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AN INTRODUCTION TO THE CHEMISTRY OF PLANT PRODUCTS

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AN INTRODUCTION

TO THE

CHEMISTRY OF PLANT PRODUCTS

VOL. I. ON THE NATURE AND SIGNIFICANCE OF THE COMMONER ORGANIC COMPOUNDS OF PLANTS

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PREFACE TO THE FOURTH EDITION.

THE original intention of this work was to provide students with an account of the chemistry and physiological significance of some of the more important substances occurring in the plant. The founding of Chairs of Biochemistry during recent years, with the consequent dissemination of biochemical knowledge, would appear to give just cause for the discontinuance of the present work, but it is not possible for all students to avail themselves of the facilities offered, and it is primarily for such students that the work is intended.

The enormous output of papers and the recent advances in knowledge have necessitated much revision, and, in the main, the present edition has been rewritten. In so doing we have borne in mind the requirements of those approaching the subject from different angles and have included a certain amount of somewhat elementary information, both botanical and chemical, and also have admitted certain rather more advanced aspects of the subject even though they be matters of controversy.

We fully recognize that this involves some disproportion, some lack of balance, but this is inevitable.

P. H.
T. G. H.

June, 1928.

PREFACE TO THE FIRST EDITION.

THE importance to the botanist of a working knowledge of chemistry can hardly be overestimated, since vegetable physiology is replete with problems awaiting solution by the combined application of botanical and chemical methods.

Teachers of vegetable physiology, however, not infrequently find that their students' knowledge is deficient in just those branches of chemistry which are of particular importance to the botanist, which is, no doubt, largely due to the fact that those compounds which are of interest to the botanist do not necessarily fit into the scheme of instruction of the chemist.

The present work is an attempt to provide such students, who are assumed to have some acquaintance with chemistry, with an introductory account of the chemistry and biological significance of some of the more important substances occurring in plants.

In compiling this book various sources of information have been laid under contribution, and although the point of view is, in the main, purely chemical and botanical, the economic aspect has not been lost sight of, and, where possible, mention has been made of the uses of plant products and of the manufacturing processes employed in their preparation.

P. H.
T. G. H.

December, 1912.

PREFACE TO THE THIRD EDITION.

THE necessity for a third edition has afforded an opportunity for making certain changes in the arrangement of the subject-matter. In order to give the more purely physiological aspect of the subject fuller treatment, without at the same time unduly increasing the size of the volume, the work now appears in two parts. Volume I. is essentially the same in scope as the earlier editions and deals primarily with the more chemical side of the subject: a sufficiency of plant physiology has, however, been retained to make the account reasonably complete and to preserve the character of the work. Volume II., which is in preparation, will be devoted to more purely physiological problems, and will contain some of the matter previously found in the original volume.

The present volume has been brought up to date as far as is possible; some portions have been rewritten, Section VIII. for example, and in other sections a certain amount of rearrangement has been deemed advisable.

P. H.
T. G. H.

October, 1920.

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SECTION I.

FATS, OILS, AND WAXES.

In ordinary parlance, no clear distinction is made in the use of the terms fat and wax, which are applied more or less indiscriminately to any solid substances which have a greasy feeling to the touch and do not mix with water. Chemically, however, there is a marked difference between the two classes; the fats are compounds of the trihydric alcohol glycerol, whereas the waxes are compounds of the higher monohydric alcohols, such as cetyl alcohol $C_{16}H_{33}OH$, myristic alcohol $C_{30}H_{61}OH$, and cholesterol $C_{27}H_{45}OH$.

The tendency to rely on physical properties only, and to regard waxes as having generally a harder consistency than fats, has given rise to several cases of incorrect nomenclature. For example, wool fat and spermaceti being compounds of cholesterol and cetyl alcohol are in reality waxes, though they are usually regarded as fats, whereas the substance ordinarily known as Japan wax is actually a fat, since it is a compound of glycerol.

The term oil, as used in the ordinary sense to imply a liquid which is immiscible with water, must not be taken to have any chemical significance, since substances having this physical property are found in almost every class of chemical compound. Used in connection with fats, the term oil simply implies a fat that is liquid at ordinary temperatures; any solid fat on melting becomes an oil, and, on the other hand, any fatty oil on solidifying becomes a fat.

OCCURRENCE.

Fats are very widely distributed in the vegetable kingdom, and occur in both vegetative and reproductive structures; in fact, it is highly probable that all living cells contain a certain

amount of fat. Amongst the Protophyta, fat is the characteristic food reserve of the Heterokontæ, Chrysophyceæ, Bacillariales, and Chloromonadales. In the Phaeophyceæ, the amount of fat, or fat-like substances, would appear to vary with the conditions of life. Thus *Pelvetia canaliculata*, var. *libera*, which is submerged only during the spring tides, may contain 8 per cent of ether-soluble material, whilst *Laminaria digitata*, which is exposed only at low water of spring tides, contains but 0·5 per cent. The fucoids of the intermediate zones contain amounts of ether-soluble substance intermediate between these extremes. The Rhodophyceæ which are characteristic of the submerged zone would appear to contain less fat, thus *Chondrus crispus* yields 0·2 per cent of ether-soluble material.* The fats of the Fungi, which are rich in fatty acids associated with lecithins and ergosterols, vary much in amount; thus the sclerotia of *Claviceps purpurea* (ergot) may contain as much as 60 per cent, whilst the mycelium of *Lactarius deliciosus* contains about 6 per cent.

In Angiosperms fats are widely distributed, especially in seeds where they may replace the carbohydrates as a reserve food-material and are not uncommonly associated with protein reserves; to mention a few examples, colza oil is obtained from the seeds of *Brassica Napus*, palm oil from the pericarp of the fruits of *Elæis guineensis*, cotton-seed oil from *Gossypium herbaceum*, linseed oil from *Linum usitatissimum*, olive oil from the sarcocarp of *Olea europæa*, castor oil from the seeds of *Ricinus*, and cacao butter from the fruits of *Theobroma*.

Oils of lesser economic importance occur in the fruits or seeds of the sunflower, almond, hemp, willow, and many other plants.

The amount of oil present in such structures may be quite considerable, thus in the kernel of the Brazil nut nearly 70 per cent may obtain, and in the almond about 54 per cent.

Oils also occur in the vegetative organs to a greater or lesser extent; substances of an oily nature are found in association with the chloroplasts and, in some cases, to a relatively large extent, e.g. in *Strelitzia*; sometimes it is present as a definite

* Authors' observations hitherto unpublished.

reserve food-material as in the tubers of *Cyperus esculentus*, where it is associated with starch, and in the roots of some orchids.

This particular form of food reserve is doubly of value since its presence may lessen the danger arising from drought, and also more energy can be stored up in the form of oil than in an equal bulk of carbohydrate; in this connection may be mentioned the fact that in some cases the appearance of oil may be transient, thus in some trees the starch stored up in the parenchyma of the stem may be converted into fat during the winter's cold; the starch, however, reappears on a rise in temperature. Also fat or fat-like substances may appear in the leaves of evergreen plants during the winter months. The fat-like substances, according to Meyer,* who studied *Vinca*, *Taxus*, and *Ilex*, do not show a seasonal variation in amount, but continually increase with the age of the leaf. A low temperature would appear to be a significant factor in this connection. Thus Tuttle † found that plants of *Linnæa borealis* exposed to a low temperature contained fat but no starch; on raising the temperature to 20° C., starch appeared in the course of a day or two in a few plants, and in all cases after the lapse of a week, during which period the plants were kept in the dark. The controls, on the other hand, kept in the dark at the low outside temperature gave no reaction for starch. Plants containing much starch, on exposure to a moderately low temperature, — 2° C., were found to lose their starch and, concurrently, fat appeared. But if such starch-containing plants were immediately exposed to very low temperatures, — 15° to — 28° C., no reconversion ensued and death took place. Lipase is present in the leaves of plants showing these changes, and this, presumably, is part of the mechanism of the change. Tuttle also found that all evergreens growing in Northern Alberta contained little or no starch but much fat by the end of October. All of the many plants examined, *Populus*, *Salix*, *Betula*, *Pyrola*, *Picea*, etc., with the exception of *Lonicera glaucescens* and *Cratægus*,

* Meyer: "Ber. deut. bot. Gesells.," 1918, **36**, 5.
† Tuttle: "Ann. Bot.," 1919, **33**, 201; "Bot. Gaz.," 1921, **71**, 146.

contained fat as a food reserve during the winter months. Even the leaves of deciduous plants at the time of leaf-fall were devoid of starch but contained fat. Whilst the power of forming fat from starch is not uncommon in plants naturally exposed to extreme winter cold, the ability to form starch on the advent of warmer weather does not necessarily follow. Thus many alpine Ericaceæ and Salicaceæ possess both starch and fat during the vegetative season, and *Gaultheria ovalifolia*, a lowland plant, has only fat. Wherefore the ability to form starch is not entirely to be associated with the climatic conditions resulting from high altitudes. These phenomena are similar to those which will be mentioned in connection with the conversion of starch into sugar under the influence of low temperature (p. 176).

The majority of vegetable fats are fluid at ordinary temperatures; a few, however, are solid, for instance, cacao butter and the fat in the seeds of *Myristica*.

CONSTITUTION OF FATS.

The naturally occurring fats are mixtures of esters of glycerol with fatty acids such as palmitic $C_{15}H_{31}COOH$ or stearic $C_{17}H_{35}COOH$ acids, or with the unsaturated acid oleic acid $C_{17}H_{33}COOH$.

A wax, on the other hand, is an ester of a monohydric alcohol as illustrated by the equation :—

$$\underset{\text{Palmitic acid}}{C_{15}H_{31}COOH} + \underset{\substack{\text{Myricyl}\\\text{alcohol}}}{C_{30}H_{61}OH} = \underset{\text{Myricyl palmitate}}{C_{15}H_{31}COOC_{30}H_{61}} + H_2O$$

myricyl palmitate being the chief constituent of beeswax.

Lapworth and Pearson * have shown that the glycerol in fats may be directly replaced by a higher polyhydric alcohol such as mannitol. This replacement may be brought about by distilling olein or stearin with mannitol under reduced pressure in the presence of sodium ethoxide. By this treatment much of the glycerol of the fat is expelled, the maximum yield being reached when the proportion of fat to the mannitol corresponds with two molecules of the former to three mole-

* Lapworth and Pearson: "Biochem. Journ.," 1919, **13**, 296. See also Irvine and Gilchrist: "J. Chem. Soc.," 1924, **125**, 10.

cules of the latter. The composition of the mannitol olein, or mannitol stearin, corresponds with that of a mixture of dioleates or distearates of mannitan and isomannide. It has been shown by feeding experiments that mannitol olein is utilized by animals to the same extent as olive oil, but there is no evidence that mannitol fats occur in nature.

The classification and identification of fats is based upon the acids which they contain. Thus it is found that whereas beef suet and mutton fat consist chiefly of esters of the higher fatty acids, such as palmitic and stearic acids, butter contains a considerable quantity of the lower members of this same fatty series such, for example, as butyric, caproic, caprylic, and capric acids ; these acids, which are low boiling liquids readily volatile with steam, are known as volatile fatty acids, and their presence or absence in a given sample of fat may be used for characterizing the fat. Thus, for example, the estimation of the amount of volatile fatty acid serves to distinguish genuine butter from its substitute margarine, which is relatively poor in volatile acids and contains chiefly higher fatty acids.

The more important members of the fatty acid series are given in the following list :—

$HCOOH$	or CH_2O_2	Formic acid †
CH_3COOH	,, $C_2H_4O_2$	Acetic acid
C_2H_5COOH	,, $C_3H_6O_2$	Propionic acid †
C_3H_7COOH	,, $C_4H_8O_2$	Butyric acid
CH_3 CH_3>$CH\ CH_2CH_2COOH$	,, $C_6H_{12}O_2$	Isobutyl acetic or caproic acid
$CH_3(CH_2)_6COOH$	,, $C_8H_{16}O_2$	Caprylic acid
$CH_3(CH_2)_8COOH$	,, $C_{10}H_{20}O_2$	Capric acid
$CH_3(CH_2)_{10}COOH$	,, $C_{12}H_{24}O_2$	Lauric acid
$CH_3(CH_2)_{12}COOH$	,, $C_{14}H_{28}O_2$	Myristic acid
$CH_3(CH_2)_{14}COOH$	,, $C_{16}H_{32}O_2$	Palmitic acid
$CH_3(CH_2)_{16}COOH$	,, $C_{18}H_{36}O_2$	Stearic acid
$CH_3(CH_2)_{18}COOH$	,, $C_{20}H_{40}O_2$	Arachidic acid
$CH_3(CH_2)_{20}COOH$	,, $C_{22}H_{44}O_2$	Behenic acid

It should be noted that these acids all conform to the general formula for the fatty acids, $C_nH_{2n}O_2$, in which " n " may have any value, odd or even, but only those in which " n " is an even number are found to occur naturally in fats ;

* Halliburton, Drummond, and Cannan : "Biochem. Journ.," 1919, 13, 301.

† These acids do not occur in fats.

the alleged occurrence in natural fats of acids with an uneven number of carbon atoms has in every case, so far recorded, been refuted on careful re-examination.

It appears probable, moreover, that all naturally occurring fatty acids have a straight and not a branched carbon chain, so that it is open to question whether the *iso*-butyl acetic acid which is said to have been found in fats was not, in reality, normal caproic acid of the formula $CH_3(CH_2)_4COOH$.

Besides acids of the fatty series whose general formula is $C_nH_{2n}O_2$, acids belonging to several other series, poorer in hydrogen than the above, are found in fats. The simplest example of such a series of acids is furnished by the acids of the Oleic series, the members of which differ from the corresponding members of the fatty acid series in having two atoms of hydrogen less.

Some of the more important acids of this group are given below.

1. Acids of the OLEIC or ACRYLIC series.

General formula $C_nH_{2n-2}O_2$.

$C_5H_8O_2$	Tiglic acid
$C_{18}H_{34}O_2$	Oleic acid
$C_{18}H_{34}O_2$	Elaïdic acid
$C_{18}H_{34}O_2$	Iso-oleic acid
$C_{22}H_{42}O_2$	Erucic acid
$C_{22}H_{42}O_2$	Brassidic acid

The most widely distributed of these acids is undoubtedly oleic acid, which, in the form of its glyceride triolein,

$$\begin{array}{l} C_{17}H_{33}COOCH_2 \\ | \\ C_{17}H_{33}COOCH \\ | \\ C_{17}H_{33}COOCH_2 \end{array}$$

forms an important constituent of most vegetable and animal oils.

2. Acids of the LINOLIC series.

General formula $C_nH_{2n-4}O_2$.

(*a*) Open chain compounds, $C_{18}H_{32}O_2$ Linolic acid and its various isomers.

(*b*) Cyclic compounds, $C_{16}H_{28}O_2$ Hydnocarpic acid.
$C_{18}H_{32}O_2$ Chaulmoogric acid.

3. Acids of the LINOLENIC series.

General formula $C_nH_{2n-6}O_2$.

$C_{18}H_{30}O_2$ Linolenic acid and its isomers.

4. Acids of the CLUPANODONIC series.

General formula $C_nH_{2n-8}O_2$.

$C_{18}H_{28}O_2$ Clupanodonic acid.

5. Acids of the RICINOLEIC series.

General formula $C_nH_{2n-2}O_3$.

$C_{18}H_{34}O_3$ Ricinoleic acid and its isomers.

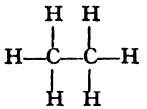
The relationship between the five series of acids, which differ from each other successively by two atoms of hydrogen, as shown by the formulæ,

$C_nH_{2n}O_2$, $C_nH_{2n-2}O_2$, $C_nH_{2n-4}O_2$, $C_nH_{2n-6}O_2$, and $C_nH_{2n-8}O_2$,

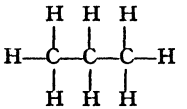
is similar to that subsisting between the three series of hydrocarbons having the general formulæ,

C_nH_{2n+2}, C_nH_{2n}, C_nH_{2n-2}.

The hydrocarbons of the first or Paraffin series are said to be saturated, by which is meant that each of the four valencies of their carbon atoms are fully satisfied, as shown by the following graphic formulæ :—

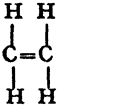


Ethane C_2H_6

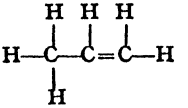


Propane C_3H_8

When, however, the graphic formulæ of the corresponding members of the second or Olefine series are written, it is found that if the tetravalency of carbon is maintained, there are not enough hydrogen atoms to satisfy all these valencies, and, in order not to leave any unsatisfied, the remaining valencies must be united to each other, thereby joining two carbon atoms to each other by more than one bond :—

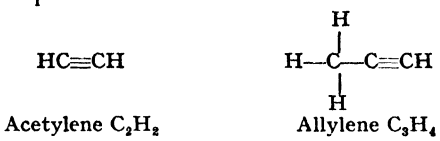


Ethylene C_2H_4



Propylene C_3H_6

In the next series of hydrocarbons, the acetylenes, by the loss of two more hydrogen atoms, the process has been carried a step farther, with the result that two carbon atoms are united by a triple bond :—



Acetylene C_2H_2 Allylene C_3H_4

All such substances containing two carbon atoms united together by more than one bond are said to be unsaturated, and are able to form additive compounds with many substances, notably the halogens.

Thus, while the saturated hydrocarbon will only react with chlorine or bromine by the replacement of one atom of hydrogen for each atom of halogen introduced into the molecule,

$$C_2H_6 + Br_2 = \underset{\text{Ethyl bromide}}{C_2H_5Br} + HBr$$

an unsaturated compound, such as ethylene, will add on the halogen directly,

$$C_2H_4 + Br_2 = \underset{\substack{\text{Ethylene}\\ \text{dibromide}}}{C_2H_4Br_2}$$

the resulting additive compound being saturated.

It will thus be seen that it requires two atoms of bromine to saturate an unsaturated compound containing one double bond, and similarly it requires four atoms of halogen to saturate a compound containing two double bonds. In this way it is shown that since the acids of the oleic, linolic, and linolenic series require two, four, and six atoms of halogen respectively for saturation, they must contain respectively one, two, or three double bonds.

A measure of the degree of unsaturation of a given acid may accordingly be obtained by determining how much bromine it will absorb ; as, however, the interaction with bromine is liable to be violent, it is found more convenient to employ iodine, which, in addition to being less violent in its action than bromine, is also easier to handle.

A description of the method employed in determining what is known as the " iodine value " of fats is given below (p. 23).

PHYSICAL PROPERTIES OF FATS.

The naturally occurring fats vary in consistency from oils to wax-like solids; the solid fats have mostly a low melting-point which is, however, rarely a sharp one, as natural fats are not simple substances, but are, as a rule, mixtures of several different chemical individuals; such mixtures never have sharp melting-points.

All fats and fatty oils are lighter than water, their specific gravity varying from about 0·900 to 0·970 at 15°. They are insoluble in water and at ordinary temperatures are sparingly soluble in cold alcohol, excepting castor oil which dissolves readily in this solvent; they, however, dissolve readily in ether, chloroform, petroleum ether, benzene, carbon tetrachloride or carbon disulphide.

CHEMICAL PROPERTIES OF FATS.

One of the most important chemical properties of fats is their decomposition by hydrolysis.

The term hydrolysis, which literally means loosening by water, is applied to any reaction in which a substance is broken up into two or more simpler ones with the fixation of water.

The following examples taken from a variety of different classes of compounds all illustrate this reaction :—

$$\underset{\text{Ethyl acetate}}{CH_3COOC_2H_5} + H_2O = \underset{\text{Acetic acid}}{CH_3COOH} + \underset{\text{Ethyl alcohol}}{C_2H_5OH} \quad . \quad . \quad (1)$$

$$\underset{\text{Methyl cyanide}}{CH_3CN} + 2H_2O = CH_3COOH + NH_3 \quad . \quad . \quad . \quad (2)$$

$$\underset{\text{Hippuric acid}}{C_6H_5CONHCH_2COOH} + H_2O = \underset{\text{Benzoic acid}}{C_6H_5COOH} + \underset{\text{Glycine}}{NH_2CH_2COOH} \quad . \quad (3)$$

$$\underset{\text{Malt sugar}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{Glucose}}{2C_6H_{12}O_6} \quad . \quad . \quad . \quad . \quad (4)$$

$$\underset{\text{Amygdalin}}{C_{20}H_{27}NO_{11}} + 2H_2O = \underset{\text{Benzaldehyde}}{C_6H_5CHO} + \underset{\text{Glucose}}{2C_6H_{12}O_6} + \underset{\text{Hydrocyanic acid}}{HCN} \quad . \quad (5)$$

It will be seen from reaction (1) that the conversion of an ester into an acid and an alcohol is an example of hydrolysis, and since fats are esters it follows that they also must be capable of hydrolysis.

The reaction—

$$\begin{array}{l} C_{17}H_{35}COOCH_2 \\ C_{17}H_{35}COOCH \\ C_{17}H_{35}COOCH_2 \end{array} + 3H_2O = \underset{\text{Stearic acid}}{3C_{17}H_{35}COOH} + \underset{\text{Glycerol}}{\begin{array}{l} CH_2OH \\ CHOH \\ CH_2OH \end{array}}$$

is, however, not readily brought about by water alone at ordinary temperatures; in the presence of enzymes, however, the hydrolysis may be effected at a moderate temperature with comparative ease.

The hydrolysis of fats for the purpose of preparing the free fatty acids may be effected in either of the following ways :—

1. By acting on the fat with superheated steam in the presence of a little lime or magnesia, which acts as a catalytic agent.

This method is the one most commonly adopted by candle-makers for the preparation of fatty acids required in the manufacture of candles. The fat is subjected to the action of steam under pressure at 170° in large copper vessels in the presence of a small quantity of lime. The resulting mixture is then treated with sulphuric acid sufficient in amount to combine with the lime, after which the free fatty acids rise to the surface in a molten condition.

2. By the action of concentrated sulphuric acid.

The molten fats are stirred up in leaden vessels with 9 per cent of concentrated sulphuric acid, the mixture being heated to about 120° C. The mixture is then warmed with water to remove the acid, and the acids are further purified by distillation with steam.

SAPONIFICATION OF FATS.

Closely related to hydrolysis is the reaction known as saponification; this reaction, which literally means "soap-making," is that which takes place when a fat is boiled with caustic alkali. The alkali acts in much the same way as water, breaking up the ester into glycerol and the fatty acid which, however, in this case, combines with the alkali to form a salt :—

$$\begin{array}{l} C_{17}H_{35}COOCH_2 \\ C_{17}H_{35}COOCH \\ C_{17}H_{35}COOCH_2 \\ \text{Tristearin, a fat} \end{array} + 3KOH = \begin{array}{l} 3C_{17}H_{35}COOK \\ \text{Potassium stearate,} \\ \text{a soap} \end{array} + \begin{array}{l} CH_2OH \\ CHOH \\ CH_2OH \\ \text{Glycerol} \end{array}$$

It so happens that the sodium and potassium salts of palmitic, stearic, and oleic acids dissolve in water, forming opales-

cent alkaline solutions which readily give a lather, and can, therefore, be used as soaps,* and hence the process by which they are made from fats is called saponification. Although alkali metal salts of other organic acids do not exhibit the characteristics of soap, the term saponification is commonly extended to include all cases of the decomposition of an ester into the corresponding alcohol and the salt of the acid, even though that salt may have none of the characteristic properties of a soap.

The saponification of a fat on a small scale † in the laboratory may be effected as follows: Boil the fat under a reflux

* The sodium and potassium salts of oleic acid and of the higher fatty acids, such as palmitic and stearic acids, when dissolved in water, are, to a large extent, hydrolysed into free fatty acid and caustic soda, according to the equation—

$$\underset{\text{Sodium stearate}}{C_{17}H_{35}COONa} + H_2O = \underset{\text{Stearic acid}}{C_{17}H_{35}COOH} + NaOH$$

The stearic acid combines with some of the unhydrolysed soap to form an insoluble acid salt, giving rise to an opalescent or turbid solution. It is this insoluble acid salt which is responsible for the formation of a lather on shaking such a solution. The detergent or cleansing action of soap is dependent on the above reaction, since the caustic soda detaches the greasy dirt which then becomes enveloped in a layer of soap solution from the lather, and is so carried away.

In this connection it is interesting to note the similar effect of soap on the formation of emulsions.

An emulsion may be defined as a mixture, under special conditions, of two otherwise immiscible liquids. Thus, for example, if olive oil is shaken up with water, the two liquids rapidly separate as soon as the shaking ceases. If, however, a little soap solution or some other substance such as gum acacia, tragacanth, saponin (see p. 261), or white of egg be added and the shaking repeated, an emulsion results owing to the oil particles being enveloped in a layer of soap or other substance which prevents their coalescing. Milk is an example of a naturally occurring emulsion; so also is latex, contained in plants.

If pure olive oil, free from oleic or other acid, is shaken up with caustic soda no emulsion is produced; on the other hand, olive oil which has been kept some time and contains free oleic acid, when shaken up with caustic soda does produce an emulsion, thus showing that the emulsifying agent is not the free alkali but the soap produced in the second case from the soda and the oleic acid.

This may be also illustrated by Bütschli's experiment, which consists in placing a drop of old olive oil containing 9 per cent of oleic acid on a little 0·06 per cent aqueous solution of sodium carbonate. If examined under the microscope it will be seen to consist of a fine honeycomb structure, consisting of particles of oil, the whole apparently exhibiting amœboid movements; these latter are due to difference in surface tension.

† For commercial soap manufacture, see p. 33.

condenser with alcoholic potash in the proportion of about 5 gms. of fat to 50 c.c. of alcohol containing from 2-3 gms. of caustic potash. The heating should be continued until on pouring a little of the solution into a large volume of water an opalescent solution free from undecomposed fat results. The time required for this may vary from a few minutes to half an hour or more.

When the saponification is complete, the contents of the flask should be heated in an evaporating basin over a water bath, and thoroughly stirred to get rid of the alcohol. If the free fatty acids are required, the residual soap is dissolved in water and sufficient sulphuric acid is then added to make the solution strongly acid, whereupon the fatty acids separate out and rise to the surface.

The aqueous layer contains the glycerol together with the excess of sulphuric acid and potassium sulphate.

In addition to the trihydric alcohol glycerol, all fats contain a small quantity of the monohydric alcohols, cholesterol and phytosterol * which constitute what is known as the unsaponifiable residue of fats (cf. p. 22).

These substances may be isolated from fats according to the following method devised by Kossel and Obermüller.†

An ethereal solution of the fat is mixed with a solution of sodium in alcohol; saponification takes place in the cold and the soap which is precipitated from solution can be filtered off; the filtrate, which is a mixture of alcohol and ether, contains the glycerol together with the so-called unsaponifiable residue consisting of phytosterol or cholesterol, which may be obtained by evaporating the solvent.

EXTRACTION OF FATS.

The isolation of fats from admixture with other substances may be effected by extraction by means of fat solvents.

* The term phytosterol, though employed by many authors to indicate a single definite substance, is beginning to be used as a generic term for a whole group of closely allied substances, the number of which is rapidly increasing as the investigation of vegetable fats proceeds.

† Kossel and Obermüller: "Zeit. physiol. Chem.," 1890, 14, 599; 1891, 15, 321.

The principle of the extraction is to treat the dried mixture with a solvent which will dissolve only the fat and leave the other substances unchanged. The solvents most commonly used for this purpose are ether, light petroleum, carbon tetrachloride, and carbon disulphide, the two latter being used chiefly on a commercial scale.

It must be borne in mind that besides extracting fats, ether will also dissolve essential oils, chlorophyll, cholesterol, lecithin, and allied substances variously known as lipoids, lipins, etc.

Moreover, other substances which are of themselves insoluble in ether may become soluble in the presence of fats.

Whatever solvent is employed must be tested before use to see that it leaves no residue on evaporation and is free from moisture.

A rough and ready method of extracting fat from a given sample is to place the finely divided and dried material on a filter paper folded into a funnel and to pour the fat-solvent on to it. The filtrate will contain most of the fat, which may be recovered by evaporating off the solvent.

When it is desired to extract the fat quantitatively, the operation is most conveniently carried out in a Soxhlet apparatus (see below).

Previous to extraction, the substance must be thoroughly dried. For this purpose it must either be gently heated in a current of dry air or else desiccated by means of alcohol or anhydrous salts.

The first method, which is the most convenient, should, however, be used with caution, as many fats may undergo chemical change during the process, as a result of which the material extracted by ether after drying may be very different from the substance originally present in the moist sample.

The second method, which consists in treating the sample to be dried with absolute alcohol for some hours and then filtering and pressing, depends on the fact that the alcohol withdraws the water without dissolving away any appreciable quantity of the fat; if treated two or three times in this way the substance will be practically free from moisture and can then be extracted under a Soxhlet with ether. The wet

alcoholic filtrates on careful evaporation yield a residue which may be separately treated with ether to extract any fat contained in them. It is obvious that the method cannot be employed if the fat to be extracted is soluble in alcohol.

The third method of drying, which involves the use of anhydrous salts such as sodium sulphate, depends on the fact that the anhydrous salt when ground up with the moist tissue withdraws the water from it, forming the hydrated crystals. In a few hours the substance is sufficiently dry to be powdered. The chief objection to this process is the fact that a considerable bulk of salt has to be employed and consequently the volume of the material to be extracted is much increased.

Whilst ether is one of the most commonly used solvents for the extraction of fats, Leathes recommends a preliminary extraction with alcohol, since this helps to dry the material and frequently renders easier the subsequent extraction by ether (see under Lipins, p. 51).

In some cases a preliminary mild hydrolysis by boiling with dilute hydrochloric acid is necessary to set free the fat in a condition in which it can be readily extracted by the appropriate solvent.

CHARACTERIZATION OF FATS.

The unequivocal establishment of the true fatty nature of a given substance is not always easy, especially if only a small amount of material is available.

1. In the first instance, the solubilities of the substance should be determined by placing it on a watch-glass and adding a drop or two of the appropriate solvent. All fats dissolve readily in the so-called fat solvents, namely, ether, petrol, chloroform, benzene, acetone, and carbon disulphide; they are sparingly soluble in cold alcohol, but more soluble in hot alcohol; all are insoluble in water. These solvents will, however, also dissolve waxes, lipins, hydrocarbons, essential oils, terpenes, resins, and chlorophyll, wherefore some further method of characterization is essential.

2. Fats leave a translucent mark on paper, and many of the aforementioned substances will do the same; but in the

case of substances which are volatile, the mark will sooner or later disappear, whereas in the case of a true fat, the mark is permanent, since fats are not volatile.

3. Fats, waxes, and lipins are all saponified by boiling with alcoholic potash. In the case of most fats 2 grams can be completely saponified by boiling for a quarter of an hour with 25 c.c. of 3 per cent alcoholic potash. The resulting mixture of potassium soap and glycerol should be completely soluble in water, and, after boiling off the alcohol and acidifying the solution, the free fatty acids should be precipitated. Waxes being, on the whole, less easy to hydrolyse, may not have been completely decomposed under these conditions, but lipins would behave like fats. To distinguish between fats and lipins, special tests have to be applied (*vide* under Lipins, p. 51).

4. The only certain way of distinguishing between a fat and a wax is to establish the presence or absence of glycerol. This may be done either by heating the substance with a crystal or two of potassium hydrogen sulphate, or, better, if sufficient material is available, by preparing a concentrated solution of glycerol free from fatty acids as follows: Saponify the material as above, boil off the alcohol, take up with a little water and acidify; filter off the precipitated fatty acids and evaporate the filtrate over a water bath; extract the residue with a small quantity of alcohol, which dissolves out the glycerol, leaving the salts in solution. Evaporate off the alcohol; if sufficient material remains divide it into two portions *a* and *b*; *a* is heated with a crystal of potassium hydrogen sulphate; the presence of glycerol is confirmed by the production of acrid vapours of acrolein—

$$CH_2OH\,CHOH\,CH_2OH = 3H_2O + CH_2 = CH\,.\,CHO,$$

which blackens a filter paper moistened with ammoniacal silver nitrate solution.

The second portion *b* is dissolved in a little water and warmed in a water bath with 10 c.c. of freshly prepared bromine water for 20 minutes; any excess of bromine is then evaporated off and the resulting solution is tested for the presence of dihydroxy acetone, $CH_2OH\,.\,CO\,.\,CH_2OH$, as follows:—

To half a cubic centimetre add 2 c.c. of sulphuric acid to which have been added 0·1 c.c. of a 5 per cent solution of either β-naphthol or resorcinol; the former should give a green colour with a marked fluorescence, while the latter should give a bright red coloration.

QUANTITATIVE ESTIMATION OF FATS.

1. *By Means of Soxhlet's Extraction Apparatus.*—The fact that oils and fats are readily dissolved by ether, chloroform, and light petroleum is made use of in their estimation; but it must be borne in mind that the method only yields correct results provided other substances, which would be extracted by the solvent employed, are absent from the material under examination.

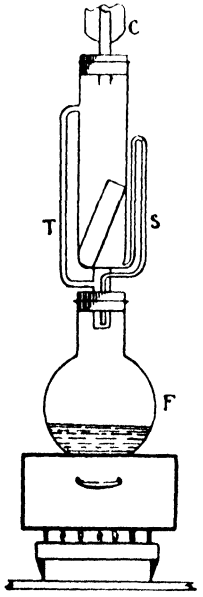


FIG. 1.

The general arrangement of the apparatus required is given in Fig. 1. The flask F, which is half-filled with the solvent to be employed, is connected to the extractor by a closely fitting cork. The material to be extracted is put into a thimble made of special quality filter paper and placed in the extractor, which is connected to a reflux condenser (C).

The method may be conveniently employed for determining the proportion of oil in the reserve food of the castor-oil seed, for example.

A number of seeds, freed from their testas, are weighed in the thimble, which is then placed inside the extractor; a few small chips of porcelain are placed in the flask F, which is then weighed and after being half-filled with freshly distilled ether it is attached to the Soxhlet. The apparatus is then connected up. The ether in the flask F volatilizes and passes up the tube T into the extractor and condenser, and gradually fills the Soxhlet; on reaching a certain level it siphons over into the flask, carrying with it the fat in

solution; once in the flask the ether is again vaporized and goes through the same process as before, the oil, however, remains behind. The ether is allowed to siphon over at least a dozen times,* and then, when most of the ether is in the extractor, the flask is disconnected. The ether in the flask is evaporated off and the flask is placed in a steam oven for half an hour, it is then allowed to cool in a desiccator and finally weighed.

Weight of seeds	x
,, flask, chips and oil	y
,, ,, and chips	z
,, oil	$y-z$

$$\text{Per cent fat} = \frac{100\,(y-z)}{x}.$$

If the ether has extracted substances other than fats, the result obtained will, of course, be too high. In such cases the ether extract may be saponified and the amount of fatty acid determined, from which the amount of fat originally present can be estimated.

Appended are a few figures giving approximately the fat content of some of the commercially exploited seeds :—

Hemp	30-35 per cent.
Rape	39-42 ,, ,,
Arachis	46-50 ,, ,,
Ricinus	45-55 ,, ,,
Sesame	50-54 ,, ,,
Cocos (copra)	64-70 ,, ,,

2. *By Saponification.*—Apart from the fact that in some cases it is not possible to extract the fat quantitatively by Soxhlet's method with less than forty-eight hours' continuous extraction, the method is open to the objection that the substance must be dried previous to extraction, and this may involve loss or alteration of the fat; furthermore, the residue which is weighed as fat may not consist entirely of fat, but may contain other substances which are extracted by the same solvents as the fats.

The following method, which is due to Liebermann and

* The number of times the liquid should be allowed to siphon off varies in every case. In order to ensure complete extraction, the only safe method to adopt is to weigh the fat extracted after a certain time, then to attach the flask again and continue the extraction for some time longer and again weigh.

Székely has the advantage of giving in a short time a reliable value for the percentage of fat in almost any substance, and is specially convenient for the estimation of fat in fodder, meat, fæces, and physiological work in general. Five grams of the sample are placed in a flask, of the dimensions given in Fig. 2, with 30 c.c. of 50 per cent caustic potash (sp. gr. 1·54). The mixture is boiled over a wire gauze for half an hour and frequently shaken. After cooling, 30 c.c. of 90-94 per cent alcohol are added and the heating is continued for another ten minutes; the mixture is then cooled again and carefully mixed with 100 c.c. of 20 per cent sulphuric acid (sp. gr. 1·145) and thoroughly shaken after each addition; the temperature must be kept low so as to avoid any loss of volatile fatty acids. When quite cold 50 c.c. of light petroleum (sp. gr. 0·6-0·7; b.p. about 60° C.) are added, and the flask is then closed with a tightly fitting rubber stopper, and is thoroughly shaken for about ten seconds; the shaking is repeated about thirty times at intervals of one or two minutes without removing the stopper. Saturated salt solution is then added until the lower aqueous layer reaches up to the 240 c.c. graduation which is marked on the neck. After shaking again a few times the flask is set aside in a vessel of cold water. When the petroleum containing the fatty acids in solution has separated, 20 c.c. are withdrawn by means of a pipette and are placed in a wide-mouthed 150 c.c. flask; 40 c.c. of 96 per cent alcohol, free from acid, are now added, together with 1 c.c. of a solution of phenolphthalein (made by dissolving 1 gram of accurately weighed phenolphthalein in 100 c.c. of 96 per cent alcohol), and the solution is titrated with N/10 alcoholic potash.

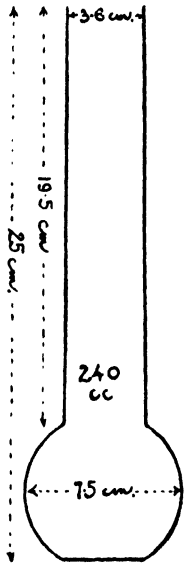


FIG. 2.

The titrated liquid is then carefully transferred in small portions at a time to a tared weighing bottle of about 80 c.c.

Liebermann and Székely: "Pflüger's Archiv," 1898, 72, 360.

capacity, which is warmed over a gently boiling water bath; when the whole liquid has been evaporated to dryness, the residue is heated in an air oven for an hour at 100°, and, after cooling in a desiccator, is weighed with the glass stopper inserted to prevent the hygroscopic soap from absorbing any moisture from the air.

The amount of fat which corresponds to a given weight of soap may be calculated as follows:—

$$\begin{array}{lll} C_{17}H_{35}COOK & & CH_2 \quad C_{17}H_{35}COOCH_2 \\ C_{17}H_{35}COOK - 3K + & CH & = C_{17}H_{35}COOCH \\ C_{17}H_{35}COOK & & CH_2 \quad C_{17}H_{35}COOCH_2 \end{array}$$

Soap Fat

From the above equation it will be seen that in order to convert three molecules of soap into one molecule of fat, three atoms of potassium, $3 \times 39{\cdot}1$, have to be withdrawn from three molecules of soap, and have to be replaced by 41 parts of $CH_2 . CH . CH_2$; this is equivalent to deducting 39·1 from one molecule of soap and adding $\frac{41}{3}$, or 13·6; or, in other words, deducting 25·5.

Hence, if " n " is the number of centimetres of N/10 caustic potash required for the titration, and since 1 c.c. N/10 KOH $\equiv$ ·00391 gram K $\equiv$ ·00136 gram C_3H_5, we have to deduct from the weight of the soap W_s

$$n \times {\cdot}00391 \text{ and add } n \times {\cdot}00136$$

which is equivalent to deducting $n \times {\cdot}00255$.

Also, since 1 c.c. of phenolphthalein solution on evaporation would leave 0·01 gram of solid, this quantity must be deducted from the weight of the soap.

Hence the percentage of fat may be calculated from the relation

$$F = \left\{ \frac{W_s - {\cdot}01 - (n \times {\cdot}00255)}{m} \right\} \times 250$$

in which " m " is the weight of the sample taken.

In estimating fat in flour or farinaceous grain by this method, it is best to subject the substance to a preliminary treatment by heating 5 grams of the sample for half an hour with 30 c.c. of dilute sulphuric acid (1 : 10), the mixture is

then diluted with 50 c.c. of 50 per cent caustic potash. Finally, the liquid is acidified with 60 c.c. of sulphuric acid (sp. gr. 1·3), as described above. After the shaking with light petroleum is completed, 50 c.c. of 94 per cent alcohol are added instead of the salt solution; this has the effect of accelerating the separation of the petroleum layer which otherwise might take a long time.

Owing to the relatively small solubility of stearic acid in light petroleum the method may give too low a result in the case of substances very rich in stearin; the result should, therefore, be checked by a second estimation in which the number of shakings with petroleum are increased two or three fold. Leathes * has modified and considerably improved this method.

Kumagawa and Suto † have found that the following method gives good results: Two to five grams of the dry substance ‡ are heated on a water bath for two hours with 25 c.c. of 5 N sodium hydroxide (20 grams in 100 c.c.) in a covered beaker. The mixture is then transferred to a separating funnel and acidified with 30 c.c. of 20 per cent hydrochloric acid. The fatty acids set free are taken up with ether, and the ethereal solution is filtered through asbestos and evaporated. The residue, which contains colouring matter, lactic acid, and other substances as well as fatty acids, is dried for some hours at 50°, and then taken up with light petroleum, whereupon the impurities separate out in resinous form. After filtering through asbestos the petroleum is distilled off and the residue, consisting of almost pure fatty acids, is dried at 50° to constant weight.

QUANTITATIVE METHODS EMPLOYED FOR THE CHARACTERIZATION OF FATS.

The following estimations are in common use for the characterization of fats:—

* Leathes: "The Fats," London, 1926.

† Kumagawa and Suto: "Biochem. Zeit.," 1908, 8, 212.

‡ Yoshitaka Schimidzu ("Biochem. Zeit.," 1910, 28, 237) recommends using unoiled material since drying leads to a loss of fat, probably from oxidation.

(1) *The Acid Number.*

This is the number of milligrams of potassium hydroxide required for the neutralization of the *free* acids in a sample of fat.

This number is determined by dissolving 1 or 2 grams of the sample in 15 or 20 c.c. of a mixture of 1 part of alcohol with 2 parts of ether, and titrating the solution with N/10 alcoholic potash in the presence of phenolphthalein.

(2) *The Saponification Value.*

This is the number of milligrams of potassium hydroxide required for saponifying 1 gram of the fat.

From 1 to 2 grams of the sample are weighed out into a 250 c.c. conical flask; 25 c.c. of approximately seminormal alcoholic potash are then added, and the flask is attached to a reflux condenser and heated over a water bath for about half an hour; the solution is then diluted with 25 c.c. of water and cooled, then the excess of potash is titrated back by means of N/2 hydrochloric acid. In order to determine the strength of the alcoholic potash, 25 c.c. of it are heated at the same time under exactly similar conditions in a second conical flask, but without any fat; in this way any error due to the effect of the alkali on the glass vessel is eliminated. The difference in the two titration readings gives the amount of acid equivalent to the potash used up in saponifying the fat, from which the number of milligrams of alkali required for 1 gram of fat may be calculated.

Since one molecule of any monobasic acid requires one molecule of potash, the magnitude of the saponification value is inversely proportional to the molecular weight of the acids contained in the fat.

	Molecular Weight.	Saponification Value.
Butyrin	302	557·3
Palmitin	806	208·8
Stearin	890	189·1
Olein	884	190·4
Coco-nut oil	—	246-260
Palm-kernel oil *	—	242-250
Palm oil †	—	196-202
Olive oil	—	185-196

* The oil contained in the kernel of the palm fruit.
† The oil contained in the pericarp of the fruit.

(3) *Unsaponifiable Residue.*

The following method, originally due to Allen and Thomson, is recommended by Lewkowitsch for the estimation of the unsaponifiable residue.

Five grams of the fat or oil are saponified by boiling under a reflux condenser with 25 c.c. of alcoholic potash containing 11·2 per cent of caustic potash for half an hour. The alcohol is then evaporated off and the residual soap is dissolved in 50 c.c. of hot water and transferred to a separating funnel of about 200 c.c. capacity, about 20-30 c.c. of water being used to rinse out the dish. After cooling, the mixture is shaken with 50 c.c. of ether and set aside until the ethereal layer has separated. The separation is accelerated by the addition of a little alcohol. The soap solution is then run off from below into a second separating funnel and shaken once more with a fresh quantity of ether. Two extractions should suffice, but it is safer to extract a third time. The ethereal extracts are then united, washed three times with 20 c.c. of water to remove any soap, and transferred to a weighed flask; after evaporating off the ether, the flask is weighed again; the increase in weight gives the amount of unsaponifiable residue in 5 grams of the sample.

The isolation and identification of the unsaponifiable residue may be carried out for the purpose of establishing whether a given sample of fat or oil is of animal or vegetable origin, since animal fats contain cholesterol, while vegetable fats contain phytosterol (see p. 48).

Fat.	Unsaponifiable Residue.
Castor oil	0·33 per cent.
Linseed	0·42-1·1 ,, ,,
Olive oil	0·46-1·0 ,, ,,
Maize oil	1·35-2·86 ,, ,,
Oil from *Pelvetia canaliculata* *	11·1 ,, ,,
Human fat	0·33 ,, ,,
Lard	0·35 ,, ,,
Beeswax	52-56 ,, ,,

* Authors' observations hitherto unpublished.

(4) *Iodine Value.*

It was first observed by Hübl that an alcoholic solution of iodine containing mercuric chloride reacted at ordinary temperatures both with the free unsaturated acids and with their glycerol esters the fats. By elaborating this reaction, Hübl formulated the so-called " iodine value " which provides a method of characterizing a fat.

For the determination of the iodine value of a fat the following solutions are required :—

(*a*) An iodine solution made by mixing together equal volumes of two solutions containing respectively 25 grams of iodine and 30 grams of mercuric chloride in 500 c.c. of 96 per cent alcohol. The two solutions should be mixed together about twenty-four hours before use, as the resulting mixture alters its strength considerably during the first few hours after it has been made.

(*b*) A sodium thiosulphate solution containing roughly 48 grams of crystallized salt in 1 litre of water ; the strength of this solution is accurately determined as follows : 20 c.c. of a potassium bichromate solution containing 3·8657 grams of the pure salt dissolved in 1 litre of water are run into a stoppered bottle containing 10 c.c. of a 10 per cent solution of potassium iodide and 5 c.c. of concentrated hydrochloric acid. The resulting brown solution, if carefully made, should contain exactly 0·2 gram of iodine ; it is at once titrated by means of the thiosulphate solution, and, supposing x c.c. were required to decolorize it then it follows that 1 c.c. of thiosulphate is equivalent to $\frac{0{\cdot}2}{x}$ gram of iodine.

(*c*) Chloroform or carbon tetrachloride, the purity of which should be tested by mixing 20 c.c. of it with 20 c.c. of the iodine solution and titrating the free iodine two or three hours after ; the amount found should be exactly the same as that contained in 20 c.c. of the iodine solution to which no chloroform or carbon tetrachloride has been added.

(*d*) A 10 per cent solution of potassium iodide made by dissolving 1 part of the iodide in 10 parts of water.

(*e*) A starch solution freshly prepared by boiling up a suspension of 0·5 gram of starch in 50 c.c. of water.

The determination of the iodine value is carried out as follows :—

From 0·15 to 0·18 gram of a drying or marine animal oil, 0·2 to 0·3 gram of a semi-drying oil, 0·3 to 0·4 gram of a non-drying oil or 0·8 to 1·0 gram of a solid fat are accurately weighed from a weighing bottle by difference into a 500-800 c.c. bottle, provided with a well-ground stopper, and dissolved in 10 c.c. of the chloroform (*c*) ; 25 c.c. of the iodine solution (*a*) are then run in, and the stopper, which is moistened with potassium iodide solution (*d*) to prevent loss of iodine by volatilization, is inserted. If a clear solution is not obtained more chloroform must be added. The bottle is then left to stand in the dark, and if the dark brown colour should disappear after two hours or less, another 25 c.c. of the iodine solution must be added, as it is essential that there should be a considerable excess of iodine. In the case of solid fats and non-drying oils the reaction can be considered as being complete after six to eight hours, but in the case of drying oils or fish oils twelve to eighteen hours are necessary. After the completion of this time from 15 to 20 c.c. of the potassium iodide solution (*d*) are added, and, after thorough shaking, the mixture is diluted with 400 c.c. of water. If a red precipitate of mercuric iodide is produced, more potassium iodide solution should be added. The excess of free iodine, part of which is dissolved in the chloroform and part in the potassium iodide solution, is then titrated by shaking with the standardized sodium thiosulphate solution until only a faint yellow colour remains. A little of the starch solution is now added, and the titration is continued until the dark blue colour is destroyed. Twenty-five c.c. of the original Hübl iodine solution, which had been left in a stoppered bottle with 10 c.c. chloroform and kept in the dark for the same length of time as the bottle containing the sample of the fat, are then titrated in a similar way with the sodium thiosulphate, and the difference in the two results gives the amount of iodine absorbed. The amount

of iodine thus absorbed by 100 grams of the fat gives the iodine value.

The values obtained by the Hübl method are generally considered to be very reliable and concordant, but the method is somewhat tedious, and for this reason the more rapid method of Wijs * is preferable.

The iodine solution required for this method is obtained by separately dissolving 9·4 grams of iodine chloride and 7·2 grams of finely powdered iodine in separate flasks in about 200 c.c. of gently warmed glacial acetic acid. The two solutions are then united in a 1 litre graduated flask and made up to the mark with more glacial acetic acid.

This solution should be standardized on the following day by mixing 20 c.c. of it with 10 c.c. of 10 per cent potassium iodide solution and titrating the free iodine by means of the standard thiosulphate.

The actual determination of the iodine value is carried out as follows :—

From 0·2-0·4 gram of fat should be carefully weighed and dissolved in 10 c.c. of pure carbon tetrachloride (which has been shown by a blank test not to absorb iodine) ; 25 c.c. of the iodine solution are then added, and the flask is stoppered and set aside in the dark for one or two hours. The liquid is then transferred to a larger flask, the smaller flask being washed out thoroughly by means of 10 c.c. of potassium iodide solution and water until the total volume is about 300 c.c. The solution is then titrated with the thiosulphate. The difference between this reading and the amount required by 25 c.c. of the iodine solution is a measure of the iodine absorbed by the amount of fat.

The values obtained by Wijs's method are, as a rule, rather higher than those obtained by the Hübl method.

Appended is a list of iodine values of some important fats :—

(*a*) Drying Oils—	
Linseed oil	173-201
Hemp-seed oil	148
Sunflower oil	119-135
Pine-seed oil	101-103

* Wijs : "Zeit. anal. Chem.," 1898, 277 ; "Zeit. Unters. Nahr. Genussm.," 1902, 497.

(*b*) SEMI-DRYING OILS—	
Beech-nut oil	104-111
Cotton-seed oil	108-110
Sesame	103-108
Rape oil (colza)	94-102
(*c*) NON-DRYING OILS—	
Almond oil	93-97
Olive oil	79-88
Grape-seed oil	96-142
Castor oil	83-90
(*d*) VEGETABLE FATS—	
Cacao butter	32-41
Palm-kernel oil *	13-17
Coco-nut oil *	8-10

(5) *The Reichert Meissl Value.*

This represents the number of cubic centimetres of N/10, caustic potash required for neutralizing the volatile acids liberated from 5 grams of a sample of fat under certain special conditions.

The determination is carried out as follows: Five grams of the sample are weighed into a 200 c.c. flask and saponified by warming with 70 c.c. of 10 per cent alcohol and 2 grams of caustic potash. The excess of alcohol is then evaporated off and the residue, after dissolving in 100 c.c. of water, is acidified with 40 c.c. of sulphuric acid (1 : 10); a few chips of asbestos are then dropped into the flask and the liquid is distilled through a Liebig condenser at such a rate that exactly 110 c.c. of distillate pass over in an hour; 100 c.c. of the distillate remaining after filtration are titrated with N/10 caustic potash in the presence of phenolphthalein. Appended are the numbers obtained for several different fats:—

Palm oil	5-6·8	Lard	0·68
Coco-nut oil	6·6-7·0	Tallow	0·5
Linseed oil	0·0	Goose fat	0·2-0·3
Olive oil	0·6	Butter fat	20·6-33·1

The determination of the Reichert Meissl value is of considerable value for the detection of adulteration in butter, since any adulterant will at once lower the value.

Though described as oils, these substances are both solid at ordinary temperatures, melting at about 25°.

(6) *The Acetyl Value.*

This is a measure of the amount of hydroxyl groups which a fat contains ; its value depends upon the fact that compounds containing an alcoholic hydroxyl group react with acetyl chloride or acetic anhydride so as to replace the hydrogen of the hydroxyl by the acetyl group (CH_3CO—) as shown by the equation—

$$ROH + \begin{matrix}CH_3CO\\CH_3CO\end{matrix}\!\!>O = ROCOCH_3 + CH_3COOH$$

If the resulting acetyl derivative is saponified by means of caustic potash it breaks up as follows :—

$$ROCOCH_3 + KOH = ROH + CH_3COOK,$$

and it is possible to determine the number of milligrams of caustic potash which are thus utilized in combining with the acetyl groups to form potassium acetate.

The number of milligrams of potash required for the saponification of the acetyl derivative obtained from 1 gram of the fat is termed the acetyl value of that fat.

Castor oil and grape-stone oil have particularly high acetyl values which in the castor oil is due to the presence of the hydroxy acid known as ricinoleic acid.

The following are the acetyl values of some of the more important oils, fats, and waxes :—

Linseed oil .	3·98	Castor oil . .	153-156
Olive oil .	10·64	Grape-seed oil .	144
Rape-seed oil	14·7	Carnauba wax .	55·24
Palm oil .	18·0	Lard . . .	2·6
Palm-nut oil .	1·9-8·4	Butter . .	1·9-8·6

The following method, due to Lewkowitsch, has been adopted as the standard process.

About 10 grams of the fat are boiled in a round-bottomed flask under a reflux condenser for two hours with twice their weight of acetic anhydride. The mixture is then poured into a litre flask and boiled for half an hour with 500-600 c.c. of water, a slow stream of carbon dioxide being conducted into the liquid all the while to prevent bumping. After cooling, the upper layer of water is siphoned off and the lower oily layer is again boiled with water as above, the whole process

being repeated three times. The oil is finally filtered and washed on the filter paper with boiling water until the filtrate is no longer acid, whereupon it is dried in an oven and weighed.

About 5 grams of the acetylated product are next saponified by boiling with alcoholic potash * as described under the determination of the saponification value. The alcohol is then evaporated off, and the resulting soap is dissolved in water.

Dilute sulphuric acid (1 : 10) is then added in excess and the solution is steam distilled until 600-700 c.c. of water have passed over. The distillate is titrated with N/10 caustic potash using phenolphthalein as indicator; the number of cubic centimetres required for neutralization multiplied by 5·61 and divided by the weight of fat taken gives the acetyl value.

Further information regarding the nature of a given fat may be obtained by investigating the relative amounts of the saturated and unsaturated acids. This may be effected by saponification and conversion of the resulting soap into lead soaps by means of lead acetate; making use of the greater solubility of the lead soaps of unsaturated acids in ether, these may be separated from the lead soaps of the saturated acids. The saturated and the unsaturated acids respectively may then be set free from their lead soaps and examined.

SPONTANEOUS CHANGES IN FATS.

Rancidity.—Most fats when exposed to air and light sooner or later become rancid, acquiring an unpleasant taste and smell. The actual cause of this change is as yet but little understood, though it appears probable that it is the result of the combined action of a number of different factors such as oxygen, light, moisture, bacteria and enzymes; the complex fats, and possibly also the small quantities of proteins and other impurities contained in them, are thereby broken down into simpler bodies such as the lower volatile fatty acids and aldehydes. Similarly, but little is known as to the chemical changes involved in the process of becoming rancid; it is frequently true that a considerable quantity of free acid is

* Prepared by dissolving about 32 grams of 90 per cent stick potash in the least quantity of water and diluting to 1 litre with 96 per cent alcohol; the solution should be filtered after standing for twenty-four hours.

liberated in fats which have become rancid, and this is especially so in the case of fats such as butter, which contain acids of low molecular weight, as butyric acid, the smell of which recalls that of rancid butter. It is, however, a fact that a fat may be acid without being rancid ; * coco-butter, for instance, has usually an acid reaction, but very rarely becomes rancid.

With regard to other constituents found in rancid fats, various authors have from time to time observed the presence of hydroxy-acids, aldehydes, alcohols, and of esters of lower fatty acids, and peroxides, but there appears to be a general consensus of opinion that glycerol does not occur.

According to Fierz,† in the case of unsaturated fats, oxidation may take place at the double bond, in the absence of micro-organisms, with the formation of aldehydes and acids of lower molecular weight which, like butyric acid, have an offensive odour and taste. On the other hand, saturated fats become rancid under the action of *Penicillium glaucum* and *Aspergillus niger* with the liberation of various odoriferous ketones. This is due to the oxidation of the β carbon atom according to the scheme—

$$RCH_2CH_2 . CH_2 . COOH + O \rightarrow RCH_2CHOH . CH_2COOH + O$$
$$\rightarrow RCH_2CO\,CH_2COOH + H_2O$$

the latter acid by loss of carbon dioxide giving a ketone—

$$RCH_2\,COCH_2COOH = RCH_2\,COCH_3 + CO_2.$$

By this means caproic, caprylic, and myristic acid, which occur as esters in coco-butter, may be regarded as the precursors of methyl amyl, methyl heptyl, and methyl undecyl ketones respectively, and which have been shown to occur in rancid coco-butter.

These compounds have been experimentally produced from their respective precursors by growing *Penicillium glaucum* and *Aspergillus niger* upon the ammonium salts of the relative acids. Methyl heptyl ketone has been isolated from Roquefort cheese.

Drying and Resinification.—Most fatty oils on exposure to

* Vintilesco and Popesco : " J. Pharm. Chim.," 1915 [iv.], **12**, 318.
† Fierz : " Z. angew. Chem.," 1925, **38**, 6.

the air tend to thicken, owing partly to polymerization and partly to oxidation; in some cases the oil actually dries up, leaving a more or less hard mass or a thin elastic film.

Those oils which only thicken, without actually becoming hard or dry, are called *non-drying* oils. They are composed for the most part of triolein (cf. p. 10), and contain only small quantities of solid fatty acids; to this class of oils belong the following: olive oil, almond oil, arachis or pea-nut oil, quince oil, cherry-, plum-, peach-, and apricot-kernel oil, wheatmeal oil, rice, tea-seed oil, and hazel-nut oil.

Two further oils, namely, castor oil and grape-seed oil, are also included in this group of non-drying oils, but they have a slightly different composition from the other members of this group. They are characterized by possessing a considerable percentage of glycerides of hydroxylated fatty acids, such as dihydroxystearic acid, a fact which is brought out clearly by their high acetyl values (p. 27).

In contrast with these non-drying oils are the so-called *drying oils*, among the more important of which are the following: linseed oil, cedar-nut oil, hempseed, walnut, poppy-seed, and sunflower oil. These oils exhibit to a greater or less degree the tendency to absorb oxygen from the air, thereby drying up and leaving an elastic skin, a property which is made use of industrially in the manufacture of oil paints. These drying oils are composed chiefly of the glycerides of the unsaturated acids of linolic and linolenic series and contain only relatively small quantities of oleic acid. Owing to the large amount of unsaturated acids which they contain, their iodine value (p. 23) is very high (120-200).

In addition to the above there is also a third group of vegetable oils, known as the *semi-drying* oils, whose iodine value and drying properties lie midway between those of the drying and non-drying oils. They differ from the true drying oils in containing no acids of the linolenic series, and from the non-drying oils in containing linolic acid. The oils belonging to this category fall naturally into two sub-groups:—

1. The cotton-seed oil group, to which belong Soja-bean oil, maize oil, pumpkin, water-melon, and melon-seed oils,

beech-nut oil, cotton-seed, sesame and croton oils, and the lesser-known oils of the apple, pear, orange, barley, and rye seeds.

2. The rape oil group comprising garden cress, hedge mustard, wild radish, black mustard seed, white mustard seed, radish seed, and rape or colza oil.

The oils of the latter sub-group have a lower saponification value (p. 21) than any other vegetable oils, and arachidic acid seems to be a normal constituent of them all.

To determine whether an oil is a drying one or not, a drop is spread on a glass plate, such as a microscope slip, and left for several days at atmospheric temperature. Non-drying oils such as olive and castor oils are unaltered after about eighteen days; semi-drying oils such as cotton-seed, sesame, and rape oil are more or less dry, but still sticky in from seven to eight days, whereas real drying oils, like poppy and especially linseed, are quite dry in from three to six days.

The mechanism of the process of drying is very imperfectly understood; it would appear to be in part a chemical change involving oxidation, with the resulting formation of a substance known as Linoxyn,* and partly a physical change.†

INDUSTRIAL USES OF VEGETABLE FATS AND OILS.

Economically, fats are of considerable value, being used for food, illumination, lubrication, soap manufacture, and for a variety of other purposes.

The following is a brief consideration of some of the more important industrial uses of the commoner fats and oils of vegetable origin.

Olive Oil is extracted from the fleshy pericarp of the fruit of the olive, *Olea europæa*, by pressure. The best quality oil, which is expressed without the application of heat, is used for food; lower grade oils, obtained by extracting the residues from the presses with fat solvents, such as carbon disulphide or light petroleum, are used in the manufacture of soap (see p. 33).

* Holden: "J. Soc. Dyers and Col.," 1927, 43, 157.
† Wolff: "Chem. Zeit.," 1924, 48, 897.

COTTON-SEED OIL is extracted from the seeds of *Gossypium herbaceum* by pressing them at a temperature of about 90°; the crude brown oil is purified by treatment with caustic soda, which removes the free fatty acids, colouring matter, and other impurities. After purification the oil is light yellow in colour. It is used for the manufacture of soap and rubber substitutes.

COCO-NUT OIL is obtained from the ripe seeds of *Cocos nucifera* and *Cocos butyracea* by pressure; the dried endosperms, known as Copra, are imported into Europe, and the oil extracted from them is commonly known as Copra oil. Soaps made from coco-nut oil have the property of absorbing large quantities of salt solutions, and can therefore be used for washing with sea water.

PALM OIL which occurs in the fruit of *Elaeis guineensis* is, when pure, a colourless substance of the consistency of lard; on exposure to air it readily turns yellow, but the colour can be removed by oxidation by means of a current of air. Both coco-nut oil and palm oil in the crude state contain free fatty acids which can, however, be removed by treatment with alcohol. When so purified they are employed in the manufacture of margarine.

RAPE OIL or COLZA OIL is a thick, yellowish oil obtained from the seeds of *Brassica Rapa* and *Brassica Napus* which is used as an illuminant.

By drawing a current of air through the oil heated to 70° a so-called "blown" oil is produced, the specific gravity of which becomes almost equal to that of castor oil, namely 0·97; in this condition it is miscible with mineral oils. The mixture which is known as marine oil is used for lubricating marine engines.

LINSEED OIL is obtained by pressing the seeds of *Linum usitatissimum* either with or without the application of heat; the residues after compression are made up into cattle food.

The drying vegetable oils, particularly linseed oil, are used in the manufacture of oil paints as vehicles for the pigments; for artist's white paints, walnut and poppy-seed oils are sometimes used. The drying properties of linseed oil used for the manufacture of paint are greatly increased by boiling with lead

oxide ; such oil is known as boiled oil. A similar effect may be produced by dissolving in it certain salts known as " driers," such as lead linoleate or the metallic salts of resin acids, etc.

Varnish consists of a mixture of boiled oil with gum resins and oil of turpentine.

CASTOR OIL is obtained by compressing the seeds of *Ricinus communis* either with or without the application of heat. The seeds contain a fat-splitting enzyme * or lipase which is employed commercially for the hydrolysis of fats ; they also contain a very poisonous toxalbumin, known as Ricin, which remains in the residues after the expression of the oil. Castor oil is a thick viscid colourless liquid ; when heated above 280° it decomposes with the formation of oenanthol, a substance having a very unpleasant odour. Castor oil is largely used in the dye industry ; for this purpose it is converted into the so-called turkey red oil, used for alizarin dyeing, by treatment with sulphuric acid and neutralization of the resulting sulphonic acid with soda.

For the manufacture of hard toilet soap the following fats and oils are used : tallow fat, palm oil, palm-kernel oil, coco-nut oil, and olive oil ; the fats are boiled with caustic soda until saponification is complete, whereupon the mixture is saturated with common salt. The soap, being insoluble in strong salt solution, rises to the surface leaving the glycerol and salt in the aqueous layer below ; the latter is then run off and the scum, which is allowed to harden in moulds, is known as hard soap. Soft soaps are prepared by boiling the cheaper oils, such as hemp-seed oil, cotton-seed oil or linseed oil with caustic potash ; when saponification is complete the mixture is allowed to set to a semi-solid without the addition of sodium chloride ; the resulting mixture contains all the gycerol together with the excess of alkali and a quantity of water.

Most of the gycerol of commerce is obtained from fats ; it is used largely for the manufacture of dynamite.

* The occurrence of a lipase is common to most fatty seeds, but the only one commercially utilized is that of the castor bean, on account of its high concentration and activity.

Hardening of Oils.—Many low-melting fats or oils are nowadays hardened by treating them with hydrogen in the presence of a nickel catalyst; the process of hydrogenation involves the removal of the double bonds of saturation with hydrogen, the resulting saturated compound having a higher melting-point.

PHYSIOLOGICAL SIGNIFICANCE OF FATS.

The great function of fats in the economy of the plant is connected with nutrition. They form one of the most important food-reserves of plants and as such may occur in vegetative or in propagative organs.

It is, however, not possible to ascribe this function to all instances of fat occurrence. Thus, in the case of the palm *Elaeis guineensis*, two distinct types of fat occur; the one in the pericarp, the palm oil of commerce, and the other in the testa adjacent to the embryo. Apart from the fact that these two fats are different, the former being of the nature of tallow and containing palmitic, stearic, and other fatty acids, and the latter containing acids of a lower molecular weight, it is difficult to see what nutritive purpose a fat occurring in the pericarp can serve in view of the fact that it is destroyed before germination actually begins; it has, moreover, been shown that germination is hastened if the pericarp is removed prior to planting. Similar considerations also apply in the case of the olive.

With regard to their origin in plants very little is known; they first appear as very small vacuoles in the protoplasm which eventually run together forming large drops.

In some cases oil has been described as owing its origin to the activity of elaïoplasts, which are colourless bodies of various shapes usually grouped around the nucleus, and, like other plastids, of a protoplasmic nature. They are, or have been, supposed to act with regard to oil formation much as leucoplasts do with respect to starch formation. Elaïoplasts have been observed in many Monocotyledons such as *Vanilla*, *Funkia*, *Gagea*, *Ornithogalum*, etc., in the flower of a Dicotyledon. *Gaillardia Lorenziana*. and in *Psilotum*.

The development of the elaïoplasts of *Gaillardia* has been followed by Beer,* who found that they are formed by the aggregation of chloroplasts which then degenerate and give origin to the oil. He considers it is most unlikely that elaïoplasts perform any function of direct importance to the life of the plant, although they may in some cases, the corolla-hairs of *Gaillardia*, for instance, serve a biological purpose.

Elaïoplasts are not, by any means, always present. Rivett † from her study of *Alicularia scalaris*, a liverwort, concludes that in this instance the fat originates as a general protoplasmic secretion, not from an elaïoplast or other special body. It is a secondary product, its production being unaffected by changes in the cultural conditions brought about by variations in illumination, temperature, and nutritive materials.

Although elaïoplasts may not perform the function originally ascribed to them, it does not necessarily follow that fats, more especially when occurring in the green parts of plants, may not be direct photosynthetic products. Thus Fleissig considers that in the case of *Vaucheria*, the abundant fat-like substance is a direct photosynthetic product comparable to the starch and sugar in ordinary green leaves. On the other hand, it is possible that the fats in such cases may have been produced by secondary changes in the original product of photosynthesis.

The fat-economy of *Vaucheria*, however, requires further investigation; thus Meyer ‡ states that the oil drops are produced by the chloroplasts and result from the photosynthetic processes; they are not, however, fats in that they do not give characteristic microchemical reactions. Similarly the oil bodies described as occurring in the mesophyll of *Ilex*, *Kalmia*, *Taxus*, *Tropæolum*, and *Vinca*, which increase in size with the age of the leaf, do not give characteristic fat reactions. Mangenot § describes two kinds of oil drops in *Vaucheria*: spherical drops of various sizes associated with

* Beer: "Ann. Bot.," 1909, **23**, 63.

† Rivett: *id.*, 1918, **32**, 207.

‡ Meyer: "Ber. deut. bot. Gesells.," 1917, **35**, 586; 1918, **36**, 5, 235, 674.

§ Mangenot: "Compt. rend. soc. biol.," 1920, **83**, 982.

chloroplasts and which he considers to be the first visible product of assimilation, and very much smaller globules, suggesting microsomes, distributed throughout the cytoplasm.

In many cases there can be but little doubt that fats are produced from carbohydrates ; the work of Schmidt,* Le Clerc du Sablon,† and others has shown that as the carbohydrates disappear so fats appear. For example, in the case of the almond the seeds when they begin to ripen are rich in carbohydrates and poor in fats, whereas the reverse is true when they are fully matured. The same holds true for the seeds of *Ricinus* and *Pæonia*. The nature of the carbohydrates used up in this process varies in different plants ; thus it is stated that in the olive mannitol replaces the carbohydrate. This statement, due to de Luca, is not accepted by other investigators of the same plant ; according to Funaro mannitol does not appear until after the oil has been formed.

In the case of *Ricinus* seeds the oil is formed from glucose, and in *Pæonia* principally from starch. The facts that fat may be translocated as such, provided it be an emulsion sufficiently fine, or in the form of fatty acid and glycerol, suggest that the fats in seeds have not been formed *in situ*, but have been conveyed there. This may be true to a certain extent, but consideration of the fact that fat will appear as the carbohydrates disappear in immature seeds removed from the parent plant, together with the facts relating to the formation of fats in vegetative organs under the influence of cold (p. 3), leads to the conclusion that the substances in question are formed at the expense of carbohydrates. Further, corroborative evidence is afforded by well-ascertained facts relating to similar problems in animals.

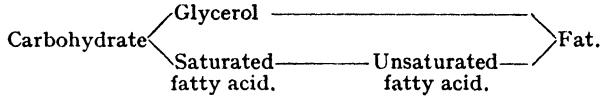
Ivanow,‡ experimenting with rape seed, has shown that they contain a lipase which may either hydrolyse a fat or may synthesize one from fatty acid and glycerol. Thus, if a glycerol extract of the seed be mixed with oleic acid, fat is

* Schmidt : " Flora," 1891, **74**, 300.

† Le Clerc du Sablon : " Compt. rend.," 1893, **117**, 524 ; 1894, **119**, 610 ; 1896, **123**, 1084 ; " Rev. Gen. Bot.," 1895, **7**, 145 ; 1897, **9**, 313.

‡ Ivanow : " Ber. deut. bot. Gesells.." 1911. **20**. 505.

synthesized, but, on diluting with water, the fat is split up again. This same author * has published important observations on the synthesis of fats in oily seeds mainly from the carbohydrates glucose, sucrose, and starch. These substances are synthesized in the order given, the last two being first hydrolysed. The initial acids to be formed are characterized by a low iodine value, showing that they are saturated. Further, since the Reichert Meissl value is constant and does not vary with the acid number, it is concluded that the acids first formed belong to the higher members of the fatty series. The saturated acids are followed by the unsaturated. Ivanow gives the following scheme to indicate the essential stages in the synthesis of fat in a typical instance such as the seed of flax :—



The iodine value of a fat is not necessarily constant, as is shown by the observations of Eyre † who found that this value steadily increased during the formation of the seed of the flax.

Days after Flowering.	Percentage of Fat in Dry Seeds.	Iodine Value.
10	2·5	114
14	15·1	119
17	31·1	127
23	37	143
28	37	170
35	39	180
51	36·3	190

The extracts of young seed have a high content of free fatty acid, which rapidly decreases as the seeds develop. This indicates that the glycerol appears later than the fatty acid, or else the combination of the glycerol and fatty acid is impeded by some factor.

After the fourteenth day there is a rapid and more or less

* Ivanow : " Beih. bot. Centr.," 1912, **28**, 159.

† Quoted by Armstrong and Allen : " J. Soc. Chem. Ind.," 1924, **43**, 207 T.

parallel increase in the amounts of carbohydrates, proteins, and fats.

During the germination of oily seeds a reversal of this process takes place. The work of Schmidt, Green,* Le Clerc du Sablon, and others, has shown that the first process is that of hydrolysis which splits the fat into a fatty acid and glycerol, lipase being the active agent.

Thus in the sunflower Miller † found that less than 1 per cent of free fatty acid was present in the oil of the cotyledons of the resting seed; as germination proceeded there was a gradual increase, thus the ether extract of the cotyledons of a seedling in which the plumule was just showing contained 30 per cent of fatty acid.

The presence of the acid may be demonstrated in such germinating seeds, but the same statement does not hold for glycerol, probably because it is translocated with great rapidity, and is quickly transformed. There can, however, be no doubt that this substance is formed because if, for example, castor oil be subjected *in vitro* to the action of lipase obtained from *Ricinus* seeds, the presence of glycerol may be detected with ease.

With regard to other changes which the original fat undergoes during germination, Schmidt found that the iodine number of the unsaturated acids and oils decreased during germination, which indicates that saturation of the acid radicles takes place. This is controverted by von Fürth,‡ who found no change in the iodine value. The observations of Schmidt, however, have been corroborated by Miller, who found that in *Helianthus annuus* the iodine value decreased from 136·2 for the seed to 67·4 for a seedling with the plumule just elongating.

Further corroboration is given by Ivanow § who, for his study on the transformation of fats during germination, selected flax, hemp, rape, and poppy seeds, since each is characterized

* Green: "Proc. Roy. Soc., Lond.," 1890, **48**, 370.
† Miller: "Ann. Bot.," 1910, **24**, 693.
‡ Von Fürth: "Hofm. Beitr. Chem. Phys. Path.," 1904, **4**, 430; 1912, **26**, 889.
§ Ivanow: "Jahrb. wiss. Bot.," 1912, **50**, 375.

by the possession of fats rich in acids of a specific series. Thus the oil of hemp seed is rich in acids of the unsaturated linolenic series, whilst poppy-seed oil is rich in acids of the saturated fatty acid series.

By ascertaining the iodine and other values of the fats of these seeds at different periods of germination, it was found that the acids disappeared in the sequence linolenic, linolic, oleic, and, finally, palmitic; in other words, the acids were consumed at a rate inversely proportional to their degree of saturation.

Ivanow considers that the fall in the iodine value of the fats is due rather to the rapidity with which the more unsaturated fatty acids are used up in the formation of carbohydrates rather than to their oxidation. He further found that the saturated fatty acids not uncommonly exist in a free state whilst the unsaturated acids occur in the form of glycerides.

Von Fürth * also found that during germination of *Ricinus*, the acetyl value decreased from 87·5 in the resting seed to 50·5 in the young seedling, from which he concluded that the normal fatty acid does not change into hydroxy fatty acid. Also, he could find no proof of the fatty acid breaking down into simpler substances as indicated by the molecular weight remaining practically constant.

This hydrolysis is the first action, but it is not the final one since carbohydrates quickly appear during the germination of such seeds. Since the days of de Saussure, who was the first to draw attention to this phenomenon, much evidence relative to this carbohydrate formation has accumulated.

In the case of *Ricinus* le Clerc du Sablon found that the resting seed contained 69 per cent of oil and 4 per cent of sugar, but in a seedling 11 cm. high the oil had fallen to 11 per cent and the sugar had risen to 14 per cent. It was further found that the sugar contained in the resting seed has a slight excess of non-reducing sugar, which increased more rapidly than the reducing sugar; finally, however, the latter variety preponderated.

* *Loc. cit.*

Le Clerc du Sablon also found the same relation between oil and sugar to obtain during germination of rape, hemp, poppy, almond, and walnut.

Similar observations have been made by Green and Jackson,* who found that in the resting seed of *Ricinus* the most abundant sugar is sucrose, which gives place to invert sugar in the early stages of germination. Subsequently the sucrose increases in amount, and occurs in quantities greater than the invert sugar; thus there is reason for supposing that the sucrose is a temporary reserve food.

The following table which summarizes the changes in the sugar content is taken from Green and Jackson's paper :—

Time of germination in hours.	Invert sugar in milligrams.	Cane sugar in milligrams.
0	1·1	10·7
45	2·7	5·17
69	2·3	0
117	6·7	19·4
168	5·2	10·5
216	19·5	35·7
240	29·01	35·8
312	40·8	52·6

Miller has found that in the sunflower, *Helianthus annuus*, the amount of ether extract of the cotyledons diminishes gradually from the beginning of germination, the most rapid depletion occurring during the period between the first appearance of the seed-leaves above ground and the point of full expansion. Also, the greatest increase in the hypocotyl and roots coincides with the period of maximum depletion from the seed-leaves. With regard to the sugar content, Miller states that the resting embryo contains about 4 per cent of sucrose, during germination there is a decrease, and this is followed by a gradual increase until the seed-leaves begin to unfold. Up to this stage the cotyledons contain only a non-reducing sugar, but as the seed-leaves assume the functions of foliage leaves a reducing sugar appears, and, in a short time, is the only sugar present. In the hypocotyl and roots

* Green and Jackson : " Proc. Roy. Soc., Lond.," B., 1906, **77**, 69.

the amount of sugar rapidly increases until in seedlings about 4 inches long it may amount to 20 per cent of the dry weight, then a gradual decrease takes place. There is also a small increase in the amount of starch.

The nature of the carbohydrate differs in different plants; thus in addition to the above-mentioned plants, during the germination of *Allium* and of *Cucumis* much glucose makes its appearance; this is also true, although to a lesser degree, for *Cannabis sativa*, in which case the glucose is quickly transformed into starch.

In other instances starch is said to be the carbohydrate formed.

The consideration of the formulæ of the substances in question shows that fats poor in oxygen give rise to carbohydrates rich in oxygen, and vice versa; but as to how this is accomplished nothing of a definite nature is known.

Many suggestions have been put forward, and before mentioning these the reader may be reminded of the large amount of oxygen which is absorbed during the germination of oil-containing seeds.

Detmer considered that starch may arise from the free oleic acid according to the equation—

$$C_{18}H_{34}O_2 + 27O = 2(C_6H_{10}O_5) + 6CO_2 + 7H_2O.$$

This change is supposed to be effected by the oxidation of the chain at the double bond setting free two unsaturated groups which by polymerization give rise to sugar.

These conclusions are based on the observations that during the germination of the seeds of *Arachis* the carbohydrate increases to 5·6 per cent of the dry weight, whilst in *Ricinus* the increase is 16 per cent. The glycerol of the fat would be sufficient to form about 5 per cent of carbohydrate; this roughly was the amount observed in the case of *Arachis*, whereas in *Ricinus* the amount of fat was about three times as great.

It has already been mentioned that glycerol so far has not been demonstrated in germinating fatty seeds; this may be owing to its powers of rapid diffusion or to the fact that it is used up in the synthesis of other substances.

Le Clerc du Sablon has put forward the idea that there might be present an enzyme which acts on the fat without liberating the glycerol.

These views are concerned chiefly with the formation of carbohydrates from fats; a reversal of the process might or might not explain the formation of fats from carbohydrates.

The whole question is of considerable difficulty and refuge may be taken in the hypothesis first put forward by Nägeli that the fats are products of the disintegration of the protoplasm. Thus the carbohydrates might be assimilated by the protoplasm which might produce the oil by some catabolic process.

With regard to the possible formation of fats from proteins very little information is available. On the animal side there is some evidence to show that substances derived from proteins may be so utilized; a possible connection may be found in the phospholipines (phosphatides) which are compounds of fatty acids containing either nitrogen or phosphorus, or both.

Leathes * points out that the fatty acid may be formed from glucose by processes analogous to the synthesis of butyric acid from lactic acid which in turn is formed from the glucose. For the underlying reasons, which are rather too complicated to be dealt with here, Leathes' monograph must be consulted. It may, however, be pointed out in this connection that the investigations of Hanriot are very significant; he found that, in attempting the oxidation of fat *in vitro*, 15 per cent of its weight of oxygen was absorbed, and in the products of its oxidation butyric and acetic acids occurred, but no carbohydrate.

In conclusion brief mention may be made of Schmidt's views regarding the translocation of fats. He considers that in many cases the oil may be transported as such to those organs requiring it, for he found that the amount of fatty acid present in the germinating seeds was smaller than would be supposed if it were hydrolysed before translocation, also that neutral oil appears in regions of the plant removed from the storage organ.

Leathes: "The Fats," London, 1926.

He considers the walls of cells are permeable to oil; provided it be an emulsion sufficiently fine, and especially if a free fatty acid be present, the permeability being directly proportional to the amount of such acid present. It is thought that the acid forms a soap in the walls, and thus facilitates the passage.

It is not improbable that both methods are adopted by the plant, viz. the translocation of the products of the dissociation of the fat, and the translocation of oil *qua* oil.

With regard to the significance of fats in the construction of cell membranes, Hansteen-Cranner * has drawn attention to the occurrence of fatty substances in the cell walls of young plants of *Ricinus*, *Vicia*, and other plants, which substances he considers to occur in the form of soaps. He regards the cell wall as a hydrogel complex, the more solid phase of which is made up of the colloidal cellulose together with pectin and soap. The matter has been pursued by Priestley † and his fellow-workers who point out that the extent to which fat compounds are held in the cell wall depend on various factors amongst which the relation between calcium, which forms an insoluble compound with soap, on the one hand, and potassium and sodium, which form more soluble compounds with soaps, on the other, appears to be all important. In soils poor in calcium the fats remain in the cell membrane in a more mobile condition and diffuse more freely towards the surface as is indicated by the thick cuticle and more suberized layers of the endodermis growing in acid soils. The deposition of fat within the cell membrane also is conditioned by the reaction of the tissue; thus in the root, the phloem, on account of its alkaline reaction, would appear to free itself from fat within its membranes as is indicated by the fact that the formation of the casparian strip and suberin lamella of the endodermis, both of which structures are formed in part from fatty acids, occur opposite the phloem before they are formed opposite the xylem rays. The position of these deposits, in the cuticle, or in the

* Hansteen-Cranner: "Jahrb. Wiss. Bot.," 1904, **53**, 536; "Ber. deut. bot. Gesells.," 1919, **37**, 380.

† Priestley: "New Phyt.," 1924, **23**, 1, and the literature there quoted,

walls of the endodermis, exodermis, or cork, depend upon a variety of factors amongst which the ratio between calcium and potassium and sodium would appear to be important. Their fate, however, is the same; the unsaturated fatty acids undergo oxidation and condensation resulting in a waterproof layer, the fat constituents of which are no longer soluble in fat solvents.

MICROCHEMICAL REACTIONS.

1. The microscopical appearance of oil when mixed with water is characteristic owing to its immiscibility with water and its different refractive index.

2. Its solubility in ether, chloroform, benzene, or other fat solvents is easily noted.

3. If oil be present in the preparation it will fairly rapidly turn brown and then black when treated with a 1 per cent solution of osmic acid. This is not absolutely conclusive since osmic acid stains proteins brown.

4. Tincture of alkannin, or a saturated solution of Scharlach R in 75 per cent alcohol, colours oil globules red or pink.

The reaction with the first-named reagent is often ill-defined and frequently fails when the alkanna used has been extracted from the root some time. The test is more satisfactory when freshly prepared tincture is used.

A similar reaction is given by Sudan III.

It is important to note that these and similar reactions are not conclusive of the chemical nature of the substances acted upon. For example, Sudan III not only stains oils red but also resins, latex, wax, and cuticle; chloroplasts are stained a pale red; cellulose, lignified walls, gelatinized membranes, starch, and tannin are unstained.

The staining tests mentioned above may be employed after extracting the oil with ether or other solvent.

WAXES.

The chief function of waxes in plants is to form a protective covering against undue evaporation of water. They are found most commonly in or on the cuticle of leaves and fruits where they give rise to the glaucous effect.

As already stated, the waxes resemble the fats in their chemical constitution in so far as they are esters, but they differ in the nature of their alcohol constituent which is not glycerol but is usually a monohydric alcohol such as cetyl alcohol $C_{16}H_{33}OH$, carnaubyl alcohol $C_{24}H_{49}OH$, pisangceryl alcohol $C_{24}H_{49}OH$, ceryl alcohol $C_{26}H_{53}OH$, myricyl alcohol $C_{30}H_{61}OH$, cholesterol or phytosterol $C_{27}H_{45}OH$.

In addition to the acids already mentioned as occurring in fats, the following are also met with in waxes in the form of esters : ficocerylic acid $C_{13}H_{26}O_2$, carnaubic acid $C_{24}H_{48}O_2$, and pisangcerylic acid $C_{24}H_{48}O_2$, as well as acids belonging to series of the general formula $C_nH_{2n-2}O_2$ and $C_nH_{2n}O_3$.

The term wax used in the chemical sense has reference only to the chemical composition of these substances, regardless of their physical state of aggregation, and consequently both liquid and solid waxes are known.

Waxes of the former class are, however, only known in the animal kingdom, they are ordinary sperm oil and arctic sperm oil.

Among the better-known vegetable waxes may be mentioned :—

(*a*) *Carnauba Wax* obtained from *Copernicia cerifera ;* this wax contains ceryl and myricyl alcohols, and two acids, cerotic acid $C_{26}H_{52}O_2$, and carnaubic acid $C_{24}H_{48}O_2$, together with a hydroxy-acid of the formula $C_{21}H_{42}O_3$. This is a very hard wax and is used in the making of gramophone records.

(*b*) *Pisang Wax* obtained from the leaves of *Cera musae* is the pisangceryl ester of pisangcerylic acid.

The following are some of the more important waxes of animal origin :—

Wool wax, better known as wool fat or lanolin (which is rich in cholesterol), beeswax, spermaceti, and Chinese insect wax.

PHYSICAL AND CHEMICAL PROPERTIES OF WAXES.

Waxes are soluble in all the ordinary fat solvents such as benzene, ether, chloroform, etc., though they are rather less soluble than the fats.

Being free from glycerides the waxes, when heated, *give no smell of acrolein ;* they do not become rancid like the fats, and are less easily hydrolysed, but they can be decomposed by prolonged heating with alcoholic potash.

Owing to the high molecular weight of their constituent acids, the saponification value of waxes is low.

	Saponification Value.
Carnauba wax	79-95

Waxes are further characterized by giving abnormally high values for the unsaponifiable residue.

WAXES.

As already stated (p. 22) all fats and waxes on saponification with caustic alkalis yield a certain amount of substance, known as the unsaponifiable residue, which is insoluble in the alkaline solution remaining after hydrolysis and may be extracted therefrom by means of ether. This material in the case of fats is composed chiefly * of a group of alcohols known as sterols, while in the case of waxes it will include in addition the higher saturated alcohols.

The sterols may occur in the uncombined state in fats, or combined with fatty acids as esters. The sterols form a group of highly complex hydro-aromatic monohydric secondary alcohols whose constitution has not as yet been completely determined. They fall into two main groups, the cholesterols and the phytosterols which are characteristic of the animal and vegetable world respectively.

REACTIONS AND PROPERTIES OF CHOLESTEROL AND PHYTOSTEROL.

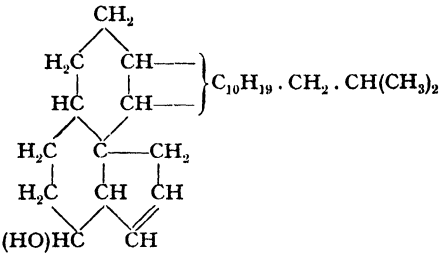
Cholesterol.

Cholesterol is a monohydric alcohol of the formula $C_{27}H_{45}OH$; its constitution is still unknown, although a great deal of work has been expended on this question;

* It may be mentioned that the unsaponifiable residue of fats contain also the fat soluble vitamin A when this substance is present.

it would appear to be a secondary alcohol containing an unsaturated group.

Cholesterol has a constitution probably represented by the formula *—



Cholesterol occurs in the bile, certain gall stones, brain, blood, and wool fat. It is insoluble in water and crystallizes from chloroform in needles and from ether or alcohol in rhombic plates, m.p. 148-150°. It may conveniently be obtained by evaporating the ethereal extract of gall stones to dryness.

Reactions.—1. Crystals of cholesterol pressed on a white porcelain surface and moistened with a drop of sulphuric acid (5 parts concentrated acid to 1 part of water) turn pink. The addition of a drop of dilute iodine causes a play of colours from red to blue or green.

2. A solution of cholesterol in chloroform gently agitated with concentrated sulphuric acid turns red, while the sulphuric acid which forms the lower layer assumes a green fluorescence.

3. On the addition of concentrated sulphuric acid drop by drop to a little cholesterol dissolved in a mixture of 2-3 drops of chloroform and about 10 drops of acetic anhydride, a transient pink colour is at first formed; on the addition of more acid, however, the colour changes to blue and finally to green.

4. Alcoholic solutions of cholesterol mixed with a few drops of 1 per cent alcoholic solution of digitonin,† give an immediate white precipitate, $C_{27}H_{46}OC_{54}H_{92}O_{28}$, a reaction employed in the estimation of cholesterol.‡

* Windaus: "Annalen," 1926, **447**, 233.

† Panzer: "Chem. Zentr.," 1912 (ii.), 540.

‡ Windaus: "Ber. deut. chem. Gesells.," 1909, **42**, 238; "Zeit. physiol. Chem.," 1910, **65**, 110; Salomon: "Ber. deut. pharm. Gesells.," 1914, **24**, 189.

Phytosterols.

The term phytosterol was at one time employed to designate a definite chemical individual of the formula $C_{27}H_{45}OH$, but it is now used more as a generic term to include a number of different substances having certain properties in common. Thus Windaus and Hauth * showed that the substance obtained from Calabar beans and commonly known as phytosterol was in reality a mixture of two substances—(*a*) Sitosterol of the formula $C_{27}H_{45}OH$, and (*b*) Stigmasterol $C_{30}H_{47}OH$, an observation which has been confirmed by Salway. †

Similarly Klobb ‡ describes a dextro-rotatory phytosterol of the formula $C_{31}H_{52}O$, $3H_2O$ occurring in *Anthemis nobilis* and a number of lævo-rotatory phytosterols of different formulæ obtained from *Matricaria Chamomilla*, *Tilia europaea*, *Linaria vulgaris*, and *Verbascum Thapsus*.§

All vegetable fats contain phytosterol, the amount varying from about 0·13 to 0·30 per cent and rising in the case of pea fat and the fat of Calabar beans to a considerably higher value. In the case of the wheat, the grain of which contains sitosterol whilst the bran contains a different phytosterol, the amounts of these substances differ in the various parts of the plant. The percentage present in the green parts is higher than the percentage occurring in the grain which is somewhat greater as compared with the percentage in etiolated plants. The fact that the highest percentage occurs in the embryo suggests a function in connection with germination and growth; not necessarily a direct nutritive function since a starved plant contains as much as the grain.‖

The sterols are widely distributed in the vegetable

* Windaus and Hauth: "Ber. deut. chem. Gesells.," 1906, **39**, 4378; 1907, **40**, 3681.

† Salway: "J. Chem. Soc.," 1911, **99**, 2154.

‡ Klobb: "Compt. rend.," 1911, **152**, 327; "Ann. Chim. Phys.," 1911, viii., **24**, 410.

§ See also Power and Rogerson: "J. Chem. Soc.," 1910, **97**, 1951; Rogerson: "Amer. Journ. Pharm.," 1911, **83**, 59; "J. Chem. Soc.," 1912, **101**, 1040.

‖ Ellis: "Biochem. Journ.," 1918, **12**, 154, 160, 173.

kingdom: in addition to the higher plants, they occur in *Sphagnum*, *Pelvetia*, *Laminaria*, *Agaricus*, *Lactarius*, and *Polyporus*.*

Ergosterol is the name given to a sterol isolated from ergot by Tanret; † this substance, melting at 154°, to which he assigned the formula $C_{27}H_{42}O$, H_2O, was accompanied by a second sterol which he described as fungisterol of the formula $C_{25}H_{40}O$, H_2O, m.p. 144°. Both these sterols are regarded as belonging to a class characteristic of crypotogams and differing from cholesterol and phytosterol in their reaction in chloroform solution with sulphuric acid; whereas in the case of the ordinary sterols the chloroform solution acquires a red colour, it is the acid which turns red in the case of the fungus sterols. Yeast has been shown to contain a mixture of sterols—namely ergosterol and zymosterol, m.p. 99-104°.‡ The work of Webster and others has shown that the irradiation of ergosterol with ultra violet light gives rise to vitamin D.

Phytosterols crystallize from alcohol in elongated plates and from ether in slender needles. The melting-point varies somewhat according to the source from which it is prepared; it lies somewhere between 135 and 137° or it may be as high as 144°. The reason for this may be that the various substances obtained from different sources and described as one and the same substance are in reality different substances but all of a phytosterol nature. The colour reactions of phytosterols resemble those of cholesterol.

DISTINCTION BETWEEN CHOLESTEROL AND PHYTOSTEROL.

In examining the unsaponifiable matter of a fat for sterols, the unsaponifiable residue remaining after evaporation of the ether is dried over a water bath and then dissolved in the least possible quantity of absolute alcohol and allowed to crystallize. The crystals which separate should be examined

* Ellis: "Biochem. Journ.," 1918, **12**, 154, 160, 173.
† Tanret: "Compt. rend.," 1889, **108**, 98, and 1908, **147**, 75.
‡ Smedley-Maclean: "Biochem. Journ.," 1928, **22**, 22.

under a microscope; cholesterol crystallizes in four-sided plates and phytosterol in elongated hexagonal plates.

Cholesterol and phytosterol cannot with certainty be distinguished by means of their melting-points, owing to the fact that phytosterol may melt at any temperature between 135 and 144° according to the source from which it is prepared. As, however, there is a considerable difference between the melting-points of the acetates of these two substances the following procedure may be adopted. After completely evaporating off the alcohol, the residue is carefully heated with 2-3 c.c. of acetic anyhdride over a free flame until the liquid boils, the remaining acetic anhydride being evaporated off over a water bath. The residue is then re-crystallized two or three times from the least possible quantity of absolute alcohol, and the melting-point of the crystals so obtained is determined.

Cholesterol acetate melts at 114·3-114·8°.

Phytosterol acetate * melts at 125-137°.

Stigmasterol acetate melts at 141°.

Since cholesterol and phytosterol are the sterols characteristic of animal and vegetable fats respectively the above procedure may be adopted for distinguishing the source of origin of a given fat, or for detecting the presence of vegetable fat in animal fat. For this purpose a melting-point of the sterol acetate up to 116° is taken to imply the absence of vegetable oil, but a melting-point of 117° or more indicates contamination with vegetable oil.

ESTIMATION OF THE STEROL CONTENT OF AN UNSAPONIFIABLE RESIDUE.

The method devised by Windaus † depends upon the formation of an insoluble compound of the sterols with digitonin.

The unsaponifiable residue obtained by the method already described, is dissolved in twenty times its weight of alcohol; it is then warmed to 65° and treated with a 1 per cent solution

* The acetyl derivative obtained by Power and Moore from the root of *Bryonia* has the melting-point 155-157°.

† Windaus: "Zeit. physiol. Chem.," 1910, 65, 110.

of digitonin in 95 per cent alcohol until no further precipitate is formed; a little chloroform is then added to prevent the separation of any excess of digitonin and the whole is allowed to stand for some hours while the precipitate of the sterol digitonide settles down; the precipitate is then filtered off on a Gooch crucible, washed with chloroform and finally with ether, dried for 10 minutes in a steam oven, and weighed. The weight multiplied by the factor 0·2431 gives the weight of sterol.

LIPINS.

The term lipin is applied to a group of glycerol esters which in their physical and chemical properties are closely allied to the fats. The nomenclature of the group has in the past given rise to much confusion, the term lipoid (from the Greek word $\lambda\iota\pi o\varsigma$ = fat) having been used somewhat loosely to include a heterogeneous group of substances which were all soluble in the ordinary fat solvents, but were not necessarily esters of glycerol.

Like the fats, the lipins are esters of glycerol with saturated fatty acids and with unsaturated acids of the oleic and other series, but they differ from the fats in containing in addition the elements nitrogen and phosphorus, or nitrogen only, as is exemplified by the formula here given for lecithin, one of the best-known representatives of the group:—

$$\begin{array}{l} CH_2 \cdot O \cdot COC_{17}H_{35} \text{ (Stearyl)} \\ | \\ CH \cdot O \cdot COC_{17}H_{33} \text{ (Oleyl)} \\ | \\ CH_2 \cdot O \cdot P(OH) \cdot O \cdot CH_2CH_2N(CH_3)_3OH \\ \qquad\quad \| \\ \qquad\quad O \end{array}$$

As already stated the phospho-lipins in general resemble the fats in being soluble in the same solvents such as ether, petrol, benzene, etc.; they are, however, generally more soluble in alcohol than the fats, but on the other hand they are insoluble in cold acetone though frequently soluble in hot acetone; the cerebrosides are practically insoluble in ether.

The fact that lipins are themselves soluble in fats and are usually found in close association with true fats in plant and

animal tissues, adds considerably to the difficulty of their preparation in a pure condition; moreover, a given lipin which may be extracted by means of ether in admixture with another lipin may, in a purified condition, be practically insoluble in this solvent.

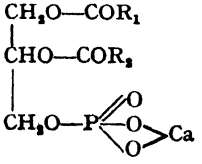
Again, it was first found by Hoppe Seyler * that when egg yolk is extracted with ether until no more extract is obtained, the residue still contains lipins which can be readily extracted by means of warm alcohol; this has since been found to be a property common to all tissues both plant and animal; no matter how long the extraction with ether is continued, a considerable quantity of the lipin is retained by the tissue only to be extracted by replacing the ether by alcohol.

In general, the first step in the purification of an ether extract from lipin consists in the addition to the concentrated ethereal solution of four times its volume of cold acetone, which will precipitate the phospholipins and probably also the cerebrosides if present. The separation of fat from lipin by this method will only be partial, and repeated solution and precipitation will be required to effect any reasonable amount of purification.†

In order to distinguish a lipin from a fat, recourse is taken to the fact that the former, unlike fats, contain either nitrogen or phosphorus or both. To establish the presence of nitrogen, it is sufficient to heat the purified substance with a little soda lime and to test for the evolution of ammonia by red litmus paper. Phosphorus may be detected by fusing with fusion mixture on a platinum foil until all carbon is burnt away, the residue is dissolved in nitric acid and tested for the presence

* Hoppe Seyler: "Med. chem. Unters.," 1869, **3**, 392.

† On applying this method to the ether-soluble extract of cabbage-leaf cytoplasm, Chibnall and Channon ("Biochem. Journ.," 1927, **21**, 233) claim to have precipitated, not an ordinary phospholipin, but the calcium salt of a diglyceride phosphoric acid, to which they assign the formula—



of phosphate by ammonium molybdate. The classification of the lipins is based upon their nitrogen and phosphorus content as follows :—

A. *Phospholipins* which contain both phosphorus and nitrogen. According to the number of atoms of phosphorus, one or two, contained in their molecule, they are classed as mono- or di-phosphatides. To this group belong lecithin and kephalin.

B. *Galactolipins* or Cerebrosides which contain nitrogen but no phosphorus and yield on hydrolysis galactose in addition to fatty acids and glycerol.

A. PHOSPHOLIPINS.

LECITHIN.

Although widely distributed in the vegetable kingdom lecithin usually occurs together with other lipins and in relatively small amount; for this reason the most convenient source for the preparation of lecithin is egg yolk. This substance is extracted with five times its volume of 96 per cent alcohol; the extract is then cooled to 0°, filtered and precipitated with an alcoholic solution of cadmium chloride; the precipitated double salt is next washed with alcohol and ether; it is then decomposed by boiling with eight times its quantity of 80 per cent alcohol and carefully adding a concentrated solution of ammonium carbonate until all the cadmium is thrown out of solution; the solution is filtered whilst hot and on cooling the filtrate to 10° the lecithin is deposited. It may be purified by dissolving in chloroform and precipitating from solution by the addition of acetone in which lecithin is insoluble.

The following are some of the more characteristic reactions of lecithin :—

1. If to an alcoholic solution of lecithin an alcoholic solution of cadmium chloride be added, a white precipitate of the cadmium chloride double salt is formed.

2. If a little lecithin is boiled with caustic soda, trimethylamine is formed, and may be identified by its characteristic

smell; the solution contains sodium salts of fatty acids; on acidifying with sulphuric acid the fatty acids are precipitated.

3. Lecithin on exposure to light and air absorbs oxygen undergoing a change which reduces its solubility in alcohol or ether and makes it increasingly soluble in water.

4. Mixed with a little water, lecithin, in common with some other lipins, swells up, forming slimy threads known as myelin forms; with excess of water these gradually produce a sort of emulsion or colloidal solution from which they can be precipitated by the addition of salts of barium or calcium.

Lecithin like many other lipins is a yellow or yellowish-white wax-like solid with a peculiar odour; the lipins are very hygroscopic, but some of them when carefully dried in a vacuum can be obtained in form of powder.

Lecithin is readily hydrolysed by boiling with alkalis, notably baryta, and is also broken up by lipase, and, less readily, by mineral acids. The products of its hydrolysis are glycero-phosphoric acid—

$$CH_2OHCHOHCH_2OP(=O)(OH)_2$$

choline $HON(CH_3)_3CH_2CH_2OH$ and fatty acids; a similar hydrolysis takes place in the germinating seed.*

Originally it was considered that the fatty acids of lecithin were either stearic, palmitic, or oleic, but it has since been found that the more highly unsaturated acids, linolic and linolenic, are also present.† The unsaturated arachidonic acid ‡ $C_{20}H_{32}O_2$, containing four double bonds which occurs in lipins of animal origin, has not hitherto been isolated from plant lipins.

To examine the products of the hydrolysis of lecithin, this substance is heated with a solution of barium hydrate in excess; a baryta soap is formed, which may be filtered off. The aqueous solution contains barium glycero-phosphate and choline; the latter may be extracted as follows:—§

* Schulze: "Zeit. physiol. Chem.," 1887, 11, 365; Schulze and Frankfurt: "Ber. deut. chem. Gesells.," 1893, 26, 2151.

† Levene and Rolf: "J. Biol. Chem.," 1925, 62, 759; 1926, 68, 285.

‡ *Ibid.*, 1921, 46, 353; 1922, 54, 91.

§ Leathes: "The Fats," Monographs of Biochemistry, London, 1910.

Treat the solution with a stream of carbon dioxide until no more barium carbonate comes down. Filter and evaporate the filtrate to dryness. Treat the residue with absolute alcohol, which will dissolve the choline but not the barium glycerophosphate. The alcoholic solution, if treated with an alcoholic solution of platinic chloride, gives a precipitate of the double platinichloride of choline.

Green and Jackson * give the following method: Allow the finely-divided material to stand for some days under absolute alcohol. Pour off the extract, and evaporate to dryness; the residue is again extracted with absolute alcohol, and finally with a mixture of alcohol and ether. These extracts are mixed, and the solvents evaporated off. The choline is contained in the residue.

In addition to the above products of the hydrolysis of lecithin of animal origin, a number of phospholipins isolated from the seeds of *Avena sativa*, *Lupinus* spp, *Pinus cembra* as well as from pollen and potato tubers yield glucose, galactose, and pentoses.†

KEPHALIN.

This is the name given to a phospholipin whose nitrogen base is aminoethyl alcohol, $NH_2CH_2CH_2OH$, in place of choline; its chemical constitution is closely allied to that of lecithin but it differs from this substance probably in the nature of the acid radicles it contains. It occurs together with lecithin in most animal and vegetable tissues; as examples of the latter may be mentioned the soya bean ‡ and yeast.§

Kephalin, unlike lecithin, is practically insoluble in alcohol, and the two substances may be separated by making use of this fact.

Betaine has likewise been described as replacing choline as the nitrogen base of a phospholipin, by Zlataroff.‖

* Green and Jackson: "Proc. Roy. Soc.," B., 1906, **77**, 69.

† Winterstein and Hiestand: "Zeit. physiol. Chem.," 1906, **47**, 496; 1908, **54**, 283; Zlatarov: "Biochem. Zeit.," 1925, **161**, 399.

‡ Levene and Rolf: "J. Biol. Chem.," 1925, **62**, 759.

§ Daubney and Smedley-Maclean: "Biochem. Journ.," 1927, **21**, 373.

‖ Zlataroff: "Biochem. Zeit.," 1925, **161**, 379.

Water-soluble phosphatides obtained from beetroot, soja bean and from *Aspergillus oryzæ* have been described by Hansteen-Cranner and Grafe and his co-workers.*

B. CEREBROSIDES OR GALACTOLIPINS.

The name cerebrosides was originally applied by Thudichum to a group of substances isolated by him from the brain of animals. They are characterized by being phosphorus free but yielding on hydrolysis a nitrogen base, a saturated fatty acid, and galactose; for this reason they are better known as galactolipins.

Substances of this type of vegetable origin were first isolated from *Lycoperdon bovista* by Bamberger and Landsiedl † and later from *Hyphaloma fasciculare* and *Amanita muscaria* by Zellner,‡ while Trier§ obtained a small quantity of a cerebroside from rice.

Unlike the phospholipins, the galactolipins are, when dry, white powders tending to crystallize. They differ also from the former substances in being insoluble in ether; they are, however, soluble in hot alcohol, benzene, and pyridine, but, like the phosphatides, they are insoluble in cold acetone.

OCCURRENCE.

Lecithin-like compounds occur in the grains of cereals, in the seeds of several Leguminosæ, *Ricinus*, and species of *Pinus;* in the leaves of *Castanea*, and in Fungi; they are also widely distributed in animals. In fact, these substances are stated to occur in small quantities in all living cells, and they appear to be more especially abundant where fats occur. Zlataroff considers that light is requisite for the formation of lecithins since he finds that the amount present in seeds increases during germination in the light.‖

* Grafe: "Biochem. Zeit.," 1925, **159**, 445; 1925, **162**, 366; 1926, **176**, 266, **177**, 16; 1927, **187**, 102.

† Bamberger and Landsiedl: "Monat. f. Chem.," 1905, **26**, 1109.

‡ Zellner: *id.*, 1911, **32**, 133, 1057.

§ Trier: "Zeit. physiol. Chem.," 1913, **86**, 413.

‖ Zlataroff: "Biochem. Zeit.," 1916, **75**, 200.

The approximate amount of lecithin contained in various substances may be seen from the following table :—

Egg yolk	9·4 per cent.
Liver	2·1 „
Blood	1·8 „
Leguminous seeds	0·8-1·64 „
Cereals	0·25-0·53 „

Pure lecithin has not as yet been obtained from vegetable sources, the substances isolated by Winterstein * and his collaborators from wheat flour and from the seeds of *Avena sativa*, *Lupinus albus*, *L. luteus*, *Vicia sativa*, from the leaves of *Æsculus hippocastanum*, etc., being mixtures which, moreover, contain a carbohydrate complex. For an account of the methods employed in the extraction of these substances the original papers should be consulted. Smolensky † found that wheat germs (i.e. the embryos which are a bye-product of the flour mills) yielded a phosphatide whose composition was much closer to that of ordinary lecithin than was that obtained from the flour.

Physiological Significance.

Lipins are, apparently, universally present in living cells and must, presumably, play an important part in the physiology of the organism: but what their function may be is unknown and as a consequence many rôles have been ascribed to these bodies.

Overton ‡ showed in many instances that those substances which are soluble in lipins readily enter the cell, whilst those which are insoluble in lipins are absorbed by the cell with difficulty. From such observations he concluded that the plasma membrane is composed essentially of lipins and formulated his solution theory of permeability. His views at first

* Winterstein and Hiestand: "Zeit. physiol. Chem.," 1907, **54**, 288; Winterstein and Smolensky: *id.*, 1908, **58**, 506; Winterstein and Stegmann: *id.*, 1908, **58**, 527. See also Schulze and Likiernik: *id.*, 1891, **15**, 405; Schulze: *id.*, 1895, **20**, 228.

† Smolensky: *id.*, 1908, **58**, 522.

‡ Overton: "Vierteljahschr. Naturf. Ges. Zurich," 1895, **40**, 159; 1896, **41**, 383; 1899, **44**, 88; "Jahrb. wiss. Bot.," 1900, **34**, 669.

found acceptance; it was, for instance, supported by Czapek * in his work on the surface tension of the external limiting membrane, and Green and Jackson † considered that lipins exercise considerable influence on the transport of material from cell to cell. On the other hand, further work on the uptake of inorganic salts and dyes by the vegetable cell and on the surface tension of solutions, indicate the imperfections of Overton's theory. It, however, stimulated investigation on the nature of the plasma membrane and, generally, on permeability, a subject which is without our present province.‡

Palladin § suggested that lipins play a part in respiration in that the more these substances are extracted with organic solvents, the more is respiration depressed as measured by the output of carbon dioxide in the presence of water during definite periods of time. This thesis involves many problems. Thus, if respiration be a matter of enzyme action, then, presumably, there must be some essential connection between lipin and enzyme. There is no doubt that fats are utilized in respiratory processes; are they, after desaturation, built up into lipins which are then oxidized for the liberation of energy? The evidence available on these points relates either to the animal or to the chemical laboratory. Vernon,‖ working on animal tissues, found that if the material were extracted with organic solvents, in order to remove the lipins, the oxidase reaction rapidly disappeared, which means that oxidase reaction is somehow dependent on the cell lipins.

Further, Gallagher ¶ isolated from the potato a lipin which in the presence of oxygen acquired the property of immediately oxidizing guaiacum in the presence of oxidase. If this be significant in respiration, it indicates that oxidase plays an essential part in the process, a conclusion which is

* Czapek: "Ueber eine Methode zur direkten Bestimmung der Oberflächenspannung der Plasmahaut von Pflanzenzellen," Jena, 1912.

† *Loc. cit.*

‡ See Stiles: "Permeability," New Phyt. Reprint, No. 13, 1924.

§ Palladin: "Ber. deut. bot. Gesells.," 1910, **28**, 120. Palladin and Stanevitsch: "Biochem. Zeit.," 1910, **26**, 351.

‖ Vernon: *id.*, 1912, **47**, 374; 1914, **60**, 202.

¶ Gallagher: "Biochem. Journ.," 1923, **17**, 515.

rendered doubtful on other considerations, at any rate for the plant.*

The following table, due to Green and Jackson,† shows the relation between the lecithin, fatty acid, and oil of the endosperm of *Ricinus*, expressed in per cent of weight of the seeds at different stages in their germination :—

Degree of development.	Oil in seeds.	Fatty acid in seeds.	Lecithin.
Resting seeds . . .	82·8	2·2	·236
Testa just cracked . .	67·5	4·6	·17
Radicle protruding 1-2 cm.	52·5	11·9	·475
Root system established .	23·6	16·89	·873

From this it appears that lecithin is formed during germination ; although there is, during the early stages of germination, a diminution in the quantity present. It was found when once the maximum was reached that this amount remained constant until the whole of the endosperm was used up.

FURTHER REFERENCE.

Maclean and Smedley-Maclean : " Lecithins and Allied Substances," Monographs of Biochemistry, London, 1927.

* See Vol. II., chapter on " Respiration."
† Green and Jackson, *loc. cit.*

SECTION II.

ALDEHYDES AND ALCOHOLS.

In view of the important part played by aldehydes and alcohols in questions relating to the carbohydrates and other compounds, it appears desirable here to draw attention to the chief properties of these substances.

It is well known that the aldehydes are the first products of the oxidation of primary alcohols :—

$$\underset{\text{Methyl alcohol}}{CH_3OH} + O = \underset{\text{Formaldehyde}}{HCHO} + H_2O$$

$$\underset{\text{Ethyl alcohol}}{CH_3CH_2OH} + O = \underset{\text{Acetic aldehyde}}{CH_3CHO} + H_2O$$

The reconversion of formaldehyde into the alcohol can be effected by means of nascent hydrogen obtained by sodium amalgam and water.

Chemically, the aldehydes are very active, undergoing a number of reactions, some of which are of biological significance, whilst others serve as valuable means of isolation or identification.

1. Aldehydes are readily oxidized to the corresponding acids by even such mild oxidizing agents as ammoniacal silver hydroxide or Fehling's solution, or even atmospheric oxygen, as is shown by the following experiments :—

(*a*) A few drops of caustic potash are added to some silver nitrate solution in a test tube, ammonia is then carefully added, drop by drop, until the brown precipitate has just redissolved. A little dilute acetaldehyde solution is poured in and the mixture is warmed gently ; if the solution be sufficiently dilute, a silver mirror will be deposited on the side of the test tube ; otherwise a black precipitate will be formed :—

$$CH_3CHO + Ag_2O = CH_3COOH + 2Ag$$

(*b*) A little Fehling's solution is gently warmed with a few drops of dilute aldehyde solution ; a change in colour takes place, from blue to green and yellow ; finally the solution becomes colourless and a red precipitate of cuprous oxide (Cu_2O) comes down.

The readiness with which aldehydes are oxidized to acids accounts for the fact that most samples of aldehydes, unless freshly prepared, contain varying amounts of free acid.

2. Aldehydes are readily reduced by nascent hydrogen to the corresponding primary alcohols, according to the equation

$$\underset{\text{Acetic aldehyde}}{CH_3CHO} + 2H = \underset{\text{Ethyl alcohol}}{CH_3CH_2OH}$$

3. Aldehydes restore the colour to Schiff's Reagent (a solution of magenta decolorized by sulphurous acid).

4. Aldehydes when warmed with caustic potash are converted into resinous substances of unknown composition. This can be readily shown with acetaldehyde ; formaldehyde, however, when treated with potash undergoes a different change, being converted into a mixture of methyl alcohol and potassium formate, according to the equation

$$2HCHO + KOH = CH_3OH + \underset{\text{Potassium formate}}{HCOOK}$$

5. Aldehydes react with ammonia to form additive compounds ; thus acetic aldehyde undergoes the following reaction :—

$$\underset{\text{Acetic aldehyde}}{CH_3CHO} + NH_3 = \underset{\text{Aldehyde ammonia}}{CH_3CHOHNH_2}$$

Here again formaldehyde behaves differently ; if ammonia is added to a formaldehyde solution, it is neutralized quantitatively according to the equation :—

$$\underset{\text{Formaldehyde}}{6CH_2O} + 4NH_3 = \underset{\text{Hexamethylene tetramine}}{(CH_2)_6N_4} + 6H_2O$$

with the formation of a crystalline solid which is used in medicine under the name of urotropine.

The reaction can be employed for estimating * the amount

* For another method of estimating formaldehyde by weighing the mercury produced by the reduction of an alkaline solution of mercuric sulphite, see Feder : " Archiv. d. Pharm.," 1907, 245, 25.

of formaldehyde in a solution by adding a known excess of standardized ammonia solution, and after some time titrating back the excess of ammonia by means of standard acid, using litmus as indicator.

Thus, for example, if 25 c.c. of the formaldehyde solution, after shaking with 50 c.c. of N/2 ammonia, required for neutralization 20 c.c. N/2 hydrochloric acid, then the amount of ammonia used up by the formaldehyde would be 50 — 20 = 30 c.c.

But 30 c.c. N/2 ammonia contain $\frac{30}{1000} \times \frac{17}{2} = \cdot 255$ gram NH_3,

and since from the equation $4NH_3$ (68) are equivalent to $6CH_2O$ (180)

$$\therefore \cdot 225 \text{ gram } NH_3 \equiv \cdot 68 \text{ gram } CH_2O,$$

$\therefore$ 25 c.c. of the solution contained 0·68 gram formaldehyde.

6. With sodium bisulphite aldehydes form crystalline addition compounds which, being sparingly soluble in water, can be used for isolating aldehydes from mixtures.

Thus if some saturated sodium bisulphite solution be added to a fairly strong solution of aldehyde and the mixture shaken vigorously, a rise in temperature takes place accompanied by the formation of a white crystalline precipitate :—

$$CH_3CHO + HNaSO_3 = CH_3CHOHSO_3Na$$

7. Aldehydes also form additive compounds with hydrogen cyanide ; these compounds are known as hydroxycyanides or cyanohydrins :—

$$CH_3CHO + HCN = CH_3CHOHCN$$

Acetic aldehyde cyanohydrin

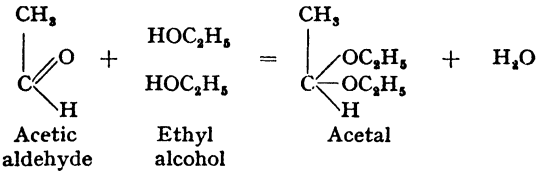
8. Aldehydes form crystalline compounds with hydroxylamine, phenylhydrazine, and semicarbazide ; in all cases water is split off between the two reacting substances :—

$$CH_3CHO + NH_2OH = CH_3CH : NOH + H_2O$$
$$CH_3CHO + C_6H_5NHNH_2 = CH_3CH : N . NHC_6H_5 + H_2O$$

The resulting compounds, which are known as oximes, hydrazones or semi-carbazones, are usually substances with a characteristic crystalline form and melting-point, which may be

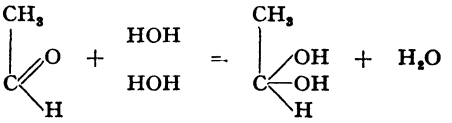
employed for the identification of the corresponding aldehydes. The use of phenylhydrazine for the identification of the sugars has already been described.

9. The aldehydes are able to react with alcohols with the formation of condensation compounds known as acetals; thus, for example, acetic aldehyde reacts with ethyl alcohol as follows :—

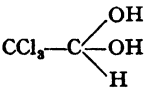


Acetic aldehyde — Ethyl alcohol — Acetal

By analogy, acetic aldehyde should also be able to react with water as follows :—



This substance does not, however, actually exist, since a compound having two or more hydroxyl groups attached to the same carbon atom is, as a rule, unstable, and at once loses water. Exceptions to this rule are, however, occasionally met with; for example, chloral CCl_3CHO forms a stable compound, chloral hydrate, of the formula :—



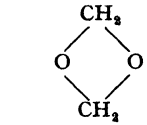
10. Aldehydes exhibit a tendency to polymerize, that is, for two or more molecules to combine together to form new compounds of higher molecular weight.

Thus two molecules of formaldehyde will combine together, forming a compound known as paraformaldehyde $(CH_2O)_2$; this substance, which is a white solid, is obtained by evaporating an aqueous solution of formaldehyde.

A second polymer formed from three molecules of formaldehyde is known as metaformaldehyde or trioxymethylene,

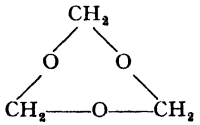
$(CH_2O)_3$. This substance is produced by the spontaneous polymerization of anhydrous formaldehyde.

In the case of both the above polymers the molecules of formaldehyde are probably connected together through oxygen atoms as under :—



Paraformaldehyde

and



Trioxymethylene or Metaformaldehyde

which accounts for the fact that they are readily broken up into the simple molecules of formaldehyde by heating.

11. A different type of polymerization, involving the linking together of molecules of formaldehyde through carbon, is also known ; this type of polymerization, which is sometimes known as aldol condensation, results in the formation of a more stable complex which cannot be reconverted into the simple substance.

The reaction takes its name from the substance produced by the action of dilute hydrochloric acid or zinc chloride on acetic aldehyde :—

$$CH_3CHO + CH_3CHO = \underset{\text{Aldol}}{CH_3CHOH . CH_2 . CHO}$$

The analogous reaction with formaldehyde is, however, brought about by dilute alkalis ; in this way two molecules of formaldehyde give rise to glycollic aldehyde,

$$HCHO + HCHO = \underset{\text{Glycollic aldehyde}}{CH_2OH . CHO}$$

or three molecules may combine together to produce glyceric aldehyde,

$$HCHO + HCHO + HCHO = \underset{\text{Glyceric aldehyde}}{CH_2OH . CHOH . CHO}$$

By repeatedly shaking a 4 per cent solution of formaldehyde for half an hour with an excess of lime water, and then filtering the solution and setting it aside for some days until the odour of formaldehyde had disappeared, Loew * was able

* Loew : " Ber. deut. chem. Gesells.," 1887, **20**, 142, 3039 ; 1888, **21**, 270 ; 1889, **22**, 470, 878.

to obtain a crude mixture of sugars called formose, from which true reducing hexose sugars have been isolated. This change may be represented by the equation—

$$6HCHO = C_6H_{12}O_6$$

Similarly H. and A. Euler * have shown that when a 2 per cent solution of formaldehyde is heated for some hours with calcium carbonate, a pentose sugar—arabinoketose—is produced ; in addition to this substance, glycollic aldehyde and dihydroxyacetone are produced, but in smaller quantity.

FORMALDEHYDE.

From the point of view of photosynthesis formaldehyde is of outstanding interest ; as is well known, it is at ordinary temperatures a colourless gas with a pungent odour ; when cooled to $-21°$ it condenses to a liquid. It is usually met with in the form of an aqueous solution, commercial formalin, which contains about 40 per cent of the gas dissolved in water and is used as a disinfectant or as a hardening medium for pathological and other specimens and occasionally as a preservative for milk. It undergoes most of the general reactions for aldehydes which have been mentioned above.

Its peculiar behaviour towards ammonia, resulting in the formation of hexamethylene tetramine, has already been mentioned ; this substance, which is used under the name of urotropine, is a crystalline base which dissolves in hot or cold water ; with bromine it forms an additive compound—tetrabromo-hexamethylene tetramine $(CH_2)_6N_4Br_4$—which has been used for detecting small quantities of formaldehyde in solution.

Formaldehyde also reacts with ammonium salts as well as with free ammonia, as follows :—

$$6CH_2O + 4NH_4Cl = \underset{\text{Hexamethylene tetramine}}{(CH_2)_6N_4} + 6H_2O + 4HCl$$

This reaction has been made use of as a means of estimating ammonium salts in solution by titrating the amount of free acid liberated according to the above equation on adding sufficient formaldehyde to a solution containing ammonium salts.

* Euler, H. and A. : " Ber. deut. chem. Gesells.," 1906, **39**, 36, 39.

For this purpose both the formaldehyde solution and the solution to be analysed must be previously neutralized, if necessary. An excess of the neutralized formaldehyde solution is then added to a known volume of the solution containing the ammonium salts, and after thoroughly shaking for one or two minutes the amount of acid set free is determined by titration with standard caustic soda, using methyl orange as indicator; the amount of ammonia can be calculated from the fact that each 36·5 grams of hydrochloric acid liberated correspond to 17 grams of ammonia.

The reactions most suitable *for characterizing small quantities of formaldehyde* are as follows:—

Rimini's test consists in adding 2 drops of phenylhydrazine hydrochloride, 2 drops of sodium nitroprusside solution and 1 c.c. of sodium hydroxide to 1 c.c. of the liquid to be tested. A blue colour is formed, which changes rapidly through green and brown to red. Schryver * has modified this test and made it much more sensitive; he recommends the following method: to 10 c.c. of the liquid to be tested add 2 c.c. of a 1 per cent solution of phenylhydrazine hydrochloride freshly made up and filtered; then add 1 c.c. of a 5 per cent solution of sodium ferricyanide, also freshly made up, and 5 c.c. of hydrochloric acid; a brilliant magenta colour is produced. The test is a very delicate one and will detect quantities of formaldehyde varying from 1 part in 1,000,000 to 1 part in 100,000. Acetic aldehyde gives no colour with this reagent.

The following test, due to Denigés,† is sensitive for formaldehyde, even in presence of acetic aldehyde up to 2 per cent; 5 c.c. of an aqueous solution of formaldehyde are mixed with 1·2 c.c. of pure sulphuric acid (sp. gr. 1·66) and 5 c.c. of Schiff's reagent. An intense violet colour having an absorption band in the orange is produced. Schiff's reagent may be prepared by adding a litre of 0·01 per cent of solution of magenta to 20 c.c. of sodium hydrogen sulphite solution (sp. gr. 1·3), and

* Schryver: "Proc. Roy. Soc. Lond.," B., 1910, **82**, 226.
† Denigés: "Compt. rend.," 1910, **150**, 529.

after five minutes adding 20 c.c. of hydrochloric acid (sp. gr. 1·18).

Kimpflir :sted for formaldehyde in the leaf of *Agave mexicana* by injecting into it, by means of a capillary tube, a concentrated solution of sodium hydrogen sulphite, containing an excess of *p*-methylamino-*m*-cresol. The presence of formaldehyde was indicated by the formation of a red precipitate on exposure to light. The precipitate is best seen by examining a section of the leaf which has been dipped in absolute alcohol. Formaldehyde is the only aldehyde giving a stable red colour with the above reagent, but other aldehydes give unstable green, yellow, or reddish-brown colours.

Occurrence in the Plant.—Since the work of Reinke, many have reported the occurrence of formaldehyde in the plant,† and its presence has been accepted as evidence of the truth of Baeyer's hypothesis of photosynthesis. It has, however, since been shown that this formaldehyde is a degradation product of chlorophyll.

Thus Warner ‡ has found that formaldehyde is produced when chlorophyll is exposed to sunlight or electric light in air; since this substance is produced both in the presence and in the absence of carbon dioxide, it would appear that the latter plays no part in the production of formaldehyde by photosynthesis outside the plant, and that the formaldehyde is in reality an oxidation product of the chlorophyll.

The above-mentioned investigations were carried out with impure chlorophyll, Jörgensen and Kidd,§ on the other hand, used chlorophyll *a* and *b* (see p. 313) in a state of purity which satisfied Willstätter and Stoll's criteria. They experimented with a chlorophyll sol with water as the dispersion medium. On exposing this sol, contained in glass vessels and in contact

* Kimpflin : "Compt. rend.," 1907, **144**, 148.

† Curtius and Franzen : " Ber. deut. chem. Gesells.," 1912, **45**, 1715 ; Reinke : " Ber. deut. bot. Gesells.," 1883, **1**, 406 ; Curtius and Reinke : " Ber. deut. chem. Gesells.," 1897, **30**, 201 ; " Sitz. Heidelberger Akad. Wiss. Math. Nat.," 1915, Abt. A.; Pollacci : " Atti. Inst. Bot. Pavia.," 1900, **6**, 1902 ; 1904, **8**, 10 ; Usher and Priestley : " Proc. Roy. Soc., Lond.," B., 1906, **77**, 369.

‡ Warner : *id.*, 1914, **87**, 378.

§ Jörgensen and Kidd : *id.*, 1916, **89**, 342.

with various gases, to light, they found that formaldehyde was only produced in the presence of oxygen. In the case of contact with carbon dioxide, phæophytin (see p. 319) was produced, and there was no further change. In oxygen the chlorophyll turned yellow, due to the presence of phæophytin, and ultimately was bleached; when the bleaching is in progress, formaldehyde occurs in but small quantity, but when the bleaching is complete, there is an increase in the amount of formaldehyde. They suggest that the formaldehyde arises chiefly from the phytol which probably is split off from the chlorophyll under the action of light and oxygen.

In conclusion, mention may be made of a simple way of demonstrating the production of formaldehyde from chlorophyll, due to Osterhout.*

A solution of chlorophyll is made with carbon tetrachloride, and in it filter paper is soaked. The filter papers are dried, moistened with water, and placed on the inner surface of a glass bell-jar. The bell-jar is inverted over a dish of water, sealed from the air, and exposed to sunlight. The chlorophyll papers are gradually bleached; when pale green in colour the water in the dish gives a positive reaction for aldehyde. The same result was obtained when the carbon dioxide was excluded or increased to 10 per cent, which indicates that the aldehyde is due to the colouring matter rather than the carbon dioxide. Like results obtained when various aniline dyes, notably methyl green and iodine green, were used in place of the chlorophyll.

To summarize, while there is much experimental proof for the presence of formaldehyde and higher aldehydes in plants, this is not evidence in support of the formaldehyde hypothesis of carbon assimilation, since it has been repeatedly shown that formaldehyde is produced by the decomposition of chlorophyll itself. The whole question is considered in greater detail in the second volume.

* Osterhout: "Amer. J. Bot.," 1918, **5**, 511.

ALCOHOLS.

OCCURRENCE OF ALCOHOLS IN PLANTS.

Methyl Alcohol has been found to occur in the aqueous distillates and in the essential oils of a very large number of different plants, amongst which might be mentioned *Juniperus Sabina, Zea Mais, Lolium perenne, Iris germanica, Euonymus europaea, Thea sinensis, Eugenia caryophyllata, Carum carvi, Anthriscus cerefolium*, etc.

Ethyl Alcohol is not quite so widely distributed as methyl alcohol, but occurs in distillates from *Cananga odorata* (Ylang Ylang), *Pyrus Malus, Mespilus germanica, Eucalyptus, Anthriscus cerefolium, Pastinaca sativa, Vaccinium Myrtillus, Betula alba*, etc.

Mention also should be made of the occurrence of this alcohol, together with lactic acid and acetone * in some cases, in the higher plants especially during anærobic respiration. Stoklasa,† for instance, found that this substance together with acetic and formic acids, was produced during anærobic respiration of potatoes and seeds. Indeed, many consider that alcoholic fermentation is the first expression of respiration, and whether alcohol is formed or not depends upon the conditions; thus under normal conditions in the presence of oxygen the first products are oxidized before the alcohol stage in the process is reached, or the alcohol may be used up in anabolic processes as soon as it is formed, or it may be oxidized to water and carbon dioxide—the normal end products of ærobic respiration.‡

Amyl Alcohol has been identified in the essential oils of geranium, eucalyptus, lavender, peppermint, and chamomile.

Several unsaturated alcohols, such as citronellol $C_{10}H_{20}O$, geraniol, and linalool, both of the formula $C_{10}H_{18}O$, occur in essential oils, such as rose oil and oil of bergamot, while

* Palladin and Kostytschew: "Ber. deut. bot. Gesells.," 1906, **24**, 273.

† Stoklasa: *id.*, 1904, **22**, 358; "Centr. f. Bakter. u. Parasit.," 1905, II., **31**, 86. Godlewski and Polzeniusz: "Bull. Acad. Sci., Cracow," 1901, 227; Stoklasa, Jelinek, and Vitek: "Beitr. z. chem. Phys. u. Path.," 1903, **3**, 460.

‡ See Kostytschew: "Ber. deut. bot. Gesells.," 1908, **26**, 565.

amongst the alcohols belonging to the aromatic series must be mentioned cinnamic alcohol, benzyl alcohol, menthol, borneol, etc. Other monohydric alcohols, with the exception of phytosterols and allied substances are of rare occurrence.

Examples of polyhydric alcohols occurring in plants are mannitol, sorbitol, and dulcitol, isomeric substances of the formula—

$$CH_2OH\ CHOH\ CHOH\ CHOH\ CHOH\ CH_2OH$$

Mannitol occurs to the extent of about 40-50 per cent in manna, the dried sap of *Fraxinus ornus*, and up to 20 per cent of the dry weight of *Agaricus integer* consists of mannitol; it also occurs in many other fungi and in leaves, twigs and unripe fruits of the olive tree and has been found in *Rhinanthus*, celery, *Syringa vulgaris*, asparagus, cauliflower, carrot, pulse, etc. Furthermore, it occurs in various fucoids where it may possibly replace sugars in the metabolism of the plant. Tutin * has shown that apple juice fermented by the bacillus responsible for "cider sickness" results in the reduction of some of the sugar to mannitol.

Sorbitol occurs in the berries of *Pyrus aucuparia* and also in apple juice from which it may be obtained by fermenting away the sugars and acetylating the residue with acetic anhydride in the presence of pyridine; the resulting hexa-acetyl sorbitol is hydrolysed with 2 per cent sulphuric acid and the regenerated sorbitol is crystallized from alcohol.†

Dulcitol occurs in the cortex of *Euonymus europaea* and in the bark of *Euonymus atropurpurea*. It has also been found to occur in *Melampyrum arvense* and *M. pratense*.

It is suggested by Braecke ‡ that the alcohols mannitol and dulcitol are the predominant nutritive compounds for the genera *Rhinanthus* and *Melampyrum* respectively, since sucrose is not found at any time as a reserve carbohydrate in *Rhinanthus crista galli*, *Melampyrum pratense* or *M. arvense*.

Adonitol is a pentahydric alcohol occurring in *Adonis*

* Tutin: "Biochem. Journ.," 1925, 19, 418.
† Tutin: *id.*, 1925, 19, 416.
‡ Braecke: "Bull. Soc. Chim. Biol.," 1925, 7, 155.

vernalis. According to Treboux* it is converted by the plant into starch. Adonitol has a sweet taste, and is used in bacteriological media.

Of recent years a number of dihydric alcohols of high molecular weight have been found to occur in plants. They belong to different series whose general formulæ are :—

$$C_nH_{2n-6}O_4,\ C_nH_{2n-8}O_4,\ \text{and}\ C_nH_{2n-10}O_4.$$

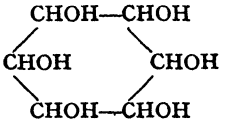
Trifolianol, $C_{21}H_{34}O_2(OH)_2$, isolated by Power and Salway,† from red clover leaves, may be taken as an example of the first group, while *Bryonol*, $C_{22}H_{34}O_2(OH_2)$, obtained by Power and Moore ‡ from Bryony root, and *Calabarol*, $C_{23}H_{34}O_2(OH)_2$, isolated by Salway § from Calabar beans, are representatives of the second and third groups respectively.

Of the polyhydric alcohols, Inositol is of particular interest, and may, therefore, receive more detailed consideration.

INOSITOL.

Inositol, which has the formula $C_6H_{12}O_6$, is isomeric with the hexoses, and, like these substances, has a sweet taste ; for these reasons, it was at one time thought to be a true sugar and was called muscle sugar owing to its occurring in muscle.

Inositol is, however, not a carbohydrate at all but a polyhydric alcohol derived from benzene and having the constitution—



Besides being found in muscle, inositol is of common occurrence in plants, in the leaves, especially when young, of *Vitis*, *Juglans*, etc. ; in the roots and rhizomes of very many plants ; in various seeds and fruits, e.g. *Phaseolus*, *Pisum*, and other leguminous seeds, *Vitis*, various cereals, and oily seeds, such as mustard, and flowers and bracts of *Cornus florida*.‖

* Treboux : " Ber. deut. bot. Gesells.," 1909, **27**, 428.
† Power and Salway : " J. Chem. Soc., Lond.," 1910, **97**, 249.
‡ Power and Moore : *id.*, 1911, **99**, 943.
§ Salway : *id.*, 1911, **99**, 2155.
‖ Sando : " J. Biol. Chem.," 1926, **68**, 403.

It may be looked upon as a plastic substance since Maquenne has found that it disappears from the young fruits of *Phaseolus* as ripening proceeds.

Preparation.

The separation of inositol from the plant juices is effected as follows :—

The sap is expressed from the organ, or, if this be impracticable, the parts are ground up very thoroughly with water. The liquid is then filtered and, if it gives an acid reaction, is neutralized by the addition of baryta water.

A solution of basic lead acetate is then added until no more precipitate comes down. The precipitate is filtered off, then washed and suspended in water, and saturated with a current of sulphuretted hydrogen. The lead sulphide is filtered off and the filtrate evaporated on a water bath to the consistency of a syrup. On the addition of alcohol, containing one-tenth of its volume of ether, inositol is deposited in prismatic crystals.

Inositol has a sweet taste, is soluble in water but insoluble in alcohol and ether. It crystallizes in prisms, it is not fermentable and it does not reduce Fehling's solution.

Identification.

1. When moistened with a little dilute nitric acid, then evaporated almost to dryness, and made alkaline with ammonia, the addition of a few drops of calcium chloride produces a rose-red coloration.

2. A solution of inositol evaporated to dryness with a few drops of mercuric nitrate produces a yellow stain which on heating turns red.

3. Solutions of inositol are not optically active.

With regard to its significance in the plant there is evidence to show that inositol is a transitory substance and is used up in the synthesis of other substances.

Inositol also occurs in combination with phosphoric acid. This compound, known as phytin, appears to be an acid

calcium and magnesium salt of inositol phosphoric acid which is a condensation compound of inositol with six molecules of phosphoric acid.

Phytin occurs especially in seeds; Arbenz † gives the following percentages of phytin, calculated as phytic acid, of the dry weight: Rice bran, 4·232; rice flour, 0·216; wheat bran, 5·073; whole meal, 0·572; wheat flour, 0·208; maize flour, 0·857; lentils, 0·326; peas, 0·561; oatmeal, 0·506; cocoa, 2·230. In vegetative organs it would appear to be absent for none was found in carrots, turnips, cauliflour, cabbage, spinach, and asparagus; also none was found in apples, pears, and figs.

According to Posternak,‡ a large amount, 80-90 per cent, of the phosphorus of certain seeds exists in the form of phytin; it occurs, for instance, in the globoid portion of aleurone grains, and the seeds, which contain it also possess an appropriate enzyme phytase for its decomposition into phosphoric acid and inositol.§

Quebrachitol is the name given to a monomethyl ether of inositol which occurs together with this substance in rubber latex.

MANUFACTURE OF ETHYL ALCOHOL.

The action of yeast on sugar is made use of in the manufacture of ethyl alcohol, which substance is prepared from potatoes, rice, and other grains rich in starch. The manufacture from potatoes is carried out as follows: Potatoes are heated in closed vessels to 125-135° by means of superheated steam under a pressure of about 3 atmospheres; by suddenly releasing the pressure the potatoes are burst, and are thus obtained in a finely divided state. The whole mass is then thoroughly stirred up with malt at a temperature of

* Cf. Neuberg: "Biochem. Zeit.," 1908, **9**, 557; 1914, **61**, 187; Winterstein: "Zeit. physiol. Chem.," 1908, **50**, 118. See also Plimmer: "Biochem. Journ.," 1913, **7**, 43; Boutwell: "J. Amer. Chem. Soc.," 1917, **39**, 491; Posternak: "Compt. rend.," 1919, **169**, 37, 138.

† Arbenz: "Chem. Zentr.," 1922, **93**, iv., 67.

‡ Posternak: "Compt. rend.," 1903, **137**, 202, 337, 439.

§ Cf. Suzuki, Yoshimura, and Takaishi: "Bull. Coll. Agric., Tokyo," 1907, **7**, 503. See also Rose: "Biochem. Bull.," 1912, **1**, 428.

about 60°, whereby the starch undergoes hydrolysis with formation of maltose and dextrin:—

$$\underset{\text{Starch}}{(C_6H_{10}O_5)_n} + H_2O \rightarrow \underset{\text{Maltose}}{C_{12}H_{22}O_{11}} + \underset{\text{Dextrin}}{(C_6H_{10}O_5)_x}$$

After about one and a half hours the mixture is rapidly cooled to 15° and mixed with yeast; fermentation at once sets in, accompanied by a considerable evolution of heat; the mixture is therefore cooled artificially, so that the temperature is maintained steady at about 27·5-30°.

During this time the maltose is converted first into dextrose and then into alcohol and carbon dioxide according to the equations—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$
$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

In order to convert the dextrin, which would otherwise be lost, into a fermentable substance, the temperature towards the end is maintained at about 26-29° in order to give the malt a further opportunity of hydrolysing the dextrin to glucose, and so rendering it capable of being fermented by yeast. When the fermentation is completed after about three days, the mixture contains about 13 per cent of alcohol by volume; by distilling the mixture through a fractionating column, so much of the water is removed that the distillate contains about 80 to 95 per cent of alcohol.*

No amount of fractional distillation without dehydrating agents will produce alcohol containing less than 4·43 per cent by weight of water, since such alcohol gives a constant boiling mixture.

Alcohol containing 0·5 per cent or less of water is, in commerce, known as *absolute alcohol*, although in a scientific laboratory the term is only correctly applied to alcohol which is quite free from moisture; such alcohol can only be obtained by careful fractionation from freshly burnt quicklime.† If

* The residue remaining after distillation contains, in addition to the solid unfermentable materials, a certain amount of other soluble products of fermentation, such as glycerol and succinic acid; it is used as a cattle food.

† Occasionally the last traces of moisture are removed by treating the alcohol with sodium wire.

the alcohol is dehydrated over quicklime to which a little barium oxide has been added, complete dehydration is marked by the formation of a yellow colour due to the production of barium ethylate, which can only be formed in the absence of any trace of moisture.

A delicate test for the detection of traces of moisture in alcohol consists in adding a few drops of the sample to a solution of liquid paraffin in anhydrous chloroform; if there is any moisture present, a turbidity will be at once produced.

SECTION III.

THE CARBOHYDRATES.

THE importance of carbohydrates becomes obvious when once it is realized that the metabolism of the green plant is essentially a carbohydrate metabolism. Carbohydrate, an essential in the food of the plant and of the animal, is synthesized from raw inorganic material only by the green plant, wherefore the maintenance of life is entirely dependent on the plant. Glucose, perhaps the simplest carbohydrate expression, is the all-important respirable material both in the animal and in the plant and it, together with other simple sugars, forms a raw material for the making of more complex carbohydrates, of proteins, of fats, and of other substances. With but few exceptions, carbohydrate in the form of cane sugar, starch, inulin, glycogen, and hemicellulose are the most significant reserve food materials of plants, whilst in the animal, glycogen alone forms a temporary reserve. In the plant, carbohydrate in the shape of cellulose, ligno-cellulose, hemicellulose, and pentosanes, form entirely or in part the structural basis of the cell wall when present,* and thus plays an important rôle in the structural mechanism. In the animal, on the other hand, carbohydrates are seldom thus employed; chitin, spongin, and chondro-mucoid, which to a limited extent enters into the composition of muscle, may be cited. Indeed, the synthesis of carbohydrate in the animal is for the most part restricted to the production of lactose, or milk sugar, from pre-existing glucose.

Notwithstanding the differences in the physiological

* It will be remembered that the Myxomycetes, many Chrysophyceæ, Euglenineæ, and other members of the lower Protophyta, together with the gametes of the majority of plants, both high and low, are naked structures with no cell wall.

significance of the various types of carbohydrates in the plant, these substances are all closely related chemically, being composed of the same elements—carbon, hydrogen, and oxygen—united together in a similar fashion.

The term carbohydrate originated through the erroneous conception that these substances were compounds of carbon with water, since the proportion of hydrogen to oxygen in most of them is the same as in water, as may be seen from the formula for grape sugar, which is $C_6H_{12}O_6$, but which might be written $C_6 . 6H_2O$.

The discovery of methyl pentoses of the formula $C_6H_{12}O_5$ shows, however, that the maintenance of this proportion of hydrogen to oxygen is not an essential characteristic of this group of compounds.

CLASSIFICATION OF CARBOHYDRATES.

On purely physical grounds such as appearance, solubility in water, taste, etc., the carbohydrates may be roughly divided into sugars and non-sugars; the systematic classification of the carbohydrates is, however, based upon their behaviour towards hydrolytic agents, such as mineral acids or enzymes. Thus there are a considerable number of naturally occurring sugars containing five and six carbon atoms which cannot be hydrolysed; such sugars form a group known as *monosaccharides.* On the other hand, many sugars are known which on hydrolysis break up into two molecules of monosaccharide according to the equation—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

Such sugars are known as *disaccharides.*

Similarly sugars which on hydrolysis give three molecules of monosaccharide as follows—

$$C_{18}H_{32}O_{16} + 2H_2O = 3C_6H_{12}O_6$$

are termed *trisaccharides.*

Finally, carbohydrates, such as starch and cellulose, which on hydrolysis yield an unknown number of molecules of monosaccharides are classed as *polysaccharides.*

The nomenclature of the monosaccharides is based on

the number of carbon atoms in their molecules, those containing five being called pentoses, while those containing six atoms are known as hexoses. For this reason the use of the terms monose and biose in place of monosaccharide and disaccharide is to be deprecated owing to the confusion which is liable to result therefrom.

A scheme for the classification of the carbohydrates is given below:—

- **Sugars**
  - **Monosaccharides**
    - Trioses ($C_3H_6O_3$)
    - Tetroses ($C_4H_8O_4$)
    - Pentoses ($C_5H_{10}O_5$). Arabinose, Xylose, Ribose, Apiose.
    - Methylpentoses ($C_6H_{12}O_5$). Rhamnose, Fucose, Quinovose.
    - Hexoses ($C_6H_{12}O_6$).
      - *Aldoses:* Glucose, Mannose, Galactose.
      - *Ketoses:* Fructose, Sorbose
    - Heptoses ($C_7H_{14}O_7$). Sedoheptose, Mannoketoheptose.
  - **Disaccharides**
    - Glucoxyloses ($C_{11}H_{20}O_{10}$). Primeverose, Strophanthobiose, Vicianose.
    - ($C_{12}H_{22}O_{11}$). Sucrose, Lactose,* Maltose, Isomaltose, Gentiobiose, Cellobiose, Trehalose, Melibiose, Turanose.
  - Trisaccharides ($C_{18}H_{32}O_{16}$). Raffinose, Melicitose, Gentianose.
  - Tetrasaccharides ($C_{24}H_{42}O_{21}$). Stachyose.
  - Unknown constitution: Agavose, Lupeose.
- **Non-sugars or Polysaccharides**
  - Pentosans ($C_5H_8O_4$)$_n$. Araban, Xylan.
  - Hexosans ($C_6H_{10}O_5$)$_n$
    - Glucosans: Starch, Dextrin, Glycogen, Lichenin, Cellulose.
    - Fructosans: Inulin, Graminin, Phlein, Triticin.
    - Mannans.
    - Galactans.
- **Derived carbohydrates containing —COOH and other groups**
  - Hemicelluloses.
  - Gums.
  - Mucilages.
  - Pectic substances.

SOLUBILITIES OF THE CARBOHYDRATES.

As might be expected of a group of substances of such varying complexity and widely different function in the plant, the solubilities of the carbohydrates present a considerable range of variation, some idea of which can be obtained from the following facts:—

1. Readily soluble in water or dilute alcohol, but insoluble in absolute alcohol and other organic solvents, e.g. sugars, inulin.

* Not found in the vegetable kingdom.

2. Sparingly soluble in cold water but more so in hot, and precipitated from solution by alcohol, e.g. gums, mucilages, starch, and pectins.

3. Insoluble in water but soluble in dilute caustic alkali, and precipitated from solution by the addition of acid or alcohol, e.g. hemicelluloses.

4. Insoluble in water, alkali, and organic solvents, but soluble in cuprammonia, e.g. cellulose.

GENERAL TEST FOR CARBOHYDRATES AND THEIR DERIVATIVES.

In attempting to characterize an unknown organic substance, there is one test which should always be employed at the outset, and that is Molisch's reaction. This test is extremely delicate, and may be applied to a substance in aqueous solution or, if the substance is insoluble in water, to a little of the liquid obtained by boiling the solid with dilute sulphuric acid. By this treatment the substance, if it contains a carbohydrate, will be hydrolysed and then will yield sufficient monosaccharide to give the test which is carried out as follows :—

A few drops of 15 per cent alcoholic solution of α-naphthol are added to about a third of a test tube full of the solution to be tested and concentrated sulphuric acid is carefully poured down the side of the tube. At the junction of the two liquids a green ring is produced * and over this a red zone; on gently agitating the colour changes to purple.

Alternatively, 1-2 drops of the solution are mixed with about 4 drops of a 4 per cent alcoholic solution of α-naphthol; about 1 c.c. of concentrated sulphuric acid is then added and the whole is gently agitated. A purple colour indicates the presence of carbohydrate.

The reaction depends upon the production of furfural, by the action of the sulphuric acid on the carbohydrate, and its condensation with the α-naphthol.

This reaction is given by all true carbohydrates and all

* No attention should be paid to the production of a green colour, which is given by the action of sulphuric acid on alcoholic α-naphthol, even in the absence of carbohydrate.

substances which contain a carbohydrate complex, such as glucosides and proteins. The further tests employed for the characterization of carbohydrates depend upon the indications obtained from the solubilities of the substance under examination, and these will be given under their respective headings in the following pages.

CONSTITUTION AND ISOMERISM OF SUGARS.

The analysis of any one of the hexose sugars, such as dextrose, levulose, galactose or mannose, would yield the same result, viz. 40 per cent of carbon, $6{\cdot}\dot{6}$ per cent of hydrogen, and $53{\cdot}\dot{3}$ per cent of oxygen; and this notwithstanding the fact that these sugars are different substances.

From the results of an analysis, it is possible to determine the simplest ratio of the atoms to each other in the molecule by dividing each percentage by the atomic weight of the corresponding element, and then determining the simplest numerical ratio between the resulting numbers:—

$$C = \frac{40{\cdot}0}{12} = 3{\cdot}\dot{3}\ ;\ H = \frac{6{\cdot}\dot{6}}{1} = 6{\cdot}\dot{6}\ ;\ O = \frac{53{\cdot}\dot{3}}{16} = 3{\cdot}\dot{3}$$

$$\therefore C : H : O = 3{\cdot}\dot{3} : 6{\cdot}\dot{6} : 3{\cdot}\dot{3}$$
$$= 1 : 2 : 1.$$

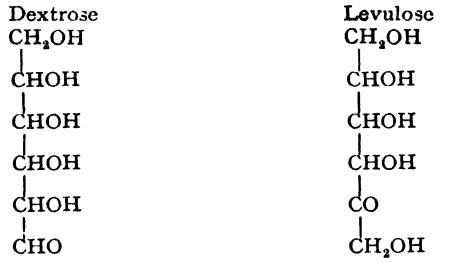
The formula CH_2O thus arrived at is known as the Empirical Formula; it indicates the *ratio* of the number of different atoms in the molecule, but does not indicate their actual number. The formula which, while maintaining the above ratio, also shows the actual number of atoms present in the molecule, is known as the Molecular Formula; and it can only be assigned correctly when the molecular weight is known. Now the molecular weight of all these sugars is 180, hence their molecular formula must be $(CH_2O)_6$ or $C_6H_{12}O_6$.

Compounds such as the various hexoses which have the same molecular formula and yet are not identical are said to be isomers.

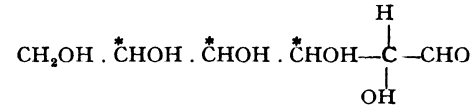
The carbohydrates exhibit two kinds of isomerism, known respectively as structural and stereo-isomerism.

Structural isomerism is well illustrated by the two sugars dextrose and levulose. A study of their reactions, which need not here be detailed, leads to the conclusion that they both

contain five hydroxyl (OH) groups; that dextrose belongs to the class of compounds known as aldehydes, which are characterized by the group —CHO; and that levulose is a ketone and therefore contains the group =CO. These facts are all explained by the following constitutional formulæ:—



Stereo-isomerism is the second type of isomerism, and is exhibited by the three sugars dextrose, mannose, and galactose, all of which are aldehydes, and have therefore the same structural formula. The possibility of isomerism in this case is accounted for by the presence in these molecules of what are known as asymmetric carbon atoms. Writing the formula for dextrose once more in a slightly different way, it will be seen that the carbon atom printed in "clarendon" (**C**) has its four valencies attached respectively to the groups (CH_2OH . CHOH . CHOH . CHOH)—, H—, —OH, and —CHO:—

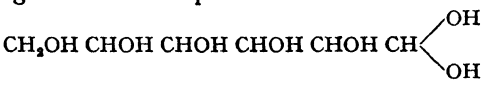


Any carbon atom whose valency is satisfied by four different groups or elements, whatever their nature may be, is said to be asymmetric, since it is possible to represent it by either of two solid models which are not super-imposable, the one being the mirror image of the other; there exists, therefore, between two modifications of such an asymmetric carbon atom a difference due to the different spacial distribution of the four substituting groups around it. Now the isomerism existing between glucose and mannose is accounted for by their each containing one of the two possible modifications of this same asymmetric carbon atom. Similar considerations will show

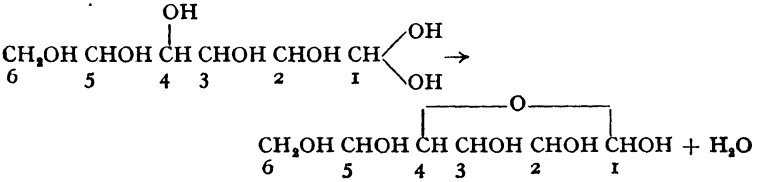
that each of the three carbon atoms marked with a star is also asymmetric, and it is therefore not surprising that it is possible to account for no less than sixteen different isomeric aldehyde sugars or aldoses; of these, however, relatively few have been found in nature.

The constitution of glucose is ordinarily represented by the formula $CH_2OH\ CHOH\ CHOH\ CHOH\ CHOH\ CHO$, which shows it to be a pentahydric alcohol and an aldehyde at the same time. When dissolved in water, however, it behaves in a peculiar manner, exhibiting the phenomenon of muta-rotation, that is to say, the optical activity of the resulting solution does not attain a steady value until some time after the solution has been made up.

The change is supposed to be connected with some alteration in its molecular configuration which may be explained by assuming that the compound



is temporarily formed,* but that water is thereupon split off again between one of the hydroxyl groups of the terminal carbon atom and the hydroxyl attached to the fourth carbon atom as follows:—



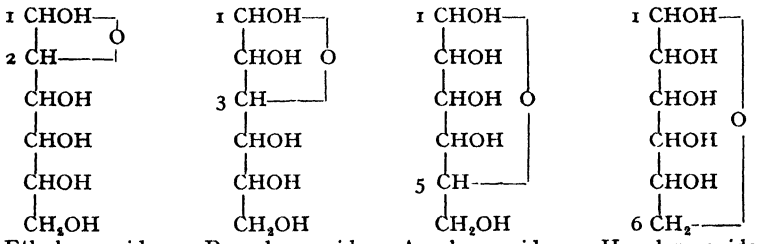
It will be seen that in this formula, sometimes known as the lactone or butylene oxide formula, the terminal carbon atom (which is conventionally regarded as carbon atom 1) has now become asymmetric, whereas it was not so before; this method of writing the formula involves the possible existence of two optically isomeric varieties of ordinary glucose, both of which are in fact known.† When glucose

* Compare the formation of similar compounds from other aldehydes (p. 63).

† Tanret: "Compt. rend.," 1895, **20**, 1060; Lowry: "J. Chem. Soc.," 1899, **75**, 213; 1903, **83**, 1314.

is crystallized from 70 per cent alcoholic solution at ordinary temperatures, a modification known as α-glucose is obtained whose specific rotation is $\alpha_D = +110°$; if crystallized from water at a temperature above 98°, another variety, known as β-glucose ($\alpha_D = +19°$), is obtained; if either α-glucose or β-glucose is dissolved in water, a gradual change in rotation is observed until a steady value of $\alpha_D = 52{\cdot}5°$ is attained, which is regarded as the specific rotation of an equilibrium mixture of α- and β-glucose. The attainment of the stable condition is accelerated by acids, and is practically instantaneous in presence of traces of alkali.

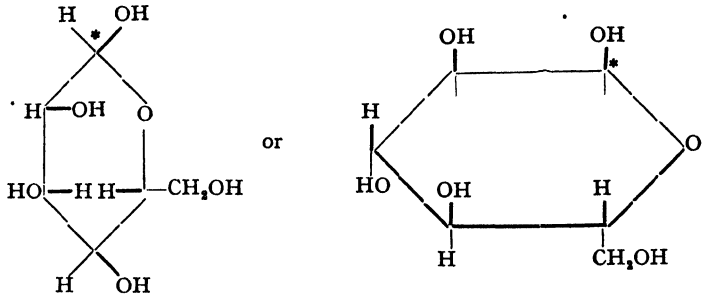
It will be readily understood that such a bridge or ring structure as is represented by the γ-lactone may also be described as a butylene oxide formula, seeing that four carbon atoms are involved in the ring. Theoretically isomeric sugars possessing an ethylene, propylene, amylene, or hexylene oxide formula should also be a possibility :—



Ethylene oxide | Propylene oxide | Amylene oxide | Hexylene oxide

It has been the task of Irvine and his collaborators to investigate this aspect of the isomerism of the sugars. Irvine's resumé * gives an account of these important investigations which cannot be further considered here owing to exigencies of space. The work of Irvine appeared to have firmly established the butylene oxide formula for glucose, but in the light of subsequent work the amylene oxide formula is now generally accepted.† This implies the recognition of the fact that glucose and the other hexoses are six-membered heterocyclic compounds whose constitution may be represented as follows :—

* Irvine : " J. Chem. Soc.," 1923, **123**, 898.
† Charlton, Haworth, and Peat : *id.*, 1926, **89**, 1858.



in which the reducing group is marked by a star and in which the thickened lines are all in the same plane : a discussion of this question, together with some important deductions therefrom, is given by Haworth,* and in consequence the interpretation of the constitution of all the polysaccharides and glucosides derived from glucose has been modified.

OXIDATION PRODUCTS OF SUGARS.

Before proceeding to a description of the methods employed for the identification of individual sugars, a brief consideration of some of their products of oxidation is appropriate in view of the fact that some are important constituents of natural products.

Oxidation by means of nitric acid under carefully controlled conditions attacks both the terminal carbon atoms of aldehydic sugars,† leaving the intermediate secondary alcohol groups unaltered.

$$CH_2OH—(CHOH)_4—CHO \rightarrow COOH—(CHOH)_4—COOH$$

In this way glucose, mannose, or galactose yield the dicarboxylic acids saccharic, mannosaccharic, and mucic acids respectively.

An intermediate stage of oxidation, in which the aldehyde group remains unaltered and only the terminal primary alcohol group is oxidized to carboxyl, is represented by the substances glucuronic and galacturonic acids of the formula $COOH . (CHOH)_4 . CHO$ derived respectively from glucose and galactose.

* Haworth, " J. Soc. Chem. Ind.," 1927, 46, 295.

† Ketonic sugars are broken down to compounds containing fewer carbon atoms.

Although it has been found possible to produce *in vitro* a small quantity of glucuronic acid from glucose by the action of hydrogen peroxide, this is not a practical method. In the animal and vegetable world, however, conditions appear frequently to arise in which the aldehyde group of the sugar is protected from oxidation by coupling with some other group as a glucoside, leaving the primary alcohol at the other end of the molecule open to attack. Such coupled glucuronic acids occur normally in the urine of animals, but may be increased in quantity by the administration of certain substances. In the plant world glucuronic and galacturonic acids appear similarly combined with other complexes; the former has been reported as a constituent of glycyrrhizin * and scutellarin † while the latter occurs in pectins.

These aldehyde acids are known collectively as "uronic" acids; when heated with hydrochloric acid they are converted into furfural with evolution of carbon dioxide.† A method for their estimation based upon the measurement of this carbon dioxide has been devised by Tollens and Lefèvre,‡ and modified by Nanji and Norman.§

When heated with Bial's reagent, glucuronic acid gives the same colour as pentoses and methyl pentoses, the colour, however, develops rather more slowly.

When boiled with an equal volume of hydrochloric acid and a small quantity of 1 per cent solution of naphthoresorcin in alcohol, the solution darkens, and on shaking up the warm solution with benzene the latter acquires a reddish-violet colour which shows an absorption band at the D line.‖ Solutions of pentoses, hexoses or disaccharides under the same conditions yield no colour or at most a faint yellow to the benzene.

A point of some importance arises in connection with the possibility of the uronic acids acting as intermediate stages

* Tschirch and Gauchmann: "Archiv. d. Pharm.," 1908, **246**, 550.
† Goldschmiedt and Zerner: "Monat. d. Chem.," 1910, **31**, 441, 476.
‡ Tollens and Lefèvre: "Ber. deut. chem. Gesells.," 1907, **40**, 4519.
§ Nanji and Norman: "J. Soc. Chem. Ind.," 1926, **45**, 337 T.
‖ Neuberg and Saneyoshi: "Biochem. Zeit.," 1911, **36**, 56; van der Haar: *id.*, 1918, **88**, 203.

in the production of pentoses from hexoses. Thus, assuming a glucose molecule to have its aldehyde group protected from attack, it would, on oxidation, give glucuronic acid which by loss of carbon dioxide would yield xylose:—

$$CH_2OH(CHOH)_4 . CHO \text{ (protected)} \rightarrow \underset{\text{Glucuronic acid}}{COOH\ (CHOH)_4 . CHO}$$

$$\underset{\text{Glucuronic acid}}{COOH . (CHOH)_4 CHO} - CO_2 \rightarrow \underset{\text{Xylose}}{CH_2OH\ (CHOH)_3 . CHO}$$

On the other hand, if a given glucose molecule were not so protected and were susceptible to oxidation at both ends, it could give rise to an isomeric glucuronic acid whose aldehyde and carboxyl groups were at the opposite ends by comparison with the previous one:—

$$CH_2OH\ (CHOH)\ CHO \text{ (unprotected)} \rightarrow CHO\ (CHOH) . COOH$$

$$CHO . (CHOH)_4\ COOH - CO_2 \rightarrow \underset{\text{Arabinose}}{CHO\ (CHOH)_3 . CH_2OH}$$

The fact that in nature xylose, rather than arabinose, is commonly associated with glucose, suggests that xylose is produced from glucose by the oxidation of the primary alcohol group,* the aldehyde group being protected from attack owing to the form of combination in the complex molecule concerned; the same explanation may account for the frequent association of cellulose with xylans. Spoehr † has isolated the lactone of glucuronic acid from cactus gum, and suggests that glucuronic acid is broken up under the influence of sunlight into carbon dioxide and xylose.

THE CHARACTERIZATION OF SUGARS.

In order to characterize a sugar, the following procedure may be followed: —

1. Ascertain whether the substance is a reducing or non-reducing sugar by adding a little of the neutral aqueous solution to a little Fehling's solution previously diluted with three times its volume of water and boiled to see that it is not changed by boiling alone. Boil the mixture for about one minute. If at the end of this time no red or brown precipitate of cuprous oxide is formed the sugar is non-reducing.

* De Chalmot: "Am. Chem. J.," 1893, **16**, 610.

† Spoehr: "The Carbohydrate Economy of Cacti," Carnegie Inst. Pub., Washington, **281**, 42, 75.

All monosaccharides reduce Fehling's solution, but some disaccharides, such as sucrose and trehalose, are so constituted that the reducing aldehydic or ketonic group is masked, and is only set free after hydrolysis.

If the sugar is non-reducing, boil a fresh portion for a short time with a little dilute hydrochloric acid; neutralize and test once more with Fehling's solution as above. The solution should now reduce owing to the hydrolysis of the di- or tri-saccharide to monosaccharides.

It must be borne in mind that other substances besides sugars reduce Fehling's solution, and consequently due precaution must be taken to exclude the presence of these before applying the test.

2. Ascertain whether the substance is a pentose (for tests see p. 90) or a hexose.

3. If a pentose is not found, distinguish between aldohexose and ketohexose (for tests see p. 96).

4. If the substance is a reducing sugar, whether pentose or hexose, its further identification usually depends upon the production of a crystalline derivative by means of phenylhydrazine or a similar compound.

Phenylhydrazine reacts with sugars containing either an aldehyde or ketone group to form, in the first place, phenylhydrazones, which in many cases are characteristic crystalline solids, but are usually soluble in water; this reaction may be illustrated thus :—

$$CH_2OH(CHOH)_4CHO + H_2NNHC_6H_5 = CH_2OH(CHOH)_4CH : NNHC_6H_5 + H_2O$$

Dextrose or Glucose. Glucose Phenylhydrazone.

If, however, an excess of phenylhydrazine be employed, a second hydrazine complex is introduced into the compound, and the resulting substance is termed an osazone. Both glucose, fructose, and mannose yield the same osazone :—

$$CH_2OH(CHOH)_3—C(=N . NHC_6H_5)—CH : NNHC_6H_5$$

which is called glucosazone.*

* This is due to the fact that the three sugars differ only in the configuration of their two terminal carbon atoms, a difference which is eliminated when they are converted into their osazones (cf. p. 95).

The osazones being, for the most part, insoluble in water, serve as a valuable means of isolating a sugar from a dilute solution; their identity can then be readily established by means of their crystalline form, melting-point, solubility, and optical activity.

Other special tests employed for the identification of individual sugars will be given under the various sugars in the following pages.

The identification of the constituents of a mixture of a number of different sugars may require special methods depending on the use of specific hydrolytic enzymes or of special yeasts which may ferment away certain hexoses, or all hexoses, leaving only the non-fermentable pentoses.

The following list, taken from a paper by Chapman,* shows the behaviour of certain species of *Saccharomyces* towards several of the more commonly occurring sugars:—

Species.	Dextrose.	Fructose.	Mannose.	Galactose.	Maltose.	Sucrose	Lactose.
S. Cerevisiæ . .	+	+	+	+	+	+	o
S. ,, *Carlsberg*	+	+	+	+	+	+	o
S. Pastorianus .	+	+	+	+	+	+	o
S. ellipsoideus .	+	+	+	+	+	+	o
S. Marxianus . .	+	+	+	+	o	+	o
S. Exiguus . .	+	+	o	+	o	+	o
S. Ludwigii . .	+	+	+	o	o	+	o
S. anomalus . .	+	+	+	o	o	+	o
S. fragilis . .	+	+	+	+	o	+	+
Kefir . . .	+	+	+	o	o	+	+

(The sign + indicates that the yeast in question is capable, and the sign o that it is incapable, of bringing about fermentation.)

An individual description of the various naturally occurring sugars will now be given.

MONOSACCHARIDES.

A. *PENTOSES.*

The pentoses, which are sugars containing five carbon atoms, have the general formula $C_5H_{10}O_5$, would not appear to be common in the free state; their presence has been

* Chapman: "J. Chem. Soc.," 1917, III, 216.

recorded in the leaves of carrot, mangold, potato, sunflower, *Tropæolum* and turnip,* and also in *Opuntia phæacantha*.† Pentosanes, however, which may be regarded as polymerized anhydrides of pentose are very widely distributed in the vegetable kingdom, forming cell wall constituents and entering into the composition of various gums, mucilages, and pectins.

With regard to their physiological significance, it is impossible to say whether they are direct products of photosynthesis; if, as Spoehr points out, the formation of sugar in a green leaf is a series of additions of molecules of formaldehyde, the presence of pentose is to be expected. There is, however, no evidence that this occurs in the green plant. On the other hand, they may have their origin in the oxidation of hexose. The facts that in the germination of seeds, the amount of total pentoses falls as development proceeds and that in some instances the amount is high at certain phases, thus in *Parthenium argentatum* a high percentage of pentose coincides with the period of growth during which the production of rubber is at its highest, suggest that pentoses are definite stages in the elaboration of other substances. As a food material the value of pentoses is variable; whilst xylose has a high nutritive value for *Aspergillus*, it, together with other pentoses, is not utilized by *Saccharomyces*. In the higher plants, Spoehr ‡ has shown that the respiration of *Cactaceæ* is not depressed when the hexoses are insignificant in amount,§ and that the formation of pentosanes is bound up with certain conditions, especially water content and temperature. Thus in the Cactaceæ, a low water content coupled with a high temperature results in a decrease in the amount of monosaccharides and an increase in polysaccharides and pentosanes. On the other hand, a high water content and a low temperature are associated with an increase of monosaccharides and a decrease of pentosanes and polysaccharides.

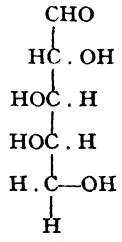
* Davis and Sawyer: "J. Agr. Sci.," 1914, **6**, 406.

† Spoehr: "Carnegie Inst. Pub.," 1919, No. 287; "Plant World," 1917, **20**, 365.

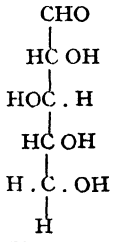
‡ Spoehr: *loc. cit.*

§ It is not uncommonly assumed that in the respiratory activity of higher plants, hexoses are the significant fuel.

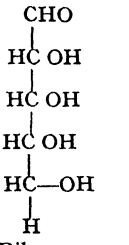
Only four pentoses have so far been recorded as occurring in the combined state and entering into the composition of plant materials; these are arabinose, xylose, ribose, and apiose. The structural formulæ of these substances is given below, in order that their relationship to the hexoses and to each other may be appreciated :—



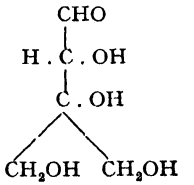
Arabinose



Xylose



Ribose



Apiose *

GENERAL PROPERTIES OF PENTOSES.

A number of colour reactions are available for the characterization of pentoses.

1. *Thomas's Reaction.*†—A freshly prepared 0·3 per cent solution of β-naphthol in concentrated sulphuric acid is carefully poured down the side of a test-tube containing a few cubic centimetres of the solution to be tested; if a pentose is present a deep blue ultramarine ring is formed at the junction of the two liquids on gently shaking; the colour gradually changes to green-brown.

2. *Bial's Reaction.*—To a few cubic centimetres of the reagent raised to boiling-point in a test-tube, add a few drops of the pentose or pentosan solution and raise again to boiling-point. A green colour indicates a pentose, a methyl pentose, or glucuronic acid. The colour is soluble in amyl alcohol, and shows an absorption band between the C and D lines.

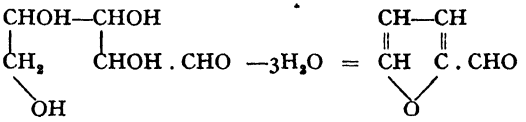
3. Add a small quantity of powdered gum-arabic to a few cubic centimetres of 18 per cent of hydrochloric acid together with a few crystals of phloroglucinol; place in a water bath and gradually raise to boiling-point; remove from time to

* It will be seen that apiose represents an abnormal type of sugar possessing a branched chain.

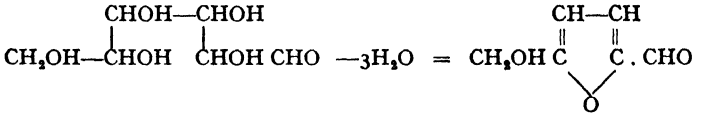
† Thomas: "Bull. Soc. Chim. Biol.," 1925, 7, 102.

time and watch for the appearance of a reddish-violet colour; when this appears, remove from the water bath, cool, and shake up with amyl alcohol; the solution in the alcohol has an absorption band between the D and E lines.

4. When boiled with 12 per cent of hydrochloric acid or sulphuric acid, pentoses give rise to furfural which is carried off by the escaping steam; if this is allowed to impinge upon a filter paper moistened with a drop of aniline acetate a bright pink colour is formed.



Methyl pentoses under these conditions give methyl furfural, while hexoses give small quantities of hydroxy-methyl-furfural.



All furfural derivatives give similar colour reactions to furfural both with aniline acetate and with phloroglucinol and orcinol (see below); were it not for the fact that hydroxy-methylfurfural is itself readily decomposed further into levulinic acid and formic acid,

$$C_6H_6O_3 + 2H_2O = HCOOH + CH_3COCH_2CH_2COOH$$

neither of which give the above colour reactions, the test described would not be specific for pentoses.

In carrying out the above test it must therefore be borne in mind that a very faint positive reaction should not be taken as evidence of the presence of pentose without further evidence. This reaction has also been made the basis of a method for the quantitative estimation of pentoses (see p. 137).

5. Pentoses reduce Fehling's solution and yield osazones but are not fermentable.

PROPERTIES OF INDIVIDUAL PENTOSES.

Arabinose.

Arabinose is best obtained by the hydrolysis of cherry gum with 4 per cent sulphuric acid ; it can also be obtained by the hydrolysis of gum-arabic and of peach gum and mesquite gum * (*Prosopis jutiflora*). Arabinose has a very sweet taste, is dextro-rotatory, α_D in 10 per cent solution $= +105°$, crystallizes in prisms, and melts at 160° ; it reduces Fehling's solution, and yields with diphenyl hydrazine a characteristic diphenyl hydrazone, melting at 204-205°.†

Xylose.

Xylose may be obtained by the hydrolysis of xylane or wood gum, and also from brewers' grains, maize, fruits, straw, and various forms of cellulose. It is a very sweet substance and shows an optical activity of $\alpha_D = +19°$ in a 10 per cent solution, it crystallizes in prisms, melting at 144-145°, and gives a phenylosazone of melting-point, 161°. When oxidized with bromine and boiled with cadmium carbonate it yields cadmium xylonate, which with the cadmium bromide in solution forms a sparingly soluble crystalline double salt

$$(C_5H_9O_6)_2Cd \,.\, CdBr_2 \,.\, 2H_2O.‡$$

Xylose may be conveniently obtained, in about a 12 per cent yield, by boiling 1 kg. of corn cobs § (previously soaked and washed in 2 per cent ammonia solution) for two hours under a reflux condenser with 8 litres of 7 per cent sulphuric acid. The solution is filtered on a Buchner funnel through cloth, and is then carefully neutralized with precipitated chalk. After filtering, the solution is treated with lead acetate, filtered, freed from lead by hydrogen sulphide, again filtered and

* Anderson and Sands : " Ind. Eng. Chem.," 1925, **17**, 1257.

† Neuberg : " Ber. deut. chem. Gesells.," 1900, **33**, 2243 ; Tollens and Maurenbrecher : *id.*, 1905, **38**, 500.

‡ Widstoe and Tollens : *id.*, 1900, **33**, 136.

§ Hudson and Harding : " J. Amer. Chem. Soc.," 1917, **39**, 1038 ; 1918, **40**, 1601. Ling and Nanji : " J. Chem. Soc.," 1923, **123**, 620. Irvine, *loc. cit.*

decolorized with animal charcoal. The filtrate is evaporated under reduced pressure and the calcium sulphate precipitated by the addition of alcohol; the filtered solution is then evaporated to a viscous syrup and crystallized from alcohol or from glacial acetic acid.

Ribose.

This pentose has been obtained as a product of the hydrolysis of yeast nucleic acid. According to Robinson it is probably not pre-existent in this substance but is produced by optical inversion during hydrolysis from the xylose contained in the nucleic acid.

Apiose.

This is a rare pentose obtained by the hydrolysis of the glucoside apiin contained in parsley; it yields a bromophenylosazone, m.p. 211-212°. Owing to its abnormal structure (see formula, p. 90) it does not yield furfural when heated with hydrochloric acid and gives no colour with phloroglucinol and hydrochloric acid.

Methyl Pentoses.

There is no evidence that methyl pentoses occur free in the plant; they are, however, associated with the pentoses as cell wall constituents, and also occur as glucosides. Their constitution is represented by the formula

$$CH_3CHOH(CHOH)_3CHO$$

When heated with 10 c.c. of concentrated hydrochloric acid and 2 c.c. of acetone, the methyl pentoses give a violet colour which is permanent, in contradistinction to pentoses, which also yield a violet colour which, however, fades within one hour.

Heated with concentrated hydrochloric acid, methyl pentoses give off methyl furfural which with aniline acetate gives a yellow colour; whereas furfural which would be

* Robinson: "Nature," 1927, **120**, 44, 656.

obtained from pentoses under the same conditions gives a pink colour.

(*a*) *Rhamnose* has been obtained by the hydrolysis of a number of glucosides, e.g. quercitrin, hesperidin, and xantho-rhamnin, and also saponins. The substance forms glistening crystals, m.p. 93°; $\alpha_D = +8{\cdot}07°$, and gives a phenylosazone melting at 180°, and a naphthyl hydrazone melting at 192°.

(*b*) *Fucose*, which is isomeric with rhamnose, may be obtained by the hydrolysis of sea-weeds by means of dilute sulphuric acid; it crystallizes in microscopic needles, and yields a hydrazone, m.p. 172-173°.

(*c*) *Quinovose*, another methyl pentose isomeric with rhamnose, is produced by the hydrolysis of quinovite, a substance formed by boiling quinovin contained in the bark of *Cascarilla hexandra* with alcohol and hydrochloric acid.

(*d*) *Isorhamnose* and *Rhodeose* are two methyl pentoses obtained by the hydrolysis of the glucoside convolvulin.

B. *HEXOSES.*

Theory accounts for the existence of no less than thirty-two sugars of the molecular formula $C_6H_{12}O_6$ having a straight six carbon atom chain. Of these sixteen are aldoses of the type

$$CH_2OH \cdot CHOH \cdot CHOH\ CHOH\ CHOH \cdot CHO$$
Aldohexose

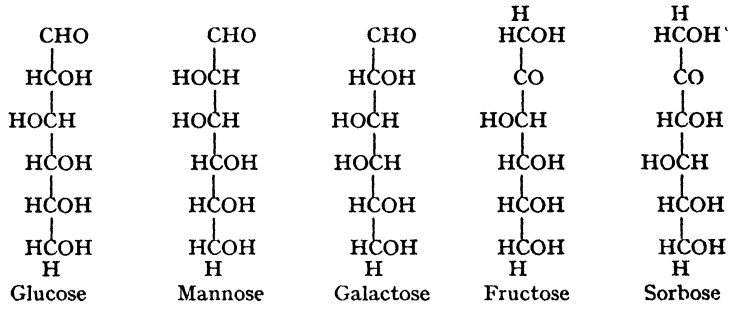
and the remaining sixteen are ketoses containing the ketonic group attached either to the second or third carbon atom of the chain—

$$CH_2OH\ CHOH \cdot CHOH\ CHOH \cdot CO \cdot CH_2OH \text{ } or$$
2 Ketohexose

$$CH_2OH\ CHOH\ CHOH\ CO \cdot CHOH\ CH_2OH$$
3 Ketohexose

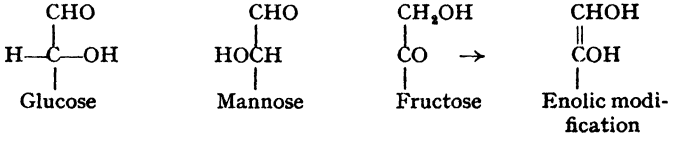
The various possible isomeric aldoses and ketoses differ only in the spatial relationships of the OH and H groups. Although most of the aldoses and a few of the ketoses have been synthesized, only three aldoses, glucose, mannose, and galactose, and two ketoses, fructose and sorbose, have so far been identified in nature.

The following formulæ illustrate the relationship between the five naturally occurring hexoses :—



From these formulæ it will be seen that the spatial arrangement of the third, fourth, fifth, and sixth carbon atoms in glucose, mannose, and fructose is identical; for this reason they all give the same osazone when once the first and second carbons have been condensed with phenylhydrazine; on the other hand, they all give different hydrazones in which only the terminal carbon atom 1 is involved, leaving the rest of the chain from carbon atoms 2-6 different in each case.

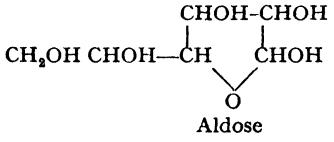
Further evidence for the close relationship existing between the three sugars, glucose, fructose, and mannose is furnished by the fact that if a 5 per cent solution of any one of these three sugars is treated with one-tenth of its volume of 10 per cent caustic potash and left in an incubator for twelve to twenty-four hours at 37° C., the solution will be found to contain all three sugars. This may be accounted for by assuming that the two terminal links in the six carbon chain of all three sugars can undergo molecular rearrangement to the so-called enolic form as under—



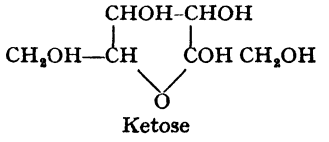
in which form they all become identical, and the change back from the enol form may give rise to any or all of the three. The significance of this lies in the explanation it offers for the possible interconversion of these sugars in the plant.

DISTINCTION BETWEEN ALDOSES AND KETOSES.

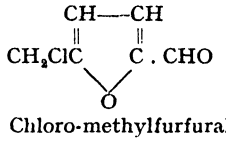
To distinguish an aldose from a ketose use is made of the fact that on heating with concentrated hydrochloric or hydrobromic acid a ketose is more readily converted into chloro- or bromo-methylfurfural than is an aldose, as may be seen from the formulæ :—



Aldose



Ketose



Chloro-methylfurfural

The production of the furfural derivative from the ketose involves much less rearrangement than from the aldose. On this fact depends the two reactions of Seliwanoff and of Fenton

Seliwanoff Reaction.—Warmed on a water bath with an equal volume of concentrated hydrochloric acid and a crystal of resorcin, a ketose solution turns rapidly red while a hexose develops a colour much more slowly.

There are no convenient general reactions for distinguishing hexoses as a class from any other group of sugars, but each of the hexoses occurring in nature is readily identified by characteristic reactions.

GLUCOSE OR DEXTROSE.

Occurrence.

The substance which is commonly known as grape sugar occurs, together with levulose or fruit sugar, in a number of sweet fruits, in honey, and in the seeds, leaves, roots, and blossoms of a great many of the higher plants. Glucose is formed by the hydrolysis of cane sugar, of glucosides, and of many polysaccharides, such as starch, cellulose, etc.

Fenton and Gostling : " J. Chem. Soc.," 1901, **79**, 361, 807.

Preparation.

The most convenient source for the preparation of glucose on a small scale is cane sugar. One hundred and twenty c.c. of 90 per cent alcohol mixed with 5 c.c. of fuming hydrochloric acid are heated at 45-50°; 40 grams of powdered cane sugar are now added, the mixture being kept thoroughly stirred. After two hours the solution is allowed to cool, and a little anhydrous glucose is added to induce crystallization. In the course of a few days the resulting crop of crystals is filtered off and washed with a little dilute alcohol; it is recrystallized by dissolving in half its weight of warm water and adding twice as much 90-95 per cent alcohol, filtering warm and setting aside to cool.

On a commercial scale glucose is best prepared by heating freshly prepared potato or maize starch freed from nitrogenous material with dilute sulphuric * acid in sealed copper vessels under 3 atmospheres pressure for half an hour. When the hydrolysis is complete, the acid is removed as calcium sulphate by the addition of powdered chalk, and the filtered solution, after being decolorized by means of animal charcoal is evaporated in a vacuum; a little anhydrous glucose is then introduced, and the syrup is allowed to crystallize, the crystals being separated from the mother liquor by means of the centrifuge.

Prepared in this way the glucose forms a rather soft cake of small crystals of the hydrate $C_6H_{12}O_6 . H_2O$; it is liable to contain small quantities of maltose, isomaltose (p. 117), and dextrin from which it may be further purified by crystallization from alcohol.

Commercial dextrose is employed as a substitute for cane sugar for the sweetening of cheap jams, etc., but its sweetness is only about two-thirds that of cane sugar.

In the United States it is used largely in the manufacture of ice cream, chewing gum, etc., and owing to its high osmotic pressure and low sweetening power it is recommended for use in condensed milk.

* More recently the use of hydrochloric acid has been recommended; this involves a modification of the technique for the removal of the acid.

Properties.

Glucose separates from alcoholic solution or from concentrated aqueous solutions at 30-35° in needle-shaped crystals, which are anhydrous; from cold aqueous solutions, however, it crystallizes with one molecule of water ($C_6H_{12}O_6 . H_2O$) in the form of plates. It is readily soluble in water, but only very slightly soluble in absolute alcohol. It is readily fermented by yeast.

Glucose is dextro-rotatory, $\alpha_D = 52{\cdot}3°$; it is sometimes known as dextrose to distinguish it from the lævo-rotatory sugar levulose with which it is frequently found associated in ripe fruits.

Reactions.

1. In the presence of ammonia, glucose can reduce silver from its salts. A little glucose is added to a solution of silver nitrate to which have been added a few drops of caustic potash and just sufficient ammonia to redissolve the brown precipitate. On warming the mixture the silver is deposited on the sides of the test tube, forming a mirror.

2. *Nylander's Test.*—When boiled with a solution of glucose Nylander's reagent turns brown and finally black owing to the precipitation of bismuth oxide and metallic bismuth.

The reagent is prepared by dissolving 2 grams of bismuth oxynitrate and 4 grams of Rochelle salt in 100 grams of 10 per cent caustic soda solution.

3. Add to the solution basic lead acetate and ammonia. If glucose be present, a white precipitate comes down, which turns red. This reaction is not given by cane sugar.

4. Add to the solution a little copper sulphate solution and an excess of caustic potash. On warming, a yellow to red precipitate is formed. This reaction also is given by levulose, maltose and other reducing sugars.

5. On warming with Fehling's solution, a red precipitate is given by dextrose, levulose, maltose, and other reducing sugars.

6. Add a little Barfoed's reagent and warm. A red

precipitate floating as a thin film on the surface of the liquid indicates dextrose. This reaction is also given by levulose and other hexoses but not by cane sugar or maltose.

The reagent, which should be freshly made up, is prepared by dissolving 5 grams of copper acetate, and 5 grams of sodium acetate, in 100 c.c. of water containing 1 c.c. of glacial acetic acid.

7. The addition to the solution of picric acid and caustic soda results in the formation of a blood-red coloration, due to picramic acid. This reaction is also given by other reducing sugars.

8. On boiling the solution of glucose with an equal volume of caustic potash, a yellow-brown colour results ; on acidifying with dilute nitric acid the colour lightens and a smell of burnt sugar is produced.

9. Glucose reacts with phenylhydrazine to give an osazone. To 5 c.c. of an approximately 5 per cent solution of glucose, add 4 or 5 drops of phenylhydrazine and about the same amount of glacial acetic acid. (If phenylhydrazine hydrochloride is used, add about enough solid to cover a threepenny piece and an equal quantity of sodium acetate.) Place the mixture in a boiling water bath for about half an hour and then remove ; a golden yellow crystalline precipitate will have been formed. On examination under the microscope the needle-shaped crystals will be seen to be gathered together in clusters resembling wheat sheaves. Glucosazone melts at 204-205° with decomposition ; it is insoluble in water but soluble in alcohol, the solution being lævo-rotatory in contradistinction to that of maltose which is dextro-rotatory.

The constitutional formula of glucose is given on p. 84.

Microchemical Tests.

For microchemical tests for sugars, the reduction of copper salts in the presence of excess of alkali is generally employed, but these are not altogether satisfactory, owing to the amount of diffusion which takes place, and also because sucrose, if its presence in a tissue be suspected, must first be hydrolysed by boiling with acid before the reduction will take place.

Mangham * and others have obtained excellent results by the use of the osazone test for microscopic work; if properly performed, it is much more satisfactory than any other, and has the advantage of being a very delicate test for some sugars. For example, a ·015 per cent solution of glucose will give a definite reaction. The main disadvantage of the method is in its comparative slowness.

Two solutions are required :—

(*a*) 1 gram of phenylhydrazine hydrochloride dissolved in 10 grams of glycerol.

(*b*) 1 gram of sodium acetate dissolved in 10 grams of glycerol.

If necessary the solution of these substances may be hastened by means of heat, and before use the solutions should be filtered.

Glycerol is used because its penetrative power is greater than that of water, and also because it will not evaporate and deposit crystals of the substances used.

For use, one drop of each fluid is placed on a glass slip and mixed thoroughly. The section, which must be more than one cell in thickness, is laid in the mixture and covered with a cover glass. The preparation is heated on a hot water oven for about half an hour, and is then allowed to cool; the osazone crystals will form in varying degrees of rapidity.

In order that familiarity with the method may be gained, the reagents may be mixed on the slip with drops of sugar solution of different concentrations heated for varying periods and examined periodically after cooling.

Maltose gives an osazone characterized by dense rosettes of lemon-yellow crystals, which are broader and larger than those obtained with dextrose and levulose.

Dextrose and levulose may be distinguished by the fact that methylphenylhydrazine gives a crystalline osazone with levulose and not with dextrose.

* Mangham: "New Phytol.," 1911, **10**, 160; "Ann. Bot.," 1915, **29**, 360.

FRUCTOSE OR LEVULOSE.

Occurrence.

Fructose occurs in most sweet fruits and in honey, together with both cane sugar and dextrose, but usually in excess of the latter two. It is formed in equal quantity with dextrose by the hydrolysis of cane sugar, and the resulting mixture, known as invert sugar, may occur in sucrose-producing plants, such as sugar beet and sugar cane, if kept for some time after gathering.

Much discussion has centred around the origin of levulose in the actively assimilating leaf. It is often considered to be chiefly employed in building up new tissue whilst the glucose is consumed in respiration (see Vol. II.). It may be more abundant than glucose as in *Galanthus nivalis* * and in oat straw, a subject which is considered on page 110.

Preparation.

The separation of pure levulose from invert sugar on a small scale is not easy to carry out, but the operation is performed on a large scale by making use of the fact that on treating invert sugar with milk of lime the levulose is converted into an insoluble calcium compound, which may be filtered off and purified, while the glucose remains in solution.

The easiest means of preparing levulose in the laboratory is to hydrolyse inulin by boiling 1 part of this substance with 5 parts of ·5 per cent sulphuric acid † for one hour; the acid is then removed by means of barium carbonate, and the solution, after being treated with animal charcoal and filtered, is evaporated at a low temperature to a thin syrup. The latter is then crystallized from alcohol after sowing with a crystal of pure levulose. A modification of this method is employed for the manufacture of pure levulose.‡

* Parkin: "Biochem. Journ.," 1912, **6**, 1.

† Düll ("Chem. Zeit.," 1895, **19**, 216) recommends the use of oxalic acid; see also Wiechmann: "Z. d. Vereins Deut. Zuckerind.," 1891, **41**, 331.

‡ Cf. Stein: "Proc. Internat. Confer. Sugar Ind.," April, 1908.

Properties.

Levulose separates from alcohol in hard rhombic crystals, which have the composition $C_6H_{12}O_6$; from concentrated aqueous solutions, however, it crystallizes in needles with water of crystallization $2C_6H_{12}O_6 . H_2O$. It is fairly soluble in hot absolute alcohol and ether, and may thus be separated from other sugars which are insoluble in these solvents. Levulose is strongly lævo-rotatory and exhibits slight muta-rotation; its rotatory power is very dependent on temperature, $\alpha_D^{20} = -93°$ in a 10 per cent solution.

Reactions.

1. To a solution of levulose mixed with an equal volume of concentrated hydrochloric acid a few grains of resorcin are added. On warming, a deep red coloration results, and finally a brown-red precipitate. The precipitate is soluble in amyl alcohol, giving a deep red solution.

This reaction is given by all keto-hexoses and by carbohydrates such as cane sugar and raffinose which give rise to them on hydrolysis.

2. Levulose gives the same reactions as dextrose with salts of copper and picric acid.

3. Levulose with milk of lime forms an insoluble compound; dextrose does not.

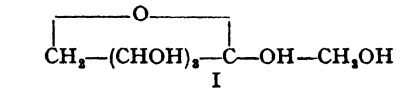
4. Levulose gives with phenylhydrazine the same osazone as glucose, namely glucosazone.

5. With methylphenylhydrazine it gives, in alcoholic solution, an osazone * crystallizing in needles; m.p. 158-160°. (Distinction from glucose and mannose.)

Constitution.

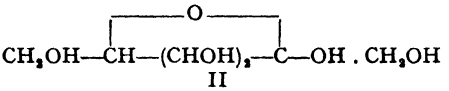
Fructose is a 2 keto-hexose whose constitution may be represented by either of the two formulæ:—

* Neuberg: "Ber. deut. chem. Gesells.," 1902, **35**, 961.



I

Normal crystalline fructose



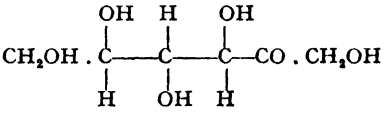
II

γ-fructose

Haworth and his fellow-workers * from their consid experimental work conclude that the normal form of hexoses, both aldehydic and ketonic and of pentoses, is the amylene oxide form, and accordingly the ordinary form of fructose is represented by the formula I. It has, however, been shown by Irvine and Steele † that fructose, as it occurs in sucrose and inulin, is present in a so-called γ-form which is more active than in its normal state. The γ-form, which has the butylene oxide configuration shown in formula II., differs from ordinary, or normal, fructose in the fact that it reduces potassium permanganate readily; it may be produced by leaving ordinary fructose in contact with acid for an hour and then neutralizing; the solution has thus acquired the power of decolorizing permanganate.

SORBOSE.

Sorbose is a 2 keto-hexose, isomeric with fructose, of the formula—



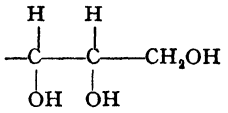
It does not occur naturally, but is produced by the oxidative action of *Bacterium xylinum* upon the alcohol sorbitol; it was, in fact, first isolated from the juice of *Pyrus aucuparia* which had been kept for some months exposed to the air. It has since been shown that the fresh juice contains no sorbose but only the corresponding alcohol sorbitol. Bertrand ‡

* Haworth and Hirst: "J. Chem. Soc.," 1926, 1858. Avery, Haworth, and Hirst: *id.*, 1927, 2308. Goodyear and Haworth: *id.*, 1927, 3140.

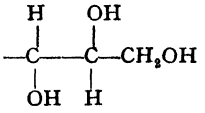
† Irvine and Steele: *id.*, 1920, **117**, 1474.

‡ Bertrand: "Ann. Chim. Phys.," 1904 [8], **3**, 200.

subsequently found that *Bacterium xylinum* had the peculiar power of oxidizing a —CHOH group to CO provided the hydroxyl was adjacent to another hydroxyl group on the same side of the molecule; thus it could oxidize mannitol or sorbitol which contain the grouping—



but not dulcitol which contains the grouping—



Sorbose is not fermentable by yeast.

GALACTOSE.

Occurrence.

This sugar has rarely been recorded as occurring free in nature. Von Lippmann * claims to have found it on ivy berries after a sudden frost, which is analogous with the increase of the raffinose content of sugar beet under like conditions. In its polysaccharide form of galactan, galactose forms a constituent of mucilages, such as agar and carrageen obtained from sea weeds; it also occurs in the gums of the peach and plum, and is a constituent of the pectic substances of carrot, turnip, and many fruits such as apple and pear. In all these instances galactose is accompanied by other sugars, which may be either hexoses or pentoses. Further, galactose is a constituent of the glucoside digitalin, of the anthocyan idæin, and forms the carbohydrate constituent of the group of lipins known as cerebrosides and galactolipins. In all cases it may be set free by hydrolysis with mineral acid.

Preparation.

A convenient material for the preparation of galactose is agar, which on hydrolysis yields a mixture of sugars amongst

* Von Lippmann: "Ber. deut. chem. Gesells," 1910, 43, 3611.

which galactose predominates. Pure galactose is, however, more easily prepared from lactose; for this purpose 1 kg. of lactose is boiled for two hours with 2·5 litres of water containing 50 grams of sulphuric acid; the solution is neutralized with barium carbonate, filtered, and concentrated; the galactose is crystallized by the addition of a mixture of 1 part of ethyl with 2 parts of methyl alcohol.*

The estimation of galactose depends upon its oxidation by nitric acid, under specified conditions, to mucic acid and weighing the latter; the results obtained vary in the hands of different workers, and considerable practice is required to obtain consistent values.

Properties.

Galactose crystallizes in minute hexagonal crystals, which melt at 164°. It is strongly dextro-rotatory, $\alpha_D = 81{\cdot}5°$, and exhibits muta-rotation. Galactose after association with ordinary yeast for some time is fermentable, but it is not acted upon by *S. Ludwigii* and *S. anomalus*.

Detection.

1. The hexagonal form of the crystals is characteristic of galactose.

2. It gives a methylphenylhydrazone (m.p. 190-191°).

3. It reduces Fehling's solution somewhat more slowly than glucose; 10 c.c. Fehling's solution ≡ 0·51 gram galactose.

4. On oxidation with nitric acid it yields mucic acid. Five grams of substance are heated in a beaker with 6 c.c. of nitric acid (sp. gr. 1·15) until two-thirds of the liquid have been evaporated off. After twelve hours the mucic acid formed will have separated, and may be washed with 10 c.c. of water. If other insoluble substances are present, place the filter paper with the solid in a dilute solution of ammonium carbonate to extract the mucic acid as ammonium salt. Filter once more, and evaporate the filtrate almost to dryness, and acidify with nitric acid; the precipitate is pure mucic acid.

* Dept. of Commerce, Bur. of Standards, Washington, No. 416.

MANNOSE.

Occurrence.

There is no record of the free occurrence of mannose in plants; in its polymerized or polysaccharide form, however, it is widely distributed as a constituent of the so-called hemicelluloses contained in the cell walls of the seeds of peas, coffee, date, etc. It is also a constituent of salep mucilage (*Orchis Morio*).

Preparation.

Mannose may be prepared by the hydrolysis of the hemicellulose contained in the endosperm of ivory nuts, *Phytelephas macrocarpa*, which are extensively used in the manufacture of vegetable ivory buttons. The turnings are added to ten times their weight of boiling 1 per cent caustic soda and allowed to stand for half an hour with occasional stirring. The liquor is then decanted off and the residue washed with water and dried; 500 grams of this material are mixed with an equal weight of 75 per cent sulphuric acid and allowed to stand for twenty-four hours. The resulting substance is dissolved in water, diluted to 5·5 litres, and then boiled for two and a half hours. The solution is neutralized with barium carbonate paste and filtered through a thin layer of animal charcoal. The last traces of barium are removed by the careful addition of dilute sulphuric acid and filtering. The filtrate is concentrated over a boiling water bath until it contains 87-88 per cent of total solids; it is then mixed with an equal volume of glacial acetic acid, seeded with a few crystals of mannose and then frozen. On allowing the mass to thaw slowly in a refrigerator, the mannose will crystallize out.*

Properties.

Mannose has a sweet taste; when dry, it is a hard crumbling substance, which, however, deliquesces and is readily soluble in water; it is only slightly soluble in hot alcohol and is

* Clark: "J. Biol. Chem.," 1922, **52**, 1. Patterson: "J. Chem. Soc.," 1923, **123**, 1139.

insoluble in ether. It is dextro-rotatory, $[\alpha]_D^{20} = +14{\cdot}36°$ in 10 per cent solution, but when freshly prepared it is lævo-rotatory. Mannose is readily fermentable by yeast.

Detection.

1. Mannose is most readily detected and estimated by means of its phenylhydrazone, which is almost insoluble in water, and forms almost at once on adding phenylhydrazine acetate to an aqueous solution of the sugar; the phenylhydrazone is soluble in a very large volume of boiling water, and separates in fine prisms from the solution on cooling. These crystals melt at 195-200°.

An excess of phenylhydrazine converts mannose into glucosazone, which is identical with the substance obtained under similar conditions from both glucose and fructose.

2. Mannose reduces Fehling's solution, 10 c.c.=·04307 gram mannose.

C. *HEPTOSES.*

A number of heptoses of the formula $C_7H_{14}O_7$ have been synthesized, but only two are known to occur naturally. One of these, mannoketoheptose, occurs in the avocado pear, *Persea gratissima*, and the other, sedoheptose, in the stonecrop; *Sedum spectabile.** Both are ketoheptoses and are not fermented by yeast.

DISACCHARIDES.

The disaccharides, as is implied by their name, give rise on hydrolysis to two molecules of monosaccharide which may both be hexoses, or one may be a hexose while the other is a pentose; the latter type of pentosehexose disaccharide, which is comparatively rare, is dealt with on page 121. The true hexose disaccharides of the general formula $C_{12}H_{22}O_{11}$ may be divided into two classes:—

(*a*) Those giving rise on hydrolysis to two molecules of the same hexose, such as maltose, isomaltose, cellobiose, isocellobiose, gentiobiose, trehalose, and isotrehalose.

* La Forge: "J. Biol. Chem.," 1920, **42**, 367.

(*b*) Those giving rise on hydrolysis to two different hexoses such as sucrose, turanose, lactose, and melibiose.

The isomerism between the various members of the first group may be due to a different mode of attachment of the two hexoses, involving in some cases the reducing groups so that, as in the case of trehalose, sucrose, and turanose, the resulting disaccharide has no reducing properties. On the other hand, two structurally identical sugars may differ in stereochemical formula, i.e. in the spatial arrangement of the two constituent sugars with the resultant production of two isomeric α- and β-disaccharides corresponding to the α- and β-glucoses; such a relationship is found to exist between maltose and isomaltose, the former of which is hydrolysed by maltase while the latter is only attacked by emulsin. Similarly, the disaccharides gentiobiose and cellobiose appear to belong to the β-glucosides, since they are not attacked by maltase but are acted upon by emulsin.

In addition to the above considerations, an exact knowledge of the nature of the anhydride ring of the constituent monosaccharides is requisite for a complete understanding of the constitution of given disaccharide; this may be seen by the alternative formulæ given for glucose on page 83.

Action of Enzymes on Disaccharides.

(*a*) *Hydrolytic Enzymes.*—The hydrolysis of disaccharides is effected by enzymes such as maltase and emulsin, which act on more than one substrate, and in some cases the hydrolysis can only be effected by a specific enzyme such as invertase (sucrase), which acts only upon sucrose.

Attempts to utilize enzymes for the synthesis of disaccharides as well as for their hydrolysis were initiated by Croft-Hill who, by acting upon a solution of glucose with a yeast extract of maltase, was able to synthesize a disaccharide to which he gave the name of revertose, but which was subsequently identified as isomaltose, the β-glucosidic isomer of maltose. Since then, largely as the result of the work of Bourquelot * and his co-workers, gentiobiose, cellobiose, and

* Bourquelot: "Ann. d. Chim.," 1920, (9), **13**, 5.

a number of similar disaccharides and glucosides have been synthesized. The two sugars, sucrose and maltose, have, however, so far resisted all attempts at their synthesis by enzymes, although both have been synthesized by chemical means.*

(*b*) *Fermenting Enzymes.*—Contrary to the assertion of Fischer that disaccharides are not attacked by yeasts until they have been hydrolysed by the appropriate enzyme contained in the yeast, Willstätter † concluded that both maltose and lactose are directly fermentable, since he was able to effect fermentation of these by distillery yeasts which contained only very little maltase and were entirely free from lactase. The fact that *Saccharomyces Marxianus*, which is known to be free from maltase, is unable to ferment maltose, he attributes to its not possessing a maltozymase rather than to any deficiency in maltase.

CANE SUGAR, SUCROSE OR SACCHAROSE.

Occurrence.

Cane sugar is one of the most widely distributed substances to be found in the vegetable kingdom. Besides forming about 20 per cent of the juice of the sugar cane, *Saccharum officinarum*, and about 10-20 per cent of that of the beetroot, it is found in varying quantities in the wood of maple and birch, and in *Sorghum saccharatum ;* it occurs, moreover, in wheat, maize, barley, in carrots, and in madder root. In most sweet fruits it is found together with a greater or lesser quantity of dextrose and levulose, which may possibly have been formed from it by hydrolysis. It also is found in the leaves of many plants associated with glucose and maltose. The following table, compiled by Kulisch, gives the relative proportions of cane sugar and hexoses found in various fruits.

	Cane Sugar.	Hexoses.
Pineapple	11·33	1·98
Strawberry	6·33	4·98
Apricot	6·04	2·74
Ripe banana . . .	5·00	10·00
Apple	1-5·40	7-13·00

* Pictet : " Bull. Soc. Chim.," 1920, [4], **27**, 650. Pictet and Vogel : " Compt. rend.," 1928, **186**, 727.

† Willstätter : " Zeit. physiol. Chem.," 1925, **150**, 287.

In honey practically only invert sugar is found, although the sugar found in the flowers by the bees is commonly cane sugar. The hydrolytic agent in this case is most probably the formic acid secreted by the bees.

Cane sugar also has been recorded as occurring in *Sphagnum*, *Hypnum*, and *Pellia*.* The relative proportions of the three sugars sucrose, levulose, and dextrose in certain plants have been studied by Collins & Gill.†

Thus in the case of *Helianthus tuberosus*, they found that during the period August-December, the period of formation of the tubers and thus of translocation, the total sugar of the stalks reaches a maximum and then falls to a low value in December. The amount of sucrose and levulose follows a similar course, but the dextrose, which is in greatest abundance in August, shows a sudden drop in September and then increases, so that in December it is the chief sugar present, being more than twice as abundant as either sucrose or levulose. The accompanying table gives the actual figures calculated to percentages of the living plant:—

	Aug.	Sept. 4.	Oct. 2.	Oct. 30.	Dec. 13.
Sucrose .	0·15	0·36	1·15	0·35	0·24
Dextrose .	0·37	0·11	0·27	0·55	0·58
Levulose .	0·29	1·18	0·90	1·37	0·24
Total sugar	0·81	1·65	2·32	2·27	1·06

In the instance of oat straw, the preponderating sugar at the end of vegetative activity is levulose not dextrose, which suggests that the nature of the reserve material determines the variety of the residual sugar. In the artichoke, the formation of inulin means the fixation of levulose, wherefore there will be a surplus of dextrose. In the oat, on the other hand, dextrose is converted into starch so that there is a residuum of levulose. In this argument Collins and Gill conclude that the hexoses have their origin in sucrose. In the

* Goris and Vischniac: "Bull. Sci. Pharm.," 1913, **20**, 390.
† Collins and Gill: "J. Soc. Chem. Ind.," 1926, **45**, 63 T.

development of the tuber, the following table gives the analysis of samples expressed in percentages of dry matter :—

	Oct. 2.	Oct. 30.	Dec. 13.
Total sugar, including inulin .	46·76	52·87	55·27
Free-reducing sugar . .	7·03	5·20	8·24
Levulose	6·72	4·82	8·54

In this connection brief allusion may be made to the work of Miller * on sorghum and maize in the leaves of which the maximum of sugars was reached between noon and 5 p.m. after which there was a gradual decrease until dawn. The water-insoluble carbohydrates reached a maximum later than the sugars and a decrease did not begin till about midnight, the minimum being at about dawn. It was also observed that the reducing sugars varied far less than the non-reducing sugars over the twenty-four hours.

The conclusion drawn by many that sucrose is the first sugar of photosynthesis is a matter of dispute, an aspect of the subject which is considered in the second volume of the present work.

Preparation.

The two chief sources for the preparation of cane sugar on a manufacturing scale are the sugar cane and the beet. The processes used in both cases are more or less similar, and consist in obtaining, purifying, concentrating and, lastly, crystallizing the juice. The juice is generally obtained from the cane by crushing, as much as 85-95 per cent of the juice being expressed in this way ; in some cases it is extracted by diffusion, which consists in immersing the cane in water, when the sugar diffuses out of the cells into the surrounding water while the indiffusible colloids remain behind. The crude juice is then boiled with milk of lime, in order to neutralize any acid present and to precipitate coagulable proteins, and is subsequently treated with sulphur dioxide. After filtering, the solution is concentrated in a vacuum and allowed to crystallize,

* Miller : " J. Agric. Res.," 1924, **27**, 785.

the mother liquor being separated by centrifugalizing; the crystals may be used at once as brown sugar, or may be refined.

When the beet is used, the roots are first cut into slices and subjected to diffusion, the same quantity of water circulating through a series of vessels in such a manner that the fresh water first passes over material from which most of the sugar has already been extracted, and as the solution becomes more concentrated, it comes into contact with material which is increasingly richer in sugar. In this way the aqueous extract attains a concentration of from 12-15 per cent.* This solution is then boiled with lime and saturated with carbon dioxide to decompose any calcium saccharosate which may have been formed; it is then filtered and again saturated with carbon dioxide or a mixture of this gas and sulphur dioxide to precipitate the last traces of calcium, and also to decolorize it; the older process of filtration through animal charcoal is thereby rendered unnecessary; the solution is then boiled and filtered and the clear filtrate is concentrated in a vacuum and allowed to crystallize. The uncrystallizable residue which remains is known as molasses; a further yield of sugar may be obtained from this residue by the addition of lime to the cold solution or of strontia to the boiling solution whereby the cane sugar in the molasses is converted into the insoluble calcium or strontium saccharosate, which may be filtered off and decomposed by a current of carbon dioxide into cane sugar and calcium or strontium carbonate. The molasses are sometimes fermented for the manufacture of rum or may be used for cattle food; they are also used in the manufacture of boot blacking.

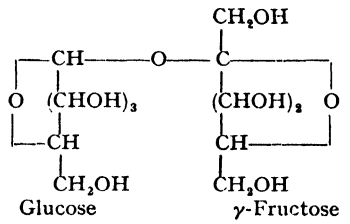
By suitable methods of cultivation, seed selection and use of nitrogenous and potash fertilizers the amount of sugar contained in the beet has been raised from 10·6 per cent in the period 1880-90 to about 15 per cent in the period 1900-10, and the beetroot is gradually displacing the sugar cane as a source of sucrose.

* The residue remaining after the extraction of the sugar is employed for cattle food.

Constitution.

Whilst it has long been known that sucrose on hydrolysis yields molecular proportions of glucose and fructose, it was first shown by Irvine and Steele * that the fructose occurred in the γ-form and not in its normal form in combination with glucose.

The constitutional formula † for cane sugar, based on the amylene oxide formula for the glucose constituent and the butylene oxide formula for the γ-fructose, is as follows :—



Repeated attempts to synthesize cane sugar from glucose and fructose failed owing to the fact that the fructose requires to be combined with the glucose in its active or γ-form.

Appreciating this fact, Pictet and Vogel ‡ prepared the acetyl derivative of γ-fructose, and uniting this with the acetyl derivative of glucose, by shaking the two in chloroform solution with phosphorus pentoxide, they obtained octacetyl sucrose which on hydrolysis yielded a compound showing all the characteristics of the natural sucrose.

Properties.

Cane sugar crystallizes from water in monoclinic crystals which do not contain water of crystallization ; it is readily soluble in water and only slightly soluble in alcohol ; it is dextro-rotatory, its specific rotation being $\alpha_D = +66{\cdot}5$.

When heated to 160° it melts to a glassy mass known as barley sugar, which gradually becomes crystalline again ; if heated to 190-200° it is converted into an uncrystallizable

* Irvine and Steele : " J. Chem. Soc.," 1920, **117**, 1474.

† Haworth and Hirst : *id.*, 1926, 1858. Avery, Haworth, and Hirst : *id.*, 1927, 2308.

‡ Pictet and Vogel : " Compt. rend.," 1928, **186**, 727.

brown substance known as caramel, which is used for colouring beer and wine.

Reactions.

1. Solutions of cane sugar heated with concentrated hydrochloric acid turn reddish-pink.

2. If warmed with concentrated hydrochloric acid and a few crystals of resorcin a deep red colour is produced owing to the liberation of levulose.

3. Cane sugar does not react with phenylhydrazine.

4. Cane sugar does not reduce Nylander's reagent.

5. Solutions of cane sugar do not reduce Fehling's solution until they have been inverted by boiling for a short time with a few drops of dilute sulphuric acid ; if then made alkaline and boiled with Fehling's solution reduction ensues.

If a solution in water is boiled with a few drops of mineral acid, the sign of the optical activity of the solution changes from + to —. This change, which is known as *inversion*, is due to the fact that the mineral acid hydrolyses the cane sugar, converting it into equal molecular proportions of the two sugars dextrose and levulose,

$$C_{12}H_{22}O_{11} + H_2O = C_6H_{12}O_6 + C_6H_{12}O_6$$

and since the optical activity of levulose is greater than that of dextrose the resulting invert sugar is lævo-rotatory.

Aqueous solutions of cane sugar, if kept for some time, gradually become inverted, the change being somewhat accelerated by prolonged boiling.

Extremely small quantities of acid suffice to effect the change in a boiling solution; thus 80 parts of cane sugar dissolved in 20 parts of water are completely hydrolysed by heating in boiling water for one hour with an amount of hydrochloric acid corresponding to 0·005 per cent of the weight of the sugar; within certain limits, however, the action is accelerated by increasing the concentration of the acid. If, however, the acid is too strong and the heating be continued too long, the solution is liable to darken and decompose. Moreover, prolonged action, even at temperatures of 10-15°, of

concentrated acids was found by Wohl * and by Fischer † to produce exactly the opposite phenomenon, known as reversion, by which the simple molecules, more especially those of levulose, are made to condense together to form complex dextrin-like substances, as well as a disaccharide iso-maltose.

6. Sucrose is fermentable by ordinary yeast, but this has been attributed to the fact that such yeast is possessed of invertase which hydrolyses the sucrose previous to its fermentation.

TURANOSE. $C_{12}H_{22}O_{11}$.

This is a disaccharide formed by the partial hydrolysis of the trisaccharide melecitose (see p. 124); it reduces Fehling's solution, and on hydrolysis yields glucose and levulose; it is therefore isomeric with sucrose.

MALTOSE. $C_{12}H_{22}O_{11}$.

Maltose does not appear to have so wide a distribution in the plant as has sucrose. The hydrolytic action of diastase on starch yields maltose—

$$\underset{\text{Starch}}{(C_6H_{10}O_5)_n} + H_2O \rightarrow \underset{\text{Maltose}}{C_{12}H_{22}O_{11}} + \underset{\text{Dextrin}}{(C_6H_{10}O_5)_n}$$

From this it might be expected that where starch is stored and subsequently digested, maltose would appear. But not infrequently maltase also is present by the action of which the maltose is converted into hexose sugars, so that if the preparation of the material is such as to destroy or to preserve maltase, maltose will or will not appear in the subsequent analysis. It is, possibly, for this reason that discrepant results have been obtained. Maltose has been described as occurring in the leaves of *Tropæolum*, *Pyrola*, *Populus*, and *Linnæa*, whilst, on the other hand, its presence has been denied in the leaves of the snowdrop, potato, and mangold.‡ Gillot § describes the occurrence of maltose in the rhizomes and roots of *Mercurialis perennis* and, from the variations in amount,

* Wohl: "Ber. deut. chem. Gesells.," 1890, **23**, 2092.

† Fischer: *id.*, 1890, **23**, 3687.

‡ See Vol. II., chapter on "Photosynthesis."

§ Gillot: "Recherches Chimique et Biologiques sur le Genre Mercurialis," Nancy, 1925.

·25-2 per cent of dry weight, which obtain in the different phases of the life-history of the plant, he concludes that maltose, in this instance, is not a transitional sugar but a true reserve material comparable to starch and sucrose. In the germination of the barley maltose is produced, but it does not accumulate owing to the action of maltase which, as has already been stated, converts it into hexose.

Maltose is also formed by the action of diastase and other enzymes on glycogen.

In preparing maltose from starch, the diastase which is employed is usually introduced in the form of malt, which is barley that has been allowed to sprout and is then killed by suddenly heating to a temperature sufficient to stop the further growth of the barley without destroying the diastase. The malt is then stirred up with starch and water, and kept at a temperature of 60-62° for about half an hour; by the end of this time about 80 per cent of the starch has been converted into maltose and 20 per cent into dextrin. Dextrin itself is also converted into maltose by diastase, but the reaction is very slow, and in practice sufficient time is not allowed to effect this change.

Properties and Reactions.

Maltose is readily soluble in water, and crystallizes from this solvent in slender white needles, having the composition $C_{12}H_{22}O_{11}$, H_2O; its aqueous solution is strongly dextrorotatory: $\alpha_D = +137°$; freshly made solutions exhibit a higher rotation than older ones, owing to a negative mutarotation.

1. Maltose reduces Nylander's reagent, but not Barfoed's reagent.

2. Maltose reduces Fehling's solution without previous hydrolysis, and can therefore be estimated directly by this means.

3. When treated with phenylhydrazine, as described under glucose, it gives an osazone (m.p. 206°), which is soluble in 75 parts of boiling water, and can be crystallized from this solvent in rosettes of plates or broad needles

resembling sword blades; alcoholic solutions of maltosazone are dextro-rotatory. (Distinction from glucosazone.)

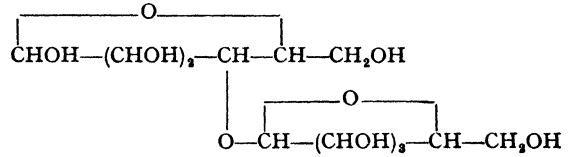
4. On hydrolysis, by boiling with dilute mineral acid, maltose breaks up into two molecules of glucose—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

the rotatory power of the solution being thereby diminished.

5. Maltose is fermentable by ordinary yeast, but not by *S. Marxianus* * and *S. Ludwigii*. As yeast ordinarily contains maltase, it was generally thought that hydrolysis by this enzyme was a preliminary to fermentation by zymase. According to Willstätter,† however, a distillery yeast free from maltase is able to ferment sucrose at P_H 4·6, which is a degree of acidity at which maltase is unable to act.

The constitutional formula assigned by Haworth and Peat ‡ to maltose is—



from which it appears that the union between the two glucose molecules is through the fourth carbon atom of one and the aldehydic carbon atom of the other; it can therefore be described as α-glucosido-4-glucose.

ISO-MALTOSE. $C_{12}H_{22}O_{11}$.

Not a little confusion exists with regard to the use of the term iso-maltose; the name was first given to a sugar obtained by Fischer § by the action of concentrated hydrochloric acid upon glucose, and this same substance has since been shown to be formed also by the action of dilute hydrochloric acid upon strong solutions of glucose. Subsequent workers,‖

* Croft Hill: "Proc. Chem. Soc.," 1901, **17**, 45.

† Willstätter: "Zeit. physiol. Chem.," **150**, 287.

‡ Haworth and Peat: "J. Chem. Soc.," 1927, 844.

§ Fischer: "Ber. deut. chem. Gesells.," 1890, **23**, 3687; 1895, **28**, 3024.

‖ Georg and Pictet: "Helv. chim. Acta.," 1926, **9**, 444. Berlin: "J. Amer. Chem. Soc.," 1926, **48**, 1107.

however, claim that the action of acid on glucose yields a mixture containing gentiobiose in addition to iso-maltose.

In a study of the reversible nature of enzyme action, Croft Hill,* in attempting to synthesize maltose by the action of maltase upon glucose, obtained some maltose and in addition an unfermentable sugar which he termed revertose, deliberately avoiding the name isomaltose "because this designation has been applied to several differing substances and revertose is different from any of these." Later Armstrong showed it to be a β-glucoside and considered it to be identical with Fischer's iso-maltose.

According to Lintner and Düll† malt diastase acting upon starch produces, in addition to maltose and dextrin, some unfermentable sugar, iso-maltose; this observation was subsequently confirmed by Ling, but in the opinion of the latter author, Fischer's iso-maltose produced by the action of acid upon starch is not identical with that produced by diastase.‖

A method for preparing iso-maltose, due to Ling and Nanji,‡ consists in allowing a solution of precipitated malt diastase to act upon crude amylopectin, or upon $\alpha\beta$-hexa-amylose prepared from it, at 50° until the rotatory power remains constant, and then fermenting away any maltose or glucose; the mixture is then filtered, evaporated, and extracted with alcohol.

Thus prepared, iso-maltose is a white, amorphous, hygroscopic power having $\alpha_D = +140°$; it forms an osazone, m.p. 150°, which is soluble in hot water or in absolute alcohol. Iso-maltose is not attacked by maltase but is hydrolysed by emulsin and is therefore a β-glucoside; it is not fermented by yeast.

It should, however, be noted that Haworth,§ who examined a sample of isomaltose prepared by Ling and Nanji, was unable to observe any structural difference between this sample and maltose itself.

* Croft Hill: "Ber. deut. chem. Gesells.," 1901, **34**, 1384. "J. Chem. Soc.," 1903, **83**, 580.

† Lintner and Düll: "Z. angew. Chem.," 1892, **5**, 268.

‡ Ling and Nanji: "J. Chem. Soc.," 1923, **123**, 2681.

§ Haworth: "J. Soc. Chem. Ind.," 1927, **46**, 300 T.

CELLOBIOSE. $C_{12}H_{22}O_{11}$.

This is a disaccharide obtained from cellulose by the action of glacial acetic acid and acetic anhydride in the presence of concentrated sulphuric acid. The resulting acetyl derivative, on treatment with alcoholic potash, yields cellobiose. It reduces Fehling's solution and gives an osazone melting at 198°. On hydrolysis, it yields two molecules of glucose and is thus isomeric with maltose, but unlike this sugar it is not hydrolysed by maltase but is attacked by emulsin. From these facts Haworth and Peat * conclude that cellobiose and maltose are structurally identical, differing only in the stereochemical configuration of their glucose residues. Thus cellobiose is represented by the same formula, as maltose (see p. 117) only is a β-glucosido-4-glucose, whereas maltose is the corresponding α-compound.

Iso-cellobiose.

An isomeric sugar, isocellobiose, was obtained in the form of its acetyl derivative together with cellobiose acetate on acetolysis of cellulose; on hydrolysis of the acetyl derivative with baryta, iso-cellobiose † was set free.

GENTIOBIOSE. $C_{12}H_{22}O_{11}$.

This disaccharide ‡ is obtained by the partial hydrolysis of the trisaccharide gentianose (see p. 125); by the action of emulsin it is converted into two molecules of glucose, from which it follows that gentiobiose is a β-glucoside. It has been synthesized by the action of emulsin on glucose,§ a method || which provides a more convenient source for its preparation, and also by the action of concentrated hydrochloric acid on glucose.¶ Gentiobiose is the biose of

* Haworth and Peat: "J. Chem. Soc.," 1926, 3094.

† Ost and Prosiegel: "Zeit. angew. Chem.," 1920, **33**, 100. Ost: *id.*, 1926, **39**, 1117.

‡ Zemplen: "Ber. deut. chem. Gesells.," 1915, **48**, 233.

§ Hérissey, Bourquelot, and Coivre: "J. Pharm. Chim.," 1913, **7**, 441.

|| Georg and Pictet: "Helv. chem. Acc.," 1926, **9**, 444. Berlin: "J. Amer. Chem. Soc.," 1926, **48**, 1107.

¶ For this method of preparation, see Harding: "Sugar," 1922, 240.

amygdalin *; as the result of its synthesis † and from other considerations its constitution may be represented by the formula—



from which it appears to be a β-glucosido-6-glucose.

TREHALOSE. $C_{12}H_{22}O_{11}$.

Trehalose is a disaccharide very widely distributed among the fungi,‡ including ergot and myxomycetes,§ in moulds such as *Aspergillus niger*, in *Selaginella lepidophylla* || and in various Florideæ.¶ It does not reduce Fehling's solution and is strongly dextro-rotatory, $\alpha_D = +199°$. When boiled with 5 per cent sulphuric acid for six hours, it is converted into two molecules of glucose.** It is also hydrolysed by the enzyme trehalase contained in many fungi.

LACTOSE OR MILK SUGAR. $C_{12}H_{22}O_{11}$.

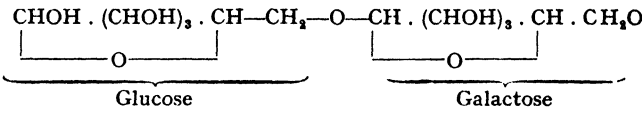
This disaccharide, though of considerable importance in the animal kingdom, is never found in plants. It reduces Fehling's solution and on hydrolysis, by the enzyme lactase or by dilute mineral acids, it yields molecular proportions of glucose and galactose.

MELIBIOSE. $C_{12}H_{22}O_{11}$.

This disaccharide †† is not a naturally occurring sugar, but is produced by the partial hydrolysis of the trisaccharide raffinose; it is dextro-rotatory, $\alpha_D = +143°$. It yields on hydrolysis molecular proportions of glucose and galactose. Owing to the fact that this sugar is hydrolysed by bottom fermentation yeasts but not by top fermentation yeasts, it

* Haworth and Wylam: "J. Chem. Soc.," 1923, **23**, 3120.
† Iwanoff: "Biochem. Zeit.," 1925, **162**, 455.
‡ Bourquelot: "Bull. soc. mycol. France," 1905, **21**, 50.
§ Helferich, Bauer, and Weigand: "Annalen," 1926, **447**, 27.
|| Anselmino and Gilg: "Pharm. Zeit.," 1913, **58**, 563; Lippmann: "Ber. deut. chem. Gesells.," 1912, **45**, 3431.
¶ Kylin: "Zeit. physiol. Chem.," 1915, **94**, 337.
** Winterstein: *id.*, 1894, **19**, 70.
†† Scheibler and Mittelmeier: "Ber. deut. chem. Gesells.," 1890, **23**, 1438.

may be used to distinguish between these varieties of *Saccharomyces*. The constitution * of melibiose is represented by the formula—

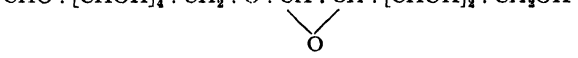


DISACCHARIDES PRODUCED BY THE UNION OF A HEXOSE WITH A PENTOSE.

Several disaccharides have been discovered which on hydrolysis yield one molecule each of a hexose and a pentose; some of the more important of these are the following:—

PRIMEVEROSE. $C_{11}H_{20}O_{10}$.

Primeverose is prepared from the glucosides primeverin and primulaverin occurring in *Primula officinalis*.† This sugar has $\alpha_D - 3{\cdot}79°$ and melts at 210°. It has a free aldehyde group and would therefore appear to have the constitution—



This disaccharide has also been found to occur in the glucosides gentiacaulin and monotropitin, the latter of which occurs in *Monotropa hypopitys*, in the bark of *Betula lenta*, and in the fresh roots of *Spiræa Ulmaria*, *S. Filipendula*, and *S. gigantea*.

The fact that this carbohydrate has thus been found to occur in five families, namely, Betulaceæ, Monotropeæ, Primulaceæ, Gentianaceæ, and Rosaceæ, would indicate that it has a much wider distribution than was formerly suspected.‡

VICIANOSE. $C_{11}H_{20}O_{10}$.

This disaccharide is obtained by the hydrolysis of the glucoside vicianin occurring in *Vicia angustifolia*, and gein

* Charlton, Haworth, and Hickinbottom: "J. Chem. Soc.," 1927, 1527. Haworth, Leach, and Long: *id.*, 1927, 3146.

† Goris and Vischniac: "Compt. rend.," 1919, **169**, 871, 975.

‡ Bridel: *id.*, 1924, **179**, 991.

obtained from *Geum urbanum*, and is found to be composed of one molecule of glucose and one of arabinose.*

STROPHANTHOBIOSE. $C_{12}H_{22}O_{10}$.

This disaccharide likewise occurs in a glucoside, strophanthin. On hydrolysis it yields mannose and rhamnose ($C_6H_{12}O_5$).†

TRISACCHARIDES.

RAFFINOSE. $C_{18}H_{32}O_{16}$.

This sugar occurs in cotton seeds, barley, eucalyptus, lotus,‡ and also in the beetroot; the juice of this latter contains on an average about 15 per cent of cane sugar but only 0·02 per cent § of raffinose. The molasses from beet sugar refineries, however, contain from 2-3 per cent of raffinose (hence the name) and form the chief commercial source of this sugar.

As the concentration of the raffinose increases it tends to crystallize out together with the cane sugar in the form of mixed crystals having a peculiar and characteristic pointed appearance quite different from ordinary cane sugar.

Numerous methods || have been described for preparing pure raffinose from molasses, but they are mostly rather tedious and a more convenient source for its preparation is cotton-seed meal ¶

Raffinose crystallizes with five molecules of water in clusters of slender glistening needles or prisms whose composition is expressed by the formula $C_{18}H_{32}O_{16} \cdot 5H_2O$. It dissolves in water and in methyl alcohol, in which latter solvent cane sugar is only sparingly soluble, but is hardly soluble in ethyl alcohol, whereas cane sugar is appreciably soluble.

* Bertrand and Weisweiller: "Compt. rend.," 1908, **146**, 1413. Herissey and Cheymol: *id.*, 1925, **180**, 384; and **181**, 565.

† Feist: "Ber. deut. chem. Gesells.," 1900, **33**, 2091.

‡ Hemmi: "J. Coll. Agr. Imp. Univ. Sapporo," 1921, **9**, 249.

§ Strohmer: "Oest. Ung. Z. f. Zuckerind u. Landw.," 1910, **39**, 649.

|| v. Lippmann: "Die Chemie d. Zuckerarten," 3rd ed., Braunschweig, Vol. II., p. 1628.

¶ Englis, Decker, and Adams: "J. Amer. Chem. Soc.," 1925, **47**, 2724. Harding: "Sugar," 1922, 240.

It is strongly dextro-rotatory, $\alpha_D = +104{\cdot}4^\circ$, in 10 per cent solution, and consequently cane sugar in which raffinose occurs as an impurity appears to contain more than 100 per cent of sucrose when estimated polarimetrically; hence raffinose is sometimes known as "plus sugar."

It does not reduce Fehling's solution, nor does it react with phenylhydrazine.

On careful hydrolysis raffinose breaks up at first into levulose and a disaccharide—melibiose.

$$\underset{\text{Raffinose}}{C_{18}H_{32}O_{16}} + H_2O = \underset{\text{Levulose}}{C_6H_{12}O_6} + \underset{\text{Melibiose}}{C_{12}H_{22}O_{11}}$$

On heating further the melibiose itself is broken up as follows:—

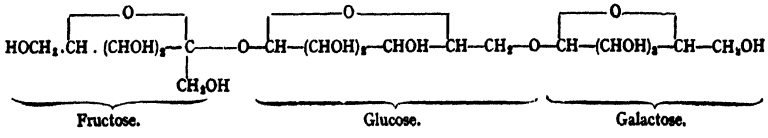
$$\underset{\text{Melibiose}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{Dextrose}}{C_6H_{12}O_6} + \underset{\text{Galactose}}{C_6H_{12}O_6}$$

If boiled with mineral acid, therefore, raffinose gives rise to a mixture of dextrose, levulose, and galactose.

According to Neuberg,* raffinose is hydrolysed by emulsin into cane sugar and galactose. (See below.)

Raffinose, unlike cane sugar, is completely fermented by bottom fermentation yeast to alcohol and carbon dioxide, whereas top fermentation yeast is only able to ferment it partially, converting the levulose complex into carbon dioxide and alcohol and leaving melibiose unattacked. These facts have been made use of by Bau † for detecting and for estimating raffinose.

From its behaviour on hydrolysis the constitution may be represented by the formula ‡:—



* Neuberg: "Biochem. Zeit.," 1907, **3**, 519.
† Bau: "Chem. Zeit.," 1894, **18**, 1797; 1897, **21**, 185; 1902, **26**, 69.
‡ Haworth, Hirst, and Ruell: "J. Chem. Soc.," 1923, **123**, 3125.

Detection.

There are no rapidly performed characteristic tests for raffinose.

The only really reliable method of identifying it is to isolate the substance by precipitating the strontium compound in alcoholic solution, filtering off the precipitate and decomposing it by a current of carbon dioxide. The resulting solution is then evaporated and the residue extracted with alcohol to remove sucrose and other sugars which are more soluble in alcohol than raffinose. The pure substance should be identified by its crystalline form and optical properties.

Another way of identifying raffinose * is to add to the solution a little decoction of fresh yeast, to act as nutriment, and then to sterilize the solution; a pure culture of top fermentation yeast is then added to the solution and the fermentation is allowed to proceed in a thermostat at 31°; when it is completed, the solution is boiled with animal charcoal, filtered, and evaporated to a syrup; the latter is then, while still hot, poured into hot alcohol and on cooling it is filtered; the filtrate is then precipitated by mixing with 1½ vols. of ether. After twenty-four hours the supernatant liquid is poured off and the residual syrup, which consists of melibiose, is converted into its osazone which is characterized by its crystalline form and melting-point, 178-179°.†

Finally, Neuberg ‡ has proposed making use of emulsin for the identification of raffinose.

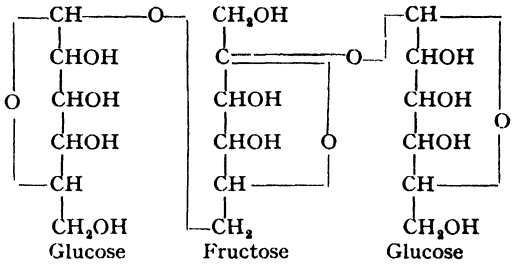
MELECITOSE. $C_{18}H_{32}O_{16}$. $2H_2O$.

This is a sugar which occurs in the sap of *Larix europæa*, in Persian manna, and especially in the manna exuded from the twigs and needles of *Pseudotsuga Douglasii*; it crystallizes with two molecules of water in rhombic prisms, and is dextrorotatory ($\alpha_D = +83°$). It does not reduce Fehling's solution, and on hydrolysis yields first a molecule of glucose and a disaccharide—turanose, $C_{12}H_{22}O_{11}$—which subsequently itself breaks

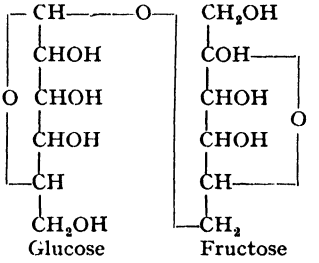
* Bau: *loc. cit.*, 1897, **21**, 185. † *Id.*, 1902, **26**, 69.
‡ Neuberg: "Biochem. Zeit.," 1907, **3**, 519 and 535.

up into one molecule of glucose and one of fructose, as is explained by the formulæ given below for these sugars :—

Melecitose.



Turanose.



STACHYOSE. $C_{24}H_{42}O_{21}$, $4H_2O$.

This substance occurs in the tubers of *Stachys tuberifera* and in a large number of leguminous seeds.† It forms plate-like crystals, which dissolve readily in water to give a faintly sweet solution, which is dextro-rotatory ($\alpha_D = +148°$). It does not reduce Fehling's solution. When boiled with dilute mineral acid it yields one molecule each of glucose and levulose, and two molecules of galactose.‡

GENTIANOSE. $C_{18}H_{32}O_{16}$.

This trisaccharide occurs in the roots of *Gentiana lutea.* On hydrolysis by mineral acids it is converted into two molecules of glucose and one of fructose. Hydrolysis by

* Kuhn and von Grundherr : " Ber. deut. chem. Gesells.," 1926, **59**, 1655. Zemplen : *id.*, 2230, 2539. Leitch : " J. Chem. Soc.," 1927, 588.

† Tanret : " Compt. rend.," 1912, **155**, 1526.

‡ Planta and Schulze : " Ber. deut. chem. Gesells.," 1891, **24**, 2705.

means of dilute acids breaks it up into one molecule of fructose and one of gentiobiose (see p. 119), while *Aspergillus niger* resolves it into one molecule of glucose and one of sucrose. Gentianose does not reduce Fehling's solution.

SUGARS OF UNKNOWN MOLECULAR WEIGHT OR SUGAR-LIKE POLYSACCHARIDES.

Of these sugars lupeose and agavose are examples. The former, which occurs in lupin seeds, does not reduce Fehling's solution, and on hydrolysis yields galactose, fructose, and glucose. It is supposed to be a tetrasaccharide.*

Agavose, obtained from *Agave americana*, is an optically inactive sugar of unknown constitution which reduces Fehling's solution.†

ABNORMAL OR ILL-DEFINED SUGARS.

Buston and Schryver ‡ have isolated from cabbage leaves a substance whose formula is $C_3H_8O_4$ and to which they assign the constitution CH_2OH . CHOH—O—CH_2OH. They suggest that this sugar may be produced by the condensation of formaldehyde with glycollic aldehyde and thus may be regarded as a simple disaccharide. It does not reduce Fehling's solution, is not hydrolysed by acids, and does not react with phenylhydrazine to form an osazone.

ESTIMATION OF SUGARS.

A. *VOLUMETRIC METHODS.*

I. ESTIMATION BY MEANS OF FEHLING'S SOLUTION.

The principle of this method lies in the fact that certain sugars are capable of reducing copper sulphate in hot alkaline solutions to cuprous oxide, the presence of which is indicated by a yellow-red precipitate.

Fehling's solution is made up in two solutions :—

A, containing 69·28 grams of pure crystallized copper sulphate in 1 litre of distilled water.

B, containing 350 grams of Rochelle salt and 100 grams of caustic soda in 1 litre of distilled water.

* Schulze : "Ber. deut. chem. Gesells.," 1910, **43**, 2233.
† Michaud and Tristan : "Amer. Chem. J.," 1892, **14**, 548.
‡ Buston and Schryver : "Biochem. Journ.," 1923, **17**, 470.

The solution A must be made up very accurately, whereas the quantities required for solution B need only be roughly weighed.

For use, 5 c.c. of A are mixed with 5 c.c. of B; the mixture is a deep blue colour, and is known as Fehling's solution. If correctly compounded, 10 c.c. of the solution contain ·11 gram of cupric oxide, which is able to oxidize ·05 gram of glucose.

This value is sufficiently correct for general purposes; it is, however, an approximation, and varies for different sugars, the factor for levulose, for instance, is ·05144, whilst that for invert sugar is ·0475. If it be desired to obtain very accurate results, it is better to standardize the solution by titrating 10 c.c. with a solution of glucose of known strength. Such a solution may be obtained by dissolving ·95 gram of pure crystallized cane sugar in 500 c.c. of distilled water and boiling for fifteen to twenty minutes with 2 c.c. of concentrated hydrochloric acid. The solution must then be neutralized by the addition of solid sodium carbonate, and made up to 1 litre; 50 c.c. of this solution contain ·05 gram of glucose, and should reduce exactly 10 c.c. of Fehling's solution.

Plant extracts frequently contain tannins and other substances which may interfere with Fehling's solution; in such cases, and likewise when the solution to be titrated is coloured, a preliminary treatment or "clarification" is necessary.

For this purpose 50 c.c. of the solution contained in a 100 c.c. graduated flask should be treated with the minimum possible amount of basic lead acetate * (carefully added until no further precipitate is formed) and then a little alumina cream.† The amounts of these reagents to be employed will naturally vary in different circumstances, though in the

* Excess of basic lead acetate is to be avoided, since a loss of levulose is liable to occur especially if this sugar is left in contact with the basic acetate for some time; there is, however, little danger of loss from this cause if excess is avoided.

† Prepared by adding a slight excess of dilute ammonia to a saturated solution of alum, and then adding more alum until the reaction becomes slightly acid.

case of relatively pure solutions of suitable strength, 1-5 drops of basic acetate followed by 3-5 c.c. of alumina cream should suffice. After the addition of these reagents, the mixture is thoroughly shaken and the precipitate allowed to settle after making up nearly to the graduation mark with water; any froth which may have formed is broken by addition of a drop of alcohol and water is then added up to the mark. After once more shaking the liquid is allowed to settle and then filtered through a dry filter paper. Such a solution is then ready for direct measurement by a polariscope, but if required for titration by Fehling's solution it must be freed from lead by hydrogen sulphide, and after filtering off the lead sulphide, the hydrogen sulphide must be removed.

Estimation of Pentoses.

When pentoses alone are present they may be estimated by determining their reducing power of Fehling's solution as in the case of glucose. The values for arabinose and xylose in terms of copper oxide have been determined by Daish,* working under the standard conditions laid down by Brown, Morris, and Millar †; the tables may be consulted in Daish's paper. If pentoses are mixed with other carbohydrates, or are present in the form of pentosanes, other methods must be used (see p. 137).

Estimation of Reducing Sugars.

The following precautions must be taken in estimating reducing sugars by these and similar methods involving the reduction of metallic salts:—

1. Any substances such as tannins which may have the power of reducing the salts used in titration must be removed.

2. The strength of the sugar solution must be weak, because the reducing power of sugar varies with the concentration, hence it is best to titrate a solution of about the same strength as that used for the standardizing of Fehling's

*Daish: "J. Agric. Sci.," 1914, **6**, 225.
†Brown, Morris, and Millar; "J. Chem. Soc.," 1897, **71**, 105.

solution. This necessitates preliminary estimation; should the strength of the solution be much above this point, add a known volume of water until the strength approximates 0·5 per cent.

The titration, which should be completed as rapidly as possible in order to avoid reoxidation of the solution by the air, is performed as follows:—

Five c.c. of each of the solutions A and B are placed in a white porcelain basin and 40 c.c. of water added; the mixture is then boiled. The sugar solution is placed in a burette and is run into the hot copper solution about 3 c.c. at a time; after each addition the solution is boiled and the precipitate allowed to settle before the next addition is made. When the blue colour has disappeared, the amount of sugar solution used is noted.

A second titration is then carried out, and all the sugar required, less 1 c.c., to effect complete reduction, is run in at once; should this prove too small an amount of sugar, more is added drop by drop until decolorization results. The process is repeated until two readings are obtained which do not differ one from the other by more than 0·2 c.c., the one being a little too high and the other a little too low; the mean of these gives the correct result.

The chief difficulty in the titration lies in the detection of the end point; * this may be ascertained by allowing the precipitate to settle, and then tilting the basin so as to view the clear liquid against the white of the dish. But if the observer's colour-sense be not very critical, an error is easily made, hence various methods have been suggested to determine accurately the end point.

1. Filter off a small quantity of the solution, acidify it with acetic acid and add a little potassium ferrocyanide; the presence of unreduced copper is indicated by the formation of a brown coloration or precipitate of copper ferrocyanide.

2. Ling's reagent consists of 1 gram of ferrous ammonium sulphate and 1·5 gram of ammonium sulphocyanide dissolved

* See also Laue and Eynon: "J. Soc. Chem. Ind.," 1923, **42**, 32; 1925, **44**, 150.

in a mixture of 10 c.c. water and 2·5 grams of strong hydrochloric acid. The solution is decolorized immediately before use by adding a few pieces of granulated zinc. A dozen drops of the reagent are placed separately on a glazed white porcelain plate and a drop of the titration mixture is, from time to time, added to one of the drops; when no pink colour is produced, the titration is complete.

3. Harrison's indicator is made by adding a little starch paste to 100 c.c. of 10 per cent solution of potassium iodide; as this solution will not keep more than a few hours, it must be freshly prepared. One c.c. of the indicator is acidified by the addition of 10 drops of acetic acid and a little of the titration mixture is added. The presence of unreduced copper is indicated by the appearance of a red or blue colour; the absence of any colour marks the end of the reaction.

EXAMPLE.—Amount of sugar solution required to decolorize 10 c.c. of Fehling's :—

11·7 c.c.	1st reading.
11·5 c.c.	2nd ,,
11·6 c.c.	Mean.

Now since

10 c.c. Fehling's ≡ ·05 gram glucose

∴ 11·6 c.c. of the solution contained ·05 gram glucose.

∴ 100 c.c. ,, ,, $\frac{\cdot 05 \times 100}{11 \cdot 6}$,,

= 4·31 per cent.

Estimation of Galactose and Mannose.

The procedure is exactly the same as for glucose :—

10 c.c. Fehling's ≡ ·0511 gram galactose ≡ ·04307 gram mannose.

Estimation of Cane Sugar.

Cane sugar does not reduce Fehling's solution; it is therefore necessary to invert it in order to make the estimation. To do this, take a known volume of the sugar solution and add a sufficiency of strong hydrochloric acid to make it about a 10 per cent solution of the acid; heat on a water bath for about a quarter of an hour, at 70° C.* Then cool, neutralize with sodium carbonate and make up to a known volume and titrate.

*Alternatively, add citric acid crystals to bring up to a 10 per cent solution and boil for ten minutes.

The inversion of cane sugar may be represented thus :—

$$C_{12}H_{22}O_{11} + H_2O = C_6H_{12}O_6 + C_6H_{12}O_6$$

The molecular weight of cane sugar is 342, and the amount of invert sugar this will give on inversion is, from the equation, 360. In other words, 1 gram of glucose corresponds to $\frac{342}{360}$ = ·95 gram of cane sugar. The titration result must therefore be multiplied by ·95 ; otherwise stated :—

10 c.c. Fehling's ≡ ·0475 gram sucrose.

Estimation of Maltose.

The reducing power of maltose is only 62 per cent of that of glucose, hence since 1 gram maltose has the same reducing power as 0·62 gram glucose, the equivalent of 10 c.c. Fehling's solution, which is 0·05 gram glucose, will be $\frac{·05}{·62}$ = ·081.

Hence 10 c.c. Fehling's ≡ ·081 gram maltose.

An alternative method of estimating maltose, suitable in the presence of glucose, is to find the number of cubic centimetres of Fehling's solution equivalent to 100 c.c. of the maltose solution before and after hydrolysis ; this number of cubic centimetres × ·005 gives the number of grams of glucose which, when multiplied by 2·32, gives the number of grams of maltose.

The figure 2·32 is arrived at from the following considerations :—

From the equation representing the inversion of maltose, it may be found that 1 gram of maltose gives 1·05 gram of glucose ; and, as 1 gram of maltose has the same reducing power as ·62 gram of glucose, it follows that 1 gram of maltose after inversion gives an increased reducing power, viz. :—

$$1·05 - ·62 = ·43 \text{ gram glucose},$$
$$\therefore ·43 \text{ gram glucose} = 1 \text{ gram maltose},$$
$$\text{and } 1 \text{ gram glucose} = \frac{1}{·43} \text{ gram maltose},$$
$$= 2·32 \text{ grams maltose}.$$

The above method of acid hydrolysis which requires heating at 100° for ninety minutes, cannot be employed if cane sugar or levulose are present, since it is not possible to hydrolyse maltose completely even at 70° in presence of cane sugar or levulose without destroying a considerable quantity

of the levulose. The most accurate way of estimating maltose is by one or other of the gravimetric methods indicated below, or by the method of Brown, Morris, and Millar.*

Estimation of Mixtures of Sugars.

To illustrate the application of the above methods in the analysis of mixtures of sugars the following examples are given :—

GLUCOSE AND SUCROSE.

1. Take 100 c.c. of the mixture and titrate with Fehling's solution.

2. Invert 100 c.c. of the mixture by the method given, and titrate.

The first operation gives the amount of glucose = a.

The second operation gives the original amount of glucose together with that due to the inversion of the cane sugar = b.

$$\therefore (b - a) \times \cdot 95 = \text{sucrose.}$$

GLUCOSE AND MALTOSE.

Proceed exactly as for glucose and sucrose :—

a = amount of sugar before inversion.
b = amount of sugar after inversion.

From the reasons already given under maltose, it follows that—

$$(b - a) \times 2 \cdot 32 = \text{maltose,}$$
$$\text{and } a - (\text{maltose} \times \cdot 62) = \text{glucose.}$$

CANE SUGAR AND MALTOSE.

Cane sugar is inverted by citric acid, while maltose is not ; this fact may be made use of in the estimation :—

1. Add to 100 c.c. of the solution 5 grams of crystallized citric acid, and heat on the water bath for about one hour. Neutralize and titrate.

$$\text{Reducing power} = a.$$

2. Completely invert another 100 c.c. of the solution with hydrochloric acid ; neutralize and titrate.

$$\text{Reducing power} = b ;$$
$$\text{then } (b - a) \times 2 \cdot 32 = \text{maltose,}$$
$$\text{and } (a - \text{maltose} \times \cdot 62) = \text{sucrose.}$$

* Brown, Morris, and Millar: "J. Chem. Soc.," 1897, **71**, 105.

GLUCOSE, CANE SUGAR, AND MALTOSE.

1. Take 100 c.c. of the solution and titrate. The result includes the glucose together with maltose.

Reducing power = a.

2. Take another 100 c.c. of the solution, invert with citric acid, and then titrate. The result includes the glucose, and the invert sugar obtained from the cane sugar, together with maltose.

Reducing power = b.

3. Take a final 100 c.c. of the solution, and completely invert with hydrochloric acid. The result represents the whole of the sugars.

Reducing power = c.

Following the same reasoning as before :—

$(b - a) \times \cdot 95$ = cane sugar,
$(c - b) \times 2 \cdot 32$ = maltose,
and a − (maltose × ·62) = glucose.

II. ESTIMATION BY MEANS OF PAVY'S SOLUTION.

The chief disadvantage connected with the use of Fehling's solution in the estimation of glucose is the difficulty in observing the end point of the titration owing to the red precipitate of cuprous oxide: moreover, if the solution to be titrated contains ammonium salts, the cuprous oxide will not be precipitated. These objections may be overcome by using Pavy's solution, which contains ammonia which dissolves the cuprous oxide with the formation of a colourless solution. As before, two solutions are necessary.

A. 8·316 grams of pure crystallized copper sulphate are carefully weighed and dissolved in 1 litre of distilled water.

B. 40·8 grams Rochelle salt.
40·8 grams caustic potash.
600 c.c. strong ammonia (·880).
Distilled water to 1 litre.

In making up the mixture B great accuracy is not essential. For titration 25 c.c. of A (very accurately measured) are

mixed with 25 c.c. of B. The complete reduction of 50 c.c. of Pavy's solution is effected by ·025 gram of glucose.

Pavy's solution may also be prepared from Fehling's solution as follows: 120 c.c. of Fehling's are mixed with 300 c.c. of strong ammonia (·880) and 400 c.c. of 12 per cent potash solution. The mixture is then made up with distilled water to 1 litre.

Method.—Fit a 250 c.c. flask with a well-fitting cork bored with two holes, one to contain an outlet tube and the other the nozzle of the burette. Pour into the flask 50 c.c. of Pavy's solution and 50 c.c. of distilled water; mix thoroughly and introduce a little powdered glass. Dilute the sugar solution with a 10 per cent solution of ammonia, in order that 50 c.c. shall be about equivalent to 50 c.c. of the Pavy solution. Bring the Pavy solution to the boil by means of a small flame, and run in the sugar solution 1 c.c. at a time. Having thus roughly ascertained the amount of sugar required, accurate readings are to be obtained by running in nearly all the requisite sugar at once, and then drop by drop until the end point is reached.

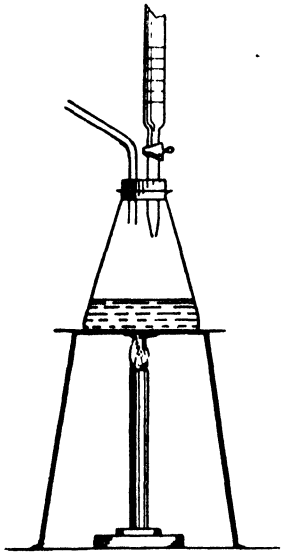


FIG. 3.

The following precautions are very important:—

1. The operation must be carried out rapidly, else all the ammonia is driven off and the cuprous oxide is precipitated.

2. The Pavy solution must be boiling throughout the titration, else air will enter the flask, owing to the lowered temperature, and the solution of cuprous oxide will be oxidized.

III. ESTIMATION BY MEANS OF BENEDICT'S SOLUTION.

In this method the difficulty of the red precipitate of cuprous oxide obscuring the end point is overcome by carrying out the reduction in the presence of potassium thiocyanate whereby the cuprous oxide is converted into an insoluble

white compound, and thus the disappearance of the last trace of blue colour from the solution is easy to observe.

The solution is prepared as follows :—

200 grams sodium citrate.

200 grams crystallized sodium carbonate or 75 grams of the anhydrous salt.

125 grams potassium thiocyanate

are dissolved in water, made up roughly to 800 c.c., and filtered.

Eighteen grams of pure crystallized copper sulphate dissolved in 100 c.c. of water are poured slowly with constant stirring into the above solution. Five c.c. of a 5 per cent solution of potassium ferrocyanide are now added as a further precaution against the formation of cuprous oxide, and the whole is then carefully made up to 1000 c.c.

The above solution, which will keep indefinitely without any special precautions, is of such a strength that

25 c.c. = 0·05 gram glucose.

The titration is performed as follows :—

Twenty-five c.c. of Benedict's solution are placed in a 4 oz. flask with 3 or 4 grams of anhydrous sodium carbonate and a few lumps of broken porcelain to prevent bumping ; the mixture is kept boiling vigorously while the sugar solution is run in until the blue colour just disappears. The sugar solution may be run in rapidly at first, but towards the end it should be run in drop by drop.

The volume of solution run in contains the equivalent of 0·05 gram glucose from which the strength may be calculated.

This method is easier to work with than Fehling's solution, and gives very accurate results.

ESTIMATION BY BERTRAND'S METHOD.

A method for the volumetric estimation of reducing sugars has also been worked out by Bertrand. This method has acquired considerable vogue owing to the ease of determining the end point since it depends upon the titration with potassium permanganate of the ferrous salt produced by the reducing action of cuprous oxide upon ferric sulphate.

The solutions required are as follows :—

A. Copper solution containing 40 grams of crystallized copper sulphate in 1 litre of water.

B. Alkaline tartrate solution containing 200 grams of sodium potassium tartrate, and 150 grams of sodium hydroxide in 1 litre of water.

C. Ferric sulphate solution containing 50 grams of ferric sulphate, and 200 grams of sulphuric acid in 1 litre of water.

D. Potassium permanganate solution containing 5 grams per litre.

The solution C should not reduce permanganate; if it does, permanganate solution should be added drop by drop until a faint permanent pink remains.

Twenty c.c. of the sugar solution to be titrated, which should contain not more than 90 milligrams in that volume, are measured into a conical flask of about 150 c.c. capacity; 20 c.c. each of the solutions A and B are added, and the mixture is boiled for exactly three minutes; the precipitate is then allowed to settle for a few seconds and is then filtered through an asbestos plug contained in a narrow vertical tube attached to the cork of a filter flask; the filtrate should be distinctly blue; the absence of a blue colour indicates that too much sugar has been used and the experiment will have to be repeated, using a diluted sugar solution. The precipitated cuprous oxide should be washed by decantation and finally transferred to the asbestos plug; after throwing away the filtrate and washing out the filter flask, the tube containing the asbestos plug with the cuprous oxide is replaced on the filter flask and 5-20 c.c. of the ferric sulphate solution C are then poured into the original boiling flask to dissolve any adhering cuprous oxide. The resulting solution is poured on to the cuprous oxide on the asbestos pad, and drawn into the filter flask; after washing out the boiling flask and the asbestos, the combined filtrate and washings in the filter flask are titrated with the permanganate solution D.

The copper value of the permanganate is determined by accurately standardizing the permanganate solution by means of oxalic acid; knowing that 1 gram of $KMnO_4 \equiv 2{\cdot}01$ grams

of Cu, the amount of copper equivalent to each cubic centimetre of the permanganate may be calculated. Tables have been drawn up giving the copper equivalents of glucose, invert sugar, mannose, galactose, sorbose, rhamnose, arabinose, xylose, maltose and lactose; these may be found in the original paper of Bertrand or in the text-book of "Practical Biological Chemistry," by Bertrand and Thomas.*

B. *GRAVIMETRIC METHODS.*

Estimation of Pentoses.

The ease with which furfural can be produced from pentoses has led to the following method of estimation, which is due to Kröber †:—

A weighed quantity of substance ‡ (usually about 5 grams) is placed in a 300 c.c. flask provided with a cork bored with two holes, through one of which passes a tap-funnel, and through the other a splash preventor, such as is used in a Kjeldahl distillation. Through the tap-funnel 100 c.c. of hydrochloric acid (sp. gr. 1·06, containing about 12 per cent HCl) are then added, and the contents of the flask are distilled briskly; when 30 c.c. have passed over, the distillation is interrupted and the contents of the receiver are poured into a beaker with a 400 c.c. graduation mark; a fresh quantity of 30 c.c. of hydrochloric acid (sp. gr. 1·06) is now added through the tap-funnel, and the distillation is continued until 30 c.c. more have distilled over; the new distillate is again transferred to the beaker, 30 c.c. more acid are added to the flask, and the whole process is repeated; altogether about a dozen distillations, each lasting ten minutes, are required to carry over the last traces of furfural. In order to ascertain whether the distillate still contains furfural, a drop of the liquid is placed on a filter paper next to a drop of aniline acetate solution; § if no

* Bertrand and Thomas: "Practical Biological Chemistry." Translated by Colwell, London, 1920.

† Kröber: "J. Landw.," 1900, **48**, 357, and 1901, **49**, 7. Tollens: "Zeit. physiol. Chem.," 1902, **36**, 239.

‡ The amount chosen should be sufficient to produce from ·03 to 0·3 gram of phloroglucide.

§ This is best prepared, according to Tollens, by shaking up equal volumes of aniline and water in a test tube and adding glacial acetic acid drop by drop until the turbid solution suddenly becomes clear.

red colour appears when the two liquids come in contact with each other, the solution is free from furfural, and the distillation can be discontinued.

The furfural contained in the united distillates is then precipitated from solution by means of phloroglucinol which reacts according to the equation—

$$\underset{90}{C_5H_4O_2} + \underset{126}{C_6H_6O_3} = C_{11}H_6O_3 + 2H_2O$$

To this end about the amount of phloroglucinol * likely to be required by the furfural obtained is dissolved in hydrochloric acid (sp. gr. 1·06), and added to the furfural solution, and the total volume is then made up to 400 c.c. with more of the same acid. The solution at once turns yellow, then becomes turbid, and, on the next day, the greenish-black precipitate of the phloroglucide is filtered off on to a tared Gooch crucible; the precipitate is washed with 150 c.c. of water, dried for four hours at 97°, then cooled in a desiccator and weighed in a weighing bottle.† From the weight (*a*) of the precipitate, which under ordinary conditions should lie between 0·03 and 0·3 gram, the weight of furfural, pentose, or pentosane may be calculated by substituting the value of (*a*) in one of the following formulæ :—

a lies between 0·03 and 0·3 gram.

Furfural = (*a* + ·0052) × ·5185
Pentose = (*a* + ·0052) × 1·0075
Pentosane = (*a* + ·0052) × ·8866

in which ·0052 is the weight of phloroglucide, which remains in solution under the conditions of the experiment as given above.

If the precipitate weighs less than 0·03 gram or more than 0·3 gram, one of the following formulæ must be employed :—

Weight of precipitate $<$ ·03 gram.	Weight of precipitate $>$ 0·3 gram.
Furfural = (*a* + 0·0052) × 0·517	Furfural = (*a* + 0·0052) × 0·518
Pentose = (*a* + 0·0052) × 1·017	Pentose = (*a* + 0·0052) × 1·0026
Pentosane = (*a* + 0·0052) × 0·8949	Pentosane = (*a* + 0·0052) × 0·8824

* The phloroglucinol employed must be pure. To ascertain this, test as follows : Dissolve a small quantity in a few drops of acetic anhydride, heat almost to boiling, and add a few drops of concentrated sulphuric acid; a violet colour indicates the presence of diresorcinol; if more than a faint coloration appears, the sample should be rejected.

† This is necessary to prevent the phloroglucide, which is hygroscopic, from absorbing moisture.

According to Böddener and Tollens,* a considerable saving in time may be effected by precipitating the phloroglucide in hot solution, i.e. between 80 and 85°. The reaction then takes place according to the equation—

$$C_5H_4O_2 + C_6H_6O_3 = C_{11}H_4O_2 + 3H_2O$$

so that the precipitate actually weighs less than the one produced in the cold; the precipitation is, however, complete in from one and a half to two hours. The weight of furfural corresponding to the precipitate so obtained may be calculated by adding ·001 (to allow for the phloroglucide remaining in solution) and multiplying the resulting figure by 0·571. The number so obtained if multiplied by 1·935 gives the corresponding amount of pentose or if multiplied by 1·703 gives the amount of pentosane. The method is, however, not suitable if it is desired to estimate the methyl-pentosans as distinct from the pentosans, in which case Kröber's method as modified by Ellett † and Mayer ‡ should be employed.

Reducing Sugars Other than Pentose.

Various gravimetric methods of estimating reducing sugars have been suggested; the outstanding feature of all these methods is that they yield reliable results only if carried out under strictly controlled conditions. One of the most reliable methods is that of Brown, Morris, and Millar; § the precipitated cuprous oxide is washed, dried, and weighed after conversion into cupric oxide by ignition; || from the weight of cupric oxide obtained, the equivalent weight of either dextrose, levulose, invert sugar, or maltose may be determined by reference to tables which will be found in the original paper.

Compared with Brown, Morris, and Millar's method, that of Allihn,¶ once extensively used, is more cumbersome. The

* Böddener and Tollens: "J. Landw.," 1910, **58**, 232.

† Ellett: *id.*, 1905, **53**, 13.

‡ Mayer: *id.*, 1907, **55**, 261.

§ Brown, Morris, and Millar: "J. Chem. Soc.," 1897, **71**, 94.

|| Alternatively, the cuprous oxide may be reduced in a current of hydrogen and weighed as copper.

¶ Allihn: "J. prak. Chem.," 1880, [2], **22**, 63.

official method of the American Association of Official Agricultural Chemists is that devised by Munson and Walker.*

Estimation of Glucose as Osazone.

The following method of estimating glucose as osazone in the products of the action of malt upon starch is recommended by Davis and Ling: † 20 c.c. of solution containing 2-3 grams of starch products per 100 c.c. are mixed with 1 c.c. of phenylhydrazine and 1·5 c.c. of 50 per cent acetic acid. After heating for an hour ‡ over a water bath, the liquid, which has by this time evaporated to a small bulk, is filtered through a tared Gooch crucible, and the crystalline osazone is washed with 20-30 c.c. of boiling water, so that the total filtrate does not exceed 50 c.c.; the precipitate is then dried in a steam oven and weighed; under these conditions, 0·1 gram of glucose gives 0·0505 gram of glucosazone.

Estimation of Natural Mixtures of Sugars.

Several methods § have been devised for the estimation of the constituents of sugar mixtures, such as occur in plant extracts and in fermentation liquors, use being made of yeasts to ferment away sugars and of enzymes, such as invertase, to hydrolyse disaccharides, etc.‖

The following method is that described by Davis, Daish, and Sawyer: ¶ The freshly plucked leaf material is dropped into boiling 95 per cent alcohol to which a little 0·88 ammonia is added, to destroy the enzymes. This leaf material is placed in a Soxhlet and is extracted with the same alcohol for eighteen to twenty hours. The extract, after evaporation to a small bulk under reduced pressure, is made up to a known volume with water. A portion is evaporated to dryness for the determination of the dry weight, and the remainder is precipitated with basic lead acetate, filtered, and made up to

* Munson and Walker: "J. Amer. Chem. Soc.," 1907, **29**, 541.

† Davis and Ling: "Journ. Chem. Soc., Lond.," 1904, **85**, 24.

‡ The heating should not be continued for more than one hour.

§ See also "Note," p. 514.

‖ Davis: "J. Soc. Chem. Ind.," 1916, **35**, 201. Nanji and Beazeley: *id.*, 1926, **45**, 220.

¶ Davis and Daish: "J. Agric. Sci.," 1913, **5**, 437. Davis, Daish, and Sawyer: *id.*, 1916, **7**, 255.

a known volume; this is solution A. A portion of this solution is freed from lead by sodium carbonate and made up to a known volume; this is solution B. Solution B is divided into portions: (1) For the direct determination of the reducing power due to dextrose, levulose, maltose, and pentose, and also the combined rotation. (2) For determining sucrose by inverting with invertase,* and with 10 per cent citric acid; each of these values should agree closely. (3) For the determination of pentoses by the Kröber method; and (4) For the estimation of maltose. To do this 50 c.c. of the solution are made slightly acid with hydrochloric acid, and hydrogen sulphide is bubbled through in order to remove the last traces of lead. Any precipitate is filtered off, and a current of air is passed through the solution to remove the sulphuretted hydrogen. The resulting solution should be absolutely free from lead, else the yeast will not grow in it, and faintly acid to litmus. To it are added 5 c.c. of yeast water and the mixture sterilized by twenty minutes heating at 115-120° C.; when cool it is inoculated with a little yeast and incubated at 25° C. for three or four weeks.† On the completion of fermentation, 5 c.c. of alumina cream are added to clarify the solution, and the whole is well boiled; it is then filtered and the precipitate washed until the filtrate and washings measure 100 c.c. An aliquot portion is used for determining the reducing power. The yeast must be a pure strain free from maltase, thus all sugars except maltose are fermented away.

C. *POLARIMETRIC METHODS.*

The presence of an asymmetric carbon atom confers upon a compound the property of optical activity, by which is meant the power of the substance to rotate to the right or to the

* The invertase required for this purpose is prepared by washing fresh-pressed beer yeast, to remove adherent wort, packing it into a large wide-mouthed bottle and adding 30-50 c.c. of toluene, which percolates through the mass. The bottle, covered with a sheet of paper, is left in a warm place at a temperature of 25°-30° C. At the end of a fortnight, nearly the whole is liquefied; it is then filtered on a Buchner funnel. The filtrate yields a highly active preparation of invertase, free from maltase and zymase.

† 0·2 to 0·5 gram of cane sugar are completely fermented in about three weeks in these conditions.

left the plane of a beam of circularly polarized light passing through it.

The polarimeter is much used in ascertaining the strength of sugar solutions, but before describing the mode of using it, it is desirable to consider briefly the principles which are involved.

When a ray of light enters a crystal of any system other than the cubical, it is broken up into two rays, the ordinary and the extra-ordinary, provided the beam of light is not coincident with the optical axis of the crystal. This phenomenon is known as double refraction.

These two rays, the ordinary and the extra-ordinary, do not behave similarly; the former conforms to the ordinary laws of refraction, but the latter does not; further, the two rays are polarized in directions at right angles to one another.

In order to make use of these facts, it is necessary to be able to examine the extra-ordinary ray alone; that is, the two rays must be separated one from the other. This is effected by a Nicol's prism, which consists of two plates of Iceland spar fixed together by means of Canada balsam. A ray of light enters one side of the prism, and is broken up into the ordinary and the extra-ordinary ray; on reaching the layer of balsam, the former is totally reflected, whilst the latter passes on through the other plate and emerges at the side opposite to its entry. If a second Nicol be placed in the path of this ray, the latter will pass through in different amounts according to the angle which the second prism makes with the first. If the interposed Nicol be parallel to the first Nicol, the ray will pass through entirely; if the second Nicol be rotated, the light passing through will be less and less in amount until, when the two prisms are at right angles to each other, no light passes at all. If the rotation be continued, the light will again pass through in gradually increasing quantities until the prism has been rotated through an angle of 180° from its original position, when the whole light will again pass through freely.

Many liquids and solutions of solids possess what is known as optical activity, which means that they can rotate the plane

of vibration of a ray of polarized light passing through them; so that, on emergence from the liquid, the new plane is inclined either to the right or to the left of the original plane.

This is known as the rotation of the plane of polarized light.

Laurent's Half-Shadow Polarimeter.—This apparatus consists of a tube containing two Nicol's prisms, of which one is fixed and is known as the polarizer, while the other can be rotated and is called the analyser. A quartz plate which covers half the field of vision is fixed just behind the polarizer.

The liquid or solution to be examined is contained within a glass tube with polished ends, and is placed in position between the quartz plate and the analyser. The analyser is fixed in a tube which can be rotated, the degree of rotation being read from a divided circle. Leaving out of consideration the quartz plate, the beam of polarized light passes through the liquid and so becomes rotated; it follows, therefore, that the vibration plane of the analyser will no longer be at right-angles to the plane of polarization of the light striking it, therefore light will enter the analyser, and in order to bring about complete extinction, the analyser must be rotated either to the right or to the left. This angle of rotation is a measure of the optical activity of the substance under observation, and according to the direction of rotation, the substance is termed dextro- or lævo-rotatory. In Laurent's polarimeter the illumination is obtained from a sodium flame, and this light before entering the tube containing the liquid must pass through the plate of quartz. When the instrument is set in the zero position, the whole field is equally illuminated, but on introducing the liquid, one-half of the field becomes the darker; equal illumination can be obtained by rotating the analyser. If this position be passed, the field is once more unequally illuminated, but in a reverse manner, that is to say, the half which was originally dark is now light, and vice versa.

As the exact position of equal illumination is somewhat difficult to determine, several readings should be made and the mean of these taken as the correct value.

The *specific rotation* of a substance is defined as the angular rotation produced by a column of liquid 1 dm. in length, which contains 1 gram of the active substance in each cubic centimetre. This quantity is expressed by the symbol α_D^{20}, the numeral indicating the temperature at which the measurements were made, and the letter D standing for the yellow line of the sodium flame which is used as the source of illumination. The use of this quantity α_D for determining the number of grams of active substance in a given solution will be rendered apparent from the following considerations.

Supposing we have a solution containing an unknown number of grams, m, of active substance per c.c., and we fill a tube of length l dm.* with this solution and observe its angular rotation to be α.

If a layer 1 dm. long containing 1 gram $\left\{\begin{matrix}\text{of substance in 1 c.c. of} \\ \text{liquid produces a rotation}\end{matrix}\right\}$ α_D

Then " l " " " 1 " " " $l\alpha_D$

$\therefore$ " l " " " m " " " $ml\alpha_D$

And this would be the observed angle of rotation (α).

$$\therefore \alpha = m \times l \times \alpha_D$$

$$\therefore m = \frac{\alpha}{l \times \alpha_D}.$$

The angle of rotation is determined as follows:—

1. Find the zero reading when no solution is between the polarizer and analyser. For this purpose the mean of at least three readings, differing by only two or three minutes, should be taken.

2. Fill the tube with the liquid, taking care to avoid the introduction of air-bubbles.

3. Insert the tube and determine the new reading at which the illumination of both halves of the field is equal. The mean of three readings should again be taken.

The difference between the initial and the final readings is the angle of rotation.

The following experiment performed on a solution known to contain glucose may be quoted in illustration of the method:—

* The length of the tube must be expressed in *decimetres*.

Initial reading of polariscope, without any solution = 0° 30′
Final ,, ,, ,, with glucose ,, = 3° 45′
Difference (α) = 3° 15′ or 3·25°
Length of tube containing the solution (l) = 2 dms.
Specific rotation of glucose ($[\alpha]_D^{20}$) = 52·5°

$$\text{From which } m = \frac{3{\cdot}25}{2 \times 52{\cdot}5} = {\cdot}0309$$

∴ the strength of the solution is 3·09 per cent.

It is of course obvious that correct values can only be obtained by this method on the assumption that the liquid contains only a single optically active substance. Plant extracts should be treated with lead acetate in the manner described above.

Some substances, e.g. glucose, exhibit the phenomenon of muta-rotation, that is to say, the rotation of their solutions varies according to the length of time that they have been made up; the maximum rotation is given by a freshly-made solution, but the rotatory power gradually decreases until it finally becomes steady. The attainment of the final condition is greatly accelerated by warming the solution in the presence of a little alkali, but the solution must of course be cooled before a reading is taken.

FURTHER REFERENCES.

Armstrong: "The Simpler Carbohydrates and Glucosides," London, 1924.

Mackenzie: "The Sugars and their Simpler Derivatives," London, 1913.

Cramer: "Les sucres et leurs Dérivés," Paris, 1927.

POLYSACCHARIDES.

The second great group of carbohydrates, namely the non-sugars or polysaccharides, are substances of high molecular weight, mostly amorphous and insoluble in water. Like the di- and tri-saccharides, the polysaccharides on hydrolysis break up into sugars containing five or six carbon atoms, and they may therefore be looked upon as anhydrides of these substances.

In the absence of any exact knowledge regarding their molecular weights, their formulæ are written $(C_6H_{10}O_5)_n$ or

$(C_5H_8O_4)_n$ according as they give rise to hexoses or pentoses on hydrolysis. The value of "*n*" has not been determined as yet for any particular case, but there is reason to believe that it is fairly high. The various methods adopted for the elucidation of this point have led to such widely different results that a description of them here would not serve any useful purpose.

HEXOSANS.

The general formula for all substances belonging to this group is $(C_6H_{10}O_5)_n$, which indicates that on hydrolysis they yield hexoses; for this reason they may be termed hexosans.

GLUCOSANS.

Starch or Amylum.

Starch is one of the most widely distributed substances in the vegetable kingdom; it may be found in green leaves as a temporary reserve of the photosynthetic products; as a more or less permanent reserve food-material it occurs in seeds and fruits, where it is not infrequently accompanied by other reserves, for instance proteins; in the vegetative parts, such as tubers, the living cells of the pith, medullary rays, and cortex of roots and stems; and also in the latex of certain plants, e.g. *Euphorbia*. When especially stored, the amount of starch may be considerable; thus in cereals it may form from 50 to 70 per cent of the dry weight of the grains, and in potatoes from 15 to 30 per cent of the dry weight of the tubers. As is well known, starch grains from different sources show much variety in size and shape, and occur in association with plastids, in which, as Schimper demonstrated, they have their origin. Not only are the microscopic characters of starch grains of diagnostic value, but the different varieties of starch can be grouped into generic, specific, and varietal classes which correspond with the classification of plants based on the ordinary morphological features.*

Brief mention may be made of the ideas held regarding the physical nature of starch grains. As is well known, the gran-

* Reichert: "Amer. Journ. Bot.," 1916, 3, 91.

ules not infrequently exhibit a more or less well-marked stratification which years ago was thought to correspond to the alternation of day and night.

The " apposition " theory held that new layers were added to those already formed, each layer being separated from the next by a thin film of air. Nägeli, on the other hand, came to the conclusion that the lamellation was due to the differences in the water-content of the several layers, and that the grain was made up of minute particles, the so-called micellæ. He held the view that the growth of the grain took place not by apposition but by a process of intussusception, that is to say, new material was intercalated between the micellæ, and either gave rise to new micellæ, or was used up in increasing the size of the old ones. Schimper expressed the idea that the grains were really of a sphæro-crystalline nature, which view was modified by Meyer, who says that the grain is made up of two kinds of needle-shaped crystals composed respectively of α- and β-amylose ; he also states that in those grains which are coloured red with iodine, for example, those found in the cells of the root-cap of *Allium Cepa*, in the seed-coats of *Chelidonium* and in *Oryza sativa*, var. *glutinosa*, dextrin and amylo-dextrin occur. On the other hand, the ordinary grains which are coloured blue with iodine, are made up almost entirely of sphæro-crystals of amylose arranged in layers.

According to Kraemer,* the starch grains of the potato are composed of colloid and crystalloid substances arranged in lamellæ which are distinct and separate one from the other. At the point of origin of growth, the hilum, and in the alternate lamellæ, the colloid preponderates and is associated with the crystalloid cellulose ; in the other lamellæ the crystalloid granulose is in the greater proportion. He also states that the peripheral layer is elastic and porous, and may be anhydride of cellulose. Dennison also has expressed the view that the outer layer of the grain is different from the more internal parts, and may be a carbohydrate not fully polymerized to starch.

* Kraemer : " Bot. Gaz.," 1902, 34, 341.

The amount of starch present in the leaf varies with the specific physiology of the plant and with the climatic conditions. Thus, in Japan, the starch content of evergreen leaves begins to diminish in November. In January, the coldest month, a minimum is reached, in fact, starch may be entirely absent, and at the end of February an increase begins. Miyake,* the maker of these observations, does not comment on the fat content, wherefore a comparison between his results and those of other workers is not possible (see p. 3).

Many monocotyledonous plants are characterized by the absence of starch, for example *Scilla nutans*, *Phleum pratense*, *Allium*, etc., but in some of these cases starch granules may occur in the guard-cells of the stomates, in the bundle sheath of the leaves, and also in the bulbs at the base of the growing shoots; further, in certain plants which normally form sugar, e.g. *Musa*, *Hemerocallis*, and *Muscari*, starch will appear when much sugar accumulates. On the other hand, many members of this same class of plants are fairly constant starch producers, e.g. *Lilium tigrinum*, *Pontederia cordata*, *Ananas sativa*, *Canna indica*, *Tradescantia virginica*, *Juncus communis*, and *Alisma Plantago*. There are many peculiarities in this occurrence of starch in the Monocotyledons; for instance, in *Scilla nutans* it is absent, whilst in *Scilla siberica* it is quite abundant; further, the former plant, if fed with cane sugar in a solution of suitable strength, does not form it, while, on the other hand, starch-free plants of *Scilla siberica* under the same treatment do form starch, the experiment being carried out in the absence of light. In the Mycetozoa, in which starch is normally absent, starch formation may be induced under the influence of acid and a supply of sugars.† In the plant starch occurs, as is well known, in the form of variously shaped microscopic bodies composed of concentric layers; the granules have an organized structure and possess the power of double refraction.

* Miyake: "Bot. Gaz.," 1902, **33**, 321.
† Boas: "Biochem. Zeit.," 1917, **78**, 308.

Preparation of Starch.

The method of preparation varies according to the source employed. From wheat flour it may be obtained by stirring up this material thoroughly with water, and allowing the mixture to stand until the gluten contained in the flour undergoes fermentation, when it dissolves and may be removed by washing. On a small scale the separation is most conveniently effected by kneading some flour in a muslin bag which is held under a stream of water. The starch granules are hereby washed through the muslin, while the gluten remains behind in the bag as a sticky grey mass.

Starch may also be obtained from potatoes by macerating them with water and separating the non-starchy material from the starch by filtration. The starch is then allowed to settle at the bottom of the water, when it is collected and dried.

Purification.

Malfitano and Moschkoff * give the following method for the purification of starch: A 1 per cent colloidal solution of starch is frozen and then allowed to melt. When melted, most of the starch is deposited in a floccular precipitate, whilst the clear liquid contains some starch and the greater part of the mineral impurities. On repeating the operation four or five times, the purified product yields less than ·02 per cent of ash. Even the purest starch yields on incineration a small amount of ash constituents chiefly of phosphates which were in organic combination with the material (see Amylopectin, p. 152). In addition to phosphates, varying quantities of silica are found in the ash, the amount depending on the source from which the starch was prepared. Silica is not a true constituent of the starch proper, but is associated with another substance, known as amylohemicellulose,† which occurs in greater quantities in some starches than in others, notably in the starches of barley, wheat, rice, tapioca, maize, and sago,‡

* Malfitano and Moschkoff: "Compt. rend.," 1910, **151**, 817.

† Ling and Nanji: "J. Chem. Soc.," 1923, **123**, 2672; 1925, **127**, 652.

‡ Clayson and Schryver: "Biochem. Journ.," 1923, **17**, 493; Schryver and Thomas: *id.*, 497.

while potato and arrowroot starch contain hardly any (cf. Amylohemicellulose, p. 153).

Properties.

Air-dried starch contains a considerable quantity of water, as much as 20 per cent being not uncommon ; it can be made to part with this water by carefully heating to 100°. If heated to about 200° it is converted into a sticky soluble substance, which is probably a mixture of isomeric substances of the empirical formula $C_6H_{10}O_5$, known as British gum or dextrin (q.v.).

Starch is insoluble in cold water, but if dry starch is finely ground for some time in an agate mortar and then stirred up with cold water and filtered through a gravimetric filter paper, such as will retain the finest suspended solids, the filtrate may be shown to have taken up some of the starch in colloidal solution since on addition of a solution of iodine a deep blue coloration results. If a suspension of starch in water is heated, the particles gradually swell and finally burst, forming an opalescent solution, known as starch paste, which is more or less mucilaginous according to the amount of starch employed ; the optimum temperature for bringing about this change varies with the starch as may be seen from the following figures :—

Rye	55° C.
Wheat	62° C.
Maize	68° C.
Rice	72° C.
Potato	72° C.

Too high a temperature tends to the formation of lumps, and it is generally best not to boil the solution but to add a fine suspension of starch in cold water to the requisite amount of warm water heated over a boiling water bath.

A solution of starch so prepared is not to be regarded as a true molecular disperse solution, but as a colloidal solution of soluble amylose thickened by a suspension of the insoluble gelatinizing material amylopectin.

A solution of starch paste undergoes a change on keeping, known as retrogradation, and deposits a white flocculent precipitate which, microscopically, resembles starch. For this

reason, the precipitate has been described as artificial starch. The change is retarded by keeping the paste at 60°. According to Fernbach and Wolff * green malt contains an enzyme " amylocoagulase " which can accelerate the change described. The precipitate is insoluble in hot water and its formation is due to a dehydration and aggregation of the colloidally suspended particles of the original solution ; certain it is that the change is influenced by the presence of electrolytes.†

In order to facilitate the preparation of starch solution for indicator purposes, a number of so-called " soluble starches " have been prepared. These are in reality starch which has been treated in a variety of ways by chemicals whereby it is rendered more soluble, without having suffered a sufficiently profound change to influence its ability to give a blue colour with iodine. Lintner's soluble starch is prepared by exposing starch to the action of 7·5 per cent hydrochloric acid for a week and then washing with cold water until free from acid.

The Composition of the Starch Grain.

Nägeli ‡ was the first to suggest that the starch grain was made up of two distinct constituents, but some years elapsed before his views were supported by reliable chemical evidence. In view of the fact that the terminology employed by the earlier investigators was irregular, a brief historical résumé is desirable before considering the present state of our knowledge of the subject.

The researches of Nägeli have shown that when starch is treated with dilute hydrochloric acid, malt extract, or saliva, a considerable portion goes into solution, leaving a transparent skeleton undissolved. The soluble portion, which gives a blue colour with iodine, Nägeli regarded as the true starch constituent of the granule, and named it granulose ; on the other hand, the undissolved skeleton, which he described as not turning blue with iodine (see below), he considered to be of a cellulose nature, and called it starch cellulose or amylocellulose.

* Fernbach and Wolff : " Ann. Inst. Pasteur," 1904, **18**, 165.
† Samec : " Kolloidchem. Beihefte.," 1912, **3**, 123.
‡ Nägeli : " Die Stärkekörner," Zurich, 1858.

On the other hand, Meyer * was of opinion that starch granules consisted essentially of two substances known respectively as α- and β-amylose. The former, which was insoluble, he regarded as an anhydride which could be converted into the soluble β variety by the action of superheated steam.

He also thought that when starch is acted upon by hydrochloric acid it is converted into amylo-dextrin, and considered that amylo-cellulose, which Nägeli regarded as an original constituent of the starch granule, was in reality identical with amylo-dextrin, and therefore a secondary product of the action of acid on the amylose.

It is to the French workers Maquenne and Roux,† and Fernbach and Wolff ‡ that we owe the first definite ideas concerning the existence of two distinct substances. They stated that starch granules consist of two substances, amylocellulose or amylose, and amylopectin; the term amylocellulose was not equivalent to Nägeli's starch cellulose but to his granulose; we thus get the following equivalents :—

Nägeli.	*Meyer.*	*Maquenne and Roux.*
Outer layer or starch cellulose.	α-Amylose.	Amylopectin.
Inner layer or granulose.	β-Amylose.	Amylocellulose or amylose.

Maquenne first stated that amylose formed 80 per cent and amylopectin 20 per cent by weight of the starch granule; the former substance was described as being soluble in water and giving a blue colour with iodine, while the latter would only swell in water without dissolving, and was erroneously stated to give no colour with iodine.

The isolation of amylopectin was first effected by Gatin Gruzewska,§ who treated starch with 1 per cent caustic soda whereby the granules burst and the amylose constituent entered into solution leaving the swollen outer shells of amylopectin; on neutralizing with acetic acid, the amylopectin shrivels and can be filtered off and washed and dried. Ling

* Meyer: "Unters. ü. d. Stärkekörner," Jena, 1895.

† Maquenne and Roux: "Compt. rend.," 1903, **137**, 88; 1905, **140**, 1303.

‡ Fernbach and Wolff: *id.*, 1904, **138**, 819.

§ Gatin Gruzewska: "Compt. rend. Soc. biol.," 1908, **64**, 178.

and Nanji have furnished further methods for distinguishing between amylose and amylopectin; they find that when starch paste, warmed to 50° C., is treated with precipitated diastase, prepared from ungerminated barley, and dried by means of absolute alcohol, the amylose constituent is converted completely into maltose whilst the amylopectin is hardly acted upon; the maltose may be removed by dialysis leaving the amylopectin. If, on the other hand, precipitated barley diastase is used, which has not been dried by alcohol, amylopectin also is attacked yielding a product to which they give the name $\alpha\beta$-hexa-amylose.

A further distinction between amylose and amylopectin was observed by Samec and von Haefft * who showed that amylopectin contains, on an average, 0·175 per cent of P_2O_5 in organic combination as a carbohydrate ester of phosphoric acid; later Samec and Mayer † were able to demonstrate that after the removal of the phosphoric acid from amylopectin, the phosphorus free carbohydrate, to which they gave the name of erythroamylose, could be re-esterified with phosphoric acid to a viscous jelly which, however, contained 2·19 per cent P_2O_5. The same authors also showed that amylose has $\alpha_D = 189°$, while amylopectin has $\alpha_D = 195\text{-}196°$.

With regard to the relative amounts of the two constituents, Ling and Nanji ‡ claim to have established an almost constant ratio of 66 per cent. amylose to 33 per cent of amylopectin, while, on the other hand, Samec and Höfft claim that the proportions in potato starch are 17 per cent of amylose to 83 per cent of amylopectin.

In addition to amylose and amylopectin, Ling and Nanji § have also found a substance described as amylohemicellulose to be associated with cereal starch grains, but the quantity varies considerably in different starches.

To summarize our knowledge with regard to the two constituents of the starch grain :—

* Samec and von Haefft: "Kolloidchem. Beihef.," 1913, **5**, 141; 1914, **6**, 23.

† Samec and Mayer: "Compt. rend.," 1921, **193**, 321.

‡ Ling and Nanji: "J. Chem. Soc.," 1923, **123**, 2666.

§ *Ibid.*, 1925, **127**, 630.

Amylose forms 66 per cent of the granule; it is a polymerized α-hexa-amylose; is soluble in water and gives a clear bright blue colour with iodine; it is converted by barley or malt diastase completely into maltose at 50° C.

Amylopectin forms 33 per cent of the granule; it is a polymerized phosphoric acid ester of αβ-hexa-amylose; when made into a paste with hot water, it gives a bluish-black coloration and precipitate with iodine (amylopectin extracted with alkali gives a violet coloration with iodine); it is dephosphated and depolymerized by barley diastase at 50°, yielding αβ-hexa-amylose.

Amylohemicellulose is the name given by Ling and Nanji to a substance associated with and, in the case of the cereal starches, apparently forming an integral part of the granule. Starches of the potato and arrowroot, on the other hand, contain hardly any of this material, although the tuber of the potato actually contains a considerable quantity which, instead of forming part of the starch granule, remains attached to the cell wall.

Amylohemicellulose contains from 1·2-1·3 per cent of ash and is regarded by Ling and Nanji as a calcium, magnesium, and iron salt of a silicic and phosphoric ester of α-amylose; it is converted by malt diastase quantitatively into maltose but, unlike amylose, it is unacted upon by barley diastase. On the other hand, like amylose it gives a blue colour with iodine, and being associated in some cases with the cell wall it is liable, when occurring in wood, to be mistaken there for starch.*

Action of Acids on Starch.

The action of acids on starch varies according to the strength of the acid, the duration of the action, and the temperature of the experiment. To complicate matters, there are considerable divergences in regard to the interpretation of the results obtained by the different workers. As an illustration of the very different effects which may be produced under different conditions, the following experiments may be quoted.

* Ling and Nanji: "J. Chem. Soc.," 1925, 127, 652.

By acting on starch at the ordinary temperature with 12 per cent commercial hydrochloric acid for twenty-four hours, Brown and Morris found that granules, while retaining their external features, had acquired the power of dissolving in hot water without the formation of paste. The addition of alcohol to such a solution caused the immediate precipitation of a white substance known as soluble starch, which is turned blue by iodine, is strongly dextro-rotatory, $[\alpha]_D = 202°$, and does not reduce Fehling's solution. On the other hand, if starch is boiled for some time with dilute hydrochloric acid, it is converted into glucose, a fact which is made use of in estimating starch.

That maltose is also produced as an intermediate product of the acid hydrolysis of starch has been shown by Fernbach and Schoen,* and also by Weber and Macpherson,† who have proved it to be present in commercial glucose (see p. 97). Accompanying the conversion of starch into glucose there is, however, the formation of varying quantities of gummy substances known as dextrins (q.v.) ; it is, however, not known for certain whether these dextrins are formed directly by the action of the acid on the starch, or whether they are produced by the condensing action of the acid on the glucose already formed ; there is, moreover, great difference of opinion with regard to the nature and number of these substances which are formed. According to Nanji and Beazeley‡ some iso-maltose is always formed during acid hydrolysis of starch.

Action of Malt Diastase on Starch.

The action of an extract of malt§ on starch paste is complex in that it involves liquefaction and saccharification.‖ These two changes are effected by different enzymes as is indicated by keeping a mixture of starch paste and malt extract at 70° C. After some minutes the paste becomes less viscous

* Fernbach and Schoen : " Bull. Soc. Chim.," 1912, [iv], **11**, 303.

† Weber and Macpherson : " J. Amer. Chem. Soc.," 1895, **17**, 312.

‡ Nanji and Beazeley : " J. Soc. Chem. Ind.," 1926, **45**, 215 T.

§ Barley malt has been shown to contain a great many different enzymes capable of acting upon lichenin, mannan, cellobiose and maltase, etc.; it is the action upon starch only which is here being considered.

‖ Ohlsson : " Zeit. physiol. Chem.," 1922, **119**, 1.

and develops but little reducing power: if now the mixture be cooled to 50° and a fresh amount of malt be added, the reducing power of the solution rapidly develops owing to the saccharifying action of the second enzyme which was all but inactivated at the higher temperature.*

The process of saccharification is essentially hydrolytic, whereby the starch molecule is successively broken down to a number of substances of lower molecular weight such as dextrins and sugars; this change may be conventionally represented as follows:—

$$\underset{\text{Starch}}{(C_6H_{10}O_5)_n} + H_2O \rightarrow \underset{\text{Dextrin}}{(C_6H_{10}O_5)_x} + \underset{\text{Maltose}}{C_{12}H_{22}O_{11}}$$

though actually other sugars such as iso-maltose or glucose may be formed according to the conditions. The production of glucose for example was demonstrated by Ling and Baker† by acting upon starch with malt diastase at 70°; its production has been attributed to the further hydrolysis of the maltose by maltase contained in the diastase, but as maltase is not active above 55° this is not possible, and, as Ling and Nanji have shown, the glucose is actually produced from the hydrolysis of β-glucosido-maltose (see below).

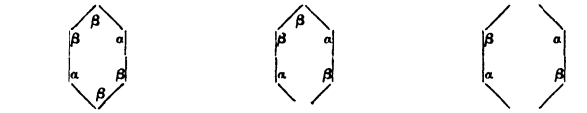
A detailed study of the action of diastase upon the two constituents of the starch grain, namely amylose and amylopectin, was undertaken by Ling and Nanji;‡ these authors find that the action of barley diastase at 50° upon amylose is to convert it quantitatively into maltose without the production of any intermediate substances. On the other hand, amylopectin is first converted into αβ-hexa-amylose and the further hydrolysis of this substance by malt diastase results in the production of a series of maltodextrins, a trisaccharide β-glucosido-maltose, iso-maltose, maltose, and glucose depending on the conditions of the experiment. Thus malt diastase acting at 70° upon αβ-hexa-amylose converts it into maltodextrin-α and thence into 2 molecules of a hexatriose β-

* Both saccharifying and liquefying enzymes are destroyed at 80°.

† Ling and Baker: "J. Chem. Soc.," 1895, **67**, 702, 739. Ling and Davis: "J. Fed. Inst. Brewing," 1902, **8**, 475; "J. Chem. Soc.," 1904, **85**, 16.

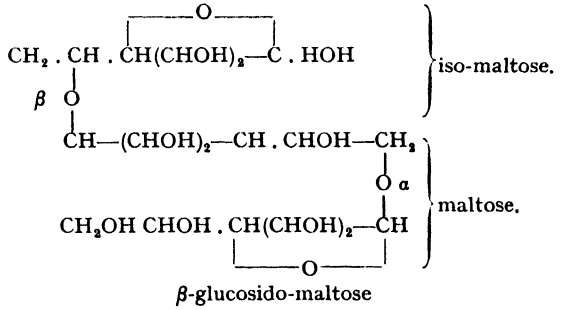
‡ Ling and Nanji: *id.*, 1925, **127**, 639.

glucosido-maltose which contains both an α- and a β-linking. These facts are best explained by the following formulæ :—



αβ-Hexa-amylose Maltodextrin-α β-Glucosido-maltose

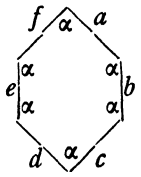
From which it appears that αβ-hexa-amylose is composed of six hexose residues united together by four β- and two α-linkages ; the conversion of this into maltodextrin-α results from the fission of one β-linkage ; the further fission of yet another β-linkage yields 2 molecules of the trisaccharide β-glucosido-maltose * which must have the constitution—



β-glucosido-maltose

since it is broken up by emulsion into glucose and maltose and by maltase into glucose and iso-maltose, showing it to have both an α- and β-linking.

On the other hand, the amylose constituent of the starch grain would in Ling and Nanji's opinion appear to be an α-hexa-amylose † of the formula—



in which the six sides, *a*, *b*, *c*, *d*, *e*, and *f*, represent glucose anhydrides, $C_6H_{10}O_5$, united together through oxygen atoms at

* β-Glucosido-maltose yields an osazone, m.p. 122°. Ling and Nanji : *loc. cit.*, p. 2679.

† Ling and Nanji : " J. Chem. Soc.," 1923, **123**, 2684.

the six corners of the hexagon; such a molecule would yield on hydrolysis 3 molecules of maltose, *ab*, *cd*, and *ef*, which is in agreement with the observed fact that amylose yields only maltose.

Action of Bacteria on Starch.

In 1903 Schardinger * isolated from retting flax a bacillus to which he gave the name *Bacillus macerans*; this organism when grown on 5 per cent starch paste, liquefies the starch and sets up an active fermentation with the evolution of carbon dioxide and hydrogen, and the production of acetone † and butyl alcohol. In the course of a few days these products give way to the formation of acids, and after about a week a liquid remains from which Schardinger isolated two crystalline substances which he described as α- and β-dextrin. The former of these gives with iodine a dark green compound crystallizing in needles, while the latter gives dark reddish-brown prisms. Schardinger ascribed to these compounds the formulæ $(C_6H_{10}O_5)_4$ and $(C_6H_{10}O_5)_6$ and named them tetra- and hexa-amylose respectively. Pringsheim and his collaborators ‡ subsequently investigated these compounds more fully and found that on treating them with acetic anhydride and zinc chloride they were acetylated and at the same time depolymerized; the product obtained from the α-dextrin $(C_6H_{10}O_5)_4$ was shown to be an acetylated diamylose $(C_6H_{10}O_5)_2$, while that obtained from the β-dextrin $(C_6H_{10}O_5)_6$ was a triamylose $(C_6H_{10}O_5)_3$. For these reasons he regarded Schardinger's α- and β-dextrin as polymerized di- and triamyloses respectively as shown by the formulæ—

α-Dextrin = Tetra-amylose $[(C_6H_{10}O_5)_2]_2$
Diamylose $(C_6H_{10}O_5)_2$
β-Dextrin = Hexa-amylose $[(C_6H_{10}O_5)_3]_2$
Triamylose $(C_6H_{10}O_5)_3$

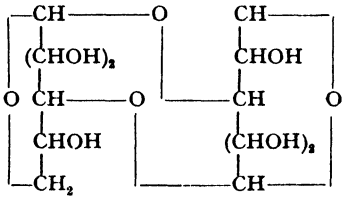
* Schardinger: "Zeit. Nahr. Genussm.," 1903, **6**, 874; "Zentr. Bakt. Parasitenk.," 1905, [ii], **14**, 772; 1911, **29**, 188.

† During the Great War this type of fermentation was first employed on a large scale for the production of acetone.

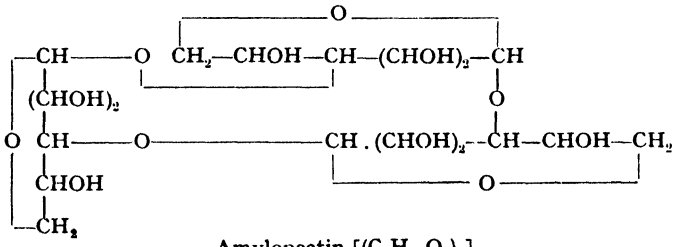
‡ Pringsheim and Langhans: "Ber. deut. chem. Ges.," 1912, **45**, 2533; Pringsheim and Eissler: *id.*, 1913, **46**, 2959.

in which the basic molecule is enclosed in a round bracket, while the degree of polymerization is indicated by the square brackets.

According to Pringsheim,* the α and β series of dextrins are derived from amylose and amylopectin respectively, and diamylose and triamylose he regards as the basal nuclei of amylose and amylopectin to which substances he assigns the following structure :—



Amylose $[(C_6H_{10}O_5)_3]_n$



Amylopectin $[(C_6H_{10}O_5)_3]_n$

Moreover, the fact that by the action of cold concentrated acid upon glycogen, he has also obtained triamylose leads him to believe that the amylopectin of starch is identical with glycogen.

In Ling and Nanji's † opinion, however, the basal unit of the polymerized amylose and amylopectin of the starch granule are α-hexa-amylose and $\alpha\beta$-hexa-amylose respectively (for formulæ, see p. 157).

Reactions.

1. The appearance of the grains under the microscope and their action on polarized light in the presence of water are well known.

* Pringsheim : " Ber. deut. chem. Gesells.," 1924, **57**, 1581.
† Ling and Nanji : " J. Chem. Soc.," 1923, **123**, 2683.

2. The most characteristic reaction of starch is the blue colour produced with iodine. The composition of this blue substance varies; it contains, on an average, about 18 per cent iodine, and cannot be formed unless a small quantity of hydriodic acid, which is always present in small amounts in ordinary solutions of iodine, be present. The blue colour is discharged on heating the solution, but reappears on cooling. The dried substance may, however, be heated to 100° without undergoing alteration.

If the starch grains are very small, or relatively so few in number that they might easily be overlooked, Meyer's procedure for their detection may be followed. A section of the material to be examined is cut, and is first treated with a fairly dilute solution of iodine in potassium iodide, the excess of the reagent is then removed, and the section is irrigated with a concentrated aqueous solution of chloral hydrate. This causes the starch grains to swell, and at the same time the other cell contents are dissolved, as are also the starch grains in time.

The fact that iodine sometimes gives a blue colour with a *soluble* cell constituent led to the assumption of the presence of a so-called soluble starch. There is, however, no need for such an interpretation, since the blue colour observed in the epidermal cells of *Saponaria officinalis*, for example, is attributable to the action of iodine on the glucoside saponarin, $C_{21}H_{24}O_{12}$, which Barger * has shown to be present and to give this reaction.

The blue colour given by starch with iodine was originally regarded by Mylius † as a definite chemical compound, and the same view is taken by Murray ‡ but by others it is considered to be a physical adsorption of colloidally dispersed iodine by the starch acting as a protective colloid; § the particular shade of colour produced probably depends upon the degree of dispersion of the iodine (cf. dextrin, glycogen, etc.).

* Barger: "Ber. deut. chem. Gesells.," 1902, **35**, 1296.
† Mylius: *id.*, 1887, **20**, 688.
‡ Murray: "J. Chem. Soc.," 1925, **127**, 1288.
§ Barger and Field: *id.*, 1912, **101**, 1394.

3. Starch grains are insoluble in cold water, but in hot water they swell up and form an opalescent solution which, if strong enough, will on cooling eventually form a paste.

4. Starch is precipitated from its aqueous solution by alcohol or by basic lead acetate (cf. Inulin and Dextrin).

5. Boil a little starch paste solution with a few drops of dilute sulphuric acid in a test tube, and from time to time remove a little of the solution, cool it and test with iodine solution ; when the starch has been converted into dextrin the blue colour at first formed will give way to a plum colour. If boiled too long only dextrose will remain which gives no colour with iodine. The solution will, however, after making alkaline, reduce Fehlings' solution.

6. Cautiously heat a little starch on a porcelain basin until it has acquired a light fawn colour. Cool and extract with cold water, and filter ; the dextrin produced being soluble in cold water is thus separated from the starch. On adding iodine to the solution a plum colour is produced.

Estimation of Starch.

The chief difficulty in estimating starch by determining the amount of reducing sugar formed after appropriate hydrolysis lies in the error caused by the presence of pentosans. Lintner overcame this difficulty by estimating the pentosans by the phloroglucinol method (see p. 137) and deducting a proportionate amount from the reducing power after hydrolysis, on the assumption that xylose and arabinose have approximately the same reducing power as glucose.

A method for the determination of starch in barley or wheat due to Ling, Nanji, and Harper * makes use of the fact that when a paste of any of the starches, or materials containing starch, is treated with barley diastase at 50°, the amylose is converted into maltose and the amylopectin into $\alpha\beta$-hexa-amylose leaving the amylohemicellulose of the cereal starches as an insoluble residue.

Cereal starches, owing to the presence of amylohemicellulose, do not give the same percentage of maltose as other

* Ling, Nanji, and Harper : " J. Inst. Brewing," 1924, **30**, 838.

starches which do not contain this substance. The ratio amylose : amylopectin though approximately 2 : 1 in most cases is not quite constant, and for this reason, in addition to the variation in the activity of the barley diastase, a control is carried out upon pure potato starch, and from the determination of the maltose as a percentage of dry starch the amylose : maltose ratio can be deduced. If this ratio has been established for one set of conditions, and the same conditions are applied to a cereal starch, it is possible to determine the amylose : maltose ratio for that cereal. The method, for the details of which the original paper should be consulted, gives the true starch content exclusive of hemicellulose, and the results are slightly lower than those given by malt diastase.

The following method depending on the hydrolysis of starch by hydrochloric acid and the subsequent estimation of the glucose produced, is only reliable if there are no pentosanes or other substances present which on hydrolysis would yield reducing sugars.

About 3 grams of the substance in as fine a state of division as possible are covered with 50 c.c. of cold water and shaken at frequent intervals; after an hour the insoluble portion is filtered off and washed with water until the total filtrate measures 250 c.c.; the addition of a little alumina shaken up with water will frequently facilitate clear filtration. The soluble carbohydrates contained in the filtrate may if desired be determined both before and after inversion.

The residue remaining on the filter paper is then transferred to a flask with a 250 c.c. graduation mark and heated for two and a half hours under a reflux condenser with 200 c.c. of water and 20 c.c. of hydrochloric acid (sp. gr. 1·125). After cooling, the solution is neutralized with caustic soda and made up to 250 c.c., whereupon it is filtered and the amount of glucose contained in an aliquot portion of the filtrate is estimated by Fehling's or Benedict's solution. The amount of glucose found when multiplied by 0·9 gives the weight of starch.

The following method for the estimation of starch in barley is due to Horace T. Brown * :—

Five grams of the powdered or crushed grain are extracted for three hours in a Soxhlet extractor with alcohol (sp. gr. 0·90); the residue is then thoroughly boiled with 100 c.c. of water, and, after cooling to 57°, 10 c.c. of active malt extract are added and the mixture is set aside for one hour; it is thereupon boiled and filtered into a flask with a 200 c.c. graduation mark; the residue is thoroughly washed with water, and, after cooling, the filtrate and washings are made up to 200 c.c. The cupric reduction of 20 c.c. of the solution is determined under the conditions laid down by Brown, Morris, and Millar,† the maltose being calculated according to Table XI in that paper (*loc. cit.*, p. 100), after correction for the reduction due to the malt extract. The starch equivalent to this maltose is then ascertained by assuming that 84·4 parts of maltose correspond to 100 parts of starch.

The malt extract is prepared by digesting 10 grams of fresh finely-ground malt for two to three hours with 200 c.c. of water and filtering.

A method of starch estimation due to von Fellenberg ‡ depends on the solution of the starch in a hot solution of calcium chloride, its precipitation by iodine and the decomposition of the iodine precipitate by alcohol.

DEXTRINS.

The term dextrin is applied to substances which are formed from starch by the action of heat alone or of diastase or mineral acids.

Occurrence.

In the plant dextrins may occur as transitory substances whenever starch is being acted upon by diastase; further, certain dextrins may occur in a more permanent form. Thus the sap of the epidermal cells of *Arum italicum* turn reddish-violet on the application of iodine. The aqueous extract of

* Horace T. Brown: "Trans. Guiness Research Lab.," 1903, **1**, 89.
† Brown, Morris, and Millar: "J. Chem. Soc., Lond.," 1897, **71**, 94.
‡ v. Fellenberg: "Mitt. Lebensm. Hyg.," 1916, **7**, 369.

such cells gives on evaporation a transparent sticky substance. This also gives with iodine a violet coloration; after boiling, the colour reaction with iodine is red, and after digestion with diastase a reducing sugar is found.

Formation from Starch.

The question of the formation of dextrins from starch by the action of diastase has been the subject of a great many researches, and has, at different times, resulted in the postulation of the existence of a large variety of dextrins and intermediate products, such as amylo-, achroo-, erythro-, and malto-dextrin, amylases, amyloins, glycoamylins, etc., many of which did not survive for long.

The chief facts observed during the action of malt extract on starch may be very briefly summarized as follows: If, say, a 10 per cent starch paste is left in contact with malt extract at 50°, the mass rapidly liquefies and the solution acquires a sweet taste owing to the conversion of starch into maltose; if the latter substance be estimated from time to time, it will be found that the reducing power of the mixture increases rapidly at first until, after about two hours, the amount of maltose present corresponds to about 80 per cent of the starch employed, when practically no further change takes place. The change in the starch paste can also be demonstrated by periodic testing with iodine solution; the blue-black coloration gradually becomes less and less marked until various shades of red are obtained, finally the iodine gives no distinctive coloration. A corresponding fall in the optical activity of the solution can also be observed, but as the activity is still greater than what it should be for maltose alone, it must be concluded that some other substance is formed at the same time as the maltose, and that its reducing power is less but its activity is greater than that of maltose. The amount of this "non-maltose" product of diastatic activity varies directly with the temperature, and increases considerably at the expense of the maltose if the temperature be kept at or above 60°; if to such a product, rich in non-maltose, a fresh quantity of malt extract be added, the non-maltose will be attacked and converted into

maltose until the amount present again attains the value 80 per cent, which is the normal maximum; this experiment, which is due to Brown and Morris,* shows that the non-maltose is composed of different constituents, some of which are converted into maltose by diastase more readily than others; moreover, experiments have shown that these substances behave differently towards yeast, some being more readily fermentable than others. This non-maltose constituent represents a mixture of the various dextrins mentioned above as having been described by several authors.

General Properties of Dextrins.

From what has been said above, it will be seen that the term dextrin comprises a number of substances some of which are not at all well defined. The following may, however, be regarded as approximately representing the characteristics of all substances included in this group :—

1. They are amorphous substances which are readily soluble in water to form gummy solutions, which are used as a substitute for natural gum; they are precipitated from aqueous solutions by the addition of alcohol.

2. Dextrins in strong solution give a precipitate with basic lead acetate.

3. As their name implies, they are strongly dextro-rotatory, in which respect they resemble starch.

4. They give either a red colour or no colour at all with iodine.

5. They are not fermentable by yeast alone, but are fermented by a mixture of yeast and diastase acting together, which is no doubt due to their slow hydrolysis in the first place by the diastase and the subsequent fermentation of the maltose so produced.

6. They do not reduce Fehling's solution when pure.

7. They are converted into glucose on hydrolysis with mineral acids.

As has already been mentioned, starch when suddenly heated to about 200° is converted into a substance commercially

* Brown and Morris: "J. Chem. Soc., Lond.," 1885, 47, 527.

known as dextrin. The use of starch for stiffening linen depends on some such similar change produced in the starch by the heat of the iron.

Although a great many different dextrins have from time to time been described, comparatively few of them are sufficiently well defined to warrant any description here.

Amylo-dextrin.—This substance is obtained by the action of ungerminated barley diastase at 50° C. and precipitated by alcohol. It is a white powder slightly soluble in cold water, but readily in hot. It is strongly dextro-rotatory ($a_D = +196$) does not reduce Fehling's solution, and gives a blue colour with iodine.

Erythro-dextrin.—This is a solid which dissolves readily in water, has a rotatory power of $a_D = +196°$, and with iodine produces a red-brown colour.

The existence of erythro-dextrin as a chemical entity is, however, disputed by Ost, who says that it is a mixture of achroo-dextrin with starch; an artificial mixture of achroo-dextrin with ½ per cent of starch also produces a red colour with iodine.

Achroo-dextrin.—This substance is optically active, has the value $a_D = +192°$, gives no colour with iodine, and has a sweetish taste.

Malto-dextrin.—In addition to the above dextrins, Brown and his collaborators, and Ling and Nanji * have described the following malto-dextrins which are non-crystalline intermediate products of the action of diastase on starch and possessing cupric-reducing power: *malto-dextrin-α* $C_{36}H_{62}O_{31}$ ($\alpha_D = 180°$), and *malto-dextrin-β*, $C_{24}H_{42}O_{21}$† ($\alpha_D = 173{\cdot}5°$), and *stable dextrin.*‡

According to Ling and Nanji, malto-dextrin-α is an intermediate product in the degradation of αβ-hexa-amylose to β-glucosidomaltose (see p. 156), and they regard the stable dextrin of Brown as a malto-dextrin of the highest type.

* Ling and Nanji: "J. Chem. Soc., Lond." 1925, **127**, 636.
† Ling and Baker: *id.*, 1895, **67**, 703; 1897, **71**, 517.
‡ Brown and Millar: *id.*, 1899, **75**, 286.

COMMERCIAL DEXTRIN.

Commercial dextrin is prepared by heating starch to about 230-260°; it is a yellowish-brown powder, while that prepared by acid hydrolysis of starch is an almost colourless solid with a choncoidal fracture, or else a white powder resembling starch. It is composed chiefly of achroo-dextrin mixed with varying quantities of erythro-dextrin and glucose. It dissolves in an equal volume of water to give a neutral sticky solution with a faint sweet taste; the solution is strongly dextro-rotatory. Dextrin is insoluble in alcohol and ether.

GLYCOGEN.

This substance, although one of the most important and widely distributed reserve foods in the animal kingdom, has a restricted distribution in plants. It occurs abundantly in certain Fungi, especially in *Saccharomyces cerevisiæ*, where it may sometimes form as much as 30 per cent of the dry weight. It has also been described as forming part of the cell-contents in Myxomycetes, Flagellates, and in certain Algæ including the Cyanophyceæ. In the yeast plant the glycogen varies in amount according to the physiological phase of the organism, and, it appears, accumulates and disappears often with great rapidity.

The glycogen appears in the cells of *Saccharomyces* during the early stages of fermentation as minute refractive granules scattered through the protoplasm; after a few hours these granules give place to small vacuoles, which in turn are replaced by one large vacuole, which may occupy the greater space in the cell.*

Wager and Peniston,† have shown that the amount of glycogen present is correlated with the periodical fluctuations in the fermentative activity.

When yeast is placed in a nutrient fluid, e.g. Pasteur's solution, fermentation may start at once, in which case it was found that the cells float and contain very little glycogen, while the cells which contain much glycogen sink to the

* Harden and Rowland: "J. Chem. Soc., Lond.," 1901, **79**, 1234.
† Wager and Peniston: "Ann. Bot.," 1910, **24**, 45.

bottom. After an hour or two the cells begin to rise, and they become distributed throughout the medium after the lapse of four or five hours. The fermentation is now much more active, and the amount of glycogen in the cells less. The next five to fifteen hours is the period of maximum vegetative activity, during which the glycogen disappears ; then it slowly reappears, and later on much more rapidly, at which phase there is a marked decrease in budding. At the height of fermentation, or immediately after, the glycogen increases rapidly, and a large number of cells sink to the bottom of the fluid. If the medium be not exhausted, the process may be repeated two or three times.

These facts suggest that the yeast, although surrounded by a medium rich in soluble carbohydrate, uses its glycogen reserve in the first instance and, moreover, is not able to utilize the free sugar without first elaborating it to glycogen and mannan. Elias and Weiss * find that the reaction of the nutrient medium has a bearing upon the amount of glycogen produced, there being a marked increase in the presence of alkali.

Although glycogen and mannan may be looked upon as a temporary reserve food,† for yeast-cells rich in glycogen retain their vitality much longer than those in which there is little or none, the fact that in the spores of species of *Mucor* and in sclerotia glycogen does not appear until growth has commenced, points to the conclusion that in these plants, at any rate, it is not primarily a storage product. Kohl considers that since it is more abundant in *Saccharomyces* during active gemmation, it is not exclusively a reserve substance, but an intermediate product in the formation of alcohol from the sugar.

In the animal kingdom, according to Hoppe-Seyler, glycogen is an invariable constituent of almost all developing cells ; it is found also in the muscles and blood, and chiefly in the liver, where it is stored in larger quantities.

It may be remarked that there is little doubt that the glycogen obtained from animal and plant sources are identical.

* Elias and Weiss : " Biochem. Zeit.," 1922, 127, 1.
† See Warkany : *id.*, 1924, 150, 271.

Preparation.

The following method of obtaining glycogen was devised by Pflüger.* Fresh finely-cut liver is stirred up with water and 60 per cent caustic potash, and heated for two hours; the filtered solution, containing 15 per cent of potash, is then mixed with an equal volume of 96 per cent alcohol, and the precipitated glycogen is collected and washed with a mixture of 1 part of 15 per cent potash with 2 parts of 96 per cent alcohol; if necessary, the substance may be redissolved and purified in the same way.

Glycogen may also be prepared from yeast, but not in a particularly pure state, in the following manner: A quantity of baker's yeast, which has been previously well washed with water, is mixed with fine well-cleaned sand and ground very thoroughly in order to rupture the cells. The mixture is then placed in a vessel with about thrice its volume of water and heated for some time, being constantly stirred. The liquid is then filtered off, cooled, and strong alcohol added to the filtrate in order to precipitate the glycogen, which is filtered off. The glycogen so obtained may be purified by redissolving it in water, adding a little acetic acid, and boiling in order to remove any proteins which may not have been removed by the initial heating, filtering, and precipitating with alcohol.

An elaborate method has been described by Harden and Young,† which has been modified by Ling, Nanji, and Paton.‡ Dried yeast, a commercial by-product of many breweries, is boiled for two hours with 2 per cent caustic soda, and after removal of the insoluble cell wall residue, the crude glycogen is precipitated by alcohol and freed from protein and nucleoprotein by heating with 60 per cent caustic potash. From this solution the glycogen is once more precipitated by alcohol, and is further purified from mannan (yeast gum) by precipitating the latter from a warm alkaline solution by means of Fehling's solution. The filtrate containing the glycogen is

* Pflüger: "Pflüger's Archiv f. Phys.," 1902, **91**, 119, and 1903, **93**, 163.

† Harden and Young: "J. Chem. Soc.," 1912, **101**, 1928.

‡ Ling, Nanji, and Paton: "J. Inst. Brewing," 1925, **31**, 316.

then acidified with acetic acid and dialysed, the last traces of copper being removed electrolytically. The further purification of the glycogen is effected by repeated alternate precipitation by alcohol and solution in water.

According to these same authors, some of the glycogen of the yeast cell occurs in the plasma and is readily extracted by water, while another portion, which is less readily extracted by water, is associated with the cell wall. The former modification, which resembles the amylose constituent of the starch granule, produces in water a faintly opalescent solution which gives a pure red colour with iodine but no precipitate. The other modification, associated with the cell wall, appears to be a phosphoric ester of the form associated with the plasma and is comparable with amylohemicellulose; its solution in water is opalescent and gives with iodine a reddish-brown precipitate.

Properties.

Pure glycogen is a snow-white amorphous solid. It is readily soluble in hot water, forming an opalescent solution, from which it may be precipitated again by alcohol, provided small quantities of dissolved salts are present; 100 c.c. of a 1 per cent solution when mixed with 200 c.c. of absolute alcohol remain clear, but on adding 0·03-0·05 gram of sodium chloride, an immediate precipitate is formed. Glycogen is strongly dextro-rotatory, $\alpha_D = + 198{\cdot}9°$, and is coloured red to brown by iodine; it does not reduce Fehling's solution, but is broken down by diastase into dextrin and maltose, and by acids into glucose.

The fact that cold concentrated hydrochloric acid converts glycogen into triamylose is considered by Pringsheim to establish the identity of glycogen with amylopectin of starch.

Identification.

1. The opalescent appearance of its aqueous solution is characteristic; it is strongly dextro-rotatory.

2. A brown coloration is given with iodine solution.

3. A white precipitate is given with basic lead acetate in strong solutions only.

4. It does not reduce Fehling's solution.

5. On boiling with mineral acids, it is converted into dextrose.

Estimation.

This is best effected by heating the aqueous solution for three hours in a boiling water bath with about 2·2 per cent HCl, and then neutralizing and estimating the resulting glucose by means of Fehling's solution; the amount multiplied by 0·9 gives the weight of glycogen.

According to Ling, results so obtained are vitiated by the presence of mannose which is produced by the partial hydrolysis of the mannan. He recommends hydrolysis by boiling for three hours with 8 per cent sulphuric acid and estimating iodometrically * the glucose and mannose produced before and after removal of the mannan.

LICHENIN AND ISO-LICHENIN.

Lichenin is the name given to a water-soluble polysaccharide extracted from "Iceland moss"—*Cetraria islandica*—and other lichens. When an aqueous extract of Iceland moss is concentrated, a gelatinous precipitate of lichenin is formed while the solution contains a substance known as iso-lichenin. The latter substance, also known as lichen starch, is dextro-rotatory and gives a blue colour with iodine, and is said by Pringsheim and others † to be identical with amylose of the starch grain. Lichenin, on the other hand, is optically inactive and gives no blue colour with iodine; according to Pringsheim it owes its gelatinizing properties to the fact that it is a carbohydrate ester of silicic acid.‡

On the other hand, Hess § claims to have prepared highly purified ash-free samples of lichenin possessing unimpaired gelatinizing properties as compared with the less purified material, from which he concludes that the gelatinizing power

* See Baker and Hulton: "Biochem. Journ.," 1920, **14**, 754.

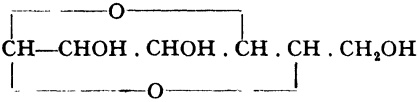
† Pringsheim and Kusenack: "Zeit. physiol. Chem.," 1924, **137**, 265; 1925, **144**, 241. Pringsheim and others: "Ber. deut. chem. Gesells.," 1924, **57**, 1581.

‡ Cf. p. 154, under Amylo-hemicellulose.

§ Hess: "Zeit. angew. Chem.," 1924, **37**, 993.

is not dependent upon the existence of an ethereal silicate as stated by Pringsheim.*

That there is a close relationship between lichenin and cellulose is shown by the work of Karrer and Joos,† and also by the fact that lichenin ‡ can be converted by the action of a lichenase contained in barley diastase into cellobiose, the disaccharide obtainable from cellulose (see p. 119), and that on acetolysis it yields, like cellulose, octacetycellobiose. When lichenin is heated in glycerol at 240° it is converted into a substance lichosan, an anhydride of glucose of the formula—



Since cellulose may likewise be converted into a glucose anhydride cellosan, lichenin and cellulose are both regarded as products of associated glucose anhydrides. By a comparison of the optical rotation in cuprammonia solution of cellulose and lichenin, however, Hess § has shown that these two substances are not structurally identical. The same conclusion is reached by Herzog and Gonell ‖; they were, however, able to establish the identity of plant cellulose with that of animal origin (Tunicin). Lichenin is widely distributed in nature and has been found by Karrer and Staub ¶ in *Evernia vulpina*, *Usnea barbata*, *Parmelia furfuracea*, and in barley, oats, maize, spinach, beans, hyacinth bulbs, and other plants. The same authors have isolated from the alimentary canal of the snail, *Helix pomatia*, the enzyme lichenase which also occurs in barley and other plants and can hydrolyse lichenin to glucose in a few hours; the enzyme is comparable with cytase which attacks reserve cellulose, and for this reason they are inclined to look upon lichenin as a reserve cellulose. The fact that a

* Pringsheim and Kusenack: "Zeit. physiol. Chem.," 1924, **137**, 265.

† Karrer and Joos: "Biochem. Zeit.," 1923, **136**, 537; "Helv. Chim. Acta," 1924, **7**, 144.

‡ Pringsheim, Knoll, and Kasten: "Ber. deut. chem. Gesells.," 1925, **58**, 2135. Pringsheim: "Biochem. Zeit.," 1926, **172**, 411.

§ Hess: "Zeit. angew. Chem.," 1924, **37**, 993.

‖ Herzog and Gonell: "Zeit. physiol. Chem.," 1924, **141**, 63.

¶ Karrer and Staub: "Helv. Chim. Acta," 1924, **7**, 159.

lichenase should occur in the snail is significant in view of the wide distribution of lichenin in the plant world.

PARA-DEXTRANE AND PARA-ISODEXTRANE.

These substances have been isolated from *Boletus edulis* and *Polyporus betulinus* respectively. The former gives a yellow colour with chlorzinc iodide, and the latter a blue when treated with iodine and sulphuric acid. Both give glucose on hydrolysis.

FRUCTOSANS.

INULIN.

This substance is commonly found as a reserve food-stuff, of the same nature as starch, existing in a state of solution in the cell sap of a number of plants belonging to the natural order Compositæ, e.g. in the tubers of the dahlia and artichoke (*Helianthus tuberosus*), and in the fleshy roots of the chicory (*Cichorium Intibus*). It has also been described as occurring in the following natural orders: Violaceæ, Malpighiaceæ, Droseraceæ, Candolleaceæ, Goodeniaceæ, Campanulaceæ, Lobeliaceæ, Myoporineæ, Liliaceæ, and Amaryllidaceæ; also in some Algæ, e.g. *Neomeris.*

Inulin, or closely allied substances, are not infrequently found in company with starch, especially in some Monocotyledons, and exhibits the same peculiarity in its occurrence, as has already been remarked upon in connection with starch in monocotyledonous plants (p. 148).

Thus in *Iris pseudacorus* starch is present but not abundant, in *Iris Xiphium* both starch and inulin are present in quantity; *Scilla nutans* has inulin but no starch, while *Scilla sibirica*, and also *Hyacinthus* and *Muscari botryoides* have both starch and inulin.

According to Grafe and Vouk * and Melchior,† inulin is found in the leaves of *Cichorium intibus* and *Marcgravia spp.*, and is considered to be a direct assimilatory product. On the other hand, Colin ‡ finds that the leaves of *Helianthus tuberosus*

* Grafe and Vouk: "Biochem. Zeit.," 1912, **43**, 424; 1913, **56**, 249.
† Melchior: "Ber. deut. bot. Gesells.," 1924, **42**, 198.
‡ Colin: "Compt. rend.," 1918, **166**, 224, 305; "Bull. Assoc. Chim. Suc.," 1919, **37**, 121; "Bull. Soc. chim. biol.," 1925, **7**, 173.

never contain inulin, but do contain dextro-rotatory sugars and starch. The formation of inulin begins in the stem and is completed in the tubers. Thus the inulin must be formed from dextro-rotatory sugars synthesized in the leaves.

Preparation.

Inulin may be obtained from dahlia tubers, of which it forms from 10-12 per cent, by crushing them and pressing out the liquid and filtering; the residue is then boiled up with a little water and some precipitated chalk and filtered again. The two filtrates are then united and once more boiled with chalk in order to neutralize any acids, and while still warm treated with lead acetate until no further precipitate is formed. The filtered solution is then saturated with hydrogen sulphide, filtered, neutralized with ammonia, evaporated to small bulk, and mixed with an equal volume of alcohol. After one or two days, crude inulin may be filtered off; it may be further purified by warming in aqueous solution with animal charcoal, filtering, and adding alcohol; the precipitated inulin is then washed with alcohol and ether, and dried over sulphuric acid.

According to Kiliani,* it may also be prepared by boiling crushed dahlia tubers with water and a little chalk, filtering and freezing the filtrate. When the water cools, the precipitate is filtered off, re-dissolved in hot water and frozen out once more. After repeating this process several times, the inulin is washed with methyl alcohol, ethyl alcohol, and finally ether.

Characters.

Pure inulin forms a white starchy tasteless powder of a sphæro-crystalline nature; it swells up and is readily dissolved in hot water, alkalis, etc., and may be recovered from the aqueous solution by the addition of alcohol, in which it is practically insoluble, or by freezing. Highly purified inulin should give less than 0·2 per cent of ash, but the removal of the last traces of inorganic substances is so difficult as to suggest that they form a definite part of the molecular com-

* Kiliani: "Annalen," 1880, **205**, 147.

plex.* Inulin is lævo-rotatory, $\alpha_D = -35°$, and is non-reducing. Unlike starch it does not give a paste with water, nor does it give a blue colour when treated with iodine. Diastase has no effect upon it; it may, however, be hydrolysed by the ferment inulase, or by mineral acids, by which reagents it is converted into fructose. Whilst the final product obtained is ordinary fructose, the initial product is, presumably, γ-fructose, since inulin has been shown to be a polymerized form of this active sugar. The low osmotic pressure which solutions of inulin exert suggests a large molecule, but its molecular structure appears to be less complex than that of starch.

Identification.

In many plants the presence of inulin is indicated by the well-known sphæro-crystals which are obtained on steeping the fresh tissues for some time in strong alcohol; this deposition is not, however, always so characteristic; thus in Monocotyledons the inulin is frequently found, after treatment with alcohol, in amorphous masses. The sphæro-crystals and the amorphous concretions of inulin are readily soluble in warm water, and thus may be distinguished from calcium phosphate which may occur in cells in shapes similar to those of inulin. These two substances may be further recognized by the fact that sulphuric acid completely dissolves inulin, whereas it forms with calcium phosphate insoluble calcium sulphate. The following tests also may be performed:—

1. *Green's Test.*—Sections of the material, which have been soaked for some time in absolute alcohol, are treated with a saturated solution of orcin in strong alcohol, and then boiled in hydrochloric acid. The masses of inulin disappear and a red colour results. If phloroglucin be substituted for the orcin, the resulting coloration will be reddish-brown.

2. *Molisch's Test.*—The sections are treated with a 10 per cent alcoholic solution of α-naphthol, then a few drops of strong sulphuric acid are added and the preparation warmed. A deep violet coloration ensues, and the inulin is dissolved.

* Irvine and Steele: "J. Chem. Soc.," 1920, 117, 1474.

These colour reactions are indicative of the formation of sugar by the hydrolysis of the inulin by the acids employed in the tests; it is therefore important, before employing these reactions, to make sure that no free sugars are present in the material to be examined, and to wash the preparations thoroughly with alcohol in order to remove them.

Since inulin does not reduce Fehling's solution, this reagent may be employed to ascertain whether any reducing sugars are present in the material before employing the above tests for inulin.

The following reactions may be carried out with a solution of inulin.

3. Basic lead acetate gives no precipitate with inulin.
4. Inulin is precipitated from solution by alcohol.
5. Hydrolyse with mineral acid and test for levulose.

Physiological Significance.

It is of interest to find that the nature of the reserve carbohydrates may often be correlated to the habitat of the plant. Parkin * points out that these reserve substances of aquatic plants and of plants inhabiting wet situations take the form of starch, e.g. *Sparganium*, *Alisma*, *Listera*, *Orchis*, and *Schizostylis*; whereas, on the other hand, inulin, generally associated with sugar, is the characteristic carbohydrate reserve in those Monocotyledons inhabiting dry situations, e.g. *Allium*, *Asphodelus*, *Anthericum*, *Yucca*, *Tritona*, *Iris Xiphium*, etc.

In this connection † reference must be made to the work of Lidforss, who showed that plants inhabiting wet situations fall into two distinct categories; those like *Elodea*, *Chara*, and *Stratiotes*, which hibernate at the bottom of the pond or stream, contain starch but no sugar; while those which live on the banks where their rhizomes, or other organs of storage, pass the winter out of the water, e.g. *Myosotis* and *Menyanthes*, contain sugar during the winter months. In the former case a temperature of $-2°$ C. to $-4°$ C. is fatal, while in the latter case the death point is about $-7°$ C.

* Parkin: "Phil. Trans. Roy. Soc., Lond.," B, 1899, **191**, 169.
† See Blackman: "New Phyt.," 1909, **8**, 354.

This peculiarity also obtains for many arctic plants; Miyake, Wulff, and others have shown that cold, which means physiological dryness, is conducive to sugar production, so that arctic plants frequently exhibit but a small amount of starch, and relatively large quantities of sugar. Stahl has shown that the leaves of mycotrophic plants, which generally show a feeble transpiration, seldom contain starch, its place being taken by glucose. Lidforss also has shown that the winter green vegetation of Sweden is characterized by the absence of starch from the leaves, the mesophyll, in its place, containing relatively large quantities of sugar, and sometimes oil during the winter months. In summer the leaves of these plants contain starch, which, on the advent of winter, is converted into sugar, from which starch is formed on the rise of temperature in the spring.*

Then, again, it is not uncommon to find sugar stored in the periderm of trees and in the leaves of evergreen plants during the winter; starch, however, may be found in the leaves of evergreen trees during the cold season, its presence being due to feeble photosynthesis.

Reference may be made here to the well-known fact that potatoes turn sweet on exposure to cold. This conversion of starch into sugar is most active at 0° C., and the action decreases with the rise in temperature, so that above 7° C. no sugar is thus produced. Also if the tubers are suddenly subjected to a temperature of −1° C., no sugar will be produced. The amount of sugar formed is not great, its maximum being about 3 per cent of the wet weight; the limit of the process depends on the concentration of sugar, and, as Czapek has shown, the transformation of the starch may be prevented, on a lowering of the temperature, if the concentration of sugar be sufficient. If these sweet potatoes be exposed to a higher temperature, all the sugar that remains—some has been used up in respiration—is reconverted into starch.

Œcologically these characters are of value to the plant; for if the water of the cell sap be frozen, the salts held in solution become concentrated and will eventually precipitate the soluble

* See also Maximow: "Ber. deut. bot. Gesells.," 1912, 30, 52.

proteins. Parkin points out that the presence of inulin * in the cell sap of the parenchymatous tissues would retard the evaporation of water. It is a well-known fact that water in the presence of oil may be much over-cooled before ice-formation takes place, and the freezing-point of water in which other substances, e.g. sugar, are dissolved is depressed, and thus the danger arising from the salting out of the proteins is minimized. But, notwithstanding these facts, plants are frequently subjected to temperatures sufficiently low to cause ice to be formed, and as the water is thus withdrawn, the sugar becomes more concentrated until it will also crystallize out. Both these processes generate heat, which may be sufficient in amount to enable the protoplasm to live. And this is, according to Mez and Lidforss, the explanation of the presence of sugar in winter leaves.

At the same time we must be careful not to push such explanations too far, for there are many exceptional cases; thus Ewart has pointed out that *Dicranum* which contains much oil is less resistant to cold that *Bryum*, and other mosses, in which such substances are absent. The beetroot also is very susceptible to cold, notwithstanding the fact that it contains much sugar; similarly the seeds of the hemp and willow, which contain much oil, are easily killed by desiccation, whereas the oil-containing seeds of the linseed are highly resistant. Such divergent phenomena must depend on the constitution of the protoplasm.

Again, oil is a convenient form of reserve food, especially in small organisms and in reproductive bodies, where space is limited and lightness is all-important and it is desirable to store a maximum of potential energy in the minimum of bulk.

INULIN-LIKE SUBSTANCES.

A number of ill-defined substances similar to inulin have been described as occurring in various plants. The chief of these are :—

Graminin in *Agrostis*, *Festuca*, *Triticum*, *Arrhenatherum*, and other grasses.

* See also Grafe and Vouk : " Biochem. Zeit.," 1913, 56, 249.

Irisin in *Iris pseudacorus.*

Phlein in *Phleum pratense* and *Phalaris arundinacea.*

Sinistrin in *Scilla maritima.*

Triticin in *Triticum repens*, *Dracæna australis* and *Dracæna rubra.*

Of these fructosans, graminin, and triticin are not precipitated from neutral or acid solutions by heavy metal salts. With barium hydroxide they give insoluble compounds, but the corresponding calcium and strontium compounds are soluble. They do not reduce Fehling's solution and yield only fructose on hydrolysis.

All these compounds possess the same characteristics ; they are lævo-rotatory, yield fructose on hydrolysis, and are fairly soluble in cold water. The majority are difficult to crystallize, and their solutions yield a gum-like substance on evaporation. It is possible that some, at any rate, of these substances may bear the same relation to inulin as dextrin does to starch.

HEMICELLULOSES.

Whilst it is possible on a physiological basis to distinguish between food reserve polysaccharides such as starch, inulin, and glycogen, on the one hand, and the typical structural polysaccharide cellulose on the other, there occur in the plant a number of related compounds whose physiological rôle is less sharply defined. These substances are associated with the structural elements of the plant and form part of the cell wall but they may, on occasion, be attacked by appropriate enzymes, secreted in the plant, and be utilized as food. Owing to their dual function, and to emphasize their relationship to cellulose, they are commonly termed reserve celluloses. Whilst they resemble cellulose in many of their physical properties, they differ from cellulose in their chemical properties. Included in the hemicelluloses are mannan, galactan, and pentosan, which have been isolated from wheat and rye bran, from beans and pea pods, and from lichens, and wood gums which have been isolated from wood.

Properties.

Schulze * first proposed for this group of substances the term hemicellulose, characterized by their insolubility in water, solubility in alkali, and precipitation from their alkaline solution by acid or by alcohol. On hydrolysis by means of dilute acid they give origin to one or more monosaccharides which may be either hexoses or pentoses, whilst cellulose, which is more difficultly hydrolysed, yields glucose only.

Although hemicelluloses are normally insoluble in water, and are not extracted from wood by hot water, they become soluble in water after extraction by means of alkali; the galactan of coniferous wood is, however, an exception, being, according to Schorger, completely extractable from the wood by hot water.

From alkaline solutions some, but not all hemicelluloses are precipitated as copper compounds on the addition of boiling Fehling's solution; among those not precipitated are the arabans of the beet and cherry gum. †

Constitution.

The earlier workers looked upon hemicelluloses as polymerized anhydrides of pentoses or hexoses or of mixed sugars as is indicated by the names xylan, araban, mannan, galactan, galactoaraban, galactoxylan, etc. It has, however, been shown by O'Dwyer ‡ for the two hemicelluloses of beech wood that they are not true polysaccharides in that they contain acid groups, one in the form of a galacturonic acid and the other a glycuronic acid residue, from which facts these compounds would appear to be more closely related to the pectins (see below) than to cellulose.

By extraction of various starches with normal sodium hydroxide, after a preliminary digestion with taka-diastase, Schryver and his co-workers have obtained solutions from which they were able to precipitate by acetic acid a hemi-

* Schulze: "Zeit. physiol. Chem.," 1892, **16**, 391.
† Salkowski: *id.*, 1901, **34**, 171.
‡ O'Dwyer: "Biochem. Journ.," 1926, **20**, 656.

cellulose to which they assign the formula $3C_6H_{10}O_5 + 2H_2O$ (see also p. 154).

MANNAN.

Mannan occurs in salep mucilage, and has been extracted by Ritthausen * and Effront † and others from wheat and barley. Mannans are also found in *Penicillium glaucum*, ergot, in the roots of several plants such as asparagus, chicory *Helianthus* and *Taraxacum;* also in the wood and leaves of many trees, such as lime, chestnut, apple, mulberry, certain Oleaceæ and conifers; the so-called reserve celluloses and hemicelluloses contained in seeds of Palmaceæ, Liliaceæ, elder, cedar, and larch, and many other plants, are also very rich in mannans. Evidence for the occurrence of a manno-galactan in the American white oak has been furnished by O'Dwyer.‡

The mannan of the vegetable ivory, the endosperm of the seeds of *Phytelephas macrocarpa*, may be prepared in 8-10 per cent yield by treating the ground ivory meal with five times its weight of 10 per cent caustic soda for half an hour; § the black alkaline liquid is filtered through copper gauze, and the residue after washing with water is boiled for half an hour with five times its weight of 20 per cent caustic soda; the solution is then precipitated by the addition of one-third of its volume of rectified alcohol spirits and the precipitate after washing with the same precipitant is dissolved in hot water; after adding sufficient acetic acid to render just acid, the solution is boiled for a few minutes when the mannan is precipitated as a white powder.

The mannan of vegetable ivory was shown by Baker and Pope ‖ to be contaminated with lævulo-mannan and galacto-mannan.

It was shown by Pringsheim ¶ that ivory nut shavings were

* Ritthausen: "J. prakt. Chem.," 1867, **102**, 321; and "Chem. Zeit.," 1897, **21**, 717.

† Effront: "Compt. rend.," 1897, **125**, 38, 116.

‡ O'Dwyer: "Biochem. Journ.," 1923, **17**, 501.

§ Patterson: "J. Chem. Soc.," 1923, **123**, 1139; Schmidt and Graumann: "Ber. deut. chem. Gesells.," 1921, **521**, 1867.

‖ Baker and Pope: "J. Chem. Soc.," 1900, **77**, 696.

¶ Pringsheim: "Zeit. physiol. Chem.," 1912, **80**, 376.

hydrolysed by certain bacteria to mannose and a trisaccharide mannotriose, and Paton, Nanji, and Ling * have found that the nuts themselves contain an enzyme capable of hydrolysing the mannan to mannose with probably the intermediate formation of mannotriose.

A substance known as yeast gum, which occurs in considerable quantities in yeasts of weak fermenting power,† is also a mannan; the amount of this substance present in yeast is in inverse proportion to the amount of glycogen (cf. p. 168), but it is not regarded as a reserve substance; its solution, which has a strong foaming power in water, does not reduce Fehling's solution.

PARAMANNAN.

Paramannan is a variety of mannan which is characterized by being much more resistant to hydrolysis; this substance, which is contained in coffee beans, is only slightly acted on by hot dilute mineral acids, potassium chlorate, and hydrochloric acid, but dissolves in a concentrated hydrochloric acid solution of zinc chloride.

CARUBIN OR SECALANE.

Carubin ‡ is the name given to a substance occurring in the seeds of *Ceratonia siliqua*, and in various cereals such as rye and barley. In its characters it closely resembles mannan, and by some authors is regarded as identical with it; when dry, it is a spongy friable substance which swells upon the addition of water. It is soluble in cold water and is optically inactive. Its sugar is fermentable and non-crystalline.

XYLAN.

This substance may be obtained by extracting sawdust from the wood of deciduous trees with dilute caustic soda after preliminary extraction of the sawdust successively with organic solvents, water, ammonia, and finally washing with water. The yield of xylan obtainable from birch wood is, according to Schorger, 19·7 per cent, but the amount obtained

* Paton, Nanji, and Ling: "Biochem. Journ.," 1924, 18, 451.
† Hashitani: "J. Inst. Brew.," 1927, 33, 347.
‡ Effront: "Compt. rend.," 1897, 124, 200, and 125, 116 and 309.

from coniferous wood is much less. Xylan also occurs in corn cobs and in straw, from which latter source it may be conveniently prepared in a state of purity * by extraction with caustic soda and precipitation by means of an alkaline solution of copper sulphate. Xylan was formerly thought to be a polymerized anhydride of xylose, since it gives rise only to this sugar on hydrolysis; the observations of O'Dwyer on the hemicelluloses of American white oak render this view no longer tenable (see p. 181).

Xylan, precipitated from alkaline solution by the addition of acid, is soluble in hot water; but water will not extract it from wood in the first instance.

ARABAN.

This substance, which on hydrolysis gives rise to arabinose, is associated with xylan in wood; it is not clear whether it occurs as a distinct individual, or whether it is combined with other material; according to O'Dwyer beech wood contains two hemicelluloses, one of which contains xylose in combination with glycuronic acid, while the other contains arabinose combined with galacturonic acid.

Another substance yielding arabinose on hydrolysis and which has been regarded as a true carbohydrate or polysaccharide form of arabinose is cherry gum; this substance is exuded from the bark of the cherry and yields on hydrolysis chiefly arabinose with a small quantity of xylose, whereas the wood of the cherry extracted with alkali yields a product which produces chiefly xylose.

Gum-arabic likewise contains an araban since it yields chiefly arabinose on hydrolysis.

An araban has also been described by Ehrlich as arising from the hydrolysis of protopectin (see p. 202).

WOOD GUM.

This term is applied to the hemicelluloses extractable from wood by caustic soda. A true gum should at least swell up if not dissolve in water, but many of the so-called wood gums

* Salkowski: "Zeit. physiol. Chem.," 1902, **34**, 162.

are not directly extractable by boiling with water, and occur in the wood in such combination that they are only soluble in water after precipitation from the alkaline solution with which they were extracted from the wood. There is a good deal of variation in the composition and properties of the various wood gums; thus while hard woods may contain up to 20 per cent of xylan, the wood of gymnosperms contains only about 1 per cent of this substance but contains, on the other hand, galactans *; amounts of galactan varying from 8-17 per cent have been found in *Larix occidentalis*.†

According to Schorger and Smith ‡ this substance is characteristically associated with coniferous wood, though it has also been reported in the wood of angiosperms such as aspen, white oak, and apple; it is not certain whether all these woods contain the same galactan. The so-called ϵ-galactan which occurs in the wood of *Larix orientalis*, to the extent of about 8-17 per cent, is a white powder which dissolves readily in cold water, and in this respect bears no true resemblance to a gum.

GALACTAN.

The term galactan is applied to any non-reducing substance which on hydrolysis gives rise to galactose; while the plant world supplies a great many substances which yield galactose on hydrolysis, the number of such substances which yield this sugar only, unaccompanied by other sugars, is small.

A number of other galactans have from time to time been described as occurring in the seeds of lucerne, lupin, and in beets; these are ill-defined substances which in the past have been distinguished by prefixing letters of the Greek alphabet to the term galactan, but the present state of our knowledge concerning them does not justify a fuller description.

* Schorger and Smith: "J. Ind. Eng. Chem.," 1916, 8, 494.

† This fact has been commercially exploited in America for the manufacture of mucic acid by the oxidation with nitric acid of hydrolysed sawdust; the galactan has also been recommended as a source of alcohol.

‡ Schorger and Smith: "J. Ind. Eng. Chem.," 1916, 8, 494.

MIXED GALACTANS.

Other sources of galactan which, however, do not yield exclusively galactose on hydrolysis are of common occurrence. Such substances have been variously described as galacto-arabans, galacto-xylan, galacto-mannan, etc., according to the sugars to which they give rise; these occur notably in the mucilaginous extracts of seaweeds and form the agar and carragheen extracts of commerce (see below, p. 191). Under this heading should be included galacto-araban, sometimes wrongly described as para-galactan, which occurs in the cell walls of the cotyledons of many plants, e.g. *Lupinus luteus* and other species, *Phœnix dactylifera*, *Cocos nucifera*, and other palms, *Soja hispanica* and *Coffea arabica*, where it forms a reserve food-material which is digested on germination.

Galacto-araban is a white solid which is insoluble in water and cuprammonia; it dissolves in hot potash. On heating with nitric acid it is oxidized to mucic acid. Microchemically it may be identified by its insolubility in the reagents mentioned, and also by the fact that with phloroglucin and hydrochloric acid it gives a red coloration on warming. No colour is given in the cold.

Its association with cellulose prevents the latter exhibiting some of its reactions; thus the cellulose is unacted upon by cuprammonia unless the galacto-araban be removed; this may be done by boiling in dilute hydrochloric acid.

Other substances giving rise to galactose are the pectins (p. 192).

AMYLOID.

Amyloid is the name given to a substance occurring in the seeds of pæonies and certain cresses,* which yields on hydrolysis with dilute sulphuric acid a mixture of galactose, glucose, and xylose. It is a colourless substance, and is insoluble in cold water, but swells up into a slimy mass in hot water; it is soluble in cuprammonia solution. Amyloid does not reduce Fehling's solution, but is oxidized by nitric acid to mucic and trihydroxy-glutaric acids. It gives a blue colour with iodine.

* Winterstein: "Zeit. physiol. Chem.," 1893, **17**, 353.

GUMS.

The natural gums were formerly thought to be carbohydrates of the general formula $(C_6H_{10}O_5)_n$; the researches of O'Sullivan, however, have shown that they are not simple carbohydrates, since on hydrolysis they give rise to sugars mixed with complex acids of high molecular weight. The gums themselves retain the character of acids, and it would appear that the molecule of a gum is composed of a number of sugar residues grouped around a nucleus acid in such a way as to leave the acid group exposed.

The gums are translucent amorphous substances, some of which dissolve in water completely, giving a sticky solution, while others merely swell up in water and form a sort of jelly; they are all insoluble in alcohol.

The natural gums must be distinguished from starch gum or dextrin, which is an artificial product obtained from starch, by the following characteristics :—

1. Solutions of natural gums are lævo-rotatory, whereas those of dextrin are dextro-rotatory.

2. Basic lead acetate precipitates natural gums from solution, but has no action on dextrin in weak solutions.

3. Natural gums on hydrolysis yield chiefly galactose and pentoses such as arabinose or xylose, whereas dextrin yields glucose only.

The hydrolysis of gums takes a long time to complete—from eighteen to twenty-four hours—whereas dextrin is easily hydrolysed.

4. On oxidation with nitric acid, natural gums yield chiefly mucic acid ($C_6H_{10}O_8$) together with some saccharic ($C_6H_{10}O_8$) and oxalic ($C_2H_2O_4$) acids, whereas dextrin yields chiefly oxalic acid together with a small quantity of saccharic and tartaric ($C_4H_6O_6$) acids.

As they occur in nature, the true gums are mostly combined with potassium, calcium, or magnesium in the form of salts, from which the free acid can be isolated by the action of a stronger acid.

The classification of gums is, for want of more accurate knowledge, based chiefly on their solubility in water :—

(*a*) Gums, such as arabin, which are completely soluble.

(*b*) Gums which are partially soluble, such as cerasin and bassorin.

(*c*) Mucilages and pectic bodies which swell up with water and dissolve, and in concentrated solution form a jelly.

The classification, however, is by no means rigid, many natural gums being composed of mixtures of several kinds of gums.

In the separation of gums from the tissues of the plant advantage is taken of their solubility in water ; it is found in practice, however, that in many cases mere maceration in water does not remove all the gum present.

Microchemical Reactions.

Microchemically, gum and mucilage may be recognized by their solubility and swelling respectively in water. Both are insoluble in alcohol and ether. With other reagents the results differ in different examples. Thus with iodine either a blue or a yellow colour may result, while in other cases the blue coloration is only obtained after treatment with chlorzinc iodide or sulphuric acid and iodine, indicating a close association with cellulose ; this type of mixed gum, e.g. gum tragacanth, is not stained by such dyes as ruthenium red (an ammoniacal solution of ruthenium sesquichloride), whereas true gums, such as those of apricot, cherry, peach, etc., are stained red. They show different degrees of solubility in cuprammonia. Many of these substances stain well with corallin soda, and they also, especially the mucilages, show a great avidity for stains such as aniline blue and aniline violet.

GUM-ARABIC.

This substance is a mixture of calcium, magnesium, and potassium salts of a weak acid of unknown constitution, to which earlier writers gave the name of arabic acid or arabin. O'Sullivan,* however, applied the term arabic acid to a

* O'Sullivan : "J. Chem. Soc.," 1884, **45**, 41 ; 1891, **59**, 1029.

substance of the formula $C_{23}H_{38}O_{22}$, which he regarded as the nucleus acid around which a number of sugar residues are grouped; by hydrolysis under varying conditions, it is possible to split off successive sugar residues with the formation of acids of gradually decreasing molecular weight, until finally the nucleus acid free from all carbohydrate residues remains, and it is this acid that he calls arabic acid; the natural gum itself would, according to him, be a diarabinan-tetragalactan-arabic acid of the formula $2C_{10}H_{10}O_8$, $4C_{12}H_{20}O_{11}$, $C_{23}H_{30}O_{18}$, which is combined with the calcium, magnesium, and potassium. The arabic acid of the earlier authors, which is the acid set free from the natural gum by the removal of the calcium, magnesium, and potassium, may be prepared by acidifying a concentrated aqueous solution of gum-arabic with hydrochloric acid, and adding alcohol. The pure substance is a white amorphous glassy mass which dissolves in water to give a lævo-rotatory solution. Ten per cent sulphuric acid converts this arabic acid into metarabic acid, which swells up in water, but does not dissolve.

Reactions.

Solutions in water (10 per cent) of arabic acid and other varieties of gum-arabic give, according to Masing,* certain more or less definite reactions.

1. They are not precipitated by (*a*) a cold saturated solution of copper acetate; (*b*) 10 per cent solution of lead acetate; (*c*) solution of ferric chloride (sp. gr. 1·2).

2. A 5 per cent solution of silicate of potash produces a cloudiness or a precipitate which is partially or wholly soluble on adding an excess. Arabic acid either does not respond to this reagent, or merely gives a slight turbidity, and the same applies to the gums obtained from certain species of *Cactus*, *Albizzia*, *Acacia catechu*, *Acacia leucophlœa*, and other plants.

3. Stannate of potash gives similar reactions, and in the case of arabic acid produces a precipitate which is soluble in excess.

* Masing: "Archiv d. Pharm.," 1879, [3], **15**, 216; 1880, **17**, 34, 41; "Year Book of Pharmacy," 1881, 191.

4. A solution of neutral sulphate of aluminium (10 per cent) generally gives a precipitate which is, in many cases, soluble in potash.

5. Basic lead acetate yields a precipitate which is entirely or partially soluble in excess.

GUM TRAGACANTH.

This gum occurs in species of *Astragalus*, and consists of about 8-10 per cent of soluble calcium, magnesium, and potassium salts, together with about 60-70 per cent of insoluble salts, which only swell up in water to a jelly. The water-soluble portion is said to contain a substance, polyaribanan-trigalactan-geddic acid, which on hydrolysis breaks up into arabinose, galactose, and geddic acid, an isomer of arabic acid. The part soluble in water, when treated with baryta water, gives two isomeric tragacanthan-xylan-bassoric acids, which on hydrolysis yield a pentose sugar tragacanthose, xylose, and bassoric acid $C_{14}H_{20}O_{13}$.

Von Fellenberg * has shown that the water-insoluble constituent of tragacanth and bassorin gums is a methoxylated compound; it dissolves in alkali undergoing hydrolysis with the liberation of methyl alcohol. The de-esterified compound is named bassoric acid and yields on hydrolysis large quantities of galacturonic acid showing it to be allied to the pectic acid derived from pectin.

WOUND GUM.

A gum-like substance, termed wound gum, is frequently found in the tracheæ of plants, in the immediate neighbourhood of wounds, and stopping up the lumina; it is secreted by the surrounding living cells. Wound gum does not swell in water, and is insoluble in sulphuric acid and in caustic soda. On oxidation with nitric acid it yields both mucic and oxalic acids, and it responds to lignin tests; e.g. on treatment with phloroglucinol and hydrochloric acid a bright red coloration results.

The origin of gums is as yet unknown; by some authors they are regarded as decomposition products of cellulose,

* v. Fellenberg: "Biochem. Zeit.," 1918, **85**, 118.

produced either by over-nutrition of certain cells or by bacterial action; * according to Wiesner, all gums are produced by a diastatic ferment acting on cellulose; although it is not possible to express any definite views on the subject, it would appear not improbable that in many cases the formation of gums and gum-like substances in the plant is a morbid condition. Mohl was able to show in the case of tragacanth gum that this substance was produced by the metamorphosis of the cells of the medullary rays.

MUCILAGE.

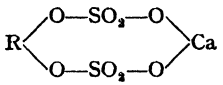
The term mucilage is applied to those substances which with water produce a slimy liquid. Mucilage is widely distributed, and occurs in all or nearly all classes of plants. Mucilage-secreting hairs, or comparable structures, occur in various Muscineæ, Filices, and especially in the Phanerogams; mucilage sacs or canals are found in certain Muscineæ, e.g. *Anthoceros*, Marattiaceæ, some Cycadaceæ, and Phanerogams; further, the external walls of plants may be generally mucilaginous; e.g. in very many Algæ, the hibernaculæ of some aquatic Phanerogams, like *Utricularia* and *Myriophyllum*, and finally in the coats of seeds and fruits, such as *Lepidium* and *Sterculia scaphigera* respectively, in which cases the superficial cell walls are mucilaginous. Mucilage is not infrequently associated with other substances; thus in the case of mucilage-secreting hairs, it is sometimes associated with tannin, and in many plants, especially in the mucilage sacs of many Monocotyledons, calcium oxalate is found.

Employed in the morphological sense the term mucilage includes a number of chemically distinct substances; thus while the mucilages from linseed, many of the Liliaceæ, and also salep yield only sugars on hydrolysis, many of the mucilages contained in seaweeds yield in addition to sugars, ash constituents which, previous to hydrolysis, were chemically combined with the carbohydrate residue. The high sulphate content of the ash of carragheen mucilage † (obtained from

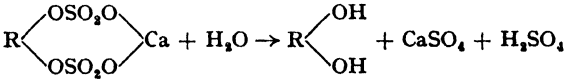
* See Greig Smith: "J. Soc. Chem. Ind.," 1904, **105**, 972.

† Haas and Hill: "Ann. App. Biol.," 1921, **7**, 352; Haas, "Biochem. Journ.," 1921, **15**, 469.

Chondrus crispus) and of agar * is accounted for by the fact that these substances have their carbohydrate residues combined with calcium sulphate in the form of an ethereal sulphate represented by the formula—



in which R represents the polysaccharide residue. Substances of this type have been shown to be colloidal electrolytes which exert measurable osmotic pressures; their solutions contain calcium ions, but the sulphate complex is masked and is only set free after hydrolysis :—



It will be seen that this hydrolysis involves the liberation of a molecule of sulphuric acid, a fact which accounts for the charring of this material which frequently occurs, owing to spontaneous hydrolysis, when the material is heated in a steam oven and even, sometimes, in the cold. Mucilages of this type have been shown to occur in a number of marine algæ † both red and brown.

Function.

Mucilage, when it is a definitely secreted product or of a definite and constant occurrence in a plant, may perform several functions, but how far these are primary functions cannot yet be stated.

When it occurs in tubers, as in the Orchidaceæ, mucilage is generally looked upon as a reserve food-material; it may serve as a check against too rapid transpiration, especially when produced in connection with developing organs, such as vegetative buds, young leaves, in the epidermis of mature leaves, the sporangia of Cryptogams, etc.; in the case of aquatic plants, such as Algæ, the hibernaculæ of *Myriophyllum*, etc., its presence may prevent a too rapid diffusion;

* Neuberg and Ohle: "Biochem. Zeit.," 1921, **125**, 311.

† Haas and Russell-Wells: "Biochem. Journ.," 1923, **17**, 696; also Harwood: "J. Chem. Soc.," 1923, **123**, 2254.

the calcareous incrustation of certain Algæ, e.g. *Neomeris dumetosa*, is dependent on the presence of mucilage; mucilage provides a water-storage mechanism in plants subjected to xerophytic conditions, e.g. *Cassia obovata*, *Malva parviflora*, *Theobroma cacao*, and *Pterocarpus saxatilis*; finally, it may be an important aid in connection with seed-dispersal and germination, as in some species of *Salvia* and *Lepidium*.

Related to the gums and mucilages are the substances known as galactans occurring in the seeds of Leguminosæ (*Lupinus*, *Medicago*, etc.); wood gum or xylan, occurring in wood, etc. These substances have already been dealt with.

PECTIC BODIES.

The term pectin was first applied by Braconnot * to the mucilaginous substance which he precipitated by means of alcohol from the juices of many fruits and from aqueous extracts of fleshy roots such as beet, carrot, swede, etc. Similar substances were later found to obtain in a great variety of plants such as onion, pea pods, leaves and stalks of cabbage, rhubarb, and flax, and also in young cells such as the root hairs of cabbage, cucumber, bean, and other plants.† In all these cases the pectin occurs in a state of solution in the cell sap or in association with the cellulose of the cell walls of parenchymatous tissues.

The name pectin was chosen because it was recognized that these substances were in some way connected with the jellying properties of fruit juices, πεκτυς being the Greek for jelly.

Frémy ‡ was the first to show that unripe fruits contain an insoluble precursor of the soluble pectin to which he gave the name pectose; as the fruit ripens the insoluble pectose is gradually converted into soluble pectin, a change which is revealed under the microscope by the swelling of the thickened walls which become translucent and exude a mucilaginous pectin.

Somewhat similar changes are brought about by boiling

* Braconnot, "Ann. d. Chim. et Phys.," 1824, [2], **28**, 173.

† Howe: "Bot. Gaz.," 1921, **72**, 313.

‡ Frémy: "J. de Pharm.," 1840, [2], **26**, 368; and "Ann. d. chim. et d. Phys.," 1848, [3], **24**, 5.

unripe fruit, whereby the acid juices exercise a hydrolytic effect upon the insoluble precursor and soluble pectin results. Prolonged boiling alters the pectin, with the result that its power to form a jelly is reduced; similarly, over-ripe fruit loses its coherence owing to the loss of the jellying qualities characteristic of the soluble neutral pectin.

When the water-soluble pectin is treated with sodium hydroxide it undergoes hydrolysis almost instantaneously, giving off methyl alcohol and leaving the sodium salt of an acid from which, on the addition of a mineral acid, the insoluble pectic acid is precipitated; this latter substance has lost all power of forming jellies which was the characteristic of the soluble pectin.

It thus becomes possible to distinguish three stages in the history of the pectins, which are represented in the following classification adopted at the Pectin Symposium of the American Chemical Society in 1925: —

1. *Protopectin* (equivalent to the older term pectose of Frémy). This represents the insoluble precursor of the true pectins and is the form in which these substances occur in the unripe material.

2. *Pectin* is the soluble substance capable of forming jellies which occurs free in the plant or is produced from protopectin in ripening or by chemical hydrolysis. Pectin is the methyl ester of pectic acid.

3. *Pectic acid* is demethylated pectin and is incapable of forming a jelly.

Isolation of Pectins from the Tissues.

Two methods of separating pectins from tissues have been adopted: Extraction by means of ammonium oxalate, and extraction by means of hot water.

(*a*) *Ammonium Oxalate Method.*—This is the method followed by Schryver and his fellow-workers: the material selected—turnips, strawberries, rhubarb petioles, apples, onions, and cabbage—is first ground and pressed to remove soluble pectins; the residue, after drying and further grinding, is extracted with warm 0·5 per cent ammonium oxalate solution

at about 80-90°; on addition of 2-3 times its volume of 95 per cent alcohol, the solution gives a precipitate of the pectin in a yield of approximately 0·1 per cent, or, in the case of the turnip, almost double this quantity.

Besides being extracted by ammonium oxalate, pectin may also be extracted by means of warm solutions of sodium or ammonium salts whose anions form insoluble salts with calcium, such as sodium carbonate or ammonium tartrate.

If the dried and ground tissues are extracted with 8 per cent sodium hydroxide, free from carbonate, previous to extraction with the ammonium oxalate, the sodium hydroxide solution will be found to contain no pectin (provided the caustic soda used was free from carbonate); it contains instead a mixture of substances which can be precipitated by an equal bulk of 95 per cent alcohol; this material reduces Fehling's solution only after hydrolysis and is coloured blue by iodine; the substances comprising this mixture yield furfural equivalent to pentose contents ranging from 40-85 per cent; they are presumably hemicelluloses and are described as Cytopectins *; though extracted from the tissues by alkali, they are not all precipitated from these solutions on addition of acid.

If the residue remaining after extraction of the tissues with caustic soda is washed free from alkali and extracted with warm 0·5 per cent ammonium oxalate, the resulting extract, on treatment with hydrochloric acid, yields a precipitate of pectic acid.

(*b*) *Hot Water Method.*—Ehrlich extracts sugar beet residues, which contain about 25 per cent of pectin, by heating with water in an autoclave under 1-2 atmospheres pressure; this treatment yields a solution of what is described as hydropectin. Hydropectin when extracted with 70 per cent alcohol yields an extract which contains an araban, while the residue insoluble in alcohol is a water-soluble calcium magnesium salt of pectic acid; a careful study of the products of the hydrolysis of this substance has shown it to be a galacturonic acid derivative (see p. 196).

* Clayson, Norris, and Schryver: "Biochem. Journ.," 1921, **15**, 643.

Until some insight had been obtained into the chemical nature of these substances, much confusion arose owing to the tendency of different authors to describe identical products under different names; the following brief summary of the development of the subject may help to clear the situation.

Frémy, acting upon pectin with acids or alkalis, obtained a number of intermediate products of hydrolysis to which he gave the names of parapectin, metapectin, pectic acid, as well as para- and metapectic acid; many of those substances were, however, insufficiently characterized and their existence is no longer credited.

Although the analyses by Tromp de Haas and Tollens of the pectins derived from a number of different sources appeared to agree fairly well with the formulæ $(C_6H_{10}O_5)_n$ or $2(C_6H_{10}O_5) \cdot H_2O$, Tollens † suspected that the pectins contained carboxyl groups; this view was finally shown to be correct when the acidic nature of these substances was established by Schryver and Haynes,‡ who prepared from pectin, by alkaline hydrolysis, a pectic acid to which they assigned the formula $C_{17}H_{24}O_{16}$.

Ehrlich § has shown that the residues from sugar beet remaining after the extraction of the sugar, provide a convenient source for the extraction of pectin; after washing with warm water to remove soluble impurities, the residue is extracted with boiling water and the filtrate after evaporation yields the pectin, though, in all probability, not in the form in which it occurred in the plant but partly hydrolysed as hydropectin (see above); extracted with 70 per cent alcohol, it yields to this solvent about 30 per cent of an araban or polymerized arabinose, while the residue consists of a water-soluble pectin. The fact that no araban is extracted by boiling beet residues for some hours with 70 per cent alcohol indicates that in the tissues the araban is combined with the water-soluble pectin, and is only hydrolysed during the boiling with water. Acid

* Tromp de Haas and Tollens: "Annalen," 1895, **286**, 278.
† Tollens: *id.*, 1895, **286**, 292.
‡ Schryver and Haynes: "Biochem. Journ.," 1916, **10**, 539.
§ Ehrlich: "Chem. Zeit.," 1917, **41**, 197; "Zeit. angew. Chem.," 1927, **40**, 1305.

hydrolysis of this soluble pectin yields galacturonic and acetic acids as well as methyl alcohol, arabinose, and galactose according to the equation—

$$C_{43}H_{62}O_{37} + 10H_2O = \underset{\text{Galacturonic acid}}{4C_6H_{10}O_7} + \underset{\text{Acetic acid}}{3CH_3COOH} + \underset{\text{Methyl alcohol}}{2CH_3OH} + \underset{\text{Arabinose}}{C_5H_{10}O_5} + \underset{\text{Galactose}}{C_6H_{12}O_6}$$

On the other hand, alkaline hydrolysis by means of calcium or barium hydroxides yields methyl alcohol, acetic acid, a galacto-araban, and a tetragalacturonic acid; the latter acid being regarded as a condensation product arising from the elimination of 3 molecules of water from 4 molecules of galacturonic acid as follows:—

$$\underset{\text{Galacturonic acid}}{4CHO(CHOH)_4 . COOH} - 3H_2O = \underset{\text{Tetragalacturonic acid}}{C_{24}H_{34}O_{25}}$$

The galacto-araban set free by alkaline hydrolysis must be distinguished from the araban separated from the original plant material by boiling with water.

Combining the information obtained from acid and alkaline hydrolysis, Ehrlich concluded that the original pectin was a triacetyl-arabino-galacto-dimethoxy-tetragalacturonic acid.

The pectins of currants and strawberries and also of orange peel are generally similar but show a rather higher content of galacturonic acid, i.e. 73-78 per cent, as compared with 68 per cent for sugar beet and 61 per cent for flax. Whereas the fruits mentioned contain about 40-50 per cent of the total pectin dissolved in the juice, the beet and orange peel contain only about 5-10 per cent in soluble form. As has been stated above, some difficulty is experienced in reconciling the various results obtained by different investigators.

Thus von Fellenberg,* who first established the presence of methylated carboxyl groups in the pectins from various fruits, came to the conclusion that the completely demethylated acid corresponding to pectin was a pectic acid of the formula $C_{70}H_{104}O_{68}$, and that the formula of the neutral pectin was—

$$\underset{\text{Arabinose}}{(C_5H_8O_4)_2}\ \underset{\substack{\text{Methyl pentose and Galactose}}}{(C_6H_{10}O_5)_2}\ \underset{\text{Galacturonic acid}}{(C_6H_7O_4 . COOCH_3)_8} . 2H_2O$$

i.e. a condensation product formed from 2 molecules of

* v. Fellenberg: "Biochem. Zeit.," 1918, 85, 141.

arabinose, 1 molecule of methyl pentose, 1 molecule of galactose, and 8 molecules of galacturonic acid.

On the other hand, Schryver * and his colleagues, who worked on turnips, onions, and pea pods, hold somewhat divergent views from those of the previous authors. Thus they disagree with von Fellenberg's statement that pectins contain any methyl pentose residue—a view which is supported by Nanji, Paton, and Ling.† Moreover, the insoluble form of pectin as it occurs in the cell wall, to which they give the name pectinogen rather than protopectin, they regard as a pectic acid in which three carboxyl groups are methylated and one is in loose combination with calcium; they obtain their pectinogen by extracting the cell wall material with 0·5 per cent solutions of either ammonium oxalate or oxalic acid; the methoxyl content of their extracts varies according to the period of extraction; the shorter the time required the more nearly does the composition correspond to that of the pectinogen as it occurs in the cell wall.

Alkalis, such as lime water, convert pectinogen into pectic acid with liberation of methyl alcohol, but accompanying this hydrolysis there is also the separation of a second substance of the nature of a hemicellulose; no mention is made of the araban obtained by Ehrlich in the sugar beet pectins, and it is not clear whether this is identical with the above hemicellulose.

As is stated previously, the formula assigned to the pectic acid obtained by Schryver and Haynes ‡ by the action of caustic soda on pectic substances, is $C_{17}H_{24}O_{16}$, while that assigned by von Fellenberg to his acid was $C_{70}H_{104}O_{68}$. The discrepancy may be explained by assuming that the latter formula is that of the normal acid containing eight carboxyl groups, while the formula of Schryver and Haynes represents an anhydride of this acid resulting by the elimination of 4 molecules of water, thus § :—

$$\underset{\text{[Schryver \& Haynes]}}{4C_{17}H_{24}O_{16} \text{ or } C_{68}H_{96}O_{64}} \equiv \underset{\text{[v. Fellenberg]}}{C_{70}H_{104}O_{68}} - 4H_2O \text{ or } C_{70}H_{96}O_{64}$$

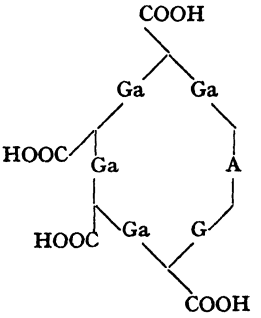
* Norris and Schryver: "Biochem. Journ.," 1925, **19**, 676.

† Nanji, Paton, and Ling: "J. Soc. Chem. Ind.," 1925, **44**, 253T.

‡ Schryver and Haynes: "Biochem. Journ.," 1916, **10**, 539. Carré and Haynes: *id.*, 1922, **16**, 60.

§ Carré: "Ann. Bot.," 1925, **36**, 818.

As regards the configuration of the pectic acid molecule, it has been suggested by Nanji, Paton, and Ling * that it contains a six-sided ring, each side of which is occupied by an appropriate carbohydrate residue as under :—



G = Galactose.
A = Arabinose.
Ga = Galacturonic acid.

a formula † which bears a striking resemblance to the basal nucleus suggested for starch (p. 157). The empirical formula of this acid is $C_{35}H_{50}O_{33}$, i.e. approximately double that proposed by Schryver and Haynes, namely $C_{17}H_{24}O_{16}$.

Properties of Pectins.

1. Neutral pectins are soluble in warm water, without boiling, up to 2 per cent, yielding a viscous solution ; such solutions do not give a jelly unless boiled with sugar and tartaric acid.

2. A 2 per cent solution of pectin is mixed with one-tenth its volume of a freshly prepared solution of pectase and a pinch of calcium carbonate ; in from one to two hours the reaction will have become acid and the solution should have set to a gel.

3. Treated with caustic soda, pectins are saponified with formation of sodium pectate which, on addition of acetic acid and calcium chloride, gives a precipitate of calcium pectate.

4. About 0·5 gram of pectin is placed in a 150 c.c. distillation flask with 20 c.c. of water ; as soon as solution is effected,

* Nanji, Paton, and Ling : " J. Soc. Chem. Ind.," 1925, **44**, 253 T.
† See also Norris and Schryver : " Biochem. Journ.," 1925, **19**, 685.

5 c.c. of 10 per cent caustic soda are added; after gently agitating the flask, the cork is inserted and the mixture is left for five minutes; now add 2·5 c.c. of dilute sulphuric acid, gently agitate once more, and distil over 17 c.c. of liquid; this is once more distilled, about 11 c.c. being collected. The resulting liquid is tested for methyl alcohol *

5. Pectin solutions are precipitated by copper sulphate or lead nitrate but not by barium chloride, ferric chloride, or lead acetate. Also they are precipitated by baryta or lime water or basic lead acetate.

Alkalis readily attack pectins with liberation of methyl alcohol and formation of the alkali metal salt of pectic acid; neutral calcium and barium salts do not precipitate unless alkali is present.

6. Aqueous solutions of pectins are precipitated by alcohol, but the precipitate can be redissolved in water.

7. Pectins are insoluble in ammoniacal solution of copper hydroxide.

Microchemical Reactions.

The fact that these pectic substances are akin to cellulose, and occur in conjunction with it, renders its identification by microchemical means somewhat difficult. Mangin † gives the following methods:—

1. Methylene blue, Bismarck brown, and fuchsin stain pectic substances, lignified and suberized walls, but not pure cellulose. If sections thus stained are treated with alcohol, glycerine, or dilute acids, the lignified or suberized walls retain their coloration, whilst the pectic substances are decolorized with rapidity.

2. Crocein and nigrosin stain lignified and suberized walls, but do not stain pectic compounds.

3. Crocein, naphthol black, and orseille red stain pure cellulose, but do not stain pectic substances; similarly, pectic compounds are unstained by congo-red and azo-blue, whilst cellulose and callose are.

* Particulars of this test will be found in Sucharipa: "Die Pektinstoffe," Braunschweig, 1925.

† Mangin: "Compt. rend.," 1889, 109, 579; 1890, 110, 295, 644.

4. The middle lamella, which consists of compounds of pectic acid, may be differentiated from the other pectic substances which are mixed with the cellulose of the cell walls by the following method: A thin section is placed in a 20-25 per cent solution of hydrochloric acid in alcohol for twenty-four hours; the section is then washed with water and treated with methylene-blue or phenosafranin. The middle lamella stains much more deeply than the rest of the wall.

5. If, after the above treatment with acid alcohol, the section be washed in a 10 per cent solution of ammonia, it is found that the cells separate with ease one from the other. According to Mangin, the combined pectic acid is freed from its bases by the treatment with acid alcohol, and is then dissolved by the ammonia. A recombination of the pectic acid may be brought about by treatment with baryta water, and after this process the cells will not separate one from the other.

6. Mehta * finds that pectic compounds stain deeply with the following dyes: Alcoholic malachite green, aqueous congo red, alcoholic eosin, alcoholic safranin, aqueous gossypimin, aqueous iodine green, and aqueous ruthenium red. None of the stains, however, is specific for pectic substances; thus ruthenium red stains oxycelluloses, hemicellulose, gums, galactans, etc. The procedure adopted by Mehta is to dissolve out the various constituents of the cell wall with appropriate reagents and then to compare their staining reactions. The sections are placed in a test tube with the reagent which is then heated for six to eight hours in a boiling water bath, the liquid being decanted off every hour and replaced by fresh reagent. The sections are then washed with hot distilled water, stained for two hours, washed with 90 per cent alcohol to remove excess of stain, dehydrated with absolute alcohol, and mounted in cedar-wood oil. The reagents used were 0·5 per cent ammonium oxalate, 0·5 per cent ammonium oxalate in 3 per cent ammonia solution, 4 per cent sodium hydroxide, 3 per cent hydrochloric acid, or 95 per cent alcohol.

* Mehta: "Biochem. Journ.," 1925, 19, 979.

7. Pectins are insoluble in ammoniacal solution of copper hydroxide (cuprammonia).

Estimation of Pectins.

The fact that pectins are readily saponified by caustic soda has been adapted by Carré and Haynes * as a means for their estimation. A dilute solution of the pectin is allowed to stand with N/10 NaOH overnight, and is then treated with N acetic acid and after five minutes with M calcium chloride; the mixture is allowed to stand for an hour and is then boiled for a few minutes and filtered; the precipitated calcium pectate is washed repeatedly until free from chloride and weighed after drying at 100° C. The composition of the precipitate was represented as $C_{17}H_{22}O_{16}Ca$, containing 7·66 per cent of calcium; but if the newer formula for pectic acid is accepted, it would be $C_{35}H_{46}O_{33}Ca_2$, containing 7·45 per cent of calcium. The method has been employed by Carré † for investigating the changes which occur in the pectic constituents of fruits during ripening.

Action of Enzymes on Pectins.

Pectase is the name given by Frémy to an enzyme which he found was able to effect the coagulation of pectin solutions. Pectases are very widely distributed in the plant world and are found in the leaves of very many green plants; the activity of the enzymes from different sources is, however, not the same as may be seen from the following table ‡ giving the time required for gelatinizing a 2 per cent pectin solution:—

Solanum lycopersicum	48 hrs.	*Daucus* (young)	15 mins.
Vitis vinifera (fruit)	24 ,,	*Zea Mais*	8 ,,
Ribes	15 ,,	*Iris florentina*	3 ,,
Rheum rhaponticum	12 ,,	*Trifolium pratense*	Less than 1 min.
Marchantia polymorpha	2½ ,,	*Medicago sativa*	
Daucus (mature)	2 ,,	*Plantago*	
Delphinium	1½ ,,	*Brassica napus*	
Gingko biloba	35 mins.	*Lolium perenne*	

* Carré and Haynes: "Biochem. Journ.," 1922, **16**, 60; also Emmett and Carré · *id.*, 1926, **20**, 6.

† Carré: *id.*, 1922, **16**, 704; "Ann. Bot.," 1925, **39**, 811.

‡ Bertrand and Mallèvre: "Compt. rend.," 1895, **121**, 726.

The optimum hydrogen ion concentration of the pectase from currants was found by Euler and Svanberg * to be P_H 4·3.

While Bertrand and Mallèvre showed that the presence of calcium salts was essential for the production of a gel, Goyaud † came to the conclusion that the activity of the enzyme was in no way dependent upon the presence of calcium salts inasmuch as it was able to break down the pectin to pectic acid even in solutions which had been deprived of calcium salts by the addition of potassium oxalate; the addition of calcium salts to such solutions, however, at once produced a gel owing to the precipitation of the insoluble calcium pectate. It is concluded from this that the function of calcium in this connection is only to reveal the products of the activity of the hydrolytic enzyme; if this be the true explanation, it is a very remarkable fact that pectase should in many cases be able to effect hydrolysis of pectin in less than one minute, although it must be borne in mind that the hydrolysis by means of caustic soda likewise is completed in a very short time (cf. p. 198).

Another enzyme, pectinase, was first described by Bourquelot and Hérissey ‡ as occurring in malt and was later found by Ehrlich in takadiastase; this enzyme also acts hydrolytically upon pectins but breaks them down further than pectase, past the pectic acid stage to the yielding of reducing sugars. This enzyme is said to be the one secreted by *Granulobacter pectinovorum*, and *Bacillus carotovorus*. An enzyme having similar properties has been described by Kylin § as occurring in marine algæ.

Pectins are also subject to attack by enzymes secreted by various fungi and other bacteria, though the exact nature of the products of their activity has yet to be studied. Thus it was shown by de Bary that *Peziza sclerotiorum* destroys the host plant by disintegrating the cell walls owing, presumably,

* Euler and Svanberg: "Biochem. Zeit.," 1919, **100**, 271.

† Goyaud: "Compt. rend.," 1902, **135**, 537.

‡ Bourquelot and Hérissey: "J. Pharm. et Chim.," 1898, [6], **8**, 145; 1899, [6], **9**, 563, and **10**, 5. Also Verdon: "J. Pharm. Chem.," 1912, **5**, 347.

§ Kylin: "Zeit. physiol. Chem.," 1915, **94**, 412.

to a solution of the middle lamella ; a somewhat similar effect was shown by Brown * to occur when an enzyme extract of *Botrytis cinerea* was allowed to act upon tissues of potato, turnip, beet, and apple. It was, moreover, shown by Winogradsky † that the retting of flax was due to the solution of pectic substances by enzymes secreted by bacteria.

Origin and Constitutional Relationships of the Pectins.

The fact that galacturonic or similar sugar aldehyde acids occur in pectins, hemicelluloses, and gums suggests that all cell wall constituents are more or less related to each other. It must be borne in mind that whilst the cell wall of unlignified elements is composed of cellulose together with pectin, in lignified elements lignin occupies the place of pectin ; for this reason pectin is regarded as the precursor of lignin, and the fact that both pectins and lignin contain acetyl ‡ and methoxyl groups would tend to support this view. Ehrlich § even claims to have isolated from the lignified tissues of the flax plant a substance which he describes as a resin-like lignic acid ; this he considers to represent a transition stage between a typical unaltered pectin and lignin. Fuchs || considers that lignin may have been produced from pectins by loss of water and oxygen.

It has been suggested by Nanji, Paton, and Ling that pectins themselves arise from the condensation of galactose to a hexagalactan which is then oxidized to uronic acids and methoxylated.

Yet another suggestion is that of Smolenski,¶ who regards pectins as intermediate stages in the conversion of hexoses into pentoses.

The Changes taking Place in Ripening.

The view originally put forward by Frémy was that softening of the tissues of fruits on ripening was due to a

* Brown : " Ann. Bot.," 1915, **29**, 313 ; 1917, **31**, 489.
† Winogradsky : " Compt. rend.," 1895, **131**, 742.
‡ But see also Nelson : " J. Amer. Chem. Soc.," 1926, **48**, 2945.
§ Ehrlich : " Zeit. angew. Chem.," 1927, **40**, 1305.
|| Fuchs : " Brennstoff Chem.," 1926, **7**, 302.
¶ Smolenski : " Chem. Soc. Abstracts," 1924, **1**, 16.

conversion of the insoluble pectose, or protopectin as it is now termed, into soluble pectin, a change which he thought was due to the hydrolytic action of the fruit acids. Evidence has, however, been obtained to show that this change is due to enzyme action.* That the middle lamella is not involved in the earlier stages of this change has been shown by Carré,† who found that the middle lamella pectic substance remained at a constant level throughout the process of ripening, and it was only in the over-ripe condition that the amount begins to decrease and finally vanishes when, owing to the absence of cementing material, the cells are entirely separated from one another. In the absence of exact knowledge concerning the nature of the relation between soluble pectin and protopectin, it is not possible to be certain what happens when the one is formed from the other. The view put forward by Mangin ‡ that protopectin is a loose form of combination between pectin and cellulose is supported by von Fellenberg, who suggests that the production of soluble pectin from protopectin involves hydrolysis of this compound; the same conclusion has been arrived at by Sucharipa §; possibly the softening of fruit on ripening is due to this same separation of soluble pectin from its combination with cellulose; it is only in the last stages of over-ripeness that still further hydrolysis of the pectin would occur with consequent disintegration of the tissues as indicated above.

Appleman and Conrad,|| working on peaches, find that the transformation of protopectin into pectin appears to be the only pectic change during ripening and softening. The sum of pectin and protopectin was practically constant at all stages of ripening, but both constituents disappear slowly in over-ripe peaches.

The everyday significance of pectins as the basis of fruit jellies and jams justifies a reference to their use in this con-

* Thatcher: "J. Agric. Res.," 1915, **5**, 103; Carré, "Ann. Bot.," 1925, **39**, 811.

† Carré: *loc. cit.* ‡ Mangin: "Comp. rend.," 1888-1893.

§ Sucharipa: "J. Amer. Chem. Soc.," 1924, **46**, 145.

|| Appleman and Conrad: "Univ. Maryland Agr. Exp. St. Bull.," 283, July, 1926.

nection. At the outset it is important to distinguish clearly between the irreversible pectin gels formed by the action of pectase, and the reversible gels concerned in the formation of fruit jellies or jams. As pointed out above, the former are most probably composed of insoluble calcium pectate which when once formed cannot be got into solution again. On the other hand, it is known that soluble pectin forms in water not a true solution but a sol which in about 2 per cent concentration is fairly viscous but does not set to a gel; in order to produce a gel from such a solution it requires to be boiled in water containing about 60 per cent of cane sugar and approximately 1 per cent of tartaric acid; on cooling the resulting mixture sets to a gel which, according to Sucharipa, is due to the fact that the pectin is insoluble in such a solution of cane sugar. Care must be taken not to boil for too long as otherwise hydrolysis may set in which will entail loss of methyl alcohol; it appears from the work of Nanji and Norman * and others that the jellying power of a pectin is a function of its methoxyl content; the authors mentioned have worked out a micro-method for the determination of methyl alcohol.

CELLULOSE.

The term cellulose should be taken in general to connote a group of substances rather than a single chemical compound; used in this generic sense, it comprises a number of substances of somewhat different origin and characters, whose chief common properties are their physiological origin and their function in forming the basis of the material which is isolated by the protoplasm of the living cell for the purpose of forming the wall or periphery of that cell. Though met with chiefly in the vegetable kingdom, its occurrence in the animal kingdom is not unknown, since a substance described as tunicin, said to be identical with cellulose, has been found in the cell walls of certain tunicates and insects. In the course of time the cellulose originally formed is altered by the addition to it of various secondary products known as encrusting

* Nanji and Norman: " J. Soc. Chem. Ind.," 1926, **45**, 337; Baker: " J. Ind. Eng. Chem.," 1926, **18**, 89.

substances; thus the process of lignification consists in the conversion of cellulose into ligno-cellulose; accompanying this change is a gradual disappearance of the protoplasm. Thus the protoplasm within the cell produces a number of different substances which are deposited in the cell wall, the nature and properties of the resulting fibre depending on the nature of these substances.

CLASSIFICATION OF CELLULOSES.

The naturally occurring celluloses were originally classified by Cross and Bevan in the following manner :—

I. *Typical or Normal Celluloses of the Cotton Type.*—These were exemplified by the cellulose obtained from cotton, flax, hemp, etc.

II. *Compound Celluloses of the Wood Cellulose, Jute and Cereal Grass Types.*—The natural celluloses occurring in jute, cereal straws, esparto grass, etc., were regarded as consisting of some form of combination of cellulose with a non-cellulose constituent, either of the nature of lignin in the case of lignocelluloses, or a pectic or gummy substance in the case of pectocelluloses, or a fatty substance in the case of adipocelluloses. This group was therefore subdivided into :—

(*a*) Lignocelluloses, e.g. jute fibre.
(*b*) Pectocelluloses, e.g. flax.
(*c*) Adipo- or Cuto-celluloses, e.g. cork.

III. *Hemi-, Pseudo- or Reserve Celluloses.*—This heterogeneous collection of substances differ structurally from the fibrous celluloses, and occur in the cell walls of the seeds of various plants such as *Coffea arabica, Soja hispida, Lupinus luteus, Cocos nucifera, Tropæolum majus, Impatiens balsamifera, Pæonia officinalis*, and in peas and beans.

In this group of celluloses were also included those which, according to the researches of Brown and Morris, are dissolved by the enzymes secreted by the germinating seed; these are sometimes referred to as reserve cellulose, though the name seems ill-chosen, inasmuch as they would not appear always to function as reserve material.

Associated with this classification was the conception that there existed in the plant several distinct varieties of cellulose. Thus Cross and Bevan found that the cellulose obtained after delignification of the straw of cereal grasses and of esparto when distilled with hydrochloric acid gave considerable quantities of furfural, from which they concluded such cellulose to be possessed of furfural-producing groups which they termed furfuroids. It has, however, been shown by Irvine and Hirst * that esparto cellulose consists of a mixture of ordinary cellulose with a pentosan, xylan, in the proportion of, approximately, 80 to 20, and that by repeated treatment with alkali the xylan could be dissolved out. The same state of affairs has been shown to hold for straw cellulose by Heuser and Haag,† and in view of the proved existence of pentosans in such cases Heuser and others consider the identity of celluloses from different sources to be established, and regard Cross and Bevan's assumption of the existence of furfuroids to be unnecessary.

One of the richest sources of cellulose in nature is the cotton plant. The following table, taken from Bowman,‡ represents approximately the composition of cotton fibre from various sources :—

Source of Cotton.	Surat.	American.	Egyptian.
	Per cent.	Per cent.	Per cent.
Cellulose	91·35	91·00	90·8
Wax, oil, and fat . . .	·40	·35	·42
Protoplasm and derivatives (Pectose)	·53	·53	·68
Mineral matter, i.e. salts of K, Na, Ca, Mg, Fe, and Al . . .	·22	·12	·25
Water	7·50	8·00	7·85

Although the raw cotton wool is amongst the purest of mature structures with respect to its cellulose, it requires treatment before it can be regarded as approximating to a

* Irvine and Hirst : " J. Chem. Soc.," 1924, **125**, 15.

† Heuser and Haag : " Zeit. angew. Chem.," 1918, **31**, 99, 103, 166, 172 ; also Heuser and Aiyar : *id.*, 1924, **37**, 27.

‡ Bowman : " The Structure of the Cotton Fibre," London, 1908, p. 147.

condition of chemically pure cellulose. For this purpose the cotton wool has to be extracted successively in a Soxhlet for six hours with 96 per cent alcohol and with ether to remove fats and waxes; it is then boiled for several hours with 1 per cent caustic soda under specified conditions involving the rigorous exclusion of air in order to avoid oxidation. After washing with water and acetic acid, and finally with water, it is dried. The resulting product, known as standard cellulose, should have the following composition:—

α-Cellulose	99·8
Ash	0·03–0·06

The term α-cellulose is applied to cellulose which is insoluble in 17·5 per cent caustic soda. In other plant material such α-cellulose is accompanied by varying proportions of two other modifications known respectively as β- and γ-cellulose; these forms are less resistant to chemicals than the α form, but may only differ from it in the degree of polymerization, of dehydration or even of dispersion.† The relative proportions in which these forms occur in a given sample of cellulose may be determined by extraction with 17·5 per cent caustic soda whereby α-cellulose remains undissolved while the β and γ modifications go into solution; on acidifying the filtrate with acetic acid, the β-cellulose is precipitated while the γ- remains dissolved. The evaluation of a cellulose for its suitability for technical purposes is largely dependent upon the results of such an analysis.

PROPERTIES OF CELLULOSE.

Pure cellulose is a white hygroscopic substance, which absorbs about 6-12 per cent of water, which it loses again when heated to 100°; it is insoluble in water at ordinary pressure, but when heated with water in sealed vessels at 500° F. it is dissolved completely with decomposition.

SOLUBILITY OF CELLULOSE.

Cellulose is insoluble in all ordinary solvents, but when treated with zinc chloride in the presence of water it is con-

* Corey and Gray: "J. Ind. Eng. Chem.," 1924, **16**, 853, 1130.
† See Schwalbe and Becker: "J. prakt. Chem.," 1919, **100**, 19.

verted into a gelatinous hydrate which, after prolonged treatment, goes into solution.

A solution of 6 parts of zinc chloride in 10 parts of water heated to 60-100° is thoroughly stirred up with 1 part of cellulose, and then digested for some time at a gentle heat. When the cellulose is gelatinized, its solution is completed by heating over a boiling water bath, and adding water from time to time to replace that lost by evaporation.

Two other salt solutions are known which dissolve cellulose :—

(*a*) *Zinc Chloride and Hydrochloric Acid.*—A solution of zinc chloride in twice its weight of hydrochloric acid dissolves cellulose rapidly in the cold.

(*b*) *Ammoniacal Cupric Oxide* (*Schweitzer's Reagent*).—The solution is most conveniently prepared by drawing a current of air through a Wolff bottle containing 0·880 ammonia and some copper turnings, until a deep blue solution is obtained. Cellulose dissolves in this solvent and on the addition of acid is reprecipitated.

ACTION OF VARIOUS CHEMICALS ON CELLULOSE.

1. *Alkalis.*—Solutions of caustic soda of 1–2 per cent strength have no action on cellulose at temperatures considerably above 100°.

When cotton fibres are immersed in a 17·5 per cent solution of caustic soda they shorten, swell up, and the lumen becomes obliterated ; the physical process of swelling is accompanied by a chemical change involving the formation of an unstable sodium compound $C_6H_9O_5Na$; on washing with water the sodium is removed, but the recovered cellulose has, as a result of its swelling, acquired a greater affinity for dyes. This observation was first made by Mercer in 1844 and technically exploited by him for dyeing cotton. Later, in 1890, it was discovered by Lowe that if the alkali treatment is carried out while the cotton was under tension the fibres acquired a lustre, a process known as mercerization.

When fused at 200-300° with a mixture of sodium and

potassium hydroxides, cellulose undergoes complete decomposition with the formation of oxalic and acetic acids.

The so-called alkali cellulose obtained by treating cellulose with 17·5 per cent caustic soda reacts with carbon disulphide to form xanthogenates; * these compounds are used in the manufacture of viscose (see below).

2. *Acids.*—Nitric acid (sp. gr. 1·25) at 180° converts cellulose into *oxycellulose*, a substance of a weak acidic character, which reduces Fehling's solution (see below). Concentrated nitric acid, or a mixture of this acid with concentrated sulphuric acid, converts cellulose into nitrates, the composition of which varies with the conditions of the experiment; di-, tri-, tetra-, penta-, and hexa-nitrates, which are of considerable technical importance, are known.

If dilute sulphuric acid is allowed to act for some hours at 100° C. on cotton, it does not alter the structure of the fibre, but makes it friable. This was at one time thought to be due to the formation of a definite substance, hydrocellulose. That this material is not a simple substance may be shown by the fact that it has acquired reducing properties, the substance responsible for which may be extracted with alkali leaving behind unchanged cellulose. The fact that the alkaline extract is yellow in colour suggests the presence of an aldehyde, possibly glucose. For these reasons it is considered that the term hydrocellulose implies a stage in the hydrolysis of cellulose rather than a definite chemical substance; it may be a mixture of cellulose, cellulose dextrins, and glucose.

Cellulose, when treated with concentrated sulphuric acid, undergoes considerable swelling, and goes into solution with the ultimate formation of dextrose. This is made use of in the preparation of vegetable parchment for which purpose paper is rapidly drawn through a mixture of 4 parts of sulphuric acid with 1 of water; the paper is then thoroughly washed with water until free from acid. If, on the other hand, cellulose is left in contact with concentrated sulphuric acid for a time sufficient to dissolve it, and the solution is immediately

* Cross, Bevan, and Beadle: "Ber. deut. chem. Gesells.," 1893, **26**, 1090; and Cross and Bevan: *id.*, 1901, **34**, 1513.

diluted, a gelatinous hydrate is precipitated; this substance is known as amyloid, since it resembles starch in giving a blue colour with iodine. The same substance is formed by the action of chlorzinc iodide, the reaction being used as a test for cellulose.

Cellulose on hydrolysis yields glucose only. Several claims to have effected the quantitative conversion of cellulose into glucose were made on the basis of observations of the change in optical activity, but the first to obtain an approximately quantitative yield of crystalline glucose from cellulose was Monier Williams * who left the cellulose in contact with 72 per cent sulphuric acid for a week and then after dilution boiled the mixture for fifteen hours.

The combined action of glacial acetic acid and acetic anhydride in the presence of concentrated sulphuric acid or zinc chloride converts cellulose into *acetyl cellulose*, which is insoluble in water but soluble in several organic solvents. Acetyl cellulose is also used in the manufacture of artificial silk.

Cellobiose,† $C_{12}H_{22}O_{11}$, is a disaccharide obtained in the form of its acetate by acting on cellulose with acetic anhydride and concentrated sulphuric acid. It stands in the same relation to cellulose as does maltose to starch.

3. *Oxidizing Agents.*—Dilute solutions of alkaline hypochlorites have very little action on typical cellulose, and can therefore be employed for bleaching this material; with concentrated solutions of hypochlorites, however, a general decomposition ensues. As already mentioned, nitric acid (sp. gr. 1·25) at 180° converts cellulose into a series of oxidation products known as oxycellulose, and similar substances are produced by the action of other oxidizing agents, such as chromic acid, potassium chlorate in the presence of hydrochloric acid, etc.

Oxygen containing 2 per cent of ozone at once attacks dry cotton with the formation of a cellulose peroxide ‡ and an acid substance; the latter, when boiled with water, dissolves,

* Monier Williams: "J. Chem. Soc.," 1921, **119**, 803.

† Skraup and König: "Ber. deut. chem. Gesells.," 1901, **34**, 1115; Schliemann: "Annalen," 1911, **378**, 366.

‡ Dorée: "J. Chem. Soc.," 1913, **103**, 1347.

leaving a neutral product which resembles a typical aldehydic oxycellulose. This is regarded as being due to the oxidation of an alcoholic group into cellulose molecule (see formulæ, p. 214).

4. *Action of Ferments.*—It has been shown by Brown and Morris, in the case of barley, rye, oat, and other cereals, that the cell wall of the endosperm cells which contain nutrient material are broken down by a cellulose-dissolving ferment, a cyto-hydrolyst, before the embryo can procure the food-stuff contained in these cells. This enzyme, which is developed during the germination of the seed, can be extracted from the malt by cold water, and precipitated from this solution by alcohol.

A cytase capable of hydrolysing hemicellulose has been extracted from *Aspergillus Oryzæ*, from the cotyledons of *Lupinus albus*, and *Phœnix dactylifera.** Cytase splits hemicellulose into glucose, mannose, galactose, and pentose.

Cellulase is an enzyme which attacks ordinary cellulose converting it into cellobiose. It occurs in *Aspergillus cellulosæ*† and in certain bacteria.‡ It is well known that many fungi, *Actinomyces*, *Aspergillus*, *Coprinus*, *Penicillium*, and *Trichoderma*, § for example, have the power of breaking down cellulose. In this activity they may be of even greater significance in the soil than the cellulose-splitting bacteria ‖ which belong to the aerobic and the anaerobic forms: of the former *Spirochæta cytophaga* ¶ and *Microspora agariquefaciens* ** may be mentioned; and of the latter those responsible for the subaquatic decomposition of cellulose with the evolution of marsh gas or, in other forms, of hydrogen.

OXYCELLULOSE.

When cellulose is exposed to the action of oxidizing agents there results a product which contains a greater proportion of oxygen than the original and is therefore known as oxycellu-

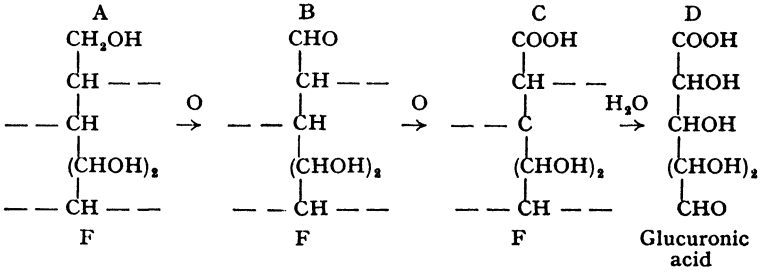
* Newcombe: "Ann. Bot.," 1899, **13**, 49.
† Ellenberger: "Zeit. physiol. Chem.," 1915, **96**, 236.
‡ Pringsheim: *id.*, 1912, **78**, 266.
§ Waksman: "Soil Sci.," 1916, **2**, 103.
‖ See Russell: "The Micro-organisms of the Soil," London, 1923.
¶ Hutchinson and Clayton: "J. Agric. Sci.," 1919, **19**, 143.
** Gray and Chalmers: "Ann. Appl. Biol.," 1924, **11**, 324.

lose; the composition of this substance varies according to the conditions under which oxidation is effected.* The fact that many oxidizing agents act in an acid medium makes it impossible to effect oxidation without a certain amount of hydrolysis, and for this reason the term oxycellulose must be taken not to signify a definite chemical individual but an indefinite mixture of oxidized cellulose, hydrocellulose, and unaltered cellulose.

Birtwell, Clibbens, and Ridge † claim that, from a technical point of view, two distinct types of oxycellulose must be recognized; those having great affinity for methylene blue and a low reducing power, and those having a high reducing power, or so-called copper number, and a marked solubility in alkali.

Properties of Oxycellulose.

The outstanding characteristics of oxycellulose are the possession of (1) aldehydic properties which are shown by the ability to react with Schiff's reagent, the production of a yellow colour on warming with alkali, and the power of reducing Fehling's solution; (2) acidic properties; (3) greater reactivity as shown by its being more easily acetylated, nitrated, etc., than cellulose; (4) greater affinity for methylene blue; (5) the ability to give off furfural when distilled with hydrochloric acid; this may be explained according to Schorger ‡ by assuming the formation of glucuronic acid (which is known to give furfural with hydrochloric acid) by the following scheme:—



* Hibbert and Parsons: "J. Soc. Chem. Ind.," 1925, 44, 473 T.

† Birtwell, Clibbens, and Ridge: "J. Text. Inst.," 1925, 16, 137.

‡ Schorger: "The Chemistry of Cellulose and Wood," London, 1926, p. 289.

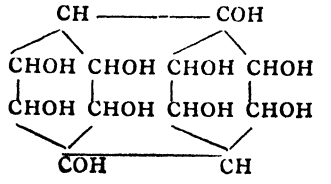
AF represents the glucose anhydride unit of cellulose, the dashes representing oxygen linkages; by oxidation the aldehydic group B is developed from A and on further oxidation gives the carboxyl group C; hydrolysis at F then sets free the glucuronic acid.

Microchemical Detection of Oxycellulose.—The investigations of Wood * and of Mehta † have shown that oxycellulose occurs naturally in the cell wall of a great variety of plant materials, and may be detected by the following means:—

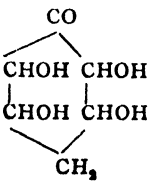
The material is washed with acid and then with water to remove all acid and then is stained with congo red; by treating again with acid, the red colour is changed to blue; on washing with water until the background becomes red, any oxycellulose present will appear dark blue or black. Oxycellulose, in common with pectic substances, hemicelluloses, and gums is stained by ruthenium red.

CONSTITUTION OF CELLULOSE.

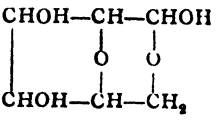
While it is agreed that cellulose is built up from a number of glucose anhydride groups $C_6H_{10}O_5$ opinions differ as to the constitution of this unit group. The formulæ proposed by Cross and Bevan, Vignon, and Green are given below:—



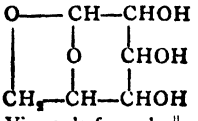
or



Cross and Bevan's formulæ.‡



Green's formula.§



Vignon's formula.‖

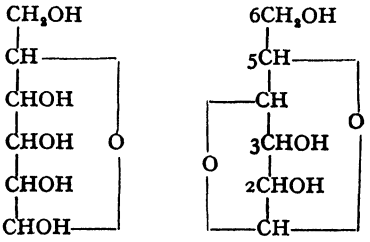
Cross and Bevan's formula implies the presence of a ketonic group for which there is no evidence; furthermore it contains

* Wood: "Ann. Bot.," 1924, **38**, 275; 1926, **40**, 547.
† Mehta: "Biochem. Journ.," 1925, **19**, 979.
‡ Cross and Bevan: "J. Chem. Soc.," 1901, **79**, 366.
§ Green and Perkin: *id.*, 1906, **81**, 811.
‖ Vignon: "Bull. Soc. Chim.," 1899, **21**, 599.

four hydroxyl groups, whereas it is known that the highest nitrate obtained from a cellulose molecule containing six carbon atoms is a trinitrate. By the nitration of cellulose, it is possible to obtain a whole series of esters representing different degrees of nitration. These various compounds may be described as mono-, di-, tri, etc., up to deca- or possibly dodeka-nitrates of a cellulose molecule containing twenty-four carbon atoms. What is commonly called cellulose hexa-nitrate, the substance employed in the manufacture of gun-cotton, is calculated on a C_{12} molecule which, therefore, corresponds to a trinitrate of a C_6 molecule.

Formulæ such as Green's or Vignon's receive some support from the behaviour of cellulose on distillation, and from the ease with which cellulose gives rise to brommethylfurfural on heating with hydrobromic acid.

On the other hand, the formula suggested by Hibbert * for the cellulose nucleus brings out clearly the relationship of cellulose to glucose as may be seen from a comparison of the two formulæ :—

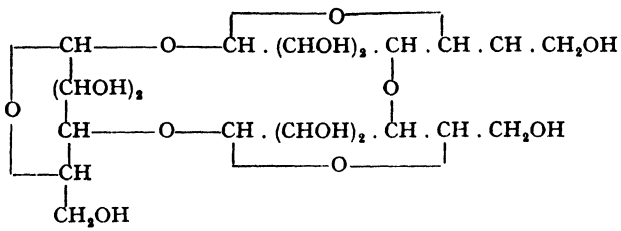


Glucose $C_6H_{12}O_6$ Hibbert's formula $C_6H_{10}O_5$

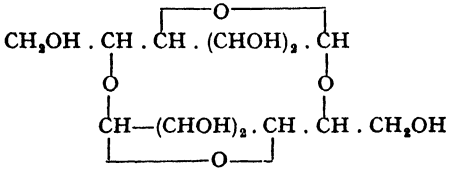
Denham and Woodhouse, by exhaustive methylation of cellulose and subsequent hydrolysis, were able to show that 2 : 3 : 6 trimethylglucose resulted, from which it would appear that carbon atoms 1 and 5 are occupied in the original cellulose molecule in uniting together the various unit groups.

The fact that the acetolysis of cellulose gives rise to cellobiose and glucose caused Irvine † and co-workers to suggest a trisaccharide constitution for cellulose as represented by the formula—

* Hibbert : " J. Ind. Eng. Chem.," 1921, 13, 256, 334.
† Irvine and Robertson : " J. Chem. Soc.," 1926, 129, 1488.



On the other hand, Karrer * favours a disaccharide basis, such as—



but much work remains to be done before a final decision is possible.

MICROCHEMICAL REACTIONS.

1. With a dilute solution of iodine a yellow coloration results.

2. After staining well with iodine, the addition of strong sulphuric acid causes the cellulose walls to swell considerably and to turn blue.

3. Chlorzinc iodide causes swelling, accompanied by the assumption of a blue colour.

4. Calcium chloride iodine solution turns pure cellulose dull pink to violet without swelling.

Zimmermann gives the following directions for making this reagent. A concentrated solution of calcium chloride is made, and for each 10 c.c. of this solution there is added ·5 gram of potassium iodide and ·1 gram of iodine. The mixture is then gently heated and filtered through glass-wool.

5. Pure cellulose is easily soluble in cuprammonia.

LIGNIFIED MEMBRANES.

Wood, for the most part, is the material used in the study of lignified tissues, and is best employed as a source of lignin and associated substances. It is well, therefore, to recall to

* Karrer and Smirnoff: "Helv. Chim. Acta," 1922, **5**, 187.

memory the more salient facts of its structure. Xylem, or wood, is not a homogeneous material but a tissue made up of various elements which differ in their structure and function, and which occur in varying amounts in the wood of different plants. These structural units are the tracheæ, the water-conducting elements, which may comprise both vessels and tracheides; the fibres which have a mechanical function; and the parenchyma, the only living cells of the wood, and which is mainly concerned with the storage of food, chiefly in the form of starch and fat, and is in communication with the outer tissues by means of the rays. These elements have their origin in merismatic tissue and all, in the first instance, are living cells with thin walls composed of cellulose and pectin. In the course of their development into permanent tissue elements, the walls of some of these cells may remain unaltered, but in the majority of those plants which undergo an extensive secondary thickening, or attain a large size, the cell walls undergo a great change, a reinforcement of the original membrane by the incorporation of a number of substances known collectively as encrusting substances, the chief of which is lignin. This lignification never occurs uninterruptedly over the whole area of the wall; pits, either simple or bordered, are left for intercommunication between contiguous elements, whilst in the first differentiated tracheæ the lignification may only occur on a relatively small area of the wall. The rate of lignification is very variable, depending on the conditions of growth and the specific physiology; Burgerstein * obtained evidence of its inception in cells but two days old. Beckmann, Liesche, and Lehmann,† in an extensive study of rye, traced the variation in the lignin content with increasing age; they found that lignin from young tissues contained a much lower percentage of methyloxyl groups than that of older tissues, and the fact that there is a considerable variation in the amount of these groups in the lignin of heart wood, or duramen, and sap wood, or alburnum, of the same tree has been shown by

* Burgerstein: "Sitz. Kais. Akad. Wiss. Wien.," 1874, **70**, i, 238.

† Beckmann, Liesche, and Lehmann: "Zeit. angew. Chem.," 1921, **34**, 285; "Biochem. Zeit.," 1923, **139**, 491.

Ritter and Fleck.* Lignification, therefore, is a progressive change. Those cells which are destined to become tracheæ and sclerenchyma lose their living contents, all of which are used up in the making of the encrusting substances. Those which develop into parenchyma, on the other hand, retain their living contents notwithstanding the fact that their walls may be considerably lignified. Not infrequently the older wood ceases its normal functions, and passes over into heart wood where the vessels may become repositories of sundry waste metabolic products such as tannins, colouring matters, and inorganic salts such as calcium carbonate and calcium oxalate. Lignification gives the cell a greater power of resistance to pressure, and a diminished power of resistance to torsion.†

The wood of gymnosperms differs from that of angiosperms in the fact that vessels are absent. Further, the xylem of many gymnosperms is characterized by the presence of resin ducts which are charged with resins and terpines which form the source of terpentine and colophony of commerce. It will be apparent from this brief consideration that the analysis of wood gives very different results, according to the nature of the material, its origin, and age. The following table gives an approximate composition of the wood of spruce :—

Cellulose	53-55	per cent.
Hemicelluloses : Hexosans	3·06	,,
Pentosans	12·25	,,
Lignin	30·00	,,
Fats and resin	2·00	,,
Protein	1·00	,,

The composition of hard angiospermic wood differs from the above in generally containing slightly less lignin; the amount and nature of the hemicellulose content is also different. Hard woods are characterized by containing considerably more wood gum or xylan, the amount being anything from 15–24 per cent, whereas from 8–9 per cent is an average figure for gymnosperms. On the other hand, hexosans are much

* Ritter and Fleck : " J. Ind. Eng. Chem.," 1923, **15**, 1055.

† Sonntag : " Ber. deut. bot. Gesells.," 1901, **19**, 138. Leon : " Zeit. Verein. deut. Ing.," 1918, **62**, 341.

better represented in the soft woods where they occur to the extent of about 13 per cent, whereas 3–5 per cent is the average for hard woods. Amongst the hexosans of the gymnosperm are mannan, which varies very considerably in amount averaging from 4·5–8 per cent, and galactan, which varies from 8 up to as much as 17 per cent; the occurrence of a high percentage of galactan is characteristic of coniferous wood, and actually this material has been commercially exploited as a source of mucic acid by oxidation with nitric acid.*

A method for the analysis of the various constituents of wood mentioned above has been worked out by Dore.†

CHEMISTRY OF LIGNIN.

Lignification is easily detected by certain colour reactions which readily distinguish lignified from unlignified tissue; of these the two following may be regarded as the most generally useful:—

(*a*) The yellow colour produced when lignified tissue is treated with a solution of 1 per cent aniline sulphate or hydrochloride acidified with the corresponding acid.

(*b*) The bright crimson produced on treatment of lignified tissue with a dilute alcoholic solution of phloroglucinol followed by a little concentrated hydrochloric acid.

The aniline employed in the first test may be replaced by a great many other primary or secondary amines or even by heterocyclic nitrogen bases such as pyrrol or indol, while the phloroglucinol of the second test may be replaced by a number of phenols both monohydric and di- or tri-hydric, but the colours obtained are not all the same and vary in intensity.

Some wood, notably that of the cherry, when moistened with hydrochloric acid alone gives a red or violet colour; the fact that an aqueous extract of this wood gives with lignified tissue a red colour on addition of acid, suggests that cherry wood contains some phenolic substance capable of replacing phloroglucinol in the test mentioned above; it

* Schorger and Smith: "J. Ind. Eng. Chem.," 1919, **11**, 556; 1920, **12**, 264, 472, 476, 984.

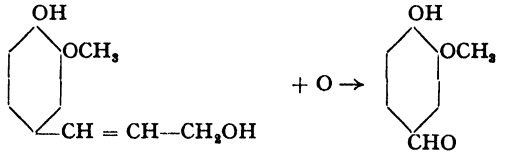
† Dore: *id.*, 1919, **11**, 556; 1920, **12**, 264, 472, 476, 984.

was suggested by Wiesner that the substance was actually phloroglucinol, but the presence of this substance in wood has not been established, and it is more likely to be substance of a tannin-like nature which, after all, is closely related to phloroglucinol, which is responsible for the reaction.

The opinion generally held is that the colour reactions for lignified tissue are not due to lignin itself—which forms about 50 per cent by weight of wood—but to small quantities of substances of an aldehydic nature which may have been adsorbed upon the surface of the lignin from the cambial sap, but their exact nature is still largely a matter of surmise.

That the colour reactions are not due to lignin itself but to small quantities of substances accompanying lignin * is supported by two facts, firstly, as shown by Wichelhaus and Lange,† pine or firwood when distilled with superheated steam at 180-200° yields a distillate which gives all the characteristic colour reactions of the original wood, and secondly, that lignin once isolated from its association with cellulose in wood generally no longer gives the colour reaction.

In attempting to find an explanation of the nature of this substance, suspicion at first fell upon vanillin and coniferyl aldehyde, since both these substances give the reaction with phloroglucinol and hydrochloric acid, but no proof has been furnished for the universal occurrence of these substances in lignified tissues. It is true that coniferin, a glucoside giving rise to coniferyl alcohol, occurs in the cambial sap of most conifers and that it is easily oxidized to vanillin.



Coniferyl alcohol Vanillin

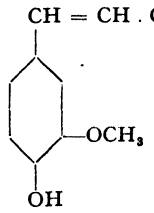
Also vanillin itself is widely distributed, having been reported in many resins, in dahlia tubers, potato peel, asparagus shoots,

* See Cross and Dorée: "Researches on Cellulose, IV.," London, 1922, p. 153.

† Wichelhaus and Lange: "Ber. deut. chem. Gesells.," 1916, **49**, 2001; 1917, **50**, 1683. Wichelhaus: "Chem. Zeit.," 1923, **47**, 865.

and also in the bark of the lime and in decayed oak wood; nevertheless, there is considerable doubt as to whether these substances are universally present in lignified tissues other than wood, although in the opinion of Klason lignin itself is a condensation product of coniferyl alcohol (see below).

It was first suggested by Nickel * that the colour reactions of lignified tissue were due to the presence of an aldehyde group in the lignin complex, more especially as the colour reactions were not given by wood which had been treated with sodium bisulphite and other reagents which would mask its aldehydic properties. Czapek,† by a somewhat drastic treatment of wood with stannous chloride, isolated a substance which was both an aldehyde and a phenol; to this substance, which gave the colour reaction with phloroglucinol and hydrochloric acid, he gave the name of hadromal, without assigning any constitution to it; his views did not attain general acceptance and were discredited especially by Grafe ‡; since then, however, Hoffmeister,§ modifying Czapek's original conditions, has isolated from oak sawdust a substance of the formula $C_{10}H_{10}O_3$, which proved to be coniferyl aldehyde of the formula—



This substance, whose constitution was definitely established by synthesis from vanillin and acetic aldehyde, gives the colour reaction with phloroglucinol and hydrochloric acid and would thus appear to be identical with Czapek's hadromal. According to the view of Hoffmeister this substance occurs to the extent of about 3 per cent as a cellulose ester in wood.

* Nickel: "Chem. Zeit.," 1887, **11**, 1520.
† Czapek: "Zeit. physiol. Chem.," 1899, **27**, 141.
‡ Grafe: "Monatshefte," 1904, **25**, 987.
§ Hoffmeister: "Ber. deut. chem. Gesells.," 1927, **60**, 2062.

Whilst it may be generally accepted that the commonly employed colour reactions for lignified tissue are given not by lignin itself but by a substance, most probably coniferyl aldehyde, which accompanies lignin, there are nevertheless two colour reactions which may be regarded as being produced by the lignin complex itself; these are known as the lignone chloride reaction of Cross and Bevan * and of Maüle's reagent. The former depends on the formation of a yellow colour when lignified material is exposed to moist chlorine gas or bromine, and which on addition of sodium sulphite changes to red; Maüle's reaction also consists in the production of a red colour when wood is treated successively with potassium permanganate, hydrochloric acid, and ammonia. Probably this is a modified form of the Cross and Bevan reaction, since permanganate followed by hydrochloric acid evolves chlorine. The colour obtained is, however, not uniform and tends, in the case of wood of deciduous trees, to be brown instead of red.

The Isolation and Constitution of Lignin.

As stated above, the process of lignification consists in the incorporation into the cell wall of a substance known as lignin; but opinions are divided as to whether such lignin is chemically combined with the cellulose or only physically adsorbed. The facts that lignified cellulose, or lignocellulose as it is called, is not soluble in cuprammonia solution and is also incapable of entering into such chemical reactions as can cellulose with carbon disulphide and caustic soda, for example, suggest that there is some kind of chemical union between lignin and cellulose. The very great technical importance of cellulose free from lignin necessitated the provision of methods for separating these two substances, the two best known and most widely employed being those of heating the wood with caustic soda or with calcium disulphite; both these methods are somewhat drastic, and it is reasonable to suppose that the lignin so isolated will differ somewhat from the form in which it existed in the original material; in the case of the bisulphite method

* Cross and Bevan: "J. Chem. Soc.," 1889, **55**, 199.

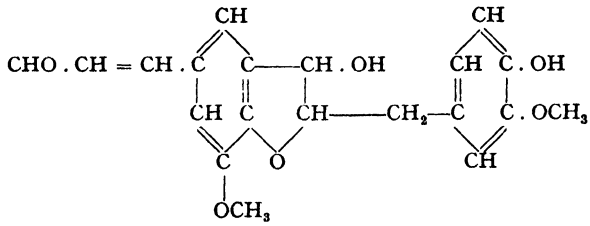
this is conspicuously so, since the material isolated from the so-called sulphite liquors contains sulphur and is, in fact, a sulphonic acid derivative of lignin ; in the case of the alkaline process, the lignin is less obviously altered. Most of the attempts made to elucidate the constitution of lignin have been carried out on material isolated by these two methods or else by concentrated hydrochloric acid. At first sight it appears strange that attempts to determine the constitution of such a complex substance as lignin should be made upon material which had undergone such drastic treatment, but this is explained by the fact that as yet no gentler methods have succeeded in separating lignin from cellulose.

In spite of the considerable literature on the subject comparatively little is definitely known regarding the constitution of this substance. The following facts are generally accepted : the presence of hydroxyl methoxyl and acetyl group ; the presence of an aldehyde or ketone group as is shown by the ability to react with hydroxylamine, while evidence of unsaturation is revealed by its ability to absorb iodine or bromine and the readiness with which it is oxidized by ozone, nitric acid ; furthermore, it appears fairly certain that some of the methyl groups are attached to phenolic hydroxyl groups and some are not.

Lignin Isolated by the Bisulphite Process.

Klason,* who worked on lignin isolated from wood by the sulphite process, and which consequently contained more or less combined sulphur, came to the conclusion that lignin exists in two forms in spruce wood, namely α-lignin which contains the acrolein group —CH = CH . CHO, and β-lignin which contains the corresponding acrylic acid group —CH = CH . COOH in the proportions 63 : 37 per cent. Klason has shown that α-lignin contains two methoxyl groups, one phenolic and one alcoholic hydroxyl, and comes to the conclusion that it has been formed from 2 molecules of coniferyl aldehyde to produce a compound of the constitution

* Klason : " Ber. deut. chem. Gesells.," 1923, **56**, 300.



The above formula is closely related to that assigned to Gambier-catechin by Freudenberg,* and lends support to the view that lignin may be related to the tannins.

Lignin Isolated by the Action of Alkali.

A study of the lignin of flax isolated by heating with 8-12 per cent caustic soda for six to ten hours at 140-160°, led Powell and Whittaker † to compare the resulting product with that isolated from various woods including pine, spruce, ash, birch, and poplar; they conclude that jute lignin is essentially different from flax lignin, to which they assign the formula $C_{45}H_{48}O_{16}$ which differs considerably from Klason's formula, $C_{20}H_{20}O_6$, for pine lignin. Flax lignin has four methoxyl groups, and five hydroxyl groups capable of acetylation, three of which are phenolic. To the parent hydroxyl compound free from CH_3 or $COCH_3$ groups, they assign the name lignol, $C_{41}H_{40}O_{16}$, and the formula—

$$C_{38}H_{30}O_4(CO)_2CHO(OH)_9$$

Powell and Whittaker disagree with Hägglund's ‡ statement that lignin contains 5 per cent of a furfural yielding carbohydrate as an integral part of the molecule; purified lignin contained only 0·3 per cent pentosan and still further purification gave no furfural at all.

Beckmann, Liesche, and Lehmann § in an investigation upon the lignin content of winter rye straw, used a 2 per cent aqueous alcoholic solution of caustic soda acting in the cold

* Freudenberg: "Ber. deut. chem. Gesells.," 1920, **53**, 1416.

† Powell and Whittaker: "J. Chem. Soc.," 1924, **125**, 357; 1925, **127**, 132.

‡ Hägglund: "Cellulosechemie," 1923, **4**, 73.

§ Beckmann, Liesche, and Lehmann: "Zeit. angew. Chem.," 1921, **34**, 285.

for forty-eight hours; they obtained a substance of molecular weight 800 to which they assigned the formula $C_{40}H_{44}O_{15}$ which they claimed contained four methoxyl and four hydroxyl groups.

Mehta,* in attempting to devise a method for the quantitative estimation of lignin, recommends heating in the autoclave with 4 per cent caustic soda under a pressure of 10 atmospheres for one hour. On precipitating with acid a lignin was obtained which, when purified by extraction with alcohol, was an amorphous, faintly acid substance with a pleasant aromatic odour and melting at 170°; its iodine value was found to be 139·7.

Dorée and Barton-Wright,† employing the above conditions and working with spruce wood previously extracted with benzene, alcohol, and water, isolated a substance with melting-point 186° to which they assign the formula $C_{20}H_{20}O_6$ which, though agreeing with that assigned by Klason to α-lignin, has approximately half the molecular weight of the formulæ suggested by earlier workers. This substance, which they propose to call meta-lignin, has one hydroxyl, two methoxyl, and two carboxyl groups, one aldehydic, and the other ketonic. They suggest that meta-lignin is the unit upon which the natural lignins are based and that, whilst the usual type isolated is of the order C_{40}, it may exist in the plant in an even more polymerized form. They suggest for meta-lignin the extended formula—

$$C_{16}H_{12}O(OCH_3)_2 \,.\, OH \,.\, CO \,.\, CHO$$

Dorée and Barton-Wright disagree with Klason's formula on the ground that no aromatic compounds are obtained from lignin on oxidation, and they suggest, as an alternative, that lignin contains hydroaromatic nuclei which would account both for the unsaturated properties of the substance and for the profound disruption undergone by the molecule with formation of oxalic and carbonic acids.

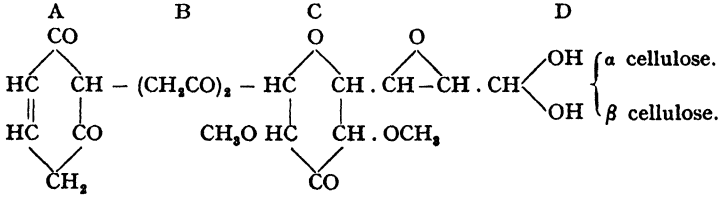
* Mehta: "Biochem. Journ.," 1925, **19**, 958.
† Dorée and Barton-Wright: *id.*, 1927, **21**, 290.

Lignin Isolated by Acid Treatment.

Methods for the isolation of lignin from ligno-cellulose have also been devised which depend on the solubility of cellulose in strong acids, the lignin remaining undissolved; for this purpose Ost and Wilkening * employ 72 per cent sulphuric acid, while Willstätter and Zechmeister † recommend concentrated hydrochloric acid, either acid being allowed to act in the cold.

Analyses of the lignin isolated by these methods, however, show that lignin has undergone some degree of hydrolysis, since it contains fewer methoxyl groups as compared with lignins prepared by other methods.

Cross and Bevan isolated from lignin, by the action of chlorine, a compound which contained chlorine and had the properties of a ketone; from this and other evidence they propose the following formula for lignin :—



in which A is the group which is attacked by the chlorine, B gives rise to the acetic acid obtained by hydrolysis or distillation, and D is the aldehyde group to whose two-hydroxyl group cellulose α and β are supposed to be attached in ester-like combination; it is, however, not easy to see how his compound which contains C_{10} should give rise to a lignone chloride containing C_{19}.

Estimation of Lignin.

The methods for estimating lignin are based upon the use of mineral acids under various conditions with the object of

* Ost and Wilkening: "Chem. Zeit.," 1910, 34, 461.

† Willstätter and Zechmeister: "Ber. deut. chem. Gesells.," 1913, 46, 2401.

dissolving out the hydrolysable carbohydrate and weighing the residual lignin; the latter substance, however, is also attacked to some extent by the acid with consequent loss of methoxyl and acetyl groups; moreover, it is liable to retain a certain amount of carbohydrate. The action of 42 per cent hydrochloric acid upon the lignified material for eighteen hours at the ordinary temperature was first suggested by Willstätter and Zechmeister *; subsequently this method was modified by Hägglund; † König and Rump ‡ employ 1 per cent hydrochloric acid under 6 atmospheres pressure for six to seven hours. Ost and Wilkening,§ on the other hand, recommend 72 per cent sulphuric acid in the cold until a portion of the solution gives no precipitate with water; the whole mixture is then poured into ten times its volume of water and the residual lignin is filtered off through cotton wool.

Methods of Estimating Cellulose in Lignified Tissues.

Cross and Bevan's || method consists in exposing the moist material to the action of chlorine for a short time,¶ whereby chlorination of the lignin complex results in the formation of a lignone chloride to which they give the formula $C_{19}H_{18}O_9Cl_4$. The lignone chloride is then dissolved out by means of a 2 per cent solution of sodium sulphite whereby a pink colour is produced which may be regarded as a true colour reaction of the lignin complex; the material is then chlorinated again, and extracted with sodium sulphite and the process is repeated until a pink colour is no longer produced by addition of the sulphite; the number of chlorinations required varies from two to five according

* Willstätter and Zechmeister: "Ber. deut. chem. Gesells.," 1913, 46, 2403.

† Hägglund: "Arkiv Kemi, Mineral Geol.," 1918, 7, 8.

‡ König and Rump: "Zeit. Nahr. Genussm.," 1914, 28, 177.

§ Ost and Wilkening: "Chem. Zeit.," 1910, 34, 461.

|| Cross and Bevan: "Cellulose," 1895, p. 102; and "J. Chem. Soc.," 1882, 41, 94.

¶ Over-exposure leads to the chlorine attacking the cellulose with formation of oxycellulose. See Heuser and Siebert: "Zeit. angew. Chem.," 1913, 26, 801.

to the nature of the material; hard woods require less than soft woods since they contain as a rule rather less lignin.

E. Schmidt and Graumann* suggested the use of an aqueous solution of chlorine dioxide† in place of gaseous chlorine; the original procedure was to employ a solution of approximately 0·3 per cent strength, but Heuser and Merlau‡ recommend a 1·5 per cent solution. For the estimation 0·5 gram of wood, which has been extracted with alcohol and benzene to remove resins, etc., is placed in a glass-stoppered flask with 100 c.c. of 1·5 per cent solution of chlorine dioxide; after forty-eight hours the residue is washed free from chlorine dioxide and then with 2 per cent sodium sulphite until the filtrate is no longer coloured; a second similar treatment is generally sufficient to remove all the lignin; the residue is washed and dried and may be weighed as cellulose. According to Schmidt and Graumann, chlorine dioxide solution has no action on the carbohydrate constituents of the cell wall, whereas small quantities of incrustive substance are readily attacked; it is thus possible to estimate quantitatively the percentage of incrustive and tissue substance in portions of plants; thus in *Pinus sylvestris* they found 63·28 per cent of tissue substance and 36·72 per cent of lignin, whereas Willstätter and Zechmeister found only 27·25 per cent of the latter.

The Nature of the Union Between Lignin and Cellulose.

Opinions are divided as to the nature of the association between lignin and cellulose; the view formerly held by Cross and Bevan§ was in favour of some form of chemical union, but later they admit the possibility of there being only a physical association, while Klason, who formerly believed in physical union, now favours combination.‖ According to König and Rump¶ the fact that wood when treated with

* Schmidt and Graumann: "Ber. deut. chem. Gesells.," 1921, **54**, B., 1860.

† Prepared from potassium chlorate and oxalic acid.

‡ Heuser and Merlau: "Cellulosechemie," 1923, **4**, 101.

§ Cross and Bevan: "Ber. deut. chem. Gesells.," 1893, **26**, 2520.

‖ Klason: *id.*, 1923, **56**, 300. ¶ König and Rump: *loc. cit.*

72 per cent sulphuric acid to remove lignin, still retains its original organised structure, shows that lignin is merely mixed with or incorporated with cellulose; the argument, however, is not convincing since cotton cellulose can be nitrated without destroying the structure of the fibres. The view of Wislicenus * on the mode of origin of lignified cell walls is that the original cellulose wall is a colloidal hydrogel which adsorbs from the sap other colloidal materials that produce lignin; the union between the lignin and the cellulose is accordingly only one of physical adsorption, possibly reinforced by supplementary valencies of oxygen atoms. Robinson † also favours the view of physical mixture from observations on the microscopical features of mechanical strains in timber which he explains as being due to displacement of films of lignin overlaying the ground cellulose of the tracheids. On the other hand, Schmidt and others ‡ have published some theoretical speculations on the relation of cellulose to the incrustation and conclude that they are united in ester-like combination, while Mehta § considers that lignin is combined with cellulose as an aromatic glucoside.

MICROCHEMICAL REACTIONS.

Lignified tissues give the following reactions :—

1. A brownish-yellow colour is given with iodine.

2. A brown colour is obtained with the use of chlorzinc iodide.

3. Calcium chloride iodine solution turns lignin yellow to yellow-brown.

4. Insoluble in cuprammonia.

5. Aniline sulphate or aniline chloride in aqueous solution and acidified with the corresponding acid turns lignified walls a bright yellow.

6. If the sections be soaked for about a minute in an alcoholic solution of phloroglucin and then mounted in a drop

* Wislicenus: "Kolloid Zeit.," 1910, **6**, 17 and 87; "Cellulosechemie," 1925, **6**, 45.

† Robinson: "Trans. Roy. Soc.," B., 1920, **210**, 49.

‡ Schmidt, Haag, and Sperling: "Ber. deut. chem. Gesells.," 1925, **58**, 139.

§ Mehta: "Biochem. Journ.," 1925, **19**, 958.

of strong hydrochloric acid, the lignified walls are turned a bright red.

7. A concentrated solution of thallin sulphate in 50 per cent alcohol gives a yellow to orange-yellow coloration.

The sections should be treated first with alcohol, and the thallin sulphate solution should be freshly prepared.

8. If lignified tissues be treated with chlorine water and then with sodium sulphite, a deep magenta colour is produced.

9. Lignocelluloses induce the formation of Prussian blue in the greenish-red solution produced by mixing ferric chloride with potassium ferricyanide.

CUTINIZED MEMBRANES.

The surface of the subærial parts of the majority of vascular plants is covered by a secretion of the epidermis. This secretion is known as cutin and forms a continuous transparent layer, the cuticle, which may be so well developed as to give a shining surface to the plant member, the upper surface of a holly leaf, for example. The cuticle may be quite distinct from the underlying cellulose membrane of the epidermal cells, to which it is closely applied, as in the leaf of the hellebore; in other cases the distinction between the cutinized and non-cutinized parts is not sharply defined, as in *Selaginella*, the one merging gradually into the other. When thick, the cuticle not infrequently shows stratification, and wax-like substances may be present. The thickness of the cuticle varies much in different plants and with the conditions of growth. Its greatest development is found on leaves and shoots which are exposed to arid conditions such as high insolation, the prevalence of dry winds, growth in soils poor in available water, together with other factors. Its chief physical property is its high degree of impermeability to water vapour and gases, its presence, therefore, impedes the evaporation of water from the surface of the plant.

Lee and Priestley * conclude that cuticle is formed by the migration of fatty substances liberated at the surface of the

* Lee and Priestley: "Ann. Bot.," 1924, **38**, 525. Priestley: "New Phyt." 1921, **20**, 17. Lee: "Ann. Bot.," 1925, **39**, 755.

protoplast, the thickness of the cuticle being proportional to the amount of fat secreted by the plant. Thus heath plants, which synthesize much fat, are characterized by the presence of thick cuticles. The authors explain the distribution of cuticle in various plants by speculations regarding the influence of external factors such as the relative proportion of potassium and calcium, light and humidity. The presence of hydroxy-acids in cuticle would appear to be established, from which the authors conclude that aeration is an important factor. The fact that the iodine value of the fat extracted from the shoot tips of *Vicia Faba* was 73 as compared with 114 for the root apices, and that the iodine values of 90 and 54 were obtained for the cuticle fat of indoor forced and outdoor grown rhubarb respectively, suggest that fats exposed to the oxidizing and drying conditions of the open air become saturated more quickly than those exposed to the atmosphere of forcing sheds. Further, the authors found that the outdoor rhubarb contained twice as much hydroxy-fatty acid as forced rhubarb.

With respect to the chemistry of cutin, it is concluded that cutin is a complex mixture of fatty acids, both free and combined with alcohols, that have undergone condensation and oxidation; soaps of fatty acids together with unsaponifiable material which probably contains some higher alcohols, and resinous substances. Cutin, unlike suberin, contains no phellonic acid, phloionic acid (see p. 233), or glycerol.

From these observations it will be seen that the term cutin does not represent a chemical individual but an aggregate of substances varying in specific composition but occurring at the same place in the plant and having the same general characters.

Other investigations of cuticle are those of Clifford and Probert * on the wax of American cotton, and of Legg and Wheeler † upon the cuticle of *Agave americana*. The former authors find the cuticle wax to contain some glycerol esters, a number of monohydric alcohols, hydrocarbons, and resin

* Clifford and Probert: "J. Text. Inst.," 1924, **15**, 8, 401.
† Legg and Wheeler: "J. Chem. Soc.," 1925, **127**, 1412.

esters and alcohols. Legg and Wheeler, after saponification of *Agave* cuticle with alcoholic potassium hydroxide, isolated cutic acid $C_{26}H_{50}O_6$ and cutinic acid $C_{13}H_{22}O_3$, which they consider to be the constituents of the acid described by Frémy and Urbain * as oleocutic acid.

SUBERIZED MEMBRANES.

In the majority of trees and shrubs and in many herbaceous plants, the superficial tissue, or tissues, is replaced by a secondary tegumentary system. This normally arises after the primary tissues are fully differentiated and, generally, soon after the beginning of secondary thickening in the vascular system. This secondary tegument is known as periderm, and its formation is instituted by the advent of a new meristem, the cork cambium or phellogen. In the stem, the phellogen may arise in the epidermis, as in *Nerium ;* in the hypodermis, as in *Sambucus ;* or in the deeper layers of the cortex, as in *Ribes.* In the root, the phellogen generally has its origin in the pericycle, although in some instances it may arise in the superficial parts of the cortex as in *Valerianella.*

The segmentation of the phellogen results in the formation of regular serial rows of closely packed brick-shaped cells towards the exterior and, more especially when the phellogen is deeply seated, a less regular and more or less extensive series of cells towards the interior. The former undergo a gradual change, suberization, lose their living contents, and finally become cork, whilst the latter retain their living contents and form a secondary cortex, known as phelloderm. The formation of cork isolates the tissues on its outer side which thus are cut off from all supplies and die.† Phelloderm thus comprises the dead cork and the dead primary tissues on its outer side, the living phelloderm if formed, and the phellogen situated between the cork and the phelloderm. The cork of commerce is mostly derived from the cork oak, *Quercus suber.*

* Frémy and Urbain : " Ann. Sci. Nat. Bot.," 1882, vi., 360.

† The term " bark " often is loosely used. Bark comprises all the dead tissues external to the phellogen.

In addition to this normal formation of cork, a phellogen may arise and form cork as a result of wounding, and suberization, without the formation of a phellogen, may take place when non-superficial cells are exposed by the removal or destruction of superficial tissue. Further, cork formation is associated with the fall of the leaf.

A mature cork cell consists of an internal suberin lamella possessed of fat-staining properties, a cellulose layer and a middle lamella both of which are more or less impregnated with fat-like bodies to which the name of suberin is given and to which is due the characteristic properties of cork, more especially relative impermeability to water and to air.

It was formerly thought that cork was a compound of cellulose and suberin. The work of Gilson,* however, shows that cellulose does not enter into the composition of cork for the following reasons :—

1. Cellulose is not attacked by prolonged boiling in a 3 per cent solution of potassium hydrate in alcohol; suberized walls, on the other hand, are dissolved.

2. Phellonic acid ($C_{22}H_{43}O_3$) has been isolated from cork, and this substance, together with its potassium salt, gives a red coloration with chlorzinc iodide. This suggests that the coloration of suberized membranes with chlorzinc iodide after treatment with potash is due to the presence of potassium phellonate and not to cellulose, for, in addition, the coloration does not take place if the corky tissue be subjected to the action of boiling alcohol after treatment with potash.

3. After treatment with cuprammonia, the chlorzinc iodide gives a yellowish-brown colour; this, according to Gilson, is due to the conversion of potassium phellonate into the copper salt, and not to the removal of cellulose, as had been supposed.

Gilson separated from oak-cork suberic acid ($C_{17}H_{30}O_3$) and phloionic acid ($C_{11}H_{21}O_4$) in addition to phellonic acid. He does not think that these occur as true glycerol esters, since

* Gilson : " La Cellule," 1890, **6**, 63. See also van Wisselingh : " Chem. Zentr.," 1892, **2**, 516 ; and Schmidt : " Monatshefte," 1910, **31**, 347.

the suberin walls are insoluble in all fat-solvents, and do not melt at a temperature below 290° C.

An investigation of the chemical nature of potato cork was undertaken by Rhodes,* who obtained a chloroform extract and an insoluble residue; the latter boiled with excess of alcoholic soda gave a solution from which he separated normal and hydroxy-acids, the latter being characterized by insolubility in light petroleum. The hydroxy-acids are not extracted from the suberin lamella until after saponification, showing that they occur there in some form of combination. He concludes that the suberin lamella arises by changes taking place in the fatty material rendering them no longer soluble in fat solvents; part of the fatty substances never undergo this change, and it is this part which is chiefly responsible for the staining properties of the lamella with the ordinary fat stains. The lamella consists in the main of relatively insoluble normal and hydroxy-fatty acid complexes which can be released by prolonged saponification as soluble soaps. Glycerol was found only in the chloroform extract and then only in traces, except in the case of regenerated cork layers.

Microchemical Reactions of Suberized and Cuticularized Membranes.

1. With chlorzinc iodide, and also with iodine and sulphuric acid, a brown or yellow colour is given.

2. Suberized and cuticularized walls are insoluble in cuprammonia and concentrated sulphuric acid.

3. Suberized walls are coloured yellow with strong potash solution; on heating the colour deepens, and on boiling yellow oily drops exude from the membranes.

4. Suberized walls are the most resistant of membranes to Schultze's macerating mixture.

5. These membranes are stained red by treatment with alcoholic solutions of Alkannin, Sudan III and Scharlach R.

6. If a section of the material be treated first with eau de Javelle, in order to destroy any tannins which may be present,

* Rhodes: "Biochem. Journ.," 1925, 19, 454.

suberized walls are stained very deeply with a solution of cyanin in 50 per cent alcohol to which an equal volume of glycerol has been added. Lignified walls, on the other hand, are not stained under these conditions.

INDUSTRIAL USES OF CELLULOSE AND CELLULOSE PRODUCTS.

One of the industries which consumes the largest amount of cellulose is that of paper manufacture. Formerly the chief sources of cellulose for this purpose were cotton or hemp fibres but with the increased consumption of paper other sources had to be found. Although straw contains cellulose which has been only slightly lignified, it is found to be unsuitable for the preparation of pure cellulose, owing to the fact that it contains a considerable quantity of silica. The employment of wood as a source of cellulose became possible with the discovery of chemical methods of destroying the non-cellulose constituent lignin, i.e. the " encrusting substances," without affecting the cellulose proper.

In the manufacture of paper from linen rags or cotton waste the material is cut up, cleaned, and disintegrated by boiling successively with dilute sodium carbonate and caustic soda under pressure ; the fibre is then bleached with chlorine, the excess being subsequently removed ; it is then treated with resin, soap, and alum, and spread in thin layers and dried, whereby the fibres become felted together in a peculiar manner, with the formation of paper. When wood is used the " encrusting substances " may be removed by boiling with calcium bisulphite, whereby the lignin remains in solution and a fairly pure form of cellulose, known as sulphite cellulose, is produced. In the preparation of inferior quality papers there is no chemical treatment of the disintegrated wood pulp ; the material is, therefore, known as mechanical pulp, and paper made from it gives reactions for lignocellulose. Cellulose used for the preparation of filter papers is, after the ordinary methods of purification, treated with hydrofluoric acid to remove silica.

COMMERCIALLY VALUABLE DERIVATIVES OF CELLULOSE.

When heated in a concentrated solution of zinc chloride, cellulose is converted into a viscid syrup. This syrup, when forced through glass nozzles into alcohol, forms threads which, after being washed and carbonized, become hard and are used for electric lamp filaments ; they have also been employed for the basis of incandescent lamp mantles.

Gun Cotton or Pyroxylin.—That a variety of different products may be obtained by the action of various strengths of nitric acid, either alone or in the presence of sulphuric acid, on cellulose, has already been mentioned. The substance known as gun cotton is a hexanitrate ; it is obtained by immersing dry cotton waste, freed from grease by treatment with alkali, in a mixture of 1 part nitric acid (sp. gr. 1·52) with 3 parts sulphuric acid (sp. gr. 1·84) ; the resulting substance is then rapidly and thoroughly washed with water, moulded into discs, and dried on heated plates. On explosion it produces corrosive gases and therefore is not suitable for use, as such in firearms ; when, however, the gun cotton is dissolved in ethyl acetate or acetone and the solution is evaporated, a new substance is obtained which has the same composition as gun cotton, but different properties ; it explodes with less violence and produces no corrosive vapours, and is therefore employed in the manufacture of smokeless powder.

Blasting Gelatine is a mixture of gun cotton and nitroglycerine. Gun cotton mixed with a variety of other substances enters into the composition of numerous explosives, such as ballastite, melanite, cordite, etc., etc.

Collodion is the name applied to a solution of cellulose tri- and tetra-nitrates in a mixture of equal parts of 95 per cent alcohol and ether.

A substance known as *artificial india-rubber* * is produced by kneading together a mixture of tri- and tetra-nitrocelluloses partially dissolved in ether alcohol with castor oil. The

* This substance must be carefully distinguished from so-called synthetic rubber, which is an artificially polymerized hydrocarbon of the formula $(C_5H_6)_n$; this substance, if not actually identical with natural rubber, is at any rate closely related to it, whereas the artificial india-rubber mentioned above is a nitrated cellulose.

resulting substance may be made to have any degree of elasticity, according to the materials which are mixed with it. It forms a more or less satisfactory substitute for rubber and possesses a high electric resistance. Though not explosive, it is inflammable, but to do away with this inconvenience the outer surface may be denitrated by treatment with alkali, whereby it is rendered non-flammable. *Artificial gutta-percha* is obtained by allowing an acetone solution of tetra-acetyl cellulose to evaporate.

Celluloid is produced by mixing the tri- and tetra-nitrates, as employed for collodion, with camphor.

Artificial Silks.—These are produced in a variety of ways by precipitating some form of cellulose from solution. The first artificial silk was prepared by Chardonnet, who obtained it by forcing collodion through fine nozzles; the thin stream of nitrocellulose solution on coming in contact with the air solidifies to a thread by the rapid evaporation of the solvent. To render it non-flammable the thread is denitrated by treatment with ammonium sulphide.

A second process for preparing artificial silk consists in dissolving bleached mercerized cotton (see p. 209) in cuprammonia solution. A fine stream of this solution is then run into a dilute sulphuric acid, whereby a continuous thread of cellulose is at once precipitated.

A third process is that in which viscose solution is forced through fine nozzles, the emerging streams being coagulated either by hot air or by a bath of ammonium chloride. The fine threads which result can be spun like silk. Cellulose acetate also is used for this purpose.

Viscose is obtained by acting on finely divided cellulose with soda and treating the resulting substances with carbon disulphide, whereby a cellulose thio-carbonate is produced; this substance on exposure to air decomposes spontaneously into cellulose alkali and carbon disulphide. Viscose solutions are employed for sizing paper and in the manufacture of wall-papers.

Mixed with metallic dust and colouring matters, viscose can be converted into an artificial leather, and may also be

employed for rendering canvas waterproof and for making cinematograph films, etc.

Viscoid, which is congealed viscose, is a hard mass obtained by mixing viscose with various substances and allowing the mixture to decompose spontaneously and harden; it is used for mouldings, cornices, statuettes, etc.

Solid Spirit.—The substance sold under this name is obtained by pouring a solution of cellulose acetate in glacial acetic acid into alcohol; a white solid is produced which does not melt, and burns when ignited without leaving any ash.

Cellulose acetate, in which there are approximately five acetyl groups to the C_{12} cellulose unit, is soluble in acetone, and is used largely as a dressing for the fabric of aeroplane wings.

Cellite is acetyl cellulose which is soluble in a mixture of ethyl acetate and ethyl alcohol. Mixed with camphor it is used in the manufacture of non-flammable cinematograph films.

Willesden Paper is paper waterproofed by treatment with cuprammonia, whereby the fibres are gelatinized, and, when dry, are impervious to water.

Finally, mention may be made of a few substances, which are made from cellulose as a starting-point, but which are produced only by the profound decomposition of the molecule. Thus by heating cellulose with a strong solution of caustic potash and soda, oxalic acid is produced, and by the destructive distillation of wood, acetic acid, acetone and methyl alcohol are obtained.

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SECTION IV.

GLUCOSIDES.

A GLUCOSIDE may be defined as a substance which on hydrolysis yields a reducing sugar, wherefore, strictly speaking, di-, tri- and poly-saccharides would be included. Custom, however, restricts the term to those compounds which in addition to reducing sugars also yield one or more other substances which, not infrequently, are of an aromatic nature. The non-sugar constituent, which is sometimes termed an aglucan, may belong to various chemical classes as is seen in the following selected examples :—

Glucoside.	*Aglucan.*
Salicin.	The alcohol saligenin.
Coniferin.	Coniferyl alcohol.
Amygdalin.	Benzaldehyde and hydrocyanic acid.
Monotropitin.	Methyl salicylate.
Phaseolunatin.	Acetone and hydrocyanic acid.
Arbutin.	The phenol hydroquinone.
Indigo.	Indoxyl.
Sinigrin.	Allyl isothiocyanate.
Anthocyanin.	Anthocyanidin.
Quercitrin.	Flavonol.

The tannins, flavones, and anthocyans, owing to their special botanical significance, will be dealt with in subsequent sections.

The carbohydrate constituent of the glucoside molecule is commonly glucose, but many other sugars may occur in place of the glucose; galactose and mannose amongst the hexoses; rhamnose and other pentoses; gentiobiose, $C_{12}H_{22}O_{11}$, the disaccharide of amygdalin; and primeverose, $C_{11}H_{20}O_{10}$, the disaccharide of monotropitin.

The glucosides are generally soluble in water or dilute alcohol which solvents may be used for their extraction from plant tissues. Owing to the fact that glucosides are not precipitated by lead acetate, their solutions may be purified by

treatment with this salt, the excess of lead being subsequently removed by hydrogen sulphide.

Aqueous solutions of glucosides frequently have a bitter taste and are lævo-rotatory; they do not reduce Fehling's solution until liberation by hydrolysis of the monosaccharide which, as has been stated, may be a hexose, pentose, or methyl pentose or a mixture of two of more of these. In the plant hydrolysis is effected by an appropriate enzyme which may be specific, or may be capable of splitting several glucosides. In the process of extraction, precautions must accordingly be taken to prevent interaction between the enzyme and its substrate; this is best effected by treating the material with boiling alcohol, in order to destroy the enzyme, prior to the extraction of the glucoside with alcohol or water.

Bourquelot's method of investigating plants for glucosides has been extensively employed; it depends on the fact that all glucosides which are hydrolysed by emulsin are lævo-rotatory, but after hydrolysis become dextro-rotatory and acquire reducing properties.

The glucoside and the enzyme may in some cases be contained in the same cell and only come into contact with each other on injury, or during certain phases in the plant's metabolism.

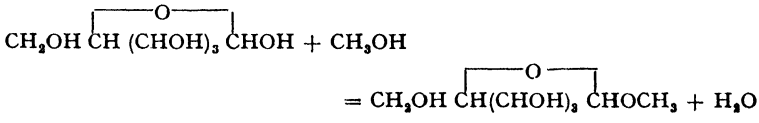
On the other hand, the enzyme and substrate may be secreted in distinct tissues; an example of this is furnished by the seeds of *Lunaria biennis* in which the cotyledons secrete the enzyme whilst the integument contains the glucoside. If the seeds are skinned and the cotyledons and testas are separately ground, no smell of mustard oil is produced; but if the two are ground together, the myrosin acting upon the sinigrin contained in the seed-leaves, liberates allyliso-thiocyanate.

THE CONSTITUTION OF THE GLUCOSIDES.

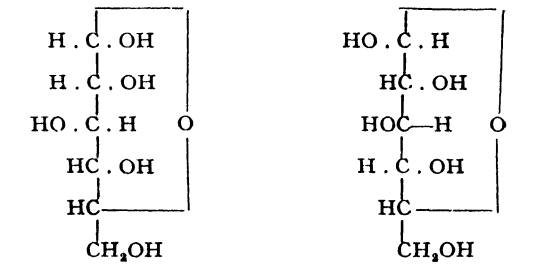
The constitution of the natural glucosides can be best understood by a brief consideration of the simplest known artificial glucosides which have been synthesized from glucose.

The lactone formula for glucose with its asymmetric

terminal carbon atom accounts for the ability of glucose to react with methyl alcohol to form two isomeric α- and β-methyl glucosides * according to the equation—



The α-glucoside, which is dextro-rotatory, is hydrolysed by maltase, but not by emulsin, while the β-glucoside, on the contrary, is unaffected by maltase, but is hydrolysed by emulsin,† there result on hydrolysis the two isomeric α- and β-glucoses, whose constitutions are represented by the following formulæ :—



α-Glucose $\alpha_D = +110°$ β-Glucose $\alpha_D = +19°$

The fact that either of these sugars tends to change at once into the ordinary form of glucose, the so-called equilibrium mixture having $\alpha_D = 52{\cdot}9°$, may be employed as a means for determining the nature of a given glucoside since the rotation of a freshly hydrolysed α-glucoside solution will tend to decrease, while that of a β-glucoside will increase.

As the result of the study of the action of maltase and emulsin upon other glucosides, Fischer divided these substances into two classes known as α-glucosides and β-glucosides, according as they are hydrolysed by maltase or emulsin respectively. Other examples of α-glucosidases besides maltase

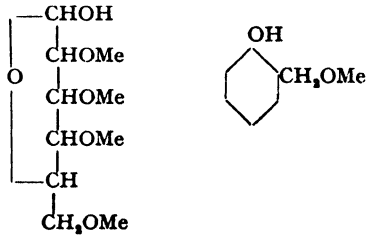
* A number of analogous compounds have since been prepared by Fischer and his co-workers from mannose, galactose, and fructose, the resulting compounds being termed mannosides, galactosides, and fructosides respectively.

† See also section on Enzymes.

are mannosidase and trehalase, while β-glucosidases are represented by amygdalase and the phenolglucosidase of emulsin, cellobiase, and gentiobiase.

For a complete elucidation of the constitution of a given glucoside it is necessary to determine not only the nature of the non-sugar residue but also to ascertain which of the hydroxyl groups of the sugar and of the non-sugar residue are involved in the union between the two complexes —more especially if the non-sugar residue contains more than one hydroxyl. For this purpose the glucoside is treated with methyl iodide and silver oxide whereby all the free hydroxyls in the molecule are methylated; the resulting methylated glucoside is then hydrolysed and the methylated sugar and non-sugar residues are examined; any free hydroxyl groups now occurring in these products must have been involved in the union of the two complexes, since if present in the original compound they could not have escaped methylation.

Thus, for example, Irvine and Rose * found that salicin yielded a pentamethyl derivative which on hydrolysis gave rise to 2 : 3 : 5 : 6 tetramethyl glucose † (I.) and a methylated saligenin (II.) containing a free phenolic hydroxyl but having a methyl group attached to its alcoholic hydroxyl, from which it follows that the parent glucoside must have had the formula III. :—



I. II. III.

2 : 3 : 5 : 6 trimethyl * glucose — Salicin

* Irvine and Rose: "J. Chem. Soc.," 1906, 89, 814; Irvine: *id.*, 1923, 123, 903.

† According to Haworth's formula for glucose, the methylated sugar is the 2 : 3 : 4 : 6 tetramethyl derivative; this formula has been adopted.

Similar methods * have been applied to the elucidation of the constitution of other glucosides, and as a result many of these have subsequently been synthesized.

The synthesis of the glucosides of a number of alcohols besides methyl and ethyl alcohols, was investigated by Bourquelot † who by means of the enzyme emulsin produced glucosides, galactosides, and mannosides of propyl- and iso-propyl-alcohols, glycol, glycerol, and cinnamyl alcohol; for this purpose the sugar was dissolved in the corresponding alcohol in the presence of a little water or acetone; all these were β-glucosides; with the use of maltase from yeast he was also able to prepare a number of α-glucosides.

It has been shown by Armstrong that enzymes can exert their synthetic action without actually being in solution, acting merely as colloids in virtue of their surface. Bourquelot, moreover, drew attention to the fact that in the presence of enzymes, insoluble alcohols could be converted into soluble glucosides by combination with glucose; from this he concluded that the plant has in the formation of glucosides a very efficient mechanism for rendering insoluble substances soluble.

In some cases the natural glucosides have been chemically synthesized; thus salicin has been obtained by the reduction of the corresponding aldehyde glucoside, helicin—

$$C_6H_{11}O_5 . O . C_6H_4CHO + 2H = C_6H_{11}O_5 . O . C_6H_4CH_2OH$$

the helicin itself having been synthesized from glucose and salicylic aldehyde.

PHYSIOLOGICAL SIGNIFICANCE OF GLUCOSIDES.

In attempting to assign the part played by these substances in the economy of the plant, it must be remembered that glucosides of natural occurrence are very numerous, and, in some cases, of a diverse nature; it is, therefore, possible that the significance of the presence of one glucoside may be quite different to that of another, but even in the case of glucosides of the same nature there is much

* Macbeth and Pryde: "J. Chem. Soc.," 1922, **121**, 1660.
† Bourquelot: "Bull. Soc. Chim.," 1913, [iv], **13**, i-xxviii.

diversity of opinion. They have been described, on insufficient grounds, as direct products of photosynthesis. Many consider them to be of value as food-stuffs on account of the sugar they contain; the occurrence of certain glucosides in seeds lends some support to this view, for in the case of the bitter almond hydrocyanic acid, in the free state, may be identified when germination starts, also the observations of Treub,* who found that in the case of some plants containing cyanogenetic glucosides the amount of the latter decreased if the plant was placed in the dark, in order that photosynthesis could not take place. On the other hand there was an increase in quantity when the plants were exposed to light, and this increase reached a maximum at about midday.

Weevers † considers that salicin, populin, arbutin and similar glucosides are of the nature of reserve food-materials, for not only do these substances form a suitable means for the storage of sugar on account of their low diffusibility, but the facts of their seasonal or diurnal variation lend support to this opinion. Thus in *Vaccinium Vitis-Idæa* the arbutin is stored in the leaves, and when the new leaves are formed in the spring it is used up; it is split by a suitable enzyme, the sugar being used up, and the hydroquinone remains behind and combines with more sugar, so that by the autumn the leaves once more contain arbutin.

In the case of the willow, salicin is formed day by day, but during the night it is split by salicase into sugar and the alcohol saligenin. The glucose is translocated, and the saligenin remains behind and is converted into salicin by combining with sugar the next day. This process stops in the autumn, by which time there is relatively much salicin in the cortex of the stem.

This translocation of glucosides from the leaves of many plants—but not of all, *Sambucus* and *Indigofera* being exceptions—is significant, and so also are the facts relating to the

* Treub: "Ann. Jard. Bot. Buitenzorg," 1896, **13**, 1; 1907, **21**, 79, 107; 1910, **23**, 85.

† Weevers: "Kon. Akad. Wet. Amsterdam," 1902; "Rec. Trav. Bot. Néerl," 1910, **7**, 1.

amount of glucosides in the bark and other parts of plants at different seasons of the year. Thus in *Salix* and *Populus* the glucoside (salicin) is most abundant in the autumn and winter, and is used up in the following spring during the period of flowering and seed formation; also in the case of *Taxus* the glucoside (taxicatin), which appears principally in the young shoots, is greatest in amount in the autumn and winter. In *Pangium edule* and other plants the amount of cyanogenetic glucosides is greatest in young leaves, with increasing age the amount diminishes.

Guignard * does not believe that glucosides, or at any rate the cyanogenetic ones, are reserve food-stuffs, since, if introduced into the food-materials of a plant, glucosides have an injurious effect, owing to the aromatic residues.

Combes,† however, finds that a glucoside is toxic only to plants in which it does not naturally occur; he thinks that glucosides do not furnish carbohydrate food, since plants grown in an atmosphere free from carbon dioxide are unable to make use of these substances.

Peche ‡ holds that hydrocyanic acid is a direct product of photosynthesis; some of it combines with sugar to form a glucoside, and some is transported in a labile form, probably in a loose combination with tannin, and stored for future use as food in various tissues.

The occurrence of certain glucosides, especially in places of active metabolism such as leaves and young shoots, may indicate that certain bye-products are fixed, either temporarily or more permanently, in this form.

The exigencies of space will permit of reference only to the following examples, which are among the more important and more interesting of the glucosides.

SINIGRIN.

Sinigrin, or myronate of potash, occurs in the seeds of certain Cruciferæ, notably *Sinapis nigra*. It is split by the

* Guignard: "Compt. rend.," 1905, **141**, 236; 1906, **143**, 451.
† Combes: "Rev. gen. Bot.," 1918, **30**, 216.
‡ Peche: "Sitz. Kais. Akad. Vienna," 1912, **121**, 33.

enzyme myrosin into glucose, potassium hydrogen sulphate, and allyl isothiocyanate—

$$C_{10}H_{16}O_9NKS_2 + H_2O = C_6H_{12}O_6 + KHSO_4 + CH_2 : CHCH_2NCS$$

Sinigrin crystallizes from alcohol in needles and from water in prisms, m.p. 126-127° C.

CONIFERIN.

This glucoside occurs in various coniferous trees, especially in young parenchyma, and also in asparagus. With concentrated sulphuric acid coniferin gives a violet coloration, while hydrochloric acid and phenol give a blue coloration; it also gives a bright coloration with phloroglucinol and hydrochloric acid (see p. 219).

Coniferin crystallizes in needle-shaped crystals, m.p. 185°, and is soluble in warm water and warm alcohol. On hydrolysis by mineral acids or by emulsin it gives glucose and coniferyl alcohol—

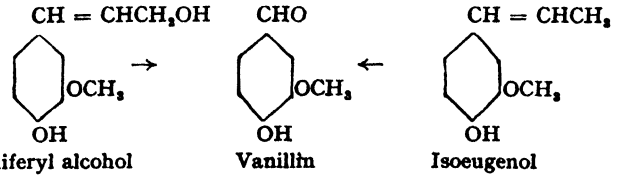
$$\underset{\text{Coniferin}}{C_{16}H_{22}O_8} + H_2O = C_6H_{12}O_6 + \underset{\text{Coniferyl alcohol}}{C_{10}H_{12}O_3}$$

The latter is a crystalline substance melting at 73°.

Both coniferin and coniferyl alcohol when oxidized with potassium bichromate and sulphuric acid yield vanillin, the aromatic constituent of the fruits of *Vanilla planifolia.*

The reaction was formerly employed for the preparation of artificial vanillin, but has now been replaced by the oxidation of isoeugenol, which is obtained by the action of dilute alkalis upon eugenol, a substance contained in oil of cloves.

The relationship between these three substances is as follows :—



Coniferyl alcohol — Vanillin — Isoeugenol

SALICIN.

Salicin, $C_{13}H_{18}O_7$, occurs in the bark of *Salix viminalis.* It has a bitter taste and crystallizes in colourless prisms and

scales. It is sparingly soluble in cold water but is more soluble in hot alcohol, especially amyl alcohol, and may be extracted from aqueous solutions by means of this solvent. Microscopically, salicin is indicated by the fact that it gives a bright red colour with strong sulphuric acid, also with Fröhde's reagent * it yields a violet coloration.

Salicin may be prepared by boiling the willow bark with water which will extract a certain amount of tannin, colouring, and other matters, together with the salicin; the solution is then treated with lead acetate and after filtering the filtrate is freed from lead by hydrogen sulphide. After removing the lead sulphide the solution on evaporation yields crystals of salicin which may be further purified by recrystallization from alcohol.

Salicin is hydrolysed by the enzyme salicase contained in willow bark and also by emulsin from bitter almonds to glucose and the alcohol saligenin according to the following equation :—

$$\underset{\text{Salicin}}{C_{13}H_{18}O_7} + H_2O = C_6H_{12}O_6 + \underset{\text{Saligenin}}{C_6H_4OH\,CH_2OH}$$

On steeping a section in a solution of emulsin, saligenin is produced which gives a blue colour with ferric chloride.

By the action of sulphuric acid and potassium bichromate salicin is oxidized to salicylic aldehyde, C_6H_4OHCHO; this substance is a fragrant colourless liquid, b.p. 196°, which occurs in the essential oil of *Spiræa Ulmaria*; it is soluble in water, the solution giving an intense violet coloration with ferric chloride.

Jowett and Potter † claim to have found a seasonal variation in the salicin content of *Salix purpurea*, and they regard it as a reserve product which is stored in the winter for use in the following spring; they also claim to have established that the reserve is drawn upon to a different extent by the male and female plants owing to their special functions, but the data they quote are hardly sufficient to warrant these conclusions; further work on this subject is

* Sodium molybdate dissolved in concentrated sulphuric acid.

† Jowett and Potter: "Pharm. Journ.," 1902, 15, 157.

desirable. According to Clark and Gillie,* the salicin content of samples of bark of *Salix sitchensis* from British Columbia varied from 2·8 per cent in the autumn to 7·38 per cent in the spring.

Weevers † suggests that the salicin formed in the leaves during the daytime is hydrolysed at night, the glucose being translocated away, while the saligenin, which remains behind in the leaf, is recombined with sugar the next day; the object of the glucoside formation would appear to be the production of a difficultly diffusible compound of sugar.

MONOTROPITIN.

This glucoside was discovered by Bridel ‡ in *Monotropa hypopitys*, and also in the fresh roots of *Spiræa Ulmaria*, *S. filipendula* and *S. gigantea* §; the same author also showed that gaultherin, occurring in the back of *Betula alba*, was identical with monotropitin. The glucoside, from whichever source obtained, is hydrolysed by the same enzyme variously described as gaultherase, betulase, or primeverase, which also occurs in *Monotropa*, giving methyl salicylate and the glucoxylose primeverose.

AUCUBIN.

The darkening of the tissues on drying of *Aucuba*, *Melampyrum*, and *Rhinanthus*, and many other plants, is due to one and the same glucoside aucubin, which is acted upon by emulsin, which also occurs in the plant yielding an aglucan, aucibigenin, of unknown constitution. The darkening of the tissues is due to the oxidation of the aucibigenin. The colour change may be readily brought about by wounding or exposing the tissues to chloroform vapour.

The similar darkening occurring in *Orobanche* is due to direct oxidation of a non-glucosidal material contained in the plant, the darkening taking place without previous intervention of a hydrolytic enzyme (see below).

* Clark and Gillie: "Amer. J. Pharm.," 1921, **93**, 618. See also Brown: "Pharm. Journ.," 1903, **16**, 588.

† Weevers: "Rec. trav. bot. Neerl.," 1910, **7**.

‡ Bridel: "Bull. Soc. chim. biol.," 1923, **5**, 918.

§ Bridel: *id.*, 1924, **6**, 679.

Bergmann and Michaelis * have re-investigated the constitution of the glucoside aucubin which is known to be identical with Rhinanthin,† the glucoside of *Rhinanthus Crista galli*, and ascribe to it the formula $C_{15}H_{22}O_9 . H_2O$ or some multiple; from this it would appear to be identical with the glucoside menyanthin contained in *Menyanthes trifoliata* and with loganin contained in *Strychnos nux vomica*. The glucoside aucubin, required for the investigation was prepared from the seeds of *Plantago lanceolata*, occurs in several species of *Plantago*. The melting-point of aucubin is 181° C., and its rotation $\alpha_D = -164{\cdot}9°$.

OROBANCHIN.

This is a glucoside typical of the orobanchs, having been found in five species of this genus ‡; it is not hydrolysed by emulsin nor by an enzyme prepared from *Rhamnus utilis* seeds, but on hydrolysis with acid gives rise to glucose and rhamnose in addition to caffeic acid or 3 : 4 dihydroxycinnamic acid—

$$C_6H_3 \begin{cases} OH \\ OH \\ CH : CH . COOH \end{cases}$$

whose close relationship to coumaric acid contained in melilotosin is interesting (see below). The orobanchs contain no aucubin and the darkening on drying is due to direct oxidation of the glucoside orobanchin without previous hydrolysis; the oxidation can be brought about by an extract of *Russula delica*, as well as by the oxidase contained in the plant itself.

ASPERULIN.

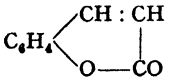
This glucoside which occurs in *Asperula odorata*, in *Galium* spp. and in many other Rubiaceæ resembles aucubin in giving on hydrolysis in addition to glucose an insoluble greenish-black substance. *Asperula odorata* also contains a second

* Bergmann and Michaelis: "Ber. deut. chem. Gesells.," 1927, **60**, 935.

† Bridel and Braecke: "Bull. Soc. chim. biol.," 1925, **6**, 665. Bourdier: "J. pharm. Chim.," 1907, [6], **26**, 454.

‡ Bridel and Charaux: "Compt. rend.," 1924, **178**, 1839; 1925, **180**, 387.

glucoside which on hydrolysis yields coumarin,* the lactone of coumaric acid—



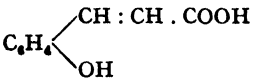
a substance which occurs also in *Anthoxanthum odoratum*, the grass which gives hay its characteristic smell, tonka bean (*Dipteryx odorata*), and other plants.

GEIN.

Gein occurs in the roots of *Geum urbanum ;* on hydrolysis by means of mineral acid or by the enzyme gease, it is broken up into eugenol and vicianose † ; the occurrence of eugenol in glucosidic combination accounts for the smell of cloves emitted by the dried roots of this plant.

MELILOTOSIN.

This glucoside, obtained from *Melilotus arvensis*, forms colourless and odourless crystals which melt at 240°. On hydrolysis by mineral acid or emulsin, it yields glucose and coumaric or *o*-hydroxycinnamic acid ‡—



INDICAN.

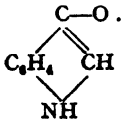
Indican,§ $C_7H_6NC \cdot O \cdot C_6H_{11}O_5$, is the name given to a glucoside which occurs not only in *Indigofera anil*, *I. arrecta*,

* Bourquelot and Hérissey : "Compt. rend.," 1920, **170**, 1545 ; Hérissey : *id.*, 1925, **180**, 1695 ; 1926, **182**, 865.

† Hérissey and Cheymol : *id.*, 1925, **180**, 565.

‡ Charaux : " Bull. Soc. chim. biol.," 1925, **7**, 1056.

§ The name indican is also applied to a compound of the formula



This substance, which is more correctly described as indoxyl potassium sulphate, occurs in small quantities in human urine and also in the urine of herbivora.

I. tinctoria, and *I. sumatrana*, but also in other plants, such as *Isatis tinctoria*, *Polygonum tinctorium*, species of *Phajus* and other orchids, e.g. *Calanthe* and *Strobilanthes*. Although the woad plant, *Isatis tinctoria*, also yields indigo, the substance giving rise to the dye is not identical with the indican of other indigo yielding plants. According to Beijerinck * the precursor in the plant is a substance isatan, of unknown composition. In the plant, indican is well distributed in the aerial organs. Thus in *Indigofera* it is found in all the tissues of the leaf except the tracheæ of the xylem; it is also abundant in the apex of the stem in all tissues except the wood vessels and the laticiferous system. The flowers also have a small quantity, but the root is characterized by its absence.†

At one time it was considered that the chloroplasts played an important direct part in the formation of indican, but Leake can find no evidence of this.

Identification.

1. The tissue may be boiled in a 2 per cent solution of ammonia. The addition of chloroform to the filtered extract may be made to separate the indigo; the chloroform will sink to the bottom of the solution, carrying with it the indigo.

2. Tissues containing indican on exposure to the vapour of alcohol for twenty-four hours will turn blue; the reaction will be better marked if the chlorophyll be subsequently dissolved out with absolute alcohol.

3. The tissue, in bulk or in section, may be boiled in strong hydrochloric acid and ferric chloride added. The indigo will separate out.

4. The tissue is cut up into pieces and quickly immersed in the following mixture:—

Glacial acetic acid	2 c.c.
Strong sulphuric acid	1 ,,
Ammonium persulphate . . .	·5 gram.
Water to	100 c.c.

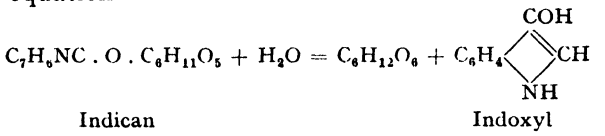
As this fluid penetrates the cells, the indigo is precipitated in blue granules. When penetration is effected fully, the material

* Beijerinck: "Proc. Kon. Ak. Wet. Amsterdam," 1900, 101; see however, Marchlewski: "Ber. deut. chem. Gesells.," 1902, **35**, 4338.

† Leake: "Ann. Bot.," 1905, **19**, 297.

is washed for twenty-four hours in water, after which sections may be cut and stained in the usual way.

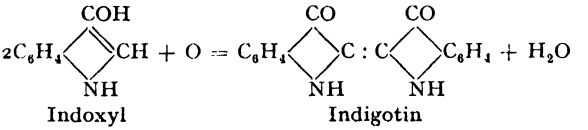
Indican is hydrolysed by indimulsin, with which it is associated in the plant, into glucose and indoxyl according to the equation—



Indican Indoxyl

The same reaction can also be effected, though more slowly, by emulsin.

The resulting indoxyl, by exposure to air, is oxidized to the deep blue colouring matter indigotin—



Indoxyl Indigotin

The production of indigotin from the indigo plant is based on these two reactions and consists in fermenting the plant material by steeping it in slightly acidified water for a few hours, and then exposing to the air the fermented extract to which a little ammonia has been added to facilitate oxidation.

Prepared in this way the natural indigo contains, in addition to indigotin, varying proportions of indirubin (a red colouring matter), indigo brown, etc., produced as by-products in the oxidation of the indoxyl.

Until a few years ago, *Indigofera* was the only source of the blue colouring matter indigo, for the obtaining of which large tracts of country were under cultivation in India. Within recent years, however, the natural production of indigo has suffered from very severe competition with the synthetic product and the planters have been compelled to improve their output. The importance of attention to fertilizing the soil has been shown by the fact that superphosphate manuring has considerably increased the yield and improved the quality of the resulting indigo.*

* Davis: "Agric. Res. Inst., Pusa," Indigo Publ., No. 4, 1918.

CYANOGENETIC GLUCOSIDES.

Among the more important glucosides are the cyanogenetic ones, so named because on hydrolysis they yield hydrocyanic acid as one of the products.

Hydrocyanic acid is of fairly common occurrence in the higher plants, and although sometimes it occurs in the free state it is, in the majority of cases, combined; the nature of many of these compounds has not yet been ascertained, but it is not improbable that generally they are glucosides.

Cyanogenetic glucosides, although widely distributed, are somewhat rare when compared with other glucosides such as the saponins. Hydrocyanic acid has been found in a few Fungi, and in certain plants of the following Natural Orders of the higher plants: Polypodiaceæ, Aroideæ, Gramineæ, Sapindaceæ, Sapotaceæ, Proteaceæ, Ranunculaceæ, Papaveraceæ, Magnoliaceæ, Lauraceæ, Droseraceæ, Rosaceæ, Saxifragaceæ, Leguminosæ, Platanaceæ, Euphorbiaceæ, Compositæ, etc. It will be observed from this list that some Cohorts, for example Rosales and Ranales, stand out in having several natural orders characterized by the presence of the substance in question.

In the individual plant the cyanogenetic glucosides occur more especially in the leaves and buds, in the seed, and also in the bark.

In *Pangium edule* Treub * found such glucosides in the phloem, pericycle, and in special cells of the leaves; Guignard † describes such compounds as occurring in the leaves of vigorous shoots, the young bark, and in the unripe fruit of *Sambucus nigra* and species of *Ribes*. The amount present in a member is not constant; Verschaffelt ‡ found that as the buds of *Prunus Padus* and *P. Laurocerasus* open, the amount of hydrocyanic compounds increases as rapidly as do the other substances present. Treub has found that in plants growing in the tropics and which contain cyanogenetic glucosides, these substances disappear before leaf-fall; in some cases this

* Treub: "Ann. Jard. Bot. Buitenzorg," 1907, 21, 107.
† *Loc. cit.*
‡ Verschaffelt: "Kon. Akad. Weten. Amsterdam," 1902.

depletion is quite sudden, in others the glucosides gradually disappear. On the other hand, in *Indigofera* and *Sambucus* the glucosides are not removed before the fall of the leaves.

Treub also states that the amount present depends on the quantity of available sugar; he observed that there obtains a daily variation, the maximum quantity occurring at about midday. On the other hand, there is no consistent daily fluctuation in *Sorghum*, and unhealthy plants may contain more than healthy. It has also been ascertained that the quantity of cyanogenetic glucosides in *Pangium*, *Phaseolus lunatus*, *Zea* and *Sorghum* may be increased by the application of manures rich in nitrates; on the other hand, it must be pointed out that in some cases, e.g. *Phaseolus lunatus*, the glucoside may be eliminated from the seed by suitable methods of cultivation. Also the amount varies in different varieties of species, e.g. *Sorghum*. In some examples of seeds which contain little or no hydrocyanic acid there may be a marked increase on germination, thus in the flax, Dunstan and Henry * found that the seeds contained ·008 per cent of the acid, whereas in the seedlings ·135 per cent obtained; the same increase also occurs in the sweet almond. Further, the percentage of hydrocyanic acid in *Linum*, *Sorghum*, *Lotus arabicus* and *Zea Mais* gradually increases to a maximum and then decreases, sometimes to zero.

The stage of development at which the maximum is reached varies in the different plants; thus, to take two extreme cases, in the flax the maximum obtains when the seedlings are between four and five inches high, whilst in *Lotus arabicus* the maximum occurs at the period of flowering.

It is clear that the actual amount of the substance in question varies pretty considerably; it may be very small or relatively large, thus in the young leaves of *Pangium* the presence of ·3 per cent of hydrocyanic acid has been ascertained.

To summarize these observations: the amount of cyanogenetic glucoside and the incidence of its maximum is very

* Dunstan and Henry: "Brit. Assoc. Rep.," York, 1906; "Phil. Trans. Roy. Soc. Lond.," 1901, B, 194, 515; "Proc Roy. Soc. Lond.," B., 1900, 67, 224; 1901, 68, 374; 1903, 72, 285.

variable, depending on the specific physiology of the plant, the age and condition of the plant member, the conditions of growth, and so on. Wherefore no general conclusion can for the time being be reached; this, in fact, may only be possible when knowledge of the sequence of metabolic events, in which the glucoside is involved, in each distinctive case is gained and correlated. This has been done, in part, in one instance. Godwin and Bishop * have traced the relations of the glucoside of cherry laurel leaves to the yellowing stage, and the respiration phenomena of starvation. When the mature green leaf is placed in the dark, the available sugars are consumed in respiration, and the hexose concentration perforce falls. With this fall there is a corresponding diminution in the respiration rate and with this disturbance in the equilibrium, there is a tendency for the glucoside to be hydrolysed. This phase is comparatively slow, but the next, marked by the yellowing of the leaf, is quick. In this, senescence is marked by the dominance of katabolic processes: the chlorophyll is destroyed, starch is hydrolysed and the glucoside disappears. The result of these hydrolytic actions is an increase in the hexose content accompanied by a rise in the rate of respiration which is maintained until the sugar is exhausted. The period of maximum loss of glucoside coincides fairly closely in time with similar maxima in the rates of yellowing and of respiration.

The younger the leaves, the smaller is the rate of loss of glucoside before yellowing and the longer is the postponement of the beginning of the second phase of rapid loss.

Godwin and Bishop draw no conclusion regarding the possible rôle of cyanogenetic glucoside in the plant. They content themselves by pointing out that the three main phases in the life of a member may possibly be explained by the changes in the condition of the protoplasm.

Reactions, Microchemical and Otherwise.

1. The presence of cyanogenetic glucosides or of free hydrocyanic acid can generally be detected by chewing a small piece of the material.

* Godwin and Bishop: "New Phyt.," 1927, **26**, 295.

2. Cut a thick section of the fresh tissue to be examined and place it in a 5 per cent alcoholic solution of potash for about a minute; transfer to a solution containing 2·5 per cent ferrous sulphate and 1 per cent ferric chloride and keep at about 60° C. for ten minutes. Place the preparation in a dilute solution of hydrochloric acid—one part of strong acid to six parts of water—for five to fifteen minutes. The presence of hydrocyanic acid is indicated by the formation of Prussian blue.

If leaves are to be tested, instead of cutting them up they may be pricked all over with a bunch of fine needles and then treated as above.

3. *Guignard's Test.*—Dip strips of white filter-paper in a 1 per cent solution of picric acid and dry; before use moisten the papers with a 10 per cent solution of sodium carbonate. The test paper turns an orange red in the presence of fumes of hydrocyanic acid. The test is very delicate, and the rapidity of the change in colour depends on the amount of prussic acid present, so that if the quantity be very small the paper may have to be suspended in the test tube containing the material to be tested, for some hours.

This test has been modified by Waller so as to give quantitative results, but it has been pointed out by Chapman * that the coloration is due to reduction, and is, therefore, not specific for hydrocyanic acid; accordingly the method must be used with caution.

Bishop † describes a convenient method for the estimation of cyanogenetic glucosides. The material, leaves of the cherry laurel for example, is treated with emulsin whereby the prulaurasin is decomposed; the hydrocyanic acid set free is carried over by a current of air into potash which is then titrated with silver nitrate.

Some of the more important cyanogenetic glucosides may now be considered.

* Chapman: "Analyst," 1910, **35**, 469. See also Francis and Connal: "J. Amer. Chem. Soc.," 1913, **35**, 1629.

† Bishop: "Biochem. Journ.," 1927, **21**, 1162.

AMYGDALIN.

Amygdalin, $C_{20}H_{27}NO_{11}$, is a lævo-rotatory bitter substance which is fairly soluble in water, and gives with concentrated sulphuric acid a pale reddish-violet coloration; this, however, is not a distinctive test, since the same coloration is given by some other glucosides.

Amygdalin occurs in the seeds of the bitter almond, *Pyrus Amygdalus;* it is, however, generally stated not to occur in the seeds of the cultivated almond, the sweet variety, although emulsin, its appropriate enzyme, is present. Dunstan and Henry have shown that traces of hydrocyanic acid occur in the seeds, and more than traces in the seedlings, of the sweet almond; it is probable, therefore, that a small quantity of amygdalin does occur in the sweet variety. This relative absence of glucoside in the cultivated plant is important, and the same phenomenon occurs in *Phaseolus lunatus.* The seeds of the wild plant yield large quantities of hydrocyanic acid, whereas those of the cultivated plants give very little or none.

Amygdalin has also been described as occurring in *Pyrus Malus, Pyrus Aucuparia, Pyrus cydonia* and other plants.

This glucoside may be obtained by crushing the seeds of the bitter almond and subjecting the mass to considerable pressure between hot iron plates in order to remove the oil. The solid cake is then digested with hot alcohol which dissolves out the amygdalin. The alcoholic extract is evaporated down when the amygdalin separates out in scale-like crystals belonging to the monoclinic system.

It has already been mentioned that the appropriate enzyme generally occurs in the same tissues as the glucoside; this being so, the bitter almonds have only to be crushed in water in order to bring the ferment emulsin into contact with the amygdalin to bring about the hydrolysis.

The study of the enzymic hydrolysis of amygdalin has revealed the fact that emulsin is not a single enzyme, as was once thought, but a preparation consisting of a number of different constituents comparable to an extract of yeast or

of pancreas.* The complexity of this enzymic mixture is shown by the fact that its activity on the substrate amygdalin can be separated into two stages :—

(1) $C_{20}H_{27}NO_{11} + H_2O = \underset{\text{Mandelonitrile glucoside}}{C_{14}H_{17}NO_6} + C_6H_{12}O_6$

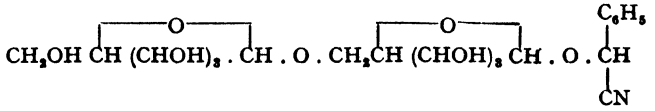
(2) $C_{14}H_{17}NO_6 + H_2O = C_6H_5CHO + HCN + C_6H_{12}O_6$

The first of these reactions is effected by the enzyme amygdalase, whilst the second is produced by the enzyme prunase.

The two stages of the hydrolyses may be demonstrated either by carefully controlling the reaction of emulsin on amygdalin and stopping the reaction at the right moment, before the prunase is able to decompose the mandelonitrile glucoside, or else by hydrolysis by means of acids.†

According to Giaja ‡ amygdalin is broken up under the action of the gastric juice of the snail, *Helix pomatia*, into benzaldehyde, hydrocyanic acid and a disaccharide which has since been identified as gentiobiose.

This disaccharide has not as yet been isolated from amygdalin, but the fact of its presence there is proved by Campbell and Haworth § and Kuhn and Sobotka || who synthesized amygdalin from gentiobiose. The constitution of gentiobiose having been determined by Haworth, Helfferich and other as a β-glucosido-*b*-glucose, the constitution of amygdalin becomes—



Some confusion originally existed as to whether amygdalin contained α- or β-glucose residues. Thus yeast extract is able to split off a molecule of glucose from amygdalin, giving mandelonitrile glucoside ; this action was wrongly attributed to maltase, the prototype of α-glucosidases ; actually this

* Oppenheimer : " Lehrbuch der Enzyme," Leipzig, 1927, p. 271.
† Caldwell and Courtauld : " J. Chem. Soc.," 1907, **91**, 666.
‡ Giaja : " Compt. rend.," 1910, **150**, 793.
§ Campbell and Haworth : " J. Chem. Soc.," 1924, **125**, 1337.
|| Kuhn and Sobotka : " Ber. deut. chem. Gesells.," 1924, **57**, B., 1767.

hydrolysis is effected by amygdalase, a β-glucosidase, which, besides occurring in emulsin, is almost always associated with maltase in yeast even when species of *Saccharomyces*, e.g. *S. Ludwigii*, contain no maltase.

The splitting of amygdalin by emulsin involves three distinct scissions: at the point of union between (*a*) the two glucose residues, (*b*) the glucose and the benzaldehyde cyanhydrin, and (*c*) the benzaldehyde and the hydrocyanic acid. Scissions at the first two points are hydrolyses effected by amygdalase and prunase respectively; the third is of a different type, whether the separation of the hydrocyanic acid from the benzaldehyde by a third enzyme, hydroxynitrilase, is not known.

PRUNASIN, PRULAURASIN, AND SAMBUNIGRIN.

The three glucosides prunasin, prulaurasin, and sambunigrin occur respectively in the twigs of *Prunus padus*, the leaves of *P. laurocerasus*, and the fruit of *Sambucus niger*. They are all isomeric with mandelonitrile glucoside obtained by the partial hydrolysis of amygdalin; they differ, however, in their optical activities and in the melting-points of their crystals :—

	Prunasin or *d*-mandelonitrile glucoside.	Prulaurasin or *r*-mandelonitrile glucoside.	Sambunigrin or *l*-mandelonitrile glucoside.
α_D . .	— 26·9	— 52·7	— 76·3
M.P. .	147°-150°	120°-122°	151°-152°

Sambunigrin has been synthesized by Fischer and Bergmann.

DHURRIN.

This is a glucoside closely allied to amygdalin, and occurs in the seedlings of *Sorghum vulgare*, but not in the older plants; it has the empirical formula $C_{14}H_{17}NO_7$ and yields, on hydrolysis, glucose, hydrocyanic acid, and parahydroxybenzaldehyde :—

$$C_{14}H_{17}NO_7 + H_2O = C_6H_{12}O_6 + HCN + C_6H_4OHCHO$$

Similar glucosides occur in the seedlings of *Panicum* and *Zea*.

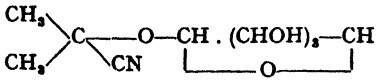
Dunstan and Henry give the following method for the

* Fischer and Bergmann: " Ber. deut. chem. Gesells.," 1917, 50, 1047.

isolation of dhurrin from *Sorghum vulgare*. The plants are dried at a low temperature and ground up as finely as possible. The material so obtained is extracted with alcohol and filtered; the alcohol is then distilled off from the filtrate and the residue dissolved as completely as possible in warm water. Lead acetate is added to this aqueous solution until no more precipitate (chiefly lead tannate) comes down. A current of sulphuretted hydrogen—a large excess is to be avoided—is then passed through the filtrate and the lead sulphide filtered off. The excess of sulphuretted hydrogen can be removed from the filtrate by passing through it a current of air. The liquid is then worked up with pure animal charcoal, sufficient in amount to convert the whole, when dry, into a powder, and dried in a vacuum desiccator. When quite dry the material is extracted with anhydrous ethyl acetate in a Soxhlet apparatus; this solvent slowly removes the glucoside, leaving most of the sugar and other impurities behind. On distilling off the solvent a syrup remains which may, if necessary, be again treated in the same fashion. The syrup will deposit crystals of the glucoside after standing for a few days in a vacuum over sulphuric acid. The crystals so obtained may be recrystallized from hot alcohol or boiling water.

PHASEOLUNATIN OR LINAMARIN.

Phaseolunatin, $C_{10}H_{17}O_6N$, occurs in the seeds of wild plants of *Phaseolus lunatus* (Burmah bean); it is present only in very small quantities, or is entirely absent from the seeds of the cultivated plants. It is also present in *Linum*, more especially that grown in tropical climates, and many rubber-yielding plants, such as *Hevea braziliensis* and species of *Manihot*. Associated with it in its natural surroundings is the enzyme phaseolunatase which is able to hydrolyse it to acetone, glucose, and hydrocyanic acid,* from which it follows that phaseolunatin is a glucose ether of acetone cyanhydrin of the formula—



* Dunstan, Henry, and Auld: "Proc. Roy. Soc.," B., 1906, 78, 145, 152.

LOTUSIN.

Lotusin, $C_{28}H_{31}NO_{16}$, occurs in *Lotus arabicus*. It is a bitter, yellow-coloured substance, which when fresh does not reduce Fehling's solution.

On hydrolysis it yields glucose, hydrocyanic acid, and lotoflavin, a bright yellow crystalline precipitate—

$$C_{28}H_{31}NO_{16} + 2H_2O = 2C_6H_{12}O_6 + HCN + \underset{\text{Lotoflavin}}{C_{15}H_{10}O_6}$$

Lotusin, like dhurrin, does not occur in old plants with ripe seeds; it is present only in the younger stages of development.

It is hardly necessary to point out the economic importance of this occurrence of cyanogenetic glucosides in the younger stages of plants like *Lotus arabicus* and *Sorghum*; much loss of stock has been sustained by their consumption by cattle.

SAPONINS.

According to the researches of Greshoff,* the saponins are very widely distributed in the higher plants; he has identified them in various plants belonging to the natural orders: Piperaceæ, Saururaceæ, Primulaceæ, Loganiaceæ, Oleaceæ, Polemoniaceæ, Proteaceæ, Caprifoliaceæ, Compositæ, Cucurbitaceæ, the majority of the natural orders of the cohort Centrospermæ, Ranunculaceæ, Magnoliaceæ, Leguminosæ, Rosaceæ, Saxifragaceæ, Pittosporaceæ, Polygalaceæ, Rutaceæ, Rhamnaceæ, Guttiferæ, Thymelæaceæ, Combretaceæ, Myrtaceæ, Lecythidaceæ, Araliaceæ, Gramineæ, Liliaceæ, and Gleicheniaceæ.

The term saponin, though originally restricted to a specific substance obtained from the root of *Saponaria rubra* and *S. alba* is now applied to a large group of compounds, all of which have properties similar to those possessed by the orginal saponin.

Physical and Chemical Properties.

The saponins are mostly amorphous colloidal substances which dissolve readily in water; their aqueous solutions, if

* Greshoff: "Kew Bulletin," 1909, 397; for summary of work on saponins, see Winterstein and Maxim: "Helv. Chim. Acta," 1919, **2**, 195.

shaken up alone, produce a froth, but if shaken in the presence of oils, fats, or resins, they produce emulsions which are characterized by their great stability.

They are insoluble in absolute alcohol, ether, chloroform, and benzene.

From their aqueous solutions they may be precipitated unchanged by the addition of ammonium sulphate. The saponins are, as a rule, neutral substances, but a few have feebly acid properties. Only a single saponin, namely, solanin, has basic properties; this substance, which occurs in *Solanum nigrum*, *S. dulcamara* and in the fruit, eyes, and young shoots of potatoes, owes its basic property to the presence of a nitrogen atom (see Nitrogen Bases), and appears to form a connecting link between the saponins and the alkaloids.

The neutral saponins are precipitated from solution by basic lead acetate, while acid saponins are precipitated by lead acetate. Similarly, barium hydroxide precipitates neutral saponins in the form of their barium compounds (see below).

The saponins are reducing agents, and will reduce ammoniacal silver nitrate to metallic silver; similarly, prolonged boiling with mercuric chloride reduces this substance to calomel; saponins also blue a solution of potassium ferricyanide containing ferric chloride, by reducing the ferric salt to the ferrous condition, and so giving rise to the formation of Turnbull's blue.

If boiled with acetic anhydride, alone or in presence of sodium acetate or zinc chloride, the saponins are converted into acetyl derivatives which are no longer toxic. On boiling the acetyl derivatives with alcoholic potash the acetyl groups are removed, but the resulting compound is not identical with the original saponin.

Isolation of Saponins.

For their preparation, the raw material may be extracted by means of hot alcohol; in some cases the saponins separate out on cooling the solution; in other cases they may be precipitated from the alcoholic solution by the addition of ether. For further purification the saponin dissolved in water is

treated with lead acetate which precipitates the acid saponins, while the neutral saponins are only precipitated by basic lead acetate. The resulting lead salts of the saponins are best decomposed by treatment with dilute sulphuric acid. Alternatively, saponins may in many cases be precipitated from their aqueous solutions by saturated baryta, and in some cases they may be salted out by the saturation of their solutions by ammonium sulphate.

From their solutions in alcohol they are precipitated by alcoholic solutions of cholesterol or phytosterol with the formation of cholesterides, a property of digitonin which is utilized for estimating sterols (see p. 50). These cholesterol compounds are, as a rule, easily decomposed; in most cases, prolonged extraction with ether will remove the cholesterol, and the saponin is recovered unchanged and possesses its original physiological action (see below).

Constitution.

On hydrolysis with dilute mineral acids * the saponins yield sugars such as glucose, galactose, arabinose, and rhamnose, together with other substances termed *sapogenins*, the constitution of which is unknown.

The nature of the sapogenin obtained from any particular saponin varies with the conditions of the hydrolysis; in some cases careful hydrolysis may yield a primary sapogenin and a sugar, while more complete hydrolysis gives rise to an other sapogenin together with more sugar.

The hydrolysis of digitonin, the saponin contained in *Digitalis purpurea*, may, according to Kiliani, be represented by the equation—

$$\underset{\text{Digitonin}}{C_{54}H_{92}O_{28}} + 2H_2O = \underset{\text{Digitogenin}}{C_{30}H_{46}O_6} + 2\underset{\text{Glucose}}{C_6H_{12}O_6} + 2\underset{\text{Galactose}}{C_6H_{12}O_6}$$

Reactions.

The following reactions are made use of in demonstrating the presence of a saponin:—

* Hydrolysis can, in some cases, be effected by bacteria, and Quillaia saponin is even said to be hydrolysed by emulsin (see Gonnermann: "Pflüger's Archiv," 1906, **113**, 185).

1. Aqueous extracts readily form a froth when shaken up.

2. Concentrated sulphuric acid gives with all saponins, either in the cold or on warming, a violet or red colour.

3. Concentrated sulphuric acid containing a little ferric chloride gives with many saponins a blue or bluish-green colour or fluorescence.

4. The hæmolytic action described below may be tried.

Although the above reactions are best carried out in the test tube, numbers 2 and 3 may be made use of in micro-chemical work.

Physiological Action.

The saponins are characterized by their strongly marked toxic properties. Fishes, particularly, are very sensitive to saponins, being killed by a solution of 1 part in 100,000 parts of water, a fact which is made use of by fishermen in the East, as the fish killed by these means are not unfit for human consumption.

Saponins have a powerful solvent action on blood corpuscles, a property which is known as hæmolysis. This property may be connected with their tendency to combine with cholesterol,* which substance they abstract from the blood corpuscles thereby effecting hæmolysis.

The action may be illustrated by adding a small quantity of a solution of a saponin † in 0·9 per cent sodium chloride to 1 c.c. of a solution made by dissolving 1 c.c. of defibrinated blood in 100 c.c. of 0·9 per cent sodium chloride; after a short time the blood corpuscles will have dissolved leaving a clear solution.

The hæmolytic action may be destroyed by shaking up some of the saponin solution with an ethereal solution of cholesterol and then warming for some hours at 36° C.; this treatment causes the saponin to combine with cholesterol to produce an inactive compound which has no hæmolytic action.

* They also combine with phytosterol.

† The saponins of *Guajacum officinale* and *Bulnesia Sarmienti* have hardly any hæmolytic action, and hence are only slightly toxic.

General Properties and Uses of Saponins.

Connected with their emulsifying property is the employment of saponins as substitutes for soaps, a fact which is indicated in the name Saponin itself and also by the names *Saponaria*, soap wort and Quillaia (meaning wash wood), etc.

The so-called soap nuts are the fruits of *Sapindus* (fructus saponis indici) and these, as well as the beans of *Entada scandens* and *Lychnis chalcedonica* or Tartary soap, are largely used in the East for washing clothes, since they have no deleterious effect on the colour or the fibre of the most delicate fabrics.

Aqueous solutions of saponins have a marked power of retaining dissolved gases, as, for example, carbon dioxide; for this reason saponins are occasionally added to effervescent drinks, such as ginger-beer or lemonade, a use which is to be deprecated owing to their toxic properties.*

Occasionally saponins are employed for making suspensions of solids in water since they exert an inhibiting effect on the precipitation or deposition of suspended solids.

FURTHER REFERENCES.

Armstrong: "The Simple Carbohydrates and Glucosides," London, 1924.

Kobert: "Beiträge zur Kenntniss der Saponinsubstanzen," Stuttgart, 1904.

Kofler: "Die Saponine," Wien, 1927.

Van Rijn: "Die Glykoside," Berlin, 1900.

* The saponin obtained from the bark and wood of *Guajacum officinale* is occasionally used for this purpose, since it is practically non-poisonous, its hæmolytic action being only very slight.

SECTION V.

TANNINS.

THE term Tannin is variously employed by different writers, sometimes to denote a particular substance better described as gallotannic or digallic acid, and sometimes as a collective term for a whole group of substances having certain characteristics in common. In order to prevent confusion it is proposed here to use the word tannin only in the latter sense.

The properties of the tannins may be summarized as follows :—

1. They are mostly uncrystallizable colloidal substances with astringent properties.

2. They precipitate gelatine from solution and form insoluble compounds with gelatine yielding tissues, a property which enables them to convert hide into leather.*)

For this purpose add 2 c.c. of 0·5 per cent of the tannin solution to an equal volume of 0·5 per cent gelatine ; an immediate precipitate or turbidity should appear.

3. They all give blackish-blue or blackish-green colours with ferric salts, a fact which is made use of in the manufacture of ink.

This test is best carried out by adding 3-5 drops of 1 per cent iron alum to 3 c.c. of a 0·5 per cent neutral solution of

* According to some authors, this property is not an essential characteristic of tannins ; on the other hand, Dekker prefers to regard those substances which do not give this reaction as pseudo-tannins and includes under this heading the tannins of *Portlandia grandiflora, Asperula odorata, Rubia tinctorum, Scrophularia nodosa, Humulus Lupulus,* etc. Similarly, Procter points out that such substances as moritannic acid, or maclurin, and lupulotannic acid, are more closely related to the colouring matters than to the tannins. In the opinion of Freudenberg, the conditions favouring the formation of a precipitate with gelatine are the possession of a sufficiency of hydroxyl groups coupled with a sparing solubility of the crystallised tannin in cold water.

the tannin; the colour is liable to be destroyed by mineral acid and to be turned green by organic acids.

4. They are precipitated from solution by many metallic salts such as copper or lead acetates or stannous chloride, etc.

5. They are precipitated from solution by a strong aqueous solution of potassium bichromate or by a 1 per cent solution of chromic acid.

6. They precipitate from solution both alkaloids and substances of a basic nature, such as basic organic colouring matters, including methylene blue.

7. In alkaline solution the tannins, and many of their derivatives, readily absorb oxygen, becoming dark in colour.

8. With a solution of potassium ferricyanide and ammonia they give a deep red colour.

It must be borne in mind, however, that none of these reactions, taken separately, are specific for tannins; they may be given by many other substances as well, but all true tannins answer them as a whole.

OCCURRENCE.

Tannin, using the word as a generic term, is generally looked upon as an aplastic substance, and is very widely distributed in the vegetable kingdom.

In certain Algæ, e.g. *Spirogyra*, *Mesocarpus*, and *Zygnema*, it occurs in the cells in the form of numerous small vesicles; in the Fungi, tannin is stated to be more abundant in parasites than in saprophytes, thus hardly any occurs in the Agaricineæ whilst in the Polyporeæ it is present in much larger amounts.

In the higher plants it occurs more or less generally throughout a tissue, for example in bark, or it may be restricted, in the more mature parts, to special cells which may be isolated or superposed one above the other in the form of chains.

Amongst the higher plants there is no great phylum in which tannin is not found; it occurs in the ferns, e.g. *Angiopteris* and *Aspidium*; in Gymnosperms, e.g. *Pinus*; and also in innumerable Angiosperms, in all parts.

Thus it obtains in the roots of *Trianea*, *Desmanthus*, and *Pistia*; in the stems, where it may be accumulated, especially

in the bark, of *Quercus*, many species of *Cæsalpinia*, *Eucalyptus occidentalis*, *Castanea*, and *Humulus*; in the leaves of *Cerasus*, *Rhus*, *Ficus*, and *Rhododendron*; in the fruit, especially if unripe, of *Terminalia Chebula*, *Cæsalpinia coriaria*, *Pyrus*, and *Phaseolus*; and more rarely in the seeds, either before or after germination, of *Areca Catechu*, *Echium vulgare*, and other Boraginaceæ.

Further, tannin is often found in more or less special structures, e.g. the cells of the pulvini of *Mimosa pudica* and *Robinia pseudacacia*; in the gland cells of *Sarracenia* and *Utricularia*; in the hairs of *Primula* and *Hedera*; and also in laticiferous tissue.*

Finally, it may be remarked that it is especially abundant in pathological growths such as galls, which may contain from 25 to 75 per cent of tannin.

Kraemer † has investigated the galls formed by the agency of *Cynips aciculata*, a gall fly, upon *Quercus coccinea*. He found that during the chrysalis stage gallic acid was produced, probably at the expense of the starch, and as the imago developed the gallic acid gave place to tannic acid.

Tannin occurs in solution in the cell sap and sometimes in distinct vacuoles. According to Lloyd,‡ in the former case the tannin is linked up or adsorbed with other cell constituents which, in some instances, is a cellulose-like body. Similarly Herszlik § on microchemical evidence concludes that the tannin vacuoles in the cortical cells of *Phaseolus* are surrounded by a membrane of a pectic nature. Probably this, or a like, protective arrangement is general for otherwise the protoplasm would be precipitated by the tannin.

The amount of tannin present in certain plants varies according to the physiological state, the season of the year, and the conditions of growth.

In *Pinus* it is stated that the amount of tannin varies with that of the resin; thus in the spring it was found that as

* For details of the distribution of tannin in *Ribes*, etc., see Dekker: "Rec. trav. bot. néerlandais," 1917, **14**, 1.

† Kraemer: "Bot. Gaz.," 1900, **30**, 274.

‡ Lloyd: "Trans. Roy. Soc. Canada," 1922, **16**, 1.

§ Herszlik: "Bull. Acad. polonaise," 1925, **3**, 315.

the tannin decreased in amount so the resin increased. Peacock * found that in *Heuchera americana* the tannin was most abundant in October and least in May, whilst the amount of starch present was greatest in March. Trimble and Peacock found that in *Geranium maculatum* the maximum amount of tannin obtained in April, i.e. just before the period of flowering. From this phase onwards there was a gradual decrease until the minimum was reached in October.

It is found that the more vigorous trees yield the most tannin, and that the character of the soil appears to be of importance. It has been found that oak trees grown in a poor dry soil yield a bark richer in tannin than those grown on the soil of damp lowlands.

It is not impossible that the different yields of tannin given by the same plant grown in different situations may be due to the relative abundance of the mineral food-materials; thus it has been found that in some instances, e.g. in *Spirogyra* and *Phaseolus multiflorus*, the formation of tannin is inhibited by the absence of chlorine.

With regard to seasonal variation in the amount of tannin in the bark of the oak, the following estimations are given by Eitner :— †

	Q. pedunculata.	*Q. sessiliflora.*
April	14·8 per cent.	12·86 per cent.
May	10·71 ,,	10·46 ,,
June	12·33 ,,	10·58 ,,
July	9·8 ,,	8·11 ,,
August	11·23 ,,	10·74 ,,

For the inner bark of the American oak, *Quercus Prinus*, Trimble ‡ found the following seasonal variation :—

December	9·33 per cent.
March	10·63 ,,
June	11·22 ,,
July	11·70 ,,
September	6·66 ,,

As a general rule the barks collected in May and June are the richest in tannin, but this does not hold for all parts of

* Peacock : "Amer. Journ. Pharm.," 1891, 172.
† Eitner : "Der Gerber, Vienna," 1878, 4.
‡ Trimble : "The Tannins," Philadelphia, 1892, 1894.

plants. Thus, Levi and Wilmer found that in the case of the horse-chestnut, *Aesculus Hippocastanum*, the youngest leaves were richest in tannin, the minimum amount obtained in June, whilst in August the quantity rapidly rose until the original value was reached; finally a diminution of tannin occurred just before leaf-fall. Weekly analyses of leaves were made from the opening of the buds to the fall of the leaves in September. The obtained percentages of tannin were: 6·5, 3·3, 3·5, 2·8, 3·7, 3·2, 1·9, 2·8, 3·5, 3·6, 3·4, 5·1, 3·1, 5·3, 4·4, 4·3, 3·4, 6·2, 6·6, 5·2, 6·1, 6·5, 4·5 per cent.

These variations in the tannin-content of parts of plants are of great interest; the value, however, of such estimations would be greatly enhanced if estimations were carried out at the same time to see whether, for instance, there is any obvious relationship between the tannin-content of leaves and of other parts of the plants such as the periderm.

MICROCHEMICAL REACTIONS OF TANNINS.

Before passing on to the detailed examination of the various tannins, the following microchemical tests may be mentioned, but it must be borne in mind that these reactions do not enable one to distinguish between the various tannins.

1. Tannins reduce Fehling's solution.

2. They are precipitated by basic lead acetate and the salts of many other metals; thus uranium acetate gives a brown precipitate or a brown or brown-red coloration, and an aqueous solution of copper acetate gives a brown precipitate.

3. Potassium bichromate in a strong aqueous solution or a 1 per cent solution of chromic acid gives brownish-coloured precipitates.

4. A red-brown to brown coloration is obtained by the use of a dilute ammoniacal solution of potassium ferricyanide. This test is very delicate, and the reagent must be used sparingly since the coloration is destroyed by an excess.

5. The addition of a neutral solution of ferric chloride gives a blue-black or greenish coloration or precipitate.

* Levi and Wilmer: "Hide and Leather," 1905.

Moeller recommends the use of a solution of anhydrous ferric chloride in anhydrous ether.

6. A solution of ammonium molybdate in a strong solution of ammonium chloride gives a copious yellow precipitate with many tannins; when added to digallic acid a red coloration results. According to Gardiner * this reagent affords a means of distinguishing glucoside tannin from tannic acid.

The red-yellow colour obtained by adding ammonium molybdate to tannic acid is destroyed by oxalic acid.

7. Lime water gives a white precipitate which turns red, brown, or blue.

8. Aqueous solutions of various organic bases such as caffeine and antipyrin precipitate the tannins.

Van Wisselingh † recommends 1 per cent aqueous solutions of antipyrine and of caffeine.

It must be remembered that several other substances besides tannins are precipitated by these reagents.

9. Pfeffer has drawn attention to the fact that tannins are precipitated by methylene blue without prejudice to the vitality of the cells. The stain must be used in very dilute solutions (1 part in 500,000 of water), and the tissue under investigation must remain in a large quantity of the solution for several hours. Van Wisselingh's experience is contrary to Pfeffer's, for he finds that even very dilute solutions of methylene blue are harmful to *Spirogyra*, the plant used by Pfeffer, and after treatment for several days only a little of the tannin was precipitated.

10. On the addition of a solution of gelatine a dirty white precipitate is formed.

11. A brilliant red colour, even when the tannins are in a very dilute solution, results from the addition of an aqueous solution of iodine in potassium iodide mixed with a little 10 per cent. ammonia.

The following are microchemical tests for gallic acid:—

1. The rapidity of the reaction with potassium chromate may provide a means of distinguishing gallic acid from tannic

* Gardiner: "Proc. Camb. Phil. Soc.," 1883, 4, 387.

† Van Wisselingh: "Konin. Akad. v. Wetensch Amsterdam," 1910, 685.

acid, for in the case of the former a precipitate immediately comes down, whilst in the case of tannic acid, according to Drabble and Nierenstein, the reaction is either very slow or entirely negative.

2. Potassium cyanide in aqueous solution gives a pink coloration with gallic acid.

3. With Nessler's solution gallic acid gives a grey-green precipitate.

With this same reagent pyrogallol immediately yields a brown precipitate; pyrocatechol forms a deep green precipitate which changes to greenish brown; and a dirty green precipitate is given by protocatechuic acid.

Vinson * gives a method of simultaneous fixing and staining of tannins *in situ*, by exposure to the vapour of sweet spirits of nitre.

PHYSIOLOGICAL SIGNIFICANCE OF TANNINS.

It is manifestly a difficult matter to ascertain the significance of tannins in the life of the plant, more especially as these substances vary in different species, so that what may be true for one is not necessarily true for all.

It is, therefore, not surprising to find that several ideas have been put forward.

With regard to the origin of tannins practically nothing of fundamental importance is known.

According to the investigations of Kraus, tannin, although not a direct photosynthetic product—as is indicated by the fact that the tannin does not increase in the leaves of plants which are able to photosynthesize in dull light—is not formed unless carbon dioxide and light are available. He found that etiolated leaves produced no tannin, and that the amount of this substance in shaded leaves was less than that contained in the leaves of the same plant fully exposed to the sun. The tannin thus formed is translocated to the stem and root.

Similarly Dekker † finds that light is requisite for the formation of tannin, and that the tannin content of leaves

* Vinson: "Bot. Gaz.," 1910, 49, 222.
† Dekker: "Rec. trav. bot. néerlandais," 1917, 14, 1.

considerably decreases in darkness owing to translocation and other processes.

This, however, is not the only origin for tannin, for if tannin-containing seeds, e.g. the oak, be germinated in darkness, there is an increase in the amount of tannin; further, the production of various aromatic compounds may be a stage in the synthesis of proteins, and some of these may eventually give rise to tannin.

The facts regarding the distribution of tannin have an important bearing on the subject. It is abundant in leaves; in parts in which growth is very active, such as growing points; in galls and other pathological growths; also it is found in association with secretory organs, such as gland cells of *Sarracenia* and *Utricularia*, and in parts in which the protoplasm is especially irritable, such as pulvini. Pfeffer found that in young fully formed pulvini no tannin occurs, but it appears soon after movements commence and gradually increases in quantity until the leaf dies.

In the case of *Robinia pseudacacia* the pulvini of the leaflets contain less tannin than the main pulvinus, which is much less sensitive than are the secondary pulvini.

The consideration of these facts supports the conclusion arrived at by Sachs that tannin results from intense metabolism such as occurs in active leaves; in rapid tissue formation, as in galls and vegetative apices; during germination and secretion; and as a consequence of particular stimulation, as in mobile pulvini.

Various facts on the relation between tannin and other substances such as starch, sugar, resin, etc., have led to various opinions.

That starch frequently is contained in the same cells with tannin suggests a connection between the two, and it is not impossible that the starch may contribute the glucose for the construction of the tannin. In the case of *Pinus*, it has already been mentioned that in the spring, when the flow of resin is most copious, the tannin decreases as the resin increases; also the cells surrounding the epithelium of resin ducts contain tannin and starch. Wiesner, therefore,

concluded that tannin is an intermediate product in resin formation.

Tannin is not uncommon in unripe fruits, and the amount of these astringent substances diminishes during ripening.

According to Bassett * " the amount of tannin in fruits varies with certain factors, such as injury, length of time between removal from tree and analysis, etc. The presence and relative amount of this tannin or tannin-like body is controlled by the presence of certain enzymes which vary in amount and activity during the growth of these fruits."

Buignet, from the fact of the diminution of tannin and starch which occurs concurrently with the increase in sugar, considered that the sugar in the ripe fruit (e.g. *Musa*) is formed from these two substances. This opinion, however, is not held by Gerber who investigated the same phenomenon. In *Diospyros Kaki* he found the young fruit to be very astringent, but not so the ripe fruit. He considers that the tannins disappear by complete oxidation without the formation of carbohydrates. One reason for his opinion is that in the conversion of tannin into carbohydrate more carbon dioxide would have to be liberated than oxygen absorbed, whereas in fruits the relation is the reverse.

Other suggestions regarding the value of tannin are not wanting; thus Moore † states that it may play an important part in the lignification of cell walls.

Drabble and Nierenstein ‡ have come to the conclusion that tannins play an important part in cork formation, and are acted upon in the plant by formaldehyde and acids and are precipitated in the walls of the cork cells.

Van Wisselingh has published certain observations from which he concludes that tannin plays an important part in the formation of cell walls in certain cases, for instance *Spirogyra*. He does not consider it a reserve food-material as such, but rather a soluble substance which the plant

* Quoted from the footnote appended to a paper on the Toxicity of Tannin by Cook and Taubenhaus: " Delaware Coll. Agric. Exp. Station," Bull. 91, 1911.

† Moore: *loc. cit.*

‡ Drabble and Nierenstein: " Biochem. Journ.," 1906, **2**, 96.

makes use of in elaborating other materials. This conclusion is in agreement with the opinions held by Wiegand and published in 1862. Van Wisselingh worked with *Spirogyra*, and the main facts on which he based his conclusions are as follows: Cells which are about to conjugate are rich in tannin, and as the process of conjugation proceeds, there is a gradual diminution in the amount of this substance, so that the mature zygospore contains nothing more than mere traces. If conjugation be interrupted at an early stage, there is still an increase of tannin, so that when death results there is relatively a large quantity present. This accumulation may be used as an argument in support of the view that tannin is a waste product. Van Wisselingh, however, remarks that this should not be a source of wonder, for in this case "it is not the intention of Nature that it should be wasted. Nature ensures a sufficient supply of tannin in *Spirogyra*, because this substance is required in development, as for instance in conjugation and spore-formation. The occasional failure to conjugate as a result of which much tannin is lost, does not prove that it is a waste product and not a plastic material."

The author in question also found that a diminution of tannin occurred during the formation of the cell wall after nuclear division, and if the tannin were precipitated during the earliest phases of cell division, the cell wall was not formed although the nucleus divided into two quite normally. *Cladophora*, which does not contain tannin, was used as a control; it was found that by keeping the filament in a solution of antipyrine, the reagent used in the experiment on *Spirogyra*, the cell-wall formation was not disturbed.

It must be mentioned that Van Wisselingh does not claim that tannin is the only substance used in cell-wall formation, nor does he maintain that the only physiological significance of tannin is its use as a plastic material.

Finally, in this particular connection, it may be mentioned that tannin may play a part in the formation of various pigments such as anthocyan, for similar decomposition products (compounds allied to the phenols) may be obtained from each.

Schell, while acknowledging that tannin may sometimes be a bye-product of metabolism, considered that at other times it might be used up in the construction of higher compounds which would serve as food. He found that, in the germination of the oil-containing seeds of *Echium vulgare* and other Boraginaceæ, as the oil is used up the tannin begins to play a part in the constructive metabolism and gradually diminishes in amount. Further, if such seeds be germinated in the light the tannin increases in quantity. For these and other reasons he concluded that such a use of tannin only obtained when there was a scarcity of the more normal foods such as starch and oil.

A consideration, however, of other facts does not tend to support the idea of tannin being of the nature of a reserve food. Hillhouse,* for example, found that if a fuchsia having an abundant supply of tannin be grown in the dark, there is no diminution in the substance in question. Then again the facts of its distribution are against this particular view; for example, it does not occur in sieve tubes which transport both sugar and other food substances; there is, in many cases, not a great discrepancy in the tannin-content of fully mature and fallen leaves, for naturally it would be expected that if tannin were of any considerable value as a food-stuff it would not be accumulated in bark and old leaves but would be translocated out of such places before they were cast off, the same as are other materials in the generality of cases. But against this argument may be cited the fact that fallen leaves may contain substances of undoubted value to the plant, such as nitrogen and phosphorus, and even glucose and starch. In evergreen leaves there is no diminution in the quantity of tannin during the winter months, which may mean that either it is of no great value or that, since growth is more or less at a standstill, the plant has more food than it requires immediately, or that it subserves some biological function.

On the other hand, the figures obtained by Levi and Wilmer, mentioned above, require some explanation; why should a minimum of tannin occur in the leaves in June

* Hillhouse: "Midland Naturalist," 1887-8.

when photosynthesis is so very active? Is it used up in the construction of other substances or is it merely translocated to other parts such as the bark? If the latter be true, the further question arises, then why should it be transferred at one time of the year and not at another?

Of course, it is possible that these and like variations may be explained by the varying conditions of, say, light, temperature and moisture; and with regard to this variation in the amount of tannin, more especially in germinating seeds, van Wisselingh points out that the amount found at any particular moment represents the balance as it were of the tannin account; that is to say, if more tannin is formed than is decomposed, an increase in the tannin-content will result and vice versa, so that in one and the same plant there will be sometimes an increase and sometimes a decrease according to the conditions obtaining. It does not necessarily follow, and this is applicable to many things besides tannin, that because there is an increase in the amount, therefore the substance is of no value in constructive metabolism.

A biological significance is not infrequently attached to tannins; thus it may be of use against animals, it may be connected with the activity of nectaries in providing sugar, and it has been suggested by Moore that when it occurs in the epidermis of leaves, it may play a part in the opening and closing of stomata.

Finally, it may be of considerable value as an antiseptic, preventing the germination and growth of parasitic Fungi. In this connection Cook and Taubenhaus * have found that in many cases tannin has a tendency to retard or inhibit the growth of Fungi, the parasitic forms being more sensitive than the saprophytic. In some cases the spores are killed, whilst in others germination is much impeded. On the other hand, low percentages of tannin may in some instances stimulate germination and also fruiting. The behaviour of Fungi towards tannin varies with the species and sometimes even with the individual, more especially in the case of spores.

To conclude, the different substances included under the

* Cook and Taubenhaus: "Delaware Coll. Agric. Exper. Station," Bull. 91, 1911.

term Tannin are so numerous as to make it improbable that they all have the same physiological significance.

THE PHENOLIC CONSTITUENTS OF THE TANNINS.

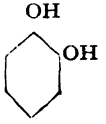
The classification and properties of the tannins will be more easily understood if preceded by a brief description of certain relatively simple phenolic substances from which the complex tannins are built up (p. 291).

The substances include the following :—

1. The dihydric phenols—pyrocatechol, resorcinol and hydroquinone.
2. The dihydroxy acid—protocatechuic acid.
3. The trihydric phenols—pyrogallol and phloroglucinol.
4. The trihydroxy acid—gallic acid.
5. Ellagic acid.
6. Digallic acid.

The above substances occur in varying proportions among the products obtained by subjecting different tannins to fusion with caustic potash or other chemical treatment; and upon their occurrence is based the chemical classification of the tannins.

CATECHOL (SYN. PYROCATECHOL) $C_6H_4(OH)_2$.



Catechol

This substance is so called from the fact that it is obtained by the destructive distillation of catechu, an extract of the bark of *Mimosa Catechu;* it is also obtained by the fusion with potash of other tanno-resins such as kino, the sap of various species of *Pterocarpus*, *Butea* or *Eucalyptus;* also it occurs in small quantities combined with sulphuric acid in the urine of horses and of human beings. It crystallizes from benzene in colourless glistening plates and melts at 140°.

Reactions.

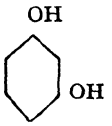
1. Catechol is precipitated from aqueous solution by lead acetate. (Distinction from resorcin and hydroquinone.)

2. With ferric chloride it gives a green colour which is changed to violet on the addition of sodium acetate.

3. Like pyrogallol it reduces silver nitrate in the cold and has therefore been used as a photographic developer.

4. It reduces Fehling's solution on warming.

RESORCINOL. $C_6H_4(OH)_2$.



Resorcinol

This is isomeric with catechol; it does not generally occur in tannins * but in certain resins, notably galbanum resin and asafœtida.

It is used commercially in the manufacture of dye-stuffs, and when heated with sodium nitrite gives the indicator known as Lacmoid.

Resorcinol crystallizes from benzene in colourless needles and melts at 119°; it is somewhat soluble in water, the solution having a sweetish taste.

Reactions.

1. It is not precipitated from solution by lead acetate.

2. With ferric chloride it gives a dark violet colour which is destroyed by the addition of sodium acetate.

3. It reduces ammoniacal silver nitrate or Fehling's solution on warming.

HYDROQUINONE. $C_6H_4(OH)_2$.



Hydroquinone

This third isomer of the formula $C_6H_4(OH)_2$ likewise is not found in tannins, but occurs combined with glucose in the glucoside arbutin and uncombined in the leaves and flowers of

* According to Nierenstein, it is produced together with protocatechuic acid and phloroglucinol from quebracho tannin by potash fusion.

Vaccinium Vitis Idæa. Hydroquinone crystallizes from water in colourless prisms and melts at 169-170°.

Reactions.

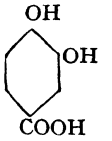
1. It gives no precipitate with lead acetate.

2. Ferric chloride gives no colour but oxidizes it to quinone.

3. It reduces ammoniacal silver nitrate and Fehling's solution.

4. It turns brown in alkaline solution when exposed to the air; its powerful reducing properties enable it to be used in photography as a developer.

PROTOCATECHUIC ACID.



Protocatechuic acid

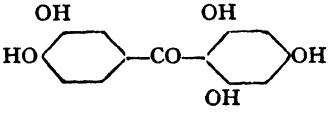
Protocatechuic acid is closely related to pyrocatechol, differing from this substance only by one atom of carbon and two of oxygen which it loses when heated above its melting-point (199°), thus:—

$$\underset{\text{Protocatechuic acid}}{C_6H_3(OH)_2COOH} = \underset{\text{Pyrocatechol}}{C_6H_4(OH)_2} + CO_2$$

It rarely occurs uncombined except, for example, in the fruits of *Illicium religiosum;* in combination, it is found in such substances as Catechin and Maclurin,* both of which give protocatechuic acid on potash fusion; it may further be obtained by a similar process from many resins such as gum benzoin, asafœtida, myrrh, and also from kino.

Finally its dimethyl ether, known as veratric acid,

* Maclurin, formerly called moritannic acid, is a colouring matter of fustic; its constitution is represented by the formula



$C_6H_3(OCH_3)_2COOH$, occurs together with the alkaloid veratrine in the seeds of *Veratrum sabadilla.*

Protocatechuic acid is soluble in water and melts at 199°.

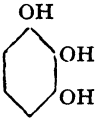
Reactions.

1. Aqueous solutions of protocatechuic acid are precipitated by lead acetate.

2. It gives a green colour with ferric chloride; on addition of very dilute sodium carbonate the green colour changes first to blue and then to red.

3. Ferrous salts produce with protocatechuic acid a violet colour.

PYROGALLOL OR PYROGALLIC ACID. $C_6H_3(OH)_3$.



Pyrogallol

This substance is so called because it is formed by heating gallic acid according to the reaction—

$$\underset{\text{Gallic acid}}{C_6H_2(OH)_3COOH} = \underset{\text{Pyrogallol}}{C_6H_3(OH)_3} + CO_2$$

It is also formed by fusing hæmatoxylin with caustic potash.

Pyrogallol crystallizes in colourless needles or plates melting at 132° and is soluble in water; its solution, in excess of caustic alkali, absorbs oxygen with great avidity, turning brown and forming carbon dioxide, acetic acid and other substances.

Pyrogallol reduces salts of silver, mercury, or gold to their respective metals.

Reactions.

1. Pyrogallol is precipitated from solution by lead acetate but not by lead nitrate.

2. It gives a blue colour with ferrous sulphate and a red colour with ferric chloride.

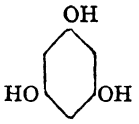
3. Aqueous or alcoholic solutions of pyrogallol, in common with those of gallic acid or tannic acid, are coloured purple by iodine.

4. Lime water added to an aqueous solution of pyrogallol produces a purple colour which rapidly becomes brown.

5. Solutions of pyrogallol give no precipitate with gelatine.

6. Potassium cyanide gives a reddish-brown coloration, which turns brown, but the red tint becomes apparent again on shaking.

PHLOROGLUCINOL. $C_6H_3(OH)_3$.



Phloroglucinol

Phloroglucinol, which is isomeric with pyrogallol, is produced by fusing a number of substances, such as catechin, kino, dragon's blood, anthocyanins, etc., with potash, and likewise from a number of glucosides, such as phloretin, phloridzin, hesperidin, etc. It crystallizes with 2 molecules of water, but loses them if heated to 100°, and melts at 218°; it dissolves readily in water, forming a sweet solution, and is readily soluble in alcohol or ether.

Reactions.

1. Phloroglucinol is precipitated from solution by lead acetate.

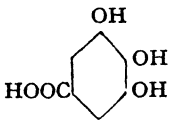
2. It gives with ferric chloride a bluish-violet colour.

3. A solution of phloroglucinol in hydrochloric acid produces a red colour on a pine wood shaving; this reaction can also be made use of for detecting lignified cell walls (p. 229).

4. It is a reducing agent, and reduces Fehling's solution.

In addition to the above-mentioned phenols, which are products of the decomposition of tannins by heat or by fusion with alkalis, there are other important substances produced by acid hydrolysis, namely, gallic and ellagic acids and the phlobaphenes.

GALLIC ACID. $C_6H_2(OH)_3COOH$



Gallic acid

Gallic acid was first prepared by Scheele in 1786 by leaving an aqueous extract of gall nuts which contain tannin to stand in a warm place, and from time to time removing the layer of mould which formed on it; the crystalline precipitate which deposited from the solution was purified by recrystallization from water.

Within recent years this change has been studied anew by Fernbach,* who isolated a tannin splitting enzyme, tannase, from *Penicillium*, and also by Pottevin,† who isolated a similar enzyme from the mould *Aspergillus*.

This change, which may be represented by the equation—

$$C_{14}H_{10}O_9 + H_2O = 2C_7H_6O_5$$

may be effected more rapidly by boiling gallotannic acid with dilute sulphuric acid.

Gallic acid, besides occurring in gall nuts, both free and in the form of its anhydride gallo-tannic acid, is also found free in sumach, divi-divi, the fruits of *Cæsalpinia coriaria*, in the leaves of *Arctostaphylos Uva-ursi*, and in tea and wine.

Gallic acid crystallizes in silken needles, and melts at 220°, forming pyrogallol and evolving carbon dioxide; it is sparingly soluble in cold water, but dissolves readily in hot water and in alkalis; alkaline solutions, like those of pyrogallol, absorb oxygen from the air, becoming brown in colour; they also reduce metallic solutions of silver or gold and Fehling's solution.

Reactions.

1. Gallic acid is precipitated from solution by lead acetate; on adding caustic potash a carmine-coloured precipitate is formed, which dissolves in excess to a raspberry-red solution.

* Fernbach: "Compt. rend.," 1900, 131, 1214.
† Pottevin: *id.*, 1900, 131, 1215.

2. Ferric chloride produces a blue-black colour or precipitate according to the strength of the solution; excess of ferric salt changes the colour to green, while excess of gallic acid reduces the ferric salt to ferrous and destroys the colour.

3. Iodine solution produces a transient red colour.

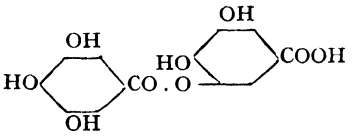
4. Gallic acid does not precipitate gelatine from solution. (Distinction from tannic acid.)

5. When heated with concentrated sulphuric acid it turns green and then purple, being converted into rufigallic acid, $C_{14}H_8O_8$, a substance used in dyeing.

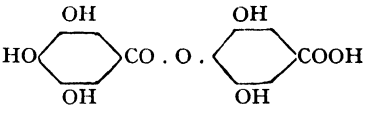
6. Potassium cyanide gives a pink colour which disappears on standing, but returns again on shaking with air.

7. Lime water gives a blue coloration or precipitate; in very dilute solutions a reddish colour is produced.

GALLOYL-GALLIC ACID OR DIGALLIC ACID. $C_{14}H_{10}O_9$.



m-Digallic acid



p-Digallic acid

As may be seen from the above formulæ two isomeric digallic acids are possible and both have been synthesized by Fischer, Bergmann, and Lipschitz * in the course of their researches on the synthesis of depsides.

A long time previous to this it had been known that gallic acid could be converted into its anhydride digallic acid by heating with phosphorus oxychloride to 130° or by boiling with arsenic acid :—

$$\underset{\text{Gallic acid}}{2C_6H_2(OH)_3COOH} = \underset{\text{Digallic acid}}{C_{14}H_{10}O_9} + H_2O$$

This digallic acid precipitates gelatine from solution, and for this reason it was regarded by Schiff † as being identical with natural gallotannic acid. This view was first shown by

* Fischer, Bergmann, and Lipschitz: "Ber. deut. chem. Gesells.," 1918, **51**, 45.

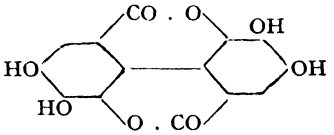
† Schiff: *id.*, 1871, **4**, 232, 967; 1879, **12**, 33; "Annalen," 1873, **170**, 143.

Walden * to be untenable, since the physical properties of the two substances are quite different, and the position was subsequently cleared up by Fischer who showed that the natural gallotannic acid occurring in oak galls was actually a pentadigalloyl ester of glucose (see p. 291).

ELLAGIC ACID. $C_{14}H_6O_8$.

Closely related to gallic acid is the substance known as Ellagic acid, its name being obtained by the inversion of the word gallic.

Its constitution is, according to Graebe, best represented by the formula—



from which it will be seen that it may be considered to be produced by the abstraction of two molecules of water from two molecules of gallic acid, with simultaneous oxidation or removal of two atoms of hydrogen.

Synthetically it may be prepared by the oxidation of gallic acid by means of arsenic acid, or better by oxidizing gallic acid in acetic acid solution with potassium persulphate and sulphuric acid.†

Whether or not this substance occurs free in nature is not definitely established; certain it is, however, that ellagic acid can be readily obtained by the hydrolysis of ellagitannic acid,‡ a substance which almost invariably accompanies gallotannic acid in the numerous vegetable products in which this latter occurs; it also occurs in conjunction with tannins of the pyrogallol class, and constitutes the bloom which is produced on leather by this type of tannin.

The most convenient natural sources are "divi-divi" (*Cæsalpinia coriaria*), "algarobilla" (*Cæsalpinia brevifolia*), "myrobalans" (*Terminalia Chebula*), etc. Aqueous extracts

* Walden: "Ber. deut. chem. Gesells.," 1897, **30**, 3153; 1898, **31**, 3167.

† Perkin and Nierenstein: "J. Chem. Soc.," 1905, **87**, 1415.

‡ Sisley: "Bull. Soc. Chim.," 1909, [4], **5**, 727.

of these fruits on long standing frequently deposit ellagic acid, most probably by the action of a ferment contained in the plant; it is, however, prepared by pouring a hot concentrated alcoholic extract of divi-divi into cold water; the acid is thereby precipitated, and may be filtered, and purified.

Properties and Reactions.

Ellagic acid is a yellow microcrystalline solid which is very slightly soluble in water, and therefore readily separates from aqueous solutions in which it is formed; it is also very slightly soluble in alcohol or ether, but dissolves somewhat readily in boiling pyridine.

The dried substance treated with 1-2 drops of nitric acid gives, on dilution with 10-20 drops of water, a blood-red colour (Griessmayer's reaction).

Catellagic, Metellagic, and Flavellagic acids are the names given by Perkin to artificially synthesized acids obtained by him. They are closely related to ellagic acid, but have not, as yet, been found to occur naturally.

THE CLASSIFICATION OF TANNINS.

With the present incomplete state of our knowledge concerning the chemical constitution of the tannins, it is difficult to make a proper chemical classification of these substances.

While a number of different classifications have been suggested * the one due to Procter † is perhaps the most generally useful, but it must be understood that it is not a rigid classification since some tannins are known which possess certain of the characteristics of each of the two groups into which he divides tannins.

Procter's classification is based upon the fact that tannins when heated to 180-200° C. yield as a general rule either pyrogallol or catechol, for which reason he has adopted the following classification :—

(A) *Pyrogallol tannins*, including oak gall tannin, oak wood

* Trimble: "The Tannins," Philadelphia, 1894, Vol. II., p. 132. Dekker: "De Looistoffen," Amsterdam, 1906.

† Procter: "The Principles of Leather Manufacture," London, 1903.

and chestnut wood tannin and sumach, divi-divi, myrobolans, valonia, and algarobilla.

These tannins have the following characteristics :—

1. They give with ferric salts a blue-black coloration.

2. They give no precipitate with bromine water.

3. They produce on leather a "bloom" consisting of ellagic acid.

(B) *Catechol tannins*, including all the pine barks, acacias, mimosas, oak barks (but not oak wood, fruits or galls), quebracho wood, cassia and mangrove barks, canaigre, cutch, and gambier.

The tannins of this class are characterized by the following properties :—

1. They give with iron alum a greenish-black colour, though the reaction is liable to be rendered uncertain by the presence of other colouring matters.

2. When treated with bromine water, until the solution smells strongly of it, they give a yellowish or brown precipitate ; in weak solutions the precipitate may form slowly.

3. The addition of concentrated sulphuric acid to a drop of the infusion produces a dark red or crimson ring at the junction of the two liquids ; on dilution the liquid turns pink.

4. These tannins deposit no "bloom," but when boiled with acids deposit red insoluble colouring matters known as phlobaphenes (see p. 297).

5. Some of the tannins belonging to the pyrocatechol group, notably gambier and cutch, contain phloroglucinol as one of their constituents ; this substance may be tested for by moistening a pine wood shaving with a little of the infusion and then adding a little concentrated hydrochloric acid ; the formation, after a short time, of a bright red or purple stain indicates the presence of phloroglucinol. (This is an adaptation of the so-called lignin reaction.)

Procter's classification should not be regarded as absolutely rigid, but it receives some support from the reaction of Stiasny * according to which pyrocatechol tannins are

* Stiasny: "Der Gerber," 1905, 186.

completely precipitated when these solutions are boiled with formaldehyde and hydrochloric acid, whereas pyrogallol tannins are only incompletely precipitated, if at all.

To carry out this test 50 c.c. of the tannin solution (0·5 per cent) are boiled for half an hour under a reflux condenser with 25 c.c. of a mixture of 100 c.c. of concentrated hydrochloric acid (diluted with an equal volume of water) and mixed with 150 c.c. of 40 per cent formaldehyde; 10 c.c. of filtrate from the above, mixed with 10 drops of 1 per cent iron alum and 1 gram of solid sodium acetate, should give no colour with a pyrocatechol tannin, whereas a blue or violet colour results in the case of a pyrogallol tannin.

PROPERTIES AND DESCRIPTION OF INDIVIDUAL TANNINS.

As already stated, the term Tannin is merely a generic name for the whole group of substances, though it has been, and still is, frequently used to mean a particular tannin, namely that contained in oak galls. This substance is, however, better named gallotannic acid, as it is customary to name the tannins after the source from which they are obtained; thus quercitannic acid indicates the tannin of oak bark, sumac-tannin that derived from sumac, and so on.

PYROGALLOL TANNINS.

Owing to lack of space it is only proposed to describe two tannins belonging to this group, namely gallotannic and ellagitannic acid.

GALLOTANNIC ACID. $C_{76}H_{52}O_{46}$.

(Syn. Tannic acid, or merely "Tannin.")

This substance, as its name implies, is the tannin contained in galls, and it is important to remember that oak gall tannin is entirely distinct from either oak wood or oak bark tannin, the latter of which is a pyrocatechol tannin.

The two chief commercial sources of gallotannic acid are—

1. Turkish or Aleppo galls, produced by the gall wasp *Cynips gallæ*, which lays its eggs in the buds of *Quercus infectoria*. These contain from 50-60 per cent of gallotannic acid.

2. Chinese galls, produced by the burrowing of *Aphis chinensis* in the leaf-stalks of young twigs of *Rhus semialata*. These galls may contain up to 70 per cent of gallotannic acid.

Gallotannic also occurs in sumach (*Rhus Coriaria*), in tea, and in many other plants.

Extraction of Gallotannic Acid.

Gallotannic acid is best prepared by extracting finely-powdered gall nuts with a mixture of 12 parts of ether with 3 parts of alcohol; 12 parts of water are then added and, after shaking, the lower aqueous layer is run off from below and evaporated. The resulting tannic acid may be decolorized by boiling with animal charcoal.

Pelouze recommends the following method: The powdered material is heated under a reflux condenser with a mixture of 30 parts of ether, 5 parts of water, and 2 parts of alcohol. On cooling three layers of liquid are formed, of which the lowest contains 33 per cent, the middle 8 per cent, and the top 2 per cent of the tannic acid present in the substance.

Gallotannic acid forms an amorphous powder * which, when pure, is almost colourless; it is readily soluble in water, forming a solution with an astringent taste and which reacts acid to litmus; it dissolves also in alcohol or glycerol, but is only sparingly soluble in ether and is insoluble in chloroform, benzene, ligroin or carbon disulphide; it is also insoluble in hydrochloric or sulphuric acids and is precipitated by these substances from its aqueous solutions; it is soluble in alkalis, and the solution, as in the case of gallic acid or of pyrogallol, rapidly absorbs oxygen from the air and darkens in colour.

When boiled with 2 per cent hydrochloric acid for some time, gallotannic acid is broken up into gallic acid.

If heated slowly from 160 to 215° and kept at the higher temperature for thirty minutes, carbon dioxide, water, pyrogallol and metagallic acid are produced. The pyrogallol volatilizes and condenses in the cooler part of the vessel.

*** What is known as "Crystal tannin" in commerce is not really crystalline; it is made by drawing a syrupy solution into threads and breaking these up after drying.**

The action of heat on tannins may also be studied by dissolving 1 gram of tannin in 5 c.c. of glycerol, heating slowly to 210° and maintaining the liquid at this temperature for half an hour. The liquid is then cooled and shaken with 20 c.c. of ether; after the addition of water the ethereal solution is separated and evaporated; the residue contains pyrogallol.

Reactions.

1. Ferrous sulphate, free from ferric salts, produces at first no change, but on exposure to air the solution darkens from oxidation.

2. Ferric chloride, or better, iron alum, produces a blue-black colour or precipitate.

3. A dilute solution of iodine in potassium iodide, added to a faintly alkaline solution, gives a transient pink colour, as in the case of gallic acid.

4. Gallotannic acid is precipitated from solution by gelatine, and similarly combines with hide powder converting it into leather. (Distinction from gallic acid.)

5. Gallotannic acid precipitates proteins, alkaloids, and many other organic substances from solution.

6. Lead nitrate or lead acetate gives precipitates of lead tannate. (Neither pyrogallol nor gallic acid is precipitated by lead nitrate, though both give precipitates with lead acetate.)

7. Potassium cyanide gives a reddish-brown colour which changes to brown, but the red tint reappears on shaking with air.

8. Lime water gives a grey precipitate.

Detection of Gallic Acid in Presence of Gallotannic Acid.

Gallic acid may be detected in the presence of gallotannic acid by dissolving the mixture in water and extracting the solution with ether; the ethereal extract on evaporation yields gallic acid which may be identified by the usual tests.

Gallotannic acid may also be separated from gallic acid by adding a solution of lead acetate strongly acidified with acetic acid; under these circumstances lead tannate is precipitated while lead gallate remains dissolved.

Similarly gallotannic acid is precipitated by many alkaloids and basic substances which have no action on gallic acid.

THE CONSTITUTION AND SYNTHESIS OF NATURAL GALLOTANNIC ACID. $C_{76}H_{52}O_{46}$.

The close relationship subsisting between gallotannic and gallic acids was first observed by Scheele, who, by allowing an infusion of gall nuts to undergo fermentation, obtained gallic acid.

When, therefore, it was found by Schiff * that gallic acid could be converted back into the anhydride by means of phosphorus oxychloride it was assumed that this substance, which was called digallic acid, was identical with natural gallotannic acid or " tannin."

This view came to be generally accepted, although objections were raised from time to time on the ground that the physical constants, such as electrical conductivity and optical activity of natural tannin and synthetic digallic acid were different.†

Until 1912 there was considerable uncertainty as to whether tannin occurred in the plant combined with glucose in the form of a glucoside, or whether the sugar which is frequently found associated with it was merely an impurity.‡

E. Fischer and Freudenberg, § on reinvestigating the question, found that gallotannic acid obtained from Chinese galls, even after repeated careful purification, yielded about 7-8 per cent of glucose on hydrolysis with sulphuric acid; from this it was concluded that " tannin " or gallotannic acid as it occurs in nature is not identical with synthetic digallic acid, since the natural product contained glucose as an essential constituent.

The rôle played by the glucose as a constituent of natural gallotannic acid of Chinese oak galls was finally established when Fischer and Freudenberg synthesized a pentadigalloyl glucose which from all appearances was identical with the natural gallotannic acid.

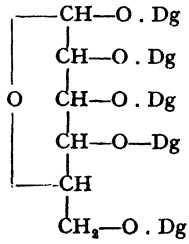
* Schiff : " Ber. deut. chem. Gesells.," 1871, **4**, 232.

† Walden : *id.*, 1897, **30**, 3151 ; 1898, **31**, 3167.

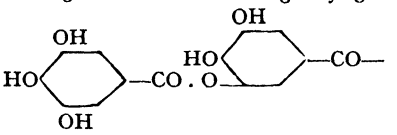
‡ Cf. Strecker : " Annalen," 1852, **81**, 248 ; 1854, **90**, 328 ; Pottevin : " Compt. rend.," 1901, **132**, 704.

§ Fischer and Freudenberg : " Ber. deut. chem. Gesells.," 1912, **45**, 915 and 2709.

The constitution of this substance is represented by the formula—



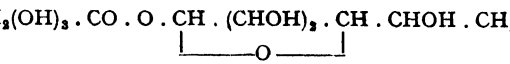
in which Dg stands for the *m*-di-galloyl group



from which it will be seen that it is composed of a molecule of glucose in which each of the five hydroxl groups have been esterified by a molecule of digallic acid.

Actually two isomeric substances of this formula, $C_{76}H_{52}O_{46}$, with a molecular weight of 1700, were synthesized.* The one derived from meta-digallic acid,† i.e. penta- (*m*-di-galloyl) β-glucose, has been found to be practically identical with Chinese tannin, and to differ from it only in regard to its specific rotation; this difference is, however, of no great significance considering the colloidal nature of the substance concerned.

Although not connected with the constitution of gallotannic acid it is of interest to mention in connection with the above synthesis that Fischer, Bergmann, and Lipschitz have also synthesized a galloyl glucose of the formula—



which is identical with glucogallin, a substance first isolated from Chinese rhubarb by Gilson.‡

ELLAGITANNIC ACID.

This tannin, which is commonly found together with gallotannic acid, is important as being the mother substance of ellagic acid, which is responsible for the bloom characteristic

* Fischer, Bergmann, and Lipschitz: "Ber. deut. chem. Gesells.," 1918, **51**, 45.

† For formula, see p. 284.

‡ Gilson: "Compt. rend.," **136**, 385. For summary of work on synthesis of tannins, see Fischer: "Ber. deut. chem. Gesells.," 1919, **52**, 13, 809.

of pyrogallol tannins. The quantity of this substance present in different plants varies considerably; it is greatest in divi-divi. Amongst the other tannins giving ellagic acid bloom may be mentioned algarobilla, myrobalans, chestnut tannin, pomegranate tannin, valonia, etc.

Ellagitannic acid, unlike ellagic acid (p. 285), is soluble in water or alcohol; prolonged boiling with water converts it into ellagic acid. It has been variously described by different authors as a glucoside, as a hydrated soluble form of ellagic acid, or as a condensation product of ellagic acid with gallic acid.*

TANNINS AS GLUCOSIDES.

Although many of the tannins are substances of a glucosidic nature and occur in the plant in combination with a carbohydrate complex such as glucose (e.g. gallotannic acid, p. 288) this has not as yet been established in all cases.

To determine whether a tannin is a glucoside or not the following procedure is recommended by Procter.†

The tannin must first be carefully purified from glucose, gums, or other bodies likely to interfere. This may be done by extracting according to Pelouze's method (p. 289), or, if the tannin is to be extracted from an aqueous solution, by agitating with ether to remove gallic acid and then saturating the aqueous solution with common salt and shaking with ethyl acetate, which extracts the tannin. The ethyl acetate is then evaporated off, the last traces being expelled by the repeated addition of small quantities of ether.

Another method is to extract with alcohol and to evaporate off the alcohol at as low a temperature as possible, and then to take up the residue with a large volume of water whereby the phlobaphenes (see p. 297) are precipitated and may be filtered off. The infusion is then precipitated with successive small quantities of lead acetate; the first and last portions are rejected and the middle fraction after washing is

* Cf. Nierenstein: "Ber. deut. chem. Gesells.," 1907, 40, 4575; 1909, 42, 353; 1910, 43, 1257.

† Procter: "Leather Industries Laboratory Book," London, 2nd ed., 1908.

suspended in water and saturated with sulphuretted hydrogen. The precipitated lead sulphide is filtered off, and the solution is warmed to drive off excess of gas and then extracted with ethyl acetate.

Thus purified the tannin, or its washed lead salt, is heated to 100° for an hour or more in a sealed tube or boiled in a flask under a reflux condenser with hydrochloric acid (2 per cent). After cooling the mixture is allowed to stand for some time and is then filtered from any deposit which may have formed. The filtrate is shaken with ether to remove gallic acid and the aqueous solution boiled, neutralized with caustic soda and precipitated with basic lead acetate to remove any unchanged tannin or colouring matter; the solution is again filtered and any lead remaining in solution is removed by the addition of dilute sulphuric acid, excess of acid being carefully avoided. The solution is then neutralized and once more filtered and the clear filtrate heated to boiling with Fehling's solution when a red precipitate proves the presence of glucose.

CATECHOL TANNINS.

As stated above the catechol tannins are distinguished from the pyrogallol tannins by their colour reaction with iron salts and more especially by their property of giving rise to red coloured substances or phlobaphenes on boiling with dilute mineral acids.

The catechol tannins comprise the various products known commercially as cutch or catechu as well as the tannins obtained from quebracho, mangrove, oak, birch, pine, larch, and fir barks and also canaigre, but only a few of these can be described.

CUTCH OR CATECHU.

The term cutch is applied to a number of somewhat similar products obtained by evaporating down aqueous extracts of various woods such as acacia, mangrove, mahogany etc.; the commercial products differ slightly in their composition according to their source of origin.

1. Gambier catechu obtained from the leaves and young

twigs of *Uncaria gambier* contains as its chief constituents catechin and catechu tannic acid.

2. Acacia catechu derived from the heart-wood of *Acacia catechu* contains, according to Perkin, a substance acacatechin which is isomeric with the catechin occurring in gambier, and presumably catechu tannic acid.

3. Areca catechu, obtained by extracting the fruits of *Areca catechu*, the Betel nut palm, contains a substance resembling catechu tannic acid, but there is some question as to its containing a catechin.

It will be seen from the above that the composition of the above products is somewhat similar; they all contain catechu tannic acid as the active tanning agent and also catechins which are not of themselves tanning materials, but may apparently be converted into such by the process of extraction and evaporation.

The work of Perkin, of Kostanecki, and of Freudenberg has shown that at least two * catechins exist, namely catechin (*b*) contained in gambier and acacatechin, or catechin (*a*), from *Acacia catechu;* these two substances would appear to be stereo-isomers, the former being dextro-rotatory while the latter is lævo-rotatory.

Gambier catechin (*b*) may be prepared by extracting powdered catechu with ether; the crude material obtained on evaporating off the ether may be purified by crystallization from water.

Catechin forms colourless glistening needles, which, when dry, melt at 175-177°. It is readily soluble in alcohol and ethyl acetate, not so readily soluble in ether, and only slightly soluble in cold water.

With ferric chloride alone it gives a green colour, but with ferric chloride and sodium acetate a dark violet.

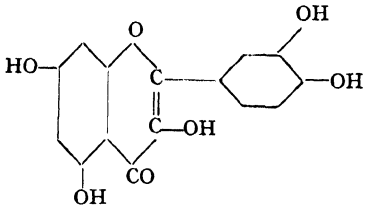
It gives the phloroglucin reaction with pine wood shaving and hydrochloric acid.

Potash fusion gives protocatechuic acid and phloroglucinol.

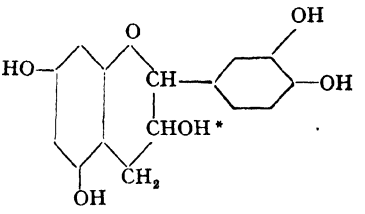
* A third *Catechin*, (*c*), melting at 235-237°, is contained in the mother liquors of the above Gambier catechin (Perkin: "J. Chem. Soc.," 1905, 87, 398).

Acacatechin (*a*) is the catechin derived from acacia catechu; it melts at 203-205° and is regarded by Perkin as being isomeric with gambier catechin.

It has been suggested * that the two catechins, *a* and *b*, are reduction products of quercetin, as may be seen from the formulæ given below and that the isomerism between the two catechins is dependent upon the position of the hydroxyl group marked with a star.



Quercetin, $C_{15}H_{10}O_7$



Catechin, $C_{15}H_{14}O_6$

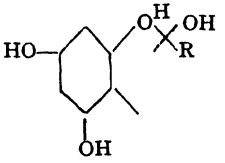
All the above catechins when boiled with dilute mineral acids readily yield red substances of a phlobaphene character.

Catechu tannic acid is the name given to the chief tanning constituents of cutch.

Little is known as to its constitution,† but it is believed to be an anhydride of catechin.

THE CONSTITUTION OF THE CATECHU TANNINS.

The catechu tannins as may be seen from the formula given above for catechin contain a potential phloroglucinol grouping—



and in fact on hydrolysis with dilute acid they yield protocatechuic acid and phloroglucinol which latter presumably gives rise by oxidation to the red phlobaphenes (see p. 298) which are ultimately obtained. The pyrogallol tannins, on

* Perkin: "J. Chem. Soc.," 1905, 87, 398.

† For a theoretical discussion of the chemistry of this substance, see v. Euler: "Chem. Zentr.," 1921, 731.

the other hand, under similar circumstances give rise to ellagic acid.

OAK-BARK TANNIN OR QUERCITANNIC ACID.

Besides the undoubted pyrogallol tannin of oak galls which is practically never used for tanning, the oak is the source of two other tannins which have in the past been used very extensively for tanning—these are oak-wood and oak-bark tannins. The former of these, which is sometimes described as quercic or quercinic acid, is probably also a pyrogallol tannin, since it gives a blue colour with ferric chloride and is not precipitated by bromine water; the tannin of oak bark, known as quercitannic acid, is, however, a catechol tannin, and is quite distinct from the tannin of oak galls; it differs from the latter in giving with iron salts a green colour instead of a bluish-black, and moreover on hydrolysis it yields no glucose.

Although much work has been done on the oak-bark tannins by various workers,* notably Etti, Böttinger, and Löwe, nothing definite is known as yet regarding their constitution.

Procter summarizes the present state of our knowledge by saying that, on the whole, it seems probable that the principal tannin of oak bark is a purely catechol tannin, and that the gallic and ellagic acids which have been detected in it are due to an admixture of the gallotannic and ellagitannic acids present in oak wood.

A great many more catechol tannins are known, but too little is known about their composition to justify their inclusion here.

PHLOBAPHENES.

Among the products of the decomposition of catechol tannins by boiling with acids must be mentioned the substances known as Phlobaphenes. The name derived from the Greek (*φλοιός*—bark, and *βαφή*—dyeing) was first given by

* Etti: "Monatshefte," 1880, **1**, 265. Böttinger: "Ber. deut. chem. Gesells.," 1883, **16**, 2712. Löwe: "Monatshefte," 1883, **4**, 515. Freudenberg and Vollbrecht: "Annalen," 1922, **429**, 284.

Stahelen and Hoffstetter,* in 1844, to a red-brown substance obtained by them by adding water to an alcoholic extract of bark which had previously been extracted with ether to remove fats or waxes. It has since been shown that aqueous extracts of oak bark, deposit from solution a substance known as oak-red or phlobaphene, and that this substance is more rapidly produced by warming concentrated solutions of tannin with sulphuric acid.

Inasmuch as phlobaphenes are produced by any process which tends to remove water, such as heating tannins to a high temperature or prolonged boiling or heating under pressure, they are regarded as anhydrides of the tannins; besides being thus produced artificially, they occur also in nature side by side with the tannins from which they can be produced.

They are red-coloured substances and are practically insoluble in water though they dissolve in solutions containing tannic acid; also they dissolve in alcohol and in alkaline solutions.

Practically nothing is known concerning the chemistry of the phlobaphenes. The term is not confined only to the products artificially produced by acid hydrolysis and oxidation, but is also applied by Freudenberg to the naturally occurring coloured decomposition products of the tannins found in the plant.

As stated above the formation of phlobaphenes by treatment of a tannin with acid is characteristic of pyrocatechol tannins (p. 288) in just the same way as ellagic acid is produced from pyrogallol tannins.

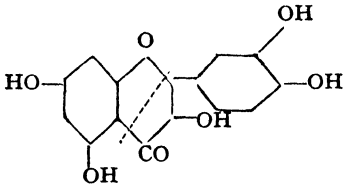
A number of different phlobaphenes are known, such as kino-red, catechu-red, oak-bark red, etc.

RELATIONSHIP BETWEEN CATECHOL TANNINS AND FLAVONOLS, ETC.

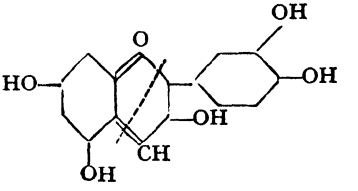
Freudenberg has drawn attention to the close relationship existing between the catechol tannins and the plant pigments belonging to the group of anthoxanthins and anthocyanins. It will be remembered that the potash fusion of anthocyanins

* Stahelen and Hoffstetter: "Annalen," 1844, **51**, 63.

yielded amongst other substances both pyrogallol and catechol, the same compounds as are obtained under similar circumstances from the catechol tannins. By examining the structural formula of either the flavonol quercetin (I.) or of the corresponding anthocyanidin cyanidin (II.), it will be seen that the formation of phloroglucinol and catechol is readily accounted for by rupture of the molecules along the dotted line :—

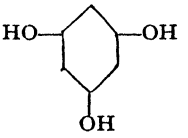


I.

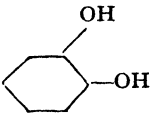


II.

whereby the left half of the molecule would give phloroglucin (III). and the left half would account for the catechol (IV.) :—

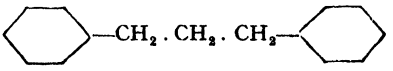


III.



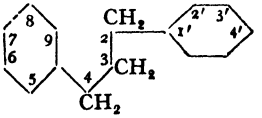
IV.

According to the same author the mother substance of all these compounds is diphenyl propane (V.) :—



V.

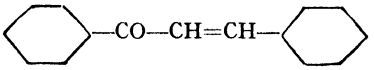
which may also be written as under :—



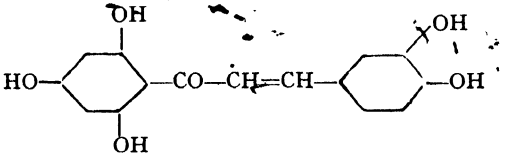
and it will readily be understood how by introduction of hydroxyl groups into positions 3′, 4′, 3, 5, 7, and 9 and slight rearrangement, it should be possible to pass without much difficulty from one compound to another.

Other substances of significance in the plant world are

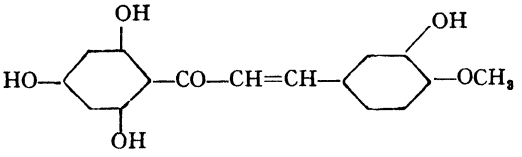
also related to the above parent substance triphenylpropane, or more closely to Chalkone, namely eriodictyol, hesperetin and phloretin :—



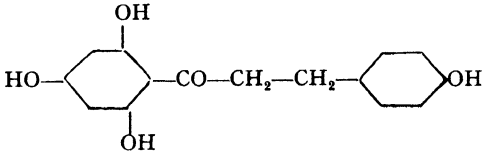
Chalkone



Eriodictyol, $C_{15}H_{12}O_6$

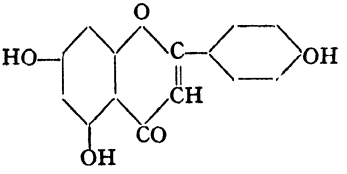


Hesperetin



Phloretin

Phloretin, which combined with glucose, forms the glucoside phloridzin ; the close relationship between this substance and the flavonol apigenin is also apparent from the formula given below :—



Apigenin

It is worthy of note that quercetin, $C_{15}H_{10}O_7$, cyanidin, $C_{15}H_{12}O_7$, eriodictyol, $C_{15}H_{12}O_6$, and gambier catechin, $C_{15}H_{14}O_6$, form a series of gradually increasing hydrogen and decreasing oxygen content.

ECONOMIC USES OF TANNINS.

A great variety of tannins are commercially exploited and it is proposed here to mention only a few representative examples classified according to their sources of origin :—

I. Tannins derived from leaves and young twigs :—
Sumach from leaves of *Rhus coriaria*, and
Gambir from leaves of *Uncaria Gambir*.

II. Tannins derived from galls :—
Aleppo or Turkey galls—*Quercus infectoria* and other species
Chinese galls—*Rhus semialata*.

III. Tannins derived from barks :—
Oak-bark tannin from *Quercus sessiliflora*, *Q. pedunculata*, *Q. cerris*.
Tannins from bark of *Salix*, *Betula*, *Picea excelsa*, *Acacia*, *Mimosa*, *Eucalyptus*, *Mangrove*.
Quebracho from bark of *Rhizophora*.
Kino from bark of *Pterocarpus*.

IV. Tannins from wood :—
Chestnut and oak.
Bengal or acacia catechu.

V. Tannins from roots :—
Canaigre from root of *Rumex hymenosepalus*.
Tannins from roots of *Geum rivale* and *Potentilla tormentilla*.

VI. Tannins from fruits :—
Myrobalans from *Terminalia*.
Divi-divi from *Cæsalpinia coriaria*.
Valonia from *Quercus ægilops* and *Q. coccifera*.

These tannins are used for a variety of purposes. The tannins from galls are employed chiefly for the production of inks, only some of them being suitable for leather manufacture.

Oak-bark tannin is particularly adapted for the production of leather.* While oak bark itself is used directly for tanning, oak wood is never so used, only extracts of the wood being

* See Procter : " The Making of Leather," Cambridge, 1914.

employed for this purpose; large quantities of leather are, however, nowadays manufactured with the use of chemicals such as chromium salts, etc. Many of the tannins, such as gambier cutch, quebracho, etc., are employed for tanning, calico printing, and dyeing, etc.

COMPOSITION OF CERTAIN DYE WOODS AND BARKS AND THEIR EXTRACTS.

The wood of a great number of tropical trees yield extracts containing mixtures of substances, some of which have tinctorial properties and others are tannins or allied substances. The substances possessing tinctorial properties are flavones or flavonols and are mostly sparingly coloured substances which only produce dyes, more or less deeply coloured, with salts of metals such as aluminium, iron, tin, etc., acting as mordants. In illustration a few examples, selected from a very large number, are given below:—

Old Fustic.

Old fustic, the wood of *Chlorophora tinctoria* (formerly known as *Morus tinctoria*), a native of Cuba, Jamaica, and Brazil, contains the flavonol morin which, though forming colourless crystals, is soluble in alkali to give a yellow solution, and with chromium copper, iron, tin, or aluminium mordants, gives various shades of olive-brown or yellow.

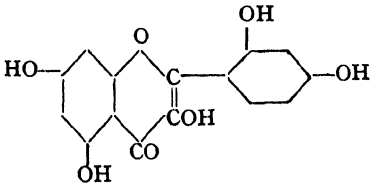
Besides the above, old fustic also contains maclurin, at one time called moritannic acid, which is a penta-hydroxybenzophenone (for formula, see p. 280); this substance precipitates gelatine from solution and thus has tanning properties, and with iron salts it gives a green-black colour.

Jack Wood.

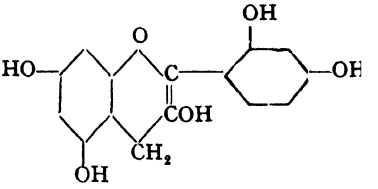
Perkin * found that the Indian dyestuff, jack wood (*Artocarpus integrifolia*) contained in addition to the colouring matter morin (I.), $C_{15}H_{10}O_7$—isomeric with quercetin (see p. 296)—a substance, cyanomaclurin (II.), $C_{15}H_{12}O_6$; this substance is colourless and has no dyeing properties but gives a violet colour

* Perkin: "J. Chem. Soc.," 1895, **67**, 939, and 1905, **87**, 715.

with ferric chloride, and when warmed with alkali gives a deep indigo blue which changes through green to yellow; this latter reaction is responsible for its name—cyanomaclurin—but as may be seen from the formula given below it is a more complex substance than maclurin (for formula see p. 280) and is in fact regarded by Perkin * as a reduction product of morin :—



I. Morin, $C_{15}H_{10}O_7$



II. Cyanomaclurin, $C_{15}H_{12}O_6$

This relationship would thus be just the same as that between quercetin and catechin which occur together in catechu (see p. 294).

Quercitron Bark.

This is the bark of *Quercus discolor* or *Q. tinctoria*, which grows in the United States of America; it contains a glucoside quercetrin which may be extracted by dilute ammonia solution after a preliminary boiling with a fairly strong salt solution to remove gummy impurities; on adding acid a flocculent precipitate is formed which is removed and the filtrate on further boiling is hydrolysed, yielding the free quercetin which crystallizes in colourless needles. Quercetin is soluble in alkali to give a yellow solution and gives various shades of yellow and brown with chromium, aluminium, tin, and iron mordants; similar shades are given by the glucoside itself.

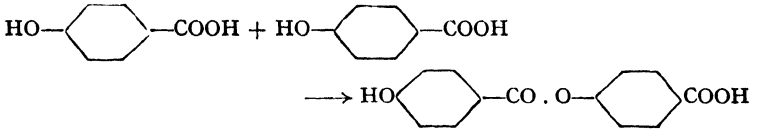
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Freudenberg: "Die Chemie der natürlichen Gerbstoffe," Berlin, 1920.
Gnamm: "Die Gerbstoffe und Gerbmittel," Stuttgart, 1925.
Perkin and Everest: "The Natural Organic Colouring Matters," London, 1918.

* Perkin: "J. Chem. Soc.," 1905, **87**, 715.

DEPSIDES.

The term depside (derived from the Greek word δεψειν = to tan) was suggested by Fischer to designate chain compounds analogous to the peptides produced by linking together the carboxyl group of one phenolic acid with the hydroxyl group of a similar one such as—

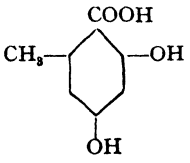


According to the number of constituent groups so linked together, the resulting products were termed di-, tri-, etc., depsides. Digallic acid according to this nomenclature would be a didepside. Although digallic acid precipitates gelatine from solution and thus justifies the term depside, many other depsides are known which do not possess this property.

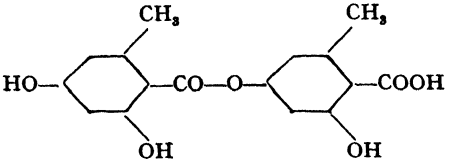
Naturally occurring depsides are found chiefly among the lichens where they are represented by the lichen acids, lecanoric and evernic acids.

Lecanoric Acid.

This substance is a didepside of orsellinic acid—



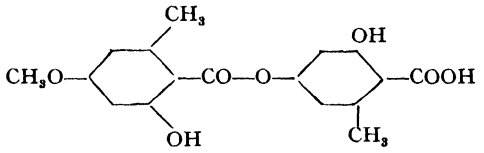
and has the constitution represented by the formula—



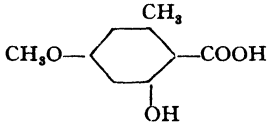
Lecanoric acid forms colourless crystals which melt at 166°; it gives a purple colour with an alcoholic solution of ferric chloride, and with dilute bleaching powder a blood-red colour.

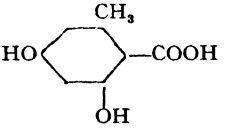
Evernic Acid.

This is a monomethyl ether of lecanoric acid of the formula—



and may be regarded as a didepside produced from the combination of the monomethyl ether of orsellinic acid (II.),

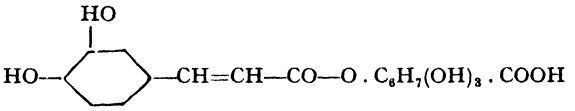

(I.)


(II.)

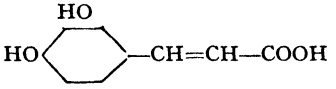
known as everninic acid (I.) with orsellinic acid (II.). Evernic acid forms needles and melts at 168-169°.

Chlorogenic Acid.

This is a didepside which, though not occurring among the lichens, is very widely distributed in the plant world.* Its constitution is represented by the formula—



from which it appears that it is a didepside formed from one molecule of caffeic acid—



with one molecule of quinic acid, a tetrahydroxy-hexahydrobenzoic acid of the formula $C_6H_7(OH)_4 . COOH$.

Chlorogenic acid occurs in green coffee beans combined with a molecule of caffeine.

* Oparin: "Biochem. Zeit.," 1921, **124**, 90.

Properties.

Chlorogenic acid is an optically active substance, $\alpha_D = -33{\cdot}1$; it is not precipitated by gelatine; with ferric chloride it gives a green colour.

Boiled with mineral acids it breaks up into caffeic and quinic acids and similar hydrolysis is effected by *Mucor* or *Penicillium* * or by the tannase of *Aspergillus.*†

According to Freudenberg ‡ the substance described in the older literature as caffetannic acid is a mixture of chlorogenic acid with other acids.

Oparin § considers chlorogenic acid to be a respiratory chromogen which is oxidized by atmospheric oxygen to a green pigment capable of acting as an hydrogen acceptor. This subject is dealt with more fully in volume ii.

FURTHER REFERENCE.

Fischer: "Untersuchungen über Depside," Berlin, 1919.

* Gorter: "J. Amer. Chem. Soc.," 1909, **247**, 184.
† Freudenberg: "Ber. deut. chem. Gesells.," 1920, **53**, 232.
‡ *Ibid.*: "Die Chemie der natürlichen Gerbstoffe," Berlin, 1920, p. 76.
§ Oparin: "Biochem. Zeit.," 1921, **124**, 90; 1927, **182**, 155.

SECTION VI.

PIGMENTS.

CHLOROPHYLL.

As is well known, chlorophyll is contained in the chloroplasts which are universally present in green plants and vary considerably in their size, shape, and number within the cell. With regard to their structure there has been much dispute. It is, however, generally agreed that the structure of the plastids is either reticulate or vacuolate.

The pigment itself is variously stated to be dissolved in some oily substance which is held in the channels and meshes of the plastids, or to exist in the form of a precipitate. Recent evidence based on the spectrum and the solubilities of chlorophyll leads to contradictory conclusions. Iwanowski * found that the absorption spectrum of leaves and of colloidal solutions of chlorophyll, although similar, are not identical in that the spectrum of the colloidal solution lies between that of the leaf and a true solution of chlorophyll; identity was obtained by the addition of an electrolyte to the colloidal solution. He concludes that spectrum analysis cannot solve the problem. Willstätter and Stoll† found the absorption bands of the green leaf and of a colloidal solution of chlorophyll to be identical. As will be seen later on, dry solvents will not extract chlorophyll from dry leaves, but immediately a little water is added, solution is effected; further, chlorophyll in a colloidal solution cannot be extracted with ether unless there is present an electrolyte such as magnesium sulphate or calcium chloride. Willstätter and Stoll for these reasons assume that the addition

* Iwanowski: "Ber. deut. bot. Gesells.," 1907, **25**, 416; "Biochem. Zeit.," 1913, **48**, 328.

† Willstätter and Stoll: "Untersuchungen über Chlorophyll," Berlin, 1913.

of water to the dry solvent dissolves some of the salts present in the dry leaf material and these precipitate the chlorophyll which is then taken up by the solvent. On treating leaves with boiling water, chlorophyll diffuses from the chloroplasts and the spectrum shows a lateral displacement towards the violet, the absorption bands being almost coincident with those of a true solution in phytol. Willstätter and Stoll explain this on the assumption that the hot water brings about a change in the sol condition, thus the wax-like substances present in the cell, which are liquefied at the temperature of boiling water, dissolve the chlorophyll, making a true solution. For these reasons Willstätter and Stoll conclude that the chlorophyll in the leaf is in a colloidal solution.

It will be remembered that solutions of chlorophyll are marked by a strong fluorescence, which property is exhibited only in true solutions, not in colloidal solutions; wherefore, if a preparation of chlorophyll or the chlorophyll in the living cell shows fluorescence, it should be in true solution. Using a culture of *Chlorella*, Stern * found that the position of the fluorescent band closely agreed with that of a solution of chlorophyll in lecithin. This lipoidal sol, compared with an alcoholic solution, shows a lateral displacement of the spectrum towards the red, a displacement due to the difference in the refractive indices of the two solvents. Stern further observed that a chlorophyll sol, shaken up with protein, sugar or glycerol, exhibits no fluorescence; but when shaken up with oil, soap, lecithin and other phytosterols, fluorescence obtains owing to the solution of the chlorophyll in the fatty material. He concludes that in the living cell chlorophyll is in true solution in a lipoid medium dispersed in an aqueous protein phase.

With regard to the distribution of the pigment within the plastid there is again some dispute. According to many, it is distributed evenly throughout the stroma, whilst, on the other hand, others maintain that it is restricted to the peripheral layers of the plastid.

* Stern: "Ber. deut. bot. Gesells.," 1920, 38, 28; "Zeit. Bot.," 1921 13, 193.

Priestley and Irving * have investigated the chloroplasts of certain species of *Selaginella* and *Chlorophytum*. They find that the pigment is restricted to the peripheral regions of the chloroplast, where it is held in the meshes of the network of the matrix. They agree with Timiriazeff's views that the function of the chlorophyll necessitates its distribution in very thin layers in order that the amount of energy set free may be as great as possible.

Zirkle,† from his study of the chloroplasts of *Elodea*, *Phajus*, *Cabomba*, *Marchantia* and other plants, concludes that the plastid is a hollow, flattened, prolate spheroid, the stroma of which is perforated by a large number of pores connecting the central vacuole of the plastid with the sheath of non-granular cytoplasm surrounding the plastid. This sheath is more or less permanent but no differentiated membrane of the plastid could be demonstrated. The pigments are intimately mixed and are evenly distributed throughout the stroma, coating the colloidal protein particles. In the leaf, starch granules are included in the central vacuole of the plastid which vacuole is thought to contain a dilute aqueous solution of sugar and protein. Zirkle is of the opinion that the chlorophyll is not in lipoidal solution since the pigment can be extracted from chloroplasts by solvents which cannot extract it from a lipoid solution. Further, the chlorophyll in plastids removed from a cell show a marked photostability, whilst chlorophyll in solution is quickly destroyed by light.

With regard to the origin of the chloroplast there is also some dispute. The general view, due originally to Schimper and Meyer, appears to be that plastids do not arise *de novo* within the cell, but by the division of pre-existing plastids, so that, in this respect, there is continuity between parent and offspring. This has led to the conception that originally the chloroplasts once had a separate individuality, and that, in a sense, ordinary plants are parasitic upon the imprisoned plastids which have become permanent members of the structures of the cell.

* Priestley and Irving: "Ann. Bot.," 1907, **21**, 407.
† Zirkle: "Amer. Journ. Bot.," 1926, **13**, 301, 321.

On the other hand, other investigators hold that the chloroplasts may arise from differentiated parts of the protoplasm, which parts are not plastids. Lewitski * draws attention to the presence of minute bodies occurring in the protoplasm, but not in the nucleus, which he calls mitochondria, chondriosomes, etc. These structures, which he considers are essential parts of the cytoplasm, increase by division, and give origin to the plastids. For instance in the pea, *Pisum sativum*, and the asparagus, *Asparagus officinale*, the mitochondria of the cells of the stem apex give rise to chloroplasts, whilst those of the apex of the root are converted into leucoplasts. Meyer,† however, is opposed to these conclusions. Miller ‡ finds that very minute chloroplasts occur in the cotyledons of the seed of *Helianthus annuus ;* they increase in size and divide by fission as germination proceeds and maturity is reached.

Mottier § agrees that some forms of chondriosomes give origin to chloroplasts and leucoplasts. He considers them to be permanent structures of the cell, and that certain kinds function as transmitters of hereditary characters.

In green plants chlorophyll may occur in places where light seemingly cannot penetrate, at any rate in any quantity, for instance, in the cortex internal to the periderm—not only in small twigs, but also of larger branches—in the medullary rays and even in the pith.‖ Chlorophyll also may be developed in roots on exposure to light, its formation being favoured by conditions advantageous to carbon assimilation in the shoot. It is developed in the cortex alone, e.g. *Triticum*, in the parenchyma of the vascular cylinder but not in the cortex as in *Rumex* and *Acer*, and in both cortex and vascular cylinder as in *Zea.*¶ Also it may occur in the cotyledons of seeds

* Lewitski : " Ber. deut. bot. Gesells.," 1910, **28**, 538.

† Meyer : *id.*, 1911, **29**, 158. See also Schmidt : " Prog. Rei. Bot.," 1912, **4**, 163 ; Forenbacher : " Ber. deut. bot. Gesells.," 1911, **29**, 648 ; Woycicki : " Sitz. Warschauer Ges. Wiss.," 1912, **5**, 167 ; and Löwschin : " Ber. deut. bot. Gesells.," 1913, **31**, 203.

‡ Miller : " Ann. Bot.," 1910, **24**, 693. A *résumé* of the literature is given by Cavers in " New Phyt.," 1914, **13**, 96, 170. See also Guilliermond : " Compt. rend. soc. biol.," 1921, **84**, 197 ; **85**, 462, 466 ; " Arch. biol.," 1921, **31**, 1.

§ Mottier : " Ann. Bot.," 1918, **32**, 91.

‖ See Scott : *id.*, 1907, **21**, 437. ¶ Powell : *id.*, 1925, **39**, 503.

before they are set free from the ovary or from the cone; *Pinus*, *Euonymus europæus*, and species of *Cucurbita* are familiar examples. In some of these cases light no doubt does penetrate through the walls of the superposed cells; this may be well seen if the seeds be removed and the lumen of the fruit of the vegetable marrow be cleaned out. It is hardly necessary to remark that if the chlorophyll in these deeply-seated tissues be functional, its contributions to the food-stuffs of the plant, as Goldflus * has pointed out, must be of considerable value.

But in some cases the pigments of such chloroplasts may not be the same as those of the ordinary chloroplasts of the leaf; thus, according to Monteverde and Lubimenko,† the seeds of many Cucurbitaceæ contain not chlorophyll, as ordinarily understood, but chlorophyllogen,‡ which may pass over into chlorophyll under the influence of light and some other factor, possibly enzymic.

Also it must be remembered that it does not follow that because chlorophyll is present, photosynthesis necessarily takes place, even though the requisite conditions, light and supply of raw material, obtain. Thus it appears probable that the chlorophyll in green parasites is not functional, and the same holds for the chlorophyll in the gynæcium of certain plants, e.g. *Ornithogalum arabicum*. At any rate, in these cases the photosynthetic power is so small as to be masked by the respiratory activity.

Attention may here be drawn to the work of d'Arbamont § who considers that the plastids containing chlorophyll may be divided into two classes, chloroplasts and pseudochloroplasts. Of these the former include those bodies usually termed chloroplasts, and are characterized by the fact that they do not swell in water, and do not, as a rule, stain when treated with acid aniline blue. On the other hand, pseudochloroplasts swell in water and do stain with aniline blue. In some cases plants may contain pseudochloroplasts only.‖

* Goldflus: "Rev. Gén. Bot.," 1901, **13**, 49.

† Monteverde and Lubimenko: "Bull. Jard. Imp. Bot. St. Pétersbourg," 1909, **9**, 27.

‡ Later described as Leucophyll.

§ D'Arbamont: "Ann. Sci. Nat. Bot.," 1909, **9**, 197.

‖ See Belzung: *id.*, 1891, **13**, 17; "Journ. Bot.," 1895, **9**, 67, 102.

With regard to the conditions necessary for the formation of chlorophyll, light is the most important, but in addition a certain degree of temperature, as well as the presence of certain substances, such as iron and magnesium, are essential. There is, however, some dispute regarding other factors. Palladin * states that chlorophyll formation, is an oxidative process, and, as a result of his experiments, finds that etiolated leaves on exposure to daylight will not form chlorophyll unless a supply of carbohydrate is available. If an etiolated leaf does not contain carbohydrate, then greening will take place if the cut leaf be placed in a solution of sugar. Almost any sugar will do, e.g. sucrose, maltose, glucose, fructose, or raffinose; success was also obtained by the use of glycerol. The solution used must be neither too weak nor too strong; a strong solution of sucrose, for instance, will retard the chlorophyll formation because it will depress oxidative processes. On the other hand, Issatchenko † finds that etiolated leaves of certain plants, e.g. those of *Vicia Faba*, when detached from the plant and placed in strong sugar solution, even 50 per cent, will form chlorophyll. He considers that light is the all-important factor.

With regard to the substances which immediately precede chlorophyll, and from which chlorophyll is formed, nothing definite is known.

The chemical study of chlorophyll dates from the year 1819, when Pelletier and Caventou ‡ first applied this name to the green leaf pigment without, however, isolating the substance. Since then, numerous workers have attempted to prepare chlorophyll in a pure condition, but the methods employed in most cases were of too drastic a nature for the substance to escape destruction. Previous to 1911, there was no chemical evidence to show that chlorophyll was not a single chemical individual, although Stokes,§ Sorby,|| and others had obtained spectroscopic evidence pointing to the

* Palladin: "Ber. deut. bot. Gesells.," 1891, **9**, 194, 229; 1902, **20**, 224; "Rev. Gén. Bot.," 1897, **9**, 385.

† Issatchenko: "Bull. Jard. Imp. Bot. St. Pétersbourg," 1906, **6**, 20.

‡ Pelletier and Caventou: "Ann. Chim. Phys.," 1819, **9**, 194.

§ Stokes: "Proc. Roy. Soc.," 1864, **13**, 144.

|| Sorby: *id.*, 1872, **21**, 442.

existence of more than one substance; confirmatory evidence was subsequently obtained by Tswett.* In 1912, however, Willstätter and Isler † definitely showed that chlorophyll as ordinarily obtained, and to which they had originally assigned the formula $C_{55}H_{72}O_6N_4Mg$, is in reality a mixture of two substances—

Chlorophyll *a* $C_{55}H_{72}O_5N_4Mg$ ‡
and Chlorophyll *b* $C_{55}H_{70}O_6N_4Mg$.

Accompanying chlorophyll are three yellow or reddish-brown pigments, Carotin, Xanthophyll, and Fucoxanthin (the latter occurring only in brown algæ), which are known collectively as the Carotinoids. Owing to the similarity in solubilities between these substances and chlorophyll, their complete separation is a matter of some difficulty; it was first effected by Willstätter and Hug.§

The average proportions in which these various constituents occur in different plants have been determined by Willstätter, and are approximately as follows :—

	In Land Plants.‡	Brown Algæ‖ (*Fucus*).	Green Algæ‖ (*Ulva*).
Chlorophyll *a* . .	·62	·16	·093
,, *b* . .	·22	·01	·066
Carotin . . .	·055	·0312	·014
Xanthophyll . .	·093	·0305	·036
Fucoxanthin . .	—	·059	—

From these figures the following interesting deductions may be made :—

1. The molecular proportions between chlorophylls and carotinoids are as 3·5 to 1 ¶ in terrestrial plants, but only 1 to 1 in the case of algæ.

* Tswett: "Ber. deut. bot. Gesells.," 1906, **24**, 326; 1907, **25**, 137; "Ber. deut. chem. Gesells.," 1908, **41**, 1352.

† Willstätter and Isler: "Annalen," 1912, **390**, 269.

‡ For the physical characteristics of these two substances, see p. 316.

§ Willstätter and Hug: "Annalen," 1911, **380**, 177.

‖ These figures are percentages calculated on the dry material.

¶ With regard to this ratio, it has been stated by Willstätter that it is remarkably constant, and that there is a greater variation between different leaves of the same plant than between corresponding leaves of different plants. This view is, however, contested by Borowska and Marchlewski ("Biochem. Zeit.," 1913, **57**, 423), who hold that it is entirely dependent on external circumstances, such as soil, stage of growth, etc.

2. In the brown algæ chlorophyll *a* predominates, only about 5 per cent of the mixture being chlorophyll *b*; in terrestrial plants, on the other hand, the proportion is pretty constantly about 3 : 1.

3. In the green algæ there is relatively more of chlorophyll *b*.

Other values have been obtained by Lubimenko * :—

	Amount of Chlorophyll.
Ailanthus glandulosa . . .	0·46 per cent. of wet weight.
Ulva lactuca	0·069 ,, ,, ,,
Dictyota fasciola . . .	0·028 ,, ,, ,,
Phyllophora rubens—	
At 19 m. depth . . .	0·037 ,, ,, ,,
,, 48 m. ,, . . .	0·024 ,, ,, ,,
,, 55 m. ,, . . .	0·032 ,, ,, ,,
Laurentia coronopus . . .	0·008 ,, ,, ,,

That the amount of chlorophyll in the algæ is not connected with the depth of immersion is indicated by the values obtained for *Phyllophora;* further, *Laurentia*, which contains the least amount of chlorophyll, grows near the surface. Lubimenko points out that the mechanism of photosynthesis of the red algæ may in some respects be different from that of higher plants, especially in view of the presence of the complementary pigment phycoerythrin. Thus *Ulva* which contains no complementary pigment has a higher chlorophyll content than *Phyllophora.*

It has been suggested by Willstätter and Stoll † that since chlorophyll *b* ($C_{55}H_{70}O_6N_4Mg$) contains more oxygen than chlorophyll *a* ($C_{55}H_{72}O_5N_4Mg$), the former compound is produced by the action of chlorophyll *a* upon carbon dioxide during assimilation, and that chlorophyll *b* is then reconverted into chlorophyll *a* with evolution of oxygen. On the other hand, the molecular formulæ of carotin ($C_{40}H_{56}$) and xanthophyll ($C_{40}H_{56}O_2$) only differ by two atoms of oxygen, and the close association between the carotinoids and chlorophyll may be explained by assuming that the function of carotin is to reduce chlorophyll *b* to chlorophyll *a*, being itself oxidized to xantho-

* Lubimenko: "Compt. rend.," 1924, 179, 1073. See also Wurmser and Duclaux: *id.*, 1920, 171, 1231.

† Willstätter and Stoll: "Untersuchungen über Chlorophyll," p. 237.

phyll, and that the latter compound is reconverted by some enzyme into carotin with evolution of oxygen.

In this connection Baly and Davies * suggest that in view of the fact that the ratio of xanthophyll to carotin tends to increase during photosynthesis, the slow recovery of the photosynthetic ability of the leaf after intense illumination, for example, is due to the slow reduction of xanthophyll to carotin.

Quantitative measurements of the relation between the amount of carbon dioxide assimilated and the weight of chlorophyll concerned have been made by Willstätter and Stoll.† A regular stream of air containing a known amount of carbon dioxide was passed over from 5 to 20 grams of leaves contained in a small illuminated glass vessel immersed in a constant temperature water bath. By estimating the amount of carbon dioxide in the issuing gas and the amount of chlorophyll in the leaves, they determined the so-called assimilation number for different leaves which was the ratio between the amount of carbon dioxide assimilated per hour and the weight of chlorophyll concerned in the assimilation. Experiments with normal, autumnal, and etiolated leaves showed that the assimilation is not always proportional to the chlorophyll content, which may be explained by assuming that some enzyme takes part in the process. The fact that in leaves rich in chlorophyll increased illumination produces no increased assimilation, whereas a rise in temperature does, is attributed to the accelerating effect of increased temperature upon enzyme action. In the case of leaves deficient in chlorophyll, on the other hand, increase of temperature has but little effect, whereas such leaves are very susceptible to increased illumination. The explanation in this case is that there is more than sufficient enzyme for the chlorophyll, but that the greatest assimilative effect can only be attained when all the chlorophyll is exerting its maximum activity. Attempts to bring about assimilation with chlorophyll outside the leaf failed, presumably owing to the absence of this enzyme. The removal of epidermis from the under

* Baly and Davies: "Proc. Roy. Soc.," A., 1927, 116, 219.
† Willstätter and Stoll: "Ber. deut. chem. Gesells.," 1915, 48, 1540.

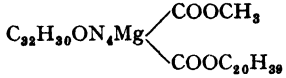
surface of leaves had no deterrent effect on assimilation, but a slight pressure applied to the leaves brought assimilation to a complete standstill.

THE CONSTITUTION OF CHLOROPHYLL.

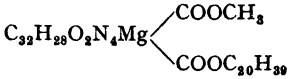
As already stated, chlorophyll was first isolated from its accompanying yellow pigments, the carotinoids, by Willstätter and Hug in 1911, and in the following year it was shown by Willstätter and Isler that the chlorophyll so obtained was not a single substance, but a mixture of two distinct substances, chlorophyll *a* and chlorophyll *b*, in the proportion roughly of three molecules of the former to one of the latter.

The separation of these two constituents was effected by repeatedly shaking a petrol ether solution of crude chlorophyll with 90 per cent aqueous methyl alcohol, whereby the chlorophyll *b* is washed out of the petrol together with a considerable quantity of chlorophyll *a*.*

The formulæ assigned to these two substances are as follows :—



Chlorophyll *a*



Chlorophyll *b*

from which it may be seen that chlorophyll *a* contains two atoms of hydrogen more, but one atom of oxygen less than chlorophyll *b*, and, accordingly, chlorophyll *b* would appear to represent a more oxidized form of chlorophyll *a* ; attempts to convert chlorophyll *a* into chlorophyll *b* have, however, not been successful. The chief difference between the two modifications are given in the following table :—

Chlorophyll (a and b).	*Chlorophyll a.*	*Chlorophyll b.*
Analysis agrees with formula $C_{55}H_{72}O_5N_4Mg$.	$C_{55}H_{72}O_5N_4Mg$.	$C_{55}H_{70}O_6N_4Mg$.
Bluish-black glistening powder, with metallic lustre.	Bluish-black powder.	Dark green or greenish-black glistening powder.
Appearscrystallineunder the microscope.	Bluish-black powder.	Dark green or greenish-black glistening powder.

* For further details of the separation see Willstätter and Hug: "Annalen," 1911, 380, 177.

Chlorophyll (a and b).	*Chlorophyll a.*	*Chlorophyll b.*
No definite m.p.	Sinters and forms a viscous mass at 117-121°.	Sinters at 86-92°, and becomes viscous at 120-130°.
Practically insoluble in cold light petroleum, but dissolves readily on addition of a few drops of methyl or ethyl alcohol.	Very sparingly soluble in light petroleum, but dissolves very easily in most organic solvents.	Quite insoluble in light petroleum, and is generally somewhat less soluble than chlorophyll *a*.
Phase Test (i.e. hydrolysis in ethereal solution, with methyl alcoholic potash), gives a transient brown coloration (cf. p. 327).	*Phase Test.* Transient pure yellow colour.	*Phase Test.* Transient brilliant red colour.

ACTION OF ACID AND ALKALI UPON CHLOROPHYLL.

The recognition of magnesium as an essential constituent of chlorophyll, which is due to Willstätter,* has proved of immense value in the study of the degradation products of chlorophyll.

By the action of alkalis and acids respectively upon the two chlorophylls, it has been found possible to divide the degradation products of chlorophyll into two groups :—

1. Those that retain magnesium, known as *Phyllins.*

2. Those that are free from magnesium, known as *Porphyrins.*

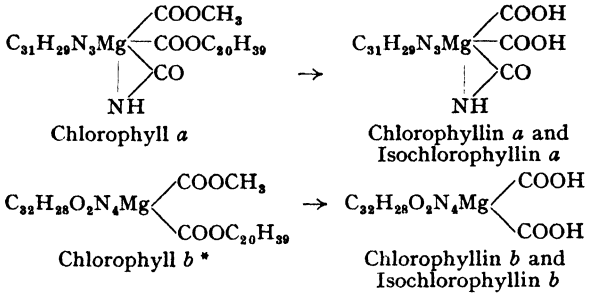
The Action of Alkalis.

Chlorophyll *a* and *b* are compounds of a tricarboxyllic acid, two of whose carboxyl groups are esterified by methyl and phytyl alcohol respectively, while the third is present in the form of a lactam grouping.

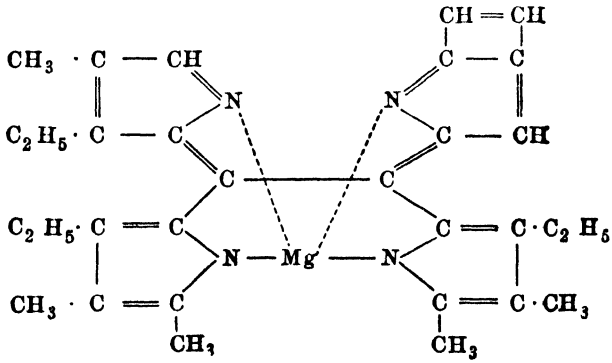
When the two chlorophylls are treated with cold concentrated methyl alcoholic potash, their ester groups are hydrolysed and at the same time a molecular rearrangement takes place accompanied by a colour change known as the Phase Test (see p. 327). This colour change is supposed to be due to a breaking open of the lactam grouping—NHCO—by the alkali which is then followed by a closing of the ring by means of one of the other carboxyl groups ; such an assumption would

* Willstätter ; " Annalen," 1906, **350**, 48.

account for the formation of four isomeric compounds, chlorophyllin and isochlorophyllin derived from chlorophylls *a* and *b*, as indicated by the following formulæ :—



Chlorophyllin *a* when heated with alkali loses carbon-dioxide, and yields two isomeric dibasic acids, glaucophyllin and rhodophyllin, $C_{31}H_{32}N_4Mg(COOH)_2$, and at a higher temperature it loses two molecules of carbon dioxide, yielding a monocarboxylic acid, pyrrophyllin, $C_{31}H_{33}N_4Mg(COOH)$. By heating with soda lime the third molecule of carbon dioxide may be removed with the formation of aetiophyllin, a substance containing no carboxyl group at all, and to which the following formula is assigned :—



Aetiophyllin, $C_{31}H_{34}N_4Mg$

Chlorophyllin *b* when heated with alkali yields the same pyrrophyllin, $C_{31}H_{33}N_4MgCOOH$, as chlorophyll *b*.

* Willstätter, in his papers, does not represent chlorophyll *b* as having a lactam grouping, but from its behaviour in the phase test there is every reason to suppose that it possesses one.

The two isochlorophyllins *a* and *b* heated with alkali go through a similar series of changes yielding dicarboxylic acids cyano-, erythro-, and rubi-phyllin, and finally both yield the same monocarboxylic acid, phyllophyllin $C_{31}H_{33}N_4MgCOOH$.

The Action of Acids.

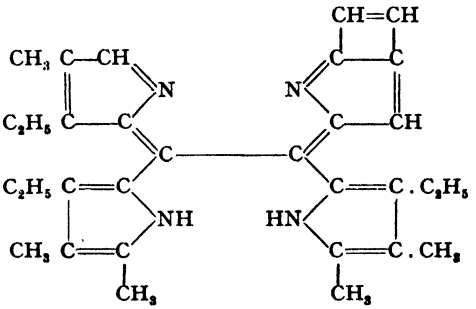
Acids, especially oxalic acid, remove magnesium from all derivatives containing this element, replacing it by two atoms of hydrogen without altering the rest of the molecule.

Thus chlorophyll *a* and *b* give by removal of Mg the compounds—

Phæophytin *a* $C_{32}H_{32}ON_4\begin{cases}COOCH_3\\COOC_{20}H_{39}\end{cases}$ and

Phæophytin *b* $C_{32}H_{30}O_2N_4\begin{cases}COOCH_3\\COOC_{20}H_{39}\end{cases}$

respectively, while chlorophyllin *a* gives phytochlorin *f* and *g*, $C_{32}H_{32}ON_4(COOH)_2$. On the otherh and, glauco- and rhodophyllin by removal of magnesium give glauco- and rhodoporphyrin $C_{31}H_{34}N_4(COOH)_2$, while pyrrophyllin yields pyrroporphyrin $C_{31}H_{35}N_4(COOH)$. By removing the last carboxyl from the latter compound a substance aetioporphyrin $C_{31}H_{36}N_4$ is obtained, which is the magnesium free analogue of aetiophyllin $C_{31}H_{34}N_4Mg$—

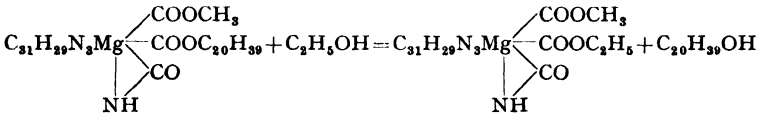


Aetioporphyrin, $C_{31}H_{36}N_4$

CRYSTALLINE AND AMORPHOUS CHLOROPHYLL.

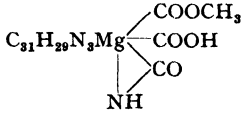
From the data in the table given on page 316, it will be seen that neither ordinary chlorophyll (*a* and *b*) nor either of the constituents of this mixture show any marked tendency

to crystallize which at first sight would appear to be in contradiction with the well-known fact first observed by Borodin * that when green leaves are moistened with alcohol, and allowed to evaporate slowly under a coverslip, crystals of chlorophyll may be observed under the microscope. Willstätter and Benz † described a method of obtaining this substance in quantity from *Galeopsis tetrahit*, and later Willstätter and Stoll ‡ showed that this so-called crystalline chlorophyll was not present as such in the plant, but was a secondary product produced by the action of the alcohol upon the chlorophyll under the action of an enzyme chlorophyllase. The phytyl group is thereby replaced by the ethyl group as illustrated by the equation—



Amorphous chlorophyll *a* — Crystalline chlorophyll *a*

For the monomethyl ester of chlorophyllin *a* Willstätter has proposed the name chlorophyllide *a*—



and adopting this nomenclature, amorphous chlorophyll would be termed phytylchlorophyllide, while crystalline chlorophyll would be ethylchlorophyllide.

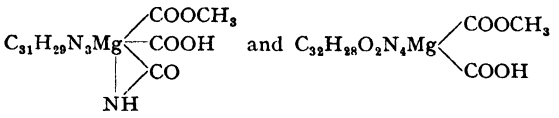
On the other hand, working with methyl alcohol and chlorophyllase, it has been found possible to replace the phytyl group by methyl, forming methylchlorophyllides, *a* and *b*—

$C_{32}H_{30}ON_4Mg(COOCH_3)_2$ and $C_{32}H_{28}O_2N_4Mg(COOCH_3)_2$

which are the methyl analogues of ethylchlorophyllide or crystalline chlorophyll; they are best obtained by treating fresh leaves with 66 per cent methyl alcohol, and extracting the mixture of methylchlorophyllides formed both from the solution and the leaf residue.

* Borodin: "Bot. Ztg.," 1882, **40**, 608.
† Willstätter and Benz: "Annalen," 1907, **358**, 267.
‡ Willstätter and Stoll: *id.*, 1910, **378**, 18.

By acting in moist 33 per cent acetone solution in the absence of alcohol, ordinary hydrolysis was effected with the formation of the monomethyl esters of the two chlorophyllins, namely chlorophyllide *a* and *b*—



which may be separated by means of ether and petrol ether.

The formation of crystalline methyl chlorophyllide is readily demonstrated by placing 1 gram of fresh leaf of *Heracleum spondylium* in a test tube with 4 c.c. of 75 per cent methyl alcohol for three to four hours ; at the end of this time yellow spots will have formed in the lamina due to the removal of chlorophyll ; if held up to the light the yellow spots will show a number of black specks which under a microscope are found to consist of deep green, almost black, pyramidal crystals.

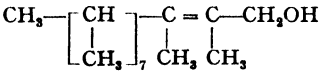
Chlorophyllase belongs to the same class of enzymes as lipase ; the latter substance, however, is only able to hydrolyse amorphous chlorophyll, replacing the phytoxyl group by hydroxyl ; it cannot effect alcoholysis.

Chlorophyll appears to be always accompanied by the enzyme, the amount increasing with the amount of chlorophyll. In *Mellitis Melissophyllum*, *Galeopsis tetrahit*, *Stachys silvatica*, *Lamium maculatum*, and *Heracleum* the amount of enzyme is comparatively large.

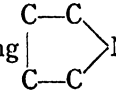
The activity of chlorophyllase may be demonstrated by placing broken-up leaves of *Heracleum* or *Galeopsis* in 70 per cent acetone (1 gm. in 3 c.c.). After a quarter of an hour the acetone solution of the pigment is diluted with water and extracted with ether; on shaking the extract with 0·05 per cent caustic soda the latter turns green owing to solution of the sodium salt of chlorophyllide. Leaves previously boiled to destroy the enzyme yield no colour to the caustic soda.

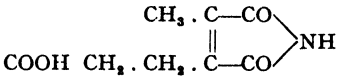
The enzyme is also able to effect the synthesis of phytyl chlorophyllide (amorphous chlorophyll) from chlorophyllide and phytol.

The constitution of this alcohol phytol has been studied by Willstätter and his pupils,* who find it to be an unsaturated primary alcohol with a double bond between the second and third carbon atoms of the chain, probably represented by the formula—

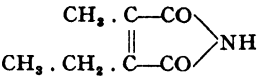


RELATIONSHIP BETWEEN CHLOROPHYLL AND HÆMOGLOBIN.

With a view to the further elucidation of the constitution of the chlorophyll molecule, especially in regard to the complex to which the carboxyl groups are attached, the oxidation of the porphyrins by means of chromic acid in the presence of sulphuric acid has been studied by Marchlewski † and by Willstätter and Asahina.‡ These investigations point to the existence of the grouping  in the molecule, since the two chief oxidation products are found to be pyrrole derivatives of the formulæ—

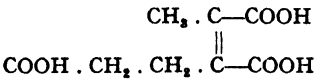


Hæmatinic acid imide



Methylethylmaleinimide

The former substance, which is the imide of a tricarboxylic acid known as hæmatinic acid, of the formula—



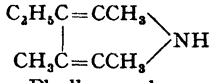
has also been obtained from hæmoglobin, the red colouring matter of the blood, and a connection between hæmoglobin and chlorophyll is thereby established.

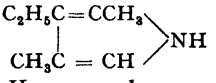
The relationship between this hæmatinic acid imide and hæmoglobin is as follows :—

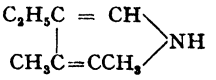
* Willstätter, Schuppli, and Mayer : " Annalen," 1919, **418**, 121.
† Marchlewski : " Chem. Zentralbl.," 1902, **1**, 1017.
‡ Willstätter and Asahina : " Annalen," 1910, **373**, 227.

Hæmoglobin is readily hydrolysed by dilute acids or alkalis with the formation of hæmatin; this latter substance contains iron, which can, however, be readily removed by treatment with hydrogen bromide in acetic acid solution,* giving an iron-free compound hæmatoporphyrin;† both hæmatin‡ and hæmatoporphyrin on oxidation yield the hæmatinic acid imide mentioned above.

Another link between chlorophyll and hæmoglobin is supplied by the fact that Willstätter and Asahina§ have obtained from chlorophyll by reduction three pyrrole derivatives—

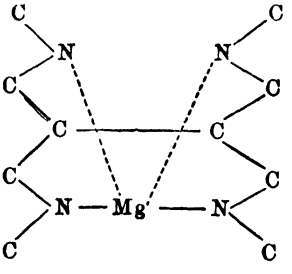

Phyllopyrrole

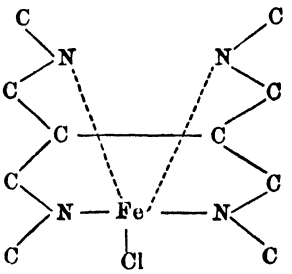

Hæmopyrrole


Iso-hæmopyrrole

one of which, hæmopyrrole, has also been obtained by the reduction of hæmatoporphyrin.

With regard to the manner in which the magnesium or iron are respectively united to the complex molecules of chlorophyll and hæmoglobin, the following skeletons, involving the assumption of subsidiary valencies, according to Werner and others, have been suggested‖:—


Chlorophyll


Hæmin¶

* Nencki and Zaleski: "Zeit. physiol. Chem.," 1900, **30**, 423.

† It should be noted that chlorophyll derivatives free from magnesium are by analogy called porphyrins: cf. Phylloporphyrin, etc.

‡ Küster: "Zeit. physiol. Chem.," 1899, **28**, 1; 1900, **29**, 185.

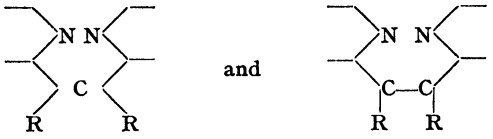
§ Willstätter and Asahina: "Annalen," 1911, **385**, 188.

‖ Willstätter and Fritzsche: *id.*, 1909, **371**, 33.

¶ See also Küster: "Zeit. physiol. Chem.," 1920, **110**, 93.

In this connection the formula assigned to aetiophyllin (p. 318) should be compared.

In the course of attempts to synthesize chlorophyll Tronow and Popow * have prepared compounds, having the structures



and find that where R is either CH_3— or C_6H_5— the compounds exhibit typical reactions of aetioporphyrin whose constitutional formula is given on page 319.

EXTRACTION OF CHLOROPHYLL.

The usual method of extracting chlorophyll from green tissues consists in first steeping the fresh material in hot water to destroy oxidizing enzymes and then extracting the colouring matter by means of warm alcohol. Willstätter, however, recommends the use of dried in place of fresh material, and extracting by shaking with organic solvents (ethyl or methyl alcohol, ether or acetone) in the cold.

The chief advantage in using dried material lies in the fact of its relatively small bulk, 100 grams of stinging nettle leaves, for example, weighing only 25 grams after drying. It has been shown, moreover, that the operation of drying produces no change of any importance in the chlorophyll, since the results obtained from dried material have been repeated and confirmed on fresh material.

On the other hand, organic solvents containing an appreciable amount of water are preferable to the dry solvents. This is attributed by Willstätter to the fact that aqueous solvents dissolve out salts, such as potassium nitrate, from the cell sap, and these affect the state † of the colloidal solution of chlorophyll in the chloroplast, thereby rendering the chlorophyll more easily accessible to the solvent. Moreover, the number of substances going into solution is thereby increased, and the

* Tronow and Popow: " J. Russian Chem. Soc.," 1927, **59**, 327.
‡ See section on Colloids.

solution is no longer effected by the solvent alone but by the solvent together with the accessory substances.

If dry solvents are used, the extract is much less pure, since it contains a larger proportion of carotinoids, lecithins, etc., whose solubilities are very similar to those of chlorophyll.

The following methods of extracting dried or fresh leaves respectively are described by Willstätter :—

1. Half a kilo of dried material is spread on a porcelain Buchner funnel in a layer of not more that 4 to 5 cms. thick, and 1·5 litres of solvent are drawn through this layer by means of a filter pump in the course of half an hour. This filtrate, measuring about 0·9 litre, contains from 4·25 to 4·5 grams of chlorophyll.

The solvent employed may be either 90 per cent (aqueous) alcohol or 80 per cent (aqueous) acetone. The former filters rather more rapidly, but acetone has the advantage over alcohol in preventing the chlorophyll from undergoing what is known as allomerization, a peculiar change which interferes with its power of crystallization, and prevents it giving the phase test.

2. Two and a half kilos of fresh leaves are ground up in a mill and shaken in a bottle with 1·5 litres of acetone to remove water and mucilage and to stop enzyme action. The acetone is then filtered off on a pump ; it contains no chlorophyll. The residue is then freed from acetone by filtering on a pump under a pressure of 200 atmospheres, and the resulting hard mass, weighing 0·8 kg., is broken up and ground again. On adding 1·5 litres of acetone the latter becomes diluted to 80 per cent by the water still remaining in the residue ; the mixture is shaken for five minutes and a further quantity of 1 litre of 80 per cent acetone is now added. The liquid is filtered off on a pump and the residue treated three times with half a litre of 80 per cent acetone. The total filtrate should measure 3·7 litres and contain 4·7 grams chlorophyll.

In order to ascertain what proportion of the total chlorophyll present has been removed in any particular extraction, another quantity of dried material, say from 100 to 200 grams, may be subjected to an exhaustive percolation with an excess

of alcohol until the alcohol comes through colourless. Both extracts are then diluted until 1 kg. of dry powder corresponds to 200 litres of extract and their strengths are compared by means of a colorimeter.

Similarly, a fairly accurate estimate of the amount of chlorophyll present in a solution can be made by colorimetric comparison with a solution containing ·025 gram of pure crystallized chlorophyll dissolved in 1 litre of alcohol. For this purpose the yellow colouring matters must, however, be removed; this is done by allowing the solution to stand for some time with alcoholic potash; the solution is then decanted from the brown resinous deposit which settles on the sides of the vessel, and, after washing the latter with a little more alcohol, the combined alcoholic solutions are diluted with water and extracted with ether to remove the yellow colouring matters.

After suitably diluting with alcohol, the solution is then compared in a colorimeter with the standard chlorophyll solution.

In this way it was found that 1 kg. of fresh stinging nettle leaves containing 25·6 per cent of total solid contained an amount of chlorophyll equivalent to 1·6 grams of the crystalline substance, corresponding, therefore, to $1{\cdot}6 \times 1{\cdot}38 = 2{\cdot}2$ grams of amorphous chlorophyll.*

The following simple experiments are selected from a number described by Willstätter and Stoll † to illustrate the properties of chlorophyll and the carotinoids:—

1. Grind up 10 grams of fresh stinging nettle leaves with silver sand in a mortar. Cover with 20 c.c. acetone and filter over a pump; wash the residue with more acetone and filter again; the filtrate will contain 0·02 gram chlorophyll.

2. Dried powdered leaves do not part with their colour on treatment with benzene or light petroleum, and only yield chlorophyll very slowly to anhydrous alcohol, acetone, or ether, but may be readily extracted by means of 90 per cent

* The factor 1·38 for converting crystalline into amorphous chlorophyll represents the ratio between the molecular weights of these two substances.

† Willstätter and Stoll: "Untersuchungen über Chlorophyll," Berlin, 1913.

alcohol or 80 per cent acetone, yielding a green solution with a strong red fluorescence.

3. *Phase Test.*—Prepare an ethereal solution of chlorophyll as follows: About 15 c.c. of an 80 per cent acetone extract of dried leaves are poured into 30 c.c. of ether contained in a tap funnel and mixed with 50 c.c. water. The ethereal solution rises to the surface. It should be washed four times with 50 c.c. of water each time by carefully allowing the water to run down the side of the funnel without shaking. If a 30 per cent methyl alcoholic solution of potash is now run under the ether layer a brown colour is produced at the junction of the two liquids. The colour gradually changes to olive-green and finally back to the original green. The reaction, which is known as the "Phase Test," is due to the saponification of the chlorophyll with formation of the potassium salt of chlorophyllin. Consequently on dilution with water the green colour remains in the aqueous layer and is no longer soluble in ether.

4. *Separation of Chlorophylls from Carotinoids.*—Shake vigorously 5 c.c. of an ethereal solution of chlorophyll (prepared as above) with 2 c.c. of concentrated methyl alcoholic potash. When the green colour has returned, dilute with 10 c.c. water, added in portions, and add a little more ether. On shaking, two layers separate; the lower aqueous alkaline layer contains the chlorophyllin, while the ether contains carotinoids.

5. *Separation of Xanthophyll from Carotin.*—Wash the ethereal solution of these two substances obtained from previous experiment with a little water and evaporate to 1 c.c. Dilute with 10 c.c. of light petroleum, and shake up two or three times with 10 c.c. of 90 per cent methyl alcohol until the latter is no longer coloured. The methyl alcohol will contain the xanthophyll, while the carotin will be in the light petroleum.

6. *Action of Acid on Chlorophyll.*—Shake 2 c.c. of an ethereal solution prepared as above with a little 20 per cent hydrochloric acid and a few drops of water; run off the lower aqueous layer and evaporate the ether solution over a water bath. Dissolve the residue in 5 c.c. of alcohol; note the

olive colour of the magnesium-free compounds, phæophytin *a* and *b* (for formulæ, see p. 319). Boil the solution with a very small crystal of copper acetate and note the bright green colour is restored when the magnesium is replaced by copper.

THE CAROTINOIDS OR YELLOW PIGMENTS ACCOMPANYING CHLOROPHYLL.

The term carotinoid or lipochrome is applied to a group of yellow orange or brown pigments which are widely distributed in the plant and animal worlds. In the plant they occur either associated with chlorophyll in the chloroplasts or else in plastids by themselves; they are to be found in all types of plant both phanerogams and cryptogams, and in the latter group, particularly in those members which are without chlorophyll, such as the fungi, they are frequently responsible for the colour. In the animal world they are responsible for the yellow colour of fats, hence the term lipochrome; the yellow pigment of the corpus luteum of the cow is identical with carotin, whilst the yellow pigment of egg yolk and of blood serum of fowls is known to be xanthophyll. The following members of this group have been described: carotin, lycopin (a red isomer of carotin), xanthophyll, which probably exists in four modifications known as α, α', α'' and β which differ slightly in their absorption spectra, rhodoxanthin (a red isomer of xanthophyll), and fucoxanthin.

CAROTIN, $C_{40}H_{56}$.

This pigment is widely distributed and, as has already been mentioned, is generally associated with chlorophyll in the chloroplasts. It also occurs in various forms, amorphous or crystalline, in various parts of many plants. The colour of yellow or orange petals is not infrequently due to it, e.g. the corona of the common Narcissus, *N. Poeticus;* similarly the presence of innumerable small intracellular crystals of carotin are responsible for most of the colour of the root of the carrot, and so also is the tint of many fruits where the carotin is often in amorphous granules.

With regard to the physiological significance of carotin

and xanthophyll, a good deal of speculation is rife in view of their close association with chlorophyll and the possibility that in the plant the conversion of carotin into xanthophyll may be a reversible process. It has been suggested by Willstätter and Stoll that chlorophyll *a* in taking up carbon dioxide is itself oxidized to chlorophyll *b* in reducing the carbon dioxide; the resulting chlorophyll *b* is then reduced back to chlorophyll *a* by carotin, which is thereby converted into xanthophyll; the reconversion of xanthophyll to carotin is supposed to be effected by a reductase. Willstätter has, however, not been able to show that carotin can be oxidized to xanthophyll. The work of Tammes and Kohl * shows that carotin absorbs certain rays of radiant energy which may be made use of in photosynthesis.

In those cases where a large amount of carotin occurs in organs of storage, such as the roots of the carrot, it may be of value as a reserve food-material. Finally, where the colours of flowers are due to its presence, carotin is important in the floral biology.

Carotin is insoluble in water and very slightly soluble in acetone or cold alcohol; in hot alcohol it is more soluble; and in ether, chloroform, light petroleum, and carbon bisulphide it is readily soluble. The colour of the solution varies from yellow to red; on crystallization flat reddish-yellow plates are formed which exhibit the phenomenon of dichroism, being orange-red by transmitted light and greenish-blue in reflected light.

According to Willstätter and Mieg,† carotin may be extracted from stinging nettle leaves by light petroleum; it has the molecular formula $C_{40}H_{56}$, and is probably identical with the substances erythrophyll and chrysophyll described by Bougarel and Schunck respectively.

It absorbs 34·3 per cent of its weight of oxygen, being converted into a colourless substance. With iodine it forms the compound $C_{40}H_{56}I_2$, which crystallizes in dark violet prisms.

* Kohl: "Ber. deut. bot. Gesells.," 1906, **24**, 222.
† Willstätter and Mieg: "Annalen," 1907, **355**, 1.

LYCOPIN, $C_{40}H_{56}$.

Under this name is described a red hydrocarbon isomeric with carotin which was isolated by Willstätter and Escher * from the tomato. Lycopin forms carmine coloured prisms or needles which melt at 168°-169°; its solution in alcohol is deep yellow in colour. Like carotin it absorbs oxygen with avidity.

XANTHOPHYLL, $C_{40}H_{56}O_2$.

This substance is closely related to carotin, having the molecular formula $C_{40}H_{56}O_2$. Ewart † claims to have shown that xanthophyll may be converted into carotin by the action of zinc dust or magnesium powder and water.

It is a neutral substance, reacting neither as an alcohol nor as an acid.

It absorbs 36·55 per cent of its weight of oxygen, and forms an additive compound with iodine of the formula $C_{40}H_{56}O_2I_2$, which crystallizes in dark violet tufts.

The more important physical constants and solubilities of carotin and xanthophyll are given in the appended table, compiled by Willstätter :—

	Carotin.	*Xanthophyll.*
Appearance . .	Copper coloured leaflets.	Pleochroic dark reddish-brown plates.
Colour by transmitted light . . .	Red.	Yellow to orange.
Melting-point . .	167·5°-168°.	172°.
Solubility in light petroleum . . .	Appreciably soluble.	Insoluble.
Solubility in alcohol	Practically insoluble in cold; very sparingly soluble in hot.	Sparingly soluble in cold; fairly readily soluble in hot.
Solubility in acetone .	Very sparingly soluble.	Readily soluble.
Solubility in carbon disulphide . . .	Very readily soluble.	Sparingly soluble.
Concentrated sulphuric acid.	Dissolves; deep blue colour.	Dissolves; deep blue colour.

RHODOXANTHIN, $C_{40}H_{56}O_2$.

This substance, which is a red isomer of xanthophyll, was discovered by Monteverde in *Potamogeton natans;* it has since

* Willstätter and Escher: "Zeit. physiol. Chem.," 1910, **64**, 47.
† Ewart: "Proc. Roy. Soc.," B., 1915, **89**, 1.

been found to occur in the arillus of the seed of the yew and to be responsible for the red colour of *Thuja occidentalis*. Its isolation has been described by Monteverde and Lubimenko.* Rhodoxanthin is sparingly soluble in petroleum ether; it dissolves in glacial acetic acid, giving a red solution, and in carbon disulphide, giving a violet-red solution.

FUCOXANTHIN, $C_{40}H_{54}O_6$.

This substance was first isolated from fresh brown algæ by Willstätter and Page.† It is more difficult to extract this substance from dried algæ. Fucoxanthin is a brownish-red substance, which crystallizes from methyl alcohol or light petroleum, and melts at 159·5°-160·5°. It absorbs iodine to form a compound $C_{40}H_{54}O_6I_4$. Unlike carotin and xanthophyll, which are neutral substances, fucoxanthin has basic properties, and forms blue salts with hydrochloric and sulphuric acids.

FURTHER REFERENCE.

Palmer: "Carotinoids and Related Pigments," New York, 1922.

ANTHOXANTHINS.

FLAVONES AND XANTHONES.

Under the headings of Flavones and Xanthones (two words derived from the Latin and Greek for yellow) are included a number of yellow pigments occurring in the vegetative organs and in the petals of many plants. Owing to their close relationship to the blue colouring matters known as Anthocyanins, Willstätter and Everest‡ have proposed the adoption for them of the generic term, Anthoxanthin, at first suggested by Marquart in 1835. They occur naturally in combination with rhamnose or glucose as glucosides and in some cases uncombined, and frequently are also associated with tannins.

The anthoxanthins in the form of their glucosides frequently are but faintly yellow in colour, the sugar-free compound

* Monteverde and Lubimenko: "Bull. Acad. Sci. Petrograd," 1913, [6], **7**, 1105.

† Willstätter and Page: "Annalen," 1914, **404**, 237.

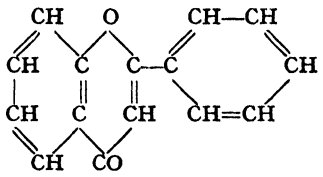
‡ Willstätter and Everest: *id.*, 1913, **401**, 189.

generally having a deeper yellow colour than the glucoside. In the plant their concentration may be insufficient to effect the colour materially ; thus they are commonly found in white petals and their presence is only revealed by exposure of the petals to ammonia fumes whereby a yellow colour is developed. This tendency to form yellow salts with ammonia or alkalis also reveals itself in the formation of deeply coloured salts with other metals ; for this reason many anthoxanthins were in the past used extensively as cotton dyes in conjunction with suitable mordants ; owing to the fact that the salt formation is associated with the phenolic groupings, the sugar-free compounds usually dye more deeply than the glucosides.

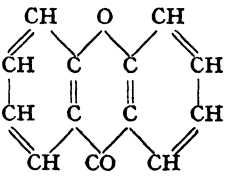
The anthoxanthins are widely distributed amongst the higher plants ; they are most abundant in plants which grow under conditions of high insolation, unless there be a protection in the form of hairs or thick cuticle. For this reason they are looked upon as affording a protection against the light rays of shorter length. There is sometimes an interchange between the anthoxanthins and anthocyanins, thus young plants often contain red anthocyanin, which gives place to a colourless flavone in the mature stage ; at leaf-fall the anthocyanin may reappear.*

YELLOW COLOURING MATTERS DERIVED FROM FLAVONE.

The mother substances from which all these compounds are derived and from which they derive their name are the two compounds Flavone and Xanthone, both of which contain the pyrone nucleus (see p. 336)—



Flavone



Xanthone

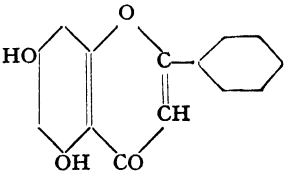
There are a considerable number of yellow substances occurring in plants derived from flavone, but only a few re-

* Shibata and Nagai : " Bot. Mag. Tokio," 1916, **30**, 149.

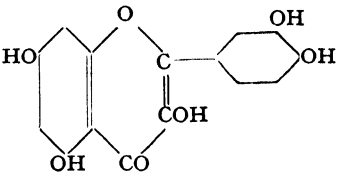
presentative ones will be mentioned here in order to give some idea of the constitution of these compounds.

Flavone in an almost pure condition is contained in the powder which may be shaken off the surface of the stem and leaves of *Primula pulverulenta;* being soluble in benzene this solvent may be used for its extraction.

Chrysin, or dihydroxy-flavone, is a yellow colouring matter occurring in several varieties of poplar, such as *Populus nigra* and *P. pyramidalis*—



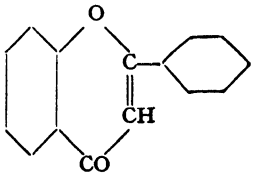
Quercetin, or tetrahydroxy-flavonol *



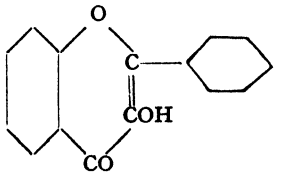
is widely distributed in the higher plants; combined with rhamnose, it exists in the form of a glucoside in quercitron bark, *Quercus tinctorius*, in onion scales, wallflower petals, leaves of horse-chestnut and hop, and in many other plants. Quercetin, in the uncombined state, also is found in the bark of *Pyrus Malus* and in the leaves of *Thea sinensis*, *Arctostaphylos Uva-ursi*, *Acacia catechu*, and many other plants.

Rhamnetin, the monomethyl ether of tetrahydroxy-flavonol or quercetin monomethyl ether, occurs in the dried berries of

* Flavonol is the hydroxyl derivative of flavone; the relationship between the two substances is shown by the following formulæ :—

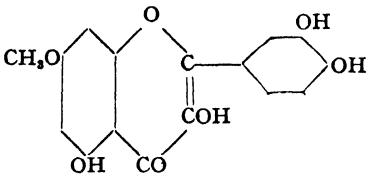


Flavone

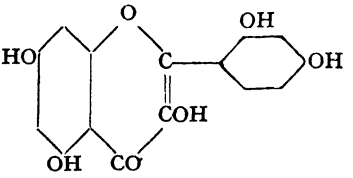


Flavonol

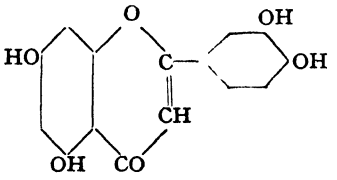
Rhamnus cathartica and R. *tinctoria*, both of which are used for dyeing cotton—



Morin.—This substance, which is isomeric with quercetin, occurs in the wood of *Morus tinctoria* (yellow wood) where it is accompanied by another colouring matter, maclurin, sometimes called moritannic acid (see p. 280)—



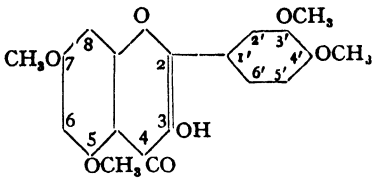
Luteolin.—This is the yellow colouring matter of *Reseda luteola*, known as "weld"; it is also contained in *Genista tinctoria* or Dyer's broom—



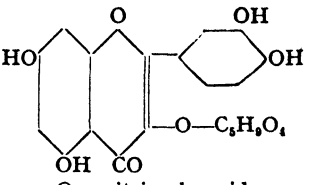
Other members of this group of substances are *Apigenin*, occurring in *Apium petroselinum*, and *Fisetin*, occurring in *Quebracho colorada*, and *Rhus cotinus* or Dyer's sumach.

The point of attachment of the carbohydrate residue has as yet only been ascertained in the case of a few of the glucosidal flavonols *; thus in the case of quercitrin glucoside exhaustive methylation yielded a pentamethyl ether which on hydrolysis gave 5 : 7 : 3′ : 4′ tetramethoxyflavonol I. from which it follows that the rhamnose was attached to the carbon atom 3 as may be seen from the attached formulæ :—

* Attree and Perkin : "J. Chem. Soc.," 1927, 234.



I.

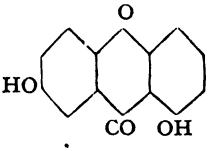


Quercitrin glucoside

YELLOW COLOURING MATTERS DERIVED FROM XANTHONE.

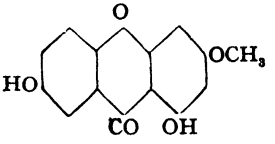
There are as yet only three colouring matters known to belong to this group, one of which, *euxanthone*, does not occur in plants, but in Indian yellow obtained from camel's urine; it has the formula (I.)—

(I.)



Gentisin (II.), a yellow colouring matter occurring in *Gentiana lutea*, is a methyoxyl derivative of the above—

(II.)



Datiscetin occurring in the form of a glucoside, *Datiscin*, in *Datisca cannabina*, whose constitution is uncertain.

Properties of Anthoxanthins.

1. These colouring matters are mostly yellow crystalline solids.

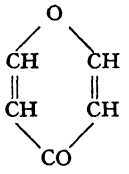
2. From their solutions they may be precipitated by lead acetate, the precipitate being yellow, orange, or red.

3. With ferric chloride a dull green or sometimes a red-brown coloration results.

4. On fusion with alkali, decomposition ensues, phloroglucinol and protocatechuic acid being commonly formed, and sometimes resorcinol, resorcylic, or hydroxybenzoic acids.

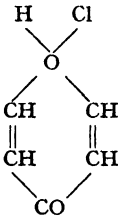
The solubility of the anthoxanthins in acids is due to the peculiar basic properties of the oxygen atom taking part in the ring formation. The basic nature of the oxygen atom in

such circumstances was first observed in the case of the simpler substance pyrone—



Pyrone

which dissolves in hydrochloric acid, forming an additive compound of the formula—



the oxygen becoming tetravalent. Such additive compounds of anthoxanthins with acids are easily dissociated and do not occur in plants, though it will be seen on page 345 that in the case of the anthocyanins analogous compounds do actually occur naturally.

FURTHER LITERATURE.

Kostanecki: "Bull. Soc. chim. Paris," 1913, [3], **29**, i-xxxvii.

Perkin, A. G., and others: "J. Chem. Soc. Lond.," 1895, **67**; 1896, **69**; 1897, **71**; 1898, **73**; 1899, **75**, etc.

Wheldale: "Proc. Camb. Phil. Soc.," 1909, **15**, 137; "Biochem. Journ.," 1914, **8**, 204, etc.

Perkin and Everest: "The Natural Organic Colouring Matters," London, 1918.

ANTHOCYANINS.

OCCURRENCE, CONDITIONS OF FORMATION, AND PHYSIOLOGICAL SIGNIFICANCE.

The occurrence of a red, blue, or purple pigment, either dissolved in cell sap—the exact colour depending on the acid, alkaline, or neutral reaction of the cell sap—or in an amorphous or crystalline state as in *Delphinium* spp., *Passiflora* spp., *Rubus* spp., and many other plants,* is a common phenomenon,

* See Gertz: "Studier öfver Anthocyan," Lund, 1906.

and is generally ascribed to the presence of the pigment anthocyanin. It is, however, doubtful whether all such colorations are due to anthocyanins ; thus Molisch found that the red colour assumed by the leaves of the aloe, on exposure to high insolation, is due to the formation of carotin within the chloroplasts.

The presence of anthocyanin is due to many causes,* light, especially when of high intensity, being important. For example, apples and other fruits and also the vegetative organs of certain plants will not assume a red colour if kept in darkness. Jonesco † observed that buckwheat seedlings when placed in the dark lost their red colour, the total amount of flavone and anthocyan glucosides falling to about one-sixth after ten days' darkening. On the other hand, light does not appear to be of such importance in the case of the roots of the beet.

In other instances the aerial vegetative organs of many varieties of plants, e.g. certain Chenopodiaceæ, are characterized by a red colour, the presence of which is seemingly independent, or nearly so, of external conditions. Thus *Salicornia ramosissima* may be found in two forms, one apple green and the other crimson, the intensity of which varies in different years. In such cases there is good reason for supposing that these colours are of an hereditary nature and come true from seed. The same also appears to be true for different forms of beet which are used for horticultural purposes. On the other hand, in the familiar example of the copper beech this is not so, the copper-coloured foliage, due to the combined effect of a red cell sap and the green of the chlorophyll, first originated, it is stated, as a sport and is propagated by means of cuttings.

According to Pick and others, anthocyanin is commonly associated with tannins, for a red sap is characteristic of tannin-containing plants, and the precipitate appearing in the palisade cells of *Hydrocharis* on treatment with caffeine and

* See also Wheldale-Onslow : " The Anthocyanin Pigments of Plants," Cambridge, 1925.

† Jonesco : " Compt. rend.," 1921, **172**, 1311.

antipyrine closely resembles the precipitates given by the same reagents with tannin. Plants in which this particular pigment does not occur are free from tannin.

The appearance of anthocyanin is closely related to the sugar-content of the tissues in which it occurs.

Ewart * has pointed out that in the case of *Elodea canadensis* and other aquatic plants the red dye will appear provided the plants be immersed in a weak solution of sugar and exposed to strong sunlight at ordinary temperatures, whilst the red colour does not appear if the plants be grown in water or in diffuse daylight.

These experiments of Ewart were much extended by Overton,† who used *Hydrocharis* and other plants. He found that, in addition to the presence of sugar, light and temperature were important factors. If the temperature be low, but above freezing-point, then the formation of the red pigment will be promoted, which accounts for the red colour prevalent in alpine plants, since under their conditions of existence sugar tends to accumulate rather than starch. This also is true for arctic plants in which, according to the observations of Wulff,‡ the leaves are very frequently sugar leaves, and are commonly characterized by the presence of anthocyanin.

In the case of *Hydrocharis* grown in water culture, Overton found that when the temperature and the intensity of light were so balanced that no colour was formed, the addition of 2 per cent of invert sugar caused its appearance in three days, not only in the young leaves but also in the old ones.

Other aquatic plants behave similarly, but in the case of cut shoots of lilies the red pigment only developed provided sugar were added to the culture solution.

Further experiments showed that the red colour is not formed in those plants, in which the pigment was restricted to the epidermis, when cultivated in sugar solution. Success only resulted in those cases where the colouring matter occurred in the mesophyll.

* Ewart: "Journ. Linn. Soc. Lond., Bot.," 1895-7, **31**, 445; "Ann. Bot.," 1897, **11**, 461.

† Overton: "Nature," 1899, **59**, 296; "Jahrb. Wiss. Bot.," 1899, **33**.

‡ Wulff: "Botanische Beobachtungen aus Spitzbergen," Lund., 1902.

In view of these facts Overton considered that anthocyanin had some connection with tannins, and was probably a glucoside. A similar view was held by Combes,* who called attention to the facts that, as compared with the green leaves, the red autumnal leaves of *Ampelopsis hederacea*, etc., contain more sugars and glucosides, the amount of anthocyanin varying directly as the sugars and glucosides; that the dextrins diminish as the sugars and glucosides increase; and that the formation of anthocyanin is not apparently dependent on the insoluble carbohydrates. For these and other reasons he concluded that the substance in question was probably a cyclic glucoside which arose, not at the expense of pre-existent sugars and glucosides nor of chromogens, but in the ordinary course of constructive metabolism; also, he concluded, it was only formed provided that oxygen be present.

The observations of Boodle † also indicate the relationship between anthocyanin and sugar. He found that in the leaves of *Rheum*, some of the veins of which had been accidentally severed, anthocyanin made its appearance in the mesophyll supplied by these veins. Boodle then experimented with species of *Oenothera;* all the species examined were not equally responsive, but in the case of *O. biennis* the severance of the midrib at about its middle caused the whole region distal to the cut to become red provided the plant were exposed to daylight. The operation interrupted the path of transport of carbohydrate from the leaf, so that sugar accumulated above the cut, and it is this concentration of soluble carbohydrates which leads to the development of anthocyanin. In this connection the work of Linsbauer ‡ may be consulted.

Keener,§ from his observations on *Diervilla lonicera*, concludes that the chief factors which affect the formation of anthocyanin are the degree of insolation, the rate of transpiration, the water content of the soil, and the composition of the soil.

That the presence of anthocyanin is connected with nutritive

* Combes: "Ann. Sci. Nat. Bot.," 1909, **9**, 274.
† Boodle: "New Phytologist," 1903, **2**, 207.
‡ Linsbauer: "Oestr. Bot. Zeit.," 1901, **51**, 1.
§ Keener: "Amer. J. Bot.," 1924, **11**, 61.

processes there can be no doubt, but other substances besides sugar may come into play; thus Dendy observed that the addition of protein to the water caused green plants of *Hæmatococcus* to turn red.

PHYSIOLOGICAL SIGNIFICANCE.

In considering the physiological significance of anthocyanin it must be borne in mind that the substance may occur in almost any organ of a plant, from the root to the flower, and in plants very remote phyletically one from the other; and that chemically this pigment may not always be exactly the same. Further, as its appearance seemingly depends upon the immediate metabolic condition of the plant, and so in some cases may be sporadic, whilst in other instances it is characteristic of the species or variety or form, care must be exercised in ascribing to it a definite function. Its presence may be due to nothing more than the particular metabolic sequence; in other words, an accident, which in some examples may be a lucky one for the plant.

It is, of course, not surprising to find that several opinions have been put forward to explain its presence.

The chief physical property of anthocyanin is its absorption spectrum. Engelmann found that it is complementary to that of chlorophyll, the main absorption bands being in the yellow and yellow-green, with minor ones in the blue end of the spectrum. Questions relating to the energy relationship between this and other pigments and chlorophyll are outside the scope of the present consideration; it may be mentioned, however, that it has been stated that leaves containing anthocyanin have relatively less chlorophyll than those which have no red pigment.

According to Pick the dye is a filter to separate from the light entering the leaf certain rays which would be deleterious to the translocation of the starch. Keeble found that in leaves which had the dye on one side but not on the other, the difference in temperature due to the anthocyanin was about 2° C., and he concluded that it may be of value as a protective mechanism against the heating effect of strong sunlight.

Stahl * thought that it absorbs heat and so increases transpiration, especially in the case of tropical plants. Ewart points out that, although this might sometimes be of value, if it were the primary function it would naturally be expected that anthocyanin would absorb the heat rays more particularly. Also Ewart cites his observations on *Elodea* against Stahl's view, and remarks that " since the plants [*Elodea*] are submerged, it cannot possibly be for the purpose of increasing what is non-existent, i.e. transpiration, nor can it perceptibly raise the temperature of a submerged plant." The first argument may no longer be valid, for it appears that transpiration current may exist in submerged aquatic plants.†

Ewart believes that anthocyanin is to protect the chlorophyll against the action of too strong light. He gives experimental data in support of his view, and cites the observations of Schröder and Klebs to the effect that the pigment is of importance in protecting the chlorophyll in *Hæmatococcus* and the resting spores of many Algæ.

Ewart does not think that the pigment is an accidental occurrence in all cases, for in *Elodea* it is not formed in diffuse light; on the other hand, in the beetroot it probably has no special function, and may be a waste product of metabolism.

Shibata ‡ and his colleagues found that derivatives of flavones are almost universally distributed in sub-aerial plants, alpine and tropical plants particularly so. They conclude that the presence of these substances, especially when in the peripheral tissues, absorb the ultra-violet rays, and thus are protective. Rosenheim § supports this view, since he found that *Leontopodium alpinum*, the Edelweiss, when grown in London, contained but a quarter the amount of the substances in question as compared with plants grown in the Alps.

Wulff considers that the pigment is of value in the absorption of extra radiant energy, and is of great importance in

* Stahl: " Ann. Jard. Bot. Buitenzorg," 1896, **13**, 137.

† See Thoday and Sykes: " Ann. Bot.," 1909, **23**, 635.

‡ Shibata: " Bot. Mag. Tokio," 1915, **29**, 118; Shibata, Nagai, and Kishida: " J. Biol. Chem.," 1916, **28**, 193.

§ Rosenheim: " Biochem. Journ.," 1918, **12**, 283.

arctic plants, for instance, which live under conditions unfavourable for metabolic activities.

Combes holds views similar to those of Palladin, that anthocyanin is closely connected with respiration. If the sugar content increases, the rate of respiration is accelerated, and this leads to the formation of the pigment.

Although it is not proposed to enter into a detailed consideration of the phenomena of respiration here, brief mention may be made of Palladin's * conceptions on the subject on account of the rôle he ascribes to colouring matters and allied substances in respiratory activity.

Occurring in plants are pro-chromogens which may be glucosides; these pro-chromogens, by the action of enzymes, give origin to chromogens. Their presence is indicated by the appearance of a reddish colour on the addition of peroxidase and hydrogen peroxide to a hot-water extract of the tissue.

Chromogens are widely distributed in the vegetable kingdom, in fact are universally present in those parts of plants which are respiring; they, however, vary in amount at different seasons of the year and according to the physiological condition of the plant. For instance, in the spring they occur in abundance in the young leaves, and in the autumn the old and dead leaves also contain much owing to the lack of co-ordination of enzymic activity.

At other times the amount of chromogens is not very great, but may be increased by suitable treatment. Thus Palladin found that leaves kept for a week in a strong solution, 20 to 30 per cent, of cane sugar showed a great increase, whereas leaves kept in distilled water and also untreated leaves of the plant showed no such increase. A bright illumination also increased the amount of chromogens.

The chromogens are acted upon by oxidases in the presence of oxygen and yield pigments which may be reduced by reducing enzymes or reductases.

A chromogen which satisfies the requirements of Palladin's respiratory pigments has been found to occur in *Mercurialis*

* Palladin: "Ber. deut. bot. Gesells.," 1908, **26a**, 125, 378, 389; 1909, **27**, 110.

and is known as hermidin.* This chromogen, on oxidation by atmospheric oxygen, is converted into a blue compound, cyanohermidin, which in turn may be further oxidized to a yellow compound, chrysohermidin. Fresh chrysohermidin is reduced to cyanohermidin, and cyanohermidin is readily reduced to hermidin by the plant, and *in vitro* by such reducing agents as nascent hydrogen or sodium hydrosulphite. These changes, therefore, are easily reversible, a lability indispensable in a respiratory chromogen.

It will be obvious that the amount of pigment produced depends on the relative activities of the oxidizing and reducing agents ; if the former be the more potent, a coloured substance will be formed, but if the latter be dominant, no pigment will appear. In the living healthy plant, if the thesis is held to be true, the balance will be justly maintained, the mechanism being one for the transference not the holding of oxygen; wherefore no colour change will be visible. The opinion of Oparin that chlorogenic acid is also a respiratory pigment has been alluded to on page 306.

In addition there is another respiratory pigment first described by MacMunn and subsequently reinvestigated by Keilin † ; it is a modified hæmochromogen, known as cytochrome, and occurs in the tissues of many animals, in aerobic bacteria, yeast, and in the non-green parts of higher plants such as the bulbs of shallot and allied plants, potato tubers and so on.

Cytochrome is an intracellular respiratory catalyst, the spectrum of which in the reduced state shows four characteristic absorption bands ; in the oxidized form, these bands disappear, a faint shading only being visible. Oxidation easily is effected by the air, and reduction is brought about by the normal activity of cells or by a simple chemical agent such as sodium hydrosulphite. These spectral changes are recognizable only under the microspectroscope ; no colour change is visible to the unaided eye.

* Haas and Hill : " Biochem. Journ.," 1925, **19**, 233, 236 ; " Ann. Bot.," 1925, **39**, 861 ; 1926, **40**, 709.

† Keilin : " Proc. Roy. Soc.," B., 1925, **98**, 312 ; 1926, **100**, 129.

PREPARATION OF PROPERTIES OF ANTHOCYANINS.

The first representative of the class to be isolated in a state of purity by Willstätter and Everest * was cyanin, the blue colouring matter of the cornflower, *Centaurea cyanus*, and of *Rosa gallica ;* closely related to this substance are the anthocyanins of the cranberry (idæin), the bilberry (myrtillin), of blue grapes (œnin), of *Delphinium consolida* (delphinin), and of *Pelargonium zonale*, var. *meteor* (pelargonin), *Althaea rosea* (althæin), and *Malva sylvestris* (malvin).

The methods employed for the extraction and isolation of the anthocyanins vary with the material employed.† In some cases the pigment is extracted with water, as in the case of cyanin from *Centaurea cyanus*, the blue cornflower, in others with alcohol, e.g. *Pelargonium*, and in others again, such as the rose and the hollyhock, by means of a hydrochloric acid solution of methylalcohol. These facts may be illustrated by the two following examples :—

The method recommended in the case of grape skins is ‡ as follows : Extract the skins in the cold with glacial acetic acid and precipitate the dark red filtrate with ether ; by heating the deposit so obtained with a solution of picric acid a crystalline picrate is formed which separates out on cooling.

For the preparation of cyanin chloride, the following method was employed by Willstätter and Mallison § : 700 grams of deep red dahlia petals were extracted with glacial acetic acid ; the extract was mixed with methyl alcoholic hydrogen chloride and precipitated with ether ; the amorphous precipitate dissolved in cold 7 per cent hydrochloric acid on standing yielded 7·4 grams of pure cyanin chloride.

The isolation of the anthocyanins depends upon the formation of sparingly soluble oxonium salts with various acids such as picric, tannic, hydrochloric and acetic. The formation of a crystalline acetate of pelargonidin is easily demonstrated by placing a petal of pelargonium on a slide, covering it with

* Willstätter and Everest : " Annalen," 1913, **401**, 189.

† Cf. Willstätter and Mieg : *id.*, 1915, **408**, 61 ; also Willstätter and Bolton : *id.*, 1915, **408**, 42.

‡ Willstätter and Zollinger : *id.*, 1915, **408**, 83 ; 1916, **412**, 195.

§ Willstätter and Mallison : *id.*, 1915, **408**, 147.

a few drops of 75 per cent acetic acid and rolling a glass rod over it to crush the tissues. The preparation is covered with a cover glass and set aside; after a few hours, needle-shaped aggregates of deep red crystals will be formed along the edge of the cover glass.

Reactions and Properties.

1. The anthocyanins are soluble in water and in alcohol,* but are insoluble in ether.

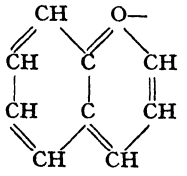
2. Solutions are turned red by acid and blue by alkalis; owing to the almost universal contamination with flavonols, the crude aqueous extracts of anthocyanins from plants usually give a green coloration with alkali due to the simultaneous production of blue and yellow.

The red juice of an unboiled beet and the anthocyanins of the Chenopodiaceæ generally, give a purple colour with acid and a yellow with alkali.

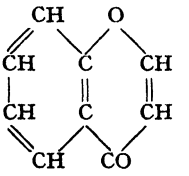
3. Lead acetate or basic lead acetate give with anthocyanins bluish-green or green precipitates; with beet juice and similar anthocyanins basic lead acetate produces a red precipitate.

CHEMICAL CONSTITUTION.

The anthocyanidins or non-carbohydrate moiety of the anthocyanins are derivatives of benzo-pyrilium which, as may be seen from the appended formula I.—



I.

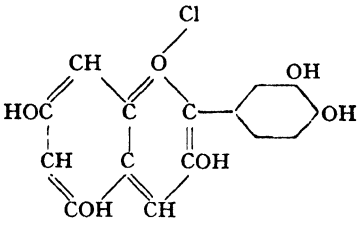


II.

is closely related to benzo-pyrone II., the mother substance of the flavones. Both these substances contain a so-called basic oxygen atom which by becoming tetravalent can form additive compounds with acids producing oxonium salts. These

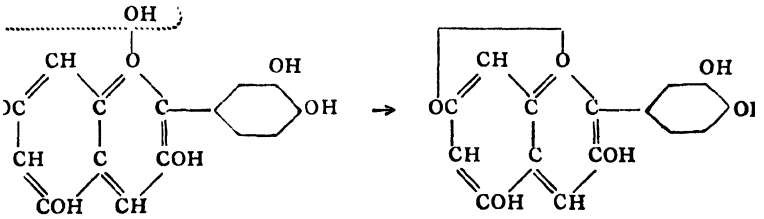
* In some cases, e.g. pelargonium, the pigment loses its colour in alcohol, but the colour may be restored by the addition of acid, or by evaporating off the alcohol and taking up the residue in water.

salts in the case of the flavones are not stable and do not occur in the plant, but the anthocyanidins yield stable oxonium salts of the type—



III.

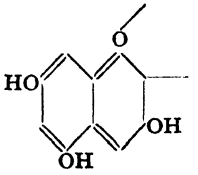
which is the formula assigned to cyanidin chloride; this represents the red form of cyanidin in acid solution; replacement of the —Cl by —OH on treatment with alkali permits the formation of an anhydride IV.—



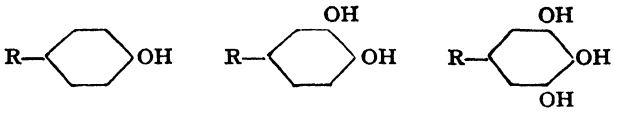
IV.

which represents the neutral form of violet tint, while the blue compound occurring in alkaline solution would be formed by introducing the alkali metal into one or more of the hydroxyl groups.

In the various anthocyanins so far examined, the hydroxy-



benzopyrilium group which may be represented by the symbol R, is the same in all cases; the variation between the different compounds is due to differences in the number and position of the hydroxyl groups in the benzene ring; thus the abbreviated formulæ V., VI., and VII.—



V. VI. VII.

represent pelargonidin, cyanidin, and delphinidin respectively.

It must be borne in mind that the anthocyanins are glucosides, and as a rule occur in the plant in this form; occasionally, however, they occur free as sugar-free anthocyanidins; thus on one occasion Willstätter found in the case of the black Alicante grape as much as 12 per cent of the pigment in the sugar-free condition.

To distinguish between an anthocyanin glucoside and a non-glucosidal anthocyanidin, Willstätter and Everest shake the solution of the pigment in 0·5 per cent hydrochloric acid with amyl alcohol; the glucoside anthocyanins remain in the aqueous layer, while the sugar-free anthocyanidins pass into the amyl alcohol.

The relations between some anthocyanin glucosides and their corresponding anthocyanidins is given in the following list:—

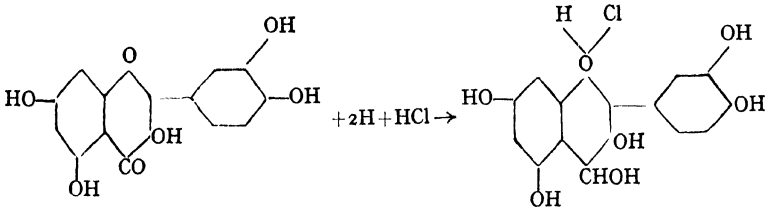
Pelargonin	is a	diglucoside of pelargonidin.
Callistephin	,,	monoglucoside of pelargonidin.
Cyanin	,,	diglucoside of cyanidin.
Peonin	,,	diglucoside of peonidin (a monomethyl ether * of cyanidin).
Idæin	,,	monogalactoside of cyanidin.
Delphinin	,,	diglucoside of delphinidin united to *p*-hydroxy-benzoic acid.
Violanin	,,	rhamnoside of delphinidin.
Myrtillin	,,	monoglucoside of myrtillidin (a monomethyl ether * of delphinidin).
Malvin	,,	diglucoside of malvidin (a monomethyl ether of delphinidin).
Œnin	,,	monoglucoside of œnidin (a dimethyl ether * of delphinidin).

The conversion of the flavonol quercetin I. into the anthocyanidin cyanidin II. was first carried out by Willstätter and Mallison,† and thus the first synthesis of an anthocyanin was effected, since flavonol had already been synthesized by

* The point of attachment of the methoxyl groups in the compounds is not known with certainty.

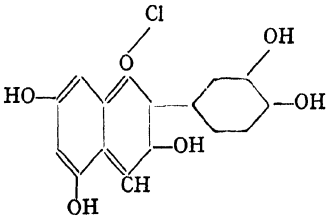
† Willstätter and Mallison: "Sitzungsber, Kgl. Preuss. Akad. d. Wiss.," 1914, 769.

Kostanecki * ; the way in which the change was effected may be seen from the following formulæ :—



Quercetin I. Intermediate reduction compound.

The latter by loss of water yielding—

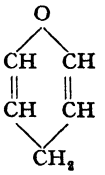


Cyanidin chloride II.

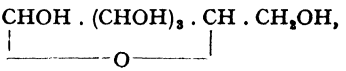
This may be experimentally verified by carefully adding magnesium powder to a solution of quercetin in a mixture of five volumes of alcohol with one volume of concentrated hydrochloric acid, when a rose-pink rapidly develops.†

It has been pointed out on page 338 that the evidence from the plant shows that there is some fairly close relationship between carbohydrates and anthocyanin production ; it was suggested by Robinson ‡ that the C_{15} nucleus of the anthocyanins, flavones and flavonols, might be derived from the union of two molecules of glucose connected together by aldol condensations with glycerose (dihydroxy acetone). On the other hand, a different point of view with regard to the significance of carbohydrates in contributing to the formation of anthocyanins is offered by Goodyear and Haworth § who draw attention to the fact that the pyran residue—

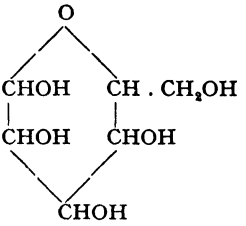
* Kostanecki : " Ber. deut. chem. Gesells.," 1904, **37**, 1402.
† Everest : " Proc. Roy. Soc.," 1914, B., **87**, 444.
‡ Robinson : " Brit. Assoc. Reports," Edinburgh, 1921.
§ Goodyear and Haworth : " J. Chem. Soc.," 1927, 3141.



which is a constituent of the anthocyanin structure, is also the basis of the normal sugars having the amylene oxide configuration—



which may also be represented as follows—



THE COLOUR OF PETALS.

The pigments contributing to the colour of petals may belong to any or all of the three groups, carotinoids, anthoxanthins, and anthocyanins.

In the case of *white* flowers, only anthoxanthins occur, and these may be detected by the yellow colour developed on exposure to ammonia.

Yellow flowers, such as daffodils, contain both carotinoids and anthoxanthins ; this may be shown by boiling the yellow petals with alcohol, filtering and evaporating the filtrate to dryness over a water bath ; the residue taken up with ether and water on shaking in a separating funnel gives an ethereal layer containing the carotinoids and an aqueous layer containing the anthoxanthins.

Brown flowers, such as wallflowers, contain anthocyanin in addition to carotinoids and anthoxanthin ; to separate proceed as for yellow petals ; the aqueous layer, which this time will be brown, contains the two types of water-soluble sap pigments ; if sufficiently concentrated, the flavonol glucoside,

quercetrin, will separate out in yellow crystals, having a deep purple mother liquor which will give the reactions of anthocyanins.

In the case of those flowers in which the anthocyanin pigment predominates, such as in most of the blue, red, or purple flowers, the particular shade of colour is due partly to the configuration of the anthocyanin concerned and partly to the reaction of the cell sap. Somewhat conflicting views on this question are held by Shibata * and others.

These facts explain the colour variations produced by the same cyanidin occurring in the same or in different flowers, it having been found, for example, that the same cyanidin was responsible for the colour of the cornflower and of the red rose.† Thus when combined, as in the case of cyanidin chloride, with mineral acid or in the plant with organic acids, the compound has a red tint. When treated with alkali, blue metallic salts are formed, while the arrangement shown in the formula IV. (p. 346) represents a neutral compound having a violet tint. The neutral violet-tinted delphinin has been isolated from *Delphinium consolida* by Willstätter and Mieg,‡ and has been shown to turn blue with alkali, and red with acids ; the colour would therefore appear to act as an indicator in the plant itself, showing whether the cell sap is neutral, acid, or alkaline.

Willstätter§ further found that the cornflower contained three modifications of the same anthocyanin, namely the purple form of cyanin itself, the blue form which is the sodium salt of this, and the red oxonium salt of the cyanin with some organic acid present in the plant.

Sometimes it is observed that the leaves of certain plants when first they unfold are bright red and that in a few days this colour fades away and the green colour is seen. Noack ‖ has investigated this phenomenon in *Polygonum compactum*,

* Shibata, Shibata and Kasiwagi : " J. Amer. Chem. Soc.," 1919, **41**, 208.

† Willstätter and Nolan : " Annalen," 1915, **408**, 1.

‡ Willstätter and Mieg : *id.*, 1915, **408**, 61.

§ Willstätter : *id.*, 1913, **401**, 189.

‖ Noack : " Zeitsch. Bot.," 1918, **10**, 561.

and thus explains it: by the action of an enzyme the anthocyanin is converted into anthocyanidin and a sugar. The anthocyanidin is then converted into a colourless pseudobase which may be oxidized to a yellow pigment. In the process, light is of importance; the pseudobase is due to the photochemical reduction of the oxidation product of the original pigment. In the dark, on the other hand, anthocyanidin is oxidized, a process accelerated by heat. Noack * also concludes that the equilibrium between flavonols and their reduction products, the anthocyanins, normally is on the side of flavonol. If assimilation is inhibited or depressed, anthocyanin is formed. Noack's conclusions based on his observations on *Polygonum compactum* and other plants is criticized by Combes † who suggests that the anthocyanidin pseudobases were probably phlobatannins, and the red substances which Noack obtained by the action of acids were probably phlobaphenes and not anthocyanidins.

CONNECTION BETWEEN ANTHOCYANINS AND ANTHOXANTHINS.

A comparison of the formula of cyanidin chloride on page 348 with that of quercetin reveals a close relationship between these two substances, and consequently between the flavones or anthoxanthins and the anthocyanins. Theoretically it should be possible to pass from anthoxanthins to anthocyanins by reduction, or conversely from anthocyanins to anthoxanthins by oxidation. In the plant no doubt this is effected readily enough by enzymes, but in the laboratory it is more difficult, and so far the only transformation effected has been the reduction of quercetin to cyanidin.‡

The view first put forward by Wheldale § was that the flavones and flavonols were the precursors of the anthocyanins; according to her the conversion of flavonols into anthocyanins was due to oxidation. Evidence in support of

* Noack: "Zeit. Bot.," 1922, **14**, 1.

† Combes: "Compt. rend.," 1922, **174**, 58, 240.

‡ Willstätter and Mallison: "Sitzungsber. K. Akad. Wiss. Berlin," 1914, 769.

§ Wheldale: "Proc. Camb. Phil. Soc.," 1909, **15**, 137; "Journ. Genet.," 1911, **1**, 10.

this view was provided by experiments which indicated that for the production of anthocyanin, two factors are requisite—the flavonol, and an oxidizing enzyme. Thus a magenta *Antirrhinum* produced two sports, an "ivory white," which contained the flavonol apigenin, a peroxidase, but no oxidase, and a "dead white" which contained no flavonol but presumably some other factor essential to anthocyanin formation, since on crossing these two varieties, magenta flowers resulted.

Keeble and Armstrong * suggested that anthocyanin formation is associated with the action of peroxidase upon a chromogen; they found that in coloured and recessive white flowers of *Primula sinensis*, the distribution of peroxidase was identical with that of the pigment, whilst dominant white varieties contain no peroxidase. This view has not found acceptance; in fact the mass of evidence leads to the conclusion that neither peroxidase nor oxidase play a part in the flavone-anthocyanin system.†

Views diametrically opposed to the idea that anthocyanin is an oxidation product have been put forward by Combes ‡ who claims to have shown that *Ampelopsis hederacea* contains both a flavone and an anthocyanin and to have converted the former into the latter by reduction and anthocyanin into flavone by oxidation. These views are criticized by Jonesco § who considers the red pigments obtained by Combes were due to the action of the acid used.

Everest and Hall ‖ examined a number of flower buds selected for the well-marked anthocyan content of their mature petals, such as auricula, apple, azalea, polyanthus, viola, etc. In all cases it was found that before anthocyanin appeared the petals were yellow or colourless, but they contained substances, presumably flavonols, which turned yellow with ammonia. On treating an alcoholic extract of a red rose and of a mauve violet, collected before anthocyanin had appeared,

* Keeble and Armstrong: "Proc. Roy. Soc.," B., 1912, **85**, 214.

† See Wheldale-Onslow: "The Anthocyanin Pigments of Plants," Cambridge, 1925.

‡ Combes: "Compt. rend.," 1913, **157**, 1002, 1454; 1914, **158**, 272.

§ Jonesco: *id.*, 1921, **173**, 850, 1006.

‖ Everest and Hall: "Proc. Roy. Soc.," B., 1921, **92**, 150.

with magnesium, a pale red colour was produced; they conclude that the young buds contained flavonols which would have developed anthocyanin by reduction of the flavonol in the course of their development.

There are, in fact, two schools of thought; Everest, Combes, and Costantin * consider that the anthocyanins are reduction products of flavones, whilst Noack, Jonesco, Wheldale, and others,† consider them to be oxidation products of glucosidal flavones. Purely chemical evidence shows that anthocyanin production from a given flavonol proceeds by reduction, but chemical proof of the relationship between the flavonols and anthocyanins occurring together in the same tissues has not as yet been provided. It is possible that the anthocyanin in a given plant material might actually contain a greater number of hydroxyl groups than the flavonol accompanying it, so that while the conversion of the flavonol into the corresponding anthocyanin would result from reduction, the introduction of an increased number of hydroxyl groups would nevertheless involve oxidation, so that both schools of thought would be justified.

PHYCOERYTHRIN.

Phycoerythrin is a red pigment commonly occurring in red sea-weeds, associated with the chlorophyll and carotin in the chloroplasts. It has been investigated more particularly by Hanson,‡ Molisch,§ and Rodio.||

Phycoerythrin is soluble in water, giving a rose-coloured solution which exhibits a well-marked orange fluorescence; the spectrum shows three absorption bands in the green, the exact positions varying with different species. Freshly prepared aqueous solutions will yield crystals on evaporation and, according to Rodio || the addition of ammonium sulphate hastens the formation of hexagonal prisms or tablets.

* Costantin: "Ann. Sci. Nat. Bot.," 1919, x., 1, 38.
† See Kozlowski: "Compt. rend.," 1921, **173**, 855.
‡ Hanson: "New Phytologist," 1909, **8**, 337.
§ Molisch: "Bot. Ztg.," 1894, **52**, 177.
|| Rodio: "Atti. Accad. Lincei," 1925, vi., 1, 188.

Preparation.

To prepare a solution of phycoerythrin the red sea-weed, *Ceramium rubrum*, which is one of the best to use, is washed in ordinary water to free it from sea salts and adhering sand. It is then soaked in distilled water; in two days most of the pigment will have diffused out. The solution is filtered through glass wool, and a few drops of eucalyptus oil or carbon bisulphide are added as an antiseptic, for putrefaction soon sets in.

It is a matter of great difficulty to obtain a pure sample of phycoerythrin, for, in an aqueous solution, it passes over into an irreversible gel,* even when kept at 0° C. This, of course, renders ordinary filtration extraordinary slow, and thus increases the difficulty of purification.

The solid phycoerythrin may be prepared from the aqueous solution by concentrating it under reduced pressure at a temperature not higher than 38° C.; any precipitate which comes down during this process must be filtered off. Methylated spirit is then added to the concentrated solution until the fluorescence disappears. The precipitated phycoerythrin is allowed to settle and the more or less clear supernatant fluid is filtered off, again treated with alcohol, and filtered. The operation is repeated until the red colour has entirely disappeared from the solution. The precipitates are washed by decantation with 70 per cent alcohol; the pigment, in a pasty mass, is placed in a clock glass and dried in a vacuum.

Reactions.

1. Phycoerythrin is precipitated from its solution by alcohol, by small quantities of mercuric chloride, and by saturation with ammonium sulphate or magnesium sulphate.

2. When dilute acids are added gradually, the fluorescence first disappears, leaving a somewhat opalescent solution of a lilac-pink tint. After the lapse of two days a pink precipitate comes down.

3. Ammonium hydrate in small quantities removes the fluorescence; in excess, a yellowish-brown coloration results.

* See Section VIII. on the Colloidal State.

4. Caustic soda or potash in small quantities causes the red colour to disappear, the solution turning opalescent and yellowish-brown in colour ; on standing, a brownish precipitate comes down.

5. The solution is immediately decolorized by bleaching powder, bromine water or a solution of iodine in potassium iodide.

6. Mercuric chloride solution in small quantities gives a lilac-grey precipitate, the solution then being yellowish in colour.

7. Ferric chloride gives a pinkish-brown precipitate.

8. Boiled with nitric acid a yellow colour results which turns to orange on adding an excess of ammonia.

9. Boiled with Millon's reagent a deep red colour results.

10. The addition of a caustic soda solution followed by a drop or two of dilute copper sulphate gives a greenish tint.

11. Digestion with pepsin, in the presence of hydrochloric acid, has no result.

12. On digestion with trypsin in the presence of sodium carbonate, the phycoerythrin loses its colour, and the solution contains a very small amount of leucin, but no tyrosin.

13. On hydrolysis with acids, tyrosin is found in very small amounts, but leucin occurs in greater quantities.

From these and other facts it is concluded that phycoerythrin is a colloidal nitrogenous substance allied to the proteins ; it is not a true protein, since its nitrogen content is too low and it does not give the biuret reaction. It is impossible to say anything more definite regarding its chemical nature until it has been prepared in a pure state in quantities sufficient for analysis.

According to Kylin,* phycoerythrin, separated from *Ceramium rubrum*, is made up of two constituents, a protein combined with a colouring matter which can be hydrolysed by acid or by alkali.

Physiologically, phycoerythrin acts as a pigment complementary to chlorophyll. It absorbs the blue-green rays,

* Kylin : " Zeit. physiol. Chem.," 1910, **69**, 169 ; 1912, **66**, 82.

and degrades them to yellow and red light of just those wavelengths which the chlorophyll can absorb.

PHYCOPHAEIN.

As is well known, a brown colouring matter may be extracted by water from the Phæophyceæ and other brown Algæ. Hitherto this has generally been considered to be due to the presence within the cells of a definite colouring matter of a protein nature. According, however, to the work of Molisch * and Tswett,† this is not the case. The brown colouring matter is really due to post-mortem changes, the oxidation of a water-soluble chromogen. An extract prepared with distilled water is at first colourless, but will turn yellow if the solution is made alkaline in reaction, e.g. by tap water, and finally brown owing to oxidation. If the reaction be made acid decolorization will result. With regard to the chemistry of this substance little, if anything, is known.

PHYCOCYANIN.

Phycocyanin is a generic term ‡ and includes several blue pigments characteristic of the Cyanophyceæ but not necessarily restricted to this group, for Kylin and Rodio § have found phycocyanin to be associated with phycoerythrin in *Ceramium*, *Chondrus*, and other members of the Rhodophyceæ.

Phycocyanin is soluble in water, giving a blue solution which exhibits a carmine fluorescence. Its absorption spectrum shows one or two bands in the orange-red.

Preparation.

To prepare phycocyanin, Molisch ‖ recommends *Oscillaria leptotricha;* the plants are rapidly washed with distilled water and placed in a beaker with enough distilled water to cover them completely. A little carbon bisulphide is added

* Molisch: "Bot. Ztg.," 1894, **52**, 181; 1895, **53**, 131; 1905, **63**, 131.
† Tswett: "Ber. deut. bot. Gesells.," 1906, **24**, 235.
‡ Molisch: "Sitz. Kais. Akad. Wiss. Wien," 1906, **115**, [1], 795. Kylin: "Zeit. physiol. Chem.," 1912, **76**, 396.
§ Rodio: *loc. cit.*
‖ Molisch: "Bot. Ztg.," 1905, **63**, 159.

and the preparation is allowed to stand for twenty-four hours. The deep indigo solution is then filtered off and ammonium sulphate added in quantity insufficient to cause precipitation; on allowing the mixture to evaporate in air in a dark place, crystals of phycocyanin will be deposited.

Little is known of its chemical constitution; it is of a protein nature and its physical properties resemble those of phycoerythrin.

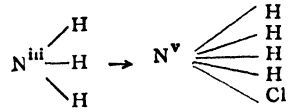
With regard to the physiological significance of these pigments of the Algæ, the work of Gaidukow * and others on complementary chromatic adaptation may be consulted.

* Gaidukow: "Ber. deut. bot. Gesells.," 1903, **21**, 484, 517; 1906, **24**, 1, 23. Richter: *id.*, 1912, **30**, 280. Boresch: *id.*, 1919, **37**, 25; 1921, **39**, 93. Harder: "Zeit. bot.," 1923, **15**, 305.

SECTION VII.

NITROGEN BASES.

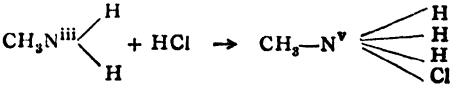
AMMONIA is said to have basic properties because it can form salts by combining with acids. This salt formation, which may be illustrated by the conversion of ammonia into ammonium chloride, is due to the unsaturated nature of the trivalent nitrogen atom, and its tendency to assume the pentavalent condition.



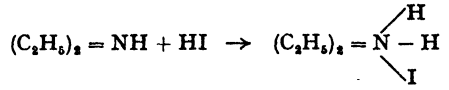
Ammonia Ammonium chloride

The replacement of one or more of the hydrogen atoms in ammonia by organic radicles, such as methyl, CH_3—, ethyl, C_2H_5—, or phenyl, C_6H_5—, gives rise to compounds known as amines or substituted ammonias, which still retain the property of salt formation possessed by the parent substance ammonia.

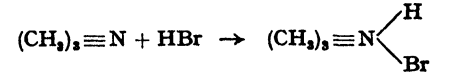
For example :—



Methylamine Methylamine hydrochloride, or Methylammonium chloride



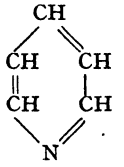
Diethylamine Diethylamine hydriodide



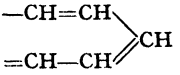
Trimethylamine Trimethylamine hydrobromide

These three substances, CH_3NH_2, methylamine, $(C_2H_5)_2NH$, diethylamine, and $(C_2H_5)_3 : N$, triethylamine, are types of three different classes of amines, known respectively as primary, secondary, and tertiary amines, according as one, two, or three of the hydrogens of ammonia have been replaced by organic radicles.

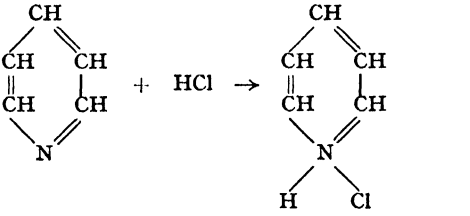
Tertiary amines are also known in which the nitrogen atom takes part in the formation of a ring, as, for example, in pyridine—



which may be regarded as being derived from ammonia by the replacement of three atoms of hydrogen by the five carbon ring—



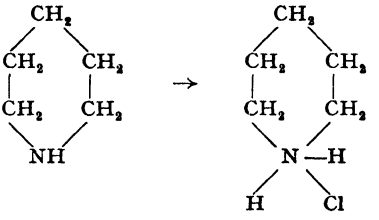
Pyridine, being a substituted ammonia, can form salts by changing the valency of its nitrogen atom from three to five, as follows :—



Pyridine Pyridine hydrochloride

Secondary amines containing a nitrogen atom in the ring are also known.

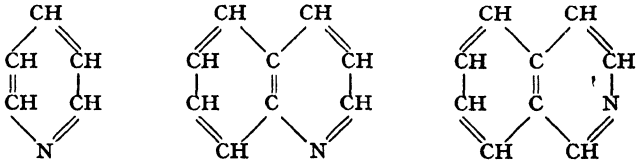
Thus, when pyridine is reduced by nascent hydrogen, six atoms of hydrogen are added on, and a substance known as piperidine is produced ; this substance is a secondary amine, since it now has a hydrogen atom attached to its nitrogen. Like pyridine, it can also form a salt with hydrochloric acid.



Piperidine Piperidine hydrochloride

From the above examples it will be seen that the presence of a trivalent nitrogen atom in a compound, whether in a ring or attached to a straight chain, will, as a rule, confer on that compound basic properties, owing to the tendency of that nitrogen to become pentavalent by combining with an acid and producing a salt. It is this property which gives rise to the term *Nitrogen base.*

The discovery and isolation from natural sources of a number of nitrogen bases, such as cinchonine, quinine, brucine, strychnine, morphine, etc., having properties analogous to those of the alkalis in being able to form salts with acids, led to their designation as alkaloids or alkali-resembling substances. As the number of such substances increased, a distinction began to be made between animal and vegetable alkaloids. The term alkaloid is, however, better reserved for nitrogen bases of vegetable origin; it was at one time suggested that the term should include only derivatives of pyridine, quinoline, and isoquinoline—



Pyridine Quinoline Isoquinoline

but this definition excludes such compounds as stachydrine and hygrine, etc., which are pyrrolidine derivatives, and also the purine bases which, according to most authors, should be included among the alkaloids.

This difficulty is, however, overcome by defining alkaloids as nitrogen bases of vegetable origin whose nitrogen atom forms part of a ring.

Even this definition is not entirely satisfactory, as it would include substances which, owing to their properties, could hardly be classed as alkaloids, and excludes others, such, for example, as hordenine.

ALKALOIDS.

Occurrence.

The alkaloids do not appear to have a wide distribution in the vegetable kingdom. Amongst the Angiosperms, the Apocynaceæ, Leguminosæ, Papaveraceæ, Ranunculaceæ, Rubiaceæ and Solanaceæ stand out in the provision of several of these substances. The Labiatæ, Rosaceæ, Orchidaceæ, and Monocotyledons and Gymnosperms very rarely contain them.

Alkaloids may occur in solution in the cell sap, especially in young parenchyma: in older tissues the substances in question may be stored in the solid state. They are found in the seeds and fruits more particularly, but in the case of the alkaloids of the Solanaceæ and some other plants they occur in the leaves, whilst the roots are the chief sources of the alkaloids of *Aconitum*, *Corydalis*, and *Hydrastis*. The cinchona alkaloids, and also pelletierine of the pomegranate, are contained in the bark of their respective trees.

With regard to their distribution in the different members of the plant, there is so much variation that a single example must serve. Stanek * found that the percentages of betaine, expressed in terms of dry weight, occurring in *Lycium barbarum*, were young leaves 3·91, old leaves 1·62, flowers without calyx 1·5, young shoots 1·55, bark of root ·49, and wood ·12.

Classification.

The classification of the alkaloids is based upon the structure of the nucleus upon which their molecules are built up. Five groups of alkaloids are accordingly recognized.

I. *Pyridine Alkaloids.*—These, as the name implies, are all derivatives of pyridine, and include—

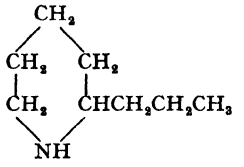
Coniine from *Conium maculatum.*

Arecolin from *Areca catechu.*

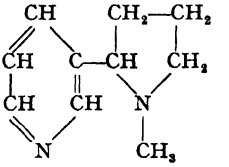
* Stanek: "Zeitsch. Zuckerind.," 1913, **37**, 385.

Trigonellin from *Trigonellum fœnum*, *Pisum sativum*, etc.
Piperine from *Piper*, and
Nicotine from *Nicotiana tabacum*.

Some idea of the structure of the molecules of alkaloids belonging to this group may be obtained from the two following constitutional formulæ, which represent coniine and nicotine respectively :—

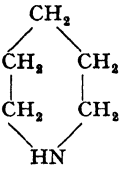


Coniine



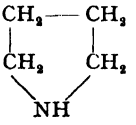
Nicotine

From these formulæ it may be seen that coniine is derived from pyridine or more strictly from piperidine—



Piperidine

whilst nicotine contains two rings, one a pyridine ring and the other a pyrrolidine ring—



Pyrrolidine

such as is also found in proline (see p. 444).

II. *Pyrrolidine Alkaloids.*—This is a small group, comprising as yet only three alkaloids, namely :—

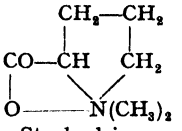
Hygrine and Kushygrine, from the leaves of *Erythroxylon Coca*, and

Stachydrine, from the tubers of *Stachys tuberifera* * and leaves of *Citrus vulgaris*.†

* Planta and Schulze : " Arch. d. Pharm.," 1893, 305 ; " Ber. deut. chem. Gesells.," 1893, **26**, 939. Schulze and Trier : *id.*, 1909, **42**, 4654 ; " Zeit. physiol. Chem.," 1910, **67**, 59.

† Jahns : " Ber. deut. chem. Gesells.," 1896, **29**, 2065.

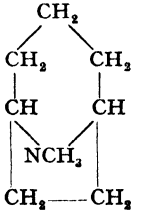
The constitution of stachydrine is as follows :—



Stachydrine

showing it to be a dimethyl betaine of pyrrolidine.

III. *Tropane Alkaloids.*—The alkaloids belonging to this group are derivatives of tropane—



Tropane

which substance, as may be seen, contains both a six-membered piperidine ring and a five-membered pyrrolidine ring.

The group includes alkaloids from the four Natural Orders :—

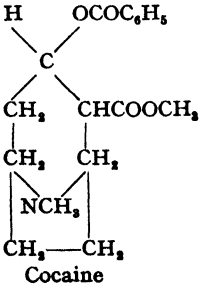
Solanaceæ, e.g. Atropine, Hyoscine, Hyoscyamine.

Erythroxylaceæ, e.g. Coca alkaloids, such as Cocaine and Tropacocaine.

Myrtaceæ : Pelletierine, Isopelletierine, etc., from *Punica granatum* (pomegranate).

Papilionaceæ : Cytisine from *Cytisus Laburnum ;* Lupinine from *Lupinus luteus*, and *Lupinus niger.*

Most of the above alkaloids have a very complex constitution, and the formula of only one will be given, namely, cocaine :—



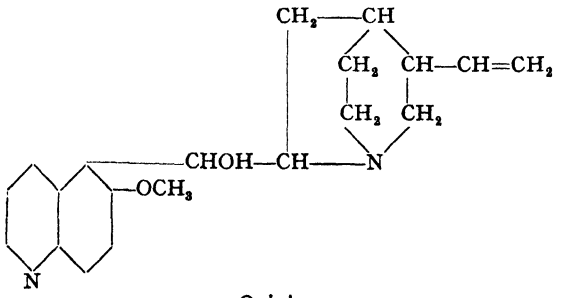
Cocaine

IV. *Quinoline Alkaloids.*—These fall into two groups :—

(*a*) Cinchona alkaloids, such as Quinine, Cinchonine, etc., from the bark of various species of *Cinchona* (Rubiaceæ).

(*b*) Strychnos alkaloids, such as Strychnine and Brucine from *Strychnos nux vomica*, *S. Ignatii*, etc., and Curarine from *Strychnos toxifera* (Loganiaceæ).

The constitution of quinine is represented by the following formula * :—



Quinine

from which it will be seen to contain a quinoline ring.

The constitution of strychnine and brucine has not yet been determined, though possible formulæ have been suggested by Perkin and Robinson.†

V. *Isoquinoline Alkaloids.*—These may be divided into the three following groups :—

(*a*) Papaverine group, including Papaverine, Narcotine, Laudanosine, etc., closely allied to which are Hydrastine and Hydrastinine from *Hydrastis canadensis.*

(*b*) Morphine group, including Morphine, Apomorphine, Thebaine, and Codeine.

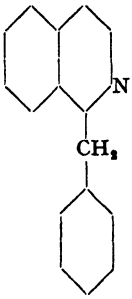
(*c*) Berberine group, including Berberine and Corydalis alkaloids.

The constitutional formulæ for alkaloids of this group are for the most part exceedingly complex, and it will suffice here

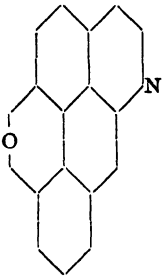
* This formula, though probably correct, has not yet been confirmed by synthesis.

† Perkin and Robinson : " J. Chem. Soc. Lond.," 1910, **97**, 305.

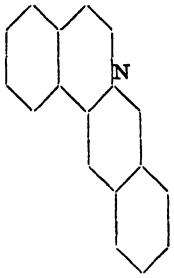
merely to show the skeleton formulæ of a member of each group :—



Papaverine



Morphine *



Berberine

In addition to the alkaloids mentioned above, there are a very large number which cannot as yet be classified, since their constitution is not entirely known ; these include amongst others ergotinine from ergot, colchicine from *Colchicum*, taxine from *Taxus baccata*, aconitine from *Aconitum Napellus*, delphinine from *Delphinium*, etc.

GENERAL PROPERTIES OF ALKALOIDS.

The alkaloids are, as a rule, composed of the four elements, carbon, hydrogen, nitrogen, and oxygen, but a few are known, such as coniine, nicotine, and one or two little-known ones, such as hymenodictine and conessine (from bark of *Wrightia antidysenterica*), which contain no oxygen.

There are a few alkaloids which are liquid, e.g., coniine, nicotine, pelletierine, sparteine, etc., but by far the greater number are colourless crystalline solids. They are, as a rule, insoluble in water, but dissolve in neutral organic solvents, such as ether, amyl alcohol, chloroform, carbon tetrachloride, etc., whereas their salts have just the opposite solubilities.

They are mostly free from smell, but coniine, nicotine, and sparteine have strong odours.

Most of them have a bitter taste and are possessed of marked physiological or toxic properties.

They are all bases, and accordingly have an alkaline reaction in solution, though it must be borne in mind that

* This formula is subject to revision.

aqueous solutions of the salts usually have a strongly acid reaction due to hydrolytic dissociation.

The majority of alkaloids are optically active, rotating the plane of polarized light to the left, though a few, such as coniine, laudanosine, pelletierine and pilocarpine, are dextro-rotatory.

GENERAL REACTIONS OF ALKALOIDS.

The alkaloids are precipitated from solution by a large number of different reagents with formation of amorphous or sometimes crystalline precipitates.

The commonest of these reagents are the following :—

1. A solution of iodine in potassium iodide, sometimes known as potassium ter-iodide, gives a chocolate-brown precipitate.
2. Mercuric iodide in potassium iodide,
3. Tannic acid,
4. Phosphotungstic acid,

   } all of which give colourless amorphous precipitates.

5. Auric chloride,
6. Platinic chloride,

   } which give crystalline precipitates often having characteristic melting-points.

The alkaloids are, however, not the only substances which are thrown out of solution by these reagents, since most nitrogen bases behave in a similar way, and the formation of a precipitate is therefore not conclusive proof of the presence of alkaloids. On the other hand, if none of the above reagents produce precipitates, it is tolerably certain that there are no alkaloids present.

In examining plant tissues for alkaloids, Errera recommends testing the fresh sections with alkaloidal reagents and also sections which have been soaked in a 5 per cent alcoholic solution of tartaric acid. In the second case no precipitate should be obtained, owing to the extraction of the alkaloid.

The final identification of the various alkaloids is usually effected by means of colour reactions.

Thus, if a section of the endosperm of *Strychnos nux*

vomica be mounted in a few drops of strong sulphuric acid, the presence of strychnine is indicated by a red coloration of the cell-contents. This colour will change to violet on placing a small crystal of potassium chromate beneath the cover-glass.

Similarly, a section of the rhizome of *Aconitum Napellus*, when treated with a few drops of 50 per cent sulphuric acid, will shown a carmine red coloration, due to the presence of aconitine, in the parenchyma surrounding the vascular bundles. This reaction is the more marked when the section has been previously warmed in a solution of sucrose.

These colour reactions are very numerous; for them the larger text-books and monographs must be consulted.

Isolation.

Most alkaloids do not occur free in the plant, but combined with some acid in the form of a salt; the acids most commonly met with are tannic, malic, citric, succinic, and oxalic, while acetic and lactic acids are rarer; some acids occur only in connection with certain alkaloids, such as meconic acid with opium and quinic acid with quinine.

In some few cases the alkaloids can be extracted from their natural sources by means of organic solvents, such as chloroform, carbon tetrachloride, ether, etc., but in the majority of cases the alkaloid requires to be set free first by the addition of an alkali, such as lime or baryta, since only the free bases, and not the salts, are soluble in the above-mentioned solvents.

The material to be extracted is mixed with slaked lime and carefully dried, and then extracted in a Soxhlet extractor with chloroform or carbon tetrachloride; the extract is then shaken up with dilute sulphuric acid, whereby the sulphate is formed; the acid layer containing the salt in solution is then run off and evaporated, when the alkaloid salt crystallizes out and can be further purified by recrystallization.

Example.—Preparation of quinine from cinchona bark. Twenty grams of quicklime are stirred up with 200 c.c. of water and then thoroughly mixed in a mortar with 100 grams

of cinchona bark which have been ground up in a coffee mill. The resulting mixture is then dried over a water bath, care being taken to prevent the formation of lumps. The dried substance is then extracted in a Soxhlet apparatus with chloroform. The extract is then shaken up with 25 c.c. of dilute sulphuric acid, the chloroform layer being run off from below; it is then shaken up with water several times and the water and acid extracts are mixed together and neutralized with ammonia. On evaporating the solution, quinine sulphate crystallizes out; the amount obtained rarely exceeds 1-2 grams in weight.

A rapid way of testing a piece of bark for quinine consists in heating it in a dry test tube. If there is any quinine present, the bark will give off a carmine-coloured vapour.

THE ORIGIN OF ALKALOIDS IN THE PLANT.

Gadamer * expresses the view that the primary products of assimilation are the same for proteins and for alkaloids. When assimilation is intense alkaloids are produced, but during periods of diminished assimilation the enzyme which synthesize proteins may break down the alkaloids, the disintegration products of which may be used in the formation of proteins.

According to Pictet,† alkaloids are produced in the plant in two successive stages, involving (1) the breakdown of complex nitrogenous substances, such as protein or chlorophyll, with the production of relatively simple basic substances; (2) the condensation of these relatively simple substances with other compounds present in the plant, with the formation of the complex molecules possessed by the alkaloids.

The processes of metabolism within the plant would therefore be strictly analogous to those taking place in the animal body, in which waste products, such as phenol, glycine, etc., are coupled up with other substances, such as sulphuric or benzoic acid, before being eliminated.

Pictet is further of opinion that among the commonest

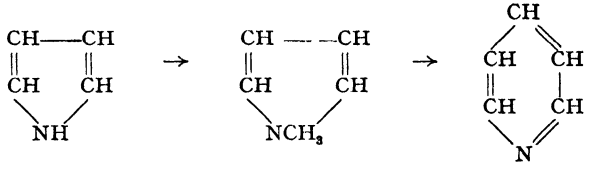
* Gadamer: "Ber. deut. pharm. Gesells.," 1914, **24**, 35.

† Pictet: "Arch. Sci. Phys. Nat.," 1905, [iv], **19**, 329; "Ber. deut. chem. Gesells.," 1907, **40**, 3771.

changes within the plant are the methylation of hydroxyl or amino groups by formaldehyde, according to the equations—

$$ROH + CH_2O = ROCH_3 + O$$
$$\text{and } RNH + CH_2O = RNCH_3 + O$$

the resulting methylated compounds being then able to undergo intramolecular transformation, by which the methyl group can enter the ring, and so produce, for example, a pyridine ring from methyl pyrrole, a reaction which he has been able to effect in the laboratory by heat—



Pyrrole Pyridine

Similar changes would also explain the formation of quinoline and isoquinoline, and it thus becomes possible to account for the origin of the pyridine and quinoline rings which occur in alkaloids, by assuming them to have been produced as above from pyrrole or indole rings, which are the normal constituents of protein (e.g. proline, histidine, tryptophane, etc.).

In support of these views, Pictet states that he was able to isolate by steam distillation from various leaves,* etc., treated with sodium carbonate, a number of simple bases which he calls proto-alkaloids ; these include pyrrolidine and methyl pyrroline—



Pyrrolidine Methyl pyrroline

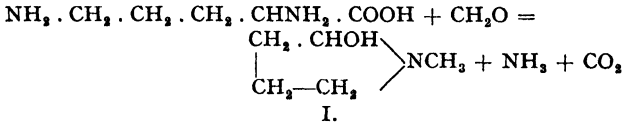
whose origin from the protein molecule is readily intelligible, in view of the fact that a similar ring occurs in proline, the cleavage product of a number of proteins. It is assumed that these proto-alkaloids are subsequently methylated, rearranged

* The leaves used were those of tobacco, carrot, parsley and coco.

and condensed as described above to form the more complex alkaloids.

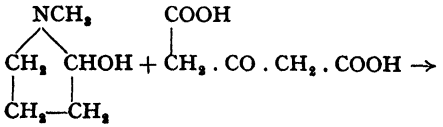
It has been suggested by Pictet that the secretion of alkaloids by plants is merely due to the inability of such plants to get rid of their nitrogenous products of metabolism by any other means than by converting them into alkaloids, which, though poisonous to animals, are not toxic to the plants themselves.

Robinson, from his work on tropinone,* offers a theory of the mechanism of the photochemical synthesis of certain alkaloids which differs fundamentally from the opinions of Pictet.† The raw materials—formaldehyde, ammonia, amino acids, and acetone dicarboxylic acid—for building up alkaloids either occur as such in the plant or in a combined state. These highly reactive bodies undergo a series of comparatively simple transformations ultimately leading to the alkaloid. Thus the condensation of formaldehyde with a diamino acid such as ornithine would account for the pyrrolidine group; a compound of the formula I. could be formed by the interaction of these two substances according to the equation—

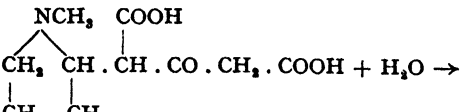


I.

This compound would yield the alkaloid hygrine (IV.) by condensation with acetone dicarboxylic acid and subsequent elimination of carbon dioxide :—

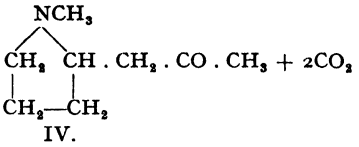


II.



III.

* Robinson : " Journ. Chem. Soc. Lond.," 1917, 111, 762, 876.
† *Loc. cit.*



IV.

Compound III. may also be the progenitor of nicotine by further condensation with formaldehyde and ammonia. Similarly, by the application of simple reactions, e.g. aldol condensations, oxidation, or dehydration, Robinson is able to account for the formation of such complex alkaloids as the pelletierines, sparteine, and the opium alkaloids belonging to the piperidine, quinuclidene, and isoquinoline groups respectively.

PTOMAINES.

Associated with the simplest form of plant life, namely, bacteria, a number of different basic substances are found, some of very simple constitution, such as methylamine, CH_3NH_2, dimethylamine, $(CH_3)_2NH$, trimethylamine, $(CH_3)_3N$ putrescine, $NH_2(CH_2)_4NH_2$, cadaverine, $NH_2(CH_2)_5NH_2$, and others rather more complex, such as choline, muscarine, neurine, collidine, etc., and some of unknown constitution, such as mydaleine and sepsine. These substances are known as ptomaines,* from the fact that they are usually associated with decomposing flesh ; some of them, such as putrescine and cadaverine, are practically non-poisonous, while others are highly toxic, producing increased salivation, diarrhœa, vomiting, etc.

On the whole, however, it is at least doubtful whether the manifestations of ptomaine poisoning are to be attributed entirely to these substances ; it would seem more likely that they were largely due to bacterial toxins, a class of substance related to the albumoses, which have the power of inducing the formation in the blood of antibodies, or, as they are better called, anti-toxins. Similar toxins or toxalbumins also occur in certain of the higher plants, as, for example, abrin, obtained from *Abrus precatorius*, and ricin, which occurs in *Ricinus*.

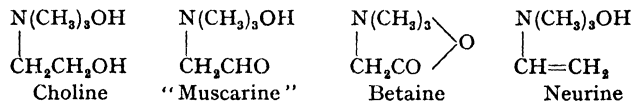
* From the Greek word πτωμα, meaning corpse.

The so-called ptomaines are all decomposition products of the complex nitrogenous substrate upon which the moulds or bacteria are growing, but are not actually found within the organisms themselves.

In the higher forms of plant life, on the other hand, these bases are actually secreted by and stored up in the plants; "muscarine," for example, occurring in *Amanita muscaria*. The term muscarine is applied to more than one substance; used to indicate the poison of *Amanita muscaria*, its constitution originally was thought to be that of a trimethylamino acetaldehyde, the formula of which is given below. King,* however, has shown that this is not so, and that the pharmacologically important constituent of the fly agaric is not related to choline and is not even a quaternary base.

Lack of space permits but the briefest reference to the chemistry of these substances.

Choline, "muscarine," betaine, and neurine are closely related, as may be seen from their formulæ—



the relationship to each other of the first three being that of alcohol, aldehyde, and acid anhydride.†

Choline and muscarine occur in the toad-stool, *Amanita muscaria*. Betaine and choline frequently occur together, as for example in the germ of *Hordeum sativum*, *Triticum sativum*, *Vicia sativa*, *Lathyrus sativus*, *Gossypium herbaceum*, and several other plants. Betaine alone occurs in the juice of the beet ‡ and in tubers of *Helianthus tuberosus*. Choline is far

* King: "J. Chem. Soc.," 1922, **121**, 1743.

† The name betaine is derived from the fact that this substance was first obtained from the beetroot (*Beta vulgaris*). It is the anhydride of hydroxytrimethylamino-acetic acid—



The alkaloid stachydrine (see p. 362) is a derivative of this substance

‡ For the preparation of betaine from this source, see "Ber. deut. chem Gesells.," 1912, **45**, 2411.

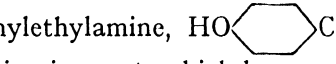
more widely distributed, and occurs in seeds and fruits of a very large number of plants, such as *Pinus cembra*, *Areca Catechu* (nut), *Cocos nucifera* (endosperm), *Acorus calamus* (root), *Fagus silvatica*, *Cannabis sativa* and *C. indica*, *Humulus Lupulus*, etc.

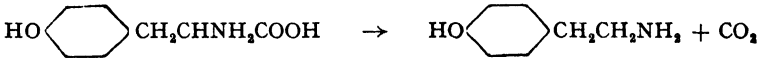
Neurine does not occur in plants, but is produced in putrefying fish and meat. "Muscarine" and neurine are both very poisonous, whereas choline is less so.

All these substances are strong bases, and answer the general reactions for alkaloids.

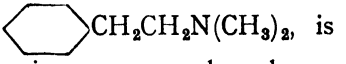
A few other bases of comparatively simple constitution which occur in plants may here be mentioned.

Trimethylamine, $(CH_3)_3N$, is a very volatile substance which occurs in the seeds of *Mercurialis annua* and in the flowers of *Cratægus Oxyacantha*, *Pyrus Aucuparia*, and many other plants, and is given off from the leaves of *Chenopodium Vulvaria*. It is also readily produced from choline and betaine, and is, therefore, commonly produced from putrifying animal or vegetable matter containing lecithin.

Parahydroxyphenylethylamine,  , is a substance occurring in ergot, which has a marked pressor action on the circulation, and causes contraction of the uterus. Its close relationship to tyrosine, from which it can be obtained by loss of carbon dioxide, is of interest—



Tyrosine — Hydroxyphenylethylamine

Hordenine,  , is the dimethyl derivative of the previous compound, and occurs in barley.

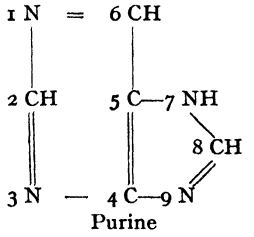
The fact that all nitrogenous bases form crystalline derivatives with such substances as platinic or auric chlorides, or with picric or picrolonic acids is frequently made use of for isolating or identifying small quantities of these substances (see choline, lecithin, p. 53); since the derivatives produced can, as a rule, be identified by their crystalline form and melting-point;

they provide a certain method of recognizing substances which do not give any characteristic colour reactions.

An additional advantage of the method lies in the fact that the reagents employed (auric or platinic chloride, etc.) being substances of high molecular weight produce crystalline derivatives whose weight is very considerably greater than that of the substance which is being isolated, and thus ponderable quantities of substance may be obtained from comparatively small amounts of material.

PURINE BASES.

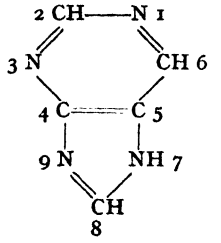
Under this heading are included such substances as caffeine, theobromine, xanthine, guanine, etc., which are called purine bases because they are all derivatives of the same substance, purine, whose formula is given below :—



Purine

This substance, which is also the mother substance of uric acid, does not occur in nature, but has been synthesized by Fischer.

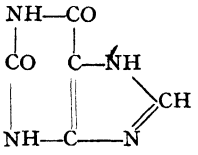
By writing the formula somewhat differently, as follows—



it will be seen that it is composed of two rings, the upper one, which is six membered, being a so-called pyrimidine ring, while the lower one, which is five membered, is an imidazol or glyoxaline ring, the same as occurs in histidine (see p. 444).

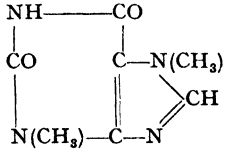
The relationship between purine, xanthine, theobromine and caffeine is best understood from the following considerations:—

Xanthine may be regarded as purine with the addition of two atoms of oxygen attached to the carbon atoms numbered 2 and 6; and it is accordingly called 2 : 6 dioxypurine, and is given the formula—

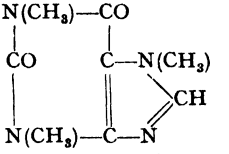


Xanthine or 2 : 6 dioxypurine

From this compound theobromine and caffeine are derived by replacing two and three atoms of hydrogen respectively by methyl groups, as may be seen from the following formulæ:—



3 : 7 Dimethyl Xanthine or Theobromine



1 : 3 : 7 Trimethyl Xanthine or Caffeine

Xanthine is widely distributed among plants, notably in sprouting seedlings, and occurs also in tea leaves and in the juice of the beetroot.

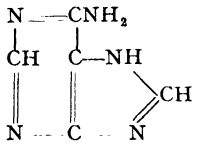
Theobromine occurs chiefly in the fruit of *Theobroma Cacao* (1·5-2·4 per cent), and a small quantity also occurs in kola nut and in tea leaves, but not in coffee; it acts as a powerful diuretic and has a stimulating effect on the central nervous system, but is less powerful in this respect than caffeine.

Caffeine occurs to the extent of about 1-2 per cent in kola nuts, 0·1-·8 per cent in cocoa beans, from 2-5 per cent in tea leaves, from 0·8-1·7 per cent in coffee beans, and from 2·5-3 per cent in the fruit of *Paullinia cupana;* the latter substance ground up into a paste is consumed in South America under the name of guarana. The so-called Maté or Paraguay tea,

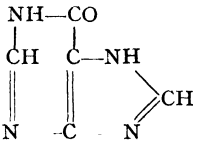
the dried leaves of *Ilex paraguensis*, contains about 0·2-1·6 per cent of caffeine.

Caffeine is a powerful cerebral stimulant, but also acts somewhat on the heart; it is furthermore a powerful diuretic.

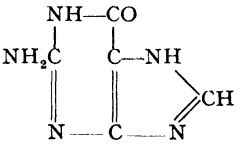
Three further purine bases deserve mention, namely, Adenine, Hypoxanthine, and Guanine, the formulæ of which are as follows :—



6 Aminopurine or Adenine



6 Oxypurine or Hypoxanthine



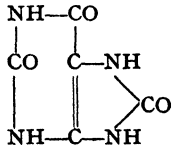
2 Amino 6 oxypurine or Guanine

All three substances have been obtained by the hydrolysis of nucleo-proteins from plants (see p. 428) and of nucleic acids from yeast * and from *Triticum sativum*.†

Guanine and Hypoxanthine are usually found together; they occur in sprouting seeds of a number of plants, notably *Cucurbita Pepo*, *Acer pseudoplatanus*, *Vicia sativa*, *Trifolium pratense*, *Lupinus luteus*, *Hordeum sativum*, and in the juice of the beet, etc.

Adenine, which is less widely distributed, likewise occurs in the juice of the beet and in tea leaves, and has also been found in leaves of *Trifolium repens*, *Chrysanthemum sinense Artemisia*, etc.‡

Uric acid, which is systematically named 2 : 6 : 8 trioxypurine, has the formula—



It does not occur in plants, but is a well-known waste product in the animal. In view of the close relationship be-

* Schittenhelm and Schröter: "Zeit. physiol. Chem.," 1904, **41**, 290.
† Osborne and Harris: *id.*, 1902, **36**, 85. Osborne: "Amer. Journ. Pharm.," 1903, **9**, 69.
‡ Yoshimara: "Zeit. Physiol. Chem.," 1913, 334.

tween this substance and the other purine bases, the assumption does not seem unwarranted that the purine bases in the plant are also waste products (see below). In this connection, it is interesting to find that the presence of urea, in very small amounts, has been observed by Fosse * in the higher plants.

In the fruit bodies of *Lycoperdon* and other higher Fungi, urea, either free or loosely combined, accumulates especially under conditions of growth involving a large supply of nitrogen.† Similarly, moulds, *Aspergillus*, for example, and bacteria *Bacillus tumescens*, for instance, grown in culture media rich in nitrogen, especially in the form of peptone or arginine, produce considerable quantities of urea.‡

The identification of individual members of the purine bases is not very easy, although the recognition of a purine base as such is rendered simple by the so-called murexide test which is given by practically all the members of this group of compounds.

The test consists in evaporating the substance (uric acid or caffeine may be used) in a porcelain basin with dilute nitric acid over a water bath. A yellowish residue remains which on the addition of ammonia or by exposure to ammonia vapour turns pink; potash changes the colour to purple.

The identification of caffeine in plants has been the subject of numerous researches ‖; it is precipitated by several alkaloidal reagents from solutions containing concentrated hydrochloric acid, but not from neutral solutions; these precipitates are, however, not characteristic. Behrens ¶ has described methods of identifying this substance with the help of mercuric chloride and of silver nitrate and nitric acid. The method is as follows:—

* Fosse: "Compt. rend.," 1912, **155**, 851; 1913, **156**, 567, 938; **157**, 948; 1914, **158**, 1374; **159**, 253; "Ann. Chim.," 1916, [ix], **6**, 13, 155. See also Fosse: "L'Urée," Paris, 1928.

† Ivanoff: "Biochem. Zeit.," 1923, **135**, 1; **136**, 1, 9; **143**, 62.

‡ *Ibid.*, 1925, **162**, 425; 1926, **175**, 181. See also Bokorny: *id.*, 1922, **132**, 197.

‖ Clautriau: "Nature et Signification des Alcaloïdes végétaux," Brussels, 1900.

¶ Behrens: "Anleitungen z. mikrochemischen Analyse d. wichtigsten organ. Verbindungen," 1897, IV., 14.

Fifty mgs. of dried tea leaves are coarsely powdered and mixed with quicklime and sufficient water to make a crumbly mass. The mixture is then dried and extracted with alcohol; the extract is evaporated drop by drop on a microscope slide and finally the residue is sublimed by heating until it turns brown, the vapour being condensed on a second slide held about 2 mm. above it. The sublimate consists of well-formed needle-shaped crystals. A drop of water containing a trace of hydrochloric acid is then placed near the sublimate and a grain of mercuric chloride is dissolved in the drop. On drawing the mercuric chloride solution through the sublimate, colourless glistening prismatic crystals are produced.

Silver nitrate in the presence of a small quantity of nitric acid produces under similar circumstances woolly aggregates.

PHYSIOLOGICAL SIGNIFICANCE OF NITROGEN BASES.

In considering the physiological significance of alkaloids, questions naturally arise with regard to their place in the metabolism of the plant. Are they connected with the elaboration of food or are they so much waste material, bye-products of metabolism, corresponding to uric acid and such-like substances excreted by the higher animals? Unfortunately, definite answers are not possible; what may be true of one group of nitrogen bases may be incorrect for another, and in any case the answers would not appear to be of general application, owing to the restricted occurrence of some of these compounds in the vegetable kingdom.

Certain organisms, more especially lower ones, can use alkaloids as a raw food-material, provided they be supplied in a sufficiently dilute state. Amongst the Algæ, Comère * found that *Ulothrix subtilis* and *Spirogyra crassa*, grown under aseptic conditions and in a solution free from nitrates, could make use of certain alkaloids as a source of nitrogen. Of the alkaloids used, this was found to be true for the sulphates and hydrochlorides of atropine, cocaine, and morphine; quinine, although it had no deleterious action, was not assimilated, whilst strychnine showed a marked toxic action.

* Comère: "Bull. Soc. Bot.," France, 1910, 57, 277.

With regard to the higher plants, De Vries considers that alkaloids are not used in assimilative processes, since in the germination of the seed of the potato, the thorn-apple (*Datura Stramonium*) and nux vomica (*Strychnos nux vomica*), little or no diminution in the substances in question occurs.

Similarly Sabalitschka and Jungermann * find but a small decrease in the lupinin content of *Lupinus luteus* and the strychnine content of *Strychnos nux vomica* in the early phases of germination; in the former plant there is a loss of about one-fifth and in the latter about one-fourth of the total amount after two weeks germination. In the lupin, this is followed by a rise which reaches a maximum at the end of fourteen weeks from germination which is about the period of seed setting. There is then a fall in the alkaloid content in the vegetative organs and an increase in the seeds which the authors think is not so much due to translocation but rather to the formation *de novo* of lupinin in the seed. In view of the small loss in the cotyledons during germination, it is not considered that these alkaloids are a reserve food. Annett † concludes that morphine is a useless end product of the metabolism of the opium poppy, *Papaver somniferum*, which is stored where its accumulation can do no harm to the plant.

These opinions are to a certain extent supported by the fact that the presence of alkaloids depends, at any rate in some cases, on the conditions of cultivation; for instance, quinine does not occur in cinchona cultivated in hot-houses in this country.

From the facts relating to the distribution of betaine in plants, Stanek ‡ concludes that this substance is not a nitrogenous reserve but is used up by the plant during its development.

Lotsy § considers that alkaloids, such as quinine, are not decomposition products of proteins, but direct synthetic

* Sabalitschka and Jungermann: "Biochem. Zeit.," 1925, **163**, 445; 1926, **167**, 479.

† Annett: "Biochem. Journ.," 1920, **14**, 618.

‡ Stanek: "Zeitsch. Zuckerind.," 1913, **37**, 385.

§ Lotsy: "Bull. Inst. Bot. Buitenzorg," No. 3, 1900.

substances. In the case of *Cinchona*, he found that the bases occur in parenchyma cells, provided that they do not contain calcium oxalate, either in solution in the cell sap, when the tissue is very young, or in a solid state in older parts. They are first formed in the leaves, and ultimately transferred to the bark.

Experience has shown that inoculation of plants with pyridine or pyrrolidine derivatives produces hardly any increase in their alkaloid content, whereas a similar inoculation of dextrose or asparagine causes a considerable increase.

On the other hand, caffeine and theobromine, which strictly speaking are purines, are generally considered to be decomposition products of proteins,* they are formed in places of great cellular activity and their disappearance is never accompanied by a concomitant increase of albuminous substances.

These particular substances may correspond to urea and uric acid of higher animals, for the purine nucleus is characteristic of xanthine bases, such as uric acid; and derivatives of xanthine, such as guanine and adenine, are found in caffeine and theobromine. In this connection one important point of distinction between animals and plants may be mentioned; in the higher animals there is a definite elimination of these waste nitrogenous substances from the organism, and the output bears a definite relation to the amount of proteins taken as food. In plants, on the other hand, there is no general elimination of nitrogenous waste, such substances being used up in anabolic processes. Thus Weevers,† whilst recognizing that caffeine and theobromine may be the products of the decomposition of proteins, considers that they are reorganized, and are therefore not to be classed as waste products in the same sense as uric acid is. It will, of course, be noticed that there is relatively much more nitrogen in these compounds than in the proteins.

Finally, not infrequently is it stated that alkaloids may be

* Clautriau: *loc. cit.*

† Weevers: "Proc. Koningkl. Akad. Wetens.," Amsterdam, 1903, 369; "Ann. Jard. Bot. Buitenzorg," 1907, **21**, 1.

of biological importance as a protection against herbivorous animals and parasitic fungi. Such teleological explanations would appear to be unwarranted in view of the facts that rabbits of the Belgian dunes consume much *Hyoscyamus*, that rats are fond of poppy heads, and that snails fed upon Sabalitschka's plants of nux vomica.

As stated above, some of the purine bases such as xanthine and guanine, although sporadic in their occurrence, have a fairly wide distribution. By far the most important form in which they occur is in combination with phosphoric acid and carbohydrate forming nucleic acid; this substance, being a universal constituent of the nucleus, would appear to be of great significance, for which reason some account of its chemistry is here given.

NUCLEIC ACID.

So far as is known, only one nucleic acid occurs in the plant world and this, presumably, is universally present in the nucleus; since yeast formed the source from which the material was first produced in quantity, it is frequently referred to a yeast nucleic acid, but the prefix yeast has no special significance except to distinguish it from the so-called thymus nucleic acid, the prototype of nucleic acids of animal origin. The two nucleic acids of vegetable and animal origin are very closely related in their composition, as may be seen by a comparison of the products of their complete hydrolysis:—

Hydrolytic Products of Nucleic Acid.

	Of plant origin.	*Of animal origin.*
	Phosphoric acid.	Phosphoric acid.
Purine bases	Adenine.	Adenine.
	Guanine.	Guanine.
Pyrimidine bases	Cytosine.	Cytosine.
	Uracil.	Thymine.
	Pentose (Ribose).	Lævulinic acid (? Hexose).

The two nucleic acids are amorphous substances which are sparingly soluble in water but dissolve readily in alkalis; with salts of the heavy metals they form precipitates and with protein solutions they likewise form precipitates of the corresponding protein nucleates.

The behaviour of the two acids on hydrolysis is substantially the same except in regard to their final products, and it is, therefore, proposed only to deal with plant nucleic acid.

When hydrolysed with ammonia under pressure at a temperature of 100-125°, yeast nucleic acid yields four so-called mono-nucleotides as follows * :—

Guanine nucleotide $\begin{matrix}HO\\ O{=}P{-}O\\ HO\end{matrix}$. $C_5H_8O_3$. $C_5H_4N_5O$

Adenine nucleotide $\begin{matrix}HO\\ O{=}P{-}O\\ HO\end{matrix}$. $C_5H_8O_3$. $C_5H_4N_5$

Cytosine nucleotide $\begin{matrix}HO\\ O{=}P{-}O\\ HO\end{matrix}$. $C_5H_8O_3$. $C_4H_4N_3O$

Uracil nucleotide $\begin{matrix}HO\\ O{=}P{-}O\\ HO\end{matrix}$. $C_5H_8O_3$. $C_4H_3N_2O_2$

From which it may be concluded that the original nucleic acid is a tetra-nucleotide.

If the hydrolyses of nucleic acid is carried out at a higher temperature, 145-155°, the mononucleotides above-mentioned undergo further hydrolysis, yielding products containing no phosphoric acid, which are known as nucleosides :—

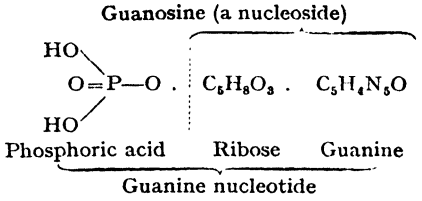
Nucleotide.		*Nucleoside formed on hydrolysis.*
Guanine nucleotide → H_3PO_4	+	Guanosine, $C_{10}H_{13}N_5O_5 . 2H_2O$
Adenine nucleotide → H_3PO_4	+	Adenosine, $C_{10}H_{13}N_5O_4 . 1\frac{1}{2}H_2O$
Cytosine nucleotide → H_3PO_4	+	Cytidine, $C_9H_{12}N_2O_6$
Uracil nucleotide → H_3PO_4	+	Uridine, $C_9H_{12}N_2O_5$

These nucleosides are each composed of a carbohydrate, ribose, united to a nitrogen base which belongs either to the group of purines or of pyrimidines, and on hydrolysis break up into their constituents :—

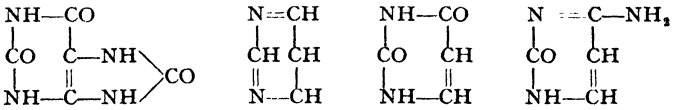
Guanosine	→	Ribose	+	Guanine	Purine.
Adenosin	→	Ribose	+	Adenine	
Cytidine	→	Ribose	+	Cytosine	Pyrimidine.
Uridine	→	Ribose	+	Uracil	

These stages of the hydrolysis of a nucleotide may be illustrated by a single example of guanine nucleotide :—

* See Jones : " The Nucleic Acids," London.

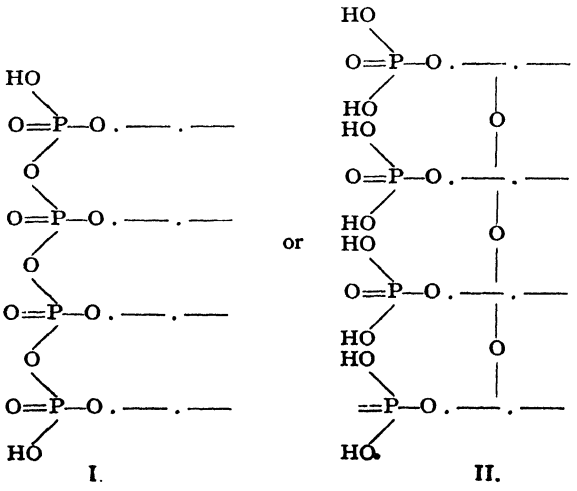


The bases uracil and cytosine are derived from pyrimidine, a substance which does not itself occur naturally; the relationship of these substances to uric acid will be seen from a comparison of the formulæ :—



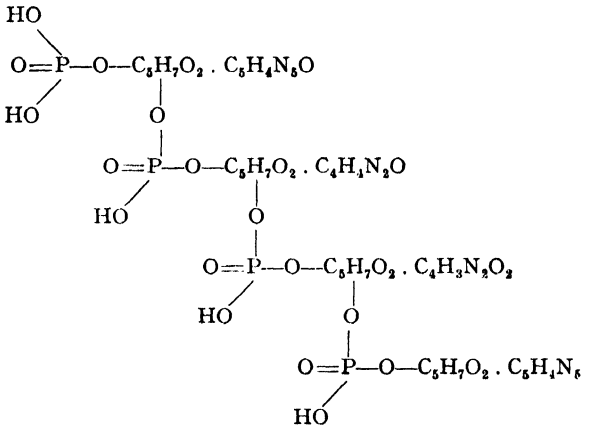
Jones and Perkins have some doubt as to whether uracil nucleotide is a true constituent of plant nucleic acid, and suggest that it is a secondary product arising from the cytosine nucleotide; they accordingly think that the distinction between animal and plant nucleic acids will in the future not be so definitely drawn.

In attempting to reconstruct the formula of nucleic acid from the constituent nucleotides, two possible configurations present themselves according as they are linked through the phosphoric acids or through the carbohydrate residues as is outlined below :—



The hydrolysis of nucleic acid of formula I. into its four constituent nucleotides should be accompanied by a marked increase in acidity but not so the hydrolysis of a nucleic acid of formula II. In his earlier experiments, Jones observed no increase and therefore accepted formula II. as correct, but later Jones and Perkins, using a weak solution of caustic soda, came to the conclusion that there was a small increase in acidity.

On the other hand, Levene,* holding that the linkage between all nucleotides is of the same order, prefers the following formula :—



FURTHER REFERENCES.

Henry : " The Plant Alkaloids," London, 1924.

Trier : " Ueber einfache Pflanzenbasen und ihre Beziehungen zum Aufbau der Eiweisstoffe und Lecithine," Berlin, 1912.

Winterstein and Trier : " Die Alkaloide," Berlin, 1927.

* Levene : " J. Biol. Chem.," 1919, **40**, 420.

SECTION VIII.

THE COLLOIDAL STATE.

A KNOWLEDGE of the properties associated with the colloidal state of matter is of the greatest importance in the study of the chemical and physical problems presented by both plants and animals; for this reason some of the more important facts concerning colloids are here set forth. To illustrate the bearing of this subject on plant chemistry, it is only necessary to point out that the protoplasmic contents of any living cell exhibit many of the properties of colloidal solutions, and, indeed, it is held by some that the chief vital function of protoplasm is due to its acting as a colloidal medium.

Apart, however, from the living cell contents, many of the reserve and waste products of the vital activity of the cell are colloidal substances. Thus, for example, the cell wall itself is composed of cellulose, a substance which exhibits all the characteristic properties of colloids, while starch, resins, gums, rubber, proteins, and enzymes are all colloidal in nature. Moreover, many of the processes of dyeing and staining employed in microscopical technique are directly due to the colloidal nature both of the material to be stained and of the staining solution; further, a number of the properties of soil and humus are directly attributable to the colloidal properties of these substances.

Before considering the properties of matter in the colloidal state, it is necessary to explain the origin of the term colloid. While studying the laws of diffusion in liquids, Thomas Graham found that water soluble substances could be divided into two classes :—

(*a*) Those that diffused relatively quickly, and

(*b*) Those whose rate of diffusion was very slow or imperceptible.

The former class, including substances such as salts, acids, bases, cane sugar, urea, etc., which for the most part crystallized readily, he called "crystalloids," while for the latter class, which comprise such substances as starch, albumen, and gum, he devised the term "colloid." Although there was this marked difference between these two classes of substance in the rates of free diffusion into pure water, it was found that the presence of a colloid, in relatively low concentration, had but little effect in retarding the rate of diffusion of a crystalloid, which accounts for the fact that diffusion experiments can be carried out in gelatine solutions, and also that crystalloids will diffuse quite readily through colloidal membranes, such as parchment, etc.

On the other hand, it was found that such membranes offered a very strong opposition to the passage of other colloids; this observation was turned to account in the dialyser, by means of which apparatus it was found possible to separate crystalloids from colloids contained in the same solution. Numerous modifications of Graham's original apparatus have been devised, but they are all ultimately based on the same principle that if a mixed solution of a colloid and a crystalloid are separated from pure distilled water by a colloidal parchment or other membrane, the crystalloid alone will diffuse out at a measurable rate, whilst the colloid will remain behind. The method is, indeed, to this day the only one known for purifying a colloid from a crystalloid since the ordinary methods applicable for the purification of crystalloids do not hold for colloids.

The origin of the terms crystalloid and colloid was, however, based on a misconception. The rate of diffusion of any substance is in no way connected with its ability to crystallize, or the reverse, since, as was subsequently shown, almost all crystalloids can be made under suitable conditions to give solutions in which they have lost their ability for rapid diffusion, and have acquired many of the characteristics of the class of substance known to Graham as colloids; similarly, many of Graham's colloids, such as egg albumen and hæmoglobin, have been obtained in crystalline form. The properties of the

colloidal solutions are, therefore, no longer regarded as being due to the intrinsic properties of the substances dissolved, but rather to the state of aggregation of the substances concerned. Only on this assumption is it possible to understand how one and the same substance can at one time produce a colloidal solution, and at another an ordinary crystalloidal solution as is, for example, the case with gallic acid, which gives a colloidal solution in water, but not in glacial acetic acid.

Graham, moreover, found that many substances which were insoluble in water in the ordinary way could, nevertheless, be made to produce colloidal solutions exhibiting the characteristic reluctance to diffuse. Since Graham's time, almost all the metals and their insoluble oxides, sulphides, carbonates, sulphates, etc., have been obtained in so-called colloidal solution, including even such insoluble substances as lead and barium sulphates.

It would appear, therefore, that the properties of a colloidal solution are not so much due to the substance itself as to the peculiar nature of the solution, or, in other words, the state of aggregation of the dissolved substance.

The evidence in support of this view is partly optical (Tyndall phenomenon, ultramicroscope, etc.) and partly direct, since it has been shown that many of the substances which are known to us as insoluble can, by a sufficient degree of disintegration, be made to yield colloidal solutions.

Thus many metals are obtained in colloidal aqueous solution by passing a powerful electric discharge between two poles of the metal held under water, and, again, a number of insoluble crystalloids, such as silica, molybdenum oxide, and vanadium oxide, have been made to yield colloidal solutions by merely finely powdering, or grinding these substances under water.

Finally, the whole question has been shown to be amenable to mathematical treatment by Von Weimarn,† who has worked out the conditions which determine whether a given substance will assume the crystalloid or the colloidal state.

* Wegelin : " Kolloid Zeitschr.," 1913, 14, 65.

† For an account of this, see Taylor's " The Chemistry of Colloids," London, 1915.

It will be seen from the foregoing that whereas in a true solution the dissolved substance is in a state of molecular dispersion this is not so in what is known as a colloidal solution, which may be regarded as a state midway between a true solution and a suspension. The evidence of the ultramicroscope * goes to support this view.

In a true suspension the particles are of varying size, but even the smallest are visible under the magnification of a high-power microscope, the limits of visibility of which are somewhere of the order of 0·1 μ, in which $\mu = \cdot001$ mm. or 1 millionth part of a meter. The particles of a colloidal solution, on the other hand, may vary between the limits 0·1 μ and $\mu\mu$ †; such particles, although beyond the limits of direct visibility by the microscope, can nevertheless be revealed indirectly by means of the ultramicroscope, the principle of which is to detect the presence of particles by the light reflected from them in a dark field—in much the same way as a beam of sunlight entering a dark room reveals the presence of dust particles by reflected light.

When the particles in a solution are of a smaller diameter than $\mu\mu$ they are no longer detectable by the ultramicroscope, and in a true solution they are assumed to have diameters of the order of 0·1 $\mu\mu$—the molecule of hydrogen being calculated as having a diameter of 0·16 $\mu\mu$.

It may be assumed then that in colloidal solutions we are dealing with non-homogeneous mixtures or two phase systems, and that the characteristic properties of such colloidal solutions are attributable to this peculiar state of aggregation.

This explains at once why the rate of diffusion of substances in colloidal solution should be slower than those in true solution, since the larger particles would naturally be expected to move more slowly than the particles of molecular dimensions found in true solution. Moreover, it accounts for the low values obtained in the measurement of the osmotic pressure of colloids by the freezing-point method.

* For a description of this apparatus and its use, see Zsigmondy: "Colloids and the Ultramicroscope." Trans. by Alexander. New York, 1909.

† $\mu\mu = \cdot001\ \mu = 1$ millionth part of a millimeter.

If osmotic pressure is ultimately caused by the impact of particles upon the walls of the containing vessel, then the more sluggish larger particles would produce fewer impacts and therefore a lower osmotic pressure than the more rapidly moving particles of molecular dimensions. Direct determinations by Starling, Lillie, Moore and Roaf, Bayliss, and others have indeed shown that colloids have a small but measurable osmotic pressure which is not due to any accidentally adhering crystalloidal impurities.

Assuming, then, that colloidal solutions are two phase systems, a phase being any particle of matter bounded by its own surface, two classes of such solutions are distinguished :—

1. Suspensoids in which, as in a true suspension, the discontinuous or disperse phase is a solid while the continuous phase is a liquid ; and

2. Emulsoids in which, as in an emulsion, both the continuous and the disperse phases are liquid.

SUSPENSOIDS.

Although the suspensoids are biologically of but slight importance, since only the inorganic colloidal solutions belong to this group, a brief description of their properties is essential in a survey of the whole subject.

Colloidal solutions of otherwise insoluble substances may be obtained in a variety of ways—such as electrical disintegration of the metals, reduction of metallic salts, the formation of the substance under special conditions or in particular solvents, etc., for the details of which one of the many text-books on Colloidal Chemistry may be consulted.

GENERAL PROPERTIES OF SUSPENSOIDS.

A. *Optical Properties.*

Suspensoid sols, as a general rule, appear more or less clear to the unaided eye, but are frequently highly coloured. This is notably so in the case of the metallic sols such as gold and silver, which may be obtained in a variety of different shades, depending on the method of preparation and the consequent size of the particles. Thus gold sols may be either blue,

purple, pink, or red, the latter containing the smallest particles, while silver sols have been obtained brownish-red, yellow, green, grey, or blue. Some of the colour effects occasionally met with in partly developed photographic plates are probably due to absorption compounds of silver particles of various degrees of dispersion with unreduced silver chloride.*

Most suspensoid sols, however, although appearing clear when examined in the ordinary way, exhibit what is known as the Tyndall phenomenon; by this is meant the fact that when a beam of light is projected into the solution, in an otherwise dark room, the path of the beam is rendered visible by the reflection of light from the particles of the disperse phase. If examined through a Nicol prism the luminous beam is found to be polarized, which distinguishes it at once from the similar effect produced by passing a ray of light into a true solution of a fluorescent substance.

Brownian Movement.—In 1827, R. Brown, the botanist, first observed, by the aid of a compound lens, the peculiar movement of pollen grains suspended in water; this movement was subsequently found to be common to all sufficiently small particles similarly suspended in a liquid of low viscosity, and the phenomenon is now known as Brownian movement. The phenomenon is generally regarded as a manifestation of molecular motion, and, as is to be expected, the smaller the particles the more rapid the movement. Thus for particles of diameter 3 μ it is only a barely perceptible oscillation, but it rapidly increases with diminishing size; in the case of particles of diameter 10-50 $\mu\mu$, which are beyond the range of visibility of the microscope, Brownian movement is manifested by a rapid rectilinear zig-zag oscillation of minute spots of light in a dark field. The general motion resembles that of the flight of gnats and the velocity is of the order of 100 μ per second.

B. *Electrical Properties.*

When a suspensoid sol in pure water free from electrolytes is subjected to the action of a powerful electric field, by dipping

* Luppo, Cramer, and Reindeers: "Kolloid Zeitschr.," 1911, **9**, 10.

into it two platinum electrodes with a potential difference of about 200 volts, the sol wanders to one or other of the electrodes, showing that it bears an electrostatic charge of opposite sign to that of the electrode in question. This phenomenon is known as Kataphoresis.

The majority of suspensoid sols bear a negative charge and consequently wander towards the anode; on the other hand, the metallic hydroxides, silicic acid and basic dyes, etc., wander to the cathode. While these statements are true for aqueous sols the conditions are exactly reversed when turpentine is the medium. This reversal of charge with the solvent is governed by the rule that "non-conductors in contact with a liquid assume a + or − charge according as their dielectric constant is $>$ or $<$ that of the liquid."

Since water has a very high dielectric constant it is natural that most other substances should assume a negative charge in relation to it.

The fact that suspensoid sols bear a recognizable electric charge renders them sensitive to electric influences, and they are consequently readily discharged by colloids of opposite sign or by electrolytes. This electrical discharge brings about a coalescing of the colloidal particles, with the formation of larger aggregates and consequent precipitation, resulting in the destruction of the colloidal solution. Such a change is irreversible, for the precipitate once formed cannot be redissolved.

(1) *Precipitation by Electrolytes.*—The precipitation is in this case, according to Hardy, effected by the ion of opposite sign; thus, for example, a negatively charged sol such as arsenic sulphide is precipitated by the metallic ion of an electrolyte; the precipitating power of such ions increases with the valency.

That the metal really enters into close relationship with the arsenic sulphide is shown by the fact that the latter when precipitated persistently retains barium hydroxide whilst the solution becomes acid due to liberation of hydrochloric acid.

The formation of a river delta by the precipitating action

of sea salts upon the positively charged suspended clay particles is an illustration on a large scale of an analogous phenomenon.

Positively charged colloids, such as ferric hydroxide, on the other hand, are precipitated by the anion of an electrolyte, the precipitating power again increasing with the valency as indicated by the series sodium chloride, sulphate, citrate.

(2) *The Precipitation of Colloids by Other Colloids of Opposite Electric Sign.*—This phenomenon was first observed by Linder and Picton, who found that certain solutions of organic dyes, on mixing, produced precipitates. Further investigations have shown conclusively that only oppositely charged colloids could mutually precipitate; thus, arsenic sulphide, which is negatively charged, is not precipitated by any other negatively charged colloid, but is precipitated by ferric hydroxide, which is positive. The resulting gel is described as an adsorption compound (see below under Adsorption).

This mutual precipitation of colloids has many very important practical applications; for example, the use of ferric salts in the purification of sewage water is probably due to the precipitation of negatively charged colloidal particles of sewage by the ferric hydroxide hydrosol and similarly with alum.

Also it has been suggested that the process of dyeing is really a mutual gel formation between the colloidal dye and the colloidal fibre; similarly the interaction between toxin and antitoxin, and the phenomenon of bacterial agglutination, etc., may be regarded as examples of the mutual precipitation of two colloids.

This same phenomenon can also be conveniently employed for determining the electric sign of a colloid. Thus, if a piece of filter paper is wetted, it assumes a negative charge and consequently if it is dipped into a positive dye sol the dye will be discharged on coming in contact with the paper, and water alone will be drawn up by capillary forces. If, on the other hand, the dye is a negatively charged one it will travel up the paper together with the water. This may be well shown by

means of two solutions of night blue and alkali blue respectively, as recommended by Wo. Ostwald. The same principle has been worked into a complicated system of capillary analysis by Goppelsroeder,* Freundlich,† and others.

PROTECTIVE ACTION OF COLLOIDS.

The sensitiveness of suspensoid sols to electric influences can be considerably reduced by what are known as protective colloids.

Many organic substances, such as gelatine, agar, etc., when added in small quantity to inorganic colloidal solutions, can prevent the precipitation of the latter by electrolytes; under these conditions the organic colloids are said to exert a protective action upon the inorganic colloid.

It is not known in what way this protective action is exerted, but it has been suggested that the particles of the suspensoid become covered with a layer of gelatine and so acquire the properties of gelatine particles.

Suspensoids, so protected, can be evaporated to dryness, and the residue when taken up with water will redissolve.

The greatly increased stability thus acquired by the inorganic colloid makes the process of value for the preparation of colloidal solutions of the metals, particularly silver and mercury, which are used for various medicinal purposes.

A measure of protective power was first worked out by Zsigmondy,‡ who defined as the gold number, the number of milligrams of colloid which, when added to 10 c.c. of a bright red colloidal gold solution containing from ·0053 to ·0058 per cent of gold, is just insufficient to prevent the precipitation (as shown by the colour change to violet) of the gold by 1 c.c. of a solution of sodium chloride, containing 100 grams of salt in 900 c.c. of water.

Appended is a list of some of the commoner colloids with their corresponding gold number taken from Zsigmondy's paper :—

* Goppelsroeder : " Kapillaranalyse," Basel, 1906.
† Freundlich : " Kapillarchemie," Leipzig, 1909.
‡ Zsigmondy : " Zeit. anal. Chem. " 1901, 40, 697.

Colloid.	Gold Number.	Reciprocal Gold Number.
Gelatine	·005-·01	200-100
Isinglass	·01-·02	100-50
Gum arabic . . .	·15-·25	6·7-4
Tragacanth . . .	2	0·5
Dextrin	6-12	·17-·08
Potato starch . . .	25	·04
Mucilage from quince kernel	∞	0

According to Oden * the humic acid of the soil exerts a protective action on clay, preventing its coagulation by electrolytes.

Electric Endosmose.—This term is applied to a phenomenon which in a sense may be regarded as the inverse of kataphoresis. Whereas in the latter case it is the disperse phase which wanders in the electric field, while the solvent, or continuous phase, remains at rest, the reverse conditions hold in the case of Endosmose. This is effected by placing the colloidal sol in a vessel the walls of which are impermeable to the colloid but permit of the free passage of the continuous phase. When this is submitted to a high difference of potential the effect is to draw the solvent out of the containing vessel and thereby to dehydrate the sol. Attempts have been made to apply this principle to the problem of the economical dehydrating of peat for the purpose of obtaining fuel but so far they have not been very successful.

It has also been proposed to dry timber by electrosmotic removal of the cell sap and to preserve the timber by replacing the sap removed by a suitable preservative. The principle of kataphoresis has also been applied to the dehydration and purification of clay.†

EMULSOIDS.

The emulsoids form the second great group of colloids and from a biological point of view they are the more important of the two.

Substances such as albumen, gelatine, gums, starch, agar, etc., which belong to this group, tend to swell up in contact

* Oden : " J. Landw.," 1919, **67**, 177.

† For a fuller account of these processes, see " Second Report on Colloid Chemistry," etc., British Ass. Reports, 1918.

with water, thus indicating a tendency for close association between the substance and its solvent; for this reason the term Lyophilic colloid has been employed by some authors to designate these substances; the term Lyophobic being, by contrast, applied to the suspensoids.

Emulsoids are in fact regarded as consisting of a liquid disperse phase composed of a concentrated solution of the substance suspended in a liquid continuous phase composed of a much diluter solution. The term emulsoid, has been adopted to indicate their general relation to the emulsions which likewise are two-phase systems produced from two liquids which are immiscible.

GENERAL PROPERTIES OF EMULSOIDS.

The outstanding feature of the emulsoids as compared with suspensoids is their much greater viscosity; this fact, however, is not surprising if the views put forward with regard to their constitution are correct, since true emulsions are known to have high viscosities, e.g. Mayonnaise sauce. The viscosity of a solution varies both with the concentration and the temperature; it is liable to be influenced by a variety of causes such as prolonged heating and by different methods of treatment. In some cases the passage through a capillary tube will alter the viscosity of a solution and in some instances the viscosity will diminish spontaneously. Viscosity is, moreover, considerably affected by the presence of dissolved salts, being increased by sulphates, phosphates, and citrates but reduced by iodides or sulphocyanides.

(*a*) *Optical Properties.*—These are in many respects less striking than those of the dispersoids since emulsoid sols, although frequently opalescent or turbid, are not as a rule highly coloured. The presence of the diffracting particles of the disperse phase may, however, in some cases cause a bluish opalescence as, for example, in a starch solution; indeed, according to Bancroft,* the blue colour † of eyes and

* Bancroft: " J. Phys. Chem.," 1919, **23**, 356, 365.

† According to Wo. Ostwald, the blueness of the sky is similarly due to the atmosphere being composed of matter in a disperse phase suspended in a continuous phase.

feathers is caused by the same phenomenon. In common with suspensoids, the emulsoids also exhibit the Tyndall phenomenon.

Examined under the ultramicroscope they also show Brownian movement, but this is not so well defined as in the case of suspensoids; this is probably due to the fact that there is not the same difference in refractive index between the disperse and continuous phases in the case of the emulsoids since, as will be seen below, the disperse phase itself contains a considerable proportion of the dispersing medium.

According to Bayliss * the pseudopodia of *Amoeba* when examined with intense dark ground illumination show numerous minute particles in Brownian movement, which may be taken as affording evidence of the colloidal nature of the protoplasm.

(*b*) *Electrical Properties.*—Compared with suspensoids, the emulsoids are relatively stable towards electrolytes; the former are liable to be precipitated from their solutions by the merest traces of electrolytes, and hence a number of precautions have to be adopted in preparing them to exclude contamination with such bodies. The emulsoids, on the other hand, are frequently contaminated by considerable quantities of electrolytes without detriment to their solubility.

The reason for their comparative indifference to electrolytes is to be found in the absence of well-defined electrical characteristics. Typical emulsoids, in fact, when pure have no electric sign, and only acquire one on the addition of either acid or alkali to their solutions. Thus, it has been shown by Hardy that whereas native albumen, when free from electrolytes, is electrically neutral, it acquires a negative charge on the addition of a little alkali, and a positive charge on the addition of acid.

According to Pauli † this accounts for the fact that positively charged metallic hydroxides are unable to precipitate electrically neutral albumen, but precipitate albumen which

* Bayliss: "Proc. Roy. Soc.," 1920, B, 91, 196.
† Pauli: "Beitr. chem. Phys. Path.," 1906, 7, 531.

has become negatively charged by the addition of a little alkali; and similarly negatively charged colloids, such as phosphomolybdic or phosphotungstic acid or certain negative dyes, are only able to precipitate albumen after it has acquired a positive charge by the addition of acid.

(*c*) *Precipitation by Electrolytes.*—The precipitation of emulsoids from their solutions by electrolytes is not to be regarded as due to the electrical discharge of the disperse phase by the ionic charges, as with suspensoids. The amounts of the salts required for precipitation are considerable, and precipitation in this case is more probably due to a redistribution of the solvent between the emulsoid and the salt added.

Metallic salts, which precipitate emulsoids, can be arranged in three groups as follows :—

(i) Sodium, potassium, lithium, ammonium, and magnesium salts.

If not left too long in contact with these salts, the precipitated colloid can be redissolved, and the process is, therefore, reversible.

Practical application has been made of this phenomenon for separating the various types of protein. Thus, for example, if an aqueous solution containing an albumen and a globulin be mixed with an equal volume of saturated ammonium sulphate solution, the globulin, being insoluble in the resulting half-saturated ammonium sulphate, is precipitated; after filtering off the globulin, the albumen may be precipitated from the mother liquor by saturating it with ammonium sulphate.

The precipitated albumen and globulin are chemically unchanged, and can be redissolved if desired.

(ii) Calcium, barium, and strontium salts.

The process in this case is reversible immediately after precipitation, but after a very short interval it becomes irreversible.

(iii) Heavy metal salts, such as those of mercury, copper, lead, or zinc.

Here the process is irreversible, owing, no doubt, to the formation of definite chemical compounds.

The case of zinc is peculiar, inasmuch as very dilute solutions of zinc salts produce irreversible precipitation of egg albumen, whereas strong solutions may either not produce a precipitate, or else cause one already formed to dissolve.*

The anion also plays an important part in influencing the precipitating power of a given salt. By arranging the various salts of sodium in the order of decreasing precipitating power, the so-called Lyotropic series is obtained as follows :—

Citrate > tartrate > sulphate > acetate > chloride > nitrate > chlorate > bromide > iodide > sulphocyanide.

Here, again, there is no relation between precipitating power and electric charge of the ion, and the fact that citric acid comes first in the list has nothing to do with its being tribasic.

The precipitating effect of a salt appears rather to be connected with its water-binding power, and it may be assumed that the presence of a citrate, tartrate, or sulphate of an alkali metal leaves less water available to the colloid.

This assumption would also explain the fact that a gelatine gel containing such salts has a higher melting-point than one containing a sulphocyanide which leaves the gelatine so much water that it is reluctant to set.

On the other hand, these salts are also known to affect the compressibility of water, and their action on emulsoids may possibly be connected with this fact.

The precipitating power of the anions when combined with one of the metals of the alkaline earths is exactly the reverse of that observed when the same anions were combined with the alkali metals. Thus the precipitating power of the anions increases in the order $C_2H_3O_2 > Cl > NO_3 > Br > I > CNS$, whereas when combined with the alkali metals the inhibiting power increases in this same order.

In conformity with the above facts, Pauli,† in studying the precipitation of albumen by various salts, came to the con-

* Pauli : " Beitr. z. chem. Phys. and Path.," 1905, 6, 233, 259.
† *Ibid.*, 1902, 3, 225 ; 1903, 5, 30.

clusion that the precipitating power of a salt was an additive property which depended on the constituent ions.

Kations, as a rule, act as precipitants for albumen, while anions tend to keep it in solution.

The precipitating power of the kations increases in the following order: Mg, NH_4-, K, Na, Li, while the inhibiting or solvent action of the anion increases in the following order: -$C_2H_3O_2$, -Cl, -NO_3, -Br, -I, -CNS.

According as the precipitating power of the kation or the inhibiting power of the anion predominates, the resulting salt will either precipitate or not precipitate albumen.

These observations are given below in tabular form. As shown by the arrows, the kations and the anions are arranged in ascending order of precipitating and inhibiting power respectively. The symbols + and — respectively signify that the salt does or does not precipitate albumen, the blank spaces meaning that the salt has not been investigated.

Kations → / Anions ↓	Mg	NH_4	K	Na	Li
Fluoride		+	+	+	
Sulphate	+	+	+	+	+
Phosphate			+	+	+
Citrate			+	+	+
Tartrate			+	+	+
Acetate		—	—	+	+
Chloride	—	—	+	+	+
Nitrate	—	—	—	+	+
Chlorate			—	—	+
Bromide	—	—	—	—	+
Iodide	—	—	—		
Sulphocyanide	—	—	—	—	

From this table it may be seen that the comparatively slight precipitating power of the kations, Mg and NH_4-, is completely neutralized by the anions -$C_2H_3O_2$ or -Cl, while the more powerfully inhibiting anions -NO_3 and -ClO_3 are able to neutralize the precipitating power of the kation K as well as that of Mg and NH_4-. Similarly the powerfully inhibiting anions -Br, -I, and -CNS, are able to counteract the precipitating power of sodium as well.

SWELLING OF COLLOIDS OR IMBIBITION.

Whereas a water-soluble crystalloid commences to dissolve as soon as it is brought in contact with water, the same is not true for most emulsoid or lyophilic colloids. Before going into solution, these substances undergo a preliminary swelling, sometimes known as imbibition; this is accompanied by the disappearance of a certain volume of water. According to an experiment described by Hatschek, 1 gram of gum-tragacanth covered with water in a specific gravity bottle kept under water for a week had increased in weight by 0·9 gram at the end of this period; this means that in the process of imbibition the gum had succeeded in drawing in to the flask 0·9 c.c. of water. In view of the resistance which water is known to offer to compression, it is clear that enormous force must have been exerted during the process.

Direct measurement of the pressures produced during swelling were made by Reinke,* on *Laminaria* contained in an apparatus known as the Oedometer.

Only by the application of an opposing pressure of 41 atmospheres was he able to reduce the amount of water imbibed to one-twentieth of the amount it would normally have taken up.

Conditions Affecting Imbibition.—(*a*) *Temperature.*—Heat is evolved during swelling, as may be seen from the following table taken from Taylor's "Chemistry of Colloids":—

	Cals. per gram of Colloid.
Gelatine	5·7
Starch	6·6
Gum-arabic	9·0
Gum-tragacanth	10·3

This being so, heat hinders imbibition, while cold and pressure favour it. For this reason it is best in making a solution of a colloid such as agar or gelatine to allow it to swell for some time in cold water without applying any heat.

(*b*) *Presence of Impurities.*—The swelling of colloids is very considerably increased by the presence of small quantities of

* Reinke: "Hanstein's bot. Abhandl.," 1879, 4, 1.

either acids or alkalis. In the case of fibrin M. H. Fischer was able to increase the normal swelling in water sixfold by the presence of 0·02 N hydrochloric acid. As a practical application of this may be mentioned the beneficial effect of the addition of a small quantity of acetic acid to the water employed for swelling agar previous to making a solution.

With regard to the action of salts, it is found that anions act in the order of the Lyotropic series mentioned on page 398.

Thus the following anions favour imbibition :—

$$CNS > I > Br > NO_3 > ClO_2 > Cl;$$

while the following inhibit—

$$- SO_4 > \text{tartrate} > \text{citrate} > \text{acetate},$$

as do also alcohol, glucose, and cane sugar.

According to Spek † salts such as lithium bromide or potassium thiocyanate, which have a strong influence on the swelling of colloids, also accelerate the rate of cell division of *Paramœcium*, while calcium chloride and sulphates, which reduce swelling, retard cell division.

Many of the epidermal tissues in plants and animals are cuticularized or otherwise hardened; this prevents their swelling when brought in contact with water, thus enabling them to maintain their shape, and it is a commonplace in histological technique to harden tissues by immersion in formaldehyde or other solutions so as to counteract and prevent this same tendency. The hardening of gelatine by means of bichromate is another example of the same principle.

Syneresis.—This is the name given by Graham to a phenomenon which may be regarded as the reverse of swelling. Most gels, on keeping, squeeze out a small quantity of liquid which is not pure water but a dilute solution of the colloid in question. The amount of liquid thus exuded varies with the concentration of the gel, and is greater for some colloids

* This author considers that much of the pathological swelling in animals and man is due to an accumulation of acid in the tissues, which, as a consequence, tend to draw fluid from surrounding tissues and so swell; he would even offer the same explanation for the swelling caused by an insect's bite.

† Spek: "Koll. Chem. Beihefte.," 1920, **12**, 1.

than for others. The phenomenon can be noticed on agar culture tubes, etc., and is familiar to bacteriologists.

GEL FORMATION.

Many colloidal solutions are able, under certain conditions, to undergo a change of state known as gel formation, in which the sol loses its liquid properties and becomes more or less rigid.

In some cases the change is reversible, meaning that by suitably altering the conditions the gel will return to a solution, and in other cases the change is irreversible.

Examples of such changes are given below :—

(*a*) *Spontaneous Precipitation.*—A silicic acid sol prepared by the addition of acid to a solution of sodium silicate will, on keeping, set spontaneously to a bluish almost transparent gel. This change is irreversible.

(*b*) *Heat Coagulation.*—This change, which may be illustrated by the coagulation of egg white in boiling water, is irreversible.

An instructive experiment, due to Hardy, consists of boiling side by side in separate beakers a fairly strong and a very dilute solution of egg white in water. The strong one coagulates while the dilute one becomes turbid only ; on the addition of a small quantity of barium chloride, however, a precipitate is produced. The explanation of this phenomenon is that, owing to the dilution of the solution, the particles of coagulated protein are too small to unite together, and therefore remain apart, forming a suspensoid which is, however, precipitated by the electrolyte.

(*c*) *Coagulation by Enzymes.*—The curdling of milk by rennet is a familiar example of this type of irreversible gel formation ; so also is the coagulation of pectic bodies occurring in fruit juices by the enzyme pectase with the formation of gelatinous calcium pectate.

Enzymes capable of coagulating milk also occur in many plants, such as *Lolium perenne, Anthriscus vulgaris, Geranium molle, Ranunculus bulbosus, Medicago lupulina, Ricinus, Datura, Pisum, Lupinus,* etc

(*d*) *Gelatinization by Altering the Concentration.*—If a dilute solution of gelatine in water be concentrated until it is about 5 per cent strength it will set to a jelly on cooling to the atmospheric temperature. Solutions of agar will gelatinize at much greater dilution. The change is, in both cases, reversible, for, by raising the temperature, or by adding more water, the gel goes into solution again.

A gel, however, once set will require a higher temperature to liquefy than its original setting temperature. Thus a 5 per cent gelatine gel setting at about 18° C. melts at about 26° C., while an agar solution which sets at about 35-40° C. will require to be heated to over 90° C. before it melts.

GENERAL PROPERTIES OF GELS.

Gels partake of some of the properties of both solids and liquids. With solids they share the property of maintaining their shape and of being more or less elastic, on the other hand their compressibility is very low like that of water of which they are very largely composed.

Owing to their rigid nature they lend themselves well for experiments on diffusion, and many interesting results have been obtained. One experiment, originally due to Liesegang, consists in placing a drop of silver nitrate solution on a gelatine gel containing a dilute solution of potassium bichromate; after a short time concentric rings of silver chromate are deposited around the original drop; this experiment has given rise to much experimental work with other reagents under varying conditions and there is much speculation regarding the true explanation of the phenomenon. There is, however, no doubt that the experiment illustrates the possibility of diffusion of a crystalloid such as silver nitrate in a gel.

The bringing about of such periodicity, as is exhibited by the alternating layers of deposit and clear solution in an inanimate system without variations in external conditions such as temperature changes, has an important bearing on biological and other natural problems; it would appear to offer a possible explanation of the stratification observable in agate and its possible significance in connection with the

many concentric ring structures or other alternating deposits found in nature will be obvious; in illustration, the formation of starch grains may be mentioned. According to Küster * many plant structures such, for example, as the banded pith of *Magnolia grandiflora*, the calcium oxalate sacs of *Ficus carica*, the regular alternation of crystal-bearing zones with those containing no crystals found in the bark of the Pomegranate and the zebra-like pigmentation of the succulent leaves of *Haworthia fasciata* and *Aloe varigata* may be due to similar causes; these structures at any rate may have their origin in some analogous internal rhythmic stimulus.

The peculiar concentric growth of certain moulds resulting in structures closely resembling the Liesegang rings have been studied by Munk.†

THE NATURE OF GELS.

As already pointed out above, emulsoids are regarded as two-phase systems in which the disperse phase is a more concentrated solution, and the continuous phase a relatively dilute one. When such a solution gives a gel, the rôles of the two phases are assumed to be changed, resulting in a sort of net- or sponge-like structure, of concentrated solution representing the continuous phase, whereas the disperse phase is represented by a dilute solution filling up the interstices.

Evidence for the existence of some such sponge-like or honeycomb structure has been obtained by Hardy ‡ in studying under the microscope the formation of a gel.

It is only by postulating some analogous structure that it is possible to understand how 1 gram of agar can cause 99 grams of water to set to a stiff jelly just as the organized cell structure of many plants enables them to maintain a rigid form while consisting of practically 90 per cent of water.

* Küster: "Beiträge z. Entwicklungsmechanischen Anatomie d. Pflanzen, Jena," 1913.

† Munk: "Centralblatt für Backteriologie," 1912, **32**, 353, and **34**, 561; also "Biol. Centralbl.," 1914, **34**, 621. See also Liesegang, "Naturwissensch. Wochenschr.," 1913, [xii], **25**.

‡ Hardy: "Proc. Roy. Soc.," 1912, A., **87**, 29.

ADSORPTION.

The phenomenon known as the occlusion of gases is an example of the adsorption of gaseous matter by a solid surface; it is exhibited to some extent by glass and platinum, but far better by wood charcoal, owing to its large superficial area; on this fact depends the use of wood charcoal, as a deodorant or for the adsorption of the last traces of gas in the production of high vacua. It is not known in what way the adsorption is effected, but the immediate effect is to produce a concentration of gaseous molecules at the surface of contact between the solid and the gas.

To all such cases of purely surface attachment the term Adsorption is generally applied, as opposed to absorption which implies something below the surface layer.

The property of adsorption is likewise one of the most important characteristics of colloidal solutions resulting directly from their great surface development. Wo. Ostwald has calculated that if a cube of material of 1 cm. edge, presenting a total surface of 6 sq. cms., were broken up it could yield 10^{18} cubes of 10 $\mu\mu$ edge ($\equiv$ ·000001 cm.), presenting a total surface of 600 square metres. Such cubes would be approximately the size of the particles of a colloidal solution, and it will therefore be seen that a comparatively small mass of the particles in such a colloidal solution must, in the aggregate, present a very considerable surface.

It has been calculated that the total surface presented by the particles of a red colloidal gold solution containing 0·5 grams of gold per litre amounts to about 8 square metres. It is, therefore, easy to understand that with such an enormous development of surface there is the possibility for a marked manifestation of adsorption by suspensoids.

In order to appreciate the effect of such surface development it is necessary to realize that all liquids tend to reduce their surface energy to a minimum; in the case of a solution this end may be assisted by increasing the concentration at the surface of any substance which lowers the surface tension. The most active substances in producing this effect are the fatty acids, soaps, albumen, enzymes, etc., and it follows,

therefore, that the surface layers will be most concentrated in aqueous solutions of these substances. Direct evidence of this may be obtained in the case of many solutions; for example, some dyes, such as methyl violet, on keeping, become so concentrated at the surface as to cover themselves with a film; the same applies to solutions of albumen. By blowing bubbles into such a solution and so increasing the surface, Ramsden * was able to remove the major portion of the dissolved substance from the solution by taking away the froth. Indeed, the tendency to froth in liquids is usually a manifestation of the greater concentration of dissolved substance at the surface with the resultant lowering of surface tension. Ramsden was further able to show that when a mixture of albumen and saponin is shaken up with water, the froth is richer in saponin since this substance lowers the surface tension of water more than does the albumen. This same phenomenon, no doubt, also explains the inactivation of some enzymes which results from mere shaking, and it has been shown that the froth of such solutions has greater activity than the rest of the liquid.

The interface between the disperse phase and the continuous phase of any colloidal solution represents a surface at which increased concentration can take place and hence the tendency for adsorption which is so characteristic a property of colloids.

The concentration of a dissolved substance upon the surface of a solid introduced into a solution may be illustrated by dipping a piece of filter paper into a dilute aqueous solution of congo red; after a short time the dye will have accumulated on the surface of the paper, leaving the solution much lighter in colour.

Moreover, since congo red itself is in colloidal solution and filter paper behaves in many respects like a colloid, this experiment also illustrates the phenomenon of mutual adsorption by colloids which is the principle underlying most processes of dyeing and staining, and also enzyme actions and other processes taking place in the living organism.

* Ramsden: "Z. physik. Chem.," 1904, 47, 336.

In this connection there is an interesting experiment due to Bayliss * which is designed to show that although in the process of dyeing adsorption upon the surface to be dyed may be the first step, yet chemical reaction between the dye and the fibre may follow as a second stage. The experiment consists in shaking up a blue solution of the acid of congo red with well-washed aluminium hydroxide; the latter at once adsorbs the blue colour from solution, and settles down on standing; if it is now heated, the physically adsorbed congo red acid combines with the aluminium hydroxide to form the aluminium salt, a chemical reaction which is marked by the change of colour from blue to red.

In the same way Bayliss holds that in the case of enzyme action adsorption of the substrate upon the surface of the enzyme is the first stage, and that then, in consequence of the intimate contact between the two, mass action accelerates the reaction.

It is, of course, easy to understand that if adsorption takes place so readily between colloids, such as filter paper and congo red, both of which bear negative charges in water, the phenomenon must take place still more easily between oppositely charged colloids in which the mutual electrical discharge facilitates the deposition.

Numerous practical applications of adsorption from solutions are known, as for example in the removal of colouring matter in the purification of cane sugar, or in the removal of fusel oil from crude spirit by filtration through charcoal.

Other substances besides charcoal, such as Fuller's earth and china clay, have been similarly used on account of the large surfaces which they present.

From what has been said with regard to the structure of gels and the assumption that they present a sort of network with a considerable development of internal surface, it is easy to find an explanation of the use of isinglass for clearing a turbid solution or for the fact that colouring matter may be extracted from a solution by precipitating gelatinous aluminium hydroxide in it.

* Bayliss: "Z. chem. Ind. Koll.," 1908, **3**, 224.

The purification of sewage by means of alum followed by alkali likewise depends on the adsorption of impurities by the colloidal gelatinous aluminium hydroxide, and also upon the precipitation of colloidally dissolved impurities by the electrolyte.

The deodorizing and generally purifying effect of the soil is likewise probably due largely to the adsorption by porous or colloidal constituents of such soil.

A very striking case of selective adsorption is to be found in the power which seaweeds * have of extracting iodine from the surrounding sea water, although the amount of this element in sea water is extremely small; again, in spite of the enormous preponderance of sodium over all other metals in sea water, the plant takes up practically none of this, but takes instead potassium, which is present in much smaller quantity.

Many natural phenomena can be attributed to the same cause. For example, the power possessed by soils rich in clay or humus to retain soluble potassium salts or phosphates which would otherwise be washed away by rain.

The hydrated aluminium magnesium and sodium silicates, known as Zaeolites, which are contained in clays are colloids and they react by double decomposition with the potassium salts which may be applied as manures, and, while retaining the potash, set free a corresponding quantity of lime or soda.†

In this connection it may be mentioned that the affinity of colloids, such as humus and clay, for certain dyes, such as methyl violet or malachite green, has been employed as a rough means of detecting or estimating the proportion of these substances in a soil. For this purpose a quantity of the soil is shaken up with the dye solution in a cylindrical vessel; on settling, the heavier particles sink to the bottom, and a band of the dyed soil constituents is formed on the surface.

Thermodynamical considerations, coupled with experimental measurements, show the fact that true adsorption takes place according to well-defined mathematical laws which enable one to decide definitely whether a certain phenomenon

* Cameron: "J. Biol. Chem.," 1914, **18**, 335.
† Cf. van Bemmelen: "Z. anorg. Chem.," 1900, **23**, 321.

is due to physical adsorption or to chemical reaction ; thus, it has been found that a relatively larger amount of the total substance in solution is withdrawn from a dilute than from a strong solution.

COLLOIDAL ELECTROLYTES.

The proteins can function as acids to form salts with the alkali metals of the type $\overset{+}{N}a\overset{-}{P}r$, in which Pr stands for a colloidal ion ; compounds of this type are known as colloidal electrolytes. Such substances, composed as they are of a diffusible metallic ion and a non-diffusible colloidal ion, are comparable with such a salt as sodium ferrocyanide, which manifests a characteristic behaviour when separated from a solution of a completely diffusible electrolyte by a membrane. Theoretical considerations led Donnan to conclude that a freely diffusible electrolyte, having a common ion with the colloidal electrolyte, if placed on the opposite side of a membrane would not, at equilibrium, be distributed uniformly on both sides of the membrane ; on thermodynamic grounds there must be a greater concentration of the diffusible electrolyte on the side opposite to the non-diffusible ion. The same condition of affairs would result if the colloidal electrolyte were mixed with a diffusible salt and separated by a membrane from pure water. This is known as the Donnan Equilibrium ; the correctness of the theoretical conclusions have been experimentally verified.

Certain writers, notably Loeb,† unduly strain this theory in the endeavour to make it explain the entire colloidal behaviour of proteins to the exclusion of all other considerations. It will be apparent that the living cell, consisting of protoplasm composed of colloidal electrolytes both in contact with and separated from diffusible electrolytes by a semi-permeable membrane (which semi-permeability may vary with altering conditions), presents just those conditions as are suitable for the establishment of a Donnan Equilibrium. Naturally these conditions

* Donnan : " Zeit. Elektrochem.," 1911, **17**, 572. Donnan and Allmand : " J. Chem. Soc.," 1914, **105**, 1963. Harwood : *id.*, 1923, **123**, 2254.

† Loeb : " Proteins and the Theory of Colloidal Behaviour," London and New York, 1922.

are of infinite complexity and it is not suggested that this is the sole controlling factor, but that it may be a contributory factor is not improbable. Many attempts have been made to apply such considerations to the observed facts and of these one example may be given. According to Butkewitsch,* phenomena associated with the Donnan Equilibrium are likely to be fairly frequent in plant cells and to take an important part in determining the distribution of diffusible ions; the rôle of the colloidal ions may be taken by ordinary molecular disperse ions if the membrane is impermeable to them. The Donnan Equilibrium may also affect the absorption of salts by the root system, and the indiffusible ion may act from the inside of the cell or possibly from the outside if such indiffusible ions happen to be present in the soil. Experiments with collodium membranes showed that silicic acid favoured the transfer of diffusible ions to the other side of the membrane, and it is suggested that silicic acid outside the cell may favour the diffusion of phosphates into the cell and thus stimulate the growth of the plant. Experiments with *Zea mais*, grown in culture solutions containing phosphates, both in presence and in absence of silicic acid, showed that when the solutions contained only small quantities of phosphates the growth of the plants was directly proportional to the amount of silicic acid present. It is further suggested that the observations of Hellriegel † and others, and more recently of Breazeale,‡ that an addition of sodium ions to the nutrient solution increased the absorption of potassium by the plant, may be attributed to the same cause; thus if the membrane is impermeable to sodium salts these would act as non-diffusible ions and so tend to favour the transfer of diffusible potassium salts across the membrane.

ENZYME ACTION OF COLLOIDS.

Associated with this enormous development of surface there is, of course, a corresponding development of surface

* Butkewitsch: "Biochem. Zeit.," 1925, **161**, 468.

† Hellriegel, Willfart, Romer, and Wimmer: "Arbeit. deut. land. Ges.," 1898, **32**.

‡ Breazeale: "J. Agric. Res.," 1923, **26**, 303.

energy, which no doubt, in part, explains the remarkable catalytic activity exhibited by colloidal solutions of the metals.

Bredig † and his collaborators have shown that a colloidal solution of platinum containing 194 grams of metal (i.e. 1 gram atom) in 70,000,000 litres of water, or a colloidal solution of gold containing 197 grams of metal in 1,000,000 parts of water, are still able to produce a distinct accelerating influence on the decomposition of hydrogen peroxide into water and oxygen.

It has long since been known that metallic platinum, more especially the variety known as spongy platinum, when left in contact with hydrogen peroxide induces the decomposition of this substance into water and oxygen, and Berzelius,‡ as long ago as 1836, pointed out an analogy between this catalytic action of platinum and the action of an "insoluble ferment," such as yeast on sugar.

This suggestion has since been borne out by a number of examples of chemical changes which could be effected equally well either by means of finely divided platinum or by a ferment, e.g. the oxidation of alcohol to acetic acid by *Mycoderma aceti*, the bleaching of indigo solution by hydrogen peroxide in presence of red blood corpuscles, the blueing of tincture of guaiacum by hydrogen peroxide in presence of red blood corpuscles, etc., all of which can also be affected by spongy platinum.

Bredig carried our knowledge of the subject a step farther; by preparing colloidal solutions of the metals and comparing their action with that of various enzymes, he traced out such a remarkable analogy between the two that he has called the colloidal metal solutions "Inorganic Ferments."

The chief points of similarity between enzymes and colloidal platinum may be summarized as follows:—

1. Both platinum hydrosol and enzymes are colloids and as such are detrimentally affected by electrolytes.

* Ostwald: "Z. physik. Chem.," 1897, **23**, 172.
† Bredig: "Anorganische Fermente," Leipzig, 1901, p. 96.
‡ Berzelius: "Jahresber.," 1836, **13**, 237.

2. Both platinum hydrosol and enzymes gradually decompose spontaneously or decompose more rapidly by heating.

3. There is an optimum temperature for both colloidal platinum and for enzymes to exert their catalytic action.

4. The activity of platinum hydrosol may be stimulated by the addition of alkali until it reaches its maximum value, after which the further addition of alkali causes it to fall again. Similar stimulation of enzymes by the addition of certain substances known as Zymo-exciters have been observed in case of emulsin acting on hydrogen peroxide, and of invertase acting on cane sugar.

5. The decomposition of hydrogen peroxide whether by platinum hydrosol or by catalase, an enzyme contained in blood, is in accordance with the laws governing a monomolecular reaction.

6. A very remarkable analogy between platinum hydrosol and the enzyme of blood is that small quantities of substances which, when added to the colloidal platinum solution, destroy its catalytic action on hydrogen peroxide, also have the same effect on the oxidase of blood. Curiously enough many of these substances are blood poisons such as sulphuretted hydrogen, hydrocyanic acid, carbon monoxide, and arseniuretted hydrogen; several other substances were also found to paralyse either the platinum solution or the enzyme.

It was further observed that platinum hydrosol when treated with very small traces of hydrocyanic acid was temporarily poisoned but recovered after a short time; a similar effect has also been observed with enzymes. The recovery is probably due to the oxidation of the hydrocyanic acid.

It was also found that the toxic effect of the hydrocyanic acid was much greater if added directly to the platinum or gold sol than if added to a sol already containing some hydrogen peroxide. Exactly similar conditions had been previously found by Schönbein * to hold in regard to the addition of hydrocyanic acid and hydrogen peroxide to blood.

In conclusion, it should be noted that Bredig, while dis-

* Schönbein: "Zeit. f. Biologie," 1867, 3, 144.

claiming any attempt to trace a fanciful connection between the colloidal metal solutions and enzymes, emphasizes the fact that the two properties of catalytic action and colloidal nature are common to both classes of compounds and regards the colloidal metals as the simple inorganic analogues of the more complex enzymes.

One further illustration might be quoted of the chemical activity which is associated with colloidal substances presenting a large surface. A calculation based on the assumption that there are five million red blood corpuscles of diameter ·007 mm. contained in 1 c.mm. of blood reveals the striking fact that the total surface presented by the blood corpuscles contained in 5 litres of blood (the amount contained in the body of a full-grown man) would be about 1875 square metres. From what has gone before it is, therefore, not surprising that these corpuscles should be endowed with special properties enabling them, in the presence of the trace of iron which they contain, to play their part in the highly complex changes involved in respiration.

THE COLLOIDAL NATURE OF PROTOPLASM.

From the foregoing consideration of the colloidal state, it is obvious that this condition endows matter with great powers, the exercise of which are dependent on the particular phase of the colloid, the presence of substance amenable to change, and the appropriate conditions for the action to take place.

From the biological point of view protoplasm is the all-important colloid, for it is in protoplasmic activity that the key to all vital actions and reactions are to be found.

In earlier days the structure of protoplasm was variously described as alveolar, reticulate, fibrillar, and so on. These views were based on the observation of living protoplasm by the ordinary methods of microscopy, and on dead protoplasm fixed and stained by the ordinary methods of cytological technique. Many of the characters observed by the latter method were artifact, and from the former pursuit too little was observable although it was realized, perhaps dimly, that protoplasm was possessed of colloidal characters.

It was not until recent years that, by the aid of the ultramicroscope * with dark ground illumination and the methods of microdissection,† a clearer knowledge of protoplasmic structure has been gained.

The structure of protoplasm is not constant; not only does it vary in different plants, but in the same plant and in the same cell according to varying conditions. It is a complex of colloids, the continuous phase of which is more or less viscous, which can change spontaneously from the hydrosol to the hydrogel condition, and *vice versa*. It has already been stated that the Brownian movement of suspended particles ceases in the gel state owing to the high viscosity. Gaidukov ‡ noticed that when the streaming of the protoplasm in the cells of *Vallisneria* temporarily stopped, only few of the particles showed Brownian movement. In time the Brownian movement became manifest, and as it increased the streaming of the protoplasm began. Also Bayliss § comments on the stopping of Brownian movement in amœba when the cytoplasm is subjected to an electric shock too weak to be fatal, presumably owing to a temporary change of state from sol to gel.

The particles in the hydrosol vary much in size, ranging from microsomes just visible under the high power with transmitted light, to submicrons only to be seen under the ultramicroscope. Suspended particles also may be present, graded in size and showing Brownian movement, the rate of which is determined by their size and the degree of viscosity of the medium. These particles tend to increase in number with the age and decreasing vitality of the cell. According to Price the hydrosol complex is always emulsoid in character but in varying degrees; the protoplasm of the hairs of

* Gaidukov: "Ber. deut. bot. Gesells.," 1906, **24**, 107, 155, 192, 581; "Dunkelfeldbeleuchtung u. Ultramikroskopie . . .," Jena, 1910. Price: "Ann. Bot.," 1914, **28**, 601.

† Chambers: "Amer. Journ. Physiol.," 1917, **43**, 1; "Biol. Bull.," 1918, **4**, 121; "Trans. Roy. Soc. Canada," 1918, **12**, 41; "Amer. Nat.," 1926, **60**, 105. Seifriz: "Biol. Bull.," 1918, **34**, 307; "Bot. Gaz.," 1920, **70**, 360; "Ann. Bot.," 1921, **35**, 269; "Amer. Nat.," 1926, **60**, 124. See also Heilbrunn: "Amer. Nat.," 1926, **60**, 143; and Mast: *id.*, 133.

‡ Gaidukov: "Dunkelfeldbeleuchtung u. Ultramikroscopie in der Biologie und in der Medizin," Jena, 1910.

§ Bayliss: "Principles of General Physiology," London, 1918.

Cucurbita, for instance, being more emulsoid than that of the cells of *Elodea*. Protoplasm also exists in a state of gel which is regarded as an active state capable of performing the functions of growth and nutrition. The change from hydrogel to hydrosol, probably by the adsorption or imbibition of water, was followed in the germination of spores of *Mucor*. The contents of a freshly mounted spore are homogeneous and show no protoplasmic movements. Water is absorbed, the spore swells, and then the protoplasm, in the gel condition, forms a peripheral layer. The contents become more opaque and the structure becomes increasingly that of a hydrogel. Just before germination the protoplasm passes into the hydrosol condition, its small particles showing rapid oscillations. The growth of the germ tube is at first rapid and into it passes most of the protoplasm which occupies less and less of the total volume as the hypha increases in size. In the hypha protoplasmic plugs are formed by the aggregation of large motionless particles, their colloidal condition being considered to be in a reversible condition intermediate between the states of sol and gel.

From the ultramicroscopic study of plasmolysed cells evidence is obtained of the presence of a delicate membrane, much finer in texture than that of the general protoplasm, surrounding the protoplast and also occurring on the inner side separating the central vacuole, when present, from the cytoplasm. This plasma membrane also has been demonstrated by the methods of micro-dissections. Experiments on many and various organisms—Protozoa, Myxomycetes, Chlorophyceæ, moulds, pollen tubes, oospheres of *Fucus*, eggs of echinoderms, etc.—indicate that all protoplasm is limited by a membrane essentially protoplasmic in nature although differing in its physical properties, and possibly also in its chemical constitution, from the general cytoplasm. This plasma-membrane is a very thin, about 0·1 μ, elastic, and highly viscous gel which can readily revert to the hydrosol condition. If rapidly torn, disintegration of the whole membrane takes place; but if slowly torn, regeneration takes place and the integrity of the protoplast is maintained.

In such instances where the general cytoplasm is in the gel state, the plasma membrane is difficult, if not impossible, visually to demonstrate since the two consistencies closely approximate. But structural differences are recognizable by their physiological behaviour. Thus Chambers found that sodium chloride or potassium chloride injected into an amœba caused quiescence and liquefaction of the internal protoplasm, but the normal state was regained in time. Calcium chloride or magnesium chloride when similarly injected, solidified the internal protoplasm and the region was permanently injured. On the other hand, when the amœba was immersed in sodium chloride, the plasma membrane was disintegrated; potassium chloride had a similar effect but to a lesser degree; like treatment with calcium chloride or magnesium chloride had no influence so long as the plasma membrane was intact.

Seifriz agrees that the outer layer of the protoplasm has a morphological identity distinct from the internal mass, but he considers that the difference in constitution is one of degree rather than of kind. The characteristic physical properties of protoplasm, capacity for imbibition, rigidity, and especially elasticity, indicate, according to Seifriz, a gel of fibrous structure.

The interaction between protoplasm as a colloid, and electrolytes dissolved in the cell sap or in the liquid medium in which the protoplasm is suspended, must also be borne in mind.

Clowes * has propounded a theory of the mechanism of permeability based upon the phase relationships between the particles of the colloidal protoplasm and the continuous medium in which they are suspended. Arguing from the fact that calcium soaps favour the formation of a water in oil emulsion, while sodium soaps favour the oil in water emulsion, he suggests that the permeability of the protoplasmic membrane might be influenced by a predominance of either sodium or calcium ions; thus the presence of an excess of sodium ions would permit the passage of water-soluble substances between the particles of an oily disperse phase, while, owing to their

* Clowes: "J. Physical Chem.," 1916, **20**, 407.

insolubility in oil, they would not be able to pass through an oily continuous phase such as would be produced by an excess of calcium ions. This theory gains support from the fact, established by Osterhout,* that the permeability of *Laminaria* is greatly increased by immersion in a solution of pure sodium chloride, isotonic with sea-water, but containing no calcium salts; moreover the addition of calcium salts to the sodium chloride restores the normal condition of the cell.

Another example of the influence of electrolytes upon protoplasm is provided by the use of the so-called Ringer solution which is a physiologically balanced solution. Ringer found that the isolated heart of the frog if perfused with pure water soon stopped beating; if a solution of sodium chloride, isotonic with blood serum, was used in place of water, the heart-beat continued rather longer. On comparing the effects of calcium chloride with potassium chloride he found that the former left the heart at rest in the contracted condition while the latter left it completely relaxed; there was thus an antagonistic action between the two metals, and on making a solution of the two salts in appropriate proportions he obtained a liquid in which the heart-beat could be maintained for several hours. The proportions of calcium to potassium in such a solution are approximately those which obtain in sea water. A point of some interest was elicited when attempts were made to replace calcium or potassium by other divalent or monovalent elements respectively; thus it was found † that the order of effectiveness in maintaining the movements of the cilia of the epithelium of the frog's œsophagus was as follows:—

$$K > Rb > Na > Cs > Li$$

This sequence, it will be remembered, is the same as the precipitating power of these kations in the lyotrope series upon colloids; thus lithium, which is least effective in maintaining the movements of the cilia, may be regarded as most toxic, an effect which may be correlated with its precipitating effect upon such a colloid as protoplasm.

* Osterhout: "Science," 1911, **34**, 187; 1913, **38**, 408.
† Overton: "Pflüger's Archiv," 1904, **105**, 176.

Further, the fact that at their isoelectric points colloids are more easily precipitated, would suggest a possible harmful consequence of an alteration of the hydrogen ion concentration of the cell sap in a direction towards the isoelectric point in view of the increased likelihood of the salts present exerting their precipitating action (see p. 437).

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SECTION IX.

PROTEINS.

THE term protein is applied to a large variety of bodies occurring in the animal and vegetable kingdoms, which occupy a pre-eminent position in the economy of life, owing to their being the chief constituents of protoplasm.

In the plant, proteins may occur either as solid bodies or in solution in the cell sap. They may be found in all living members; in roots, stems, leaves, sieve tubes, laticiferous tissue, etc. Reserve proteins commonly are found in the solid state, especially in seeds and in vegetative organs of propagation.

These protein bodies may be either quite amorphous or crystalline; sometimes the grains are partly amorphous and partly crystalline, as in the well-known aleurone grains of the seed of *Ricinus*.

Protein crystals may be cubical, as in the potato, falciform as in the carpellary walls of *Gratiola officinalis*, and other shapes; they may occur quite free within the cell, as in the potato, or embedded in other bodies. These embedded crystals may be found in nuclei, e.g. in the leaves of *Melampyrum arvense* and in the ovary wall of *Campanula trachelium;* in chloroplasts, e.g. *Hedera* and *Canna;* and in amorphous protein, e.g. in the seeds of *Ricinus* and *Bertholletia*.

These last, generally known as aleurone grains, are often somewhat complicated; the grain is surrounded by a protein membrane, which is less readily soluble than the remaining amorphous protein of the matrix. Embedded in the matrix is the crystalloid, and also a globoid consisting of a double phosphate of calcium and magnesium. The crystalloids vary in shape; commonly they are hexagonal and stain brown with iodine and are readily soluble in dilute alkali, also they

may readily be stained in fuchsin. To do this, the sections should be placed in a 0·2 per cent aqueous solution of acid fuchsin for twenty-four hours, washed in running water and mounted in Canada balsam in the usual way.

Several proteins may occur in aleurone grains and may be recognized by their different solubilities in water, salt solution, alkali, and alcohol. Also, the details of the composition of these grains are not the same for all plants in which they occur; for instance, in the pæony the matrix is soluble in water, whereas in the castor-oil plant it is insoluble in water but soluble in a strong aqueous solution of sodium phosphate.

According to Bokorny,* globulins are the common proteins occurring in the aleurone grains and crystalloids of seeds. It should be remarked that the term aleurone grain is frequently used in a generic sense to include all non-crystalline reserve protein bodies of a more or less definite shape; they are not always of the complicated nature described above, thus in the grain of wheat they are quite simple in structure and do not contain a crystalloid nor a globoid.†

The seed proteins which are soluble in salt solutions are deposited from their solutions when the concentration of the salt is reduced by dialysis, by dilution, or by other means. According to Osborne,‡ the deposition of proteins in a crystalline form within the aleurone grain may be attributed to a similar diminution in the salt content of the cell sap resulting from the formation of the globoid, the calcium magnesium salt of inosite phosphoric acid known as phytin.

In contrast to the proteins of the seed, which are reserve proteins, those of the leaf are of a more labile nature and may be expected to differ from the former in composition. The method initiated by Osborne and Wakeman § for the investigation of the leaf proteins of spinach, consisted in grinding the leaves with water, centrifuging, and coagulating the proteins contained in the resulting colloidal solution by warming to

* Bokorny: "Bot. Centrbl.," 1900, **82**, 289.

† For an account of the artificial production of protein grains, see Thompson: "Bot. Gaz.," 1912, **54**, 336.

‡ Osborne: "The Vegetable Proteins," London, 1924.

§ Osborne and Wakeman: "J. Biol. Chem.," 1920, **42**, 1.

40°. Chibnall * has devised a method for distinguishing between the proteins of the cell sap of the central vacuole and those contained in the cytoplasm of the cell. The method consists in treating the fresh leaves with water containing ether, to effect cytolysis, and pressing in a hydraulic press; the cake is then allowed to imbibe water and is once more pressed without rupturing the cells. The liquid so obtained presumably contains the proteins of the cell sap; these are precipitated with 30 per cent alcohol. In the case of the spinach, this product contained 2·4 per cent nitrogen and consisted of either globulin or albumin. The residual cake of leaf tissue is ground with water, in order to break up the cells, and the resulting colloidal solution is treated with acetic acid which throws out a floccular precipitate. This precipitate after purification, gave a white powder which contained 14·9 per cent of nitrogen; it represents the cytoplasmic protein and appears to be a type of protein hitherto unrecognized, differing from other native proteins in being soluble in small excess of either acid or alkali.

Osborne, Wakeman, and Leavenworth † are of the opinion that the protein in alfalfa leaf may be combined with a colour complex of a flavone nature and thus belongs to the class of conjugated proteins; their evidence for combination is the fact that the protein is extracted only after prolonged heating with alkali, during which period hydrolysis takes place

EXTRACTION OF PROTEINS.

The main facts relating to the solubilities of the common vegetable proteins are as follows:—

1. Proteoses, albumins, and some globulins are soluble in water.

2. Globulins, together with most of the proteins soluble in water, dissolve in 10 per cent sodium chloride.

3. Prolamins are soluble in alcohol (70-90 per cent).

4. Glutelins and prolamins dissolve in dilute acid and in dilute alkali.

* Chibnall: "J. Biol. Chem.," 1923, **55**, 333.

† Osborne, Wakeman, and Leavenworth: "J. Biol. Chem.," 1921, **49**, 63.

These facts are made use of in the extraction of the substances in question from vegetable tissues such as seeds, which may contain several proteins; and although the products so obtained are anything but pure, a brief outline of the method may be given. The separation of the proteins removed by these means from the seed by a given solvent is a very lengthy and tiresome process, and the details must be sought for elsewhere.*

Before proceeding with the extraction, the material must be ground up as finely as possible, in order that all the cells may be broken; if needs be, the tissue must be carefully dried beforehand, but too high a temperature must not be used.

In all cases the initial procedure is much the same; the powdered material is well mixed with the solvent, which is allowed to act for some time; the mixture should be well shaken periodically † The solid is then filtered off and well washed with fresh solvent, and is again treated until the extract gives no protein reaction. The temperature may be raised during the extraction, but it should not be high enough to alter the proteins. If the extraction, especially with aqueous solvents, be prolonged, it may be necessary to add a little antiseptic, such as chloroform, in order to stop bacterial action.

When it is desired to make successive extracts, in cases such as seeds where several proteins may be present, the order may be water, 10 per cent sodium chloride, alkali (·1 to ·2 per cent caustic potash or ·5 to 1 per cent sodium carbonate), and finally alcohol (70 to 80 per cent).

The initial extraction may be made with salt solution, the albumins being afterwards separated from the globulins, and this course is recommended when both are present on account of the saving of time.

The proteins isolated by these means may be roughly purified as follows:—

* See Osborne: "The Vegetable Proteins," London, 1924, on whose account the following is based. For a method for the preparation of plant globulins, see Reeves: "Biochem. Journ.," 1915, **9**, 508.

† If the material employed is a fatty seed, a preliminary extraction with ether is essential to remove the fat before treating with aqueous solvents.

1. Albumins and globulins.—These will nearly always be contaminated one with the other. A separation may be effected in the following ways :—

(*a*) The solution is saturated with magnesium sulphate, whereby the globulins are precipitated and the albumins remain in solution (but see below, under albumins and globulins).

(*b*) Dialysis. The extract is placed in a dialyser and floated in water which is continually changed. The precipitated globulins are filtered off from the salt solution, which, of course, is getting weaker and weaker and contains the albumins. The precipitated globulins are re-dissolved in warm saline solution, which may on cooling deposit globulins in a crystalline form ; if this does not occur, the solution may be saturated with magnesium sulphate. The albumins may be precipitated by saturating the solution with ammonium sulphate. Further purification may be effected by a repetition of the process and by fractional precipitation with magnesium sulphate or by ammonium sulphate, according to the protein to be purified.

2. Glutelins.—The proteins soluble in dilute alkali may be precipitated by very carefully neutralizing the solution and then further adding only just sufficient acid to cause the precipitation of the glutelins. The precipitate may be well washed with a dilute neutral saline solution, in which it is insoluble, in order to remove any globulins which may be present.

3. Prolamins.—The extract, which is made by treatment with hot alcohol, is either mixed with water sufficient in amount to precipitate the proteins, or the filtered solution may be evaporated under a reduced pressure at a temperature not higher than 50° C. The precipitate is filtered off and may be re-dissolved in as little alcohol as possible. From this solution the protein may be recovered by the addition of absolute alcohol, in which prolamins are insoluble, and ether. The

ether is added in order to make the precipitation more complete and also to hold any fats which may have been extracted by the alcohol.

CLASSIFICATION OF PROTEINS.

The classification of the proteins was originally, for want of chemical knowledge, based on their different physical properties, such as solubilities, coagulation by heat, precipitation by neutral salts, etc.

Now that, from a study of their products of hydrolysis, a little more is known of the chemistry of the proteins, it is found that, on the whole, the physical method of classification is more or less in accordance with the chemical evidence.

Appended is the scheme of classification generally adopted in this country :—

Protamines.—These are the simplest proteins known, and are represented by such substances as salmine, sturine, cyclopterine, etc., which have been isolated from fish sperm.*

They usually occur associated with nucleic acid in the form of salts.

No compounds resembling the protamines have as yet been isolated from plants, although they may possibly occur in pollen.

Histones.—The histones, of which the best known one is that obtained from blood corpuscles, are characterized by being precipitated from solution by ammonia ; they are related to the protamines, but are more complex than these substances.

Albumins.—This group includes egg-albumin, serum-albumin ; and such vegetable albumins as legumelin of the pea and leucosin of wheat and other cereals.

The albumins are typically soluble in water and are coagulated by heat. They are not precipitated by saturation with sodium chloride or magnesium sulphate, nor by half saturation with ammonium sulphate, but, like

* Kossel : " Bull. soc. chim. Paris," 1903, [23], **29**, i-xviii.

all proteins, are precipitated by complete saturation with ammonium sulphate.

Traces of albumins occur in practically all seeds, but no seeds, so far examined, have been found to contain large quantities.

While plant albumins resemble those of animal origin in regard to the two essential features of this group, namely, solubility in water and coagulation by heat, they differ in regard to their behaviour towards strong solutions of inorganic salts. Thus animal albumins are not supposed to be precipitated by half saturation with ammoniun sulphate or saturation with sodium chloride or magnesium sulphate, but this is not always found to be the case for vegetable proteins, many of which are precipitated under these conditions.

Globulins.—These are exemplified by serum globulin, fibrinogen and myosinogen, and also the derivatives of the two latter, fibrin and myosin. Examples of vegetable globulins are furnished by conglutin from the seeds of *Lupinus*, edestin from the seeds of *Cannabis sativa*, excelsin from the seeds of *Bertholletia excelsa*, legumin from the seeds of *Pisum sativum*, *Vicia Faba*, and other Leguminosæ, juglansin from the seeds of *Juglans spp.*, vicilin from the seeds of *Pisum sativum*, *Vicia Faba*, *etc.*, and vignin from the seeds of *Vigna sinensis*. In brief, globulins are amongst the commonest protein reserves of the higher plants.

The typical globulins are insoluble in pure water and are coagulated by heat. They are soluble in dilute salt solutions, but are insoluble in stronger salt solutions; thus, unlike the albumins, they are precipitated by saturation with magnesium sulphate or by only half saturation with ammonium sulphate.

* These differences in solubilities between albumins and globulins may be illustrated by dissolving some of the white of an egg in water and placing it in a dialyser; as the small quantity of sodium chloride contained in the egg-white diffuses out, the globulin is precipitated out of solution; or, again, if the solution is mixed with an equal volume of saturated ammonium sulphate solution, the globulin will likewise be precipitated out, owing to the solution now being half saturated with ammonium sulphate, but the albumin will remain in solution.

The vegetable globulins, which form the major portion of the reserve proteins of all seeds except cereals, do not always conform to these conditions of solubility. Thus, whereas animal globulins are insoluble in water and are precipitated by half-saturation with ammonium sulphate, a great many globulins from plants are precipitated at less than half saturation, and, on the other hand, some are not precipitated until the solution is almost saturated with ammonium sulphate. It must, however, be noted that globulins extracted from seeds are nearly always obtained in the form of salts with a small amount of acid, and so long as they are in this form they have the characteristic solubilities of animal globulins. As soon, however, as the acid is removed they lose these and become completely soluble in water.

A further point of difference between animal and vegetable globulins is that many of the latter are only coagulated by heat with considerable difficulty.

The albumins and globulins are the only classes of proteins which are coagulated with heat.

Glutelins.—This is a small class represented by two proteins, both of vegetable origin, namely, glutenin found in wheat and oryzenin in rice. Similar substances probably occur in other cereals as well, but owing to the difficulty of obtaining them in a pure condition, they have not as yet been investigated.

Glutelins are insoluble in water and neutral saline solutions, but dissolve in dilute alkali.

Gliadins or *Prolamins.*—These also are represented only by vegetable proteins, namely, gliadin from wheat or rye, hordein from barley, and zein from wheat or maize. Up to the present they have only been found to occur in cereals. The gliadins differ from all other proteins in being soluble in 70-90 per cent alcohol, the solutions being unaltered by boiling; they are insoluble in water or in salt solutions, but are soluble in dilute acids or alkalis.

On hydrolysis they yield a considerable quantity of proline (hence the name prolamins), glutamic acid and

ammonia, but only small amounts of arginine and histidine, and no lysine.

Glutelins and gliadins are the chief protein constituents of the substance known as gluten.

Sclero-proteins.—This term is synonymous with the older term albuminoid, and includes substances of skeletal origin, such as keratin from hair, horn, etc., gelatin, elastin, and silk fibroin.

No representative of this class has as yet been found among vegetable proteins.

Phospho-proteins.—This group, which is probably not represented in the vegetable world, contains such substances as caseinogen and vitellin, obtained from milk and egg yolk respectively. The phosphorus of these proteins is in intimate organic combination with the protein molecule, and is not contained in the "prosthetic group" (see below) as in the case of the nucleo-proteins, which are composed of proteins with the phosphorus-containing nucleic acids.

The phospho-proteins are insoluble in water, but soluble in alkalis.

The phospho-proteins resemble the nucleo-proteins in their solubilities, but they differ from them in their behaviour on hydrolysis; they yield at first a so-called pseudo- or para-nuclein, corresponding to the formation of a nuclein from a nucleo-protein, but whereas a nuclein on further hydrolysis yields nucleic acid, and ultimately purine bases, the pseudonuclein yields no corresponding pseudonucleic acid, but on the other hand is broken up by baryta water into phosphoric acid, but gives no purine bases.

Conjugated Proteins.—Conjugated proteins are characterized by the fact that on hydrolysis they break up, yielding a true protein and a substance of a different nature, for which Kossel has proposed the name "prosthetic" group.

Thus, for example, a chromo-protein like hæmoglobin breaks up into globin (a protein) and a pyrrole derivative, hæmatin (cf. chlorophyll, p. 323). Similarly, a

glucoprotein such as mucin yields a protein and a carbohydrate, glucosamine.*

The conjugated proteins appear to be rarely found in plants; they may be divided into three sub-groups :—

1. *Gluco-proteins*, represented by mucin and possibly by the mucilage of the roots of *Dioscorea japonica* which in many of its characters resembles mucin obtained from animal sources.

2. *Chromo-proteins* represented by hæmoglobin and possibly by phycoerythrin (p. 353) together with some of the chromoproteins described by Osborne, Wakemann, and Leavenworth † as occurring in alfalfa.

3. *Nucleo-proteins*.—With regard to the occurrence of nucleo-proteins among plants, it is undoubtedly true that nucleic acid has been repeatedly found in plants, and compounds of proteins with nucleic acid have been isolated by Osborne, but it is not certain whether these substances actually occurred pre-formed in the seed, or were produced during the process of their isolation. Osborne ‡ is of the opinion that the small quantities of nucleo-protein which occur in the seed are chiefly in those parts of the embryo which are rich in nuclei, rather than in the places of food storage, such as the cotyledons and endosperm.

A nucleo-protein, when subjected to peptic digestion, or treated with dilute acid, gives a protein and a nuclein; this latter with caustic alkali breaks up still further into a second protein and a nucleic acid; the nucleic acids on further hydrolysis yield phosphoric acid, a carbohydrate residue, either a pentose or glucose, and purine

* Glucosamine is a peculiar nitrogen containing sugar of the formula $CH_2OHCHOHCHOHCHOHCHNH_2CHO$ or $CH_2OHCHOHCH\overset{O}{\diagdown\diagup}CHOH \cdot CHNH_2CHOH$ It has all the ordinary reactions of sugars as regards reduction of Fehling's solution, reaction with phenylhydrazine, etc., but is not fermentable by yeast. Owing to the presence of the amino group, it is also able to form salts with acids such as hydrochloric acid. It was first obtained by the hydrolysis of chitin contained in the shell of lobsters, and has since been obtained by the hydrolysis of several gluco-proteins such as serum mucoid, etc.

† Osborne, Wakeman, and Leavenworth: "J. Biol. Chem.," 1921, 49, 63.

‡ Osborne: "The Vegetable Proteins," London, 1924.

bases, such as guanine, adenine, xanthine, etc. (see p. 376). The view of Jones * on the nature of nucleo-protein is that the term connotes a salt of protein with nucleic acid in which the protein is in excess; when submitted to peptic digestion, part of the protein is removed, leaving a salt containing rather less of the base and consequently of a more acid nature to which the name nuclein was at one time given. He concludes that the term nucleo-protein "means rather a method of preparation than a chemical substance."

Derivatives of Proteins.—In this group are included a number of substances obtained by the hydrolysis of proteins; they may be sub-divided as follows :—

1. Meta-proteins, consisting of acid albumin and alkali albumin, produced respectively by the action of acid or alkali on proteins.

2. Proteoses, represented by albumose, globulose, gelatose, etc. These substances are produced from proteins by the action of digestive juices such as pepsin and trypsin.

Pepsin, which acts in an acid medium, breaks up the protein as follows :—

PROTEIN.
META-PROTEIN (acid albumin).
PRIMARY PROTEOSE (precipitated by half-saturated ammonium sulphate and by potassium ferrocyanide in the presence of acetic acid).
SECONDARY PROTEOSE (precipitated by saturated ammonium sulphate, but only slowly by potassium ferrocyanide in the presence of acetic acid).
PEPTONE (not precipitated by saturated ammonium sulphate nor by potassium ferrocyanide in the presence of acetic acid).
POLYPEPTIDES AND AMINO ACIDS.

The formation of amino acids from peptones takes place only after prolonged action.

Trypsin, which acts in an alkaline medium, produces substantially the same series of changes, only that the meta-protein in this case is alkali albumen; furthermore the decomposition into amino acids takes place more rapidly than with pepsin.

* Jones: "Nucleic Acids," London, 1920, p. 7.

A great many seeds have been found to contain proteoses after the removal of the other proteins, and substances resembling the proto-, hetero-, and deutero-proteoses obtained from animal proteins have been described; but in all cases it is difficult to say whether these substances were not produced by some secondary action of enzymes upon the protein, during the process of isolation.

3. Peptones.—Substances belonging to this class still give the biuret reaction, but unlike all other proteins they are not precipitated from solution by saturation with ammonium sulphate.

4. Polypeptides, which include such substances as leucyl glutamic acid, obtained by Fischer and Abderhalden from gliadin by hydrolysis with 70 per cent sulphuric acid, and glycyl tyrosine and glycyl leucine, obtained by the same authors from silk fibroin and elastin respectively.

COMPARISON BETWEEN VEGETABLE AND ANIMAL PROTEINS.

From the foregoing it will be seen that, in the main, the animal and vegetable proteins conform sufficiently well with regard to their general properties and solubilities that they may be included in the same scheme of classification. The greatest irregularities are exhibited in the groups of albumins and globulins, but even these are not sufficiently serious to suggest any fundamental difference between the proteins derived from animal and vegetable sources. These views are confirmed by chemical evidence: with the single exception of di-amino trihydroxy-dodecanic acid, a substance as yet only obtained from casein, all the known products of hydrolysis of animal proteins have been obtained from vegetable proteins, and there is no real reason for assuming that there is any fundamental difference in the structure of the protein molecule from the two sources.

On the whole, vegetable proteins yield more glutamic acid, and many also yield rather more proline, arginine, and ammonia than do animal proteins.

The comparatively large quantities of proline and arginine which occur in some cases may be responsible for the slightly higher nitrogen content which characterizes proteins of vegetable origin.

Further, it should be noted that the prolamins, or alcohol soluble proteins, form a distinct class; they are found only in the vegetable kingdom, and have no analogues amongst proteins from animal sources.

Of twenty-three different seed-proteins which have so far been systematically hydrolysed, all were found to contain leucine, proline, phenylalanine, asparagine, glutamic acid, tyrosine, histidine, arginine, and ammonia; two gave no glycine; two gave no alanine, four gave no lysine; and one gave no tryptophane. One, namely zein, gave neither glycine, lysine nor tryptophane. Three gave no cystine, and two others only traces.

It is, on the whole, unlikely that there is any protein entirely free from sulphur, although in the case of vicilin the amount is actually as low as 0·1 per cent. If it is assumed that the sulphur is contained in the molecule in the form of cystine, it follows that there must be as least two atoms of sulphur present. Calculations based on this assumption give a value for the molecular weight of at least 15,000, but although from other considerations the molecular weight of proteins is known to be high, it is unlikely that the value is as high as this.

While it is possible by means of general reactions to place a given protein in the class of albumins or globulins, there are no distinctive chemical or physical methods by which the identity of any particular albumin or globulin may be established; thus it not infrequently occurs that two substances which have been obtained from different sources, and are described under different names, are eventually found to give the same figures on analysis, and are therefore regarded as identical. This is notably the case with albumins obtained from different plant seeds, and the serum albumin derived from different animals. Within the last few years, however, a biological method has been discovered which promises to become of the very greatest

value in distinguishing the various compounds from each other. Following upon the researches of Wassermann and Uhlenhuth, Tstistowitch found that serum drawn from a rabbit, which had been inoculated for some time with the serum of a horse, had acquired the property of producing a precipitate when added to normal horse serum; this is due to the formation in the rabbit's blood of a substance known as a precipitin, which belongs to a class of compounds described by Hofmeister as pseudo-globulins; the precipitate formed is a compound of the precipitin with the albumin contained in the serum to which the precipitin was added. The precipitin so prepared should only react with horse serum and not with the serum of any other animal; the reaction is, however, not absolutely specific, inasmuch as a precipitin may react with the serum of an animal closely related to the one from whose serum it was prepared. It has, moreover, been found that substances which had been isolated from natural fluids, as well as native sera, were able to incite a precipitin formation when injected into the blood of some living animal, and it has been thus possible to show that the albumin contained in milk is not identical with that obtained from blood. The method has been employed by Kowarski and Schütze * for distinguishing the various plant albumins, and by Rickmann,† Uhlenhuth,‡ and others for distinguishing between horse flesh and the meat of other animals. An attempt has also been made to employ the same principle for the estimation of proteins by a comparison of the precipitates formed under various conditions.§

As illustrating the very close connection existing between albumins and globulins, it is worthy of note that Moll claims to have converted serum albumin into serum globulin by warming a 3 per cent solution of serum albumin for one hour to 60° C. with N/66 sodium carbonate, but it is difficult to say whether true serum globulin was actually produced. According to

* Kowarski and Schütze: "Deut. med. Wochenschr.," 1901, **27**, 442; 1902, **28**, 804.

† Rickmann: "Chem. Zeit.," 1907, **2**, 1983.

‡ Uhlenhuth and Weidanz: "Praktische Anleitung z. Ausführung des biolog. Eiweissdifferenzierungsverfahren," Jena, 1909.

§ Schulz: "Deut. med. Wochenschr.," 1906, **32**, no. 26; "Zeit. Unters. Nahr. u. Genussm.," 1906, **12**, 257.

Chick and Martin,* however, the conversion of albumin into globulin may be explained merely by assuming a difference in the state of aggregation.

THE PHYSICAL AND CHEMICAL PROPERTIES OF PROTEINS.

A. Physical Properties.

1. Indiffusibility.

Proteins are colloids and therefore are unable to diffuse through a parchment or animal membrane; it is thus frequently possible to purify a protein from salts by dialysis. The purification is, however, not complete, and it has, hitherto, not been found possible to remove from any protein the last traces of adhering inorganic salts, so that a perfectly pure protein, which on ignition yields no ash, has not as yet been obtained by this means.

2. Optical activity.

The solutions of all proteins are lævo-rotatory, the degree varying from $-33{\cdot}5°$ in the case of egg albumin to $-80°$ in the case of casein.

3. Irreversible precipitation.

Soluble proteins by the action of various agents may undergo a physical change whereby their solubility properties are altered without any demonstrable chemical change. This is known as denaturing.

The change may be effected by (*a*) heat and by (*b*) alcohol.

(*a*) The solutions of all animal albumins or globulins may be coagulated by heating; the temperature at which the change takes place is characteristic for each substance, and varies from 56° C. in the case of fibrinogen to 70-80° C. for serum albumin.† The reaction of the solution as well as the presence of dissolved salts are factors which exercise a powerful influence, a slightly acid solution being most favourable for the phenomenon, whereas an alkaline reaction may prevent coagulation entirely.

The plant globulins, on the other hand, are less readily

* Chick and Martin: "Journ. Physiol.," 1912, **45**, 261.

† The coagulation temperature is not sufficiently well defined to be employed as a means of identification.

coagulated, and many are not coagulated even by boiling water.

Heat coagulation is best effected as follows. The solution is first boiled, and from 1-3 drops of dilute acetic acid are added for each 10 c.c. of liquid, the liquid being boiled before the addition of each drop.

(*b*) The addition of absolute alcohol to a neutral or faintly acid solution of a native protein * will precipitate it from solution unchanged. If, however, it be left in contact with the alcohol for some time, the protein is rendered insoluble and is coagulated.

4. Reversible precipitation.

Certain salts, such as sodium chloride and the sulphates of sodium, magnesium, and ammonium, etc., have the property of throwing proteins, except peptones, out of solution. This is, however, purely a physical phenomenon, and must be distinguished from the chemical precipitation described below, inasmuch as the proteins are precipitated unchanged, and retain all their original properties and solubilities. Absolute alcohol, also, as mentioned above, precipitates the proteins unchanged, though the precipitate must not be left in contact with the alcohol, or else it will become coagulated.

With regard to the precipitating power of these various salts, it should be mentioned that saturated ammonium sulphate will precipitate *all* proteins except peptones, and consequently a solution which on saturation with ammonium sulphate remains clear, can be regarded as free from protein.

Furthermore,	zinc sulphate is approximately equivalent to	ammonium sulphate.
	saturated sodium chloride is approximately equivalent to	saturated magnesium sulphate, or 1/2 saturated ammonium sulphate.

Solubilities of Proteins and their Physiological Significance.

In view of the number of proteins in the plant and their different characteristic solubilities, it is easy to see the impor-

* The term native protein is applied to proteins which have been isolated from the tissues by some simple process which does not involve any material alteration in their original properties.

tance to the well-being of the plant of factors which have a bearing on these properties. Thus any cause which removes water, not immediately replaceable, from the cell, and so leads to a concentration of the cell sap, may be a determining factor in the existence of a plant. Cold is one such factor; * a fall in the temperature may cause the water to crystallize, so that the salt solutions in the cell become stronger, with the result that some of the proteins of the protoplasm may be dissolved and other proteins in solution may be precipitated. The importance of soluble carbohydrates and of oils in the cell sap in this connection has already been pointed out.

It is unnecessary to remark that this effect of cold must vary pretty considerably in different plants, and depends upon the nature of the salts dissolved in the cell sap and the proteins upon which they can act. To take a few examples: It was found that in *Begonia*, soluble proteins were precipitated when the temperature reached $-3°$ C.; on the other hand, in the leaves of *Pinus*, a temperature of $-40°$ C. was required to obtain a similar result.† This may, in part, be due to the paucity of crystalloids in the cell sap, for it is stated that plants which are subject to periodic drought possess only small amounts of soluble crystalloids in the cell sap.

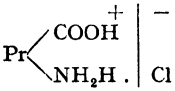
In the case of the barley, it was observed that an exposure for one night to a temperature of $-7°$ C. reduced the yield of soluble proteins by about one-third as compared with a control experiment in which the temperature was not so lowered. This salting-out effect is much increased if the cell sap becomes acid on cooling, as is not infrequently the case.

If the low temperature be long continued, the precipitated proteins will not again enter into solution when the amount of water is increased by raising the temperature; on the other hand, if the temperature be suddenly raised, the precipitated proteins will re-dissolve, provided that they have not stood too long, and thus the plant will not be greatly harmed.

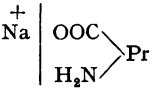
* See Blackman: "New Phytol.," 1909, **8**, 354.
† Gorke: "Landwirth. Versuchs. Stat.," 1906, **65**, 149.

The Isoelectric Points of Proteins.

The proteins having both amino and carboxyl groups are amphoteric electrolytes and can function either as bases or as acids according to circumstances. Thus in acid solution the proteins tend to assert their basic function, occupying the position of kations I., while in alkaline solutions they tend to function as acids :—



I.



II.

It is therefore evident that the behaviour of proteins is largely influenced by the reaction, or, in other words, the hydrogen ion concentration of the solution.* Assuming that a given protein in neutral solution is functioning as the anion, as indicated by formula II., the addition of acid will gradually tend to reduce this state and to drive over the protein into the position of cation ; as the acidity is gradually increased, a certain hydrogen concentration will be attained at which the number of protein anions and kations are exactly equal. This hydrogen ion concentration is known as the isoelectric point; it does not coincide with the point of neutrality $P_H = 7$ and will vary for different proteins.

Appended are some values for the isoelectric points of various plant materials taken from a paper by Pearsall and Ewing :— †

Substance.	*Isoelectric Point.*
Edestin	5·3-5·6
Legumin	4·4-4·6
Globulin (yeast)	4·6
Albumin (yeast)	4·6
Glutenin (wheat)	4·4-4·5
Leucosin (wheat)	4·5
Tuberin (potato)	4·4
Globulin (carrot)	4·1-4·4
,, (tomato)	4·0
Nitella extract	4·6-4·7
Yeast cells	3·1-3·3

* See Appendix I.

† Pearsall and Ewing : " Biochem. Journ.," 1924, **18**, 329. See also Atkins : " Proc. Roy. Dublin Soc.," 1922, **16**, 414.

At the isoelectric point the amount of ionized protein is at a minumum, or, in other words, the protein is mainly electrically neutral or uncharged. In this condition it will not wander in an electric field or, as it is usually expressed, exhibit cataphoresis; moreover, in consequence of the absence of electric charge, the protein is most readily precipitated at the isoelectric point.

In this connection attention may be drawn to the hydrogen ion concentration of the cell sap of various plants; the values obtained vary from 6·87 for *Ficus carica* to 3·19 for *Rumex acetosella*, as may be seen by reference to a paper by Chibnall and Grover,* but except in the case of unripe fruits or such as normally contain much acid the value lies somewhere about 5·5-6·5. Chibnall† gives the following comparison between the isoelectric points of some leaf cytoplasmic proteins and the hydrogen ion concentrations of the contents of the leaf cells:—

	Isoelectric Point of Protein.	P_H of Cell Contents.
Spinach	5·0-4·0	6·57
Hogweed	5·0-4·3	6·19
Broad bean	5·1-4·3	5·69
Cabbage	4·7-4·0	5·6
Rhubarb	3·5	4·00
Vine	4·8-4·4	3·02

It would appear from these figures and from similar observations by Pearsall and Ewing, that the proteins of the plants are bathed in a solution whose reaction is on the alkaline side of their isoelectric points, except in the case of the vine; such proteins therefore function as anions. The conclusion may be drawn that if the reaction of the cell sap is made more acid, so as to approach the isoelectric point of the proteins, these latter would tend to be precipitated with serious consequences to the cells concerned. Hoagland and Davis‡ have indeed shown in the case of *Nitella* that the reaction of the sap remained constant and the cells suffered no injury so long as the P_H of the external medium exceeded 4·4, but when the acidity was increased beyond this value to 4·4 or less,

* Chibnall and Grover: "Biochem. Journ.," 1926, **20**, 108.
† Chibnall: "J. Amer. Chem. Soc.," 1926, **48**, 728.
‡ Hoagland and Davis: "J. Gen. Physiol.," 1923, **5**, 629.

injury resulted. Pearsall and Ewing,* moreover, have shown that rapid outward diffusion of chlorine ions results when the living plant tissue is brought to hydrogen ion concentrations equal to or greater than the isoelectric point of the chief proteins.

As the result of a detailed study of the colloidal properties of the mycelium of *Fusarium lycopersici*, Scott † comes to the conclusion that the living tissue of the fungus behaves analogously to an amphoteric protein colloid with an isoelectric point near $P_H = 5{\cdot}4$.

B. Chemical Properties.

1. Precipitation reactions.

The proteins have both acid and basic properties; thus, casein may be looked upon as typically acid, seeing that it dissolves in alkalis to form sodium and potassium salts, whilst the histones and protamines are powerful bases. All proteins, however, have basic properties, which enable them to form insoluble salts with a great many of the ordinary alkaloidal reagents, such as phosphotungstic, tannic, picric, ferrocyanic, and trichloro-acetic acids. Pittom,‡ however, finds that many of the simpler polypeptides are not precipitated by phosphotungstic acid. They are also precipitated by potassium teriodide (a solution of iodine in potassium iodide) and by the double iodides of potassium with mercury, bismuth, and cadmium. The strong mineral acids also precipitate proteins. In consequence of this dual nature of proteins they are classed as amphoteric electrolytes.

The salts of the heavy metals also produce insoluble precipitates with the proteins, a fact which is made use of in the administration of egg albumin as an antidote in cases of poison with salts of these metals. Moreover, the antiseptic action of mercuric chloride is most probably connected with this formation of insoluble salts.

Amongst the salts most frequently used as precipitants for proteins are the chloride and acetate of iron, colloidal iron

* Pearsall and Ewing: "New Phytol.," 1924, **23**, 193.
† Scott: "Missouri Agric. Exp. Sta. Res. Bull.," 1926, **92**, 1.
‡ Pittom: "Biochem. Journ.," 1914, **8**, 157.

(ferric hydroxide), the sulphate and acetate of copper, the chloride of mercury, and the acetates of lead and zinc.

2. Colour reactions.

These reactions depend on the fact that certain groups or radicles in the protein molecule produce characteristic colours with suitable reagents. The reactions may also be employed for detecting these same groups in the decomposition products of the proteins, with the object of determining how far the decomposition has gone, and whether it has been sufficiently deep-seated to destroy this grouping or not. The following is a list of the more important colour reactions :—

(i) *Biuret Reaction.*—This is the bluish-violet colour produced by adding dilute copper sulphate to an alkaline solution of a protein. Unchanged proteins give a bluish-violet, whilst altered proteins, such as the peptones, give a pink.

The colour is given by the substance biuret itself, whose composition is expressed by the formula $NH_2CO.NH.CONH_2$, and by similarly constituted compounds containing two —CO . NH— groups connected together through a carbon, nitrogen, or sulphur atom.

(ii) *Millon's Reaction.*—A solution of mercuric nitrate containing nitrous acid added to a solution of a protein produces a precipitate which turns pink or red. This reaction is connected with the phenolic group of the tyrosine complex in the protein molecule ; it may also be used as a test for tyrosine. The reagent may be prepared by dissolving some mercury in twice its weight of nitric acid (sp. gr. 1·42), the operation being performed in a fume cupboard. When the action has ceased, the solution is diluted with twice its volume of water.

(iii) *Xanthoproteic Reaction.*—Protein solutions treated with concentrated nitric acid develop a yellow colour which is intensified by heating, and is changed to orange by ammonia. This reaction is likewise connected with the tyrosine complex.

(iv) *Tryptophane Reaction.*—This consists in mixing the suspected solution with a little glyoxylic acid * and carefully

* Made by adding magnesium powder to a saturated solution of oxalic acid.

adding concentrated sulphuric acid so that the latter forms a separate layer at the bottom of the test tube. After a short time a pink ring is produced at the junction of the two liquids, and on careful agitation the colour extends over the whole solution.

According to an experiment devised by Molisch, the above tests may be employed for demonstrating the distribution of protein in leaf tissue. The leaf is freed from chlorophyll by steeping in boiling water and then in boiling 75 per cent alcohol; separate portions of the leaf are then left to soak for half an hour in (*a*) nitric acid (1 : 2), (*b*) Millon's reagent, and (*c*) 5 per cent copper sulphate. Portion (*a*) is then transferred to a solution of ammonia, while portion (*c*) is placed in 10 per cent caustic potash. The characteristic colour changes are produced.

Microchemical Reactions.

The following are the usual microchemical tests employed for the indication of proteins within the plant :—

1. Iodine gives a yellow to brown coloration.

2. With osmic acid a brown coloration results.

3. *Biuret Reaction.*—A solution of copper hydrate in caustic potash may be added direct to the preparation ; or the section may be steeped for some time, say twenty to sixty minutes, in 0·2 per cent solution of potash, washed, placed in a 10 per cent solution of copper sulphate for thirty to sixty minutes, washed in water and mounted in a 2 per cent solution of caustic potash. A mauve to violet coloration indicates the presence of proteins.

4. *Millon's Reagent.*—The section or scraping is mounted in a few drops of the reagent and warmed. A brick-red coloration results when proteins are present.

5. *Xanthoproteic Reaction.*—A yellow to orange coloration results with proteins. The preparation is warmed on the slip with a few drops of strong nitric acid. The proteins acquire a yellow colour which is changed to orange on moistening with strong ammonia.

THE DECOMPOSITION PRODUCTS OF THE PROTEINS.

The most direct way of obtaining an insight into the probable groups or groupings which occur in the molecule of some complex substance, is to break it up into simpler ones, whose constitution is already known, or may be determined with comparative ease. This is the method which has been employed to elucidate the very complex structure of the proteins.

Various processes have been employed for breaking down the protein molecule, such as acid hydrolysis, fusion with alkalis, the action of enzymes or putrefactive bacteria, oxidation, etc. As a result of all these various methods, a number of simple compounds have been obtained, which fall primarily into two main groups :—

1. *Biuretic Derivatives*, such as albumoses, peptones, etc., which are still very complex substances, but have, at any rate, a lower molecular weight than the original unaltered protein. These substances all give the Biuret reaction.

2. *Abiuretic Derivatives.*—In this group of cleavage products, which give no Biuret reaction, are included the various amino acids.

By an amino acid is meant an acid in which one or more of the hydrogen atoms other than the carboxylic hydrogen are replaced by the amino group $-NH_2$. Thus acetic acid CH_3COOH gives rise to the amino acid known as glycine CH_2NH_2COOH. Theoretically it should be possible to replace two or even three atoms of hydrogen in acetic acid by the $-NH_2$ group to produce diamino acetic acid $CH(NH_2)_2COOH$ and triamino acetic acid $C(NH_2)_3COOH$; these compounds are, however, not known, and appear to be incapable of existing.

The next homologue after acetic acid, namely, propionic acid CH_3CH_2COOH, can give two mono-amino acids CH_3CHNH_2COOH and $CH_2NH_2CH_2COOH$ known respectively as α- and β-amino propionic acids, according as the amino group is attached to the α-carbon atom, adjacent to the carboxyl group, or to the β-carbon atom, which is next but one from the carboxyl.

In the case of the higher homologues, diamino acids are

known which have two amino groups attached to different carbon atoms, such, for example, as α- δ- diamino valeric acid $CH_2NH_2CH_2CH_2CHNH_2COOH$ derived from valeric acid $CH_3CH_2CH_2CH_2COOH$.

The dicarboxylic acids also can give rise to amino derivatives such as aspartic acid $COOHCH_2CHNH_2COOH$ derived from the dicarboxylic acid succinic acid $COOHCH_2CH_2COOH$ and glutamic acid $COOHCH_2CH_2CHNH_2COOH$ derived from glutaric acid $COOHCH_2CH_2CH_2COOH$.

It is important to note that all amino acids which are known to take part in the building up of the protein molecule are α-substituted acids, as will be seen from the list of protein cleavage products given below.

The presence of the $—NH_2$ group in amino acids confers upon these substances basic properties, in addition to the acid properties which they already possess. Thus, for example, glycine CH_2NH_2COOH is able to react with hydrochloric acid to produce glycine hydrochloride $CH_2NH_2HClCOOH$, just as ammonia reacts with hydrochloric acid to give a hydrochloride; on the other hand, being an acid, it is also able to form metallic salts, such as CH_2NH_2COOK. It is not surprising to learn that the mono-amino acids, such as glycine and its homologues, have no very pronounced acidic or basic properties. On the other hand, the mono-amino derivatives of the dicarboxylic acids, namely, aspartic acid $COOHCH_2CHNH_2COOH$ and glutamic acid $COOHCH_2\ CH_2CHNH_2COOH$, are strong acids, owing to the predominating influence of the two carboxyl groups, while the diamino derivatives of the monocarboxylic acids, such as lysine $CH_2NH_2CH_2CH_2CH_2CHNH_2COOH$, ornithine $CH_2NH_2CH_2\ CH_2CHNH_2COOH$, etc., have strongly marked basic characteristics owing to the two amino groups.

A class of substances which have to be carefully distinguished from the amino acids are the acid amides. These are derived from carboxylic acids by replacing the hydroxyl group of the carboxyl by $—NH_2$. Thus acetic acid CH_3COOH gives the amide CH_3CONH_2 known as acetamide, while aspartic acid $COOHCH_2CHNH_2COOH$ gives the amide $CONH_2CH_2CHNH_2COOH$ known as asparagine.

AMINO ACIDS OBTAINED AS CLEAVAGE PRODUCTS OF PROTEINS.

(I) *Aliphatic Compounds.*

Mono-carboxylic mono-amino acids.

Glycine or α-amino-acetic acid CH_2NH_2COOH

Alanine or α-amino propionic acid CH_3CHNH_2COOH

Amino-butyric acid $CH_3CH_2CHNH_2COOH$

Amino-caproic acid $CH_3CH_2CH_2CH_2CHNH_2COOH$

Valine or α-amino-isovaleric acid

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!\rangle CH \,.\, CHNH_2COOH$$

Leucine or α-amino-isocaproic acid

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!\rangle CHCH_2CHNH_2COOH$$

Isoleucine or α-amino-β-methyl β-ethyl propionic acid

$$\begin{matrix} CH_3 \\ C_2H_5 \end{matrix}\!\!\rangle CHCHNH_2COOH$$

Serine or α-amino β-hydroxy propionic acid.

$CH_2OHCHNH_2COOH$

Dicarboxylic mono-amino acids.

Aspartic * or α-amino-succinic acid

$COOH\,CH_2CHNH_2COOH$

Glutamic * or α-amino-glutaric acid

$COOH\,CH_2CH_2CHNH_2COOH$

Mono-carboxylic di-amino acids

Ornithine or α-δ-di-amino-valeric acid

$NH_2CH_2CH_2CH_2CHNH_2COOH$

Arginine or δ-guanidine α-amino-valeric acid

$$HN{=}C\begin{matrix} NH_2 \\ NHCH_2CH_2CH_2CHNH_2COOH \end{matrix}$$

Lysine or α-ε-di-amino-caproic acid

$NH_2CH_2CH_2CH_2CH_2CHNH_2COOH$

Di-amino-trihydroxy-dodecanic acid † $C_{12}H_{26}O_5N_2$

Dicarboxylic di-amino acid.

Cystine or di [β-thio α-amino propionic] acid

$$\begin{matrix} CH_2{-}S{-}S{-}CH_2 \\ | \qquad\qquad | \\ CHNH_2 \qquad CHNH_2 \\ | \qquad\qquad | \\ COOH \qquad COOH \end{matrix}$$

* The amides corresponding to these two acids, namely, asparagine $CONH_2CH_2CHNH_2COOH$ and glutamine $CONH_2CH_2CH_2CHNH_2COOH$ are of considerable importance in plants. The former occurs in asparagus and is produced in seeds which are allowed to germinate in the dark (Schulze, "Landwirtsch. Gahrb.," 1878, 411), while the latter has been found in the seeds of *Cucurbita* and many other plants (Schulze and Barbieri, "Ber. deut. chem. Gesells.," 1877, **10**, 199; Schulze, *id.*, 1896, **29**, 1882). Asparagine and glutamine being readily hydrolysed by mineral acids, are not obtained as cleavage products of proteins by the ordinary methods of chemical hydrolysis, and for this reason are not quoted in the above list of cleavage products.

† The constitutional formula of this substance has not yet been determined.

(2) *Aromatic Compounds.*

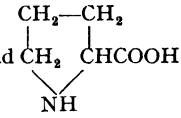
Mono-carboxylic mono-amino acids.

Phenyl alanine or β-phenyl α-amino propionic acid
$C_6H_5CH_2CHNH_2COOH$

Tyrosine or β-parahydroxyphenyl α-amino-propionic acid
$HOC_6H_4CH_2CHNH_2COOH$

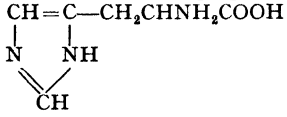
(3) *Heterocyclic Compounds.*

Proline or α-pyrrolidine carboxylic acid

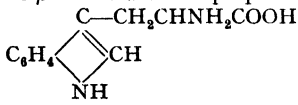


Hydroxyproline or hydroxy α-pyrrolidine carboxylic acid

Histidine or β-imidazol α-amino propionic acid



Tryptophane or β-indole α-amino propionic acid



The above list comprises most of the more important cleavage products of proteins, the constitution of which has been definitely established.

Since different proteins give rise to different amounts of these various substances, it is obvious that a careful quantitative determination of the amounts of these acids produced by the hydrolysis of different proteins must be of considerable value.

To this end Fischer, in 1901, introduced his so-called "Ester method," which consisted in converting the mixed amino acids obtained by hydrolysis of proteins into their corresponding esters, and then separating these by fractional distillation.

The method * is best illustrated by an example. Casein was decomposed by hydrolysis with concentrated hydrochloric acid, the hydrochloride of glutamic acid being separated by filtration. The filtrate was then evaporated under reduced pressure, taken up with alcohol and saturated with dry gaseous

* Fischer: "Zeit. physiol. Chem.," 1901, **33**, 151.

hydrogen chloride; in order to remove the water formed by the reaction, the solution was once more evaporated down, and the residue taken up with alcohol and again saturated with hydrogen chloride. The esters were next liberated from their hydrochlorides by evaporating the solution down to a syrup in a vacuum, diluting with water and approximately neutralizing by means of strong caustic soda solution while keeping thoroughly cooled in a freezing mixture. Concentrated potassium carbonate was now added, and the esters of aspartic and glutamic acid were extracted by ether; after adding more 33 per cent caustic soda and potassium carbonate and extracting again with ether, the combined extracts were dried with anhydrous sodium sulphate, evaporated and distilled under 8-15 mm. pressure. The various fractions were then separately hydrolysed, either by boiling with water or by warming on the water bath with 20 per cent baryta water.

This method, with slight modifications, has been applied by several workers, more especially Abderhalden and Osborne, to a considerable number of different proteins, with the result that there are now more or less reliable data for comparing the composition of proteins from various sources, both animal and vegetable.

Dakin * has suggested a method of separating the products of the acid hydrolysis of proteins by extracting with such solvents as butyl and ethyl alcohols, and Schryver and his fellow-workers † have elaborated an alternative method depending upon the conversion of the amino acids into carbamates.

A second method for gaining some insight into the composition of proteins consists in studying the distribution of nitrogen in the molecule with a view to ascertaining whether it is present in the form of mono- or di-amino acids, etc. A method for distinguishing between the different types of nitrogen-linking occurring in the molecule was first suggested by Hausmann,‡ and has since been modified by Gümbel; § it

* Dakin: "Biochem. Journ.," 1918, **12**, 290.

† Buston and Schryver: *id.*, 1921, **15**, 636; Kingston and Schryver: *id.*, 1924, **18**, 1070.

‡ Hausmann: "Zeit. physiol. Chem.," 1899, **27**, 95; 1900, **29**, 136. See also Osborne and Harris: "J. Amer. Chem. Soc.," 1903, **25**, 323.

§ Gümbel: "Beitr. chem. Phys. u. Path.," 1904, **5**, 297.

depends on the fact that di-amino acids, in virtue of their strongly basic character, are precipitated from solutions by the addition of phosphotungstic acid, whereas mono-amino acids are not.

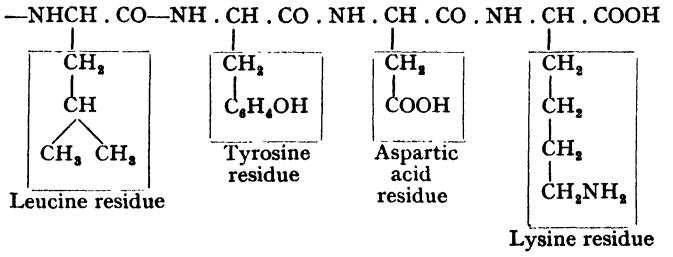
A method for obtaining an insight into the composition of proteins on a comparatively small quantity of material, was devised by van Slyke * and has since been modified from time to time.†

The substance to be examined is first hydrolysed by boiling with concentrated hydrochloric acid for several hours under a reflux condenser. The amount of amide nitrogen and ammonia in the resulting mixture is then determined by distillation with magnesia in vacuo at 40° C.

2. The di-amino acid nitrogen is next determined by precipitating the residue in the flask with excess ‡ of phosphotungstic acid and estimating the amount of nitrogen in the precipitate by Kjeldahl's method.

3. The nitrogen combined as mono-amino acids may be determined directly in the filtrate or by the difference between the total nitrogen and the sum of the nitrogens separately determined by the above methods.

The fact that proteins on hydrolysis yield such a large number of amino acids, all of which have the amino group attached to the α-carbon atom (i.e. the carbon atom adjacent to the carboxyl), has led to the conclusion that the protein molecule is really composed of a long chain of these acids linked together in some such way as is represented below :—

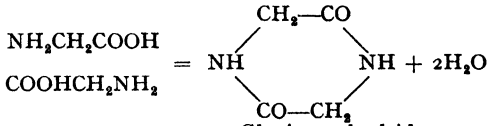


* Van Slyke: "J. Biol. Chem.," 1911, 10, 15.
† Plimmer and Lowndes: "Biochem. Journ.," 1927, 21, 247.
‡ In order to ensure complete precipitation of arginine.

Such a compound would give the biuret reaction and contain but few free carboxyl groups or amino groups, which is entirely in agreement with the properties of proteins. Acting on this assumption, Fischer has synthesized a number of compounds containing such a structure, with the object of studying their properties and comparing them, if possible, with natural proteins. To these synthetic substances he has given the general name of Polypeptides.

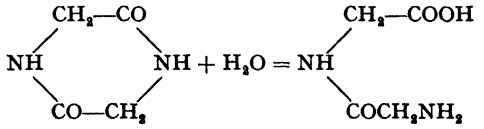
The simplest polypeptide known is glycylglycine; this substance was obtained as follows:—

Glycine, when kept for some time in aqueous solution, loses water from two molecules, giving an anhydride—



Glycine anhydride or diketopiperazine

This substance, when boiled with hydrochloric acid, is hydrolysed, the ring being opened with the formation of the dipeptide glycylglycine—



To give anything like a complete account of the methods employed in the synthesis of polypeptides is outside the province of this book. It may, however, be mentioned that a very fruitful method of synthesizing these substances consists in acting on an amino acid, or a polypeptide with chloracetyl chloride, thus:—

$$CH_2ClCOCl + NH_2CH_2CONHCH_2COOC_2H_5 = CH_2ClCONHCH_2CONHCH_2COOC_2H_5 + HCl$$

The latter, after conversion into the acid, and treatment with ammonia, yields a tripeptide—

$$CH_2ClCONHCH_2CONHCH_2COOH + NH_3 = \underset{\text{Diglycylglycine a Tripeptide}}{CH_2NH_2CONHCH_2CONHCH_2COOH} + HCl$$

Another valuable method consists in treating an amino acid suspended in acetyl chloride with phosphorus pentachloride and so obtaining an acid chloride R_1CHNH_2COCl. This

latter is then allowed to act upon the amino group of a second acid as follows :—

$$R_1CHNH_2COCl + NH_2\underset{\displaystyle R_2}{\underset{|}{C}}H \cdot COOH = R_1CHNH_2CONH \cdot \underset{\displaystyle R_2}{\underset{|}{C}}HCOOH + HC$$

The resulting polypeptide may be of considerable complexity, according to the nature of R_1 and R_2.

By these and similar methods, employing other combinations of amino acids, polypeptides containing a great many different groupings have been synthesized. The one with the longest chain as yet obtained is an octodecapeptide leucyltriglycyl-leucyltriglycyl-leucyloctoglycyl-glycine of the formula

$$NH_2CHC_4H_9CO[NHCH_2CO]_3NHCHC_4H_9CO[NHCH_2CO]_3NHCHC_4H_9CO[NHCH_2CO]_8NHCH_2COOH$$

The more complex of these polypeptides resemble the proteins in being colloidal substances which give the biuret reaction, and in being precipitated from solution by phosphotungstic or tannic acids and by ammonium sulphate.

The action of digestive ferments upon them has been studied by Abderhalden and others ; they are not readily attacked by pepsin, but are hydrolysed by pancreatic or intestinal juice.

A striking confirmation of Fischer's view concerning the close connection existing between the polypeptides and the natural proteins is to be found in the fact that the hydrolysis of proteins, under suitable conditions, yielded four substances which could be identified with synthetic polypeptides. Thus, a solution of silk fibroin in hydrochloric acid was allowed to stand for several days ; on evaporating, a residue was obtained which, when digested with trypsin, yielded a peptone-like substance ; the latter on hydrolysis with barium hydroxide gave glycylalanine, which was identified by its naphthaline sulphonic acid derivative.* Subsequently, the hydrolysis was repeated under somewhat altered conditions, with the same result that glycylalanine was obtained.† In a later communication, the same authors described the isolation of glycyltyrosine from the products of hydrolysis of silk fibroin, and

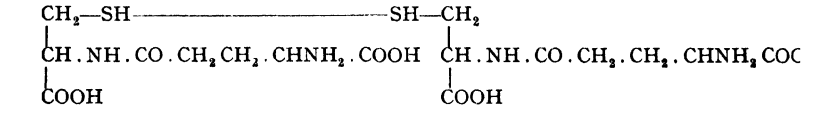
* Fischer and Abderhalden : " Ber. deut. chem. Gesells.," 1906, **39**, 752.

† *Ibid.*, 2315.

of glycyl-leucine from elastin. Levene and Beatty also claim to have obtained prolyl-glycine from the hydrolysis of gelatine.

Abderhalden † also mentions certain substances of a polypeptide nature which he found amongst the products of pancreatic digestion of a number of proteins such as casein, edestin, hæmoglobin, serum globulin, egg albumin and fibroin.

A polypeptide of considerable importance is that known as Glutathione; this substance, whose constitution has been established by synthesis,‡ has the formula—



and is thus a diglutaminyl cystine. The significance of this substance as an oxidizing mechanism is discussed in Vol. II.

OCCURRENCE OF AMINO ACIDS IN PLANTS.

Leucine occurs as such in the buds of the horse-chestnut § and many other plants. Isoleucine has been discovered by Felix Ehrlich || in the residual molasses obtained from sugar refineries.

Lysine and *histidine* have been isolated from sprouting plants by Schulze.¶

Arginine has been observed in the cotyledons of lupin seeds and in etiolated pumpkin seeds,** and also in several species of conifers.

Phenylalanine was discovered by Schulze and Barbieri †† in etiolated germinating lupin seeds.

* Levene and Beatty: "Ber. deut. chem. Gesells.," 1906, **39**, 2060.

† Abderhalden: "Zeit. physiol. Chem.," 1905, **44**, 28, 33.

‡ Stewart and Tunnicliffe: "Biochem. Journ.," 1925, **19**, 207.

§ Schulze and Barbieri: "J. prakt. Chem.," 1882, **15**, 145. Schulze and Winterstein: "Zeit. physiol. Chem.," 1902, **35**, 299.

|| Ehrlich: "Ber. deut. chem. Gesells.," 1904, **37**, 1809.

¶ Schulze: "Zeit. physiol. Chem.," 1899, **28**, 465.

** Schulze and Steiger: *id.*, 1887, **11**, 43; "Ber. deut. chem. Gesells.," 1886, **19**, 1177.

†† Schulze and Barbieri: *id.*, 1881, **14**, 1785.

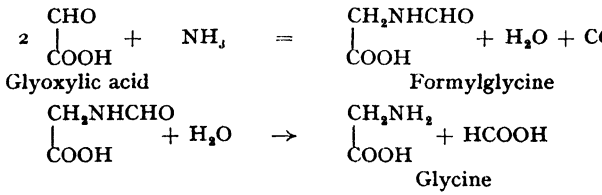
Tyrosine, according to Shibata,* occurs in considerable quantity in rapidly growing shoots of Japanese bamboos, and in small quantity in seedlings of *Lupinus albus* † and *Vicia sativa*; ‡ it has also been described as occurring with asparagine in the root-tubers of *Dahlia variabilis*.

Tryptophane has been found in seedlings of *Lupinus albus*, *Vicia sativa*, and in *Pisum sativum*.§

Proline is obtained by the hydrolysis of a number of proteins of vegetable origin, notably the prolamins, but has not so far been found to occur as such in any plants.

SYNTHESIS OF AMINO ACIDS IN THE PLANT.

With regard to the synthesis of amino acids within the plant, it is of interest to note that in the laboratory Erlenmeyer and Kunlin || have been able to synthesize the acetyl and formyl derivatives respectively of alanine and glycine by the action of ammonia on glyoxylic acid, both of which substances are known to occur in plants. The changes involved may be represented by the following formulæ :—



Furthermore, Fischer and Schlotterbeck ¶ synthesized a diamino acid by the action of ammonia on sorbic acid, an unsaturated acid occurring in the unripe berries of the mountain ash; also another unsaturated acid belonging to the same series as sorbic acid, namely, β-vinyl acrylic acid, has by the action of ammonia been converted into diamino valeric acid,** and further, aspartic acid †† has been obtained by the action of ammonia on fumaric acid.

* Shibata : " J. Coll. Sci. Tokyo," 1900, **13**, 329.
† Schulze and Castoro : " Zeit. physiol. Chem.," 1906, **48**, 387, 396.
‡ Gorup Besanez : " Ber. deut. chem. Gesells.," 1877, **10**, 781.
§ Schulze and Winterstein : " Zeit. physiol. Chem.," 1910, **65**, 431.
|| Erlenmeyer and Kunlin : " Ber. deut. chem. Gesells.," 1902, **35**, 2438.
¶ Fischer and Schlotterbeck : *id.*, 1904, **37**, 2357.
** Fischer and Raske : *id.*, 1905, **38**, 3607.
†† Engel : " Compt. rend.," 1887, **104**, 1805, and 1885, **106**, 1677.

Baly and his co-workers claim to have synthesised α-amino acids by exposing an aqueous solution of formhydroxamic acid and formaldehyde to ultraviolet light.

From the plant physiological point of view, however, the interest of these latter discoveries is dependent on the occurrence in the plant both of unsaturated acids and of ammonia.

The researches of Ehrlich † upon the action of yeast on amino acids showed that the addition of leucine or isoleucine to a fermenting sugar solution gave rise to the production of inactive or active amyl alcohol respectively, according to the following schemes :—

$$\begin{matrix}CH_3\\CH_3\end{matrix}\!\!\rangle CHCH_2CHNH_2COOH + H_2O = \begin{matrix}CH_3\\CH_3\end{matrix}\!\!\rangle CHCH_2CH_2OH + CO_2 + NH_3$$

Leucine — Amyl alcohol

$$\begin{matrix}CH_3\\C_2H_5\end{matrix}\!\!\rangle CHCHNH_2COOH + H_2O = \begin{matrix}CH_3\\C_2H_5\end{matrix}\!\!\rangle CHCH_2OH + CO_2 + NH_3$$

Isoleucine — Active amyl alcohol

The amounts of these alcohols produced are proportional to the quantities of leucine or isoleucine added and rose, under favourable conditions, to as much as 7 per cent ; furthermore, it was found that although the leucine parted with its nitrogen in the form of ammonia, the latter substance was not lost, but appeared to be taken up by the yeast in the production of new protein material ; this observation led to trying the effect of adding ammonium salts, when it was found that the yeast, finding these latter to be an easier source of nitrogenous food, gave up attacking the leucine, and consequently less amyl alcohol was produced.

These experiments, therefore, prove that amino acids can be fermented by yeast with the production of alcohols in much the same way as sugars can be fermented. The amino acids of the protein of the yeast cells are the source of the amyl alcohol and succinic acid found among the products of the fermentative activity of such living yeast cells. When yeast juice is employed, these bye-products are not formed. The practical importance of these discoveries can be gauged from

* Baly, Heilbron, and Hudson, " J. Chem. Soc.," 1922, **121**, 1078.

† Ehrlich : " Ueber die Bedeutung des Eiweisstoffwechsels, etc.," " Sammlung chem. u. chem. tech. Vorträge," Stuttgart, 1911.

the fact that the production of amyl alcohol or fusel oil by the yeast fermentation of sugar has always been a source of trouble to spirit distillers, necessitating elaborate processes for refining.

Since, moreover, other amino acids besides the leucines are also found to be attacked in a similar way with the production of a number of widely different products, some of which are aromatic, it is easy to account for the different flavours which are peculiar to the various alcoholic beverages, all of which are ultimately produced by alcoholic fermentation of sugars in presence of different proteins.

The destruction of amino acids by enzymes derived from yeasts, fungi or bacteria with the formation of different bye-products, may also account for the flavours of different cheeses, as well as the odour of flowers ; the substance phenyl ethyl alcohol, for example, which is produced by the fermentation of phenyl alanine—

$$\underset{\text{Phenyl alanine}}{C_6H_5CH_2CHNH_2COOH} + H_2O = \underset{\text{Phenyl ethyl alcohol}}{C_6H_5CH_2CH_2OH} + CO_2 + NH_3$$

being the chief odoriferous constituent of rose oil.

These researches would therefore lead to the conclusion that the proteins, through the breaking up of various amino acids derived from them, are ultimately responsible for the production of a variety of nitrogen-free alcohols, aldehydes and acids as bye-products, which go to produce the different essential oils, etc.

The metabolism of proteins in the animal world is, as is well known, a very important process and results in their very complete decomposition with the formation of urea, carbon dioxide and water. Although little is known concerning the metabolism of proteins by plants, there is good reason for believing that the destruction of the protein molecule is far less complete ; the occurrence of urea has in fact been recorded in small quantities in higher plants, namely, *Cichorium endiva, Cucurbita maxima, Cucumis melo, Brassica olereacea, B. nigra*

* Amyl alcohol is required for the preparation of amyl acetate, used as a flavouring material for confectionery, and as a solvent in the manufacture of varnish, smokeless powder, etc.

and *B. napus*, *Daucus carota*, *Solanum tuberosum* and *Spinacia olereacea*.* In lower plants, the bacteria and fungi more especially, it has likewise been shown to be a product of metabolism.† It has been suggested that many of the simpler nitrogenous compounds, as, for example, caffeine, theobromine, the alkaloids, skatol and allied substances such as indoxyl, etc., may be products of protein metabolism.

A NOTE ON THE CHEMICAL COMPOSITION OF PROTOPLASM.

Protoplasm is the living substance of all organisms, and being the centre of metabolic activity it is continually undergoing change, wherefore it may not have precisely the same composition from moment to moment. Further, since there are specific, and even individual physiological processes, the protoplasm of different species and of particular plants may not be the same. Any attempt chemically to analyse protoplasm destroys its vital activity, and therefore the material is no longer protoplasm; it is true that analysis of the dead protoplasm shows it to contain various elements—carbon, hydrogen, oxygen, nitrogen, phosphorus and sulphur—and that these are combined to form various complicated structures.

Thus the analysis of naked masses of protoplasm, the plasmodia of myxomycetes, for example, gives various results. For instance, Kiesel ‡ found that the plasmodium of *Reticularia lycoperdon* contained the following percentages of materials estimated on the dry weight: protein, 26·65; plastin, 8·42; nucleic acid, 3·68; nitrogenous extractives, 12·00; fat, 17·85; lecithin, 4·67; cholesterol, 0·58; reducing sugar, 2·74; non-reducing soluble carbohydrate, 5·32; glycogen, 15·24; other polysaccharides, 1·78; together with some substances of undetermined composition. Similar analyses have been made by other authors.§ It is to be remembered that all

* Fosse: "Compt. rend.," 1912, **155**, 851; 1913, **156**, 567, 1938; 1914, **158**, 1374; **159**, 253. Verschaffelt: "Pharmaceut. Weekblad," 1914, **51**, 189.

† Ivanoff: "Biochem. Zeit.," 1923, **135**, 1; **136**, 1, 9; **143**, 62; 1925, **162**, 425; 1926, **175**, 181.

‡ Kiesel: "Zeit. physiol. Chem.," 1925, **150**, 149.

§ See Lepeshkin: "Ber. deut. bot. Gesells.," 1923, **41**, 179. Ivanow: "Biochem. Zeit.," 1925, **162**, 441.

such analyses include not only the dead protoplasm but also substances mixed with the protoplasmic matrix but not necessarily forming an integral part of the protoplasm. Further, chemical analyses can only identify the more stable substances; there is no technique available to enable the identification of transient substances, the recognition of which would go far to solve many physiological problems.

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SECTION X

ENZYMES.

It has long been known to chemists that the velocity of chemical reactions could, in many cases, be increased by the presence of relatively small quantities of certain substances which do not appear to take any immediate part in the reaction.

This is well illustrated by the familiar example of the effect of a small quantity of manganese dioxide in bringing about the liberation of oxygen from potassium chlorate at a temperature much lower than would be possible by heating this substance alone.

Other examples of the accelerating influence of foreign substances on the velocity of reactions are to be found in the use of cuprous chloride in Deacon's chlorine process, and of spongy platinum, either in the manufacture of sulphuric acid by the contact process, or for effecting the explosive combination of hydrogen and oxygen.

Similarly, the hydrolysis of cane sugar according to the equation—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

takes place very slowly in neutral aqueous solutions, but may be greatly accelerated by warming the solution with a little mineral acid.

A feature common to all the above reactions is the fact that the substance which produces the accelerating influence is unaltered by the reaction, and can usually be recovered from the reaction-product unchanged in quality and quantity.

Substances which have this remarkable property of being able in some way to influence the velocity of a reaction, without apparently undergoing any change themselves, and

which act in quantities which bear no particular relation to the weights of the reacting substances, are called catalytic agents.

The process of catalysis has been defined by Ostwald as "The acceleration of a chemical change by the presence of some foreign substance," and it must be clearly understood that a catalytic agent only accelerates a reaction, but is not capable of bringing about a reaction which would not take place at all in its absence.* Berzelius,† in 1850, drew attention to the similarity between the decomposition of hydrogen peroxide, under the influence of insoluble inorganic catalysts such as platinum or silver, and the decomposition of sugar into alcohol and carbon dioxide under the influence of substances known as ferments. Thus, in view of the ease with which so many complex reactions are effected within the living organism at a low, or a comparatively low, temperature, the idea is suggested that nature likewise makes use of catalysts.

As a matter of fact a large number of complex organic substances, capable of exerting catalytic action, have been isolated from plants and animals; and to these substances the name of enzymes has been applied.

The food of plants, carbohydrate, protein, fats, etc., is, in many cases, valueless unless it can be brought into a condition suitable for assimilation and, very often, translocation. Thus the starch in a leaf must be rendered soluble before it can be transported to other parts of the plants, and, similarly, the starch in a potato before it can be used for the nutrition of the young shoots.

In the living organism these changes are brought about by the enzymes.

With regard to the mode of the formation of enzymes nothing is known; they are generally described as being due to the activity of the protoplasm, a phrase which contains no information. Sometimes the enzymes are secreted in specialized organs or in tissues more or less remote from the

* Ostwald's definition, on various grounds, is not always accepted. Thus Willstätter objects to its rigidity, and points out that the facts of catalysis are so numerous and so diverse that it is futile to attempt to explain all these phenomena by means of a single definition.

† Berzelius: "Jahresber.," 1850, **15**, 237, 240, 278.

cells containing the material to be acted upon. In other cases they are formed in the same cells as the substrate.

A few examples may be given. In *Zea Mais* the cells of the surface of the scutellum next the endosperm have a distinct gland-like appearance, and here and there they dip down into the deeper layers of the scutellum, giving an appearance not unlike the crypts of Lieberkühn of the intestine. These secretory glands of the maize, however, have no lumina. In *Phœnix dactylifera* the secretory organ of the seed is the rounded structure situated opposite the furrow. In *Nepenthes* and other insectivorous plants special glands occur in appropriate places, e.g. in the lining membrane of the pitchers, or in special tentacles, as in *Drosera*.

The pericarp of the fruit of *Rhamnus infectorius* contains a glucoside, xanthorhamnetin, which, on hydrolysis, breaks up into glucose and rhamnetin, a yellow compound. This hydrolysis is brought about in nature by an enzyme which is contained in the parenchyma of the raphe of the seed. To illustrate this, the following experiment may be tried:—

An aqueous extract of the separated pericarp is made and placed in a glass vessel, then into the solution is thrown the raphe of a seed. A golden yellow precipitate comes down. A parallel case is furnished by the seeds of *Lunaria biennis* (p. 240).

In other cases the enzyme and substrate are contained in different cells of the same tissue, so that it is only necessary to crush up the tissue, or to macerate it, in order to obtain the reaction; the bitter almond, containing emulsin and amygdalin, may be given as an example.

The enzyme-secreting cells of *Zea* and *Phœnix* have been studied by Reed.† He finds that in the resting condition these elements are crowded with granules of a protein nature which disappear as secretion begins. At the beginning of secretion, the nucleus is poor in chromatin, but this material increases in amount as germination proceeds, the nucleolus

* Ward and Dunlop: "Ann. Bot.," 1887, **1**, 1.

† Reed: *id.*, 1904, **18**, 267; see also Huie: "Q.J.M.S.," 1897, **39**, 387; 1899, **42**, 203.

becoming smaller and smaller. Finally, at the end of the secretory activity, the protoplasm of the gland-cells breaks down, and the products of its disintegration disapppear from view.

It may be remarked that in the dried condition enzymes may retain their characteristic power for a considerable time; thus White * found that the ferments—diastase, protease, and ereptase—of the resting fruits of wheat and barley retained their activity after twenty years, by which time the power of germination is lost. Also, that the subjection of the dry grains to certain extremes of temperature did not destroy the enzymes. Thus the heating of dry oats to 100° C. for four and a half hours was without effect in the destruction of the enzymes; an exposure to a temperature of 130° for one hour, however, did destroy the ferments. On the other hand, a temperature of — 200° C. did not destroy the dry diastase of barley.

The number of enzymes which a plant may contain is surprising; thus in *Beta vulgaris*, the leaves contain invertase, diastase, and maltase, the stem possesses invertase, diastase, inulase, and emulsin, and the root diastase, maltase, inulase, and emulsin, but not invertase.†

The moulds—the digestive activities of which are, to a great extent, extra-cellular—also exhibit marked powers of secreting different enzymes. Thus *Monilia sitophila* may form maltoglucase, trehalase, raffinase, invertase, cytase, diastase, lipase, tyrosinase, and trypsin. These, according to Went,‡ are secreted according to the nature of the food; Dox,§ however, who has demonstrated the presence in moulds of protease, nuclease, amidase, lipase, emulsin, amylase, inulase, raffinase, sucrase, maltase, lactase, catalase, and phytase, considers, from the data at hand, that these enzymes are formed regardless of the chemical nature of the substrate.

Observations such as these open up many questions relating

* White: "Proc. Roy. Soc. Lond.," B., 1909, **81**, 417.

† Robertson, Irvine, and Dobson: "Biochem. Journ.," 1909, **4**, 258.

‡ Went: "Jahrb. Wiss. Bot.," 1901, **36**, 611; see also Pringsheim and Zempter: "Zeit. physiol. Chem.," 1909, **62**, 367.

§ Dox: "Plant World," 1912, **15**, 40.

to the nature of enzymes; are all these different ferments really specific, or are there only a few enzyme-nuclei which, before they can attack any particular substance, have to have attached to them certain molecular complexes according to the nature of the substrate?

There may, in certain cases, be made out a curious association of different enzymes. Thus Vines * found that when a tissue gave the guaiacum reaction, with or without the addition of peroxide, that same tissue also exhibited proteolytic activity and vice versa. Thus in the fruit of the orange, neither the juice nor the pulp gives the guaiacum reaction, whilst, on the other hand, the peel does. The peel is actively proteolytic, but not the pulp and juice. Similarly the latex of the fig, papaw, lettuce, and spurge, has proteolytic qualities and also gives the peroxidase reaction. The meaning of this association is not clear.

CLASSIFICATION OF ENZYMES.

The following classification of enzymes, based on the chemical reactions in which they exert their accelerating influence, indicates the extensive use made by nature of these catalysts :—

1. HYDROLYTIC ENZYMES.
   - (*a*) Ester or fat-splitting enzymes (esterases) : Lipase, chlorophyllase.
   - (*b*) Carbohydrate-splitting enzymes (carbohydrases) :—
     - Invertase, which hydrolyses cane sugar to dextrose and levulose.
     - ,, ,, ,, raffinose to levulose and melibiose.
     - Maltase ,, ,, maltose (malt-sugar) to dextrose.
     - Lactase ,, ,, lactose (milk-sugar) to dextrose and galactose.
     - Amylase or Diastase, which hydrolyses starch to maltose and dextrin.
     - Inulase, which hydrolyses inulin to levulose.
     - Pectinase, which hydrolyses pectins to arabinose and galactose.
     - Cytase, which hydrolyses hemicellulose to mannose, galactose, etc.
   - (*c*) Glucoside-splitting enzymes :—
     - Emulsin, which hydrolyses amygdalin to glucose, hydrocyanic acid and benzaldehyde.
     - ,, ,, ,, β-methylglucoside to glucose and methyl alcohol.
     - Myrosin ,, ,, potassium myronate to allylisothiocyanate, glucose, and potassium hydrogen sulphate.
     - Phytase ,, ,, phytin to inosite and phosphoric acid.

* Vines : "Ann. Bot.," 1903, 17, 257.

(*d*) Protein-splitting enzymes :—
Protease, which hydrolyses proteins to albuminoses and peptones.
Peptidase ,, ,, albuminoses, peptones and other polypeptides to amino acids.
(erepsin)
Amidase ,, ,, amino compounds to ammonia.
(*e*) Urea-splitting enzymes :—
Ureases, which hydrolyse urea into ammonia and carbon dioxide.

2. FERMENTING ENZYMES.
Alcoholic fermentation of glucose, fructose, mannose, etc., by zymase.
Lactic acid fermentation of lactose by lactic acid bacteria.
Butyric acid fermentation of lactose by the butyric bacteria, etc.

COAGULATING ENZYMES.
Rennin (Chymosin), which curdles milk.
Thrombin which coagulates blood.
Pectase ,, ,, soluble pectic bodies.

4. OXIDISING ENZYMES.
Oxidases which oxidize phenols and chromogens.
Oxygenase, which oxidizes catechol with the formation of a peroxide.
Peroxidase, which sets free active, or atomic, oxygen from hydrogen peroxide, or other peroxides.
Catalase, which splits molecular oxygen from hydrogen peroxide only.

ISOLATION AND PURIFICATION OF ENZYMES.

To obtain an active enzyme from a given source, the material is thoroughly ground in water, dilute alcohol or glycerol, sand being added, if needs be, to break up the cell wall. The filtrate can be used in qualitative work and to it antiseptics may be added if the experiment be protracted. The antiseptics commonly used are chloroform, toluene, tri-cresol, thymol, or sodium fluoride.

The solution obtained as above is a crude preparation. If it is likely to contain crystalloids, these may be removed by dialysis. By pouring the solution into absolute alcohol the enzyme is precipitated; the precipitate is filtered off, dissolved in water and again precipitated with alcohol. The enzyme is filtered off and dried in a vacuum desiccator. The preparation will still be impure, being contaminated with protein and other substances.

Enzymes are colloidal substances which exhibit in a marked degree the phenomenon of adsorption, as a result of which they tend to be removed from solution by adsorption on any precipitate formed in their presence or by the addition to their solutions of substances presenting a large surface such as animal charcoal. Making use of these facts, Willstätter

has developed a technique of adsorption on kaolin, alumina, lead phosphate, and other substances, whereby not only may enzymes be largely separated from ordinary impurities but also from their activators.* In general terms, the smaller amount of adsorbent required, the higher is the degree of purity attained. The adsorbed enzyme is removed from its adsorbate by such gentle chemical means as dilute alkali, alkaline phosphate, or weak acid according to the relation between the enzyme and its adsorbate, which relationship cannot be foretold.

The adsorption is influenced by various factors, the chief of which are as follows :—

1. The nature of the solvent in which the enzyme is dissolved; peroxidase, for example, is not adsorbed from an aqueous solution, but is from an alcoholic solution. Likewise papain is better adsorbed from an alcoholic solution.

2. The reaction of the medium is not without influence: papain, for example, is but feebly adsorbed from an acid solution, better from a neutral solution, and best from a feebly alkaline solution. It is freed from its adsorbent by a weak acid.

3. Concentration. In certain instances, e.g. saccharase, the use of a very dilute solution results in a preparation of greater purity.

4. The nature of the adsorbent on which depends the degree of selectivity. Its significance may be illustrated by the use of various preparations of aluminium hydroxide in the gel state.† Autolysed yeast contains maltase and saccharase; these may be separated by the use of β-alumina, $Al(OH)_3$, or of metahydroxide of alumina, AlO_2H, both of which adsorb maltase readily and saccharase very sparingly. The maltase may be recovered by treatment with alkaline phosphate.

* See Willstätter : " J. Chem. Soc.," 1927, 1359.

† Aluminium hydroxide is precipitated in the form of a gel from aluminium salts. It first comes down in the form of an unstable gel, $Al(OH)_3$, which is termed α; α changes quickly into a β variety, which changes slowly into a stable variety, termed γ. By heating $\gamma Al(OH)_3$ with ammonia to 250° C., a gelatinous hydroxide of the formula AlO_2H is obtained.

In addition to purification by selective adsorption, selective elution sometimes may be effective. Thus γ-alumina will adsorb both saccharase and maltase, both of which are liberated by weakly acid or neutral phosphate solution. A primary phosphate, however, will liberate saccharase completely, most of the maltase remaining behind, and may be recovered by elution with a secondary phosphate.

The adsorption method of purification tends to show that what was hitherto supposed by some to be responsible for the catalytic action are removable impurities. Thus Willstätter and his collaborators found that they were able to reduce the iron content of their peroxidase preparations from 0·5 to 0·06 per cent without loss in activity. Similarly it has been shown that phosphorus is not responsible for the activity nor is an essential constituent of saccharase which, by adsorptive methods, has been freed almost completely from carbohydrate, protein, and phosphorus without loss of stability or activity.

Owing to the absence of chemical criteria for determining the effect upon enzymes of the process of purification, special methods have been devised for ascertaining the alteration in activity of an enzyme in the course of its purification; some of these methods are outlined under the respective enzymes (see under Lipase and Peroxidase). The criterion suggested by Euler and Josephson for saccharase * which has been adopted by Willstätter, is known as the "Time Value" (Zeitwert); this is the time in minutes required by 0·05 gram of the enzyme preparation dissolved in 5 c.c. of 1 per cent sodium phosphate and added to 20 c.c. of 20 per cent sucrose to reduce the rotation of the solution to zero, the temperature being 15·5° C. By a number of alternate adsorptions and elutions, including the use of lead phosphate, Willstätter † has prepared from yeast a saccharase preparation having a "time value" of 0·1, compared with a value of 300 for the yeast from which he started, which implies a 3000-fold increase in activity. The purest product is free from carbo-

* Euler and Josephson: "Zeit. physiol. Chem.," 1925, **145**, 130.

† Willstätter: *id.*, 1926, **151**, 1; "Annalen," 1922, **427**, 111, and earlier papers.

hydrate and protein, but may contain from 4 to 10 per cent of nitrogen and a trace of phosphorus.

CHEMICAL CONSTITUTION.

The chemical constitution and nature of enzymes is, as yet, largely a matter of speculation, owing to the fact that it is very difficult to obtain enzymes in a pure condition; it is particularly difficult to purify them from proteins and this may, to some extent, account for the fact that all enzymes were formerly supposed to be of a protein nature. Willstätter* however, points out that the purification of lipase, saccharase, and peroxidase has been carried so far that the final preparations give no protein reactions and are free from carbohydrate.

The work of Willstätter indicates that enzymes consist of a chemically active group and a colloidal carrier. The specific nature of the enzyme is associated with the chemically active group which can be transferred from one colloidal carrier to another; separation of an active group from its colloidal carrier involves a loss in activity of the enzyme.

Willstätter† is of the opinion that for the proper functioning of saccharase, a colloidal carrier is essential; different substances may function in this capacity according to the process of purification adopted, but a complete absence of carrier may involve a molecular rearrangement in the active saccharase complex with a destruction of its catalytic properties.

Considerable difference of opinion exists in regard to the special class of enzyme known as oxidases. These, according to some authors, as for example Dony-Henault,‡ are not organic compounds at all, but owe their action to the presence of certain inorganic salts, more especially manganese salts, in colloidal solution. Bertrand,§ on the other hand, considers that the laccase of *Rhus succedanea* is a protein, whilst Euler and Bolin‖ are of the opinion that the laccase of

* Willstätter: "Ber. deut. chem. Gesells.," 1926, **59**, [B.], 1591.
† *Ibid.*, **59**, 1591.
‡ Dony-Henault: "Bull. Acad. Roy. Belg.," 1908, 105.
§ Bertrand: "Ann. Chim. Phys.," 1907, [7], **12**.
‖ Euler and Bolin: "Zeit. physiol. Chem.," 1909, **61**, 1.

Medicago sativa is composed of the calcium salts of glycollic, citric, and malic acids.

According to Wolff,* moreover, a very dilute ferrocyanide solution mixed with a colloidal iron solution gives all the reactions of an oxidase and is partly destroyed by boiling or mixture with traces of metallic salts. But the fact that an inorganic complex may bring about the same result as an oxidase, does not militate against the organic structure of naturally occurring oxidases. Finally, as has been mentioned above, peroxidase has been purified to such a degree that the final preparation contained but ·06 per cent of iron and showed no decrease in its activity.

MODE OF ACTION OF ENZYMES.

To explain the mode of action of inorganic catalysts, it is frequently supposed that they form labile additive compounds with one of the reacting substances which then react more readily than the original substance would have done.

Similarly, in the case of the enzymes, it is now generally assumed that they enter into some form of loose combination with the substrate; in spite of this the enzyme is, in general, not altered by the reaction but retains its original activity after having completed its work, unless the products of the reaction have any deleterious effect on it.

In the group of carbohydrates the action of the enzymes is usually regarded as being more or less specific, each disaccharide being hydrolysed only by its own enzyme, e.g. cane sugar by invertase, milk sugar by lactase, and malt sugar by maltase.

That this specific activity is in some way connected with the molecular structure of the substances would appear from the researches of Fischer on the action of enzymes upon artificial glucosides. Fischer, by the action of methyl alcohol and hydrochloric acid on glucose, obtained two stereoisomeric methyl glucosides known respectively as the α and β variety. Now these two substances differ from each other only by the arrangement in space of the groups attached to the terminal

* Wolff: "Compt. rend.," 1908, **147**, 745.

carbon atom, and it is found that while the α modification is readily converted by maltase into glucose and methyl alcohol, the β modification is not affected by maltase at all, but is, on the other hand, hydrolysed by emulsin, which has no action on the α compound.

It would appear from this that the structure of the molecule which is to be decomposed is the determining factor.

Incidentally it may be mentioned that the fact that emulsin and maltase are complementary in their action upon α and β methyl glucosides, enables one to classify a glucoside as belonging to the α type if it is attacked by maltase and not by emulsin, or to the β type if it is attacked by emulsin and not by maltase.

Several other examples of this selective action on the part of enzymes for different optical isomers have been described by Fischer and Abderhalden, who found that whereas d-alanyl-d-alanine, d-alanyl-l-leucine were split up by enzymes, their stereoisomers d-alanyl-l-alanine and l-alanyl-d-alanine were not.

This peculiar dependence upon structure led Fischer to suggest that the relationship which exists between the substance to be decomposed and its enzyme is similar to that existing between a lock and its key ; or, in other words, unless the molecular structures of the two substances fit each other no interaction can take place.

These facts give strong support to the theory of the formation of some sort of compound between the enzyme and the substrate.

It should, however, be noted that the action of enzymes is not entirely specific, inasmuch as the one and the same enzyme may be able to hydrolyse two or more substances. Thus maltase is able to hydrolyse both maltose and α-methyl glucoside ; and emulsin is able to decompose β-methyl glucoside, β-methyl galactoside, milk sugar, amygdalin (the glucoside of bitter almonds, and with which it is primarily associated in nature), arbutin, salicin, and coniferin and most naturally occurring glucosides.

The specific nature of the interaction between enzymes and

other substances is, however, only really strongly marked in connection with optically active substances. For, taking the case of the fat-splitting enzymes or lipases, practically all esters are broken up by pancreatic lipase, although the ease with which the hydrolysis is effected may vary considerably in different cases.

CONDITIONING FACTORS.

The rate of enzyme action is the resultant of various factors the chief of which are temperature, reaction of medium, concentration of enzyme and of substrate, accumulation of end products, paralysers, and radiation. The sensitivity of many enzymes is so great that any one of these factors may inhibit their activity, thus certain animal proteases are inactive in an alkaline medium, pepsin for example; or in an acid medium, trypsin for example. On the other hand, certain plant proteases are active in media irrespective of its reaction. The appraisement of these factors, particularly temperature and the reaction of the medium, is a matter of some moment, for it is only when they are precisely ascertained that the full value of the activity of an enzyme is available in the laboratory and in industrial processes. They are, however, often definable only within wide limits, since an indisputably pure enzyme has yet to be obtained, and these impurities may be effective in altering the value of the factor in question. Willstätter * and his fellow-workers by special methods of yeast cultivation and of preparation have isolated an invertase (saccharase) possessed of high activity. The preparation was effected by fractional autolysis of the yeast, followed by dialysis, and purification was effected by adsorption on kaolin. The resultant enzyme was associated with less impurity, was more stable, and was possessed of an activity 28 per cent greater than any other previous preparation.

A general consideration of these factors follows; a more detailed account must be sought in manuals devoted to

* Willstätter, Schneider, and Bamann: "Zeit. physiol. Chem.," 1925, 147, 248.

enzymes, some of which are mentioned at the end of this section.

1. TEMPERATURE.

1. In general terms, the velocity of a reaction, enzymic or otherwise, increases with a rise of temperature in accordance with van't Hoff's law. Enzymes are thermolabile; they are destroyed at 100° C., and in the majority of cases cannot be heated with safety above 60° C. This sensitivity to heat may in part be explained by attributing it to the colloidal nature of the enzyme and the consequent tendency to coagulation by heat. Even at low temperatures enzymes become inactive at varying rates; an increase in temperature hastens this inactivation which obtains at various degrees and marks the thermal death-point. This point is difficult to determine since it depends on various factors such as the reaction of the medium; the degree of purity, which may be indeterminate; the presence of various substances in the substrate, protein, for example, which may act as a protective colloid; the presence of various salts, particularly phosphates and chlorides, which may enter into combination with the enzyme; together with other factors. For these reasons it is essential to have an arbitrary definition of the thermal inactivation point of enzymes: Euler * defines it as that temperature at which the activity of the enzyme is halved when heated for one hour in an aqueous solution free from the appropriate substrate and having a definite hydrogen ion concentration. Enzymes inactivated by heat may in some cases recover their activity by suitable treatment; thus Falk † observed the partial recovery of lipase on adding manganese to the solution, similarly Biedermann ‡ found that the diastatic activity of saliva, after heating to 100° C., could be restored by vigorous shaking with air. Gallagher,§ moreover, found that the peroxidase of the mangold could be temporarily inactivated by heating for a little less than two minutes at 100°, but recovered

* Euler: "Chemie der Enzyme," München, 1925.
† Falk: "J. Amer. Chem. Soc.," 1913, **35**, 601.
‡ Biedermann: "Biochem. Zeit.," 1922, **129**, 582.
§ Gallagher: "Biochem. Journ.," 1924, **18**, 39.

its characteristic properties on standing for some time in the cold. The preparation of the enzyme contained some iron and also gave several reactions for aldehyde even after the peroxidase had been temporarily inactivated by heat; it was found that a trace of iron contained in a solution of an aldehyde added to a peroxidase increased its activity; from this it was concluded that the precursor of the peroxidase is an aldehyde which, under the catalytic influence of iron, is converted into the peroxidase.

The above hypothesis coupled with the fact that Falk reactivated lipase by means of manganese perhaps places the significance of iron and manganese on a general basis; their influence would thus appear to be not so much upon the enzyme itself as upon the production of the enzyme from its precursor or zymogen.

Below the temperature of inactivation, there is for enzymes a temperature which is most favourable for their activity; this is termed the optimal temperature and varies within a wide range for different enzymes; for papaïn it is in the neighbourhood of 60° C., whilst for maltase it is around 40° C. Constant values are hard to obtain, for the optimal temperature hastens inactivation and, further, it is dependent on the reaction of the medium, on the length of time of exposure to the temperature, together with other factors such as impurities.

2. REACTION OF MEDIUM.

The reaction of the medium has an important influence on the activity of an enzyme. The optimum reaction is that P_H value * at which the enzyme exhibits its greatest activity. It varies with different enzymes and with their degree of purity. This is illustrated by Willstätter,† who points out that the lipase of the human stomach shows its optimum activity at P_H 5-6; but if purified by an adsorption process with kaolin, the optimum activity is at P_H 8.

Willstätter, however, points out that the optimum P_H is not only dependent upon the enzyme but upon the substrate

* See Appendix.
† Willstätter: "J. Chem. Soc.," 1927, 1359.

as well since; for example, the proteases papaïn and bromelin of the papaw and pineapple respectively hydrolyse fibrin best as P_H 7·2, but peptone or gelatine at P_H 5.

3. CONCENTRATION OF ENZYME AND OF SUBSTRATE.

According to the Law of Mass Action enunciated by Guldberg and Waage, the rate at which a body undergoes chemical change is dependent on the concentration as measured by the number of gram molecules of substance present in the litre; consequently the amount of substance changed in unit time will be greater at the beginning of the reaction than towards the end, since the amount of unchanged substance is continually decreasing.

The relationship between the amount of substance x (measured in gram molecules per litre) changed in time t (measured in minutes) and the original concentration a of the substance is given by the equation—

$$K = \frac{1}{t} \log \frac{a}{(a - x)}.$$

The above formula holds only for the decomposition of a single substance, and it is, therefore, characteristic of what is known as a monomolecular reaction or a reaction of the first order, and as such is applicable to all cases of hydrolysis, as for example—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

Although from the left-hand side of the equation it would appear that two substances are reacting, the quantity of water present is so large, as compared with the amount of cane sugar, that its concentration is practically unaltered, and therefore, for all intents and purposes, only a single substance is undergoing alteration in concentration.

Now the hydrolysis of cane sugar which takes place slowly in aqueous solution is catalytically accelerated by the addition of dilute mineral acids, the effect being greater in proportion to the amount of acid used, without, however, altering the order of the reaction. In reactions acting in accordance with the logarithmic equation above given, the amount of substance changed in a given time bears a constant

ratio to, or is a constant fraction of, the amount of substance unchanged; on plotting the amounts changed as ordinates against the time as abscissæ there is accordingly obtained what is known as a logarithmic curve.

In the case of organic catalysts, the rate of the reaction is dependent on the proportional relationship between the enzyme and substrate, provided that the conditioning factors, other than those under consideration, are kept constant and that no disorganization of the reaction, such as autocatalysis and reversibility, takes place. It is not infrequently supposed that there is a union between the enzyme and its substrate; and since the enzyme does not itself enter into the reaction, its part is that of an accelerator, this union is dissolved when the change in the substrate, hydrolysis, for example, has been effected, and the molecule of enzyme is available to accelerate the hydrolysis of another molecule of substrate. If a fermentation be set up by adding a small amount of enzyme to an excess of substrate, the proportionate relationship between them will change with time. In the beginning the substrate will be in excess, midway parity will be reached, and beyond this point the proportion of enzyme to substrate will increase until there is no more substrate to be hydrolysed.

As regards the rate of the reaction no one law is applicable throughout; in the earliest phase the rate of the reaction is in linear proportion to the amount of enzyme, i.e. the rate will be doubled if the amount of enzyme be doubled, this also obtains at the later phase where the enzyme is in great excess, but here the substrate must be increased to obtain an increased rate. To mention a few examples: Horace Brown and Glendinning * found that equal amounts of starch were hydrolysed by diastase in equal times during the earlier part of the reaction, in other words, the course of the reaction was expressed by a straight line; as the reaction proceeded, however, it became logarithmic, or, in other words, at the commencement, when the concentration of the substance being hydrolysed is great as compared with that of the enzyme, the reaction is linear and not in accordance with the law of mass

* Brown and Glendinning: "J. Chem. Soc., Lond.," 1902, 81, 392.

action, but where the concentration of the enzyme increases the reaction obeys the law of mass action up to a certain point.

Similar results were obtained by Adrian Brown * in the study of the action of invertase on cane sugar; he also expresses the view that, in the case of alcoholic fermentation and other enzyme actions which do not apparently conform with the law of mass action, the exceptional action "is due to a time factor accompanying molecular combination and change which limits the influence of mass action . . . this theory demands not only the formation of a molecular compound of enzyme and reacting substance, but the existence of this molecular compound for an interval of time previous to final disruption and change."

Similarly E. F. Armstrong † in studying the action of lactase and maltase upon their respective sugars found that while the reaction was in the main logarithmic, both the initial and final stages were linear.

The law of mass action only is applicable at those stages where the concentration of the enzyme is more or less equal to that of the substrate. As the relative concentration of the enzyme increases, the activity falls off and other laws have been propounded to express the rate of reaction.

4. INFLUENCE OF END PRODUCTS.

The velocity of enzyme action may be retarded by the interference of an end product of the reaction. Thus the alcohol produced by the fermentation of sugar by yeast ultimately stops the reaction, and the same applies to the production of acetic acid by *Mycoderma aceti*. The hydrolysis of amygdalin is retarded by the addition of glucose, benzaldehyde or hydrocyanic acid, which are products of the reaction. Similarly glucose interferes with the action of maltase. These retarding influences are due to various causes; the specific action of the end product on the organism, the alteration of the hydrogen ion concentration, and mass action. The subject is intimately connected with the action of paralysers.

* Adrian Brown: "J. Chem. Soc. Lond.," 1902, 81, 379.

† Armstrong: "Proc. Roy. Soc. Lond.," B., 1904, 73, 500, 516, 526;

5. PARALYSERS.

Amongst substances having a retarding effect on the activity of enzymes may be mentioned inorganic substances such as mercuric chloride or cyanide, arsenious oxide, sulphuretted hydrogen, ozone, and organic compounds such as chloroform, chloral, formaldehyde, hydrocyanic acid, phenylhydrazine, aniline, alcohol, etc.; the influence of these substances on different enzymes varies considerably; thus, for example, alcohol usually acts as a paralyser, but on lipase it has a stimulating effect.

The majority of the substances included in the above list also act as poisons to colloidal solutions of metals; the peculiar phenomenon of the recovery of metallic colloidal solutions from poisoning by hydrocyanic acid, is also met with in the case of the enzymes, and is likewise attributed to the oxidation of the poison.

The mechanism of these toxic actions is as yet unexplained; it is assumed that some form of chemical combination between the paralyser and the substrate enzyme or activator takes place.*

6. RADIATION.

With regard to the action of light rays on enzymes it appears, according to Iodlbauer and v. Tappeiner,† that there exist two distinct kinds of action :—

(*a*) Those produced by ordinary light in the presence of oxygen, and (*b*) those produced by ultra-violet light independently of oxygen.

The destructive action which has resulted from exposure to bright sunlight therefore appears to be dependent on the presence of oxygen, and is greatly increased by the presence of fluorescent substances, such as eosin, quinoline red, etc.‡ It is most destructive at the optimal reaction.§

It was first shown by Green ‖ that ultra-violet light de-

* Cf. Loewenhart and Kastle : " Amer. Journ. Chem.," 1903, **29**, 397, 563.

† Iodlbauer and v. Tappeiner : " Deut. Archiv. Klin. Med.," 1906.

‡ Tappeiner : " Biochem. Zeit.," 1908, **8**, 47.

§ See Pincussen : *id.*, 1923, **134**, 459.

‖ Green : " Trans. Roy. Soc. Lond.," 1897, **188**, 167.

stroyed diastase, and since then several other authors have described similar effects for other enzymes.*

The action of radium and radium emanation on enzymes has been studied by Wilcock,† by Loewenthal and Edelstein,‡ by Bickel, by Loewenthal and Wohlgemut, and others.§

REVERSIBILITY OF ENZYME ACTION.

Comment has above been made on the decrease in the velocity constant of enzyme action after a certain point has been reached; the enzyme appears to become less active. This may be accounted for in one of two ways: either by the assumption that the products of the reaction combine with the enzyme or, by their concentration, exercise some inhibiting influence upon the enzyme; or else by assuming that the tendency for the reverse action to take place has a retarding effect.

That there should be a tendency for the reverse reaction to take place is a perfectly legitimate conclusion; in fact van't Hoff long ago pointed out that a catalyst which accelerates a reaction in one direction must also be able to exert an accelerating effect on the reverse reaction. Consequently the same enzymes which effect hydrolyses should also, under suitable conditions, be able to synthesize.

The first experimental proof of this was given by Croft Hill,‖ who showed that when maltase was allowed to act on a concentrated solution of glucose, the disaccharide maltose was produced; later it was shown ¶ that the disaccharide isolactose could be synthesized from galactose and glucose by the action of lactase from Kefir. Since then a large number of enzymatic syntheses have been effected.

* E.g. Burge, Fischer, and Neill: "Amer. Journ. Physiol.," 1916, **40**, 137, 426.

† Wilcock: "Journ. Physiol.," 1907, **34**.

‡ Loewenthal and Edelstein: "Biochem. Zeit.," 1908, **14**, 484.

§ Loewenthal and Wohlgemut: *id.*, 1909, **21**, 476. Laborde and Lemay: "Compt. rend. Soc. biol.," 1921, **85**, 497.

‖ Croft Hill: "J. Chem. Soc. Lond.," 1898, **73**, 634.

¶ Fischer and Armstrong: "Ber. deut. chem. Gesells.," 1902, **35**, 3144.

ANTI-ENZYMES.

The term anti-enzyme is applied to a class of substances occurring in the animal organism or produced in it by subcutaneous injection with an enzyme. The anti-enzymes are antagonistic in their action upon the enzymes, and their action is quite specific, the relationship between an enzyme and its anti-body being similar to that existing between a toxin and an anti-toxin. The first example of immunity against an enzyme was recorded by Hildebrandt,* the enzyme being emulsin.

Since then, anti-enzymes have been discovered for lipase, amylase, pepsin, papaïn, and urease. Anti-trypsin and anti-rennet occur normally in the blood, and, according to Weinland,† anti-pepsin and anti-trypsin occur in the mucous membranes of the stomach and intestine respectively.

A CONSIDERATION OF SELECTED ENZYMES.

LIPASE.

Lipase is widely distributed in the plant world and may be expected to occur where fats are of significance as a reserve food. It also has been described as occurring in many moulds such as *Aspergillus*, *Eurotium*, etc., in agarics, and in the latex of higher plants such as *Ficus* and *Euphorbia*.

In 1890 Green ‡ found that germinating seeds containing fat or oil, when macerated with water and left for some time, gradually acquired an acid reaction. This observation was subsequently confirmed and extended by Connstein, Hoyer, and Wartenberg,§ with the result that it has been found that the seeds of Euphorbiaceæ, and especially castor-oil seeds, whether germinating or not, contain an enzyme capable of hydrolysing fats. Lipase may occur in the seed, as in the castor oil, or it may develop during germination, as in linseed.

* Hildebrandt: "Virch. Arch.," 1893, **131**, 12, 26.

† Weinland: "Zeit. f. Biol.," 1903, **44**, 45.

‡ Green: "Proc. Roy. Soc. Lond.," 1890, **48**, 375.

§ Connstein, Hoyer, and Wartenberg: "Ber. deut. chem. Gesells.," 1902, **35**, 3988; Hoyer: *id.*, 1904, **37**, 1441; "Zeit. Physiol. Chem.," 1907, **50**, 414.

The fact that hydrolysis is slow at first and then suddenly increases from 5 per cent after one day to 58 per cent after two days and to 95 per cent after four days led Connstein to the conclusion that for rapid hydrolysis a certain minimum amount of free acid must be present, and it was found that when a little free acid was added at the beginning, hydrolysis could be completed within a few hours. Similar observations regarding the curve of the hydrolysis of fats during the germination of *Ricinus* seeds have been made by Deleano.*

A simpler way of demonstrating the action of the lipase of the castor-oil seed is to shell about 10 grams of the seeds and to divide these into two portions A and B ; A is pounded up in a mortar with 4 grams of castor oil and 5 c.c. of water, while B is treated in the same way, 5 c.c. of N/10 sulphuric acid being used in place of the 5 c.c. of water. After about an hour 25 c.c. of alcohol are added to each and the free acid is titrated with N caustic soda in presence of a few drops of phenolphthalein indicator. The quantity of acid developed in B, after allowing for the 5 c.c. of acid originally added, will be found to be much greater than that in A, showing that the enzyme works more efficiently in an acid medium.

Willstätter and Waldschmidt-Leitz † found that the dormant seed of the castor oil contained a variety of lipase, the activity of which was confined to P_H 4·7, no activity being manifest at P_H 7. On the other hand, young seedlings were found to contain a lipase which was active over a wide range of hydrogen in concentration, from P_H 4·7 to P_H 8. These facts indicate two different varieties of lipase ; to the former the name of spermatolipase has been given and to the latter, blastolipase. Willstätter and Waldschmidt-Leitz suggest that at the outset of germination proteolysis of the original enzyme complex takes place and leads to the formation of blastolipase. The amount of spermatolipase thus falls off as germination proceeds, but there is no equivalent increase in the amount of blastolipase, which, as a matter of fact, is unstable and is

* Deleano : " Centrlbl. Bakt.," 1909, **24**, 130.

† Willstätter and Waldschmidt-Leitz : " Zeit. physiol. Chem.," 1924, **134**, 161.

destroyed. The rapidity of the disappearance of the blastolipase depends on the hydrogen ion concentration; at P_H 4·7 the decline is faster than at P_H 7.

In support of their conclusion that the blastolipase arises from the proteolysis of the original enzyme complex, Willstätter and Waldschmidt-Leitz found that the treatment of spermatolipase with pepsin yielded a product having the same properties as blastolipase.

THE PREPARATION OF LIPASE.

Ricinus lipase has certain peculiar properties which make it extremely difficult to obtain an active preparation in a state of relative purity. It is, for example, insoluble in water, and its separation from fat renders it inactive; further, it is sensitive to such organic substances as alcohol, glycerol, etc. Willstätter and Waldschmidt-Leitz have elaborated a method of preparation which involves dialysis, centrifuging, adsorption on kaolin and drying, for the details of which the original paper must be consulted.

For commercial purposes the enzyme is prepared as follows: * Castor-oil seeds are ground up with water and then centrifuged; the resulting emulsion, which contains castor oil, proteins, and the enzyme, is then allowed to ferment at a temperature of 24°, whereby a scum containing the ferment rises to the surface and can be separated from the aqueous layer. This scum is then allowed to act upon the molten fat in the presence of water and a little manganese sulphate as a catalytic agent.

PROPERTIES.

Ricinus lipase, comprising both spermato- and blastolipase, is insoluble in water, and if it be rendered free from fat, its activity is lost; according to Willstätter and Waldschmidt-Leitz this is due to an alteration in the colloid state of aggregation both of the enzyme and its carrier, since this loss of activity can be considerably reduced by using finely divided carriers, such as kieselguhr, as diluents in the process of fat extraction.

* Cf. Hoyer: "Die Seifenfabrikant," 1905, **25**, 649.

The optimum temperature of spermatolipase is 35°; in a water fat emulsion a temperature of 50° C. is lethal. The optimal reaction of spermatolipase is at P_H 4·7; at P_H 6 it is quite inactive; blastolipase, on the other hand, shows a much wider range from 4·7 to 6·8; even at P_H 8 its activity is measurable. Further, blastolipase is much more stable in the dry condition, and has a greater synthetic power than spermatolipase. For the measurement of the lipolytic activity of enzyme preparations, Willstätter and Waldschmidt-Leitz have devised certain standards of reference termed phytolipase units and the phytolipase value, the latter being the number of phytolipase units in one centigram of the preparation. For details the original papers should be consulted.

DIASTASE (AMYLASE).

The term diastase must be regarded as generic: it includes a number of enzymes which are characterized by their power of attacking starch dextrin and like substances; they are of wide distribution and those from different sources have not precisely the same characteristics. The amount present in any particular organ varies according to the conditions obtaining; thus when the temperature and other factors are most favourable for growth and for the germination of starchy seeds, diastase is much more abundant than when growth and germination are slow. Also, the amount of diastase is always greater in starch leaves than in sugar leaves and the same holds for insolated leaves containing much starch, as compared with shaded leaves containing little or no starch.* The abundance of amylase in regions of active growth suggest to Sjöberg † that amylase also is concerned in synthethic activity.

It has already been stated (p. 155) that the action of diastase on starch is twofold and that it is possible to distinguish between a liquefying action on the one hand and a saccharifying action on the other.

* Eisenberg: "Flora," 1907, **97**, 347.
† Sjöberg: "Biochem. Zeit.," 1923, **142**, 274.

ISOLATION OF DIASTASE.

Diastase commonly is prepared from malted barley which yields in addition to amylase, the polysaccharide-splitting enzymes lichenase and mannanase, together with the disaccharide-splitting enzymes cellobiase, mannobiase, etc.* Lintner's † method may be used. One part of malt is extracted with 2-4 parts of 20 per cent alcohol for twenty-four hours. The solution is filtered and to the filtrate is added 2-3 times its volume of absolute alcohol. The precipitated enzyme is filtered off, washed with ether, and dried in vacuo. A certain degree of purification is effected by dissolving in water, dialysing and reprecipitation with alcohol.

The optimal temperature of amylase is 40-56° C.; it varies according to the source and the amount of impurities. The optimal reaction of plant amylases in general is P_H 5·0-5·4, that of malt amylase is P_H 4·3-4·5.

QUANTITATIVE DETERMINATION OF THE ACTIVITY OF DIASTASE.

The diastatic value of malt extract may be determined by the method of Lintner as follows: 25 grams of malted barley are ground and mixed with 500 c.c. of water and kept at room temperature for six hours, after which period the extract is filtered. Into a series of ten test tubes are delivered 0·1, 0·2, 0·3 . . . 1 c.c. of the filtered extract, and then to each tube are added 10 c.c. of 2 per cent soluble starch. The tubes are shaken up and kept at 21° C. for one hour, at the expiration of which 5 c.c. of Fehling solution are added to each. The tubes are then placed in a boiling water bath for ten minutes. The tube which contains the least amount of enzyme and in which the copper salt is completely reduced is then selected. The diastatic power is calculated on the assumption that, under these conditions, 0·1 c.c of malt extract produces just enough sugar to reduce completely 5 c.c. Fehling solution, and this diastatic value is taken as 100. From this it follows, from proportion, that if the second tube which contained 0·2 c.c.

* Pringsheim, Genin, and Perewosky: "Biochem. Zeit.," 1925, **164**, 117.
† Lintner: "Zeit. Brau.," 1908, II., **32**, 421.

of extract is the end point, the diastatic power is 50; if the tube containing 0·3 c.c. of extract is the end point, the diastatic power is 33·3 and so on, for the more enzyme required to hydrolyse the unit of starch in unit time, the lesser is the diastatic activity. Since no two samples of malt will exactly contain the same amount of moisture, the amount of moisture in the sample must be found from the dry weight and allowed for in the calculation; moreover malt prepared from oats has only about 30 per cent of the diastatic power of barley malt, while maize malt is still weaker. The diastatic power of brewers' malt is about 20-40° on the Lintner scale.

A convenient method for comparing the diastatic activity of two malt extracts consists in determining the relative times taken by the two extracts in hydrolysing a given starch solution up to the point at which it gives no colour with a dilute iodine solution; this is the so-called achromic point method. For this purpose, into a number of serially labelled test tubes are pipetted 5 c.c. of a 1 per cent solution of soluble starch and the series are placed in a water bath at 40° C. To these tubes are serially added 1, 3, 5 . . . c.c. of a stock solution of diastase heated to 40° C., the time of addition being noted. At frequent intervals a drop of the mixture is placed on a white glazed tile together with a drop of dilute iodine solution. When a colour is no longer produced, the time is noted. That tube in which the reaction is completed in rather less than three minutes should be chosen for an accurate redetermination. The conventional diastatic value, D, is given by the formula—

$$D = \frac{n}{\text{no. of c.cs. of diastase}} \times \frac{5}{t}$$

in which n is the number of cubic centimetres of starch solution employed, and t the time taken to effect decoloration.

From this D becomes the number of cubic centimetres of starch solution which can be hydrolysed by 1 c.c. of diastase.

TAKADIASTASE.

In addition to malt diastase, another variety, known as takadiastase, is commonly used in biochemical work; it is a product of *Aspergillus oryzæ* and is prepared from the mycelium

by extraction with water. The solution is concentrated *in vacuo* at 30-40° C., and then treated with 2·5 times its volume of 95 per cent alcohol. The precipitate is filtered off and dried. The crude takadiastase may be purified by dissolving in water, precipitating with ammonium sulphate, redissolving the precipitate in water, dialysing off the salts, and precipitating with alcohol.* In addition to amylase, takadiastase contains invertase, maltase, protease, lipase, sulphatase, together with a number of other enzymes.†

MALTASE.

This enzyme has a wide distribution and may be expected to occur wherever starch and maltose are significant in the metabolism of the plant. Thus it obtains in the leaves of the potato, beet, mangold, dahlia, sunflower, turnip and other phanerogams ‡ and also is widely distributed among the moulds and yeast, which latter form the best source for its preparation. The amount occurring in different yeasts is, however, variable; very little occurs in *Saccharomyces marxianus*, and distillery yeast generally provides but a small yield. It occurs also in the ungerminated barley grain and in green malt, i.e. germinated barley dried without the application of heat. In the ungerminated grain it is insoluble in water and its presence can only be demonstrated by allowing finely ground barley to act on maltose.§ During germination the insoluble maltase undergoes some change whereby it becomes partly soluble.

Maltase converts maltose into glucose; it has a delicate constitution, for which reason its presence is easily overlooked. Maltase is readily destroyed by heat, alcohol, and acid; owing to its destruction by alcohol it is not found with the diastase precipitated from aqueous solutions by this reagent. Its optimal temperature is 40° C., and it is rapidly

* Sherman and Tanberg: "J. Amer. Chem. Soc.," 1916, **38**, 1638.
† Nishimura: "Chem. Zelle Gewebe," 1925, **12**, 202.
‡ Daish: "Biochem. Journ.," 1916, **10**, 49.
§ Ling and Nanji: "Biochem. Journ.," 1923, **17**, 593; Maquenne: "Compt. rend.," 1923, **176**, 804.

destroyed at 55° C. Its optimum reaction is P_H 6·8,* but this would appear to vary, for Pringsheim and Leibowitz has described a maltase from barley whose optimum reaction is P_H 4·5-5·0.†

PREPARATION.

In view of the instability of maltase, its preparation cannot follow the ordinary course; active preparations are obtained by extracting dried yeast with water, the rate of extraction being increased with a rise in temperature; a low temperature, 15° C., is, however, preferable since the destruction of enzyme is less. The reaction should be neutral. If fresh yeast be used, the amount of maltase obtained is less and the best temperature to use is 30° C.‡ Willstätter and Bamann § autolysed yeast with ethyl acetate, keeping the reaction neutral and the temperature low. The enzyme may be separated from the filtered extract by adsorption on alumina, from which it can be recovered by ammonium phosphate.

PROTEOLYTIC ENZYMES.

OCCURRENCE.

The proteolytic enzymes of plants fall into three main groups: protease,‖ which hydroylses protein to albumins and peptones; peptidase (erepsin), which hydrolyses albumins and peptones into amino acids such as leucine and tyrosine; and amidase, which hydrolyses amino acids to ammonia.

Protease when present in the plant is usually associated with peptidase (erepsin); *Drosera* ¶ provides one of the few examples in which it occurs alone. Even where proteases might be expected, in protein containing seeds for example, they may be wanting. Dean's work on *Phaseolus vulgaris* may be taken as an example.** The seeds of this plant

* Willstätter, Kuhn, and Sobotka; "Zeit. physiol. Chem.," 1924, **134**, 24.

† Pringsheim and Leibowitz: "Biochem. Zeit.," 1925, **161**, 456.

‡ Krieble, Skaw, and Lovering: "J. Amer. Chem. Soc.," 1927, **49**, 1728.

§ Willstätter and Bamann: "Zeit. physiol. Chem.," 1926, **151**, 242, 273.

‖ Vines: "Ann. Bot.," 1905, **19**, 171; 1908, **22**, 103.

¶ White: "Proc. Roy. Soc. Lond.," B., 1910, **83**, 134.

** Dean: "Bot. Gaz.," 1905, **39**, 321.

contain much protein which undergoes proteolysis before translocation takes place. But no enzyme has been discovered in the seed which is capable of digesting these proteins; peptidase, however, which can hydrolyse the products derived from the digestion of these seed proteins, is abundant. Dean considers that the protoplasm plays the part of a protease, whilst the peptidase may carry the digestion further.

The observations of Blagoveschenski * indicate that the protease of a plant splits the globulins characteristic of that plant more actively than the globulins from other sources.

Peptidase (*ereptase*), often associated with protease, is more common and is, in fact, almost universally present in the vegetable kingdom. Peptidases are found in abundance in the fruits of *Musa*,† of *Carica papaya* (papaïn),‡ of *Ananas sativa* (bromelin), and in the latex and fruit of *Ficus* (cradeïn). They occur in the seeds or seedlings of *Cannabis*,§ *Hordeum*, *Lupinus*, *Medicago*, *Phaseolus*, and *Ricinus*.‖ Also their presence has been noted in *Agaricus*, *Saccharomyces* ¶ and other fungi.

Amidases would appear to be more elusive than either protease or peptidase: they may be classed as aminases or amidases according as to whether they split off ammonia from amino groupings or from amide groupings.

Kato ** found that the juice of bamboo shoots was able to act on asparagine with the liberation of ammonia. Shibata †† separated from the mycelium of *Aspergillus niger* an amidase which acted on amides and asparagine; Dernby ‡‡ found that the press juice of yeast set free ammonia from asparagine, but was inactive on amino acids; and Grover and Chibnall §§ have isolated an enzyme from the roots of barley which is

* Blagoveschenski: "Biochem. Journ.," 1924, **18**, 795.
† Bailey: "J. Amer. Chem. Soc.," 1912, **34**, 1706.
‡ Vines: "Ann. Bot.," 1908, **22**, 103.
§ Vines: *id.*
‖ Butkewitsch: "Zeit. physiol. Chem.," 1901, **32**, 1. Jacobson: "J. Amer. Chem. Soc.," 1912, **34**, 1730. Dean: "Bot. Gaz.," 1905, **39**, 321.
¶ Vines: "Ann. Bot.," 1905, **19**, 171.
** Kato: "Zeit. physiol. Chem.," 1911, **75**, 456.
†† Shibata: "Hofmeister's Beitr.," 1904, **5**, 384.
‡‡ Dernby; "Biochem. Zeit.," 1917, **81**, 107.
§§ Grover and Chibnall: "Biochem. Journ.," 1927, **21**, 857.

able to attack the amide group of asparagine, yielding aspartic acid.

ISOLATION OF THE ENZYMES.

The methods followed in isolating these enzymes differ in details according to the material used; the principle, however, is the same in most cases. The enzyme is precipitated from its solution by strong alcohol, filtered off, and washed with absolute alcohol. It may be partly purified by dissolving in water and re-precipitating with alcohol, filtered and washed with absolute alcohol followed by ether, and then dried in a vacuum desiccator. Following are some methods which have been pursued in particular cases.

To isolate the enzymes from the fluid contained within the pitchers of *Nepenthes*, Vines * added to the liquid an equal volume of absolute alcohol, then phosphoric acid followed by lime water in order to increase the bulk of the precipitate. Ammonium carbonate was added until the liquid gave a neutral reaction, and the precipitate filtered off. For use, the precipitate was shaken up with a 0·2 per cent solution of hydrochloric acid and filtered; the clear filtrate actively digests fibrin.

If it be desired to examine the contents of a tissue for these ferments, the expressed juice may be used, or an aqueous extract, the enzyme being separated as above if necessary. But sometimes this is unsatisfactory for various reasons—a syrup-like consistency or high coloration, for example. In such cases the tissues may be bruised in a mortar and placed with water in the vessel in which the experiment is to be carried out, together with the material—fibrin, for example—to be acted upon.† Buscalioni and Fermi ‡ used sterilized gelatine, with 0·5-1 per cent carbolic acid as an antiseptic, in a Petri dish. Fragments of the tissue to be tested are placed upon the jelly; the liquefaction of the gelatine in the neighbourhood of the pieces indicates the presence of proteolytic enzymes, but inasmuch as all proteases do not attack gelatine,

* Vines: "Ann. Bot.," 1897, 11, 573.
† Vines: *id.*, 1903, 17, 237, 597.
‡ Buscalioni and Fermi: "Ann. R. Inst. Bot. Roma," 1898, 7, 99.

a negative result does not necessarily indicate the absence of these enzymes.

Deans * prepared peptidase from the seeds of beans by extracting the cotyledons with water, filtering, and half saturating the filtrate with ammonium sulphate. The precipitate thus obtained is filtered off, dissolved in water and separated from ammonium sulphate by dialysis. The solution of enzyme thus purified may be dried at a temperature below 50° C.

Vines † separated protease from peptidase by making use of the fact that the former is hardly soluble in water but readily so in a dilute solution of sodium chloride, whilst peptidase is easily soluble in water. The material, e.g. seed of *Cannabis sativa*, is ground and extracted with a 10 per cent solution of sodium chloride. The solution is filtered and rendered just acid by the addition of acetic acid, whereby a white precipitate of protein is formed, which is filtered off. The acid filtrate has marked proteolytic qualities but has no action on fibrin; it therefore contains the peptidase. The fibrin-digesting protease is in the precipitate; to recover it, wash the precipitate with a 10 per cent solution of sodium chloride slightly acidified with acetic acid. The precipitate is next treated with distilled water and filtered; the filtrate, which has an opalescent appearance, digests fibrin but has no effect on Witte peptone. In order to ensure the best results, the temperature should be kept as low as possible during filtration.

Grover and Chibnall ‡ prepared the enzyme (asparaginase) responsible for the deamidation of asparagine from the young roots of germinated barley as follows: after 8-9 days germination, the seedlings were dried in an incubator at 37° C. for three days. By vigorous shaking the dried roots were broken from the grain and were separated by sifting through a coarse sieve; 200 grams of root were ground with 1·5-2 litres of water for two hours and the liquor pressed out in a Buchner press. The residue was ground for another hour with 1·5 litres of water and again pressed. The combined extracts were centrifuged and the clear liquid precipitated with 4·5

* Deans: "Bot. Gaz.," 1905, **39**, 321.
† Vines: "Ann. Bot.," 1908, **22**, 103. ‡ *Loc. cit.*

litres of ice cold 90 per cent alcohol. The precipitate was filtered off, washed with absolute alcohol and twice with ether, and then air-dried. The last traces of ether were removed in a vacuum desiccator. The resulting powder represented 4·4 per cent of the original dried roots.

GENERAL CONSIDERATIONS.

Proteases are slightly soluble in water and in 50 per cent alcohol; they are, however, readily soluble in 10 per cent sodium chloride solution in water. Peptidases are readily soluble in water whilst amidases are but sparingly soluble.

The proteolytic enzymes of plants are less rigid than those of animal origin in respect to their activity in acid or alkaline media. Thus it is stated that the proteolytic enzyme of *Drosera* is active in acid, alkaline or neutral media; papaïn is active both in acid and alkaline media, thus differing from animal pepsin; and some proteases will only work provided the reaction be acid, e.g. *Nepenthes*,* malt, mushroom, and yeast.† For this reason it is not possible to divide plant proteases into a pepsin group, which are active only in an acid medium, and a trypsin group which are active only in an alkaline medium.

In general terms the proteases are most active at P_H 5·0. There is, however, much variation; thus the optimum reaction of malt protease varies between P_H 3·7 to P_H 4·2; of the peptidases, the optimum reaction of papaïn is about P_H 5·0; whilst that of asparaginase is P_H 7·0. These values, however, vary with the substrate; thus Willstätter found that the peptidases, papaïn and bromelin, hydrolyse fibrin best at P_H 7·2, and peptone best at P_H 5·0, which indicates that the optimum P_H is not entirely a function of the enzyme itself but is dependent also on the nature of the substrate.

It will be noticed that these values are either neutral or acid; the reaction of plant juices likewise are neutral or acid, wherefore the natural reaction of the plant juice is the best to maintain for experimental purposes.

* Fisher: "Biochem. Journ.," 1919, **13**, 124.
† Vines: "Ann. Bot.," 1905, **19**, 171.

A like variation obtains in the optimum temperatures of the plant proteolytic enzymes; for papaïn it lies between 65-70° C., for bromelin it is about 60° C., and for asparaginase it is about 38° C.

Some doubt has been expressed as to the status of amidases distinct from peptidases. Grover and Chibnall * found that the asparaginase which they isolated from barley roots broke down *l*-asparagine into aspartic acid, but it had little or no effect on the dextro form of asparagine, a result concordant with that of Ravenna and Bosinelli, † who found that when moulds were grown on a mixture of *d*- and *l*-asparagine, the dextro form is unaffected, whilst the laevo form is attacked. The fact that this enzyme also acts on a dipeptide, in the shape of glycylglycine, suggests that asparaginase is in reality a peptidase. And this fact, together with observations on corresponding animal material, suggest that there is no sharp line between peptidase and amidase.

ZYMASE AND ALCOHOLIC FERMENTATION.

The formation of alcohol from fluids containing sugar has been known and practised from the earliest times, and the use of yeast in the manufacture of alcoholic beverages and of bread is an ancient industry. As is well known, when yeast is placed in a sugar solution, fermentation begins sooner or later, the principal end products being alcohol and carbon dioxide; substances other than ethyl alcohol, however, are formed, especially glycerol, succinic acid, and amyl alcohol,‡ the last

* Grover and Chibnall: *loc. cit.*

† Ravenna and Bosinelli: "Atti. R. Accad. Lincei.," 1919, **28**, 113.

‡ Amyl alcohol, using the term in its general acceptance, is a mixture of two isomeric primary alcohols, isobutyl carbinol $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CH.CH_2.CH_2OH$

and secondary butyl carbinol $CH_3—CH_2—\underset{\displaystyle CH_3}{\underset{|}{CH}}—CH_2OH$. The two substances together form "fusel oil," which is the harmful constituent of cheap spirit made from potatoes.

They appear to be produced from leucine

$$(CH_3)_2CH—CH_2—CHNH_2COOH,$$

and isoleucine $CH_3CH_2—\underset{\displaystyle CH_3}{\underset{|}{CH}}—CHNH_2COOH$, which are constituents of the

more particularly in the fermentation of the sugars obtained from wheat and potato starch. Alcoholic fermentation is due to the activity of the enzyme zymase which was first separated from the yeast cell by Buchner, whose work * marks the beginning of an epoch of vigorous investigations into this and kindred subjects.

The occurrence of zymase is not confined to *S. cerevisiæ*, which as a result of selection and high cultivation is able to produce a maximum alcoholic fermentation. This activity, which in cultivated yeast is not dependent upon atmospheric oxygen, preponderates over the ordinary aerobic respiration of the plant: in wild yeasts, however, the conditions are reversed; these plants respire oxygen freely and only manifest slight fermentative activity. Although oxygen is not necessary for alcoholic fermentation, yeasts are not disturbed in this activity by free aeration, the only effect of which is to stimulate their growth and reproduction, while they continue to form alcohol.

Zymases have also been identified in other Fungi such as *Mucor stolonifera* † and *Aspergillus niger*.‡ Germinating seeds in the absence of air produce appreciable quantities of alcohol and have been shown to yield a juice containing zymase; on admitting air the alcohol, if formed, does not accumulate, and there is an increased output of carbon dioxide as compared with the anaerobic condition.

The Activity of Different Species of Yeast.

Saccharomyces cerevisiæ is the species of yeast employed commercially in the production of alcohol from a sugar solution; this yeast secretes a number of enzymes in addition to zymase such as invertase, maltase, amygdalase, trypsin,

protein molecule, by loss of CO_2 and replacement of the NH_2 group by OH (see p. 451). The mixture is optically active owing to the asymmetric carbon atom of the secondary butyl carbinol.

The succinic acid likewise is formed not from carbohydrate, but from the amino acid, glutamic acid, supplied by the protein present.

* Buchner: "Ber. deut. chem. Gesells.," 1897, **30**, 117, 1110; 1898, **31**, 568. Buchner and Rapp: *id.*, 1897, **30**, 2668; 1898, **31**, 209, 1084, 1090; 1899, **32**, 127.

† Kostytschew: "Ber. deut. bot. Gesells.," 1904, **22**, 207.

‡ Maximow: *id.*, 1904, **22**, 225.

catalase, carboxylase, peroxidase, etc. Of the common hexoses only three, namely, glucose, fructose, or mannose, are readily susceptible to direct attack by this yeast, while galactose is only slowly acted upon; ordinary yeast may, however, be trained to acquire the power of fermenting galactose more readily by cultivating it for some time in a solution containing galactose; some strains such as *Saccharomyces apiculatus*, *S. Ludwigii*, and *S. anomalus* are, however, without action on galactose.

The ability of *S. cerevisiæ* to ferment disaccharide sucrose and maltose was formerly thought to be due to their possessing the enzymes saccharase (or invertase) and maltase, which were supposed to effect a preliminary hydrolysis of the corresponding disaccharides; Willstätter* has, however, found distillery yeasts, containing very little maltase, which were able to ferment maltase quite readily, and, to a lesser extent, lactose though entirely deficient in lactase; it is even suggested by Willstätter that sucrose may be susceptible of direct attack without the intervention of saccharase.

From what has gone before it will be noted that practically all yeasts contain maltase, but as a general rule brewers' yeasts are richer than distillers. Yeasts are without action on pentoses. Technically a distinction is made between brewery and distillery yeast; the strains employed by distillers are selected for their so-called high attenuating power, which means their ability to effect the alcoholic fermentation of practically all the saccharified carbohydrate in the substrate, and thus produce a maximum yield of alcohol; yeasts of this type go by the name of "Frohberg," from the name of the distillery from which the most marked representative of the class was first produced. Brewers, on the other hand, desire to leave in the fermented liquor an appreciable quantity of unfermented carbohydrate both sugar and dextrin, and for this purpose make use of a yeast which yields a higher gravity liquid after fermentation; this type of yeast is known as the "Saaz" type.

In addition to the above two types of yeast, brewers further distinguish between top and bottom fermentation yeasts;

* Willstätter: "Zeit. physiol. Chem.," 1925, **150**, 287.

the former of which grow on the surface of the wort, and are generally employed for the brewing of the stronger varieties of beer such as are produced in this country; the bottom fermentation yeasts, on the other hand, are used chiefly on the Continent for brewing of Lager beer.

Great care has to be taken to ensure the purity of the yeast employed in fermentation, since contamination with wild yeasts, which produce substances other than alcohol, may result in the introduction of strange and unwanted flavours in the resulting brew.

MECHANISM OF FERMENTATION.

Gay Lussac proposed the following equation to represent the action of the living yeast cell upon sugar :—

$$(1)\quad C_6H_{12}O_6 = 2C_2H_6O + 2CO_2$$

The mechanism of this reaction, involving the production of carbon dioxide without the intervention of atmospheric oxygen, has given rise to much speculation as to the association of the phenomenon with the vital activities of the yeast cell, especially from the point of view of its life without oxygen. The significance to the yeast plant of its fermentative activity and its relation to the whole chain of phenomena known by the name of respiration will be dealt with elsewhere.

Various theories have been put forward to explain the formation of alcohol from glucose. Kostytschev * considers that pyruvic acid and acetic aldehyde are intermediate compounds.

$$(a)\quad C_6H_{12}O_6 = 2CH_3CO\,.\,COOH + [4H]$$

This acid is then decomposed by a carboxylase present in the yeast to form acetic aldehyde—

$$(b)\quad 2CH_3CO\,.\,COOH = 2CO_2 + 2CH_3CHO$$

The aldehyde is then reduced to ethyl alcohol—

$$(c)\quad 2CH_3CHO + [4H] = 2CH_3\,CH_2OH$$

A combination of these three equations gives equation (1) above.

It was first observed by Pasteur that in addition to carbon dioxide and alcohol there are formed small quantities of

* Kostytschev : " Zeit. physiol. Chem.," 1912, **79**, 130, 359.

succinic acid, fusel oil, and glycerol. The formation of glycerol was explained by Neuberg and Kerb * by a series of equations according to which a molecule of glucose gives rise to two molecules of pyruvic aldehyde (methylglyoxal) as follows:—

(1) $C_6H_{12}O_6 - 2H_2O = 2CH_3CO.COH$ (pyruvic aldehyde)

The two latter then undergo a Cannizarro reaction, one molecule being oxidised and the other reduced, with the formation of a molecule each of glycerol and pyruvic acid.

(2) $\begin{matrix} CH_2=C(OH).CHO \\ CH_2=C(OH).CHO \end{matrix} + H_2O + \begin{matrix} H_2 \\ \| \\ O \end{matrix} = \begin{matrix} CH_2OH.CHOH.CH_2OH \\ \text{glycerol} \\ CH_3.CO.COOH \\ \text{pyruvic acid} \end{matrix}$

The pyruvic acid so formed is converted by carboxylase into acetic aldehyde and carbon dioxide.

(3) $CH_3.CO.COOH = CH_3CHO + CO_2$ (acetic aldehyde)

Finally the acetic aldehyde undergoes another Cannizarro reaction with more pyruvic aldehyde with formation of ethyl alcohol and pyruvic acid.

(4) $\begin{matrix} CH_3.CHO \\ CH_3.CO.CHO \end{matrix} + \begin{matrix} H_2 \\ \| \\ O \end{matrix} = \begin{matrix} CH_3CH_2OH \\ \text{ethyl alcohol} \\ CH_3.CO.COOH \\ \text{pyruvic acid} \end{matrix}$

The pyruvic acid being converted into acetic aldehyde again according to equation (3).

The shortage of glycerol experienced by the Central Powers during the World War led to a reinvestigation of this reaction with a view to its technical exploitation. This was successfully accomplished by the observation that addition of sodium sulphite to a fermenting mixture was able to increase the yield of glycerol from its normally very low value up to about 20 per cent of the weight of the sugar employed; by employing concentrations of sodium sulphite equivalent to 200 per cent of the sugar concentration, a yield of 36·7 per cent was obtained.†

To explain this increased formation of glycerol, it is sug-

* Neuberg and Kerb: "Biochem. Zeit.," 1913, **58**, 158.

† Connstein and Lüdecke: "Ber. deut. chem. Gesells.," 1919, **52**, 1385. Zerner: *id.*, 1920, **53**, [B.], 325. Tomoda: "J. Fac. Eng. Tokyo," 1924, **15**, 193.

gested by Neuberg that the action of the sodium sulphite is to combine with the acetic aldehyde as soon as it is formed, thus preventing it from acting as an hydrogen acceptor ; some other three carbon compound of unknown composition then functions in its stead producing glycerol as follows :—

$$C_3H_6O_3 + 2H = C_3H_8O_3$$

The effect of the added sulphite is, therefore, to produce a modification of the original Gay Lussac equation to account for the production of acetic aldehyde and glycerol as follows :—

$$C_6H_{12}O_6 = CO_2 + CH_3CHO + C_3H_5(OH)_3$$

If it is desired to represent the action of the sulphite, the equation becomes—

$$C_6H_{12}O_6 + Na_2SO_3 + H_2O = NaHCO_3 + CH_3CHO\,.\,NaHSO_3 + C_3H_8O_3$$

Neuberg and Reinfurth * describe the following experiment for demonstrating the production of acetic aldehyde during alcoholic fermentation. Two tubes containing 20 c.c. of 10 per cent cane sugar or glucose are shaken up with 2 gms. of yeast and to one tube are added 2 gms. of calcium sulphite (prepared by double decomposition from sodium sulphite and calcium chloride, cf. p. 1690). The two tubes are then immersed in a water bath at 38-40°. After a quarter of an hour acetic aldehyde can be demonstrated in the tube containing the sulphite by removing 3 c.c. and adding to them, without filtering, 0·5 c.c. of 4 per cent sodium nitroprusside solution and 2-3 c.c. of 3 per cent piperidine solution, when a deep blue colour indicating acetic aldehyde is produced. The control solution gives no colour under the same conditions.

A third mode of fermentation is that produced in the presence of alkaline salts such as ammonium carbonate or other soluble carbonates or phosphates. In this case the acetic aldehyde, produced as before, is acted upon by an enzyme known as aldehyde mutase which causes it to undergo a Cannizzaro reaction, producing acetic acid and ethyl alcohol as follows :—

$$\begin{matrix} CH_3CHO & O \\ \downarrow & \| \\ CH_3CHO & H_2 \end{matrix} \rightarrow CH_3COOH + CH_3CH_2OH$$

* Neuberg and Reinfurth : " Ber. deut. chem. Gesells.," 1919, **52**, 1677.

The net result is, therefore, that, as before—

$$2\{C_6H_{12}O_6 = CO_2 + CH_3CHO + C_3H_8O_3\} \quad . \quad . \quad (1)$$

$$2CH_3CHO + H_2O = CH_3COOH + CH_3CH_2OH \quad . \quad . \quad (2)$$

On combining these two equations—

$$2C_6H_{12}O_6 + H_2O = 2CO_2 + 2C_3H_8O_3 + CH_3COOH + CH_3CH_2OH$$

Thus it is seen that according to the conditions, *Saccharomyces cerevisiæ* growing on a sugar substrate can bring about three distinct types of fermentation :—

(1) $C_6H_{12}O_6 = 2C_2H_6O + 2CO_2$.
(2) $C_6H_{12}O_6 = CH_3CHO + C_3H_5(OH)_3 + CO_2$.
(3) $2C_6H_{12}O_6 + H_2O = 2C_3H_8O_3 + CH_3COOH + CH_3CH_2OH + 2CO_2$.

CO-ENZYME OF ZYMASE.

Harden and Young * showed that if yeast juice is dialysed or subjected to ultrafiltration it can be separated into two constituents ; a non-dialysable enzyme which does not pass through the filter and a dialysable constituent, the co-enzyme, which does pass through the filter. The enzyme constituent is thermolabile, whilst the co-enzyme is thermostable.

Neither of these two substances is able alone to bring about alcoholic fermentation, but when reunited after separation by dialysis or ultrafiltration, the mixture is able to ferment sugar even if the co-enzyme has been boiled.

The nature of the co-enzyme is unknown ; Neuberg and Schwenk † found that washed zymin could be reactivated by the addition of a mixture of α-ketonic acids and phosphate, whilst Harden ‡ found that zymin prepared from top fermentation yeast deprived of its co-enzyme by washing, was reactivated by sodium and potassium pyruvate, or by aldehydes, provided potassium ions are present. If sodium pyruvate is used alone no activation results, but a mixture of sodium and potassium pyruvate, or the latter salt alone, are effective. With respect to bottom yeasts, Neuberg § considers that ketonic acids are of most importance, and attaches but little significance to the potassium ions. According to Neuberg

* Harden and Young : " Proc. Roy. Soc.," B., 1906, **78**, 369.
† Neuberg and Schwenk : " Biochem. Zeit.," 1915, **71**, 135.
‡ Harden : " Biochem. Journ.," 1917, **11**, 64.
§ Neuberg : " Biochem. Zeit.," 1918, **88**, 145.

and Sandberg,* the rôle of the co-enzyme would appear to be that of a hydrogen acceptor since aldehydes, ketonic acids, nitro-bodies and disulphides can act as co-enzymes; he suggests that the action of all such substances consists in accepting the hydrogen from pyruvic aldehyde hydrate until sufficient acetic aldehyde has been formed to act as the hydrogen acceptor, thereby becoming reduced to ethyl alcohol. Harden summarizes the position in stating that the effect of the co-enzyme can be reproduced by the addition of substances which are able to yield aldehydes under the action of the enzyme carboxylase, which acts according to the equation—

$$R\,CO\,COOH = R\,CHO + CO_2$$

In this connection it is interesting to note that the co-enzyme of yeast is also found in the hot-water extract of animal muscle, especially frog's muscle, and of other tissues; it is also present in milk but is absent from serum. Meyerhof † has also shown that the co-enzyme of yeast plays an essential part in the respiration of muscle as well as in that of yeast.

According to von Euler and Myrbäck,‡ the activity of the co-enzyme can be increased by successive precipitation with lead acetate and silico-tungstic acid.

THE ISOLATION OF ZYMASE.

The following is the method pursued by Buchner in isolating zymase from *Saccharomyces*. One kilogram of compressed yeast is mixed with 250 grams of the infusorial earth known as kieselguhr and a quantity of fine quartz sand. The mixture is ground in a mortar until the microscope shows the majority of the yeast cells to be broken. To this paste-like mixture are added 100 c.c. of water which is very thoroughly stirred in; the mass is then wrapped in a cloth, placed in a press and gradually subjected to a very high pressure—Buchner used a pressure as high as 500 atmospheres—the liquid extracted being collected in a glass vessel. The residue is then removed from the press, broken up, and again

* Neuberg and Sandberg: "Biochem. Zeit.," 1920, 109, 290.
† Meyerhof: "Zeit. physiol. Chem.," 1918, **101**, 165; **102**, 1.
‡ von Euler and Myrbäck: *id.*, 1924, **139**, 281.

mixed with 100 c.c. of water and subjected to pressure. The extracts are united, shaken up with a little kieselguhr, and filtered. The filtrate contains the zymase, but in an impure condition; it may be purified by precipitating with alcohol and dissolving the precipitate in water. The aqueous solution rapidly loses its ability to ferment owing to the destructive action of a tryptic enzyme. It may, however, be preserved for a longer time—but not indefinitely—by drying the extract under reduced pressure, the solid substance so obtained being kept in a cold desiccator and dissolved in water as occasion demands.

In preparing extracts of yeast, it must be remembered that the potency of the extracts depends upon the physiological state of the yeast used. Thus, if brewers' yeast be taken from the wort whilst fermentation is at its height, a high quality zymase will be obtained; if, however, fermentation of the wort be over, the yeast taken from it will yield an extract of little or no fermenting power.

A more stable preparation than zymase is zymin, which is prepared by stirring a bottom fermentation brewer's yeast with acetone for some minutes, filtering, treating again with acetone, draining and finally extracting with ether. The material is dried and kept at a temperature of 45° for twenty-four hours. This zymin is more active than yeast juice but is less active than living yeast, fermenting at about one-eighth the rate of an equivalent weight of the living cells. The optimal temperature of zymase is between 28-30° C., and in solution it is destroyed at 40-50°; its optimal reaction lies between P_H 6·2 and 6·8.

RÔLE OF PHOSPHATE IN YEAST JUICE FERMENTATION.

Wroblewski * was the first to observe that the addition of an alkaline phosphate increased the rate of fermentation by yeast juice, which fact he attributed to the reaction of the medium. The work of Harden and Young,† however, provides

* Wroblewski: "J. prakt. Chem.," 1901, [2], 64, 1.
† See Harden: "Alcoholic Fermentation," London, 1923.

a very different interpretation of the phenomenon. They found that the addition of phosphate may increase the velocity twenty-fold, but this increased rate falls off with time to its original value ; the addition of a second quantity of phosphate brings about a repetition of the phenomenon. Measurement of the increased amount of carbon dioxide over that produced in the absence of phosphate indicates that, within the limits of experimental error, the increase in the amount of carbon dioxide is equivalent to the amount of phosphate added, i.e. $R_2H \cdot PO_4 \equiv CO_2$, the amount of alcohol formed being in proportion.

If the solution is boiled directly the fermentation velocity has fallen to its initial value, it is found that practically the whole of the added phosphate is no longer precipitable by uranium acetate, it is, in fact, in organic combination as a hexose diphosphate, $C_6H_{10}O_4(R_2PO_4)_2$.

Fermentation by yeast-juice therefore takes place in stages, the first of which is the formation of a hexose phosphate which takes place during the first period of temporary acceleration :—

(1) $2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O$

The above equation, however, may not be a complete statement in view of the fact that the hexosediphosphate is accompanied by a hexosemonophosphate. Thus Robison * found that when fructose or glucose is fermented by yeast juice, an hexosemonophosphate is formed together with the hexosediphosphate.† In order to throw some light on this, Harden and Henley ‡ have redetermined the amounts of carbon dioxide evolved and the hexosediphosphate and hexosemonophosphate produced. They find that the ratio of carbon dioxide to total phosphorus esterified is on the average 0·9; it is invariably below unity, which indicates that some esterification of phosphorus, about 10 per cent, takes place without the evolution of carbon dioxide. The product of

* Robison : " Biochem. Journ.," 1922, **16**, 809.

† A third hexosemonophosphate was prepared by Neuberg (" Biochem. Zeit.," 1918, **88**, 432) by the hydrolysis of hexosediphosphoric acid.

‡ Harden and Henley : *id.*, 1927, **21**, 1216.

this esterification, in all probability, is a monophosphate. The ratio of carbon dioxide and hexosediphosphate required by the equation is 2, but the redetermined figures give an average value of 2·38. This may be explained on the assumption that the carbon dioxide and the diphosphate are produced in accordance with the equation, but part of the hexosediphosphate is subsequently hydrolysed by the enzyme hexosephosphatase, with the formation of hexosemonophosphate and an inorganic phosphate which again enters into the reaction according to the first equation—

$$(2)\quad C_6H_{10}O_4(PO_4R_2)_2 + H_2O = C_6H_{11}O_5(PO_4R_2) + R_2HPO_4$$

The rate at which this second reaction takes place is one determinating factor in the fermentation rate when glucose is fermented by yeast-extract. There is an optimum concentration of phosphate which produces a maximum initial rate of fermentation; beyond this optimum a further addition of phosphate depresses the fermentative activity. If the available amount of phosphate in a mixture of sugar, ferment, and co-ferment be very small, the total fermentation is greatly reduced, but if to such a mixture a little phosphate be added, there is an enormous increase, as much as 700 per cent, in the total fermentation, even after discounting an amount of carbon dioxide equivalent to the phosphate added.

With regard to other sugars, Harden and Young found that mannose and fructose are freely fermented by yeast-extract, fructose being fermented more quickly than mannose and mannose rather more quickly than glucose. Also the total weight of carbon dioxide given off from an excess of sugar by the action of a given volume of yeast-juice was slightly greater with fructose than with glucose, whilst that evolved from mannose was less than from glucose. No matter what sugar is used, glucose, fructose, or mannose, the hexose phosphate is the same, namely, fructose phosphate, from which it may be concluded that glucose and mannose undergo molecular rearrangement to the enolic modification (cf. p. 95). The behaviour of fructose is qualitatively the same as glucose, but quantitatively there is a considerable difference. Thus

the optimum concentration of phosphate for the fermentation of fructose is from 1·5 to 10 times as great as the optimum for glucose, and the maximum rate of fermentation of fructose is 2 to 6 times as great as that of glucose.

Harden and Young also find that the addition of a suitable amount of arsenate to a fermenting mixture of yeast-extract and sugar (glucose, fructose, or mannose) causes a marked acceleration in the rate of production of alcohol and carbon dioxide, which is continued long after a chemical equivalent of carbon dioxide has been evolved. In this, the action of arsenate differs from that of phosphate and, further, the arsenate occurs in the free state throughout the period of fermentation. This increased rate of fermentation is due to the accelerating influence of the arsenate on the hexose-phosphatase; the arsenate, however, cannot replace phosphate in the fundamental reactions of alcoholic fermentation.*

Whilst it is difficult to explain the precise significance of phosphorus in alcoholic fermentation, it is interesting to note that Embden and his fellow-workers † have found that phosphoric acid is formed simultaneously with lactic acid during muscle contraction, and have traced the formation of these two acids to the same precursor, lactacidogen, which substance they consider to be identical with the hexosediphosphate of zymase fermentation. The conclusion is not unwarranted that the formation of phosphoric acid esters is an intermediate stage in the metabolism of carbohydrates both in the plant and in the animal.

That phosphate is a necessity for alcoholic fermentation by zymase is generally agreed, it would not, however, appear to be a requisite in all alcoholic fermentations by the living yeast cell. Thus Euler ‡ finds that top fermentation yeasts do not produce hexose-phosphate, nor do the living yeast cells respond to the addition of phosphate which may be due to the requisite balance naturally obtaining in the plant.

* Harden and Young: "Proc. Roy. Soc.," B., 1911, **83**, 451.

† Embden, Griessbach, and Schmitz: "Zeit. physiol. Chem.," 1915, **93**, 1. Embden and Lacqueur: *id.*, 1914, **93**, 94; 1917, **98**, 181; 1921, **113**, 1. Embden and Zimmermann: *id.*, 1925, **141**, 225.

‡ Euler: "Biochem. Zeit.," 1918, **86**, 337.

Neuberg * even goes so far as to suggest that the phosphate relations of yeast juice, represent an artificial condition, and states that bottom fermentation yeast will only synthesize hexosephosphate when treated with a protoplasmic poison such as toluene.

OXIDASES.

The oxidases are enzymes which have the power of oxidizing various aromatic compounds and chromogens, which action is, in the latter case, indicated by a change in colour. The change in colour in vegetable tissues on exposure to air is an everyday phenomenon ; the exposed surfaces of an apple, especially cider varieties, will rapidly turn brown. The darkening in the colour of raw rubber is also due to an oxidase which is associated with the protein of the coagulated latex.†

These changes are often of considerable economic importance ; thus the discoloration of sap wood markedly depreciates the value of timbers,‡ while the lacquer industry of China and Japan has been built up on the facts relating to the action of the oxidase, laccase, on the expressed sap of species of *Rhus* (see below).

Oxidases are very widely distributed in the vegetable kingdom ; in the higher plants they may occur in any organ —stem, root, leaf, laticiferous tissue, petals, and fruits.

According to Wheldale-Onslow,§ oxidases are present in about 63 per cent of the higher plants. As a rule all the genera of an order contain oxidases or peroxidases ; thus oxidase orders are Gramineæ, Labiatæ, Umbelliferæ, Boraginaceæ, Solanaceæ, and Compositæ, while peroxidase orders are Liliaceæ, Cruciferæ, and Crassulaceæ ; orders containing both oxidases and peroxidase plants are Ranunculaceæ, Rosaceæ, and Leguminosæ.

A distinction is made between oxidases and peroxidases in

* Neuberg : "Biochem Zeit.," 1920, **103**, 320. Neuberg, Farber, Levite and Schwenk : *id.*, 1917, **83**, 244.

† Spence : "Biochem. Journ.," 1908, **3**, 165, 351.

‡ Bailey : "Bot. Gaz.," 1910, **50**, 142.

§ Wheldale-Onslow : "Biochem. Journ.," 1921, **15**, 107.

ordinary practice. The former are characterized by giving the so-called direct action with an alcoholic tincture of guaiacum, blueing it at once ; * the peroxidases, on the other hand, only give an indirect action, i.e. they only produce a blue with guaiacum after the addition of a little hydrogen peroxide. A difficulty in the way of recognising a fundamental difference between oxidases and peroxidases based upon their behaviour towards guaiacum, is the fact that no distinction between these two groups is noticed when they are allowed to act upon the following reagents: benzidene, α-naphthol, and p-phenylene diamine, each dissolved in 1 per cent strength in 50 per cent alcohol; in each case no colour results until after hydrogen peroxide has been added, a fact which suggests that neither oxidases nor peroxidases are sufficiently strong to oxidise these substances without the assistance of hydrogen peroxide (but see p. 500).

GENERAL CONSIDERATIONS.

Up to comparatively recent times an oxidase was considered to be a single enzyme, but according to Bach and Chodat,† what used to be termed oxidase is really a mixture of peroxidase and peroxide. According to them, there are three categories of oxidizing enzymes:—

(*a*) Oxygenases which produce the peroxide.

(*b*) Peroxidases which transfer oxygen from peroxides to the substance to be oxidized.

(*c*) Catalases which act specifically upon hydrogen peroxide with evolution of oxygen.

In the colour reactions mentioned above two actions are possible. Either the plant juice, e.g. of the potato, gives the blue coloration with the guaiacum tincture alone, or, the blue colour will not occur, as, for example, in the sap of the cucumber or of the horse radish, unless a peroxide, such as hydrogen peroxide, be added.

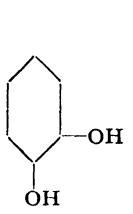
On Bach and Chodat's hypothesis, there are present in

* It should be noted that the presence of a large quantity of tannin or sugar may interfere with the reaction and inhibit the production of the blue colour.

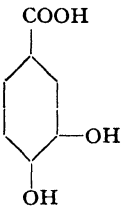
† Bach and Chodat: "Biochem. Centrbl.," 1903, 1, 416. Bach: "Ber. deut. chem. Gesells.," 1906, 39, 2126; 1907, 40, 230; 1908, 41, 216.

the potato oxygenase, peroxidase, and peroxide; the peroxidase transfers oxygen from the peroxide to the guaiacum, and the oxygenase re-oxidizes the reduced peroxide. This may be termed the direct action. On the other hand, in the cucumber juice, only peroxidase is present, so that in order to obtain the blue reaction with guaiacum, hydrogen peroxide, or other peroxide, must be added. This is the indirect action.

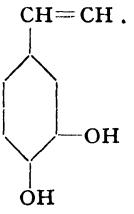
According to Wheldale,* the direct oxidase reaction is dependent upon the plant containing an orthodihydric phenol grouping such as catechol (I.), protocatechuic acid (II.), or caffeic acid (III.) :—



I.



II.



III.

Such substances when exposed to the air undergo slow autoxidation with the formation of brown oxidation products and a simultaneous formation of peroxides; according to Wheldale it is the function of the enzyme oxygenase to catalyse this oxidation and the resulting peroxide then sets free active oxygen in contact with the peroxidase, which active oxygen blues the guaiacum or, alternatively, is available for oxidation of other substances in the plant.

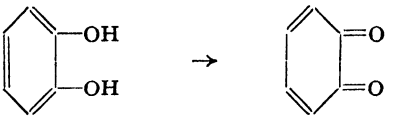
The following experiments due to Wheldale-Onslow illustrate the nature of the direct acting oxidase system. Slices of peeled potato are pounded under 96 per cent alcohol avoiding undue exposure to air. The peroxidase and oxygenase are thereby precipitated upon the tissues while the catechol containing complex remains in the solution which may be filtered off on a filter pump. In order to remove it completely, the extraction is repeated several times until a colourless powder consisting of tissue residues and peroxidase and oxygenase remains. An aqueous extract of this residue will not blue guaiacum directly, but does so on addition of hydrogen per-

* Wheldale: "Biochem. Journ.," 1920, 14, 353.

oxide. On the other hand, it will blue guaiacum without addition of hydrogen peroxide if treated with an aqueous solution of the purified catechol constituent extracted from pounded potato by means of boiling alcohol, the complete oxidase system being thereby restored.

A dissentient opinion with regard to the significance of phenolic substances in the oxidase mechanism is held by Gallagher * who points out that plant juices, especially in the absence of phenols, on exposure to air may form peroxides from autoxidizable substances in the tissues, which reaction is independent of enzyme action. There is no necessity, therefore, to invoke the action of an enzyme to oxidize a phenolic substrate. Gallagher isolated from the potato such an autoxidizable substance which was able to blue guaiacum immediately in the presence of peroxidase. This substance appeared to be related to the lipins; terpenes also can combine with oxygen and so effect the oxidation of guaiacum in the presence of peroxidase. These views are, however, disputed by Wheldale-Onslow.†

On the other hand, Szent-Györgyi ‡ sees no necessity for assuming the existence of a peroxidase; in his opinion the only requisites for the direct acting oxidase system which blues guaiacum are an oxidase and a substrate containing catechol or a derivative; by the oxidase the catechol (I.) is converted into orthoquinone (II.),



I. II.

a substance which he has shown to give a blue colour with guaiacum without the intervention of any enzyme. The only difference between a direct and an indirect acting system is, according to this view, that the indirect acting system has the oxidase but no catechol substrate, but has instead possibly

* Gallagher: "Biochem. Journ.," 1923, **17**, 515.
† Wheldale-Onslow: *id.*, 1924, **18**, 1549.
‡ Szent-Györgyi: "Biochem. Zeit.," 1925, **162**, 399.

a hydroquinone substrate which on oxidation gives *p*-quinone, a compound that is unable to blue guaiacum.

According to Ewart * there is no real distinction between oxidases and peroxidases other than a difference in strength.

A technical application of oxidases is furnished by laccase, an enzyme which was first investigated by Yoshida,† and was employed in China and Japan in the making of lacquered articles. The latex of many species of *Rhus* rapidly turns brown and finally black on exposure to the atmosphere; if the juice be evenly spread out, the final product is black and shiny. The extract of the plant contains urushic acid (laccol) which is oxidized into oxyurushic acid—

$$C_{14}H_{18}O_2 + O = C_{14}H_{18}O_3$$

The action takes place best at 20° C. in the presence of moisture and oxygen; at higher temperatures it is destroyed, at 63° according to Yoshida, and at 70° according to Bertrand. Bertrand ‡ also has given much attention to this oxidase, and the most important fact ascertained by him in this connection is that the presence of manganese is all-important. He found that the activity of the ferment is directly proportional to the amount of the metal present. But whether manganese is essential for all oxidase reactions is uncertain.

Isolation of Oxidases.

The isolation of oxidase may be a difficult matter when it exists in a tissue together with its substrate and other enzymes. Bourquelot and Bertrand give the following method for Fungi such as *Russula*. The tissue is chopped up, extracted with water—which may be warmed—and filtered as quickly as may be. The filtrate is then poured into an excess of strong alcohol, whereby the enzyme is precipitated. The precipitate is then filtered off and dissolved in water.

* Ewart: "British Assoc. Rep.," 1915. See also Moore and Whitley: "Biochem. Journ.," 1909, **4**, 136.

† Yoshida: "J. Chem. Soc. Lond.," 1883, **43**, 472.

‡ Bertrand: "Compt. rend.," 1895, **120**, 266; 1895, **121**, 166; 1896, **122**, 1132; 1896, **123**, 463; 1897, **124**, 1032, 1355. See also Rippel: "Biochem. Zeit.," 1923, **140**, 315.

PEROXIDASE.

Peroxidases which set free active or atomic oxygen from hydrogen peroxide, or organic peroxides, are very widely distributed, being, according to Wheldale-Onslow almost universally present in the higher plants, indeed, they have even been described as occurring in coal.*

Preparation of Peroxidase.

A crude preparation of horse-radish peroxidase † may be obtained by mincing the material and setting it aside for twenty-four hours in order to allow the myrosin to destroy the sinigrin. The material is then extracted for some days with three changes of 80 per cent alcohol and filtered. The residue is washed with strong alcohol, pressed dry, and extracted with 40 per cent alcohol in which the peroxidase is soluble; on adding a mixture of three parts of alcohol and one part of ether to the filtrate, the peroxide is precipitated.

Willstätter and his fellow-workers ‡ have elaborated a method for obtaining a highly purified enzyme; for this purpose the horse-radish root is left in running water to remove dialysable impurities; it is then treated with dilute oxalic acid whereby the enzyme is precipitated upon the protein; the peroxidase is then removed from the protein by elution with alkali and is then successively adsorbed on to kaolin, which removes carbohydrates, including a very tenacious glucoside, and alumina, and is subsequently precipitated by tannin; a further treatment with alumina, followed by precipitation with alcohol, completes the process.

In order to check the activity of the enzyme preparations during the course of purification, they are allowed to act upon an aqueous solution of pyrogallol in the presence of hydrogen peroxide; at the end of a given interval the reaction is stopped by the addition of acid and the purpurogallin formed by oxidation of the pyrogallol is extracted by means of ether and estimated colorimetrically. The "purpurogallin number" is

* Stoklasa, Ernst, and Chocensky: "Ber. deut. bot. Gesells.," 1907, **25**, 38.

† Bach: "Ber. deut. chem. Gesells.," 1904, **37**, 3787.

‡ Willstätter and Stoll: "Annalen," 1918, **416**, 21; 1920, **422**, 47; Willstätter and Pollinger: *id.*, 1923, **430**, 269.

the number of milligrams of purpurogallin which would be produced by 1 mgm. of the vacuum-dried preparation. This number which is about 0·25 for well-pounded horse-radish has been raised in the purest samples to over 3000.

Practically nothing is known regarding the chemical nature or mode of action of peroxidases.

The view formerly expressed by Willstätter that horse-radish was a nitrogenous glucoside containing over 30 per cent of pentose and an equimolecular proportion of glucose has been entirely modified owing to his obtaining more highly purified preparations; these latter are free from both carbohydrate and protein, and contain only 0·06 per cent of iron and 0·027 of phosphorus, probably only present as impurities, but contains from 9·37-13·57 per cent of nitrogen.

Regarding the mechanism of the action of peroxidase, Gallagher * has found that peroxidase prepared by precipitation with alcohol is usually associated with aldehydic substances † and appears to have an oxide group of the type $R_1 = O$; this group in conjunction with the ordinary peroxide grouping $R_2\begin{matrix}O\\|\\O\end{matrix}$ gives rise to an oxidation potential higher than that possessed by either alone, and he suggests that the first step in the oxidation process is the formation of a complex of the type—

$$R_1{=}O\begin{matrix}O\\\|\\O\end{matrix}R_2$$

Reagents Used for Detection of Oxidases and Peroxidases.

(*a*) *Oxidases*—

1. Guaiacum tincture (freshly prepared and dissolved in absolute alcohol diluted with water) gives a deep blue coloration.

2. One per cent α-naphthol in 50 per cent alcohol and 0·75 per cent *p*-phenylene diamine in the presence of a little sodium carbonate give on addition of a drop of hydrogen peroxide a deep blue colour.

3. One per cent benzidine in 50 per cent alcohol, followed

* Gallagher: "Biochem. Journ.," 1924, 18, 29.

† The idea that peroxidases were aldehydes was first suggested by Woker: "Ber. deut. chem. Gesells.," 1914, 47, 1024; 1917, 50, 672, 677.

by a drop of dilute hydrogen peroxide, gives a deep blue turning to brown.

4. One per cent α-naphthol in 50 per cent alcohol and a drop of hydrogen peroxide, gives a green colour.

(*b*) *Peroxidases*—

1. Guaiacum used as above gives a deep blue colour only after the addition of hydrogen peroxide.

2. Ten per cent aqueous solution of pyrogallol, followed by a drop or two of dilute hydrogen peroxide, gives a reddish-brown colour.

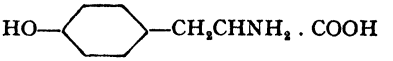
TYROSINASE.

This is the name given to another oxidizing enzyme which is distinct from oxidases and peroxidases though it frequently occurs in the same plants with these; thus it occurs in many Fungi, notably *Russula*, and amongst the higher plants it may be found in wheat bran and in the potato, especially in the peripheral layers adjacent to the skin.

The distinction between tyrosine, on the one hand, and the oxidases and peroxidases, on the other, is that the latter have no action upon monohydric phenols or their derivatives either in presence or absence of hydrogen peroxide, whereas tyrosinase acts upon *p*-cresol (I.) or tyrosine (II.) as well as upon phenol and aminophenol even in the absence of hydrogen peroxide—



I.



II.

producing in the case of *p*-cresol an orange-red colour and with tyrosine a series of colours through yellow, red, and brown to black. On the other hand, tyrosinase has no action upon pyrogallol in the presence of hydrogen peroxide in which respect it differs from peroxidase.

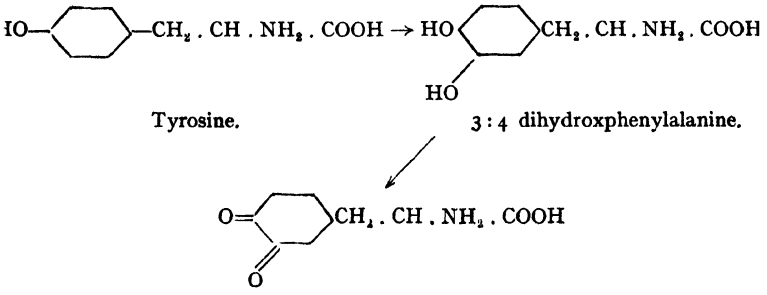
The mechanism of the reaction which takes place when tyrosinase acts upon tyrosine has been investigated by Raper and his fellow-workers.*

It appears from their results that there are three stages in the reaction, the first involving the production of the red

* Raper and others: "Biochem. Journ.," 1923, **17**, 454; 1924, **18**, 84, 92; 1925, **19**, 69; 1926, **20**, 735; 1927, **21**, 1370.

colour is an oxidative process which takes place at P_H 6; this red compound changes spontaneously by molecular rearrangement, more rapidly on warming, into a colourless substance without the intervention of tyrosinase; finally, this colourless substance is oxidized by oxygen to the black pigment melanin, a change which is probably catalysed by a phenolase present in the tyrosinase.

The course of events is probably represented by the following formulæ:—



Tyrosine. 3:4 dihydroxphenylalanine.

3:4 Quinone of phenylalanine.

The dihydroxy phenylalanine is then further converted through the 3:4 quinone of phenylalanine into the red substance whose constitution is not yet definitely determined, and this in turn gives rise to the colourless compound which is probably an indole derivative, and it is this latter which produces the black pigment melanin.

CATALASE.

This enzyme is widely distributed in aerobic plants and animals; it functions in the liberation of free molecular oxygen from hydrogen peroxide and hence removes this toxic substance from the cells. The action of catalase differs from that of peroxidase which does not liberate molecular but active atomic oxygen (see vol. ii.).

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APPENDIX.

HYDROGEN ION CONCENTRATION.

THE reaction of a medium not infrequently is described in terms of the colour change, acid, neutral or alkaline, effected in respect to litmus. In many instances this indication is sufficient, but in much biological work a more precise definition of reaction is requisite, and this is made possible by the conception known as the hydrogen ion concentration.

A normal solution of any acid or salt is defined as one containing 1 gram of hydrogen or its equivalent dissolved in 1 litre of water. According to this definition, the weights of hydrochloric, nitric, acetic and any other monobasic acid contained in a litre of normal acid would be the respective molecular weights in grams, namely $HCl = 36{\cdot}5$, $HNO_3 = 63$, $CH_3COOH = 60$. In the case of a dibasic or tribasic acid, it would be the molecular weight divided by two or by three—

$$\frac{H_2SO_4}{2} = 49, \frac{H_3PO_4}{3} = 32{\cdot}6.$$

From this reasoning it follows that whilst normal solutions of all these acids contain in the litre different quantities of acid, they all contain the same quantity of hydrogen, namely, 1 gram per litre. Whilst, however, they all contain potentially the same amount of hydrogen, it does not follow that the whole of this quantity is ionized ; and inasmuch as the actual acidity of a solution at any given moment is measured by the proportion of ionized hydrogen atoms it contains, it follows that the actual acidity of equinormal solutions of these various acids may be very different. This does not mean that their actual titratable value, as measured by their power of neutralizing alkali, will be different. Thus for the complete neutralization of the 1 gram of hydrogen contained in 1 litre of each of the

above-mentioned normal solutions, exactly the same quantity of caustic soda will be required, namely, 40 grams :—

$$\underset{36\cdot5}{HCl} + \underset{40}{NaOH} = NaCl + H_2O$$

$$\underset{60}{CH_3COOH} + \underset{40}{NaOH} = CH_3COONa + H_2O.$$

As a matter of fact, hydrochloric acid, which is a strong acid, is almost entirely ionized in dilute solutions, whilst acetic acid in solutions of equivalent strengths is ionized to a much smaller degree. In actual figures, about 97 per cent of the hydrogen in a ·001 N solution of hydrochloric acid is ionized and only about 84 per cent in a N solution, whilst in ·001 N acetic acid not more than 13·6 per cent of the hydrogen is ionized. Thus in the case of these two acids of the same normality, although the total amount of titratable hydrogen, as determined by the alkali-neutralizing power, is found to be the same, the actual percentage of ionized hydrogen is seven times as great in the case of the hydrochloric as in that of the acetic acid.

That the comparatively feebly ionized acetic acid ultimately requires the same amount of alkali for neutralization as the more strongly ionized hydrochloric, is due to the fact that as the ionized hydrogens in the acetic acid are neutralized a fresh quantity of previously un-ionized hydrogens become ionized to take the place of those which have been neutralized, and so on until all have been satisfied.

In practice it is the ionized hydrogen only which is responsible for the acidity of a solution at any given moment and so it comes about that the hydrogen ion concentration for a solution is, for biochemical purposes, a much more valuable criterion of the actual conditions prevailing in any given circumstances than is the potential alkali neutralizing power.

The concentration of hydrogen ions may be expressed as follows : In a decinormal solution of hydrochloric acid there would be 0·1 gram in 1000 c.c., presuming it to be completely ionized. In actual fact, however, a decinormal solution of hydrochloric acid is only ionized to the extent of 97 per cent, consequently the concentration is only $0\cdot1 \times 97$ or $9\cdot7 \times 10^{-2}$.

This concentration is more conveniently expressed as a logarithm: $\log_{10} 9{\cdot}7 = {\cdot}9868$, wherefore $9{\cdot}7 \times 10^{-2} = 10^{{\cdot}9868-2} = 10^{-1{\cdot}01}$. It has been agreed to express hydrogen ion concentration as the exponent to the base 10 of the concentration with the negative sign omitted, and this is represented by the symbol P_H. Hence the hydrogen ion concentration of the above N/10 hydrochloric acid would be $P_H = 1{\cdot}01$; if completely ionized it would be $P_H = 1$.

On this principle the following are synonymous methods of expression:—

$$N = \frac{N}{10^0} = P_H\,0 \qquad \frac{N}{1{,}000{,}000} = N \times 10^{-6} = P_H\,6$$

$$\frac{N}{10} = N \times 10^{-1} = P_H\,1 \qquad \frac{N}{10{,}000{,}000} = N \times 10^{-7} = P_H\,7$$

$$\frac{N}{100} = N \times 10^{-2} = P_H\,2 \qquad \frac{N}{100{,}000{,}000} = N \times 10^{-8} = P_H\,8$$

$$\frac{N}{1000} = N \times 10^{-3} = P_H\,3 \qquad \frac{N}{1{,}000{,}000{,}000} = N \times 10^{-9} = P_H\,9$$

$$\frac{N}{10{,}000} = N \times 10^{-4} = P_H\,4 \qquad \frac{N}{10{,}000{,}000{,}000} = N \times 10^{-10} = P_H\,10$$

$$\frac{N}{100{,}000} = N \times 10^{-5} = P_H\,5$$

It will be seen from the above that the greater the value of P_H, the lower is the actual hydrion concentration. Moreover, t is an established fact that the product of the concentrations of the hydrogen and hydroxyl ions in any given solution, is a constant, namely—

$$C_H \times C_{OH} = 10^{-14{\cdot}14}$$

and consequently at exact neutrality, when the concentrations of the two are exactly equal, $C_H = 10^{-7{\cdot}07}$ and $C_{OH} = 10^{-7{\cdot}07}$, whence it follows that for absolute neutrality, in which the concentration of hydrogen ions is exactly equal to that of the hydroxyl ions $P_H = 7{\cdot}07$.

There is no need to determine the OH ion concentration since it is easily found from the difference between 14·14 and the hydrion concentration.

Thus for $P_H 1$ the hydroxyl ion concentration would be P_{OH} 13·14, and for $P_H 10$ it would be P_{OH} 4·14.

Since for P_H 7·07 there is exact equality between H and OH ions, it follows that on either side of this value one

or other will be in excess. Thus values of P_H below 7·07 indicate acid solutions, while values of P_H above 7·07 are alkaline.

The most accurate method of determining P_H is the electrical method depending upon conductivity determinations. For practical purposes, however, a colorimetric method has been devised depending upon the fact that a series of indicators have been found whose colours depend upon the prevailing P_H and which are sensitive to changes in P_H within certain well-defined limits.

Taking for example the commonly used indicators, the range for methyl orange is from P_H 3·1 to P_H 4·4 red to yellow.

litmus	P_H 5·4 to P_H 7·8 red to blue.
phenolphthalein	P_H 8·3 to P_H 10 colourless to red.

It will be seen from this table that owing to the fact that these indicators each have their clearly defined range of sensitiveness, it follows that one and the same liquid, such as urine, with a P_H 5 may have an alkaline reaction to methyl orange and yet be acid to litmus or phenolphthalein, and for the same reason a solution which is neutral to litmus may still be acid to phenolphthalein. This is well illustrated by the fact that many media which require to be neutralized previous to use require more alkali for neutralization if phenolphthalein is used as indicator than if litmus be employed.

Within recent years the importance of hydrogen ion concentration to the well-being and growth of plants has been more and more recognized.

In most living organisms provision is made for securing that the P_H of the medium shall not be easily disturbed; this is effected by the presence of certain salts such as the phosphates of the alkali metals or sodium bicarbonate, etc. These salts exert what is known as a buffer action in counteracting any considerable increase in P_H on the introduction into the solution of a small quantity of acid. This principle may be illustrated as follows: If a single drop of dilute hydrochloric acid is added to a quantity of distilled water, the P_H of this water, which should be 7·07, may be very considerably altered, and the same would apply if instead of pure water, a dilute

solution of sodium chloride had been used. If, however, the water had contained, in the place of the sodium chloride, an equivalent amount of sodium phosphate, the effect of the addition of the hydrochloric acid would merely have been to displace a corresponding amount of feebly ionized phosphoric acid whereby the P_H would have been hardly altered at all. This may be expressed by saying that sodium chloride has no buffer action whereas sodium phosphate and the salts of other feeble acids, such as boric, citric, and amino acids, have strong buffer action.

The blood, as a typical physiological fluid, is provided with a complex system of sodium phosphate and bicarbonate which has a most efficient buffer action preventing the fluid from having its P_H appreciably altered in the event of the sudden abnormal development of acid.

Acting upon this principle, standard solutions of known P_H are best made from suitable concentrations of salts of known marked buffer action; such solutions may be kept without fear of alteration through contamination with atmospheric carbon dioxide or alkali from the glass bottle, whereas solutions made from salts with little or no buffer action would rapidly alter and be useless.

In practice it is found convenient to keep a number of such standard buffer solutions of known P_H for the purpose of determining the P_H of a given liquid by comparison of the colours given with the same indicator. For this purpose a small quantity of the liquid under examination is treated with a few drops of the appropriate indicator and its colour is matched against that buffer solution which gives the closest approximation to its own with the same indicator. It should be noted that the indicators employed in this work are sensitive only over a certain range of P_H, say from P_H 2·8 to P_H 4·6 for bromphenol blue and from P_H 4·4 to P_H 6·0 for methyl red, and from P_H 6 to P_H 7·6 for bromthymol blue and so on; hence if no match was obtained with one indicator, the P_H of the solution lies outside that range and another indicator has to be employed until the correct one has been found and the P_H fixed with the greatest possible degree of accuracy.

With a little practice it becomes possible to detect differences of 0·1 in the value of the P_H.

For more accurate determinations, electro-metric methods are employed, for the details of which special textbooks must be consulted.

The application of these principles has not only given to biochemical problems a much greater precision but have led to the recognition of new facts in the organism itself and to a new factor in its environment. To take a few examples for illustration: Gustafson * found a gradient in the hydrogen ion concentration, varying with age, in a number of plants, the direction of which varied in different individuals of the same species, and in the same organs of different species. Thus the older leaves of *Zea*, *Phaseolus*, and *Cucurbita pepo* (squash) have a higher hydrogen ion concentration than the young leaves, whilst in *Helianthus* and *Cucurbita pepo* (pumpkin) the reverse is the case. In the stems of the maize, sunflower, and pumpkin, the hydrogen ion concentration increases from the base to the apex. In the instance of the *Vigna sinensis*, Clevenger † observed a variation in the same organ during the course of a day. With respect to environment, Salisbury ‡ found that the soil of natural woodlands shows a stratification in which a definite gradient of hydrogen ion concentration obtains, the maximum being at the surface. The surface of the soil is poorest in bases which increase in amount with increasing depth, the buffer action being greatest in the layer of maximum organic content. These points are significant in that they play a part in the distribution and density of the bacterial flora.§ Interesting and important observations have also been made by Atkins || with regard to the sea, the hydrogen ion concentration of which varies at different places in response to depth, season, and degree of carbon assimilation by the algal plankton :—

* Gustafson : " Amer. J. Bot.," 1924, **24**, 11.

† Clevenger : " Soil Sci.," 1919, **8**, 227.

‡ Salisbury ; " J. Ecol.," 1922, **9**, 220. See also Atkins : " Proc. Roy. Dublin Soc.," 1922, **16**, 369.

§ See Thornton : " Ann. Appl. Biol.," 1922, **9**, 241.

|| Atkins : " J. Marine Biol. Assoc.," 1922, **12**, 717 ; 1923-5, **13**, 93, 437.

Locality	P_H	Range
Rock pool	8·57-8·01	0·56
Shallow water	8·42-8·01	0·41
Plymouth Sound	8·29-8·01	0·28
,, Breakwater . . .	8·27-8·07	0·20
Open sea	8·27-8·14	0·13

With respect to season, Atkins finds that, in general terms, the P_H maxima may be correlated with the phytoplankton maxima in early summer and autumn.

NOTE

The Estimation of Natural Mixtures of Sugars.

WHILE going to press a paper on the estimation of the sugar in apples was published by Evans ; † the method of analysis, which is adapted to the accurate determination of sucrose, fructose, and glucose, is as follows : 20 c.c. of the juice (obtained by freezing the cut slices and expressing in a hand press) were placed in a graduated flask, almost neutralized with soda and diluted to 400 c.c. with distilled water ; 4 c.c. of basic lead acetate were then added and the mixture allowed to stand for ten minutes ; the excess of lead was thereupon removed by the addition of an 18 per cent solution of potassium oxalate and the mixture made up to 500 c.c. and subsequently filtered.‡

The estimation of *reducing sugars* was carried out upon the above filtrate, diluted to half strength, employing the method of Lane and Eynon.§

The *total sugars* were estimated by adding to 100 c.c. of the cleared solution, obtained as above, a sufficient quantity of citric acid crystals to have 10 per cent of the crystalline acid ($C_6H_8O_7 + H_2O$) present ; the solution was then boiled for ten minutes, cooled, neutralized with concentrated alkali, and made up to 200 c.c., so that the dilution was the same as that used for the determination of the reducing sugar. The reducing power of this solution was then determined and the difference between the total sugar and reducing sugar so found was taken as the value for the sucrose present, expressed in terms of invert sugar.

For the estimation of *glucose and fructose* the method of Willstätter and Schudel ‖ was employed. A quantity of sugar solution containing approximately 0·08 gram of glucose was

* From page 140. † Evans : " Ann. Bot.," 1928, **42**, 1.

‡ According to a footnote attached to the paper subsequent work has shown that this method of clearing entails a loss of sugar of the order of 3 per cent.

§ Lane and Eynon : " J. Soc. Chem. Ind.," 1923, **42**, 32.

‖ Willstätter and Schudel : " Ber. deut. chem. Gesells.," 1918, **51**, 780.

treated with 20 c.c. of 0·1 N iodine solution and 5 c.c. of 0·5 N sodium hydroxide, the temperature being kept at 5° C.; * after forty-five minutes the mixture was acidified by the addition of 5 c.c. of 2 N sulphuric acid and the excess of iodine titrated with ·05 N sodium thiosulphate.

The equation representing the change is as follows :—

$$C_6H_{12}O_6 + I_2 + 3NaOH = \underset{\text{Sodium gluconate.}}{C_5H_{11}O_5COONa} + 2H_2O + 2NaI.$$

The proportions of glucose (x) and fructose (y) can then be determined from the equations :—

$$Cx + C_1y = \text{iodine equivalent of the solution.}$$
$$Kx + K_1y = \text{reducing power of the solution.}$$

in which K and K_1 represent the grams of CuO equivalent to 1 gram of glucose and fructose respectively, at the dilution used (as obtained from Lane and Eynon's tables), and C and C_1 represent the grams of iodine equivalent to 1 gram of glucose or fructose respectively, namely 1·404 and 0·02.

The iodine titrations must be carried out on cleared uninverted solutions as citric acid vitiates the results.

* At this temperature the oxidation of fructose is small.

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