalysing the decomposition of hexose-phosphate has been shown by Neuberg & Kobel (61) to be present in the leaves of the Tobacco (*Nicotiana Tabacum*). It was found that the pounded leaves hydrolysed sodium and magnesium hexose-diphosphates.

Bodnár & Hoffner (6) repeated the investigations of the earlier workers described in the last section on the stimulation by phosphate of post-mortem respiration. As material, they used peas, soaked for 1–2 hours, dried at 30–40° C. after removing the testas, and then ground in a mill. Toluol was used as an antiseptic.

The chief results of Bodnár & Hoffner confirm earlier work, and may be summarised in the following terms. They found both the CO_2-output and alcohol formation from the Pea meal to be greater in air than in hydrogen; the amount of both products formed was increased by addition of 2 % K_2HPO_4.

GLYCOLYSIS; SPLITTING OF HEXOSE

It is now known that, as in fermentation by Yeast, there are a number of stages succeeding phosphorylation in the biochemistry of respiration. Formerly, the whole anaerobic process, now termed zymasis, was regarded as one catalysed by the enzyme zymase, though, even for the higher plants, it was recognised, as early as 1913, that zymase contains another catalyst, carboxylase, responsible for the production of carbon dioxide. Before dealing with the individual stages of respiration, some account must be given of the first observations on the existence in the higher plants of this complex of catalysts—zymase.

It was originally shown (Stoklasa & Czerny, 104) that such an enzyme is present in potatoes, beet and peas. The tubers, roots or seeds were first kept in a stream of hydrogen under sterile conditions for 5–10 days, and thus shown to be capable of anaerobic respiration. The crushed or ground tissues were then subjected to a pressure of 300 atmospheres, and the expressed juice precipitated with alcohol. The crude precipitate, containing zymase among other enzymes, fermented, in some cases very strongly, hexose, saccharose, maltose and starch paste, with the production of carbon dioxide and alcohol in theoretical proportions.

To show that zymase is present in normal aerobic tissues, 20 day-old Pea seedlings grown in air were ground and pressed under 300 atmospheres. The precipitate from the expressed juice contained in this case an equally potent enzyme. The same result was obtained with beet which had been kept under aerobic conditions.

The following are the values found for the ratio $\frac{CO_2}{\text{alcohol}}$ as a result of the fermentation of sugar by the various preparations (theoretically from the equation

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

the ratio is 100:104·5):

Beet zymase	Potato zymase	Pea zymase
97·9	137·0	103·2
148·0	89·8	110·3
108·1	Av. 113·4	110·2
109·6		Av. 107·9
120·0		
123·5		
103·9		
Av. 115·8		

Later, Stoklasa (102) showed the presence of zymase in seedlings of Barley, in leaves of Sugar Beet and in the plant of Herb Paris (*Paris quadrifolia*).

Though the existence of zymasis in the higher plants is now regarded as a certainty, the validity of the results in connection with the extraction of zymase and its action *in vitro* as shown by Stoklasa was questioned on the ground that the fermentations might have been contaminated with bacteria. Later work confirming Stoklasa's results, however, was carried out by Bodnár (3) on potatoes and beet in which the presence of zymase was demonstrated by extraction of the juice in a Buchner press (300–400 atmospheres pressure), filtration through linen, and precipitation by alcohol. The enzyme was added to 15 % glucose, and kept at 37° C., considerable precautions being taken to have sterile apparatus and to demonstrate, by trial, the absence of bacteria during the experimental period. The values obtained by Bodnár for alcohol per 100 gm. carbon dioxide were as follows:

Potatoes	Beet
105·4	100·8
104·4	101·1
92·1	118·6
103·8	105·7
Av. 101·4	Av. 106·5

Attention may now be turned to the first of the series of reactions which, as we have said, was formerly termed zymasis, and considered to be due to the action of zymase. This first process can be regarded as the splitting of sugar. Though in the higher plants the substrate for respiration appears to be the γ-fructose from sucrose, yet an unstable γ-hexose may also be formed as a result of phosphorylation. It can be assumed, however, that whichever hexose may be concerned, it is split into 3-carbon molecules, probably of methyl-glyoxal or an isomer of this compound.

Recently (Kobel & Scheuer, 134) evidence has been obtained—on analogy with the processes in Yeast—of the formation in the higher plants of methyl-glyoxal by the splitting, or glycolysis, of hexose by a catalyst, **glycolase.**

The detection of methyl-glyoxal is rendered possible in Yeast if apo-zymase (i.e. zymase without co-enzyme) is employed. For, according to Neuberg, a co-enzyme is not only necessary for phosphory-

lation, but also for a subsequent process, namely the dismutation of methyl-glyoxal. If hexose-phosphate is used as the substrate, and dismutation of methyl-glyoxal is prevented owing to absence of co-enzyme, then methyl-glyoxal accumulates and can be identified and estimated.

In the case of *Nicotiana* the enzyme used was contained in either an alcohol-ether or an acetone preparation from the fresh leaves. By this treatment the co-enzyme is largely destroyed, and accumulation of methyl-glyoxal was obtained when magnesium hexose-phosphate was used as substrate.

THE DISMUTATION OF METHYL-GLYOXAL; METHYLGLYOXALASE (KETONALDEHYDEMUTASE)

The next stage in the series of reactions is not clearly indicated for the higher plants. If we refer to the schemes given on p. 261 it will be seen that there are various possibilities; a Cannizzaro reaction can take place either between two molecules of methyl-glyoxal giving rise to pyruvic acid and glycerol; or acetaldehyde, from the decarboxylation of pyruvic acid, can serve as a hydrogen acceptor, being thus reduced to alcohol. The latter contingency does not arise in the higher plants in normal respiration. Other possibilities can be suggested, but without, necessarily, any experimental support. Glycerol, to some extent, must be formed as it is a constituent of fats, and evidence given in the following sections supports the view that pyruvic acid and acetaldehyde are intermediate products in the respiration of higher plants. The precise series of reactions, however, between formation of methyl-glyoxal and the evolution of carbon dioxide remains as yet undetermined.

The presence of a catalyst, **ketonaldehydemutase**, or **methylglyoxalase**, discovered by Neuberg in Yeast, has also been demonstrated in the higher plants (Neuberg & Gorr, 57). These authors used, as a source of enzyme, acetone-treated peas, water extract of ground peas or the alcohol-ether precipitate of such extracts. By this catalyst, methyl-glyoxal was converted into racemic lactic acid. The same result was obtained with similar preparations made from seeds of *Vicia Faba* and *Lupinus albus* (58).

More recently, the presence of such a mutase has been demonstrated by Neuberg & Kobel (61) in Tobacco leaves and by Pi-Suñer Bayo (135) in Lime leaves.

The decomposition of pyruvic acid: carboxylase

The existence of such an enzyme catalysing the decomposition of pyruvic acid was first indicated in the higher plants by Zaleski & Marx (121, 122) in peas, beans (*Vicia Faba*) and seeds of *Lupinus luteus*. In all three cases, the ground seeds were allowed to act on a solution of pyruvic acid (or sodium pyruvate) in presence of an antiseptic. The carbon dioxide formed was estimated, and was found to be considerably greater from the pyruvic acid (or pyruvate) solution than from the control. The decomposition took place either aerobically or anaerobically. Unripe peas, however, produced less carbon dioxide with pyruvic acid than in the case of the control.

Tests were also made for acetaldehyde in the resting liquid. It was identified by Rimini's test, and estimated approximately as acetaldehyde-*p*-nitrophenylhydrazone. Only comparatively little was present in the control.

Further work (Zaleski, 115) was carried out showing the presence of carboxylase in Wheat grains, and Wheat seedlings, and also its action on keto-acids other than pyruvic acid (Zaleski, 118).

Bodnár (3) also found that his zymase preparation from potatoes and beet contained carboxylase.

Bodnár & Hoffner (6) have confirmed the action of Pea, Wheat and Lupin meal on solutions of pyruvic acid and sodium pyruvate. In addition, they found that carboxylase could be extracted by water from the respective meals. They also tested for acetaldehyde, and pointed out that a negative reaction for this substance may be given, especially in the case of the alkaline pyruvate and Pea meal, owing to dismutation of acetaldehyde (see p. 290).

Kobel & Scheuer (134) however were unable to demonstrate the presence of carboxylase in leaves of *Nicotiana Tabacum*.

Evidence for the formation of acetaldehyde as an intermediate product

Kostytschew, Hübbenet & Scheloumoff (43) give evidence for having detected small amounts of acetaldehyde in Poplar catkins kept under anaerobic conditions. Thomas,[1] also, detected and identified acetaldehyde in apples; later (Thomas, 110) he estimated the amounts found in normal apples and in those which had been kept in nitrogen, and showed that there was an increase under anaerobic

[1] *Report Food Investigation Board*, London, 1923.

conditions, as well as in certain concentrations of oxygen and carbon dioxide (see p. 295).

A fixation of aldehyde on the lines employed in the case of Yeast has been obtained in the higher plants by Neuberg & Gottschalk (59, 60). They used coarsely ground peas as material. These were placed in 2·25 % glucose solution containing a certain proportion of calcium sulphite; a control was set up without the sulphite. Both were kept in an atmosphere of hydrogen and, after a certain period of time, the aldehyde was estimated. The amount obtained in the case of added sulphite was 722 mg., whereas the control contained only

TABLE LXVII. Acetaldehyde in tissues of higher plants (Klein & Pirschle)

Material		Mg. aldehyde as % fresh wt.	
		Fixed by "dimedon"	Fixed by sulphite
Flowers.	*Hydrangea*	2·6	2·5
	Tulipa	5·6	6·8
	Matthiola	2·3	—
	Narcissus	2·0	—
	Taraxacum	0·6	—
	Arum	1·4	1·3
	Paeonia	1·6	2·7
	Iris	0·6	—
Leaves.	*Syringa*	4·2	5·5
	Aster	0·3	—
	Myriophyllum	1·4	2·5
Seedlings.	Wheat	7·6	—
	Wheat (1 % glucose)	12·6	—
	Maize. Plumule	0·1	—
	Seed	6·2	5·6
	Radicle	1·4	2·2

9 mg. Such a result is strong evidence for the view that aldehyde is formed as an intermediate product of respiration in the higher plants.

Bodnár, Szepessy & Ferenczy (7) claim also to have shown the fixation of acetaldehyde, using whole peas as material. The peas were allowed to respire anaerobically in presence of sodium or calcium bisulphite. Acetaldehyde was fixed and estimated, together, in some series of experiments with the carbon dioxide and alcohol formed.

More recent work of Klein & Pirschle (30) has demonstrated the accumulation of acetaldehyde in the living plant under aerobic conditions, a demonstration which is more convincing if we are to regard this substance as an intermediate compound in normal respiration.

These authors put their plants into solutions of the reagent for fixing aldehyde. They found rapidly respiring organs, such as seedlings, flowers, etc., to be most useful. The flowers or leaves were placed with their stalks in a weak solution of "dimedon" (dimethylhydroresorcinol)—which forms a condensation product with acetaldehyde—of concentration 1/1000 or in 1 % sodium sulphite. The flowers were kept in good insolation, and the leaves in darkness, for 6–8 days. The condensation product was subsequently extracted from the tissues, decomposed and the aldehyde estimated.

Klein & Pirschle obtained in this way definite evidence of the accumulation of acetaldehyde in respiring flowers and leaves, the controls in each case giving a negative result (see Table LXVII).

They demonstrated further that, in shoots and, especially, etiolated seedlings, the aldehyde formed is increased by sugar-feeding. Also, by separating the plumule and radicle from the seed of etiolated seedlings, they showed how the formation of aldehyde may be dependent on the presence of reserve materials. From these results Klein & Pirschle draw the conclusion that in normal aerobic respiration acetaldehyde is an intermediate metabolic product.

The fate of acetaldehyde; (i) alcohol formation

The fate of acetaldehyde depends primarily in the higher plants on whether respiration is normal. Under anaerobic conditions, it may be reduced to alcohol; in fact in many of the higher plants, in absence of oxygen, alcohol and carbon dioxide are formed in theoretical proportions. It can be assumed that scheme II (see p. 261) possibly represents the reactions taking place.

In Table LXVIII are given the values of carbon dioxide and alcohol found by different observers (41) for various materials under anaerobic conditions.

Thomas (110) has shown that when apples are kept in certain gas mixtures of carbon dioxide and oxygen, a form of zymasis occurs in which, apparently, less aldehyde is reduced to alcohol, so that the ratio alcohol/aldehyde is 2/1 as contrasted with the ratio 50/1 in anaerobic zymasis.

The fate of acetaldehyde; (ii) aldehydemutase

According to Neuberg there is present in Yeast an enzyme, aldehydemutase, which brings about a Cannizzaro reaction between two molecules of acetaldehyde, with the formation of ethyl alcohol and

acetic acid. In fermentation of sugar under ordinary conditions, little acetic acid is formed, but in the presence of alkaline salts, the reaction takes place to a greater extent (see p. 261).

Various investigators have tested for the presence of aldehyde mutase in the higher plants. Bodnár & Hoffner (6) added aldehyde to Pea, Lupin and Wheat meal as well as to extracts of the meal, and found that a certain amount of aldehyde disappeared. No evidence, however, was given that actual dismutation had taken place.

TABLE LXVIII. Alcohol in tissues of higher plants

Material	CO_2 : Alcohol
Pisum, seedlings (Godlewski & Polzeniusz)	100 : 100–105
Pisum, seeds (Nabokich, 55)	100 : 104·4
Ricinus, seeds („)	100 : 69·3
Acer, flowers (Kostytschew) ...	100 : 107
Daucus, root („) ...	100 : 102
Apple (sweet) („) ...	100 : 80
Lepidium, seedlings („) ...	100 : 57
Acer, leaves („) ...	100 : 58
Syringa, leaves („) ...	100 : 56
Prunus, leaves („) ...	100 : 51
Brassica, root („) ...	100 : 49
Apple (sour) („) ...	100 : 42
Potato, tuber („) ...	100 : 7 : 0
Pisum, cotyledons (Boysen-Jensen) ...	100 : 81 : 65
Daucus, root („) ...	100 : 91 : 86 : 72
Grape (green) („) ...	100 : 86 : 74
Grape (blue) („) ...	100 : 95 : 88 : 81 : 74
Potato, tuber („) ...	100 : 7 : 2 : 0
Tropaeolum, leaves („) ...	100 : 45 : 24 : 17
Sinapis, seedling („) ...	100 : 50 : 32

Zaleski & Schatalowa-Zaleskaja (125) showed that dialysed meal from peas and Pea seedlings does not affect aldehyde unless a co-enzyme from hefanol is added (see also p. 298). The authors give no evidence for the fate of the aldehyde.

Kertész (133) also claims that the aldehydemutase of Barley increases in amount on germination, but, again, there is no evidence that mutation has taken place.

Later, Bodnár & Bernauer (5) investigated the actual products formed when acetaldehyde was added to various tissues under anaerobic conditions. Juice from potatoes gave a negative result, but with Pea meal a disappearance of aldehyde was obtained, which was increased by the presence of sodium bicarbonate:

Material	2 % $NaHCO_3$ solution (c.c.)	Water (c.c.)	Time (hrs.)	Aldehyde added (mg.)	Aldehyde remaining (mg.)	Aldehyde used (mg.)
5 gm. Pea meal	—	7	20	22·5	7·2	15·3
,,	1	6	,,	24·4	2·5	21·9
,,	—	7	24	25·0	6·2	18·8
,,	1	6	,,	,,	1·5	23·5

In no case could a trace of acetic acid be detected, though a special examination was made. The same negative result was obtained with whole peas, and leaves of Spinach, Potato and Sugar Beet were used. They therefore conclude that no Cannizzaro reaction has taken place. Tests were made, however, for alcohol in view of the possibility of a simple reduction of acetaldehyde having taken place, but the results were negative. Bodnár & Bernauer point out that Zaleski & Marx (122) obtained alcohol by the action of Pea meal on pyruvic acid. They repeated and confirmed this observation, and as a result suggest that aldehyde is capable of being reduced to alcohol only when attacked in the nascent condition. They further conclude from certain tests (formation of addition products from components of the distillate) that aldol condensation may have taken place.

The co-enzymes of zymasis, glycolysis, etc.

The question of the existence of a co-ferment which activates one or more of the component enzymes of zymase has received attention from several investigators.

Bodnár & Hoffner (6) demonstrated that Pea meal, after dialysis, loses the power of evolving carbon dioxide and forming alcohol; this power is restored by adding an extract of the original meal, as shown below:

Material	CO_2 evolved (mg.)	Alcohol (mg.)	Time (hrs.)
Dialysed meal (5 gm.) + 7 c.c. water	1·2	0	20
Ditto + ext. of meal (20 gm.)	18·4	18·5	,,
Ditto	11·4	12·3	,,

The same result was obtained with a meal from Lupin seeds, and can, moreover, be demonstrated in an atmosphere of hydrogen.

Zaleski & Pissarjewsky (124) also confirmed Bodnár's result, that the formation of carbon dioxide and alcohol from dialysed Pea meal can be stimulated by addition of a boiled extract of the normal meal.

The employment of extracts of hefanol (dried Yeast) by the earlier workers, as mentioned previously (p. 281) in connection with stimulation of post-mortem CO_2-output, has brought up another point for consideration, namely, as to how far stimulation by these extracts may not be due to a co-enzyme. Such a suggestion, in fact, was made by Iwanoff (24, 25). Zaleski (116), though he confirmed the fact of stimulation, is doubtful as to whether hefanol extract contains a co-enzyme or some fermentable substance. He noted a parallelism between stimulation of CO_2-output of certain plants by hefanol extracts and the power to decompose pyruvic acid.

Several of the later investigators have pursued this line of research. Though it would appear to confuse the essential point which concerns us here, namely, whether co-enzymes are involved in the respiration of the higher plants, to test the enzymes of the latter with Yeast extracts, yet it may be worth while to enumerate briefly some recent developments in this direction.

Bodnár & Hoffner (6), as a result of experiments with hefanol extract and Pea and Lupin meal, arrived at a similar conclusion to that of Zaleski. They maintain that hefanol extract cannot replace the Pea and Lupin co-enzymes, but that it contains a substance from which the carboxylase of the meal employed forms carbon dioxide and acetaldehyde, the latter being reduced under some conditions to alcohol.

Zaleski & Pissarjewsky (124), unlike Bodnár & Hoffner, found that the extract of dried Yeast will act as a co-enzyme in the case of dialysed Pea meal.

From the above considerations, yet another question has arisen, namely, as to which enzyme components of the complex series involved in the first (anaerobic) stage of respiration, the co-enzyme is essential; for certain controversies have arisen in connection with Yeast, as to how many of the enzymes, phosphatase, mutase, etc., are activated by the same co-enzyme.

Zaleski & Pissarjewsky (124) maintain that the Pea co-enzyme does not activate the phosphorylation of hexoses. Zaleski & Schatalowa-Zaleskaja (125) also made an investigation, using ground peas (both

ungerminated and germinated), on the relationship of the co-ferment in Pea meal extract to an enzyme in the meal, termed by them "aldehydrase" (≡aldehydemutase). The latter enzyme catalyses some reaction which leads to the disappearance of added acetaldehyde; if dialysed meal is used, then no aldehyde disappears unless extract of meal (or hefanol) is added. They give no information as to the products derived from the aldehyde.

Bodnár & Bernauer (5) however are of the opinion that there is no real evidence for the existence of aldehydemutase in the cases among the higher plants examined by them (see p. 297).

Finally, Kobel & Scheuer (134), as pointed out previously (p. 291), assume that there is a co-enzyme for hexosephosphatase and for methylglyoxalase.

The fate of acetaldehyde; (iii) aerobic oxidation

Evidence quoted in the previous pages is in favour of acetaldehyde as an intermediate product in the respiration system. Should this be so, then, since it does not accumulate, one would suppose it to be oxidised in normal respiration; that is, in presence of air, it apparently does not serve as a hydrogen acceptor as in anaerobic respiration, but its place is taken by oxygen acting either directly or indirectly. How, precisely, this occurs is at present undetermined.

Thomas (110) formerly held the view that, in the case of apples, acetaldehyde, once formed, could not be reoxidised. He worked, however, with material in the later stages of senescence.

The fate of acetaldehyde; (iv) condensation to fat

It has been suggested that fats may arise in the following way from the products of glycolysis of sugar: Acetaldehyde and pyruvic acid first condense to form a higher ketonic acid:

$$CH_3 \cdot CHO + CH_3 \cdot CO \cdot COOH = CH_3 \cdot CH = CH \cdot CO \cdot COOH + H_2O.$$

This ketonic acid then decomposes in a similar way to pyruvic acid:

$$CH_3CH = CH \cdot CO \cdot COOH = CH_3CH = CH \cdot CHO + CO_2.$$

This aldehyde again condenses with pyruvic acid to give a ketonic acid with a longer chain, and so forth.

From the unsaturated ketonic acid, we can obtain an unsaturated fatty acid by oxidation:

$$CH_3 \cdot CH = CH \cdot CO \cdot COOH + O = CH_3CH = CH \cdot COOH + CO_2,$$

and, from this again, by reduction, a saturated fatty acid.

Apart from the production of a certain amount of fatty substance throughout the plant, in many seeds, on ripening, large quantities of carbohydrates are converted into fat. In certain deciduous trees, starch in the woody tissues is replaced by fat during the winter. Such a synthesis of fats from sugars, which needs, in addition, the co-operation of glycerol, might be regarded as an arrested stage of respiration, the products of glycolysis being condensed, instead of being oxidised in the usual way. In the germination of fatty seeds the respiratory quotient $\frac{CO_2}{O_2}$ is very low, there being a large absorption of oxygen. In the development of oily seeds, the converse is true; in the respiration, for instance, of ripening Poppy fruits the quotient $\frac{CO_2}{O_2}$ may be 1·5.

THE PART PLAYED BY MOLECULAR OXYGEN IN RESPIRATION

The interpretation of this line of metabolism is beset with considerable difficulties. Before applying the more modern views of the present day, some account will be given of the suggestions made by one of the earlier workers. A coherent scheme, representing the whole course of respiration including the preliminary anaerobic stages and the later oxidation of the anaerobic (primary) or intermediate products, has been formulated by Palladin, and is well known as his "respiration-theory" involving the function of *Atmungspigmente*. The main outline of the theory may be briefly stated in the following way.

There are, widely distributed throughout the vegetable kingdom, aromatic compounds—frequently in the form of glucosides—which he regards for the purpose of his theory as chromogens, and which on oxidation produce coloured oxidation products. Examples of such substances would be phenols, tannins, aromatic amino-acids, i.e. tyrosine, and many compounds of unknown constitution.

The chromogens are capable of being oxidised by molecular oxygen through the medium of oxidising enzymes (oxidases) to red, reddish-brown, brown, black or even blue pigments—*Atmungspigmente* (see p. 135). In the absence of air, the pigments may be reduced through the medium of reducing enzymes (reductases), and thereby (in modern terminology) act as hydrogen acceptors in the anaerobic (primary) stage of respiration.

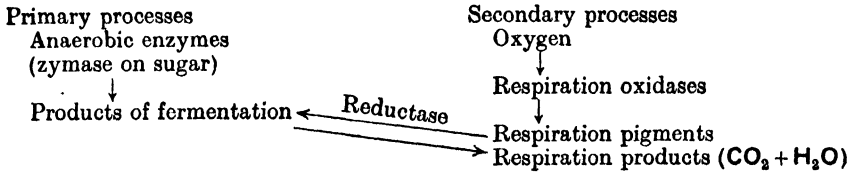
In the living plant, the *Atmungspigmente* do not accumulate, for they are postulated as being constantly reduced. After the death of

the protoplasm by mechanical injury, chloroform vapour, etc., the reductases apparently are weakened or destroyed, and oxidation proceeds without the counteracting reduction.

Some of Palladin's experimental evidence is as follows: Extracts of many plants, on the death of the protoplasm, do in fact produce these coloured products. More exact knowledge of distribution of chromogens was obtained by him in a wide range of plants. The tissues were quickly extracted with boiling water, and to the extracts, peroxidase (from Horse-radish) and hydrogen peroxide were added. In practically all cases, a red, reddish-brown, brown or black product was formed to a greater or less extent. It should be noted that Palladin adds hydrogen peroxide, so that the difference between oxidase and peroxidase plants is thus eliminated.

If such chromogen-containing tissues with direct oxidase, for instance, Wheat seedlings, are allowed to autolyse under sterile conditions (in presence of chloroform or toluol) in air, dark brown or black *Atmungspigmente* are formed. On shutting off the supply of air, these pigments are reduced, and become colourless. Re-oxidation takes place on admitting air, which may again be followed by reduction, and so forth.

Palladin represents his scheme for respiration in simple form as follows:



He regards all carbon dioxide as being of anaerobic origin, and the respiration pigments are reduced by the substances necessarily formed in anaerobiosis. The chromogens are then oxidised by oxidising enzymes, again with production of *Atmungspigmente*:

$$C_6H_{12}O_6 + 6H_2O + 12R = 6CO_2 + 12RH_2 \text{ (anaerobic stage)},$$
$$12RH_2 + 6O_2 = 12H_2O + 12R \text{ (aerobic stage)}.$$

He considers the above reactions to be analogous to the well-known reaction of methylene blue, which can act as a hydrogen acceptor.

Under anaerobic conditions, since respiration pigments are not re-oxidised, other substances would be reduced, such as acetaldehyde to alcohol. There is, however, one outstanding difficulty in regard to

Palladin's theory, and that is the apparent lack of oxidase systems in a certain percentage (about 40 %) of the higher plants. Some other mechanism may take its place, though general considerations lead one to expect uniformity in the process of respiration.

We may now turn to some of the later views on the oxidation by molecular oxygen in respiration. The immediate problem before us in the higher plants may be expressed in the following way. We have

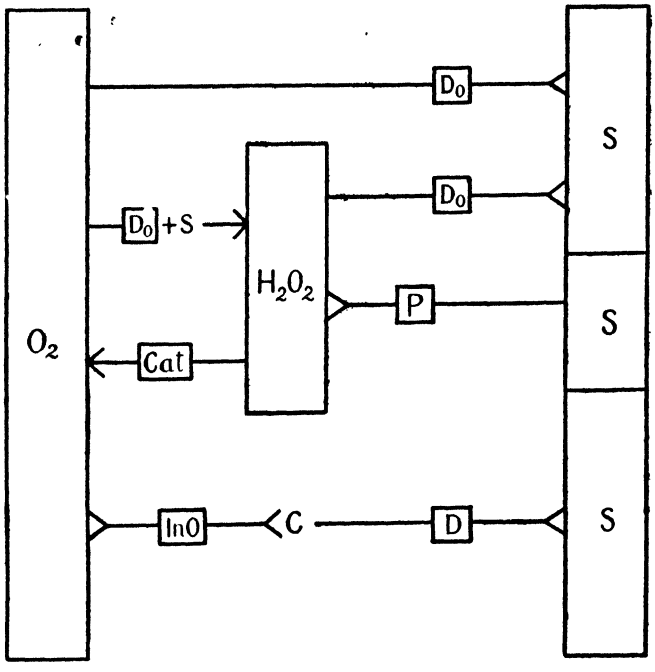


Fig. 11. Schematic representation of oxidising systems (Dixon).
S = substrates, i.e. organic substances undergoing oxidation.
D = anaerobic dehydrases.
D_0 = aerobic dehydrases.
P = peroxidases.
Cat = catalase.
InO = indophenol oxidase = respiratory enzyme.
C = cytochrome.
A>—[B]—C reads "A, being activated by the enzyme B, reacts with C".
A—[B]—>C reads "A, under the influence of B, is converted into C".

attained to the production of carbon dioxide and acetaldehyde from sugar. As glycerol is only formed to a limited extent, and alcohol does not accumulate in normal respiration, either these intermediate products are oxidised or some other substance or substances must be reduced in the anaerobic stages. In any case, whether the whole anaerobic stage is prolonged by reduction of a number of intermediate metabolites or not, oxidation by molecular oxygen must ultimately

take place, or respiration would cease. There is, it would appear, in addition to the oxidation of the intermediate acceptors, the oxidation of acetaldehyde to carbon dioxide and water.

As we have noted in Chapter III, many oxidation systems have been identified in plant and animal tissues; many attempts also have been made to fit these into schemes of respiration. Such a scheme (Dixon, Bib. VI, 162) is set out below. Based on observations with animal tissues, bacteria and yeast, this scheme does not take into account the special case of the higher plants. It is reproduced in Fig. 11, however, as showing the trend of thought among a certain group of investigators.

It is not in place here to discuss theories of either oxidation or oxidase reactions. Certain outstanding points, however, must be noted in connection with the aspect of such problems expressed in the scheme above (see also Chapter III). The advocates for this scheme emphasise the fact that practically all oxidations of organic compounds actually produce a loss of hydrogen atoms. In cases where there is addition of oxygen (for example, oxidation of aldehyde) the lack of concordance is overcome by assuming the preliminary formation of a hydrate. Oxidation of metabolites is regarded as an activation and removal of hydrogen as postulated by Wieland, this activation being catalysed by enzymes termed dehydrases. Certain of these enzymes, the aerobic dehydrases (for example, xanthine oxidase), can not only oxidise their substrates anaerobically in presence of a hydrogen acceptor, but also in air, molecular oxygen acting then as the hydrogen acceptor. In so doing, molecular oxygen is reduced to hydrogen peroxide, and the latter, acting again as a hydrogen acceptor, may be further reduced to water:

$$\underset{\text{Xanthine}}{X} + OH_2 = \underset{\text{Xanthine hydrate}}{XOH_2}$$

$$XOH_2 + \text{MethBl} = \underset{\text{Uric acid}}{XO} + \text{MethBl } H_2$$

$$H_2O_2 + H_2 \rightarrow 2H_2O$$

or hydrogen peroxide may be activated by peroxidase, giving rise to indirect oxidation; or, again, may be decomposed by catalase.

The scheme given above can now be explained in the author's words:

"According to this scheme, cell oxidations proceed along two main lines initiated by the two types of dehydrase respectively. Corresponding to these two lines the absorption of oxygen (respiration) is due to two different processes. In one the oxygen oxidises cytochrome under the influence of indophenol oxidase; in the other the oxygen is

converted into hydrogen peroxide in oxidising the substrates of the aerobic dehydrases.

"Making the fairly probable, but perhaps not absolutely justifiable, assumption that the respiration of tissues consists entirely of these two processes, we can obtain some information as to their relative importance in respiration from the effect produced by cyanide. Cyanide does not inhibit the aerobic dehydrases and those systems which react directly with oxygen, but it inhibits completely the indophenol oxidase system."

Dixon and his co-workers have found that cyanide inhibits the respiration of animal tissues, in general, to the extent of 50–80 %. Hence he concludes that the cytochrome system cannot contribute more than two-thirds of the total respiration, the remaining third being due to aerobic dehydrases and similar systems.

To continue the explanation of the scheme in detail:

"The top line represents reactions such as the oxidation of hypoxanthine, etc., by the xanthine oxidase. The second line shows on the left the formation of hydrogen peroxide during these reactions, and on the right its subsequent reduction to water by the same system. Below this is represented the various coupled oxidations in presence of peroxidase, and on the left the protective action of catalase. These systems are connected with the first main line of events, where oxygen acts as a direct hydrogen acceptor, and perhaps account for about one-third of the total oxygen uptake.

"The second main line of events, accounting probably for a considerable part of the remainder, is represented by the indophenol oxidase oxidising cytochrome...which in turn is reduced by the anaerobic dehydrases plus their substrates. This chain of events is prevented by cyanide, etc."

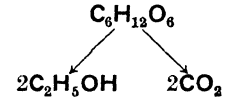
Applying, as far as possible, the suggestions in the previous paragraphs to the case of the higher plants, certain comments can be made. Catalysts of the type of dehydrases of course exist, as, for instance, the mutases of the anaerobic stages of respiration; also those catalysts which have been identified by Thunberg. The dominating system, however, investigated in the higher plants has been that of Bach & Chodat, with the later modification introducing the catechol-oxygenase-peroxidase components, which has been discussed fully in Chapter III. The mechanism of this system is still doubtful; it does not conform to the type of aerobic dehydrases, since oxidation by it has not yet been brought about in the presence of any hydrogen

acceptor other than molecular oxygen. It is, moreover, inhibited by cyanide. It has long been known as a system forming peroxide, which would be capable of producing secondary oxidations under the influence of peroxidase, so that the exhibition of a similar phenomenon in connection with aerobic dehydrase has not the novelty claimed by its discoverer.

The oxygenase plus catechol compound constitutes an indophenol oxidase, though the oxygenase alone is without action on this reagent. To regard this system as a component of the respiratory complex is unsatisfactory, since it is absent from about 40 % of the higher plants in which no such indophenol oxidase is detected, though there is every reason to believe that respiration takes place, broadly speaking, on similar lines throughout the group. The impression left, after consideration of all available facts, is that some relatively simple universal mechanism is responsible for the catalysis of oxidation by molecular oxygen in respiration.

Oxidative anabolism

From the recent studies of respiration of apples, Blackman (2) has demonstrated, like Meyerhof for Yeast, that there is a certain amount of oxidative anabolism in respiration in air. He points out that glycolysis in air and nitrogen can be assumed to be the same in amount, as oxygen has no effect on zymase activity. Further, in anaerobic respiration of sugar, the following relationship holds:

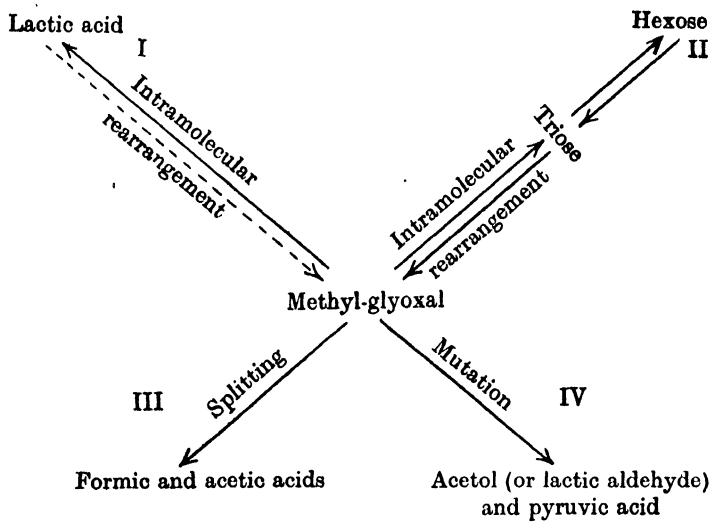


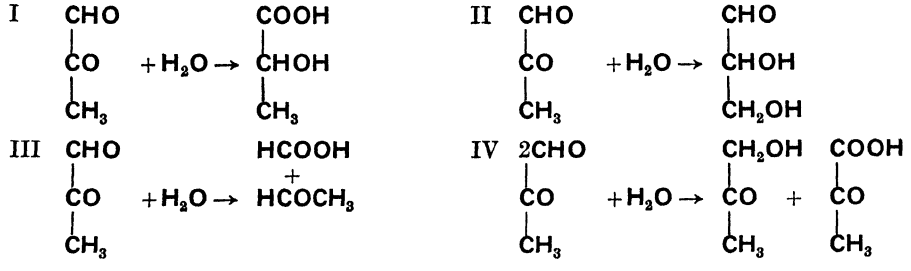
The loss of carbon dioxide in respiration in air has been found by him to be considerably less than that in nitrogen, while glycolysis can be assumed to be the same for both. Hence the carbon loss is three or more times greater in nitrogen than in air. As no carbon-containing product of glycolysis is known to accumulate during aerobic respiration, the only conclusion to be drawn is that a proportion of the products of glycolysis is built back in aerobic respiration by oxidative anabolism to sugar or some other complex. The proportion of that built back to that respired is 3 or 3·5 to 1.

Conclusion

In a recent survey of the relationship of changes which have actually been achieved by chemical means to those occurring in the metabolism

of the living organism, Bernhauer[1] gives the following scheme for the rearrangement of the methyl-glyoxal molecule by chemical means:





We do not know at present the precise route taken nor the exact kind and amount of intermediate products formed in the decomposition of the sugar molecule in the respiration of the higher plants. It is obvious that under special conditions special products predominate, or divergent courses arise for the transmission of these products. It is hoped that the résumé set out in the preceding pages may stimulate investigation upon these, as yet undefined, stages.

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