SOME RECENT RESEARCHES IN PLANT PHYSIOLOGY

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WITH 28 ILLUSTRATIONS

PREFACE

THE general aim of the book is to present to senior students and investigators the results of recent work in a few of those branches of plant physiology which are at present attracting attention. Matter already to be found in text-books has been almost entirely excluded.

In order to preserve to some extent a historical method of treatment, the date is inserted after the name of each worker, and is followed by a numeral to show to which paper reference is being made in cases where more than one were published in a year.

By such a presentation of portions of the science which are still in a state of rapid growth, it is hoped that further investigation will be stimulated. The choice of material by the author was, to a considerable degree, influenced by his familiarity with certain subjects of general interest, portions of which are being studied experimentally by the staff of the School of Botany, Trinity College, Dublin. Upon these, rather than upon other researches of equal or greater importance, he has felt qualified to write, on account of his first-hand knowledge of many of the methods employed. A small amount of hitherto unpublished work has also been included.

Throughout the book quantitative data have been

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quoted wherever obtainable, as their employment tends to counteract vagueness.

It will be noticed that many of the researches dealt with are as yet incomplete, but in a rapidly advancing science one is compelled to agree with James Stephens's Philosopher's remarks concerning his stirabout: "Finality is death. Perfection is finality. Nothing is perfect. There are lumps in it."

In conclusion, I wish to express my great indebtedness to Professor H. H. Dixon for his continuous advice and criticism throughout the preparation of the book, and for his kindness in reading the proofs. My thanks are also due to Professor Sydney Young for revising Chapter V., which deals with osmotic pressure. In addition I am under an obligation to those of my friends who gave me help by sending me copies of their papers or in other ways, and to Miss R. R. A. Colthurst for reading the proofs.

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SOME RECENT RESEARCHES IN PLANT PHYSIOLOGY

CHAPTER I

THE CARBOHYDRATES OF THE ANGIOSPERM LEAF IN RELATION TO PHOTOSYNTHESIS

THE presence of sugars and of starch in green leaves exposed to light has been established for many years, yet the relationship of the various carbohydrates to one another is as yet undecided. Sachs pointed out that starch is the first visible product of assimilation, though he did not deny that the immediate source of the starch granules was of the nature of a sugar dissolved in the chloroplast. The changes preceding starch formation, as well as those concerned in its dissolution, were the subject of the classical researches of Brown and Morris (1893). These authors give a critical summary of previous work, and as this is both readily accessible in the original, and has been incorporated in text-books, it will not be considered here at any length.

As the work of Brown and Morris laid the foundations of the accurate analysis of carbohydrate mixtures occurring in plant tissues, and has also been the starting-point for several subsequent researches, it is advisable to discuss its results in detail even though they are well known. The analytical methods employed will be treated of in a separate chapter for the sake of clearness.

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The Starch of the Leaf.

When the above-mentioned research was begun, it had
been already proved by the work of Bokorny (1891) that
starch could be produced in the chloroplasts of Spirogyra
from formaldehyde by supplying the alga with a nutrient
solution containing a compound of formaldehyde united
with sodium bisulphite, which on warming with water is
hydrolyzed to give the original components in the free
state. At air temperature the amount of free formaldehyde
in such a solution is very small, but the supply is con-
tinuous, for as the substance is removed by the plant more
is set free by hydrolysis to restore the disturbed chemical
equilibrium. By thus ingeniously avoiding poisonous con-
centrations of the aldehyde, Bokorny showed that starch
was formed by *Spirogyra majuscula* in the absence of any
other possible source. This afforded evidence in favour of
Bayer's view that plant carbohydrates are formed by
polymerization and condensation of formaldehyde. But
the intermediate steps remained quite unknown, while even
the first one could not be regarded as conclusively estab-
lished. Accordingly the problem which presented itself to
Brown and Morris was to determine what sugars were
utilized by the chloroplasts in the formation of starch, and
what the down-grade products were when starch was
brought into solution for respiration or translocation. In
addition to this they had to examine the possibility of the
formation of starch without intermediate sugar, for there
was no conclusive evidence on this point, although J. A.
Böhm (1877, 1883) and A. Meyer (1886) had shown that
starch could be elaborated by leaves in the dark and in
absence of carbon dioxide. Böhm found that the chloro-
plasts of the leaves of seedlings of *Raphanus sativus,
Lepidium sativum*, and *Phaseolus multiflorus*, which still

contained reserve materials of the seed within their other tissues, could form starch under the above-mentioned conditions. Both Meyer and Böhm demonstrated the existence of starch in leaves, which had been completely depleted of it, when they were subsequently cut into strips and floated on the surface of sugar solutions. Experiments of this type have proved that both the chloroplasts of the mesophyll and the amyloplasts of tissues devoid of chlorophyll are able to form starch from sugars. These results however, do not negative the possibility of direct starch formation, though they render it highly improbable.

Brown and Morris first of all turned their attention to the determination of the starch in the leaf, and the proportion it bears to other products of assimilation. To obtain the material for comparative experiments they adopted Sachs's half-leaf and template method, thereby obtaining similar and equal areas. In the case of *Helianthus annuus*, it was shown that the probable error of the method amounted to about 1 per cent., the leaf tissue of the right-hand halves weighing 40·53 grammes per square metre, the remaining halves giving 40·96 grammes.

As an example of the degree of accuracy of their chemical manipulations the following experiment is quoted: Working with 10 grammes of dry leaf powder, two similar analyses afforded 6·408 per cent. in one case, and 6·545 per cent. of starch in the other. The leaves were those of *Tropæolum majus*, picked after a sunny day and quickly dried. They were usually found to contain from 3·5 to 7·5 per cent. of starch calculated on the dry weight. Leaves which were kept for sixty-three hours in darkness, with their petioles in water, showed a loss in starch amounting to over two-thirds of that present at the time of picking, and are therefore rapidly depleted of their stored starch under such conditions.

Other experiments proved that during a period of eight hours' sunshine on a sunny day in August the leaf of Tropæolum stored the products of assimilation at the rate of 0·9 gramme per square metre per hour. Comparison of this result with the determinations of starch per square metre (per day of twelve hours)—viz., from 1·33 to 2·01 grammes— shows that the latter must represent only a small proportion of the material assimilated. In one case the observed increase in starch between 5 a.m. and 5 p.m. amounted to 1·40 grammes; the increase in total products of assimilation was 8·56 grammes per square metre, and in a parallel experiment on cut leaves this exceeded 12 grammes. These figures furnish strong evidence against Sachs's view that all the assimilated products pass through the form of starch. But as to the intermediate steps no evidence was adduced.

It has since been shown by Thoday (1909) that there are serious errors in Sachs's template method, owing to the alterations in size undergone by any given area when the turgor conditions of the cells change. Thoday advocates the marking out of equal areas in some sort of ink upon the leaves when under similar conditions, and considers that comparisons should be made between such well-defined portions at the end of insolation. The corrections that such modifications of the technique would occasion could not, however, alter the argument based upon the researches of Brown and Morris, though the quantitative results would be slightly different.

THE DIASTASE OF THE LEAF.

They next examined leaves for the presence of an enzyme, diastase, capable of hydrolyzing starch, and obtained evidence of its existence, though the variations in its activity from one genus or species to another were very

great. Adopting as a unit the amount of maltose, expressed in grammes, which 10 grammes of the air-dried leaf will produce from soluble starch by hydrolysis in forty-eight hours at 30° C., they compiled a table in which the relative diastatic activities varied from 240·30 for *Pisum sativum* to 0·267 for *Hydrocharis morsus-ranœ*. The latter is, however, unusually low, and this abnormality was subsequently found to be due to the marked inhibitory action of the tannin contained in the leaves. With the exception of the case of Hydrocharis, far more diastase was present in the leaves examined than was requisite to transform all the starch in them within a moderate period of time. Thus, leaves of *Pisum sativum* contain sufficient diastase to hydrolyze twenty-four times their own dry weight of starch within forty-eight hours. Comparative experiments showed that this represents about one-third the activity of an average quality of barley malt. The Leguminosæ have high diastatic activity; they also form starch readily. On the other hand, certain of the Liliaceæ form little or no starch, and are very poor in diastase. Thus there is a relation between the starch-producing and the starch-dissolving power of plant tissues. The presence of tannin is not as effective an inhibition in the living plant as in dried leaves, for during life it is retained in separate cells or portions of cells.

Further experiments showed that the diastatic activity of leaves undergoes periodic variations, increasing during darkness or when the sugar content of the leaves is small. Brown and Morris brought forward an hypothesis to account for the production of the enzyme by the protoplasm, basing it on the observed relation between diminution in sugar content and increase in diastatic activity. They regard a poor sugar-supply as a hunger stimulus, prompting the protoplasm to form or liberate diastase. It was, however,

found that the action of leaf diastase upon solid starch granules was often not very marked, especially when the starch and diastase were obtained from the same plant. Thus the first stages of the action on the starch granules seem to be dependent upon the life of the protoplasm, or the presence of an enzyme not easily extracted. To sum up, however, it seems justifiable to conclude that the diastase of the leaf is largely concerned in the dissolution of starch for the four reasons which follow: (1) Diastase occurs constantly and abundantly in leaves. (2) Its amount appears to depend upon the occurrence of starch. (3) Its increase and decrease shows a remarkable periodicity. (4) This periodicity is correlated with the appearance and disappearance of starch.

THE SUGARS OF THE LEAF, WITH SPECIAL REFERENCE TO *TROPÆOLUM MAJUS*.

IDENTIFICATION AND ESTIMATION OF LEAF SUGARS.— At the time when Brown and Morris began their researches it was known from the work of Kayser (1883), A. Meyer (1885), Schimper (1885), and Keim (1891), that reducing sugars and a non-reducing sugar, cane-sugar (sucrose or saccharose), existed in leaves. Estimations of these had been made by means of Fehling's solution, which is reduced by glucose, fructose, and maltose, but not by cane-sugar. Meyer had also shown that leaves which contain little or no starch, such as those of *Iris germanica, Allium cepa*, etc., are relatively rich in sugars when compared with starch-forming leaves.

Brown and Morris selected the leaves of *Tropæolum majus* as suitable for their object, to determine the up-grade and down-grade products of assimilation, and to ascertain the forms of sugar employed in translocation, respiration, and cell growth. In this leaf they detected only the four

sugars—glucose, d-fructose, sucrose, and maltose. Two separate analyses of the dried leaf gave the following results:

TABLE I.

PERCENTAGES OF SUGARS IN TROPÆOLUM LEAF, CALCULATED ON THE DRY WEIGHT.

	a.	b.
Sucrose	3·24	3·39
Glucose (dextrose) } d-Fructose (levulose) }	4·69	{ 2·41 } { 2·18 } 4·59
Maltose	0·76	0·61
	8·69	8·59

This table shows the nature of the accuracy to be expected in other analyses.

The presence of maltose was proved not only by the analytical details of the sugar determinations, but also by the preparation of its characteristic osazone, and by the determination of the nitrogen content of the latter. The increase in reducing power of the mixture of sugars, when treated with maltase after previous complete inversion with invertase, affords additional evidence of the existence of maltose in the leaf, for maltase has no hydrolyzing action on any carbohydrate other than maltose.

CHANGES IN CARBOHYDRATE CONTENT DUE TO INSOLATION.—The diurnal changes in the carbohydrate content may be seen from Table II., in which under a is given the analysis of leaves picked from the plant at 5 a.m.; under b, that of leaves plucked at 5 a.m., and insolated with their petioles in water till 5 p.m.; and under c that of leaves plucked at 5 p.m. The day chosen was fairly bright and sunny.

It will be noticed that the amount of starch in the cut leaves, has not increased to so great an extent as in those attached to the plant, though the sugars have increased very markedly. This result was always obtained

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It will be noticed that the amount of starch in the cut leaves, has not increased to so great an extent as in those attached to the plant, though the sugars have increased very markedly. This result was always obtained

by Brown and Morris. It is not clear why the starch should be less in the plucked insolated leaves than in those still attached, and the large amount of maltose in the attached leaves is equally hard to interpret. Perhaps it entered through the petioles. No mention is made of the possible passage of sugars from the petioles of the plucked leaves into the water in which they were standing. This loss, whatever its magnitude, would only go to increase still further the differences between *b* and *c*. The altered conditions of turgor in *b* as compared with *a* and *c* may have

TABLE II.

CARBOHYDRATES IN TROPÆOLUM LEAF.

						a.	*b.*	*c.*
Starch..	..	..	..	..	..	1·23	3·91	4·59
Sugars:								
Sucrose	..	..	..	..	..	4·65	8·85	3·86
Glucose	..	..	..	..	..	0·97	1·20	0·00
Fructose	..	..	..	..	..	2·99	6·44	0·39
Maltose	..	..	..	..	..	1·18	0·69	5·33
Total sugars per cent.			..	..		9·79	17·18	9·58

some influence on the results, through a change in the concentration of the cell sap. This effect could, however, hardly reverse the relative concentrations in *b* and *c*, for *b* is more than twice as rich as *c* in sucrose.

The great rise in the sugar content of *b* is due to the sucrose and fructose, as the maltose has diminished and the glucose remained almost constant. This the authors regard as pointing to sucrose as the principal up-grade sugar, rather than a hexose sugar as commonly believed at the time. They explain the increase in fructose as due

to hydrolysis of sucrose, the glucose produced by that reaction having been largely used in respiration. To test this latter point they record two sets of analyses of leaves picked after a bright warm day—*a*, dried at once; and *b*, kept in the dark for twenty-four hours with petioles in water. One of these tables is quoted below:

TABLE III.
CARBOHYDRATES IN TROPÆOLUM LEAF.

	a.	*b.*
Starch	5·425	0·906
Sugars:		
Sucrose	7·33	3·35
Glucose	0·00	1·34
Fructose	2·11	3·76
Maltose	2·71	1·28
Total sugars per cent.	12·15	9·73

Total loss of sugars and starch in *b* = 6·93 per cent.

The great loss in carbohydrates experienced by the leaves kept in the dark is accounted for as due to the respiration of the cells. In addition the relative amounts of the carbohydrates have altered considerably. The loss has fallen chiefly upon the starch, though sucrose and maltose have sunk to less than half their original quantities. On the other hand, both glucose and fructose have increased. Thus, during darkness starch is hydrolyzed to maltose, and finally to glucose, which, together with glucose and fructose obtained by the inversion of sucrose, is used for respiration· The accumulation of fructose in excess of glucose points to the latter as being respired or used for tissue formation more readily than the former.

Previous work by these authors (1890) had shown that sucrose far surpasses all other carbohydrates in bringing about starch formation, when it is used as a nutrient for the

cultivation of barley embryos.   This pointed to sucrose as
an antecedent of the formation of starch, and the experi-
ments on Tropæolum strengthen the theory.   Thus, Brown
and Morris regard sucrose as the first sugar to be formed
in the green leaf as a product of assimilation.   Its removal
is effected either by its conversion into starch as a more
stable reserve substance, or by its inversion to give the
easily respirable hexoses.

It may here be pointed out that this transformation of
sucrose into starch sets a major limit to the osmotic pressure
obtainable in the leaf cells; for whereas sucrose is a crystal-
loid, starch is a colloid, and exerts a negligible osmotic
pressure.   Measurements of the changes in pressure caused
by the insolation of plucked leaves were made by Dixon
and Atkins (1910) by determining the freezing-point of the
expressed sap by the thermo-electric method.   The follow-
ing table is typical of the results obtained.   Under Δ are
recorded the depressions of freezing-point in degrees, under
P the osmotic pressures calculated from Δ, while under M
are tabulated the mean molecular weights of the sap
solutes.

TABLE IV.

Syringa vulgaris : LEAVES.

Description of Sample.	Δ.	P.	M.	Water.
(a) Gathered and pressed im- mediately	1·423	17·12	202	Per Cent. 69·16
(b) Gathered, insolated for 1½ hours in a breezy posi- tion, with petioles in water, then pressed ..	2·135	25·68	202	63·50
(c) Gathered and kept in dark for two days, with petioles in water, then pressed..	1·183	14·23	211	—

Here the rise, owing to insolation, is very marked—viz., from 17·12 to 25·68 atmospheres. Some of this must be attributed to concentration by loss of water, as at the end of the experiment a few of the leaves had begun to wilt. The last column, however, shows that this concentration can only account for a small portion of the observed rise, from 17·12 to 18·66 atmospheres. It is interesting to see that the mean molecular weight of the sap solutes hardly alters at all, for sucrose and maltose have identical molecular weights, 342, whilst that of glucose and fructose is 180. Other constituents of the sap lower the mean, as values far below those of the sugars are frequently obtained. The diminution of osmotic pressure is not, of course, a measure of the total consumption of carbohydrates during darkness, for hydrolysis of starch keeps up the supply of maltose. From the table of sugar analyses just quoted it may be calculated that the ratio of the number of gramme molecules of the sugars present before and after twenty-four hours in the dark is 0·0411 to 0·0419 respectively. This would, of course, produce a rise of pressure, and would quite parallel a similar experiment by Dixon and Atkins in which the osmotic pressures before and after twenty-four hours' darkness were 14·61 and 14·76 atmospheres. In another table given by Brown and Morris the alteration in molecular concentration of sugars is from 0·0436 to 0·0381 after twenty-four hours, which accordingly causes a fall in osmotic pressure. Table V. which follows further illustrates these changes. The leaves were picked, and each leaf was halved. The halves were then employed in the comparative experiments.

The most striking result is that the osmotic pressure in the control has not varied at all, though from the work of Brown and Morris it is certain that a complicated series of changes has been taking place in the leaf sugars. The

small increase in mean molecular weight in the last two experiments is probably due to the production of maltose, in the unfiltered sap and intact leaves, at a rate sufficient to mask the decrease due to inversion of sucrose and respiration of the hexoses. The fall in pressure of the unfiltered as compared with the filtered sap is explicable by the action of respiratory enzymes. Such comparisons between the analyses of sugar content on the one hand, and determinations of osmotic pressure and mean molecular weight on the other, serve to impress one with the delicate balance

TABLE V.

Syringa vulgaris : LEAVES.

Description of Sample.	Δ.	P.	M.
(a) Half-leaves:			
(1) Sap pressed and examined immediately	1·424	17·13	211
(2) Sap pressed and filtered, kept, and examined next day ..	1·540	18·52	210
(3) Sap pressed, not filtered, kept, and examined next day ..	1·505	18·10	216
(b) Half-leaves kept in dark, examined next day	1·424	17·13	217

maintained by the living cells of the leaf throughout the cycle of chemical transformations taking place from day to day.

IDENTIFICATION OF SUGARS BY MEANS OF PHENYL-HYDRAZINE AND ITS DERIVATIVES.

The identification of sugars in plant tissues was later studied by Senft (1904), who employed phenylhydrazine with aqueous acetic acid. As is well known, glucose and fructose afford the same phenylosazone, which crystallizes

out even from hot solutions in characteristic clusters and sheaves of yellow needles. The monosaccharides yield these crystals in the cold, the disaccharide sucrose only after inversion by prolonged heating of this acetic acid reagent. Maltose, on the other hand, gives a phenylosazone which crystallizes in very minute rosettes of needles, or narrow plates, which are soluble in hot water. The reaction with maltose necessitates heating on a water-bath for an hour and a half. If a large excess of sodium acetate be added to the solution it may be boiled without fear of inverting the sucrose, and so the time required for obtaining the glucosazone is greatly diminished. This affords a method for the microchemical detection of sugars. Grafe (1905) further enlarged the scope of this method by the introduction of secondary asymmetrical methylphenylhydrazine $\begin{matrix} C_6H_5 \\ \diagdown \\ \diagup \\ CH_3 \end{matrix} N - NH_2$, which gives osazones only with ketonic sugars after five hours at room temperature. For examination of microscopic sections of tissues the reagents may conveniently be made up with glycerine instead of water, as the sugars are less soluble in the former, and it does not evaporate so readily. In this manner Mangham (1911) proved the presence of maltose in sieve tubes of the bast of certain angiosperms. [See also Mangham (1915).]

THE DISTRIBUTION OF SUGARS AND SUCROCLASTIC ENZYMES IN THE BEET.

Making use of the microchemical method just described, Strakosch (1907) studied the distribution of sugars in the sugar beet, *Beta vulgaris*. He found that glucose is contained in the cells of the mesophyll, and that it is the only sugar present. Diffusion of glucose into the veins is followed by the appearance of fructose in them. Sucrose is

then formed there from the two hexoses. Furthermore, when leaves have been kept in the dark till depleted of all their starch, on exposure to light it is again formed in the chloroplasts before sucrose appears. Maltose occurs only in the leaf petiole, along with glucose, fructose, and sucrose. The latter is the most important, and travels to the root, where it is stored. The transformation of the hexose sugars into sucrose takes place in the light, and ceases when the leaf is placed in the dark. This, it may be remarked, is probably rather an effect of the cessation of the production and accumulation of glucose than of the action of light as a protoplasmic stimulus.

The above conclusions as to glucose being the first sugar of photosynthesis are confirmed by a few quantitative estimations of the sugars. An extract of the mesophyll, from which even the finest veins had been excluded, was found to contain 0·154 per cent. of dextrose, but only 0·025 per cent. of sucrose, the latter probably being derived from very fine veins which had been overlooked. On the other hand, an extract of the veins with some of the mesophyll lying between them contained 0·118 per cent. of glucose and fructose combined together with 0·538 per cent. of sucrose. Thus, the results obtained by Strakosch are quite opposed to the conclusion of Brown and Morris, that sucrose is the first sugar of photosynthesis.

The glucose theory received further support from the researches of Robertson, Irvine, and Dobson (1909), on the sucroclastic enzymes of *Beta vulgaris*, from whose paper Table VI. is quoted.

It is noteworthy that invertase is absent from the beet-root, in which sucrose is stored. Kastle and Clark (1903), however, demonstrated its presence in the potato and artichoke, where starch and inulin respectively form the major part of the carbohydrate reserves. Diastase and

inulase were also found in these storage organs, in presence of their respective substrates, but in smaller quantities than was invertase.

TABLE VI.

ENZYMES OF *Beta vulgaris.*

					Leaf.	Stem.	Root.
Invertase	..	..	..	..	+	+	0
Diastase	..	..	..	..	+	+	+
Maltase	..	..	..	..	+	0	+
Inulase	..	..	..	..	0	+	+
Emulsin	..	..	..	..	0	+	+

Robertson, Irvine, and Dobson, obtained from 4 to 6 per cent. of sucrose by treating invert sugar with a turbid liquid or sludge prepared by straining leaves macerated with water through fine muslin and precipitating with alcohol. The change was studied with the polarimeter, and after inversion with acid the original rotatory power was recovered; thus there can be little doubt that sucrose was produced. Hudson (1914) has, however, shown that pure invertase has absolutely no synthetic action.

From the results of his analyses of beet leaves picked before sunrise and late in the afternoon, Girard (1883, 1884) concluded that sucrose was the principal sugar to be directly formed in sunlight, and that it alone was stored in the root. From his table quoted below it may be seen that, while the hexoses remain fairly constant, sucrose increases to a marked degree upon insolation.

It may be mentioned that in a recent paper Colin (1914) controverts Girard's statement that sucrose only is found in the beetroot, for he has found in it a small quantity of a reducing sugar, which decreases in amount as the plant grows older. It may possibly be that in some varieties of

beet there is no reducing sugar in the root, whilst in others such a sugar is present. This would be comparable to the occurrence of maize grains with and without sugar. The striking results obtained by Pearl and Bartlett (1911) upon the segregation of such qualities as starch content, moisture content, sugar content, ash content, nitrogen content, fat content, etc., according to Mendelian laws of heredity, are of great interest in this connection. Colin also found that at the base of the petiole of the beet the ratio of reducing sugar to sucrose was greater than unity, and he concludes that the root receives both types of sugar, though only sucrose is stored in quantity. Ruhland (1912) had previously noticed this, and pointed out that in the beet sugar is translocated mainly as invert sugar.

Girard heads his last column, " To 100 of Glucose "— viz., hexoses—not being aware of the possibility of the existence of maltose in the leaf when starch is being dissolved during the night. The maltose present would tend to make the value given for the reducing sugars too low, for they were calculated as hexoses; and also to lower the true value of the ratio of sucrose to hexoses, for the latter now appear higher in amount owing to the erroneous inclusion of maltose. These errors are, however, not of great magnitude, for Strakosch has detected maltose only in the petiole, though, as starch exists in small quantity in the leaf, maltose must be present also; the presence of maltase and diastase point to its existence there too.

The results of Table VII. do not necessarily bear the interpretation given to them by Girard, but fit in quite as well with that of Strakosch. For it is obvious that, once a certain concentration of glucose is reached, it will diffuse into the veins, and may there result in an equilibrium mixture of glucose and fructose. Combination of these hexoses to form sucrose, and the translocation of the latter to the

TABLE VII.

Analyses of Beet Leaves.

Date and Hour.		Water.	Sucrose.	Reducing Sugars.	Other Organic Substances.	Mineral Matter.	Ratio of Sucrose to 100 of Reducing Sugars.
Aug. 18-19	4 p.m.	88·07	0·92	1·54	6·57	2·90	60
	4 a.m.	88·96	0·35	1·14	6·62	2·89	30
„ 20-21	4 p.m.	87·44	0·42	0·95	7·85	3·34	45
	4 a.m.	88·06	0·26	0·98	7·42	3·28	26
„ 22-23	4 p.m.	87·86	0·40	1·60	7·40	2·71	25
	4 a.m.	88·35	0·18	1·14	7·35	2·98	16
„ 25-26	4 p.m.	87·65	0·49	1·83	7·14	2·89	27
	4 a.m.	88·88	0·22	1·55	6·27	3·08	14
„ 27-28	4 p.m.	88·53	0·42	1·86	6·67	2·52	22
	4 a.m.	87·74	0·19	1·76	7·15	2·56	10
„ 29-30	4 p.m.	87·47	0·72	1·40	7·43	2·98	51
	4 a.m.	88·08	0·48	1·55	7·03	2·86	31
Sept. 1-2	4 p.m.	88·30	0·65	2·07	6·37	2·61	31
	4 a.m.	88·47	0·23	1·84	6·70	2·76	12
„ 3-4	4 p.m.	87·50	0·32	2·22	7·31	2·65	14
	4 a.m.	87·95	0·27	2·04	7·17	2·57	13
„ 5-6	4 p.m.	87·37	0·71	2·26	6·87	2·79	31
	4 a.m.	87·63	0·51	2·91	6·11	2·84	17

root, sets a limit to the osmotic pressures attainable by the cells of the leaf parenchyma.

Maquenne (1895) investigated the causes of the passage of sucrose from leaf to stem in the beet, and found that, by pressing sap from each organ and determining its freezing-point, values were obtained for the osmotic pressures in which those of the leaf were usually a little lower than those of the root. His table of results shows, moreover, how closely osmotic equilibrium is maintained in this plant. It must be noted, however, that his figures are only relative, not absolute, for it has been pointed out by Dixon and Atkins (1913, 1) that, unless the protoplasmic membranes of cells are rendered permeable by some method, treatment with liquid air being the most reliable, the concentration of the expressed sap varies with the degree and duration of pressure. In contradistinction to Maquenne these authors found that as a rule the osmotic pressure obtaining in the cells of leaves is greater than that in the roots. Maquenne gives it as a general rule that any substance formed in a cell may accumulate when its formation occasions a fall in osmotic pressure.

TABLE VIII.

Date.		Δ.	P.	M.
March, 1910	*Beta vulgaris*, root ..	$\begin{cases} 1\cdot082 \\ 1\cdot090 \\ 1\cdot041 \end{cases}$	13·01 13·11 12·52	214 215 202

Maquenne's determinations were made, presumably, in the height of summer. The above measurements by the author (1910) agree closely with them. The point of special interest is the low value for the mean molecular

weight, M, of the sap solutes. This shows that the total osmotic pressure can only be due in part to sucrose, though it is the only sugar present normally in the mature root in any considerable quantity.

These measurements were made without previous treatment of the roots with liquid air. Accordingly they are somewhat too low, as previously pointed out. Inspection of Table 9 makes this point clear. In it is also recorded the electrical conductivity of the sap, expressed as reciprocals of the conductivity in ohms. Under Δ_e is given the depression of freezing-point occasioned by the electrolytes; this is calculated from the conductivity C, as explained in a subsequent chapter. $\Delta - \Delta_e$ therefore represents very approximately the depression produced by the non-electrolytes, which consist almost exclusively of sucrose in this case.

TABLE IX.

Date.		Δ.	Δ_e.	$\Delta - \Delta_e$.	P.	$C \times 10^5$.
Dec., 1912	*Beta vulgaris*, root: Pressed untreated	1·473°	0·285°	1·188°	17·72	570
	Pressed after freezing in liquid air	1·761°	0·277°	1·484°	21·18	555

Since a 1 per cent. solution of sucrose* lowers the freezing-point of water 0·054°, it may be seen that a depression of 1·484° due to non-electrolytes points to the existence of slightly over 27 per cent. of sucrose in the sap. The difference of under 3 per cent. in the electrical conductivity in favour of the untreated tissue may be put down to a local difference, or it may be partly due to the

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* The depression produced by a 1 per cent. solution of glucose or fructose amounts to 0·106°.

greater viscosity of the solution richer in sucrose tending to give too low a value for the conductivity.   However, even if the conductivity were as high as 800×10^{-5} mhos, a very high value for a root, the calculation would still show over 25 per cent. of sucrose.   This result has been arrived at on the assumption that even for such considerable depressions the simple relationship of molecular concentration to depression still holds.   This, of course, is not strictly accurate.

THE CARBOHYDRATES OF THE SNOWDROP.

In 1911 appeared the final results of Parkin's work on the carbohydrates of the foliage leaf of the snowdrop (*Galanthus nivalis*).   This plant and some other monocotyledons are peculiar, inasmuch as they store no starch in the leaf, except a negligible amount in the guard cells of the stomata.   Accordingly, as starch is not present, it is to be expected from the results of Brown and Morris that maltose will be absent.   This surmise was completely borne out, and it was ascertained that in this leaf no carbohydrates were present other than glucose, *d*-fructose, and sucrose.   The problem of the origin of the first carbohydrate of photosynthesis may thus be very directly attacked.

Preliminary analyses made it clear that the snowdrop leaf is very rich in sugars, which amount to from 20 to 30 per cent. of the weight of the dried leaf, or from 4 to 6 per cent. of the fresh leaf.   The total carbohydrate content of the Tropæolum leaf, including starch, is from 14 to 18 per cent. of the dry weight.   Thus there is a large balance in favour of Galanthus.   It was also found that in any leaf the amount of sugar increases from above downwards, and at the same time the ratio of sucrose to the hexoses diminishes.   This may be seen from Table X. subjoined.

In leaves picked from plants growing in clumps the

hexoses are usually more abundant than the sucrose, but the change in the ratio from above downwards remains much the same. This excess of hexoses in leaves not brightly illuminated may perhaps be taken as supporting Strakosch's view that transformation of hexoses to sucrose takes place only in light. As before pointed out, the present writer is of the opinion that this conclusion is

<div align="center">TABLE X.</div>

<div align="center">LEAVES OF SNOWDROPS GROWING IN OPEN ORDER.</div>

Portion of Leaf taken.	Total Sugar, in Grammes.	Ratio of Sucrose to Hexose.	Date and Time of Picking.
Experiment 1: Upper halves	22·93	1:0·57 ⎫	April 11, 1906,
Lower ,,	24·46	1:0·6 ⎭	7 p.m.
Experiment 2: Upper halves	19·26	1:0·5 ⎫	April 12, 1907,
Lower ,,	21·40	1:0·75 ⎭	noon.
Experiment 3: Upper halves	24·03	1:0·65 ⎫	March 30, 1910,
Lower ,,	24·36	1:0·82 ⎭	2.30 p.m.

not justified by the experimental data, for the accumulation of dextrose seems a more immediate cause of the condensation to sucrose.

THE RATIO OF SUCROSE TO HEXOSES.

Another result of great interest was established by Parkin —namely, that the proportion of sucrose to hexose decreases as the season advances. This Table XI. makes very clear. The ratio recorded is calculated, taking the amount of sucrose as unity.

The ratios cannot be correlated with the daily temperatures, and no very satisfactory explanation is forthcoming. Girard's work on the beet leaf shows that in it hexoses are always largely in excess of sucrose. His analyses were made in August and September.

TABLE XI.

SUGARS IN SNOWDROP LEAVES.

Date and Hour.	Maximum Shade Temperature.	In 100 Grammes Dry Leaf.			Ratio of Sucrose to Hexoses.
		Sucrose.	Hexoses.	Total Sugars.	
	Degrees F.	Grammes.	Grammes.	Grammes.	
Feb. 16, 1906, 3 p.m. ..	48·9	19·80	3·56	23·36	1:0·2
„ 26, 1907, 4-5 p.m. ..	45·0	15·07	2·53	17·60	1:0·2
Mar. 7, 1906, 4 p.m. ..	66·9	14·55	5·69	20·24	1:0·4
„ 30, 1905, 5-6 p.m. ..	49·6	15·50	11·40	26·90	1:0·7
April 5, 1906, 4-4.30 p.m.	60·0	14·64	11·17	25·81	1:0·8
„ 5, 1907, 4-4.30 p.m.	58·0	14·64	11·61	26·25	1:0·8
„ 24, 1905, 4-4.30 p.m.	51·0	14·84	17·29	32·13	1:1·2
May 4, 1905, 3-3.30 p.m.	53·0	10·30	12·78	22·08	1:1·2

One as yet unnoticed deduction from the above table is that it points to a progressive increase in osmotic pressure as the season advances even though the total percentage of sugars may remain unchanged.   This follows because for a given weight of sugar a hexose exerts nearly twice as great a pressure as sucrose, owing to its smaller molecular weight. The assumption is here made that the sugars are dissolved in equal weights of water, which is approximately true, for the leaf cells are always almost fully distended.   Part of the increase in osmotic pressure with the age of the leaf, attributed by Dixon and Atkins (1912, 2 and 3) to the accumulation of salts, may possibly be due to this change in the nature of the stored sugars.

DIURNAL CHANGES IN THE SUGARS OF THE SNOWDROP.

The analyses of beet leaves by Girard previously quoted are quite paralleled by those obtained by Parkin for the snowdrop, for he finds that during any single day of the spring the percentage of hexose sugars in the leaf remains fairly constant, no matter what hour out of the twenty-four the leaves may be examined.   The sucrose, on the other hand, fluctuates greatly.   It increases during the day and diminishes during the night.   Further, leaves detached and insolated contain decidedly more sucrose than their controls.   Parkin performed comparative sets of experiments on the following:

(a) Leaves picked in the morning with those gathered in the afternoon.

(b) Leaves picked in the evening with those gathered the following morning.

(c) Leaves detached from the plants with those attached, both being kept in the dark overnight.

(d) Leaves detached and insolated with those left attached

The following typical experiments, quoted from a large number recorded by Parkin, formed the data from which the previously quoted conclusions were drawn:

(a) April 8, Carlisle: Maximum shade temperature, 58° F.; minimum temperature previous night, 32° F.

TABLE XII.

					8.15 a.m.	4.15 p.m.
Sucrose	..	..	..	..	8·88	12·92
Hexose	..	..	..	..	9·40	10·74
	Total sugar		..	..	18·28	23·66

The total increase is nearly $5\frac{1}{2}$ grammes, very largely due to sucrose.

(b) April 5 and 6, Carlisle: Maximum shade temperature, 58° F.; minimum during the night, 39° F.; maximum shade temperature on 6th, 52° F.

TABLE XIII.

					4.30 p.m.	Noon Next Day.
Sucrose	..	..	..	..	14·65	7·80
Hexose	..	..	..	..	13·66	13·29
	Total sugar		..	..	28·31	21·09

The plants were covered, when the first lot of leaves was gathered from them, and kept in darkness till the time of taking the second batch the following noon; thus no assimilation could take place.

Nearly 7 grammes of sugar have disappeared, due solely to reduction in the sucrose. The hexose has remained almost constant. The conditions were favourable for the translocation of sugar.

The following experiment gives an indication of the amount of sugar disappearing from the leaf otherwise than by translocation. The plants were in a darkened frame overnight.

(c) March 7 and 8, Cambridge:

TABLE XIV.

Sugars in Snowdrop Leaves, as Percentages of Dry Weight.

	4 p.m., 7th.	9.30 a.m., 8th.	
		Attached Leaves.	Detached Leaves, with Petioles in Water.
Sucrose	14·65	9·64	12·51
Hexose	5·48	5·67	5·65
Total sugar	20·13	15·31	18·16

Here the hexose is again practically constant, while the sucrose has diminished by nearly 5 grammes in the attached leaf, and by 2 grammes in the detached. Thus 3 grammes has certainly been lost by translocation, leaving 2 grammes to be accounted for by respiration and diffusion from the cut ends of the petioles.

(d) April 5, Carlisle: Maximum shade temperature, 58° F.; sunny morning and dull afternoon. Insolation after cutting of leaves, from 9.30 a.m. to 4.30 p.m.

TABLE XV.

	Attached Leaves.	Detached Leaves.
Sucrose	14·65	20·42
Hexose	11·66	10·50
Total sugar	26·31	30·92

In this series the plucked leaves were insolated while standing in dishes containing water placed beside the control plants. The leaves were spaced so as to permit of good illumination, and were maintained in an upright position. The object of the experiment was to ascertain to what extent sugar accumulated in the leaf when translocation was prevented, and to see which sugar was most affected.

It may be seen that the accumulation of sucrose owing to prevention of translocation is considerable, and that the hexose has fallen somewhat.

Another important conclusion reached by Parkin is that as a rule *d*-fructose is in excess of glucose in the leaf of Galanthus. Thus, out of fifty-two duplicate analyses the fructose and glucose were present respectively in the ratio of from 1 : 0·4 to 1 : 0·76 in forty-seven, whilst in the remaining five the ratio was from 1 : 1·01 to 1 : 1·06. From the details of the separate estimation of each hexose, which he did not publish, he concludes that the proportion of fructose to glucose tends to rise during the night, when naturally photosynthesis is in abeyance, and also to increase from above downwards in the leaf, being especially large in the colourless basal portion. This excess of fructose over glucose agrees with the results of Brown and Morris obtained with Tropæolum. These authors consider that glucose is more readily respired than is fructose. That glucose should be less plentiful than fructose is hard to account for, according to the views of Strakosch, though on the assumption made by Brown and Morris, that sucrose gives rise to both by inversion, and that glucose is used up more readily, the experimental results are easily explicable. Presumably the excess of fructose goes to form the inulin which is stored in the bulb. On the other hand, the increase in the proportion of fructose from above downwards is quite in accordance with the work of Strakosch, as this change is supposed to take place in the veins.

THE EFFECTS OF PROLONGED DARKNESS UPON THE CARBOHYDRATES OF LEAVES.

Further experiments showed that prolonged darkening of the leaves never results in anything approaching complete exhaustion of the sugars, as the supply is evidently made

good by translocation upwards. The depletion never amounted to more than two-thirds of the original quantity of sugar present. The results of Dixon and Atkins (1910) on the osmotic pressures of leaves kept in darkness for many days are quite in accord with Parkin's sugar analyses, as may be seen from their measurements subjoined:

TABLE XVI.

Syringa vulgaris : LEAVES.

Date of Gathering.	Description of Sample.	Δ.	P.	M.	Water.
Sept. 17	(a) Covered 2 days, gathered sunny morning	1·798	21·63	244	Per Cent. 59·9
	(b) Exposed control. Sunshine on previous days, 5·4 and 7·5 hours	2·043	24·57	259	61·0
„ 22	(a) Covered 7 days, gathered dull morning	1·328	15·97	253	—
	(b) Exposed control. Sunshine on previous days, 6·5, 3·4, 7·4, and 0·7 hours	1·589	19·12	234	—
„ 30	(a) Covered 12 days, gathered bright morning	1·010	12·15	156	—
	(b) Exposed control. Previous two days wet	1·608	19·34	250	—
Oct. 9	(a) Covered 21 days, gathered bright morning	0·963	11·58	249	—
	(b) Exposed control. Sunshine on previous days, 1·9, 4·4, 5·5, 7·3, 3·8, and 5·2 hours	1·505	18·10	256	—

Here translocation, and in the earlier stages the production of maltose, made good the sugars lost by respiration. The low values for the darkened leaves gathered on September 30 may be explained as due to the failure of the other leaves to supply their deficiencies, owing to conditions unfavourable to photosynthesis having prevailed. Thus

the osmotic pressure falls, and also the mean molecular weight, since the percentage of sugars in the sap solutes has diminished. That the exposed control has normal values is probably due to rapid assimilation during the early morning.

SUMMARY OF RESULTS OF RESEARCHES ON THE SNOWDROP.

On the whole, Parkin's work confirms that of Brown and Morris in indicating sucrose as the first sugar to be formed in photosynthesis. It must, however, be pointed out that mere accumulation of sucrose is not in itself direct evidence, as this may be equally well explained according to the views put forward by Strakosch. With his work, that of Parkin on the constancy of the hexoses is in good agreement. To the present writer the excess of fructose over glucose, and the almost entire absence of the latter sugar at times of rapid photosynthesis, are more telling facts in favour of the sucrose theory. The experiments of both Strakosch and Parkin point to sucrose as the sugar which is chiefly concerned in translocation downwards, whereas Brown and Morris believe that before translocation the sucrose is inverted.

THE DIURNAL CARBOHYDRATE CYCLE OF THE MANGOLD.

The first investigation on the cycle of changes taking place daily in the foliage leaf was that of Campbell (1912), carried out in 1910, though not published till over a year later. He analyzed the carbohydrates of the mangold (*Beta maritima*) leaf. The methods employed for this purpose were such as to give comparable results, though they were not rigorously accurate. One possible source of error, affecting all the results, appears to have been overlooked. This occurred in the concentration of the extract

from 50 grammes of chopped-up leaves, which was evaporated down to about 20 c.c. The great liability to hydrolysis of sucrose under these conditions has been pointed out by C. A. Browne (1912). This error would vary with the time occupied by each extraction, and would lower the sucrose content and raise that of the hexoses. Bearing in mind this possible source of a fluctuating error, it remains to consider the results obtained, which are of great interest.

Leaves were collected every two hours of the twenty-four in the middle of September. The quantities of carbohydrates found are recorded in Tables XVII. and XVIII., from which the graphs have been constructed.

From the tables and graphs it is seen that the hexoses do not appear to fluctuate very much, but there is an approximately constant percentage throughout the day, and a somewhat lower percentage during the night. The curve rises suddenly between 4 a.m. and 6 a.m. (sunrise being at 5·30 a.m.), and falls between 6 p.m. and 8 p.m. This points to a quick response of the leaf towards light, shown by hexose formation. The constant level at night seems to indicate that the hexoses are not largely concerned in translocation.

The sucrose varies from about 0·5 to 2·5 per cent. The rise begins about sunrise, and continues till sunset, when there is a rapid fall. The curve, however, lags behind that of the hexoses by about an hour.

It is also found that the starch varies much in the same way as sucrose, though its curve lags slightly behind that of the latter in the morning, and continues to rise till midnight. As to its formation during darkness two views must be considered—that it arises from (a) the hexoses, thus producing the fall to their night level, and from the hexoses originating from inversion of the sucrose; or (b) the sucrose present at nightfall, together with

TABLE XVII.

CARBOHYDRATES IN MANGOLD LEAF. PERCENTAGES AT TWO-HOUR INTERVALS, CALCULATED ON DRY WEIGHT OF LEAF.

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

TABLE XVIII.

CARBOHYDRATES IN MANGOLD LEAF, RECALCULATED AS PERCENTAGES OF CARBON.

	6 p.m.	8 p.m.	10 p.m.	Mid-night.	2 a.m.	4 a.m.	6 a.m.	8 a.m.	10 a.m.	Noon.	2 p.m.	4 p.m.
Hexoses	1·87	1·52	1·59	1·57	1·52	1·03	1·88	1·77	1·37	1·73	1·43	1·86
Sucrose	0·92	0·61	0·52	0·62	0·23	0·19	0·33	0·26	0·72	0·74	0·81	0·92
Maltose	0·20	0·19	0·33	0·66	0·93	1·01	0·57	0·55	0·39	0·37	0·40	0·22
Starch	3·82	3·98	4·14	4·41	3·26	2·86	2·36	2·16	2·62	3·02	3·85	4·11
Total	6·81	6·30	6·78	7·26	5·94	5·09	5·14	4·74	5·10	5·86	6·49	7·11
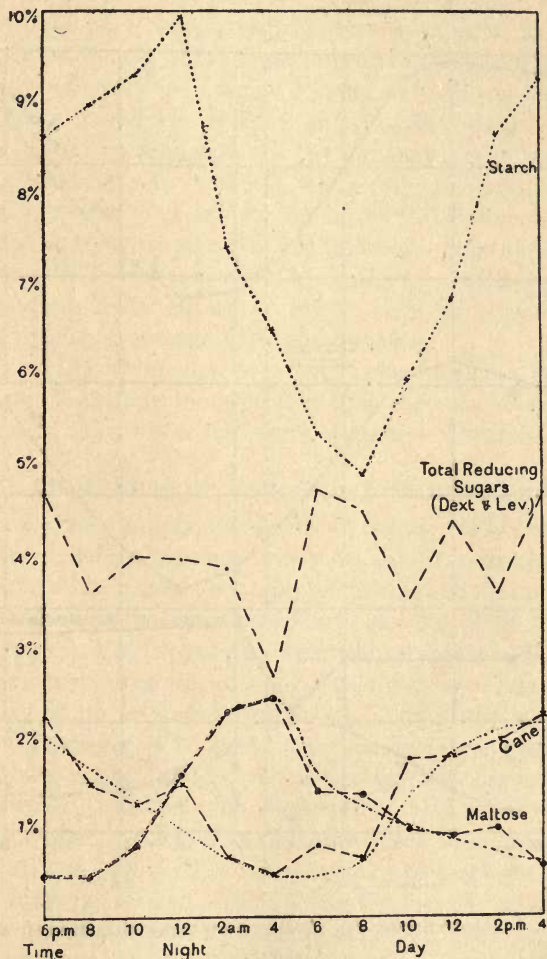
FIG. 1.—CARBOHYDRATES IN MANGOLD LEAF: PERCENTAGES AT TWO-HOUR INTERVALS.
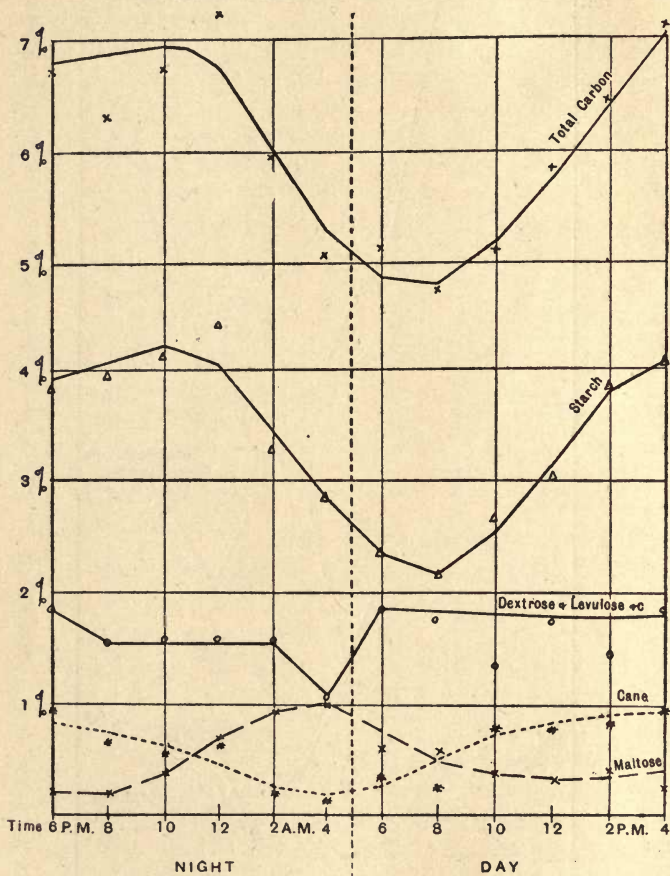
FIG. 2.—CARBOHYDRATES IN MANGOLD LEAF CALCULATED AS
CARBON.

that formed from the hexoses in falling to their night level. These two views are diametrically opposite to each other, but it is clear that the first involves the direct transformation of some hexose into starch, and regards sucrose either as a temporary reserve or transport sugar. The second looks upon sucrose as the primary sugar on the direct road to starch, or, if not the primary, at any rate as an indispensable prerequisite of starch formation. The former view is practically that of Strakosch. The apparent increase in the total carbon after darkness seems to be due to the material having an unusually high starch content through want of uniformity in the samples.

The behaviour of maltose, which increases as starch decreases, is entirely in agreement with the view of Brown and Morris, that it is a down-grade product of starch.

REVIEW OF RESULTS OF RESEARCHES ON THE MANGOLD.

On the whole Campbell's researches indicate that hexoses are formed before sucrose, and soon attain a maximum. Then sucrose increases, and, after reaching a certain limiting value, begins to be transformed into starch. This agrees with Strakosch's theory in regarding a hexose as the primary sugar of photosynthesis, but regards sucrose as a necessary stage in starch formation. The latter appears to the present writer to be an unwarranted conclusion, as the limiting concentration attained to by the sucrose may be readily explained as due to its rapid translocation. The ease with which darkened leaves form starch when supplied with nutrient solutions of various sugars suggests that starch may be formed directly from different sugars in different species of plants. It has, however, been emphasized by Lundegård (1914) that the system Sugar ⇌ Starch is not a simple reversible reaction governed by the laws of mass action; for increase of the sugar content of the

vacuoles brought about by plasmolysis does not necessarily lead to an increase in starch, and may even lead to its disappearance. The regulation of the process appears rather to be one of a very complicated nature depending upon the amount of sugar and synthetic enzyme in the cytoplasm, and upon the quantity of such enzyme secreted by the latter under the influence of stimuli of which we are as yet totally ignorant.

The researches of Campbell on the mangold are being continued and extended by Davis and Daish (1913 and 1914), who have as yet only published details of their preliminary work on an exhaustive examination of the many sources of error in such analyses.*

SUMMARY OF EVIDENCE AS TO THE FIRST SUGAR OF PHOTOSYNTHESIS.

It has been established that leaves supplied with glucose, fructose, galactose, sucrose, maltose, or lactose, all form starch, but those supplied with sucrose produce starch somewhat more readily than do those which are placed in solutions of the other sugars. Thus, unless the protoplasm or enzymes bring about very rapid isomeric changes and condensations resulting in the appearance of sucrose from each of the other sugars, there is reason to believe that starch may be built up from many different sugars.

Maltose is universally regarded as a down-grade product, arising from starch and giving rise to glucose.

Of the hexoses, fructose and glucose, it appears that the latter is more readily respired than is the former, and so in the absence of any source of either the ratio of glucose to fructose decreases.

Sucrose on hydrolysis by invertase yields d-fructose and

* Mr. Daish has since been wounded in Flanders.

glucose, and there is a certain amount of evidence in favour of the view that this process is reversible, and that sucrose may be formed in the plant from glucose, with the isomeric rearrangement of glucose to give d-fructose as an intermediate step.   It has, however, been shown by Hudson (1914) that pure invertase has no synthetic action.

Whether glucose or sucrose is the first sugar to be formed in photosynthesis must be regarded as an open question for the present.   Furthermore, it has never been proved that the behaviour of all plants is similar in this respect.

The presence of both hexoses and sucrose in the choloplasts of the leaf of *Elodea canadensis* has recently been demonstrated by Dixon and Mason.

CHAPTER II

METHODS OF ESTIMATING CARBOHYDRATES IN PLANT EXTRACTS

In forming an opinion of the value attaching to the results of carbohydrate analyses, due regard must be paid to the accuracy of the methods employed in obtaining them. It is with the object of doing this, as well as because the methods themselves are frequently of biological interest, that the present chapter has been inserted.*

Preparation of Material and Analysis according to Brown and Morris.

The estimations may be based upon the fresh or dry weight of tissue taken. In either case it is necessary to extract with a solvent. The particular method of extraction employed is of great importance, since the carbohydrates are readily attacked both by the naturally occurring tissue enzymes and by acids.

Confining the problem to the analyses of mixtures of starch, maltose, sucrose, glucose, and fructose, it is obvious that the first-mentioned can readily be separated, so that extracts contain only the sugars.

Brown and Morris (1893) tried to make use of juice expressed from the leaves, but, finding that there was a difficulty in calculating the sugars back to the dry weight,

* For detailed information a textbook such as Browne's "Handbook of Sugar Analysis" should be consulted, or the original memoirs should be read.

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and also because enzyme actions were in progress in such sap, they abandoned the method.

More recently Dixon and Atkins showed by physical methods (1913, 1) that sap pressed out never represents the true composition of that in the cells, unless their protoplasm has been made permeable by immersion of the tissue in liquid air. For the application of pressure, whilst rupturing some cells, results in a progressive increase in concentration of the sap solutes in the intact ones, as the liquid pressed out through the cytoplasmic semi-permeable lining is an approximation to pure water.

Brown and Morris, abandoning the use of pressed sap, finally adopted the following procedure: The leaves were dried quickly on wire-bottomed trays in a steam-oven. The high temperature soon checked enzyme action, though this had a short period of considerable activity. Also the natural acids of the leaves became greatly concentrated during the desiccation. Rapidity of drying lessened, but did not entirely get rid of, these sources of error.

Extraction of the dried material with ether removed fat and chlorophyll. About 10 grammes was then extracted with 80 per cent. alcohol for twenty-four hours at 40-45°. Further extraction for a like period was followed by washing by decantation. The spirit was then distilled off after neutralization with ammonia to prevent any invertive action of vegetable acids. The residue, after addition of water and evaporation to remove all alcohol, was cleared with basic lead acetate. The latter served to remove tannin, amino-acids, and proteid materials, which would affect the subsequent determinations.

After filtration, the excess of lead acetate was removed by treatment with hydrogen sulphide and further filtration and washing. The solution thus obtained was analyzed as described in the next section.

ANALYTICAL METHODS OF PARKIN.

The procedure adopted by this and all subsequent workers followed in the main the general outlines of that of Brown and Morris. But owing to the fact that starch is absent from snowdrop leaves, the analyses were greatly simplified, as this involves the absence of maltose also. The material used was dried as rapidly as possible. To check this method, similar leaves were frozen in liquid air and analyzed after extraction. The results quoted here show that drying has not resulted in any very appreciable inversion of sucrose, for the proportion of sucrose to hexose is greater in the liquid-air-treated material than in the air-dried. It is, however, to be noted that the small loss in sugars shown by the air-dried sample, falling as it does chiefly on the hexoses, is probably to be explained as due to respiration of the leaves before the destruction of the oxidases, especially as this type of enzyme is stable up to comparatively high temperatures. Parkin (1911) attributes it to a more complete extraction of the fresh pulp. The amounts recorded are the weights of sugar for 100 grammes of dry leaf.

Cold water was used for the extractions, and after extracting four times it was found that only about one-fortieth part of the sugars was left in the tissues. This does not interfere with the comparative nature of the results, but they are not absolute values. It was found that in the extraction about 0·5 gramme of sucrose, per 100 grammes of dry leaf, was inverted by the small quantity of invertase which had escaped destruction at the high temperature. This error affects the absolute values, and it may have a small effect upon the relative figures.

Another point in which Parkin's procedure differed from that of his predecessors was in his omitting to remove

excess of basic lead acetate, added as a clarifying agent. He took the precaution of adding this reagent cautiously, so that there was but little excess. Under these conditions he found that the rotation even of the fructose was not appreciably affected, whilst on the other hand a small loss in total sugars followed the treatment with hydrogen sulphide. Thus, apparently, Parkin's results are free from any serious objection on the score that they were obtained with leaded solutions. At the same time my own experience has been that, when dealing with sap pressed from leaves and centrifuged to remove débris, it is quite impossible to avoid adding excess of the basic acetate, for the whole solution becomes a yellow slimy mass.

TABLE XIX.

COMPARISON OF AIR-DRIED AND FROZEN LEAVES.

	Fresh Leaf. (Ground up after Liquid-Air Treatment.)		Air-Dried Leaf, Control.	
	Expt. I.	Expt. II.	Expt. I.	Expt. II.
Sucrose	12·84	10·46	12·74	10·42
Hexoses (reducing sugars) ..	5·94	12·87	5·67	12·38
Total sugar	18·78	23·33	18·41	22·80
Ratio of sucrose to hexose ..	1 : 0·46	1 : 1·23	1 : 0·44	1 : 1·19

Parkin employed the tables of Brown, Morris, and Millar (1897), for his reducing sugars, and, since maltose was absent, inverted the sucrose with hydrochloric acid under Clerget conditions—namely, by heating 50 c.c. of solution with 5 c.c. of concentrated hydrochloric acid for fifteen minutes at a temperature rising to 68°.

These reduction methods were checked by fermentation with yeast, when it was found that no substances optically active remained in the solutions. The error arising from the slight cupric reducing-power of sucrose in presence of hexoses was avoided by first estimating the sucrose from the loss of rotation through hydrolysis.

THE METHODS OF THE ROTHAMSTED EXPERIMENTAL STATION.

Since the researches of Strakosch (1907) and Mangham (1911) were almost entirely microchemical and qualitative, the next reasearch requiring mention is that on *Beta maritima,* the mangold. This was begun by Campbell (1912) and continued by Davis and Daish (1913, 1914).

Campbell found that drying at 100°, or killing with chloroform, ether, or toluene, before drying, greatly influenced the sugar content. The plan finally adopted was to immerse the chopped-up leaves in boiling alcohol, after removal of the midrib, and to begin the extraction at once. The percentage of dry matter was determined in another portion. Extraction was continued till all colouring matter was removed from the leaves, as it was found that this corresponded to complete extraction of the sugars. The solution was then concentrated with removal of the alcohol, and estimated by Pavy's solution, after clarifying by heating for a few seconds with alumina cream or *kieselguhr* and filtering. Inversion of sucrose was effected by boiling for seven minutes with 2 per cent. citric acid, and that of maltose by further hydrolysis of a diluted portion with 10 c.c. of concentrated hydrochloric acid for an hour. The results so obtained are admittedly not absolute, but it is claimed that they are comparable. The starch was estimated by O'Sullivan's diastase method after gelatinization. It must be noted that all these determina-

tions were made volumetrically and without use of the polarimeter, and so glucose and fructose are classed together as hexoses. The method of clarification adopted, too, was not thorough. However, as the very interesting results obtained depend for their significance on fairly large carbohydrate fluctuations, they are probably substantially accurate.

Davis and Daish, in continuing the mangold experiments, began with a critical examination of the methods employed in carbohydrate estimations. They tested the tables of Brown, Morris, and Millar, employing pure and carefully dried sugars, and obtained agreement with the tables to within 1 milligramme on the weight of copper used, which the authors regard as the probable degree of accuracy of their method.

In doing this they prepared their asbestos much in the manner recommended by Munson and Walker as described in C. A. Browne's "Handbook of Sugar Analysis." Special attention was drawn to the seriousness of the errors arising from neglect of this precaution. Davis and Daish, however, do not seem to have been aware of Munson and Walker's procedure, and arrived at it independently.

Their manner of weighing the copper as cupric oxide, after heating for half an hour in a Gooch crucible protected from reducing gases by a large outer crucible of porcelain or nickel, is both accurate and very convenient. It was adopted by Wilson and Atkins in testing Kendall's (1912) sugar tables. The agreement obtained with the tables was very close, though Kendall estimated the copper by means of his modification of the iodide volumetric method (1911).

With regard to volumetric methods, Davis and Daish criticize that of Bertrand adversely both on the score of accuracy and rapidity. That of Ling and Rendle (1905), however, was found to be accurate to at least 0·3 per cent., and also quite rapid.

To invert a pure 0·5 per cent. sucrose solution, these workers found it sufficient to boil for ten minutes with 2 per cent. crystalline citric acid, and their analyses by Ling's solution substantiate this statement. However, Davis and Daish pointed out that the sodium acetate usually present in de-leaded plant extracts so lessens the ionization of citric acid as to require a concentration of 10 per cent. to effect inversion by boiling for ten minutes. This 10 per cent. is over and above an addition of sufficient sulphuric acid to cause the solution to have a faintly acid reaction to methyl orange.

ESTIMATION OF MALTOSE.

Experiments with maltose showed that digestion of 25 c.c. of a dilute solution with 1 c.c. of autolyzed yeast for three hours at 40° did not result in the hydrolysis of any of this sugar. Treatment with 10 per cent. citric acid produced slight hydrolysis, of the order of 1 per cent. In the presence of sodium acetate and sulphuric acid, as before described, the hydrolysis is considerably less. In their analyses, however, they adopted both the invertase and citric acid methods of hydrolysis for sucrose, and thus obtained a double check on their accuracy. Both the Clerget and Herzfeld conditions result in the hydrolysis of considerable quantities of maltose, so they were not employed. Kluyver (1914) has stated that this criticism of the Herzfeld method is not justified, but Davis in his reply (1914) has completely upheld his own position.

The action of hydrochloric acid upon maltose was very exhaustively studied by Davis and Daish, for, as is well known, only 98 to 99 per cent. of the theoretical yield of d-glucose is obtainable owing to the formation of levulinic acid and humus substances. As a result of their work with various concentrations of acid and at several tempera-

tures, they state that this method is totally inapplicable to the analysis of plant extracts, owing partly to this destruction of glucose, but chiefly to the far more serious loss of fructose which takes place.

Their maltose estimations were carried out, therefore, as follows: The extract, clarified as usual, is de-leaded by sodium carbonate and hydrogen sulphide in succession, though the former affords a sufficiently complete removal of lead for ordinary chemical methods. The excess of hydrogen sulphide is removed by bubbling air through the solution, and finally by addition of ferric hydroxide. Sterilized portions are then fermented with pure cultures of the maltase-free yeasts, *Saccharomyces marxianus*, *S. anomalus* and *S. exiguus*, and in duplicate with baker's yeast. Fermentation is complete at the end of three to four weeks at 25°. The reducing-power of the solution is then determined after clarifying with alumina cream and boiling to remove alcohol.

The fermentation in the first three cases leaves the maltose untouched, the two with baker's yeast are carried out to remove the maltose also, and thus to enable a small correction to be applied for the reducing-power of the unfermentable pentoses present.

Of these *d*-ribose has been shown by Levene and Jacobs (1909) to be an essential constituent of the nucleus, and arabinose, xylose, and methyl-pentoses, may also occur in small quantities. On the whole the methods of Davis and Daish leave little to be desired on the score of accuracy, but are very laborious. It cannot, however, be expected that such complicated mixtures will ever be analyzed with both rapidity and accuracy, and in research work the latter is essential. More recently Daish, Davis, and Sawyer, (1914, 2 and 3) have studied the reducing-power of pentoses and their estimation in plant extracts.

ESTIMATION OF STARCH.

For the estimation of starch they make use of powdered taka-diastase (1914), for this converts it into maltose and glucose, without leaving any dextrin. By a combination of reduction and polarimetric methods it is possible to obtain the data for equations of the type employed to calculate the proportions of each present in a mixture of glucose and fructose. After the substitution of the maltose constants for those of fructose, the separate amounts may be determined, and the starch originally present may be found from these.

The objection to O'Sullivan's method is that though basic lead acetate (when carefully prepared, I venture to add) does not itself precipitate dextrin, yet when the latter is present in solutions in which a precipitate is produced, as in the purification of those obtained from diastase conversions, a portion of it is carried down with the precipitate. The Sachsse method, as modified, and adopted as official in the United States of America, is unreliable, as actual destruction of glucose occurs during the prolonged treatment with dilute hydrochloric acid. Furthermore, such a procedure is especially objectionable in the case of plant material, for pentoses arise during it from the pentosans of the cell walls.

Davis and Daish also point out the necessity for special care in sampling the material, and for the removal of substances soluble in water, such as gums, which are optically active and introduce errors into the polarimetric readings.

THE ESTIMATION OF REDUCING SUGARS BY THE METHODS OF BENEDICT AND KENDALL.

For the volumetric determination of reducing sugars, the citrate solution proposed by Benedict (1908) has been

found to be accurate and rapid. Kendall (1912) tried to adapt this solution for gravimetric determinations, but found it unsatisfactory. As it seemed to him desirable to obtain a reagent of a more stable character than the ordinary Fehling solution as used by Brown, Morris, and Millar, also by Munson and Walker, and one which would give a larger yield of cuprous oxide for the weight of glucose employed, series of trials were carried out. A solution of copper sulphate, potassium carbonate, and salicylic acid, was found to be very suitable, as it showed no reduction even after seven hours at 100°. The amount of copper reduced by Kendall's method is about half as great again as with Fehling's solution. Furthermore, the solvent action of the carbonate upon cuprous oxide is very much less than that of the hydroxide. Kendall prepared tables for glucose, invert sugar, lactose, and maltose. These have been supplemented by one for fructose constructed by Atkins and Wilson.

Kendall's solution is, I believe, capable of giving very accurate results. For example, a certain weight of pure glucose, of known moisture content, afforded a figure for the weight of cupric oxide, which differed by 0·0002 gramme from that recorded in the table. This was obtained without any previous experience of the technique, at a first trial. It is not advisable, however, to employ this solution in cases where citric acid has been used to invert sucrose, for low results are always obtained. These, however, are strictly comparable *inter se*.

THE LIMITED OXIDATION OF SUGARS BY BROMINE.

It has recently been shown by Nef (1914) that ketonic and aldehydic sugars may be separated by the action of bromine in the cold. In this way he states that the latter

may be quantitatively oxidized to aldonic acids, while the former remain nearly or quite unattacked. Atkins and Wilson have tested the possibility of employing oxidation with bromine in the quantitative estimation of mixtures of sucrose, maltose, glucose, and fructose. They found that, after standing eight days in the dark in a solution saturated with bromine, fructose remains almost entirely untouched, as shown by its reducing action on Kendall's solution. The removal of excess of bromine was effected before analysis, by the cautious addition of sulphurous acid till the solution became colourless. Blank experiments show that, in the absence of sugars, the above procedure does not give rise to any reduction.

Maltose, on the other hand, is completely oxidized, and glucose is almost entirely destroyed. Sucrose is inverted during (or before) the bromine treatment, and the glucose it yields is oxidized, but the fructose remains.

Thus it is clear that, by means of bromine, glucose and maltose may be largely removed, also the glucose resulting from sucrose. Accordingly the reducing-power of the final solution is due mainly to fructose. Since the sucrose can be determined separately by inversion with invertase, it is possible to subtract from the total fructose that which arose from sucrose. In this manner the reducing-power due to the fructose originally present can be approximately determined, and since the sucrose is already known, it remains only to determine the glucose and maltose. For these, after making the proper allowances for sucrose and fructose, both the values of optical activity and reducing-power are known, and so, having two equations and two unknowns, a solution can be obtained by the ordinary algebraical methods.

For purposes of accurate analysis, however, it is not allowable to assume that fructose is untouched. For

under conditions such that all the maltose (and about 98 per cent. of the glucose) is destroyed, it is found that about 3 per cent. of fructose has also disappeared. Accordingly it is necessary to proceed as follows: Quantities of the three reducing sugars, glucose, fructose, and maltose, are weighed out and treated separately with bromine, maintained as a saturated solution, for some convenient period—about sixty-six hours—at 16°. By this procedure the major part of the glucose and maltose is destroyed, whilst the fructose is only slightly diminished in quantity.

The following three equations may then be obtained, so it is possible to solve for the three unknowns:

The original rotation of the solution—

$$(1) \quad 100 \, a = a \, [a]_{20}^{D} g + b \, [a]_{20}^{D} f + c \, [a]_{20}^{D} m,$$

where a, b and c are the original amounts of the sugars.

The initial reducing-power, in terms of cupric oxide—

$$(2) \quad w = a k'_g + b k'_f + c k'_m,$$

in which k'_g, k'_f and k'_m represent the weights of oxide obtained when definite quantities, say 0·1 gramme, of the various sugars are used.

After treatment with bromine, only quantities $a k_g$, $b k_f$, and $c k_m$, are left. The final reducing-power will then be—

$$(3) \quad w' = a k_g \, k' + b k_f \, k'_f + c k_m \, k' \, .$$

It is more convenient to determine the final reducing-power than the final rotation, since the oxidation products are optically active and corrections have to be made for the rotation due to them.

If in other experiments the time or temperature is varied slightly, it is possible to substitute new values of k_g, k_f, and k_m, or to correct k_f and k_m by a fresh determination of k_g. This method is being carefully tested. [See note, p. 52.]

THE DETERMINATION OF MOISTURE AND
THE PREPARATION OF ANHYDROUS SUBSTANCES.

In making out tables of the reducing-powers of sugars, it has been usual to dry the material to be tested in a desiccator over sulphuric acid, and to introduce a correction for the small amount of moisture still retained in it. The latter is only removed after special treatment, such as heating *in vacuo* to temperatures from 60° to 110°, according to the substance being dried, and by using phosphorus pentoxide to absorb the water vapour. It is, however, obviously preferable that the whole sample should be anhydrous, as, especially with crystalline substances, it is never quite certain that the moisture is uniformly distributed. For example, sodium chloride, when prepared as a fine dry powder, crackles when heated, owing to the inclusion in the minute crystals of still more minute droplets of water. In this case the moisture can of course be removed by fusing the salt, but with many organic substances even prolonged drying in air at 100° results in oxidation to a more or less serious extent.

To remove water under conditions which preclude oxidation and the destruction of thermolabile substances, Shakell (1909) recommends the freezing of the tissue or extract, and its evaporation as ice under reduced pressure. This method appears to be a very perfect one for drying the material sufficiently to prevent bacterial action, and it may even be possible to obtain anhydrous tissues in this manner, though, so far as the writer is aware, Shakell's aim was only to preserve the constituents of serum and antitoxins unaltered.

When dealing with substances which are not injured by heating to 80° in absence of air, Atkins and Wilson (1915), however, have found it possible to remove water

entirely by the following modification of Young's well-known method for the preparation of alcohols and other liquids in the anhydrous state. Young distils the alcohol, containing a little water, with benzene, through his evaporator stillhead with eight or more sections. At first a turbid ternary mixture of constant boiling-point comes over at 64·85°. This contains almost all the water, a large percentage of benzene, and some alcohol. Then the temperature rises to about 68°, and the last traces of water are removed as a little of the ternary mixture passes over, together with a small quantity of a binary mixture of alcohol and benzene. At 68·25° the distillate is the pure binary mixture of constant boiling-point. Finally the temperature rises to 78·3°, the boiling-point of the pure alcohol; or if benzene has been added in excess it rises to 80·2°, the boiling-point of pure benzene. To obtain anhydrous fructose, for example, it is only necessary to add some of the powdered solid to the distillation flask. This is dissolved by hot alcohol (99 per cent.), and is then distilled from a water-bath with benzene. If the quantities have been suitably chosen, by reference to Young's "Fractional Distillation," at the end of the distillation anhydrous fructose remains partly as a solid and partly in solution in hot alcohol. The alcohol can be readily removed in a vacuum desiccator. In some cases it is preferable to have benzene in excess, so that at the end the solid is dissolved or suspended in the liquid. With a substance which remains suspended in alcohol, such as starch or cellulose, the method is equally applicable. Its use over a more extended range of substances is being examined. It is of course desirable to test the anhydrous nature of the products by carrying out moisture determinations in the usual manner by drying over phosphorus pentoxide *in vacuo*.

The determination of the percentage of water in a tissue may accordingly be carried out by placing it in a weighed flask, and distilling with alcohol and benzene. In such cases it is advisable to protect the end of the stillhead from being splashed by the alcohol containing extracted matter. When the flask has been distilled as nearly to dryness as possible, it is disconnected and the remainder of the liquid distilled away without a condenser, the entrance of moisture being prevented by a drying tube attached to the cork. The difference between the original and final weights is the weight of water and other volatile substances, if any, lost during the treatment.

This method appears to offer possibilities for determining water of crystallization in many cases, and may be of use in deciding whether it is present as " water of crystallization " or as " water of constitution."

NOTE.—It has been found that since errors are largely magnified in the equations for three unknown quantities given on p. 49, the best results appear to be obtained by choosing the conditions so that glucose is almost entirely removed as well as maltose. The residual reducing-power of the glucose compensates for the slight loss of fructose when the two are present in anything roughly approaching equal amounts. A small correction can be introduced for the residual glucose.

CHAPTER III

THE CARBOHYDRATES OF THE THALLOPHYTA AND BRYOPHYTA IN RELATION TO PHOTOSYNTHESIS

THE relationships of the carbohydrates of the angiosperm leaf to one another are already known, though by no means completely. At the present time, however, the question of the first product of a carbohydrate nature to arise during photosynthesis in the lower groups of plants has hardly been seriously discussed at all. Yet it is a problem of great interest, not only in itself, but also from a consideration of the light it may be expected to throw upon the more general discussion of carbohydrate synthesis and the functions of the various members of this class. For in thallophytes the distinction between assimilating and conducting tissues is frequently a very indistinct one, and, indeed, is sometimes non-existent. Accordingly, it is quite possible that the disaccharides sucrose and maltose, which are the forms in which sugars are most frequently translocated, may be absent in some cases, and that starch may by the action of a special type of diastase afford glucose only on hydrolysis. For example, whilst ordinary malt diastase generates dextrin, maltose, and glucose, by its action on starch, taka-diastase, derived from the fungus *Eurotium oryzæ*, hydrolyzes starch to maltose and glucose only, and from its mode of action has been shown to consist both of a diastase and maltase. Speculations of this type, however, are only useful in so far as they lead to the acquisi-

tion of analytical data which have a direct bearing on the subject. Researches with this end in view are at present in progress in the laboratories of Trinity College, Dublin.

Now, while it is quite possible that there is no one sugar which is, in every class of plants, the first to arise during photosynthesis, yet, since the intake of inorganic carbon is a fundamental necessity, it is tempting to conceive of it as following a uniform course. It may, therefore, be profitable to inquire into the nature of the carbohydrates occurring in the lower groups with this idea in mind. For a full summary the reader is referred to Czapek's " Biochemie der Pflanzen." A good account of the chemistry of seaweeds is given in " Fertilizer Resources of the United States."

CARBOHYDRATES OF THE PHÆOPHYCEÆ.

The recent investigations of Kylin (1913) have added considerably to our knowledge of the algal carbohydrates. To begin with, he proved that the brown algæ *Ascophyllum nodosum* and *Fucus vesiculosus* contain fructose. His data for cupric reductions and polarimeter readings both before and after inversion enable one to calculate the amount of glucose present also, as well as that of a small amount of a lævorotatory substance affording glucose on hydrolysis. This is probably laminarin.

Curiously enough, Kylin himself does not make any calculation by the algebraic expression employed by Brown and Morris, but attributes the discrepancy between the calculations based on polarimeter readings and on titrations with Fehling's solution as due to traces of fucosan, though this difference varies from 0·14 to 1·09 per cent. in terms of reducing hexose in the four algæ he examined. In *Laminaria digitata* and *L. saccharina* he records the presence of glucose, but his readings prove that fructose

is also present. Here, too, after inversion there is an increase in the dextrorotatory power of the solution accompanied by a rise in its reducing-power, both pointing to the presence of laminarin. The traces of fucosan Kylin mentions could not show an increase in the discrepancy between the values obtained by reduction and by polarization before and after inversion. None of his quantitative results lend themselves to the view that sucrose is ever present in these algæ, for inversion invariably increases the dextrorotation of the solution. It is possible, however, that the presence of a little sucrose might be quite masked in this respect by a preponderating quantity of laminarin.

His work on fucosan has shown that this substance is not, as was believed, a carbohydrate reserve material occurring in highly refractive globules. It is, however, a tannin, but not a typical one, since it is not precipitated by ferric chloride, though it does give a dark brown colour with this reagent. In solution it has an astringent taste, a strong reducing action, and is precipitated by neutral lead acetate and by lime-water even when acids are present. In alkaline solution it is quickly oxidized to a brown substance, which under the name of phycophæin was formerly considered to be a water-soluble chromatophore pigment. This, however, Molisch (1905) and Tswett (1906) had already shown to be a post-mortem oxidation product, though its relation to fucosan was not suspected. When warmed with dilute mineral acid fucosan yields no sugar, and accordingly cannot be a glucoside.

As a reserve material the polysaccharide laminarin plays the part in this group that starch does in the higher plants and in the red algæ. Kylin prepared this substance in a pure condition, and found it to have the specific rotation $a_D = -13 \cdot 15°$, presumably at the temperature of his laboratory; as before mentioned, laminarin gives only glucose on

hydrolysis. Recently Duggar and Davis (1914) investigated the enzymes of *Fucus vesiculosus*, but could detect none except catalase, which Atkins (1914, 2) had previously located. This poverty in enzymes is very remarkable, for one might reasonably expect to find one capable of splitting carbohydrates.

CARBOHYDRATES OF THE RHODOPHYCEÆ.

In the Florideæ the osazone of a simple sugar was obtained by Tihomirow (1910). Kylin, however, failed to confirm this, and both he and Kolkwitz (1900) are in agreement as to the absence of reducing sugars from the members of this group examined by them.

A considerable amount of work has been done upon the nature of Floridean starch.

With iodine the granules give various shades of colour. Thus, Kolkwitz divides the class into those which give a rose-red, as Laurencia and Cystoclonium, and those affording a blue-violet, as do Furcellaria and Delesseria. These modifications of starch are described at length by Oltmanns in his "Morphologie und Biologie der Algen, Bd. II." It may, however, be remarked that some at least bear a strong resemblance to the starch of the phanerogams, in that they are hydrolyzed by dilute acids to yield glucose, and are also rapidly acted upon by malt diastase. *Furcellaria fastigiata* was Kylin's source for the material he used. Saiki (1906) found that strong solutions of malt extract failed to digest the carbohydrates of certain commercial food preparations made from Gelidium sp., Porphyra sp., and *Chondrus crispus*. The influence of the indigestible cell wall may perhaps be very appreciable in these cases. By hydrolysis of similar preparations with acids, König and Bettels (1905) were able to identify galactose, fructose, and glucose. Whilst from *Chondrus crispus*

Müther and Tollens (1904) obtained galactose and probably other hexoses.

The grains in the red algæ are in appearance quite similar to those of land plants, showing frequently a hilum and striation. They, however, occur in the cytoplasm outside the plastids, and sometimes independently of the latter. Their appearance and disappearance at various times led Bartholomew (1914) to seek for a diastatic enzyme in these algæ, for if this were found to act both upon algal and phanerogamic starch it would go to show a certain similarity in the structure of the two.

Polysiphonia variegata Ag., *Dasya elegans* Ag., *Agardhiella tenera* (J. Ag.) Schmitz, and *Ceramium* spp., were obtained in quantity, dried at 35° to 40° after treatment with alcohol and acetone, pulverized, and allowed to stand in 20 per cent. alcohol. From this solution enzymes were subsequently precipitated with 95 per cent. alcohol, and dried after washing with alcohol and ether. Experiments carried out with careful controls showed that this precipitate contained a diastase capable of digesting maize starch. In the course of this process the grains were found to be corroded, so that in this respect the behaviour of the enzyme is similar to that of a translocation diastase. From the various stages of the reaction, as judged by the iodine and copper reduction tests, it is probable that the diastase of the red algæ resembles that of the higher plants in being composed, not of a single enzyme, but of a series of amylases and dextrinases.

In comparison with the action of approximately equal weights of malt diastase, that derived from the algæ was found to be rather a slow-working enzyme. All the evidence afforded by this research is in favour of the view that the starch of the red algæ is very similar to that of the higher plants.

THE PRIMARY SUGAR OF PHOTOSYNTHESIS IN THE BROWN AND RED ALGÆ.

Notwithstanding the close resemblance between the ultimate forms in which the products of photosynthesis are stored in the red and brown algæ and in the higher plants, there is as yet no evidence of the occurrence of sucrose in the two former classes. Glucose and fructose are, however, certainly present in the Phæophyceæ, and though their identification in the Rhodophyceæ has not as yet been confirmed, it is very likely that it will be, when due regard is paid to the variations which may be expected to take place owing to differences in illumination, temperature, and age, of the plants. Thus, up to the present the marine algæ afford no support to the view that sucrose is the first sugar to be formed in photosynthesis; nevertheless, that which regards this position as being occupied by a hexose is by no means surely established. Much work remains yet to be done in this domain of physiology before any definite conclusions can be reached

OCCURRENCE OF MANNITOL.

Kylin and Segers-Laureys (1913) also confirmed the presence of the polyhydric alcohol mannitol in the brown algæ. It is found to form a large proportion of the white incrustation appearing on Laminarias when dried. This substance has also been reported as occurring in many other plants—for example, *Syringa vulgaris, Fraxinus excelsior, Polypodium vulgare, Agaricus campestris*. Among mushrooms it is very widely distributed, but Bourquelot (1903) has shown that its origin is the trisaccharide trehalose. The latter is hydrolyzed to *d*-glucose by trehalase, and then by an ill-understood biological reduction this substance gives mannitol.

Busolt (1913) has shown that sap from French beans yields a considerable amount of mannitol when kept for a week, though none was detected in fresh sap or in sap kept for a week after sterilization while fresh. Cauliflower heads, on the other hand, afford mannitol when quite fresh, though it is possible that it may have been formed during evaporation of the juice. This change, either bacterial or enzymic, casts doubt on the reality of presence of mannitol in brown algæ. It is not impossible that in them trehalose may occur instead of sucrose, being an intermediate step in the formation of laminarin. The identification of trehalose by any means other than by obtaining it in a pure crystalline condition is very uncertain. Its presence could of course be deduced by quantitative methods. This suggestion as to the occurrence and rôle of trehalose is, it must be remembered, purely speculative.

CARBOHYDRATES OF THE BRYOPHYTA.

With regard to other holophytic members of the Thallophyta and Bryophyta little quantitative work appears to have been done, but the presence of glucose, fructose, sucrose, and starch, has been demonstrated in many cases. Marchal (1906) has investigated the distribution of starch in this group, and has recorded its absence from a considerable number of mosses. From these maltose is also absent. Mason * (1915) has noted the absence of starch from the leaves of *Thuidium tamariscinum* and *Sphagnum cymbifolium*. Marchal, however, found it in the young leaves of the former, and its presence in the upper part of the stem of the latter was demonstrated by Mason. As additional evidence for the presence of sucrose in certain mosses, Mason has shown that invertase is present in the

* The publication of Mr. Mason's results was delayed for a year owing to his departure for France on active service.

leaves of the following both by day and by night: *Sphagnum cymbifolium, Brachythecium rivulare, Dicranum majus,* and *Thuidium tamariscinum.* Quite in keeping with the evidence as to the absence of starch from the leaves of *S. cymbifolium* is the complete absence of diastase also, but this enzyme is present in those of *Polytrichum commune* which contain starch.

As an illustration of the quantities in which the sugars are found in *P. commune,* Mason's analyses are quoted. Reductions were carried out with Benedict's solution, and the inversion of maltose was effected by boiling with hydrochloric acid according to the procedure of Brown and Morris. This, though convenient, is admittedly faulty. The stems and leaves in their normal proportions were taken for analysis, results being given as percentages of the moist weight.

TABLE XX.

Polytrichum commune.				*Examined when Fresh.*	*Examined after Three Days in Dark Press.*
				Per Cent.	Per Cent.
Hexoses	..	..	..	2·17	2·00
Sucrose	..	..	..	1·36	0·59
Maltose	..	..	..	1·38	1·58

From the above it may be seen that much sucrose has been used up during storage in the dark, and also a little hexose has disappeared in spite of the replenishing of glucose and fructose by inversion of sucrose, and of glucose by that of maltose. The last-named sugar shows a small increase owing to the mobilization of starch reserves.

The ratios which the various sugars bear to each other in the leaves and stems of *P. commune* are given in the following table. The actual percentages could not be determined owing to the time required for separating the

leaves from the stems.   The tissues had therefore to be immersed in absolute alcohol to check inversion changes. The green portions of the stems were rejected, and are not included in the analyses of either leaf or stem.

TABLE XXI.

Sugars of Polytrichum.

P. commune from—	Ratios.		
	Hexoses.	Sucrose.	Maltose.
Tibradden Pine Forest (Co. Dublin): Leaves	1·00	2·14	0·45
Kippure Mountain (Co. Wicklow): Leaves ..	1·00	2·19	0·51
Tibradden Pine Forest: Stems	1·00	0·19	0·66
Kippure Mountain: Stems	1·00	0·24	0·72

The abundance of sucrose in the leaves as compared with the stems appears to favour the view of Brown and Morris, that it is the first sugar to be formed in photosynthesis.   The analyses, however, furnish no evidence for or against Campbell's suggestion that hexoses are found at first, but soon reach a maximum due to their transformation into sucrose.   In the stem sucrose appears to be rapidly converted into starch, or it is perhaps inverted to a considerable extent.

TABLE XXII.

Sugars of Thuidium.

T. tamariscinum from Dublin Mountains.	*Hexoses.*	*Sucrose.*
Examined in the afternoon	1·00	1·66
Examined after three days in dark press ..	1·00	0·53

That the behaviour of the starch-free moss *Thuidium tamariscinum* is similar to Polytrichum as regards the diminution in sucrose consequent upon storage in the dark is also shown by Mason's results.

The distribution of sugars in *Sphagnum cymbifolium* is illustrated by the following table, from which it appears that in it, too, the assimilating portions are richest in sucrose. To avoid enzymic changes during transit from the bog to the laboratory, jars of alcohol were carried up, and the material was placed in them directly. All these analyses of mosses were performed on material gathered during the months of July and August. In addition to the hexoses there are perhaps traces of maltose, so the term reducing sugars is employed.

TABLE XXIII.

SUGARS OF SPHAGNUM.

S. cymbifolium from Dublin Mountains, collected at 11 a.m.	Reducing Sugars.	Sucrose.
Upper green portion	1·00	2·16
Lower colourless portion	1·00	0·91

It is desirable that further work should be done upon the carbohydrates of this group, and the completion of Mason's researches may be expected to throw some light upon the vexed question of the first sugar to arise in photosynthesis.

CHAPTER IV

THE PECTIC SUBSTANCES

OCCURRENCE AND PROPERTIES OF PECTIN AND PECTASE.

IN a very great number of fruits and fleshy tissues in general there exists a substance which is ordinarily soluble, but is precipitated as a gelatinous mass on the addition of alcohol. This is known as pectin, and was first fully described by Frémy (1840), though Bracannot (1825) had previously obtained it. From its relationship to the material which forms the middle lamella of cell walls, it is clear that it must be of considerable physiological importance. In many fruit juices as well as in the sap pressed from woody stems, gelatinization takes place on standing, this being due to the action of an enzyme, pectase, upon the pectin of the juice.

The extensive literature of the subject has been summarized by Czapek (1913), whilst the common properties and reactions of these bodies have been described in the recent publications of Molisch (1913), Browne (1912), Euler (1912), and of Haas and Hill (1913). There is, therefore, no need to give more than an outline here, to serve as an introduction to the study of the action of pectase by viscosity measurements which was carried out by Ball (1915).

A survey of the work done so far upon the pectic sub-

stances leads one to realize that their constitution and physiological functions are as yet very imperfectly understood.

The middle lamella of cell walls consists of pectose, or possibly of calcium pectate. Pectose can be distinguished from cellulose by its insolubility in ammoniacal cupric hydroxide, and by the fact that when treated with hot 2 per cent. hydrochloric acid followed by sodium hydroxide it goes into solution. This pectose is by some considered to be a calcium salt of pectin, but the fact that it requires somewhat prolonged treatment with acid to bring it into the condition in which it dissolves in an alkaline solution with formation of a salt seems to weigh against such a view. It is accordingly more probable that the change from pectose to pectin involves hydrolysis. During the ripening of fruits and the retting of flax, the breaking down of the middle lamella of pectose is said to be brought about by an enzyme, pectosinase. The pectin produced in this reaction may be precipitated by alcohol or by calcium salts, to form in the latter instance a calcium pectinate which is soluble in 0·2 per cent. hydrochloric acid. But when pectin is acted on by pectase, a substance, one of the many pectic acids, is produced, the coagulation of which is induced by the presence of calcium salts; this gelatinous material is not dissolved by the previously mentioned concentration of acid.

The end product, obtained by heating these pectic substances with hot dilute alkali, is arabinic acid. When hydrolyzed with dilute acid they all yield d-galactose and l-arabinose, the proportions of the two varying with the kind of pectin. In the cell this change is supposed to take place through the activity of yet another enzyme, pectinase.

The careful analyses of pectin from different sources which have been performed in recent years by Tromp de

Haas and Tollens (1895) leave no doubt as to its carbohydrate nature, though, owing to the great difficulty of purifying such gelatinous material, there is a certain amount of fluctuation in the ratio of hydrogen to oxygen. Tollens (1914) thinks it possible that the small amount of oxygen found in excess of that required for a carbohydrate may be due to the existence of one or more carboxyl groups. He suggests for pectin the formula $5(C_{10}H_{18}O_9) + C_{10}H_{18}O_{10}$.

To sum up, three enzymes are said to be concerned in the changes undergone by pectic substances. Pectose, which occurs as the middle lamella of cell walls, is split up by pectosinase to form pectin. Pectin, a soluble substance, is transformed into a pectic acid by pectase. In presence of traces of salts this gelatinous substance becomes aggregated into clumps which sink in water. Soluble pectin may also be acted upon by pectinase, with the production of sugars.

For the obvious reason that there is no ready chemical method for studying the changes brought about by these enzymes, it has resulted that their action is but little known.

The rôle played by calcium salts in the bringing about of the coagulation of pectin by pectase was demonstrated by Bertrand and Mallèvre (1894, 1895), as was the fact that calcium could be replaced by strontium or barium. They also ascertained that acids have a retarding effect upon coagulation, 0·088 per cent. hydrochloric acid prolonging the time required for the process from one to forty hours, whilst 0·1 per cent. completely stopped all action. The behaviour of other mineral and organic acids was similar. With increased concentrations of calcium salts or of enzyme the effect of the acid is not so powerful. The time necessary for coagulation was thus found to depend upon the proportions of pectase, pectin, calcium salts, and free acids.

The recent work of Haynes (1914) on the action of salts of the alkalies and alkaline earths upon pectin will be considered farther on.

THE ACTION OF PECTASE.

Since there is no chemical method at all suitable for following the course of the gelatinization of pectin by pectase, recourse was had, at the suggestion of the author, to the measurement of the viscosity of a mixture of enzyme and substrate at regular intervals of time and at various temperatures. It is at once evident that the chemical change is not proportional to the alteration in viscosity. In spite of this the method enables one to obtain much information as to the character of the reaction and the factors which influence it, as will be seen from a study of the viscosity—time curves given by Ball.

PREPARATION OF PECTIN.

As a source of pectin, Ball employed the roots of the carrot, *Daucus carota*. These were finely minced and heated for two hours on a water-bath. Sufficient water was added to cover the material. The pulp was then pressed, and a little oxalic acid added to precipitate calcium ions. After filtering the pectin was brought down by mixing an equal volume of spirit with the extract. This was collected on a filter and redissolved in a small quantity of hot water. Further addition of alcohol caused the pectin to be precipitated in its usual mucilaginous form, and its removal from the liquid was effected by means of a centrifuge. In this manner it was obtained in a fairly pure state, and was then dried on a water-bath. On addition of hot water it readily redissolved, and was made up to a 2 per cent. solution for use with the viscosimeter. A few drops of toluene were added as a preservative.

Source of Pectase.

To obtain the enzyme, leaves of *Syringa vulgaris* were pressed in a vice, and the resulting liquid was either employed directly, after removal of débris by the centrifuge, or its pectase was precipitated by alcohol and redissolved in distilled water. The fresh sap contained both calcium salts and other electrolytes, whereas the precipitated enzyme mixture was to a great extent free from both. Attempts were made to correlate the activity of the sap with the age of the leaf, but fluctuations from sample to sample obscured the influence, if any, of this factor.

Electrical Conductivity of Gelatinizing Pectin Solutions.

Since the electrical conductivity of a solution at constant temperature depends both upon its concentration of electrolytes and the viscosity of the medium, it was thought that measurements of this character might throw light upon the phenomena of gelatinization. For whereas a liquid such as glycerol has a high viscosity both as regards its time of flow through a capillary tube and its effect upon electrolytes, another, such as a gelatin solution,* may have an equal viscosity as measured by time of flow, but when its effect upon electrolytes is the criterion the difference between it and the pure solvent may be negligible. Thus, leaving out of account possible changes in the chemical character of the pectin leading to increase or decrease in conductivity during gelatinization, it is clear that, if but little change occurs in the resistance of the solution while the pectase is acting on it, there can be no doubt that the structure of the product is that of a jelly, a meshwork of a colloid containing a colloidal liquid solution, rather than

* This, of course, is not a homogeneous solution.

of a liquid such as glycerol. From the fact that small quantities of pectin could give rise to a mass of considerable consistency, it seemed highly probable that the structure was that of a gel. To test the point experimentally, 1 c.c. of 2 per cent. pectin solution was mixed with an equal volume of distilled water and with 1 c.c. of freshly pressed sap. The solution was then placed in a Hamburger conductivity tube at air temperature, 12·5°; its resistance was measured from time to time in the usual manner, but up to the time at which it set to a firm jelly, about two hours after mixing, there was no change whatever in the readings. This furnishes definite proof of the gel structure of the gelatinized pectin.

THE ACTION OF PECTASE STUDIED BY THE VISCOSIMETER

To study the action of pectase by viscosity determinations, Ball constructed a small apparatus of the Ostwald type. The size of the bulb was such that 3 c.c. of solution sufficed for each experiment, the proportions of the constituents being the same as in the conductivity determinations—viz., 1 c.c. each of 2 per cent. pectin, distilled water, and Syringa sap. Thus the experiments are comparable as regards the pectin content, but it is certain that the amount of enzyme could not have been identical in the different samples of sap, and the electrolytes also were probably not quite constant in quantity.

To standardize the apparatus, the time required for pure water to flow out of the bulb between the two marks was measured. For purposes of comparison one instrument was chosen, and the slightly different times of flow for the others were made the basis of factors by which the times of flow of the pectin solutions could be recalculated for the standard viscosimeter. As the absolute amounts of en-

zyme and calcium salts were unknown, there was nothing to be gained by calculating the measurements as absolute values. Accordingly, they were all recorded as seconds of time for the emptying of the bulb of the standard at the temperature of the experiment. The times of flow for water at 0° were from 3·0 to 3·8 seconds, and the initial

FIG. 3.

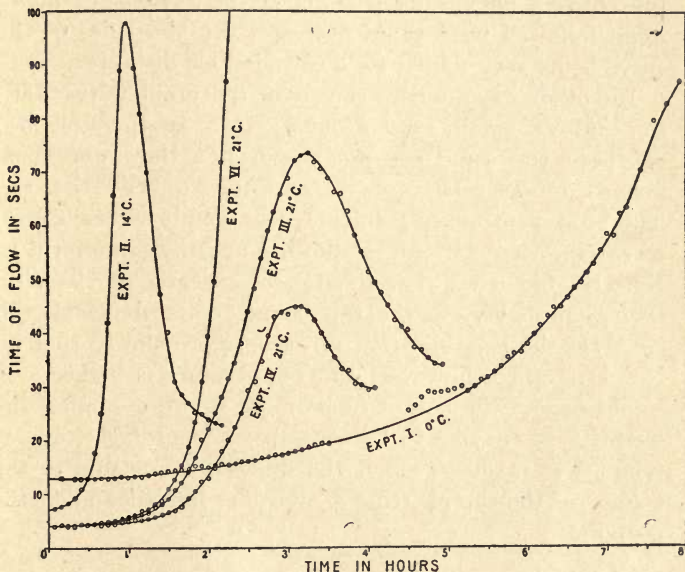values for the solutions, measured five minutes after mixing, were from 3·8 to 7 seconds. The results of five experiments are shown in the accompanying viscosity—time graph.

The first series of determinations (Experiment I.) was carried out at 0°. In it, after several hours had elapsed, but little change in the viscosity could be perceived. It then increased rapidly, till after eight hours, when the

experiment had to be brought to a close, the curve gave indications of approaching a maximum. One slight irregularity during the fifth hour was occasioned by an accidental elevation of the temperature.

Experiment II., at 14°, furnished evidence of the great rapidity of the action at this temperature, and revealed the entirely unexpected attainment of a viscosity maximum followed by a rapid decrease, the two sides of the curve being very similar for a considerable distance.

The other experiments shown in the graph—Nos. III., IV. and VI.—were carried out at 21°. In the first two of these the same sap was used, but they were performed on successive days, and in No. IV. the sap had been neutralized ; toluene was employed as a preservative. No. IV. curve shows much the same form as No. III., but never rises to so great a degree of viscosity. Owing, probably, to the sap being poorer in pectase in No. III., the gelatinization proceeds more slowly than in No. II. Since, however, the temperature is higher, one would expect the action to be more rapid, assuming the quantity of enzyme to be constant. Accordingly, an attempt was made to see if the difference was due to the acidity of the sap exerting a retarding influence upon the pectase.

THE INFLUENCE OF IONS UPON THE MAXIMUM VISCOSITY OF THE SOLUTION.

To test this point, the sap was rendered neutral to litmus, but in the curve of No. IV. it is shown that the maximum, though reached somewhat sooner than in No. III., is much below it. This seemed to be due to the acceleration of the reverse process occasioned by the increase of the metallic ions of the sodium hydroxide added. Such an alteration of the relative rates of the two processes would naturally lead

to the maximum value of the viscosity becoming smaller. There is, however, an alternative explanation possible, that the low value is due to a decrease in the activity of the enzyme during its storage for one day. The form of the curve, however, is against this view, for it shows that the initial velocity is not very much less than in No. III.

In order to obtain decisive evidence, the enzymes of 3 c.c. of freshly pressed sap were precipitated by the addition of 15 c.c. of 95 per cent. spirit. After centrifuging, the precipitate, which was almost free from electrolytes, was redissolved in 3 c.c. distilled water, and, as in other experiments, 1 c.c. was added to the pectin solution. The curve No. VII., obtained in this way, shows the rapidity of the coagulation to be great. The viscosity was such that about 500 seconds was required for the emptying of the bulb, whereas at the start, less than four hours previously, 3·8 seconds sufficed. After four hours no flow took place. This furnishes direct and conclusive evidence that removal of electrolytes, including of course the calcium salts, results in the increase of the viscosity of the solution of transformed pectin up to a point far in excess of that attained to by any similar solution containing electrolytes. Traces of electrolytes were, however, still present, having been adsorbed by the precipitated enzymes. In view of the minute traces of calcium salts ordinarily found in plant juices, the amount remaining must have been negligibly small. Direct experiments are being undertaken to test this point.*

On the whole, it seems to be well established by Ball's results that the two effects, the production of a gel by the action of pectase and the clumping of the material of the gel by electrolytes, are distinct, and were probably confused

* This work has had to be postponed, for Mr. Ball has received a commission in the army for the duration of the war against Germany.

by the previous workers on the subject. The marked acceleration in the clumping caused by calcium, strontium, or barium, need not mean that their ions are in any way connected with the action of pectase, but is apparently fully explained by the well-known property of divalent ions in causing the clumping of colloids with a far greater rapidity than do monovalent ions. The presence of calcium salts in quantity evidently causes flakes of coagulum to appear even when the action of the enzyme has been in progress for a short time only.

DISCUSSION OF THE FORM OF THE VISCOSITY CURVES.

Ball has suggested that the product of the action of pectase on pectin consists at first of separate colloidal particles, and has pointed out that during their formation there is practically no change in viscosity, as shown by the initial portions of the curves being almost parallel to the time axis. This is succeeded by a portion of the curve which shows rapid increase in viscosity, the physical interpretation of which he considers may be the union of adjacent particles to form a loose network, the individual meshes of which are continually becoming smaller by subdivision through the attachment of freshly formed material. Since Bertrand and Mallèvre have shown that gelatinization does not occur when all traces of electrolytes have been removed, it is possible that the building of the network is due to their activity. As an alternative explanation, the view has been advanced that the enzyme itself looses its activity when electrolytes are entirely removed, for it is well known that other enzymes become enfeebled when their ash content is reduced below a certain point. The constituents of the ash may of course be in combination, and not dissociated, though in most cases it is probable that they are partly in the state of dissociable metallic

salts of organic complexes. The process of gelatinization continues till the resulting jelly is of such a consistency as to cease to flow in the viscosimeter, unless the condition becomes complicated by the action of appreciable quantities of electrolytes.

The effect of this class of bodies appears to be the clumping of the colloid particles by a metallic ion. By this means, the amount of transformed pectin available for meshwork formation is continually decreased. Thus the maximum viscosity can never be as great in the presence of any appreciable quantity of electrolytes as in their absence. Whether any increase in viscosity occurs will, according to this view, depend upon the relative rates of pectin transformation and of clumping. When the former preponderates the viscosity rises, but in time the two rates become equal and the viscosity attains a maximum. The downward portion of the viscosity curve is explained as representing the progress of clumping at the expense of the already formed network. When the clumps reach a certain size they tend to unite into visible flakes, causing discontinuity in the gel and consequent diminution in viscosity accompanied by irregularity owing to the plugging of the capillary. It may be seen that in the curves of Fig. 3 this breaking up occurs usually when the viscosity of the solution in the different experiments has about the same value.

By means of the above hypothesis given by Ball, the interesting nature of the curves seems to be qualitatively explained in a simple and rational manner, while at the same time the need of further work is indicated.

Gelatinization of Pectin by Alkalies.

Contemporaneously with Ball's research, which was completed in July, 1914, Haynes's study of the gelatiniza-

tion of pectin by the alkalies and alkaline earths was being carried out, and has come to the author's notice since the foregoing pages were written.

Haynes employed parapectin derived from limes and lemons, and measured the rate of precipitation of the jelly by the reagents, assuming the latter process to begin when a definite amount of parapectin had been gelatinized. Thus the time required for precipitation to begin in the jelly could be taken as a measure of the rate of gelatinization of the solution. With this assumption, Haynes found that the reaction could be represented by the equation $c^2/t = k$ for dilute solutions, where t is the time required for the formation of precipitate, and c is the concentration of the hydroxides of calcium, strontium, or barium. The interpretation of this, according to Haynes, is that the reaction is a chemical one between the pectin, from which hydrogen is eliminated, and the positive ion of the alkali, as follows:

$$\bar{P}\cdot OH + Ba(OH)_2 = \bar{P}\cdot OBaOH + H_2O.$$
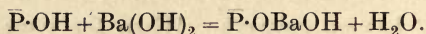
The original paper should be consulted for a complete consideration of this equation, and for details of much interest. It is hard to correlate the changes with those studied by Ball, since both the methods and sources of material were so entirely different. Ball, however, noticed that his stock solution of pectin, which had remained over for four months, in the absence of a sufficient quantity of toluene, gave immediate precipitates with a drop of various laboratory reagents, sodium hydroxide, copper sulphate, ferric chloride, etc. A comparative study of the two methods would be of interest, and in its absence speculation does not seem to be profitable.

CHAPTER V

OSMOTIC PRESSURE IN PLANTS

BETWEEN a pure solvent and a solution there is a difference in free energy. This is rendered apparent in several ways, by their disparity in vapour pressure, boiling-point, freezing-point, and osmotic pressure. As these values vary with the molecular concentration of the solution—*e.g.*, the cell sap in the present discussion—their measurement affords a ready means of studying, in the gross, changes which occur in the metabolism of vegetable cells.

The connection between the above-mentioned properties of solutions becomes apparent when one considers the work necessary to separate a given quantity of the pure solvent from a solution in a reversible manner and isothermally. It is evident that there must be a quantitative relationship between the various properties, since when the process is completed the same amount of work will have been done in each case, irrespective of the method adopted. Hence it is immaterial whether the removal of solvent be effected by evaporation, by crystallization or by the movement of a semi-permeable membrane through the liquid.

As is well known, the pioneer measurements in this domain were carried out on plant cells by the plasmolytic method, in which the osmotic pressure of the cell sap is balanced against that of an external solution of known concentration. Direct measurements of the osmotic pres-

sures of plant juices with artificial semi-permeable membranes have never been carried out, owing to manipulative difficulties and to the fact that by calculation from cryoscopic data the desired values may be more readily obtained. However, by balancing an external gas pressure against the internal osmotic pressure of leaves on a severed branch, and noting when wilting supervened, Dixon (1896) has been able to obtain approximate values. The method is fully described in his book on " Transpiration and the Ascent of Sap," so will not be given here.

As in other branches of physiology, cryoscopic measurements have recently been extensively employed, to the almost complete exclusion of the plasmolytic. The work of the Neapolitan physiologists Cavara (1905), Trinchieri (1909), and Nicolosi-Roncati (1907), was carried out with the Beckmann apparatus, which as usually constructed requires about 15 c.c. of liquid, though tubes of smaller capacity are also in use. The introduction of thermocouples for these determinations by Dixon and Atkins (1910) has effected a considerable saving in the volume of sap necessary, 2·5 to 3 c.c. being sufficient for each experiment. A description of the modification would be superfluous here, as it may be found in " Transpiration and the Ascent of Sap."

Owing to the liability of all liquids of animal or vegetable origin to undergo changes when heated, the determination of boiling-points has never assumed any importance in physiology.

An elegant though tedious method based upon vapour pressure differences has been worked out by Barger (1906) in Errera's laboratory. In it drops of a solution of unknown concentration are drawn up in a capillary-tube between drops of various solutions of known strength. Isothermal distillation or condensation takes place from

or upon a drop according as its vapour pressure is greater or less than that of its neighbours. This causes the drops to decrease or increase in size, an effect which can be observed by a measuring microscope. The method has recently been employed by Halket (1913) for plant saps, and its value is obvious, as very minute quantities can be employed.

QUANTITATIVE LAWS OF OSMOTIC PRESSURE.

Before proceeding to trace the influence of various factors upon the osmotic pressure of cell sap, it seems advisable to give the term a precise meaning, and briefly to describe its nature and the conditions under which it acts. For fuller information Findlay's " Osmotic Pressure " should be consulted, and to it the author is much indebted in the following account. According to Findlay, the osmotic pressure of a solution may be defined as " the equivalent of the hydrostatic pressure produced when the solution and solvent are separated by a perfectly semipermeable membrane; or as the equivalent of the excess pressure which must be imposed on a solution in order to prevent the passage into it of solvent through a perfectly semi-permeable membrane." To be exact, the solution itself does not have any osmotic pressure, but if solvent and solution were separated by a membrane permeable to the former only, then such a pressure would be produced. Thus it is the excess of the tendency of the solvent to diffuse inwards over that to diffuse outwards that produces the osmotic pressure.

Van't Hoff, stimulated by Pfeffer's direct measurements, undertook the thermodynamical deduction of the laws of solutions, and arrived at the equation $P = \dfrac{nRT}{V}$, where P is the osmotic pressure, T the absolute tempera-

ture, R a constant almost identical with the gas equation constant, and n is the number of gramme-molecules of solute in the volume V of solution. But this he directly states to be applicable only in the case of infinitely dilute solutions, for in them the heat effect of further dilution is negligible. Thus it is not surprising that the equation was not found to hold good for concentrated solutions, and though by Morse's substitution of the volume of the pure solvent for that of the solution a better agreement is obtained between observed and calculated values, this is in reality due to its being an approximation to a more recent equation expressly constructed for concentrated solutions.

Owing to the formal identity of the gas laws and those of dilute solutions, it was thought that a modification of the equation might be capable of correctly representing the behaviour of concentrated solutions, just as those of the Van der Waal's type were found suitable for gases. Two equations of this kind have been shown to give a fair degree of agreement between calculated and observed values of osmotic pressure over a moderately extended range.

The most generally applicable equation, however, is that deduced thermodynamically for ideal solutions—namely, those composed of two unassociated liquids which are completely miscible without occurrence of heat, volume, or chemical changes. This general equation is :

$$P = \frac{RT}{V_o}[-\log_e (1-x)] - \tfrac{1}{2}aP^2.$$

In this x is the ratio of the number of molecules of solute to the total number of molecules present; V_o is the molecular volume of the solvent under the standard pressure; whilst a represents the coefficient of compressibility of the solvent. For moderate pressures the last factor may be

neglected, and the equation can be thrown into the form

$$P = \frac{RT}{V_o}(x + \tfrac{1}{2}x^2 + \tfrac{1}{3}x^3 + \ldots).$$

Now, when n represents the number of gramme-molecules of solute, and N those of solvent, $x = \frac{n}{N+n}$. Accordingly, for infinitely dilute solutions, n becomes negligible in comparison with N, and $x = \frac{n}{N}$; thus, the fraction being now very small, x^2 and higher powers may be neglected, the equation becoming $P = \frac{RT}{V_o} \cdot \frac{n}{N}$. But the water in the solution occupies the volume NV_o, which for very dilute solutions is practically the volume of the solution V. Thus one arrives at the Van't Hoff equation $P = \frac{nRT}{V}$. The latter is clearly a limiting case of the more general form.

When allowance is made for the association of water and for the formation of a pentahydrate in aqueous sucrose solutions, the values calculated by the equation for ideal solutions agree well with the direct determinations of Lord Berkeley and E. G. J. Hartley over a wide range of concentrations.

THE NATURE OF OSMOSIS.

When a solution and the pure solvent are placed in direct contact, diffusion proceeds till the concentration is uniform throughout. But when a semi-permeable membrane is interposed between them, such uniformity can never be reached. By increase of pressure, however, the vapour pressure of a solution may be raised so as to equal that of the solvent. Thus when under sufficient pressure a solution may be in equilibrium with pure solvent. The inter-

position of the membrane permits of this condition being realized, for the solution can be subjected to an additional pressure, to which the pure solvent is not exposed. In the natural course of events, this excess pressure is produced by the entrance of the solvent into the solution by the process of diffusion which is here termed osmosis.

The foregoing considerations may perhaps be rendered more easily intelligible by reference to the accompanying figure, which represents a tube in the form o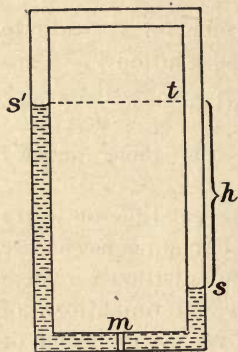f a rectangle with two sides vertical. In it a solution of a non-volatile substance is separated from the pure solvent by a semi-permeable membrane below, and by a space containing only the vapour of the solvent above. It is evident that passage of the solvent into the solution will take place till a position of equilibrium is attained. At this stage it is clear that the vapour pressure of the solvent at s must be greater than that of the solution at s' by an amount equal to the pressure exerted by the column of vapour of height h, which is the difference in level between the liquid in the two limbs. Under these conditions the vapour pressure at s' and at t, a point at the same level in the other limb, will be equal, and no isothermal distillation will take place.

FIG. 4.

Turning now to the semi-permeable membrane m, separating the liquids, since a condition of equilibrium has been reached, the net amount of transference of solvent across the membrane must be zero. This result is, of course, quite independent of the nature of the membrane, pro-

vided only that it is truly semi-permeable. It is therefore permissible to consider the membrane as a bubble of vapour contained by two rigid walls which prevent the hydrostatic pressures of the liquid columns from acting upon it. Since distillation of vapour does not take place, the vapour pressures of solvent and of solution must be equal on either side of m; but as that of the solution is normally lower than that of the solvent, it is evident that their equality in this case is due to an increase in the vapour pressure of the solution brought about by the application of a hydrostatic pressure. This is occasioned by the difference in level of solvent and solution in the tubes.

Starting from the deduction that for dilute solutions the osmotic pressure was equal to the gaseous pressure which the solute would exert if considered as occupying the actual volume of the solution, Van't Hoff suggested that such a pressure might be similar in nature to that exerted by a gas—viz., that it was due to the impinging of the solute molecules upon the membrane. His equation was, however, in no way dependent upon this hypothesis, and he clearly recognized that the phenomenon might be explained by the attraction between solvent and solute. The gas pressure theory has now been largely abandoned, as it fails to account for the behaviour of concentrated solutions satisfactorily. Its simplicity and applicability to dilute solutions both contributed to its continued employment in explaining the phenomena of osmosis.

The generally accepted view now is that osmotic pressure is a hydrostatic pressure, of such a magnitude as to bring the vapour pressures of solvent and solution into equilibrium. For the numerous hypotheses as to the manner in which this pressure is produced Findlay's book should be consulted. At present there is some evidence in favour of the view that it is due to the inactivation of the solvent

6

molecules by union with those of the solute, but this could not be a satisfactory explanation in the case of an ideal solution in which there is no such combination.

THE NATURE OF SEMI-PERMEABLE MEMBRANES.

A semi-permeable membrane is one which is permeable to the solvent only, and does not permit of the passage of solutes. Some membranes show selective permeability in that they allow certain solutes to pass through, but restrain others. It has frequently been urged that no membrane is absolutely semi-permeable, but that some small quantity of solute always penetrates. In view of the perfection to which the preparation of copper ferro-cyanide membranes has been brought by Morse and Frazer (1911), the Earl of Berkeley and Hartley (1906), and others, this objection can be no longer maintained except as a purely theoretical belief beyond the limits of experimental verification. Morse (1911), for instance, working with a sucrose solution, found that a pressure of over 12 atmospheres was sustained without any leakage for sixty days at the temperature of 15°.

The earliest view of the structure of semi-permeable membranes was that they acted as sieves, allowing the passage of molecules which were below a certain size. This conception is certainly too crude.

H. E. Armstrong (1906, 1909, 1912), using as an illustration A. J. Brown's (1912) experiments on the selective permeability of the seed coats of *Hordeum vulgare*, has sustained his theory that such membranes permit the passage of substances which are hydrated feebly or not at all, whereas those which form stable hydrates are unable to penetrate. This view is in many respects a very tempting one, as in solvents other than water quite similar

complexes are known to occur. On the whole, however, it cannot be regarded as at all well established.

The theory which has been found to command the greatest degree of credence is that which considers the membrane as a solvent, permitting the passage of those substances which are soluble in it. Though propounded as far back as 1855 by L'Hermite, it has only come into prominence comparatively recently through the work of E. Overton (1895) and others.

CHAPTER VI

THE OSMOTIC EQUILIBRIUM BETWEEN THE CELL AND ITS SURROUNDINGS

NAKED AND WALLED CELLS.

WHEN a naked cell is placed in a medium the osmotic pressure of which is lower than that of its vacuoles, water is taken up, causing distension of the protoplasm, and the process may ultimately cause the cell to burst. This occurs commonly in the lower marine animals when transferred suddenly to water of a lesser degree of salinity than that of their normal surroundings.

It has been suggested by Dixon that one function of the contractile vacuole is to remove crystalloids from the interior of the cell, for by their accumulation these would cause the vacuole to increase greatly, and so stretch the protoplasm unduly. Contractile vacuoles are never met with in cells which have a continuous wall of cellulose or other similar substance, for in these the wall sets a limit to the distension of the protoplasm. The latter lines the wall, and owing to its semi-permeable nature may be pressed against it and subject it to a considerable distending force. In some cases, as, for instance, when certain kinds of pollen grains fall into water, this force may be sufficient to rupture the cellulose wall also. As a general rule, however, the wall is able to withstand the osmotic

pressure of the cell sap.* The majority of vegetable cell walls are permeable to dissolved substances as well as to water, but some peculiar exceptions to this have recently been investigated.

Since the cellulose wall is usually in a distended condition, it follows that when attempts are made to ascertain osmotic pressure by the plasmolytic method, a very appreciable volume change occurs in many instances, before there is any perceptible forcing back of the protoplasm from the cellulose. This is owing to the diminished turgor of the cells and contraction of the cellulose walls if they belong to young tissue.

In addition to the source of error arising from the distension of the walls, there is always uncertainty as to how far the agents employed for plasmolysis penetrate the cells, for in various groups of plants marked differences are shown in this respect.

Cryoscopy of Sap.

For the reasons just mentioned, it is desirable, whenever possible, to investigate the relationship between the cell sap and the medium with which it is in equilibrium, by determining osmotic pressures by cryoscopy, combined with the equation as given by Nernst:

$P = 12.03 \ \Delta$ atmospheres at $0°$, where Δ is the depression of freezing-point of the liquid under examination.

To obtain the cell sap required, it has been shown by Dixon and Atkins (1913, 1) that it is not sufficient merely to apply pressure to plant tissues, for this results at first in the forcing out of water through the protoplasm, which is later on mixed with the contents of many burst cells.

* Determinations of the breaking stress of cellulose carried out by Dixon (1897) show that an osmotic pressure of 100 atmospheres can be withstood by cellulose walls of the dimensions usually encountered.

From its appearance, the sap might be judged to represent the composition of the vacuoles correctly, as it consists so largely of cell débris. However, numerous experiments have proved that a progressive concentration of the sap is effected by pressure, so that the first liquid expressed is more dilute than the last, whilst what remains in the tissues is even still more concentrated. Accordingly, it is necessary to render the protoplasmic membranes permeable before applying pressure. To this end the employment of narcotics such as chloroform and toluene was tried, as was also exposure to a high temperature for a short time. All of these methods were found open to objection. Finally it was ascertained that the object in view could most efficiently and accurately be attained by immersing the tissues in liquid air. As an illustration of the study of the equilibrium between the cell and its surroundings, the work of Dixon and Atkins (1913, 3) on yeast is quoted below:

The Relationship of the Yeast Cell to its Medium.

1. CRYOSCOPIC RESEARCHES.—In view of the rapid metabolism of the yeast cell as regards carbohydrates, a study of the osmotic equilibrium between it and the solution which it ferments seemed to be of interest.

It has recently been demonstrated by Paine (1911) that alcohol penetrates the yeast cell readily, a state of equilibrium being soon reached in which the ratio of alcohol in the cell to that outside is a constant, deviating only slightly from 0·85. Salts, on the other hand, penetrate to a small extent, the ratio of the internal and external concentrations being no more than 0·1 to 0·25, except in the case of poisonous substances. Indeed, it is an open question how much of this apparent absorption is really due to adsorption on the surface.

To determine the osmotic pressures, the method of thermo-electric cryoscopy was employed. The unaltered yeast-juice was obtained by freezing the solid material in liquid air and centrifuging the resulting liquid mass.

The electrical conductivities of the juice, beer, and wort, were also determined, to give an idea of the relative proportions of electrolytes and non-electrolytes concerned in the production of osmotic pressures. The apparatus was the usual one, employed by the authors in previous work. All specific conductivity measurements were carried out at 0°, and are recorded as reciprocals of the resistance in ohms, not in Siemens's units.

Both pressed yeast and that skimmed from the vats with adhering beer were employed in the investigation. The beer was removed by centrifuging or by pressing through a linen cloth by hand.*

In Table XXIV. are recorded the results thus obtained, Δ being the depression of freezing-point, P the osmotic pressure in atmospheres calculated from Δ, and C the specific electrical conductivity at 0°.

TABLE XXIV.

OSMOTIC PRESSURE OF A YEAST AND WORT.

No. of Expt.	Liquid.	Δ.	P.	$C \times 10^5$.
582	Sap of washed bakers' yeast	1·064	12·80	780
583	Sap of pressed brewers' yeast	4·082	49·10	596
593	Sap of pressed brewers' yeast	3·370	40·53	671
598	Sap of pressed brewers' yeast	4·600	55·34	607
585	Wort	1·177	14·16	149
594	Wort	1·247	15·00	150
597	No. 594 fermented 7 days in open vessel in laboratory..	1·545	18·58	207

* The brewers' yeast was obtained from Guinness's Brewery, Dublin, through the kindness of Mr. A. McMullen, of the Research Laboratory.

From the above figures it may be seen that, both in osmotic pressure and electrical conductivity, pressed yeast gives values which are much higher than those of wort. Baker's yeast, however, gives a low osmotic pressure, but a high conductivity even after washing.

The figures afforded by the sap of yeast and by the surrounding nutritive fluid may be seen in Table XXV.

On comparing the results given by beer with those of wort, it is at once apparent that, while the electrical conductivity remains much the same, the osmotic pressure becomes approximately three times as great during fermentation, when interrupted at the usual stage in the commercial process. Very complete fermentation, however, judging from the single experiment performed, occasions a fall in osmotic pressure after the initial rise, and is accompanied by a marked increase in the conductivity (see No. 597). It is, however, possible that the conditions of this fermentation were abnormal, and there was probably considerable loss of liquid by evaporation. The above-mentioned experiment is substantiated by No. 609, which is the beer of No. 606 allowed to stand at air temperature in a closed vessel with a little yeast. It will be noted that there is a fall in pressure, but a slight rise in conductivity.

Turning now to the yeast in Nos. 595, 598, 612, the osmotic pressure of the juice is much higher than that of the beer, corresponding in two cases to a difference in freezing-point of about 0·5°. In these cases the yeast was separated from the beer by centrifuging to remove adherent liquid as completely as possible, and was then frozen. This process occupies some time. In No. 612, where it was effected as rapidly as possible, less than one hour elapsed between the separation and the freezing in liquid air. In this case the divergence between the os-

motic pressure of the yeast and of the beer was the greatest observed. In Nos. 607 and 610 yeast was allowed to stand for six hours and twenty-four hours respectively after separation before freezing. The results here show a diminution in pressure, owing most probably to respiratory changes, so that it has fallen slightly below that of

TABLE XXV.

OSMOTIC PRESSURE OF YEAST AND BEER.

No. of Expt.	Liquid.	Δ.	P.	$C \times 10^5$.
595	Sap of yeast	3·907°	47·00	518
598	Sap of yeast	3·815°	45·88	558
607	Sap of yeast which was kept separated from beer for 6 hours before freezing ..	3·367°	40·51	608
610	Sap of yeast separated from beer kept 24 hours before freezing	3·243°	39·00	742
611	Sap of yeast (same sample as in 610) suspended 24 hours in running water	3·166°	38·09	653
612	Sap of yeast	3·730°	44·85	562
592	Beer of No. 595	3·417°	41·10	123
596	Beer of No. 598	3·655°	43·96	125
606	Beer of No. 607	3·417°	41·10	132
609	No. 606 kept 6 days	2·996°	35·22	145
615	No. 606 kept 7 days	—	—	146
608	Beer of No. 610	3·460°	41·62	146
614	No. 608 kept 24 hours ..	—	—	146
613	Beer of No. 612	3·188°	38·34	147

the beer from which it was removed. From Nos. 610 and 611 it appears that this decrease in osmotic pressure takes place whether the yeast is kept dry or suspended in water. This rapid falling off shows very likely the normal rate of consumption of carbohydrate with the resulting increase in conductivity. Such a lessening of pressure is under ordinary circumstances made good by the diffusion

inwards of sugar from the wort, hence this carbohydrate must be able to pass freely into the cell, while the alcohol produced passes out, maintaining a constant ratio, as shown by Paine (*loc. cit.*). A well-marked but relatively small extra fall in pressure was observed in No. 611, where the yeast, after separation from the beer, was suspended in a linen cloth in a large vessel of water with a delivery tap and overflow.

The small degree of permeability of the yeast as regards electrolytes is clearly brought out by the conductivity of the juice being from four to five times that of the beer. Even allowing for fluctuations from sample to sample, there is a well-marked rise in conductivity in yeast after its separation. While this may be due in part to decreasing viscosity of the sap owing to sugars having been used up, yet, quantitatively considered, this explanation seems insufficient, and Nos. 610 and 611 make it more probable that such a result is partly due to the retention of an acid produced in fermentation, which in the normal course would diffuse very slowly outwards. Succinic acid, for instance, and its more highly ionized ammonium salt have been shown by Ehrlich (1909) to arise during fermentation from glutamic acid.

To avoid the possibility of error in the comparison of yeast-juice and beer owing to the expulsion of gases by freezing the former solid, measurements were made of both freezing-point and conductivity of beer as separated from yeast and after freezing solid. No appreciable difference was observed between the two sets of figures.

2. RESEARCHES BY THE METHOD OF PLASMOLYSIS.—The plasmolysis of the yeast cell has lately been investigated by Euler and Palm (1914), using solutions of glycerol. It had previously been shown by Bokorny (1903) that yeast retains its fermentative power in solutions of sugars which

inhibit its inverting action, for the former process can be carried out in glucose solutions of great concentration, but not in sucrose of similar strength. Thus solutions of glucose were fermented even when containing over 70 per cent. of the sugar.

As was pointed out previously, the yeast cell must be permeable to sugars, so is not injured by such concentrations. Bokorny found the upper limit for the carrying on of fermentation by the yeast cell to be a 3·7 normal glucose solution, which possesses an osmotic pressure of about 80 atmospheres.

Euler and Palm ascertained that with the bottom yeast they employed scarcely any plasmolysis took place inside twenty hours in 15 per cent. mannitol. Glycerol of the same concentration (not the same molecular concentration, however *) produced plasmolysis of the oldest cells within fifteen minutes. With 25 per cent. glycerol this occurred almost instantaneously. Multiplication of the cells took place in 15 but not in 20 per cent. glycerol.

They also showed that a certain percentage only of the cells were plasmolyzed after two hours in solutions of various strengths. Each result is based on ten counts of plasmolyzed and unplasmolyzed cells. The figures so obtained are tabulated below and shown graphically in Fig. 5.

TABLE XXVI.

Percentage of Glycerol.	Percentage of Yeast Cells plasmolyzed after Two Hours.
10	17·1
15	25·7
20	38·4
25	71·0

* The molecular concentration of 15 per cent. glycerol is 1·66 per litre; that of 15 per cent. mannitol is only 0·82 per litre.

The 25 per cent. solution is 2·77 normal, and has a calculated osmotic pressure of about 70 atmospheres. Owing to the association of the glycerol molecules, the osmotic
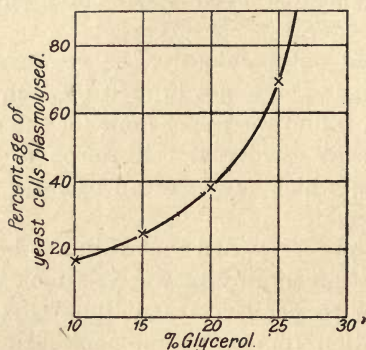

FIG. 5.

pressure may, however, be considerably less in reality. Inspection of the figure makes it clear that, whilst some
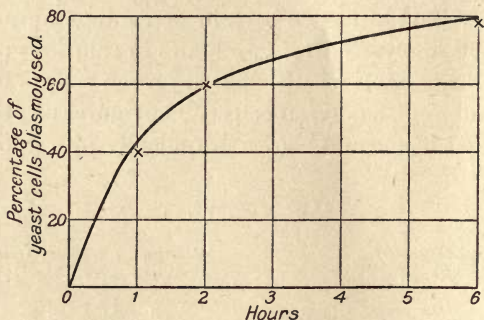

FIG. 6.

of the cells, the oldest ones, had comparatively low osmotic pressures, others could withstand very great external pressures without plasmolysis. The values obtained by

Dixon and Atkins (*loc. cit.*) are of course mean values, for the sap of millions of cells was taken for each freezing-point determination.

Culture experiments in 15 per cent. glycerol made up with Lindner's solution showed that the number of yeast cells approximately doubled itself in two days. When, however, the cells used to sow this solution had themselves been in it for some days, it was found that their increase was much more rapid. Thus after four days' treatment the number sown afresh increased to 4·3 times their original number, as against 2·0 times without treatment. This curious result is probably due to the much larger number of young cells in 15 per cent. glycerol solution, for old cells are plasmolyzed by this concentration.

Furthermore, Euler and Palm obtained evidence that, when yeast cells cultivated for twelve hours in Lindner's solution containing 5 per cent. glycerol are subsequently placed in 25 per cent. glycerol, the curve obtained by plotting percentages of cells plasmolyzed, against time in hours, is of a logarithmic nature as shown in Fig. 6. The results are tabulated here.

TABLE XXVII.

Time of Experiment in Hours.	Percentage of Yeast Cells plasmolyzed.
1	41
2	60
6	78

It was previously shown that without the preliminary treatment 71 per cent. were plasmolyzed in two hours. The difference between this figure and that in the above table is probably due to the cells being on the whole younger in this experiment, but perhaps it is in part occasioned by a slight penetration of the glycerol.

The above investigation makes it clear that, whilst it

is not as accurate as the cryoscopic method for the determination of osmotic pressures, the method of plasmolysis is nevertheless of great value in obtaining information quite inaccessible by cryoscopy.

It seems that the hæmatokrit of Köppe (1895), employed by Hedin (1895) and Hamburger (1902) and others for investigations on the volume changes in blood-corpuscles, might also be used for yeast. Euler, however, preferred to use the plasmolytic method, for the hæmatokrit gives average results just as the cryoscopic method does.

COMPARISON OF THE CRYOSCOPIC AND PLASMOLYTIC METHODS OF MEASURING OSMOTIC PRESSURES OF PLANT CELLS.

As has been pointed out on p. 85, the application of pressure alone is not sufficient to obtain the unaltered sap of the vacuoles. The cells must be rendered permeable by intense cold. Through want of knowledge of this factor, the cyroscopically determined values recorded in all the papers by Dixon and Atkins up to 1912 inclusive are too low, by amounts varying from a few per cent., in soft tissues such as potato tubers, up to several hundred per cent. in a tough leaf such as that of *Chamærops humilis*. The same criticism applies equally to the experiments of Sutherst (1901), Cavara (1905), Heald (1902), Nicolosi-Roncati (1907), Trinchieri (1909), Marie and Gatin (1912), and Ohlweiler (1912). It is of course obvious that determinations of electrical conductivity and chemical analyses of such pressed saps are no less vitiated than are those of osmotic pressures. The results obtained with a particular organ of any plant under varying conditions seem, however, to be quite truly comparable.

Since the source of error due to alterations in concentration induced by pressure can now be avoided, cryoscopy

affords the readiest and most accurate method of measuring osmotic pressures when sufficient material is available.

The plasmolytic method is, on the other hand, applicable to the study of individual cells, and for this reason alone has an extended range of usefulness. Contraction of the distended cell before actual plasmolysis ensues may be, however, a very considerable source of error. For if the diameter of a cell, assumed to be spherical, decreases by 10 per cent. from this cause, its volume is diminished by 27·1 per cent., whilst even a shrinkage of 2 per cent. in diameter occasions a volume change of 5·9 per cent. De Vries (1877) has shown that the linear dimensions of young and turgid cells may be diminished by as much as 20 per cent. before plasmolysis is apparent. It has been pointed out by Schwendener and Krabbe (1898) that scarcely any extension is normally produced in the walls of grown cells.

In addition to this channel for the entrance of inaccuracies, there is the possibility that the cell may not be completely impermeable to the substance used to produce plasmolysis, which tends to raise the result still more. Also it must be remembered that even the same cell may alter its permeability under various conditions of external medium, of illumination, or at different periods of its life. The changes induced in the permeability of the cells of the pulvinus in *Mimosa pudica* by stimulation furnish a striking example of this.

CHAPTER VII

THE PERMEABILITY OF PROTOPLASM

THE YEAST CELL.

VERY numerous researches have shown that protoplasm is freely permeable to some substances, and practically impermeable to others. That yeast cells (see Table XXV.) should yield a sap the electrical conductivity of which is $C_{0^\circ} \times 10^5 = 742$, whereas the beer from which they were removed affords the value $C_{0^\circ} \times 10^5 = 146$, shows clearly how impermeable these cells are to electrolytes. But to alcohol they are freely permeable, also to glucose, as this is the source of the alcohol which is formed inside the cell. Paine's investigations on this subject have already been mentioned in the preceding chapter.

THE SECRETORY ACTIVITY OF COLOCASIA LEAVES AND OF THE PITCHER OF NEPENTHES.

Other cells appear to be completely impermeable to both electrolytes and sugars. As an instance of this may be cited the examination by Dixon and Atkins of the liquid secreted by the leaf-tips of *Colocasia antiquorum*. When in a warm saturated atmosphere the leaves give a continuous succession of small drops from the extreme ends. These may amount to several per second under favourable conditions. A large quantity of this liquid was collected on various occasions in the greenhouses of the Botanic Gardens

of Trinity College, Dublin. On evaporation of 20 c.c. only 0·012 per cent. of dry residue was left. The secretion, when examined by the thermo-electric method of cryoscopy, was found to freeze at almost exactly the same temperature as distilled water. This method, it should be remembered, is differential, as one thermocouple is placed in the secretion, and the other in distilled water. Below are recorded the values obtained for the freezing-point and electrical conductivity of the secretion. It will be noticed that the [conductivity is less than that of the Dublin tap-water. The latter is extremely pure and soft, and is obtained from the reservoir in the Wicklow Mountains. No trace of sugars could be detected in the secretion, even when a large volume was concentrated. A trace of a chloride was, however, found.

When the tip of the leaf was cut off, it was found that the conductivity of the secretion was at first three times as great as before. But after twenty-four hours it had fallen to something less than twice as great, probably because electrolytes from the injured cells had all been removed. At this stage the conductivity was slightly greater than that of Dublin tap-water, so there is no doubt that there is a separation of electrolytes as the secretion passes out from the uninjured leaf.

In the unopened pitcher of Nepenthes sp. there is, on the other hand, a secretion of liquid which contains a quite considerable quantity of solutes, mainly electrolytes, as may be seen in the table on p. 98. The liquid lodged in the leaf-bases of Dipsacus also contains appreciable quantities of electrolytes.

Under Δ_e are recorded the values of the depression of freezing-point due to electrolytes calculated from the conductivity, as explained in Chapter IX. Owing to the small values of Δ, as directly determined, errors of experi-

ment become of serious importance. This is not felt to anything like the same extent in the conductivity measurements, and they are seen to be very uniform in the table below.

TABLE XXVIII.

OSMOTIC PRESSURES AND ELECTRICAL CONDUCTIVITIES OF SOME SECRETIONS.

Description of Sample.	$\Delta.$	$\Delta e.$	$P.$	$C \times 10^5.$
Colocasia antiquorum, leaf secretion, October 16	0·000	0·0015	—	3·02
Dublin tap-water, October 16 ..	0·000	0·0016	—	3·30
C. antiquorum, leaf secretion, October 18	0·042	0·0023	0·50	4·84
C. antiquorum, young leaves, secretion, March 23	0·006	0·0015	0·07	3·05
C. antiquorum, old leaves, secretion, March 23	0·003	0·0016	0·03	3·23
C. antiquorum, secretion from tip of intact leaf, June 22 ..	0·003	0·0012	0·04	2·56
C. antiquorum, secretion from same leaf, after cutting off the tip, June 23	0·033	0·0036	0·40	7·93
Dublin tap-water, June 23	—	0·0019	—	3·85
C. antiquorum, secretion from same leaf, June 24	0·008	0·0020	0·10	4·02
Dipsacus sp. fluid lodged in expanded leaf-bases, June 21	0·039	0·0289	0·47	57·79
Nepenthes sp., unopened pitcher, secretion, May 15	0·154	0·1270	1·86	277·5

The measurements carried out upon Colocasia secretion show that normally the cell solutes do not pass outwards even when there is a rapid stream of water.

THE PENETRATION OF SALTS INTO LEAVES.

Concerning the entrance of salts some interesting facts have been brought to light by Lewis (1912). This worker studied the effect of immersing the leaves of various non-

halophytes in sodium chloride solutions for considerable periods.

In preliminary experiments leaves were immersed in sea-water, and after some hours sections were cut with a dry razor and mounted in $\frac{N}{10}$ silver nitrate. These were then exposed to the light of an electric lamp for twenty minutes at a distance of one foot. Leaves so treated always showed a much more intense reaction for chloride than similar leaves examined as a control. The treated leaves were in every case thoroughly rinsed before testing. The chloride appeared in greatest quantity in the upper and lower epidermis and in the palisade cells. This observation shows that the quantitative results, to be quoted shortly, are not explicable by the entrance of salt solution into the intercellular spaces.

The leaves used for the quantitative determination of chloride were thoroughly washed and dried at 100° C. for an hour and a half. They were subsequently incinerated at a temperature sufficiently low to avoid any appreciable volatilization of sodium chloride. Titration was then carried out in the usual way with silver nitrate after neutralization of the slightly alkaline ash with nitric acid. The same individual plants were used throughout all the experiments, and the leaves were kept in darkness, between the successive weighings for water determinations. In every case the leaves were cut freshly from the plant and their petioles sealed with paraffin wax. At three-hour intervals they were removed from the salt solutions and weighed after drying quickly. By this means both the variations in total weight of the leaves were studied, as well as the final change in chloride content.

The subjoined table shows the amount of sodium chloride in the ash, calculated as a percentage of the dry weight of the leaves. Three experiments with each type of leaf are

recorded. In 1 and 2 the tissue was placed in 3·042 per cent. sodium chloride, whereas in 3 sea-water containing 2·5155 per cent. of this salt was used. The duration of experiment was in each case twenty-seven hours. At the end of this time the cells were not plasmolyzed, for they had recovered initial plasmolysis owing to penetration of the salt. They were, however, rapidly plasmolyzed when placed in 10 per cent. sodium chloride. Transference to tap-water resulted in speedy recovery. This shows that the protoplasm was not seriously damaged.

TABLE XXIX.

ABSORPTION OF SODIUM CHLORIDE BY THE LEAVES OF SOME NON-HALOPHYTES.

Plant.	NaCl in Ash of Untreated Leaves	NaCl in Ash of Treated Leaves.
	Per Cent.	Per Cent.
1. *Camellia japonica*	0·2	0·9
2. „ „	0·2	0·6
3. „ „	0·2	0·3
1. *Ilex aquifolium*	0·3	1·2
2. „ „	0·5	0·8
3. „ „	0·3	0·9
1. *Syringa vulgaris*	0·8	4·5
2. „ „	0·7	4·6
3. „ „	0·9	4·3
1. *Cavendishia acuminata*	0·2	1·7
2. „ „	0·2	2·6
3. „ „	0·3	2·4
1. *Arum maculatum*	0·9	8·5
2. „ „	1·3	5·1
3. „ „	0·8	6·7

By these experiments Lewis found that *Camellia japonica*, *Syringa vulgaris*, and *Arum maculatum*, showed at first a decrease in weight both in sea-water and in the salt solution of approximately the same strength. After the first

three to six hours this was succeeded by a progressive increase in weight (except in the case of Arum, where there was a loss). *Ilex aquifolium* and *Cavendishia acuminata* showed a progressive increase in weight from the time of first immersion to the end of the experiment. It must, however, be noted that the first weighing was not made till three hours had elapsed, so the initial loss may have been missed. An increase in sodium chloride was found in all the treated leaves. The subsequent recovery of the initially plasmolyzed cells was due, as previously pointed out, to gradual penetration of the salt.

Of surpassing interest in this connection is the work of Overton, Osterhout, and Czapek, by the method of plasmolysis and by electrical conductivity and surface tension measurements. These researches have imparted to such investigations a degree of exactness hitherto unknown.

Overton's Theory of the Lipoid Surface Film.

It was shown by Overton (1895) that the simple alcohols, aldehydes, ketones, esters of fatty acids, and alkaloids, do not as a rule produce plasmolysis. Accordingly, it is evident that to them the protoplasmic surface is permeable. With glycols plasmolysis is more easily effected, whilst glycerol and erythrite act even still more readily. On the other hand, all sugars, amino-acids, and salts of organic and inorganic acids, bring about plasmolysis with rapidity.

As pointed out by Overton, his results as to permeability may be summarized in the statement that substances which are soluble in fat pass through the protoplasmic membrane with readiness. From this he concluded that a thin film of lipoid substances constitute the outer layer of the protoplasm. The term lipoid has been used by some authors to denote any fat-like substance soluble in

ether and chloroform, whilst others restrict it to those which are non-saponifiable. As all substances which lower the surface tension of a liquid tend to become more concentrated at the surface than throughout its bulk, it is only to be expected that such fatty bodies would accumulate on the outer surface of protoplasm, and also on the vacuole wall. A summary of these researches is given by Czapek in " Chemical Phenomena in Life."

It must be added that Ruhland (1909) has shown that many dyes fail to penetrate the plasmatic surface though freely soluble in lipoids, whilst others insoluble in lipoids and forming colloidal solutions in water enter readily. Methyl orange is an example of the latter class.

Surface Tension of Protoplasm.

Czapek (1910) measured the surface tension of plant cells by noting the diffusion from them of tannin, anthocyanin, etc., when in contact with various solutions; for he found that media which brought about such changes always had equal surface tensions, amounting to about two-thirds that of water—viz., that of 11 per cent. aqueous ethyl alcohol. It was also shown by Czapek that emulsions of fatty bodies can cause injurious effects if their surface tensions are sufficiently low, as in the case of lecithin or cholesterin. Czapek's view of the constitution of the surface film of protoplasm is that it consists of fat in the form of a very fine emulsion. The aqueous phase filling up the spaces between the globules of fat contains a colloidal protein solution.

Differential Permeability.

It has been usual, in studying permeability, to assume that one surface only need be considered—namely, the outer plasmatic membrane. Osterhout (1913, 3) em-

phasized the fact that several surfaces may be involved, and prefers to speak of surfaces rather than membranes, since a definite membrane is not essential for semi-permeability.

To investigate such surfaces the marine alga Griffithsia was employed. Within its cell wall a thin layer of protoplasm surrounds a central vacuole, which expands or contracts according as water is taken in or given out by osmosis. Such cells diminish in volume when placed in hypertonic sea-water, but regain their normal size when returned to their original surroundings. If the cell be placed in hypertonic ammonium chloride, instead of in hypertonic sea-water, contraction takes place, but the inner wall shrinks to a greater extent than does the outer. Thus the space between the two surfaces, which is normally very small, may increase until in places it is equal to one-third of the length of the cell. Thus a distinction must be drawn between the outer surface of the protoplasm, the plasmatic membrane, and the inner surface, the vacuole wall. The explanation of such behaviour may be that the outer is more permeable to ammonium chloride than the inner. The salt would therefore have a stronger plasmolyzing action on the inner surface, since the more readily a substance penetrates, the smaller is its efficacy as a plasmolyzing agent. The alternative interpretation is that the permeability of the surfaces may be altered by the ammonium chloride, and the contraction may be due to " false plasmolysis," as certain appearances of plasmolysis produced by injurious actions are termed. The behaviour of Griffithsia could then be accounted for by the supposition that the false plasmolysis of the inner surface was greater than that of the outer. Osterhout inclines to the opinion that both effects contribute to the result.

With a lower concentration of ammonium chloride a marked contraction of the inner surface can be produced while the outer still maintains its turgidity. That such a condition is due, in part at least, to false plasmolysis is shown by the fact that it may be brought about by hypotonic solutions, tap-water, or even distilled water.

In the protoplasm of this alga are embedded numerous chromatophores, containing chlorophyll and a red pigment soluble in water. When separation of the inner and outer protoplasmic surfaces has proceeded to a certain degree, the surfaces of the chromatophores becomes permeable to the red pigment, as is shown by its diffusion into the protoplasm. The outer and inner protoplasmic surfaces still remain impermeable to it, so the vacuole and surrounding solution are uncoloured. This condition may be maintained for a couple of hours, when suddenly the pigment begins to diffuse from the protoplasm. At the start the nuclei are not penetrated by the colouring matter, but soon after diffusion from the chromatophores has taken place they also become red.

From such researches it is clear that the various surfaces of a cell exhibit differences in permeability. For these phenomena Osterhout has suggested the term differential permeability. This conception may be extended to all intracellular surfaces, down to those which are ultramicroscopic.

FALSE PLASMOLYSIS.

Appearances almost or quite indistinguishable from true plasmolysis may be brought about by hypotonic solutions, or even by distilled water. These Osterhout (1913, 2), to whom the investigations on this subject are due, has termed false plasmolysis.

The root-tips of *Zostera marina* were employed as suitable for study, and were mounted in their normal medium,

sea-water. They were attached to the cover-glasses which formed the roof of an irrigation chamber. After definite root-hairs had been sketched by the aid of a camera lucida during irrigation with sea-water, the latter was changed for distilled water. This had been carefully prepared by using glass vessels only, and plugs of absorbent cotton as stoppers. It was not toxic to sensitive species of Spirogyra or root-hairs of Gypsophila, whereas water distilled from metal stills is poisonous to such cells.

Within half an hour of the application of distilled water, the Zostera cells were plasmolyzed, so as to remain in contact with the walls at the lower side only. This slowness of action is not, however, a characteristic of false plasmolysis, for the colourless terminal hairs of *Polysiphonia violacea* contract rapidly, a marked reaction being visible at the ends of the cells within two minutes from their immersion in distilled water. Additional proof that this was not due to any toxic action of the distilled water was furnished by the fact that pond, river, or spring water could be substituted for distilled with the same result.

The phenomenon may in part be accounted for by an increased permeability of the cell surfaces, as the result of which the substances which maintain the osmotic pressure diffuse out. That the effects are not due to an absorption of water is shown by the failure of the cells to increase in size, as well as by the action of isotonic solutions of cane-sugar, which produce the same alterations as distilled water, though not so rapidly.

The increase in permeability appears to be occasioned by the loss of substances upon which the maintenance of normal conditions depends. Of these, the inorganic salts are the most important, for in sea-water to which an equal volume of distilled water has been added the cells remain turgid, whereas when three volumes of fresh water are

added to one of sea-water shrinkage takes place in seven hours, and increases in rapidity if the proportion of fresh water be further augmented. This change in permeability has been quantitatively studied by an electrical method to be described later.

Osterhout points out that the observed shrinkage in volume of the protoplasm itself when transferred from sea to fresh water directly negatives the theories of those biologists who seek to explain effects, usually attributed to osmotic pressure, as being due to imbibition or to the giving up of water by the protoplasm.

THE PERMEABILITY OF PROTOPLASM TO SALTS AND IONS.

Though salt solutions, when sufficiently concentrated, bring about plasmolysis readily, yet it is not correct to suppose that the protoplasm forms an impenetrable obstacle to the entrance of the solute. The passage of sodium chloride into leaves was demonstrated by the work of Lewis described previously. Quantitative researches by the author (1909) on the absorption of water by the seeds of *Phaseolus vulgaris* showed that living dry seeds took up as much water from a decinormal solution of sodium chloride as did those which had been killed by chloroform vapour. There was no concentration of the solute in the surrounding liquid, which shows that it penetrated in the same proportions as did the solvent.

Among the most complete studies upon the penetration of salts into protoplasm must be considered those of Rufz de Lavison (1911). This investigator studied their entry into the central cylinder of roots through the protoplasm of the cells of the endodermis and found that with weak concentrations the kations of salts of the alkalies and alkaline earths, united to non-toxic anions, penetrated the

protoplasm readily. Salts of barium and of cæsium, and iodides in general, only entered with difficulty. Certain salts of aluminium, yttrium, and the majority of the salts of the heavy metals, did not penetrate at all.

He further ascertained that the permeability of protoplasm to solutions of low concentration is quite different to that with respect to high concentrations. The latter enter easily, without killing the protoplasm. Thus it acquires certain conditions of dead protoplasm, and considered as a membrane it is a modifiable one. In this connection Osterhout's researches quoted farther on are of interest.

Endler (1912) also has investigated the permeability of protoplasm by following the influence exerted by various salts upon the penetration of a dye, such as neutral red. He showed that neutral salts, in dilute solution, favoured the entry of the dye into living cells of *Ulva lactuca*, Vaucheria sp., *Nitophyllum punctatum*. Increase in concentration, however, resulted in a maximum for dye absorption being reached, after which the penetration of the colouring matter fell off and finally ceased. The curves given by various salts resembled one another in form, but exhibited qualitative differences. A table showing the order in which the various anions came with regard to their effect upon stopping the entry of the dye was also drawn up by Engler. The behaviour of the anions lent itself more readily to comparison *inter se* than did that of the kations, for the differences between the latter are not so sharply defined. Endler later on (1913) tried to measure the iso-electric point of protoplasm, by a method based upon the influence of hydrion and hydroxylion upon the coloration of cells.

The penetration of dyes, acids, alkalies, and other substances, has been studied by various physiologists, but

to such experiments the objection has been raised that the permeability of the protoplasm had been thereby altered, and that it was not shown by subsequent observation that the cells remained uninjured. Osterhout (1913, 1) investigated the absorption of calcium salts by growing roots in distilled water, whereby the root-hairs were obtained free from crystals. On being placed in tap-water or dilute solutions of calcium salts, the appearance of crystals of the oxalate could soon be observed, thus proving the penetration of the calcium salts. These cells continued to develop in a normal manner. The absorption of oxygen and excretion of carbon dioxide by plant cells shows that the protoplasm is permeable to these substances.

PRECAUTIONS NECESSARY IN PLASMOLYTIC INVESTIGATIONS.

In order to guard against errors in the plasmolytic method, previously overlooked, attention was directed by Osterhout (1913, 1) to the following points:

1. The necessity of finding a solution which could be used as a standard in comparative studies. The chief requirement of such a medium is that it should have a purely osmotic action, involving no alteration of the normal permeability of the plasmatic membrane. Quantitative measurements by the electrical method showed that reagents such as solutions of sucrose, sodium chloride, or potassium nitrate, were not without an injurious action, though previously considered to be quite free from objection on this score. It was shown, however, that sea-water fulfilled the requirements of such a solution, for in it the various salts are present in such proportions that their toxic effects mutually balance each other. Accordingly sea-water, suitably concentrated or diluted, was taken as the standard medium for investigations upon plasmolysis.

2. It is important to make frequent observations, keeping the same individual cell under observation throughout.

3. A distinction must be drawn between true and false plasmolysis, and where this is impossible the results must be rejected.

4. In experiments in which the recovery of the cell after plasmolysis is the proof of permeability, care should be taken to maintain a constant temperature, for the process takes place more rapidly with increase of temperature. Controls in sea-water should be examined at various temperatures where constancy cannot be secured. Plasmolysis should be carried to the same degree in each case, as the rate of recovery is reduced when the action has proceeded to any considerable extent.

Uniformity of material is also necessary. Osterhout employed long filaments of Spirogyra, of Chætomorpha, or portions of the leaf of Elodea.

RATE OF PENETRATION OF SALTS AND IONS.

Since the more rapidly a plasmolyzing agent penetrates a cell the shorter will be the time required by the latter to recover from plasmolysis, it is clear that a measurement of these times furnishes a comparison of the relative rates of penetration of various substances. This may also be determined by the following method: When two compounds are used to produce the same degree of plasmolysis, it is evident that the molecular concentration, and consequently the osmotic pressure, must be the same in each case, provided both fail to penetrate or do so to the same degree. If, however, one enters the cell more readily than the other, it will require a higher concentration to produce plasmolysis than does the more slowly penetrating body. Accordingly, by obtaining the ratios of the osmotic pressures of the solutions with respect to that of a balanced solution

which plasmolyzes to the same extent in the same time and at the same temperature, a measure of the relative rates of penetration is furnished. The results of these two and of the electrical method agree well.

CRITERIA OF PENETRATION OF SALTS AND IONS.

Overton considered inorganic salts as incapable of penetrating living cells. They are, it must be remembered, almost entirely insoluble in lipoids. Osterhout (1913, 1), repeating such experiments on Spirogyra, the material used by Overton, found that very many salts enter cells readily. That they should do so is quite easily understood if Czapek's view of the nature of the protoplasmic surface is adopted. The recovery from plasmolysis of cells left in solutions of salts of ammonium, rubidium, sodium, cæsium, potassium, lithium, magnesium, calcium, strontium, and aluminium, shows that all these salts can enter.

The most striking proof of the penetration of the salt is given by the following experiment devised by Osterhout: A filament of Spirogyra, divided into several portions, was found to be plasmolyzed by 0·2 N calcium chloride and by 0·38 N sodium chloride, but not in solutions of these salts of normalities 0·195 and 0·375 respectively. On mixing 100 c.c. of the sodium salt with 10 c.c. of the corresponding calcium solution, other portions of the same filament underwent rapid plasmolysis on immersion. Thus by mixing two solutions, neither of which is able to plasmolyze, there results a solution which plasmolyzes strongly. The calcium chloride solution has, be it noted, a much lower osmotic pressure than the sodium chloride solution, but owing to its slower rate of penetration a smaller concentration is as efficient a plasmolyzing agent as a solution of sodium chloride having a higher concentration.

Evidently the remarkable result produced by the mixture is brought about by a mutual hindrance of each salt upon the entrance of the other into the cells. The antagonistic action of various metallic ions has been studied in detail by Loeb (1906), who has summarized many of his researches in " Dynamics of Living Matter." The experiment just described shows that the toxic action of each salt upon the protoplasm is diminished by the presence of the other because their rate of penetration into the protoplasm is greatly reduced.

This conclusion is supported by the fact that recovery from plasmolysis is very slow in a balanced solution of the chlorides of sodium and calcium as compared with a solution of the sodium salt only. Thus in one species of Spirogyra recovery requires ten hours when in a solution of the two chlorides in which the molecular proportion of sodium to calcium is a hundred to one, whereas when in pure sodium chloride half an hour is sufficient.

It is highly probable that the outer surface of the protoplasm is not the only one at which the sodium and calcium ions exert a retarding influence, but that at all the other surfaces, down to those which are ultramicroscopic, a similar delay in penetration is occasioned. It is not clear why, since the salts ultimately penetrate the protoplasm through and through, a retardation in the rate of entry should be so important. However, there are several instances in colloid chemistry of the influence which the rate of addition of an electrolyte has upon the ultimate condition of the system.

Osterhout summarizes the results of these researches as follows:

1. Overton's hypothesis is untenable, since it requires that only those salts which are soluble in lipoid should be able to penetrate.

2. Evidence has been given of the correctness of Loeb's suggestion that the antagonistic action of one salt on another is due to the fact that the one hinders the other from entering the cell.

3. The facts brought to light are not at variance with the view that the plasmatic membrane is proteid rather than lipoid.

4. The usual plasmolytic methods of determining osmotic pressure by salt solutions are at fault.

5. The confusion between true and false plasmolysis has often led to error.

These conclusions have been tested by the electrical method also, in which the resistance of living tissue is measured.

The Electrical Conductivity of Living Cells.

The work just described shows that salts enter living cells, but throws no light upon the question of whether penetration is effected by the ions or by the undissociated molecules. To decide this question, Osterhout (1912, 1) made conductivity measurements upon living cells in various solutions. For this purpose material which is not injured by weak currents must be employed. It should also be uniform in texture. In addition it is desirable that the current should pass through as large an area as possible to minimize the effect of local irregularities. This is most readily secured by using thin sheets of tissue closely packed together and separated only by films of the solution. The sheets should also be sufficiently rigid to permit of manipulation involving the application of pressure to insure that the solution films are very thin.

Such conditions being all fulfilled by the fronds of varieties of Laminaria, these were accordingly used by Osterhout throughout the investigations.

For each experiment from 100 to 200 discs were cut from the fronds and packed together, like a pile of coins, to form a cylinder 5 to 10 centimetres long and about 1·3 centimetres in diameter. These were clamped by a ring of glass rods attached to a block of hard rubber at each end. Each rubber disc carried a platinum electrode, covered with platinum black, against which the algal tissue was pressed by means of a screw. The area of the discs of living cells amounted to from 265 to 530 square centimetres. The measurements of resistance were carried out in the usual manner by means of a Wheatstone bridge, and the figures recorded refer to readings taken between 18·0° and 18·2°.

The procedure adopted was to immerse the cylinder in a solution for some time, and then to measure the resistance after the apparatus had been removed from the bath and superfluous liquid had been allowed to drain away.

Repeated determinations upon the same roll of discs immersed in sea-water showed by their consistency that even prolonged treatment did not damage the cells, for further work demonstrated that injury is accompanied by a fall in resistance.

The resistance of such a cylinder was found in one case to be 1,100 ohms, whereas that of a cylinder of sea-water of equal dimensions was 320 ohms. Thus the additional resistance was due to the living protoplasm and walls of the cells, for the salts within the tissue are here closely similar to those of the sea. That the difference was occasioned almost entirely by the living cells is made clear by the fact that when these are killed by a 2 per cent. solution of formalin in sea-water, or by drying carefully, the resistance fell to about 320 ohms. This observation further shows that there is no doubt that the ions, which carry the current, penetrate more rapidly into dead than into living protoplasm.

The two following experiments are described as typical of a number which were performed to determine the rates of penetration of various ions:

A cylinder of Laminaria discs was found to maintain a resistance of 1,100 ohms unchanged for four hours when in sea-water. It was then transferred to 0·52 N sodium chloride, which has the same conductivity as sea-water at the same temperature. Each disc was carefully rinsed and replaced in the clamp; such manipulation, when carried out in sea-water, only altered the reading slightly.

Immersion for five minutes in 0·52 N sodium chloride sufficed to lower the resistance to 1,000 ohms; after ten minutes it had fallen to 890, after fifteen to 780, after sixty to 420 ohms. This fall continued till a resistance of 320 ohms was reached, at which point a constant reading was obtained. Thus at the end the tissue had practically the same conductivity as sea-water. When replaced in sea-water it failed to recover any of its resistance, even when allowed to stand for several days. As the sodium chloride was nearly isotonic with sea-water, none of the observed toxic action could have been due to osmotic effects.

It was shown, however, that replacement in sea-water, at the stage at which the resistance of the tissue had fallen only about 100 ohms below its original value, always resulted in complete recovery, the resistance rising to the initial figure.

Thus the rapid alteration in permeability brought about by sodium chloride solutions is reversible when not permitted to proceed too far.

When living tissue is placed in a solution of calcium chloride having the same conductivity as sea-water, instead of decreasing, the resistance rises in a very marked manner. Thus it may increase from 1,100 ohms to a maximum of

1,750 within fifteen minutes, remaining stationary at this for some hours. It then slowly sinks, finally reaching the value of about 320 ohms as in sodium chloride. If replaced in sea-water, soon after the maximum has been reached, the original resistance is regained and maintained unchanged.

When the two chlorides are combined in the same proportions as those in which they are present in sea-water, their antagonistic action is clearly shown. For when placed in such a mixture, diluted till its conductivity was the same as that of sea-water, the Laminaria discs neither gained nor lost in resistance within twenty-four hours.

Thus it is evident that the presence of a relatively small number of calcium ions greatly delays the penetration of sodium ions.

Further experiments proved that the chlorides of potassium, magnesium, cæsium, rubidium, lithium, and ammonium, also the bromide, iodide, nitrate, sulphate, and acetate of sodium, act in general like the sodium chloride, though with different degrees of rapidity, whereas the chlorides of barium and strontium act like that of calcium. Apparently only the positive ions are effective in these changes.

With regard to the increase of resistance occasioned by transfer of tissue into calcium solutions two hypotheses have been formed. One regards the plasmatic membrane as unchanged, and explains the increased resistance as due to the protoplasm being normally less permeable to calcium than to sodium ions.

Certain facts, however, favour the other hypothesis, that the plasmatic membrane itself undergoes an alteration as the result of the transfer. Thus Osterhout calls attention to certain changes in the appearance of the protoplasm brought about by calcium solutions. Alum,

too, when added in the solid condition to sea-water, greatly increases the resistance of protoplasm, although it decreases that of the water.

As pointed out in connection with the plasmolysis experiments, the antagonistic action of various ions may be satisfactorily explained by Loeb's suggestion, that they mutually hinder penetration into the cells. It must be remembered, in connection with objections which have been raised to the method of plasmolysis, that the relative powers of penetration and of antagonism of various salts have been shown to be independent of the concentration within wide limits.

VARIATIONS IN THE PERMEABILITY OF THE LIVING CELL —INFLUENCE OF SALTS AND ANÆSTHETICS.

It is held by some that permeability is a fixed property of the cell, being only altered as the result of injury, in which case the change is irreversible.

The researches of Osterhout (1912, 2), however, afford conclusive evidence that permeability may be increased or decreased within certain limits, and that no injury results from such alterations. For by means of sodium solutions a decrease of over 10 per cent. was effected in the electrical resistance of tissue, whereas the normal value was recovered by restoration to sea-water. This was repeated for fifteen days in succession without any alteration in the normal resistance. Similarly, treatment with salts of calcium or lanthanum resulted in a larger resistance being met with, but this, too, was reversible and capable of repetition for several days without evil effects.

With regard to the action of anæsthetics very conflicting views have been held. They were generally believed to increase permeability, though some physiologists thought the opposite to be the case. Undoubtedly the effect of

applying ether, toluene, and chloroform, in quantity to plant cells is to kill them and render them permeable. Thus, Giglioli (1911) drew attention to their effect, and that of essential oils, upon transpiration of water from leaves and upon the permeability of the yeast cell (1912). Armstrong (1910) and his co-workers have shown that under the influence of anæsthetics, and of certain substances termed by them hormones, actions take place within the cells by which enzymes and their substrates are brought into contact. Varied phenomena ensue, such as liberation of hydrocyanic acid from certain glucosides, and oxidations resulting in pigmentation. Dixon and Atkins (1913, 1) also have found that anæsthetics greatly increase permeability, for cell sap is much more readily pressed out after their application. It must, however, be considered whether these are toxic and irreversible, as distinct from true anæsthetic effects, which are reversible.

To decide this important question, Osterhout (1913, 1) measured the resistance of tissue after exposure to anæsthetics. He found that very low concentrations of these substances produce little or no effect, but a point is soon reached at which a decided decrease of permeability occurs. With still further increase in concentration this diminution becomes more pronounced, and may be maintained for several hours in presence of the anæsthetic. This change of permeability is completely reversible and without injury to the cells.

If, however, a larger amount of anæsthetic be administered, an additional decrease in permeability is observed. This is followed by a rise to far above the normal, and if the concentration be sufficiently high the increase continues till death supervenes.

In these experiments it is seen that the decrease in permeability is easily reversible, whereas the increase is not.

For it is only in exceptional cases, when it has proceeded but a very little way, that the process can be even partially reversed. These facts all favour the view that the true anæsthetic action is due to a decrease in permeability.

Similar investigations by the electrical method have demonstrated that sucrose, glucose, and glycerine, substances so frequently employed in plasmolytic researches, all cause a marked, but reversible, increase in permeability.

TABLE XXX.

EFFECT OF LIQUID AIR IN RENDERING PROTOPLASM PERMEABLE.

Sap from—	Δ.	$C \times 10^5$.	$\dfrac{C \times 10^3}{\Delta}$.
Hedera helix, leaves untreated	0·767°	403	5·2
Same sample frozen	1·255°	605	4·8
Ilex aquifolium, roots untreated	0·531°	563	10·6
Same sample frozen	0·682°	629	9·2
Leaves untreated ..	0·651°	433	6·6
Same sample frozen	1·130°	619	5·4
Pyrus malus, fruit untreated	1·507°	171	1·1
Same fruit frozen ..	1·919°	161	0·8
Solanum tuberosum, tuber untreated	0·523°	555	11·0
Same tuber frozen	0·588°	583	9·9
Vitis vinifera, fruit untreated	2·567°	132	0·5
Same sample frozen	3·185°	112	0·3
Chamærops humilis, leaf untreated ..	0·365°	298	8·1
Same leaf frozen ..	1·529°	752	4·9
Beta vulgaris, root untreated..	1·473°	570	3·9
Same root frozen ..	1·761°	555	3·2

There can now be no doubt that Osterhout has shown that the permeability of the plasmolytic membrane is variable, and that it depends on the nature of the substances with which it is in contact. Thus the internal surfaces, of the nuclei, vacuoles, and plastids, may have a permeability which differs considerably from that of the

external surface of the cell. The effect of various sugars, before alluded to, has very probably an important bearing on the translocation of these carbohydrates, and systematic researches by the electrical method may go far towards explaining the functions of the different members of this group.

Osterhout's researches have afforded very valuable information on this difficult branch of physiology, and have demonstrated that the permeability of a tissue is a delicate index of its vitality. The preservation of a normal degree of permeability is, therefore, of the greatest importance to every cell.

As previously mentioned, anæsthetics increase the permeability of the protoplasm, thereby allowing the cell solutes to be pressed out in the same proportion as that in which they normally exist. The same effect is produced by intense cold. From results obtained by chemical analyses, André (1906 and 1907) concluded that the total concentration, but not the relative proportions of the constituents, of such expressed saps underwent alteration during the application of the force. With this result the experiments of Dixon and Atkins (1913, 1) are at variance, for, instead of remaining constant, the ratio of the electrical conductivity to the freezing-point of the sap was higher in that from organs pressed direct than when obtained from those rendered permeable by immersion in liquid air. This demonstrates the greater permeability of the protoplasm to electrolytes, upon which the conductivity depends, than to the solutes as a whole, including the sugars. The conductivities recorded for the saps of treated tissues may be slightly too low owing to the increased viscosity occasioned by the sugars. This, however, could not reverse the results, for Heald (1902) has shown that the ash of plant sap, when diluted to the correct concentration,

affords closely the same conductivity as the sap itself. Table XXX. substantiates this selective permeability of the protoplasm.   In it Δ denotes the depression of freezing-point, and C the electrical conductivity in mhos at 0°, and a few typical results are shown.   It should be mentioned that the pressed sap upon which these measurements were made by Dixon and Atkins was freed from cell débris, by means of a powerful centrifuge.

The Measurement of the Antagonism of Ions.

Osterhout (1914, 1) has quite recently still further increased the precision of investigations on the antagonism of ions by introducing suitable quantitative criteria.   As a basis he advocates the mixing of equally toxic solutions. This may give rise to one of three possible effects:

1. The toxicity is unaltered, which means that the toxic action of the two salts is additive.

2. The toxicity is diminished, in which case the salts are said to have an antagonistic action.

3. The toxicity is increased.

To represent these actions the molecular compositions of the mixtures of equally toxic solutions of two salts, A and B, may be taken as abscissæ, and the amounts of growth in any solution marked off as ordinates.   Then in the graph (Fig. 7) the additive effect will be represented by the line LJM parallel to the composition axis; LKM is the antagonism curve, and LHM shows the behaviour of mixtures of increased toxicity.

By the method of mixing two equally toxic solutions disturbances due to variations in osmotic pressure are eliminated.   For if one substance, A, is twice as toxic as another, B, molecule for molecule, it is evident that a solution of A $^M/_{20}$ will be isotoxic with a solution of B $^M/_{10}$. But if the osmotic pressure of the solution of A is less than

that of the solution of B, a better growth may in some cases occur in the former. To compensate for this the concentration of A must be slightly increased, in order to obtain truly isotoxic solutions. When the effects of the two salts used are additive, a straight line parallel to the concentration axis represents their action, as in Fig. 7.
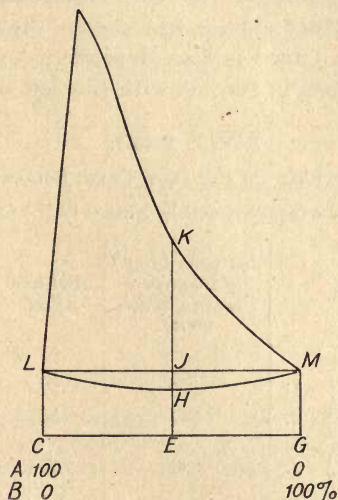
Fig. 7.—Curves showing the Growth of Roots in Mixtures of Equally Toxic Solutions of Two Salts, A and B.

The ordinates represent growth and the abscissæ represent the composition of the mixture in molecular percentages.

The antagonism of any mixture may then be expressed as the height of the curve, at any concentration, which rises above this straight line, divided by the distance of the straight line from the base. Thus, in Fig. 7, $\dfrac{KJ}{JE}$ represents the antagonism of an equimolecular mixture of A and B. This may be expressed conveniently as a per-

centage of JE—namely, as $\dfrac{KJ}{JE} \times 100$. Similarly, the increase of toxicity which is occasionally met with may be represented by $\dfrac{JH}{JE}$.

Osterhout has illustrated his method by the following table. The percentages refer to molecular proportions. More recently (1914, 7) he has shown that the form of the antagonism curve is also dependent upon how long the cells have been in contact with the salt solution.

TABLE XXXI.

MIXTURES OF EQUALLY TOXIC SOLUTIONS.

WHEAT (*growth during thirty days*)—NaCl 0·12 N + CaCl₂ 0·164 N.

Culture Solution.		Aggregate Length of Roots per Plant in Milli- metres.	Additive Effect.	Antagonism.
CaCl₂		55	55	—
75 per cent. CaCl₂ 25　,,　NaCl		105	55	$\dfrac{105-55}{55}=0.91$
50　,,　CaCl₂ 50　,,　NaCl		180	55	$\dfrac{180-55}{55}=2.27$
25　,,　CaCl₂ 75　,,　NaCl		298	55	$\dfrac{298-55}{55}=4.42$
15　,,　CaCl₂ 85　,,　NaCl		370	55	$\dfrac{370-55}{55}=5.73$
5　,,　CaCl₂ 95　,,　NaCl		435	55	$\dfrac{435-55}{55}=6.91$
1　,,　CaCl₂ 99　,,　NaCl		300	55	$\dfrac{300-55}{55}=4.45$
NaCl		55	55	—

Osterhout emphasizes the fact that the growth of parts not in immediate contact with the solutions does not furnish a trustworthy criterion of antagonism.

To represent the antagonism of mixtures of three salts, Osterhout (1914, 2) made use of Rooseboom's method, which is fully described in Findlay's " Phase Rule." It consists in the construction of an equilateral triangle, each side of which is made equal to 100 units. The apices represent 0 or 100 per cent. of one of the three components. Thus a point on any side denotes a percentage of a mixture of two substances, whereas a point inside the triangle defines the composition of a mixture of all
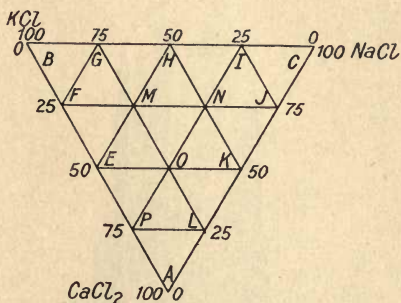
Fig. 8.—Diagram representing the Composition of Various Mixtures of the Chlorides of Sodium, Potassium, and Calcium, obtained by mixing Isotoxic Solutions of the Components

The numerals refer to molecular percentages. This serves as the base of the solid model shown in Fig. 9.

three components. This is at once apparent on referring to Fig. 8. Osterhout found that the roots of wheat grew equally well in solutions of sodium chloride 0·12 M, potassium chloride 0·13 M, and calcium chloride 0·164 M. The apices of the triangle, C, B, and A, represent respectively pure solutions of the salts of the above concentrations. Thus G denotes a mixture of the chlorides of potassium and sodium in which the molecular composition of the first-mentioned salt is 75 per cent.

Within the triangle the point M, for example, denotes a molecular concentration of 25 per cent. $CaCl_2$, as it is on the line FJ parallel to the base which is opposite the $CaCl_2$ apex, and begins and ends at points which are distant from the base 25 per cent. of the length of a side, and situated in the sides BA and CA. The point M is also on the lines EH and GL denoting concentrations of 50 per cent. potassium chloride and 25 per cent. sodium chloride
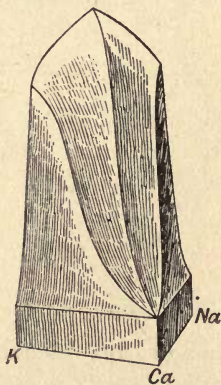
FIG. 9.—SOLID MODEL SHOWING THE FORMS OF THE ANTAGONISM CURVES IN ALL POSSIBLE MIXTURES OF NaCl 0·12 M, KCl 0·13 M., AND $CaCl_2$ 0·164 M.

respectively. Accordingly M represents a mixture of 25 per cent. calcium chloride, 50 per cent. potassium chloride, and 25 per cent. sodium chloride.

At any point on or within this triangle ordinates may be erected to denote the growth of the plant organ under investigation. When a sufficient number of points have been obtained, a solid model may be constructed. This describes completely the amount of growth taking place in any of the solutions. Fig. 9 shows such a model. In the solid figure the additive effect is represented, not by a

line, but by a plane parallel to the base. To determine the antagonism of any of the mixtures, it is only necessary to measure the height of the ordinate at the point representing the particular concentration under consideration, and, having also measured the height of the plane of the additive effect (which is 55 for wheat roots, as shown in
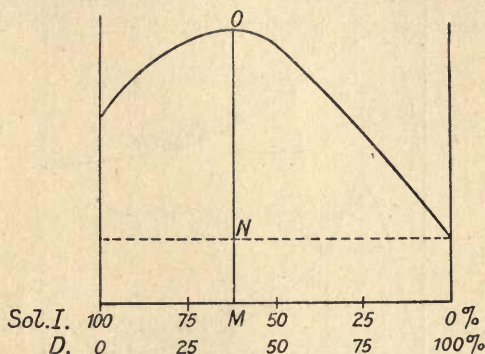
Fig. 10.—Method of expressing Antagonism in Mixtures containing more than Three Components.

Three of the components (A, B, and C) are combined in Solution 1, and various amounts of the fourth component (D) are added; the ordinates represent growth; the abscissæ represent the composition of the mixtures. Thus, at the point M the mixture contains 62·5 c.c. of solution 1 to each 37·5 c.c. of Solution D. The antagonism at M is $\dfrac{ON}{MN}$.

Table XXXI.), to subtract it from the total, and to divide the remainder by the amount subtracted.

When solutions of more than three components are employed, Osterhout recommends the following procedure: Starting with isotoxic solutions A, B, C, and D, a mixture of the first three may be made, and named solution 1. To this various amounts of D may be added; taking the growth in the mixtures as ordinates, a graph may be constructed
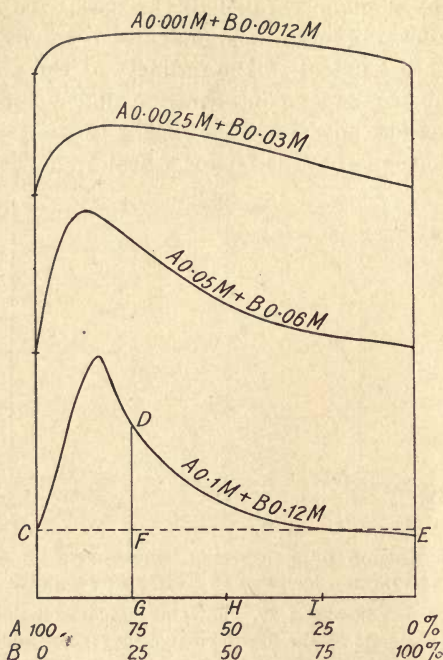
FIG. 11.—EFFECT OF DILUTION ON THE FORMS OF ANTAGONISM CURVES.

The ordinates represent the growth of roots in solutions, the composition of which is represented by the abscissæ. For example, on the curve *CDE* the ordinate at *G* represents the growth in a mixture of A 0·1 M and B 0·12 M in such proportions that 75 per cent. of the dissolved molecules are *A*, and 25 per cent. are *B*. In the curve immediately above *CDE* the ordinate at *G* represents the growth in a mixture of A 0·05 M and B 0·06 M in the proportions denoted by the point *G*.

such as Fig. 10. In it the dotted line denotes the additive effect. Antagonism may be measured as usual; for example, at the point M it is $\dfrac{MO - MN}{MN}$.

Though Osterhout illustrated this quantitative method by data obtained by the measurement of the roots of wheat seedlings, this might have been equally well done by measurements of electrical resistances of tissues and their alterations in isotoxic salt solutions of various composition. The underlying phenomena of all such experiments are alterations in the permeability of the protoplasm,
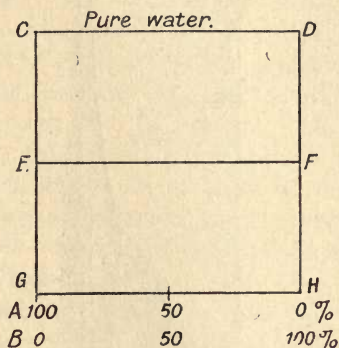
FIG. 12.—DIAGRAM REPRESENTING THE COMPOSITION OF SOLUTIONS.

(This serves as a base of the solid model shown in Fig. 13.)

The lowest line represents mixtures of solutions of two salts A and B; the line EF represents the same mixtures diluted with an equal volume of water. Any line drawn parallel to EF will express the same mixtures diluted to a degree corresponding to the position of the line. On the line CD all points represent distilled water.

which by the constructions described in the preceding paragraphs can now be expressed in a quantitative manner.

The effect of variation of the concentration of the isotoxic solutions has also been studied by Osterhout (1914, 3). The forms of the curves were found to become much flatter as dilution proceeded, but the proportions showing the greatest degree of antagonism appear to remain practically unaltered as shown in Fig. 11. To illustrate the results

obtained by diluting mixtures of isotoxic solutions, A and B, Osterhout employs a solid model in which the ordinates are lengths of growth and the base is a square, one side of which, CD, represents pure water, and the other side, GH, the solution. This can be diluted with water, the effect of
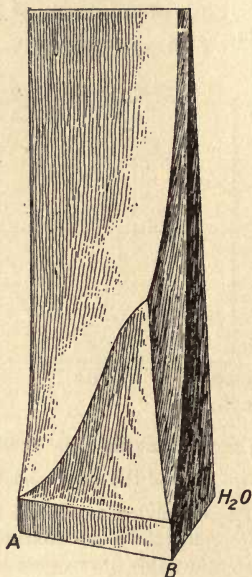

FIG. 13.—A SOLID MODEL WHICH GIVES A COMPLETE DESCRIPTION OF THE CHANGES PRODUCED IN THE FORM OF THE ANTAGONISM CURVE BY ALTERING THE CONCENTRATIONS OF THE SOLUTIONS.

which is to shift the line representing it inwards halfway towards the side CD. It then will occupy the position EF, as shown in Fig. 12.

By altering the composition of the isotoxic mixture also, a number of lengths of growth may be obtained, and a solid model may then be constructed, as in Fig. 13.

The alteration in permeability produced by an alkaline medium has formed the subject of a recent paper by Oster-hout (1914, 4). Warburg (1910) noticed that the rate of oxidation in the egg of the sea-urchin increased when the surrounding sea-water was made faintly alkaline, even though the sodium hydroxide used to produce this effect failed to penetrate into the egg, as was shown by previously staining the latter with an indicator. This peculiar occurrence was thought by Osterhout to be due to an alteration in the permeability of the egg, for oxidation would be increased if either the entry of oxygen or the exit of the products of oxidation were facilitated. This surmise was fully borne out by experiment. For example, it was found that the electrical resistance of discs of the thallus of *Laminaria saccharina* decreased rapidly when placed in sea-water rendered alkaline to the extent of containing 0·0052 gramme - molecule of sodium hydroxide per litre. The fall amounted to about 70 per cent. of the original value. When placed in a solution of the chlorides of sodium and calcium containing considerable quantities of the latter, the resistance increased at first, and then decreased. The addition of sodium hydroxide to such a solution caused the initial rise to be less noticeable, and diminished the time required for the fall to take place. Very dilute alkaline solutions were, however, without effect; thus 0·001 M sodium hydroxide and all weaker solutions are harmless. With these results the conclusions of Loeb and Wasteneys (1913) are in agreement. These authors repeated Warburg's experiments, and found that 0·001 M sodium hydroxide had no accelerating action upon the oxidations taking place in the egg of the sea-urchin. Higher concentrations injured the egg and raised the rate of oxidation.

The possibility that the antagonistic action of salts might

be due to oppositely charged ions has been carefully considered by Loeb (1914), and much evidence has been adduced by him against this view. Some years previously Loeb (1899) drew attention to the fact that the action of the ions $Na^{\cdot}$, $K^{\cdot}$, and $Ca^{\cdot\cdot}$, with regard to the absorption of water by soaps and by muscular tissue, was very similar. Hansteen Cranner (1914) showed that the same parallel held good for their influence upon the absorption of water by roots and by isolated plant cells. These facts lend support to Robertson's (1910) view that the action of calcium in antagonizing sodium is due to its forming insoluble calcium soaps with the plasmatic surface, whereas those of sodium are soluble.

The effect of acid upon permeability has also been investigated by Osterhout (1914, 5), who found that, while alkali merely increases permeability, an acid, such as hydrochloric, produces a rapid decrease, followed at once by a rapid increase which continues until the death-point is reached. It had been pointed out by Loeb that the effect of acid upon the imbibitional swelling of muscular tissue and upon the duration of the life of Fundulus could be antagonized by sodium chloride. Osterhout, too (1914, 6), working as before with Laminaria, proved by means of conductivity measurements that acid could antagonize the action of sodium chloride, though the degree of antagonism was not as great as between this salt and calcium chloride. He further showed that life cannot be maintained as long in the most favourable mixture of acid and sodium chloride as in that composed of the latter and calcium chloride. These results are considered by Osterhout as affording additional evidence for the view that the plasmatic membrane of plants is protein in character.

In addition Osterhout (1915, 1) has shown that the permeability of protoplasm may be greatly increased or

diminished without injury.   A rapid alternation of increase
(amounting to 20 per cent. above normal) and decrease
(amounting to 39 per cent. below normal) failed to
produce any evil effects.

He has also (1915, 2) drawn attention to the remarkable
difference between monovalent and bivalent kations in
their effects on permeability.   While none of the mono-
valent kations except hydrion are able to decrease per-
meability, all the bivalent kations so far investigated are
able to do so to a marked degree.   These include the
bivalent ions of magnesium, calcium, barium, strontium,
manganese, cobalt, iron, nickel, zinc, cadmium, and tin.
Furthermore, he has demonstrated (1915, 3) that the
trivalent kations of lanthanum, cerium, yttrium, iron,
aluminium, and the tetravalent kation of thorium, are
all able to decrease permeability to a considerable extent.

THE EFFECT OF ALTERATIONS IN TEMPERATURE
UPON PERMEABILITY.

This subject has recently been investigated by Eckerson
(1914), and the results obtained lead to the conclusion
that thermotropism is intimately connected with altera-
tions in permeability, and consequently in turgor.

Rysselberghe (1901) had previously shown that the
permeability of epidermal cells of *Tradescantia discolor* to
dissolved substances such as glycerol, carbamide, and
potassium nitrate, increases with rise of temperature from
0° to 30°.   Lepeschkin (1905), working with another plant,
found an increase of permeability from 0° to 20°, and a
decrease from 20° to 35°.   This worker also proved that
the permeability of protoplasm to sucrose does not change
within a moderate range of temperature, though that of
potassium nitrate increases, as a consequence of which
higher concentrations are necessary to bring about plas-

molysis. To test whether changes of permeability had taken place in various roots exposed to a succession of temperatures, Eckerson plotted the molecular concentrations of sucrose, glucose, and potassium nitrate solutions necessary to produce slight plasmolysis against the several temperatures at which experiments were conducted. The results obtained with *Raphanus sativus* are shown in Fig. 14. Similar experiments were also performed with other plants, and the temperature at which permeability ceased to increase or began to decrease was noted in each
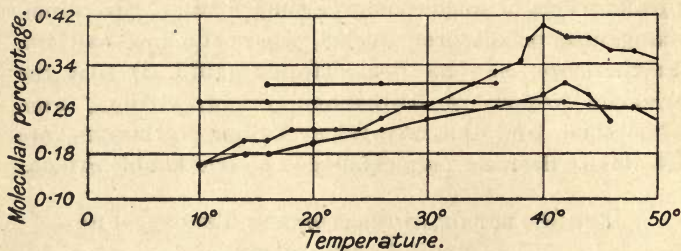
FIG. 14.—*Raphanus sativus* : CURVES SHOWING THE EFFECT OF TEMPERATURE ON THE PERMEABILITY OF ROOTS TO POTASSIUM NITRATE AND GLUCOSE; ORDINATES INDICATE THE PERCENTAGE WT.-MOL. SOLUTIONS PRODUCING SLIGHT PLASMOLYSIS, AND THE ABSCISSÆ REPRESENT TEMPERATURES. THE HORIZONTAL GRAPHS REPRESENT GLUCOSE.

case. When these are tabulated and compared with the limits of positive and negative thermotropism of the same roots a very striking similarity is revealed. The following table, quoted from Eckerson's paper, illustrates this. The temperatures recorded are those inside the vessel containing the roots.

In the table + denotes positive curvatures and increasing permeability; 0 denotes no curvature and no change in permeability; − denotes negative curvatures and decreasing permeability. (K) and (W) refer to results obtained by Klercker (1891) and Wortmann (1885) respectively.

TABLE XXXII.

THERMOTROPIC CURVATURES AND PERMEABILITY VARIATIONS
OF ROOTS.

		+	0	+	−
1. *Raphanus sativus*	Curvatures	7-15°	16-23°	24-36°	38-51°
	Permeability	10-14°	18-24°	24-40°	40-50°
2. *Pisum sativum* ..	Curvatures	8-15°	17-29°	—	34-50°
	Permeability	6-15°	15-35°	—	35-45°
3. *Sinapis alba* ..	Curvatures (K)	14-29°	—	—	None
	Permeability	10-30°	30-45°	—	None
4. *Helianthus annuus*	Curvatures (K)	None	—	—	15-40°
	Permeability	None	12-20°	—	20-40°
5. *Phaseolus multiflorus* (primary roots)	Curvatures (w)	None	8-22°	—	22-50°
	Permeability	None	13-20°	—	25-40°
6. Ditto (secondary roots)	Curvatures (w)	10-?°	—	—	?-40°
	Permeability	6-25°	—	—	25-40°

The interpretation of the results of this investigation appears to be that, since there is such close agreement between the temperature limits of thermotropic curvature in a positive direction and increase of permeability, and a similar relationship exists between negative thermotropism and decrease of permeability, it is to be concluded that the occurrence of curvature in any direction is due to the diminished turgor of the cells on that side owing to their increase in permeability. When the curvature is positive the effect of heat is to increase permeability, for the cells on the side next the source of heat are at a slightly higher temperature than are those on the opposite side, and as a consequence are more permeable and less turgid. When a negative curvature takes place, it is because at the temperature in question the effect of heat is to diminish permeability, and so the side remote from the source possesses the smaller turgor.

This interesting research definitely removes thermotropism from the class of mysterious phenomena which are said to be due to the action of stimuli.

THE EFFECT OF VARIATIONS IN THE INTENSITY OF ILLUMINATION UPON PERMEABILITY.

A very elaborate research upon the alterations in permeability brought about by variations in illumination was carried out by Tröndle (1910). He concluded that the alteration in permeability which takes place under the influence of light is a typical stimulus action, for in the narcotized condition no change in permeability takes place. In view, however, of the fact that Osterhout has shown that the effect of small quantities of anæsthetics is to decrease the permeability, it is possible that Tröndle's verdict on this point may need revision, as a decrease due to illumination might just balance an increase due to a narcotic.

Tröndle found that prolonged illumination (about twenty-four hours) led to a decrease in permeability with strong intensities. With medium intensities an increase was observed, whereas with weak intensities a decrease again resulted. Exposure to a strong light for a short time was, on the contrary, seen to produce an increase. Storage in the dark leads to a return of normal permeability.

Under natural conditions of growth the permeability of plant cells increases with the intensity of illumination. The physiological importance of this change appears to be that it permits of the translocation of assimilates from the leaves. It does not, however, appear probable that all phototropic curvatures are directly attributable to alterations in permeability due to the action of light, as cases are known in which a stimulus is transmitted to a region shielded from light.

THE CONSTITUTION OF THE PROTOPLASMIC SURFACE.

It has been already pointed out that Overton supposed the surface of protoplasm to be covered over with a layer of lipoid substances, such as lecithin or cholesterin. This hypothesis was based upon the behaviour of such bodies, which, in accordance with the well-known thermodynamic deductions of Willard Gibbs, tend to accumulate on the surface.

The view was subsequently advanced by Czapek that the true structure of the surface was that of a fatty emulsion in a colloidal solution of protein. In answer to Lepeschkin's criticism (1913), Czapek (1914) has recently emphasized the fact that he does not regard the fat as being in globules of any considerable size, for such a mixture would have a surface tension approximating to that of pure water. An emulsion, however, the droplets of which are for the most part less than a micron in size can have a low surface tension. Particles which are smaller than 6 $\mu\mu$—the limit of visibility with the ultra-microscope—are termed amicronic. Czapek accordingly assigns to the protoplasmic surface the structure of an amicronic emulsion.

With regard to Czapek's measurements of the surface tension of living cells, Vernon (1913) has raised the objection that, out of twenty-nine solutions of substances which lower surface tension, twenty-two have no toxic action till the surface tension is about 0·68 (that of water to air being taken as unity), as shown by Czapek. The remaining seven, however, are injurious even when their surface tensions are only from 0·82 to 0·99. In reply the argument has been brought forward that such exceptions may readily be accounted for as due to a chemical poisonous action, and that, when a single case can be instanced in which a

solution with a smaller surface tension than 0·68 is without a destructive action upon living cells, a really valid objection will have been brought forward. Czapek's work, showing the similarity in surface tension of fat emulsions and living cells, gives much support to the view that they have structurally similar surfaces. The profound influences exerted by various salts upon the permeability of protoplasm, recently revealed by the researches of Osterhout, seem to have their origin in the action of the solutes upon the degree of dispersion of the proteid and fatty colloids of the surface. It has also been pointed out that the intake of salts by the living cell is regulated largely by the laws of adsorption, and that in this the specific selective action of the surface colloids is of the greatest importance.

An interesting account of researches on osmotic pressure and the permeability of protoplasm, carried out before 1903 has been given by Livingstone.

NOTE.—Osterhout (1915, 4) has since studied the effect of dilution upon the isotoxicity of isotoxic mixtures, and has given a correction to be applied for the change, which is generally of negligible magnitude. An important research by Stiles and Jørgensen (1915) has also just appeared. They measured the rate of diffusion outwards of cell electrolytes by determining the change in conductivity of the external medium. They conclude that, within limits, the rate of exosmosis is a measure of the toxicity of the medium.

CHAPTER VIII

THE PERMEABILITY OF ORGANIC MEMBRANES OTHER THAN PROTOPLASM

A NUMBER of animal and vegetable membranes are now known which exhibit to a greater or less degree the phenomenon of semi-permeability, and a few have been investigated within recent years which disclose a remarkable selective action towards substances in solution.

These are of interest, not only in themselves, but also because they afford macroscopic demonstrations of osmotic phenomena.

PERMEABILITY OF SOME ANIMAL MEMBRANES.

One very simple experiment illustrative of these phenomena consists in boiling a sausage. The animal membrane which constitutes its covering is freely permeable to water, but does not admit of the outward passage of salts at all readily. The result is that, both on account of the osmotic action of the salts inside and of the imbibitional intake of water by the colloidal animal tissues, a stretching of the membrane results to such a degree that bursting usually occurs. If punctured before rupture of the membrane has ensued, a quantity of liquid will, in the majority of cases, be squirted out.

The membranes of the egg of the common fowl were also found by the author (1909, 2) to show features of interest. Immediately against the shell lies a tough mem-

brane, in contact with which is one of a more delicate nature. The yolk, too, is enclosed in a very delicate membrane.

At one end of the egg the two membranes are separated by the air space. Careful removal of the shell permits of the outer one being placed in contact with distilled water by standing the egg on end in a small dish. In this position the two membranes are adpressed. On testing some of the surrounding water with silver nitrate from time to time, it is seen that chlorides only diffuse through slowly. When, however, the outer membrane is ruptured, a relatively rapid diffusion of chloride takes place, showing that the inner is far less resistant to the passage of salts than is the outer membrane.

The membranes, both outer and inner, are readily penetrated by water; for when the shell has been removed from one end in such a way as not to injure the membrane, and a long, narrow glass tube has been passed through the membrane and cemented in at the other end of an egg, it is seen that the contents rise in the tube when the membrane is placed in contact with water. This experiment, described by Bergen and Davis (1906), has been found to be of considerable value as a lecture illustration. The yellow colour of the column, secured by puncturing the yolk, renders it easy to be seen, and a rise of over a metre may be obtained. This height is sometimes maintained for several days. Semi-permeability may also be demonstrated by means of the yoke of an egg. The white and yolk of the egg of the common fowl, *Gallus bankiva*, are isotonic, and possess an osmotic pressure of 5·5 atmospheres as determined by the cryoscopic method. When the yolk is transferred to water, it swells and at the same time becomes paler in colour. In this distended condition the membrane is very easily ruptured, and cannot even sus-

tain the weight of its own contents, for on draining off the water very slowly rupture always occurs. Before this osmotic intake of liquid, the yolk membrane is, if the egg is fresh, quite able to remain on a flat surface unsupported by surrounding liquid.

Osmotic and imbibitional absorption of water may also be illustrated by the following experiment : The shell of an egg is dissolved away completely by dilute hydrochloric or acetic acid, which penetrates slowly into the interior. When placed in pure water the egg increases in volume to a very marked degree, the membrane becoming tense. In a strong salt or sugar solution the volume diminishes only slightly, partly because the membrane is by no means strictly semi-permeable, and partly because much of the increase is due to the imbibitional swelling of the contents of the egg, which have been coagulated by the acid.

It may be remarked that for demonstration purposes semi-permeable membranes of collodion mounted on silver gauze, and on which copper ferrocyanide has been precipitated, seem very suitable and easy to prepare. Fouard (1911), who introduced the method quite recently, has even used it for quantitative work, such as molecular weight determinations.

PERMEABILITY OF SEED COATS.

While engaged in an investigation on barley seeds, A. J. Brown (1909) found that the seed coat exhibited a very peculiar selective permeability, in that whereas water passed through it freely, and ionized salts, sugars, and other substances, were held back, yet certain feebly ionized salts, such as mercuric chloride and cadmium iodide, together with alcohols, aldehydes, ketones, esters, and the simple fatty acids, were able to enter the seeds more or

less rapidly. This discovery gave rise to much experimental work which is now too well known to be described here. The suggestion was made by Armstrong (1909) that the peculiar grouping of substances brought out by this research might be due to the relation of the various compounds to water, those which formed hydrates being held back, whereas the remainder, which were not hydrated or only feebly hydrated, possessed the power of penetrating the seed coats. It is certainly remarkable that whereas aqueous alcohol can pass into the seeds very freely, yet when in the anhydrous condition this substance is unable to enter.

It was shown by Schröder (1911) that wheat possessed the same type of selectively permeable coat.

Quite recently Shull (1913) has added considerably to our knowledge of the subject by his experiments on *Xanthium glabratum*. Seeds of this plant, carefully dried at 40° over phosphoric anhydride, were found to germinate vigorously after prolonged immersion in ether, chloroform, acetone, and absolute alcohol. It was not necessary to remove the last traces of water from these liquids, this point being tested directly. Moreover, even 95 per cent. commercial alcohol did not kill air-dried seeds till after over four days' immersion. This confirmed the previous work of Giglioli (1882 and 1895), Dixon (1902) and Becquerel (1907), who, with the exception of the last-named, had even treated seeds with saturated solutions of mercuric chloride in methylated spirit. The dried coats of Xanthium seeds were also proved to be impermeable to oxygen.

When placed in water, air-dried seeds of Xanthium quickly absorbed over 50 per cent. of their original weight, the process being rapid at first, and almost complete in fifteen hours. In strong salt solutions the forces of capillarity and imbibition, as well as the attraction of colloids

and cell solutes, cause water to enter at almost as great an initial rate as when pure water is the surrounding medium. When, however, the osmotic pressure of the external solution balances the various internal forces, the entrance of water ceases. Any further dilution or concentration of the liquid on the outside quickly leads to
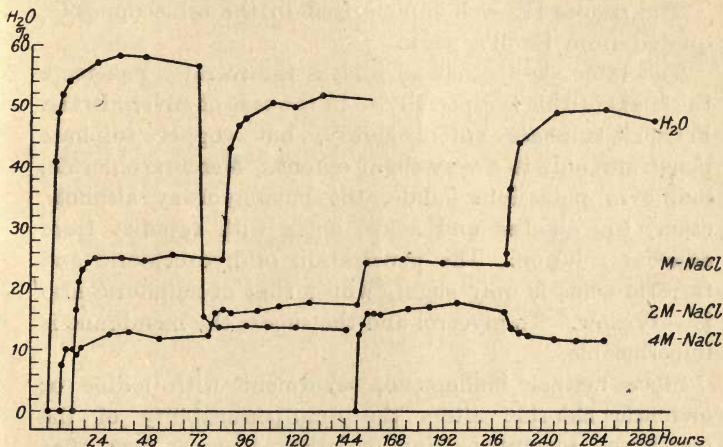

FIG. 15.—CURVES SHOWING ENTRANCE AND WITHDRAWAL OF WATER IN XANTHIUM SEEDS ON SHIFTING FROM WATER TO SALT SOLUTIONS, OR VICE VERSA, AT THREE-DAY INTERVALS.

The semi-permeability of the membrane is well illustrated by the behaviour recorded in these curves; Curves 2, 3, and 4 are displaced to the right to avoid confusion.

a readjustment, water passing in or out till equilibrium is again established. The accompanying figure with legend taken from Shull's paper makes this clear, and illustrates well the efficiency of the coat as a semi-permeable membrane. Sodium chloride solutions containing from 1 to 4 gramme-molecules per litre were employed.

It was, in addition, proved by chemical tests that

no passage of sodium chloride through the membrane occurs, though other substances are able to penetrate.

The capability or incapability of a substance to enter the seeds was judged by the amount of water taken up by them from various solutions, which were usually gramme-molecular.

The results are well summarized in the table opposite, quoted from Shull's work.

The table shows that as a class the nitrates penetrate the coats. This is especially so in the case of silver nitrate. Ferrous sulphate enters slowly, but copper sulphate penetrates only to a very slight extent. Mercuric chloride, iodine in potassium iodide, the monohydroxy alcohols, ether, the alkalies and acids, enter with rapidity from aqueous solution. The penetration of hydrochloric and tartaric acids is only slight, whilst that of sulphuric also is very slow. To glycerol and the sugars the membrane is impermeable.

Since neither boiling nor treatment with iodine or mercuric chloride alters the semi-permeability of the membrane towards sodium chloride, it is evident that this property depends, not on living cells, but upon the chemical and physical properties of the cell walls.

Shull has recorded that a number of other seed coats also behave as semi-permeable membranes, and gives the list which follows: Alismaceæ (*Alisma plantago-aquatica*), Gramineæ (barley, wheat, oats, etc., probably most grasses), Chenopodiaceæ (sugar beet), Rosaceæ (peach, apple), Leguminosæ (*Vicia faba*, scarlet runner, Lima bean), Compositæ (*Xanthium glabratum*, *Helianthus annuus*). These however, vary considerably in their degree of semi-permeability, and some of the number permit of the passage of salts to a noticeable extent.

TABLE XXXIII.

To show Percentages of Water, calculated on the Air-dry Weight, taken up by the Seeds of *Xanthium glabratum*.

Osmotic Pressure* in Atmospheres.	Liquid.	Percentage absorbed.		Remarks.
		Three Days.	Ten Days.	
—	Water	54·94	—	—
38·02	N. sodium chloride	26·0	—	—
130·62	4 N. sodium chloride	12·16	—	—
—	Saturated sodium chloride	7·12	—	End of two days
35·53	N. silver nitrate	49·83	70·89	—
36·53	N. potassium nitrate	37·89	41·29	—
28·25	N. copper sulphate	34·48	35·59	—
—	N. ferrous sulphate	34·39	44·73	—
—	5 per cent. mercuric chloride	70·2	—	—
—	Methyl alcohol	66·6	—	10 per cent. by volume
—	Ethyl alcohol	57·14	—	Ditto
—	n-propyl alcohol	69·25	—	Ditto
—	Glycerol ..	29·32	—	Ditto
—	Ether saturated	53·95	—	End of five hours
—	N. sucrose ..	34·41	—	—
—	N. fructose ..	35·54	—	—
—	N. glucose ..	36·51	—	—
—	N. lactose ..	35·47	—	—
—	N. hydrochloric acid	32·87	—	—
—	N. sulphuric acid	42·06	—	—
—	N. tartaric acid	36·87	—	—
—	N. acetic acid..	57·3	—	In eleven hours

* Calculated from the Landolt-Börnstein tables.

IMBIBITIONAL FORCES IN DRY SEEDS.

To measure the capillary and imbibitional forces of air-dry seeds, Shull employed saturated solutions of lithium chloride, which gives the highest osmotic pressure of any known neutral salt. Such seeds contain 8 to 9 per cent. of moisture, and when placed in this saturated solution undergo no permanent change in weight.

An experiment the converse of the above was then
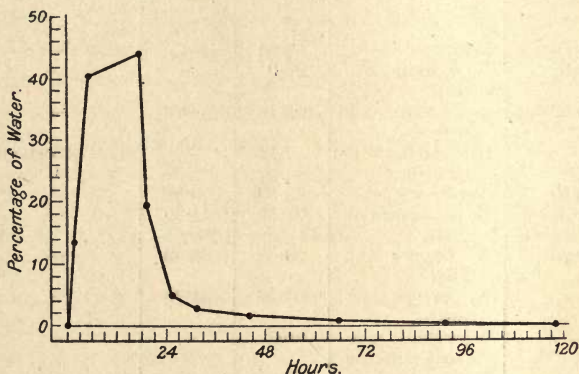
FIG. 16.—CURVE SHOWING LOSS OF WATER FROM SEEDS SOAKED IN WATER BEFORE TRANSFERRING TO SATURATED LiCl SOLUTION; IMBIBITION FORCE OF AIR-DRY SEEDS AND OSMOTIC PRESSURE OF SATURATED LiCl SOLUTION APPROXIMATELY EQUAL.

carried out, for the seeds were first placed in water till they had taken up about 44 per cent. of their dry weight. Being subsequently placed in the chloride solution, which contained the solid in excess to maintain saturation, they rapidly lost weight, so that after 100 hours they retained only their original amount of water, as shown by their weight having been recovered. A glance at the accompanying figure permits of the changes being readily comprehended.

These two experiments, taken together, show that, since there is a state of equilibrium between a saturated solution
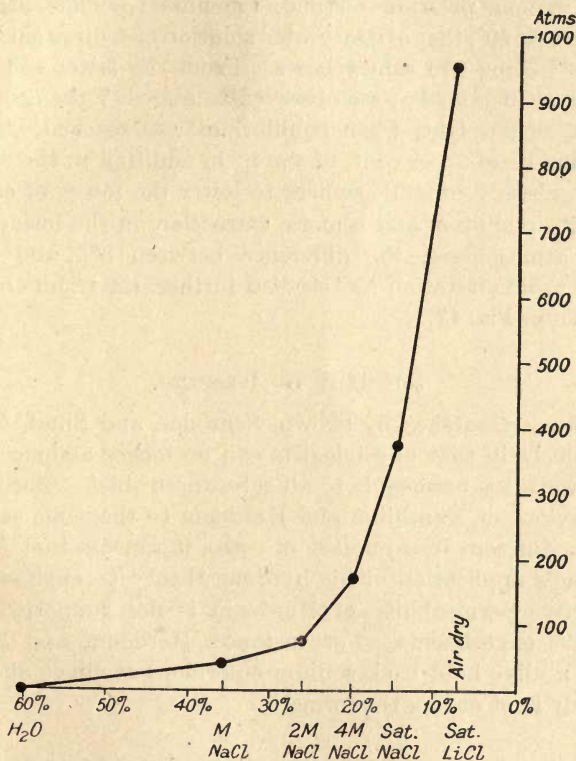
FIG. 17.—CURVE SHOWING INCREASE OF IMBIBITION FORCE IN SEEDS WITH DECREASE OF WATER-CONTENT.

Percentage of H_2O is based on absolute dry weight; air-dry seeds are considered to have 8 per cent. of hygroscopic moisture in this diagram.

of lithium chloride and Xanthium seeds containing about 8 per cent. of water, the internal forces must be balanced

10

by the osmotic pressure of the external medium. This, according to the measurements of Raciborski (1905) by the vapour pressure method, amounts to 965·3 atmospheres, at 20°, that of a saturated solution of sodium chloride at 18° being 375 atmospheres. From the latter solution these air-dry seeds were observed to absorb 7 per cent. of their own weight, when equilibrium was reached. Thus the intake of 7 per cent. of water, in addition to the 8 per cent. already present, suffices to lower the forces of capillarity, imbibition and osmotic attraction on the inside by 590 atmospheres, the difference between 965 and 375 This rapid alteration was studied further, the results being shown in Fig. 17.

SUMMARY OF RESULTS.

These researches by Brown, Schröder, and Shull, show conclusively that physiologists can no longer assume that cell walls are permeable to all substances alike. Since the behaviour of Xanthium and Hordeum to the same salt is quite different in a number of cases, it appears that Armstrong's application of his hydrone theory to explain the selective permeability of Hordeum is not supported by Shull's experiments. For instance, Hordeum and Xanthium alike hold back sodium chloride, but silver nitrate is only kept out by the former.

LOCALIZATION OF THE SEMI-PERMEABLE LAYER IN SEED COATS.

In Xanthium the semi-permeability has been traced to the inner and middle layers of the testa. The outermost is incapable of this function. The middle layer is several cells thick, whereas the inner is only one cell thick, except in the chalazal region. This last layer is believed

to be the nucellar epidermis.   It consists of nearly pure cellulose, and is unsuberized.   Both layers contain some tannin, but as this does not form a continuous layer, and as semi-permeability is not destroyed by treatment with tannin solvents, the evidence is adverse to Reichard's (1909) theory, which regards semi-permeability in such cases to be due to the deposition of tannin compounds.

CHAPTER IX

THE MAGNITUDES OF OSMOTIC PRESSURES AND ELECTRICAL CONDUCTIVITIES IN PLANTS, AND THE FACTORS WHICH INFLUENCE THEM

Owing to the many inaccuracies to which the method of plasmolysis has been open, reference will be made in this chapter only to determinations by the cryoscopic method, except in a few cases where such data are unobtainable.

Renner (1912) has pointed out that the discrepancies between the results of cryoscopic and of plasmolytic investigations may be in part due to the fact that, whereas cryoscopic determinations used for calculating osmotic pressures are made with solutions containing weight-normal quantities of solute—viz., a certain weight in 1,000 grammes of water—it has always been customary to employ volume-normal solutions in plasmolytic investigations. Morse advocates the use of the former mode of making up solutions, as with it a better agreement is obtained between the direct measurements of osmotic pressure and those calculated by the simple Van't Hoff equation. Thus in this respect cryoscopic determinations are really of greater accuracy than are plasmolytic. Renner counsels the adoption of weight-normal solutions for the latter method also, as it leads to a better agreement. It is to be remembered that cryoscopically determined osmotic pressures relate to 0°, whereas those measured by the plasmolytic method are at air temperatures—10° to 20°. This,

however, is merely a matter of the form in which the results are expressed.

Mention has already been made of the fact that, owing to the method by which the sap was expressed, all but the most recent cryoscopic determinations are minor limits, for in these only were the cells rendered permeable.

Electrical conductivity determinations show the variations in the organic and inorganic salts and acids of the sap. It has been pointed out by Dixon and Atkins (1915, 1) that by finding the freezing-point of solutions of some standard substance, such as potassium chloride, which have conductivities equal to those of the plant juices under examination, and by subtracting these values respectively from the freezing-points of the saps as actually observed, it is possible to get a very approximate measurement of the proportion of the osmotic pressure due to non-electrolytes. The values thus obtained for the conductivities are probably somewhat too low, owing to the increased viscosity of the sap occasioned by the sugars in solution.

Leaves and Other Aerial Organs.

In Chapter X. of "Transpiration and the Ascent of Sap," Dixon analyzes the most recent cryoscopically determined data for the osmotic pressures of leaves, and draws attention to the following points:

1. The concordance of the results obtainable by examining leaves from different branches of the same tree growing under similar conditions. This agreement is even found with leaves not rendered permeable, but pressed to the same degree, as far as was feasible.

2. The chance of slightly low values being afforded by leaves plucked from a tree in the sap of which considerable tension had existed. For if the cells are not fully dis-

tended, the slackening of the tension consequent upon rupture results in their engorgement with water from the adjoining tracheæ.

3. The rise in osmotic pressure which occurs as leaves increase in age.

4. The marked influence of exposure to sunlight, especially in deciduous leaves. For the sugars formed during insolation increase the pressure greatly.

5. The possibility of the osmotic pressure of a leaf being more than doubled by the combined effects of exposure to light and evaporation to the stage of incipient wilting.

6. The maintenance of a slightly higher pressure, in evergreens such as *Hedera helix* which grow in a southerly sunny aspect, as compared with the same species when facing north and shaded from direct sunlight.

7. The want of correlation between osmotic pressure and height above ground-level in the leaves of tall trees, any such apparent connection being due rather to the increase in sugar formation occurring in well-illuminated positions.

Dixon has also compiled a table showing the maximum pressures found in the plants examined by him and the writer. In unwilted leaves of *Syringa vulgaris* a pressure of 25·68 atmospheres was found as a maximum. In this determination liquid air was not used in the extraction, so the actual pressure may have been higher.

Subjoined are some of the most interesting of the values obtained by Cavara (1905), Nicolosi-Roncati (1907), Trinchieri (1909), Marie and Gatin (1912), and Ohlweiler (1912). These are all under-estimates, as previously explained, owing to the fact that no attempt was made to render the protoplasm permeable. In view of this fact, the numerous determinations carried out to compare the osmotic pressures met with in the leaves of various natural orders of

plants have but little claim to consideration except as minor limits. However, as they stand, Cavara's values for the osmotic pressures of leaves vary on an average from about 6 to 14 atmospheres. Extreme values range as high as 30 atmospheres. This estimate does not include the salt lagoon plants, which will be considered later. The following table is quoted from Cavara:

TABLE XXXIV.

OSMOTIC PRESSURES OF LEAVES AND OTHER ASSIMILATING TISSUES.

	Δ.	P.
Aloe arborescens	0·14	1·68
Agave americana	0·52	6·25
,, ,, (Dixon and Atkins) ..	0·84	10·11
Hœmanthus coccineus	0·60	7·22
Ficus rubiginosa	0·70	8·42
Tournefortia fruticans	0·81	9·74
Mesembryanthemum acinaciforme ..	1·04	12·51
Plantago maritima	1·12	13·47
Myoporum debile	1·34	16·12
Statice limonium	1·52	18·28
Bupleurum fruticosum	1·72	20·69
Gendarussa adatoda	2·26	27·19
Statice globularioides	2·49	29·95

Cavara's comparative studies of the variation of osmotic pressure in the zones from the apex downwards are also of interest. He examined in this manner *Opuntia amyclœa*, *O. ficus-indica* and *Agave americana*, and ascertained that in each case the pressure fell slightly from the region of the apex downwards for some distance, but rose to about its former value in the basal portions. Somewhat similar experiments were carried out subsequently by Nicolosi-Roncati (1907), who in addition measured electrical conductivities; accordingly, his values only are quoted.

From among them the following were selected. They show how the electrical conductivity of the internodes of the stem of *Begonia semperflorens* increases from below upwards. In the freezing-points obtained there is no such regularity. For all these the whole of the stem tissues were pressed.

TABLE XXXV.

Begonia semperflorens.

Region of Stem.	$C_{28} \times 10^4.$	Δ.
Three apical internodes	159	0·57°
Fourth internode	131	0·43°
Fifth ,, ...	129	0·48°
Sixth ,, ...	110	0·50°
Seventh ,, ...	95	0·46°
Eighth ,, ...	93	0·50°
Ninth ,, ...	91	0·49°

The conductivity determinations were carried out at 28°, and are written thus: $C_{28} \times 10^4 = x$.

The freezing-points of the leaves of ninety trees and shrubs were determined by Ohlweiler (1912) by means of the thermo-electric method. In nineteen other cases no sap could be pressed from the leaves. Dixon and Atkins (1910) also found this difficulty in certain cases. Treatment with liquid air was found by them to make it possible to obtain sap in many cases in which it was previously impossible.

Ohlweiler's numerous measurements show that pressures of from 10 to 24 atmospheres exist in leaves of a large number of species. Treatment of the leaves with liquid air would certainly have in many cases largely raised the results. The material for these experiments was collected in the Missouri Botanic Gardens in full sunlight,

between 2 p.m. and 4 p.m. during dry weather in the third week of May. The research was carried out with the object of ascertaining whether there was any correlation between the freezing-point of the sap and the resistance of the plants to a severe frost which occurred at the end of the previous April. In view of the many factors which influence osmotic pressure in leaves, sufficing as they do to cause alterations of nearly 100 per cent. within a few hours in extreme instances, it is not surprising that no very close parallel could be found between the two sets of records.

The small number of observations recorded by Marie and Gatin (1912) also fail completely to make good the validity of any such general relation between osmotic pressure and resistance to cold. The not very marked differences recorded by them between plants from the mountains and the same species from the plains may be readily accounted for as due to inequalities of illumination, temperature, or age. In addition Dixon and Atkins (1910) have shown that whilst storage of the pressed and filtered sap alters its freezing-point but little within twenty-four hours, yet the keeping of leaves in a closed vessel in the dark usually leads to an increase in osmotic pressure, probably owing to the mobilization of starch resulting in the production of maltose. To such storage the plants used by Marie and Gatin were subjected during transmission from the mountains to Paris.

The physiology of resistance to cold has, however, been worked out by Maximow in a series of papers, which he has recently (1914) summarized.

PRESSURES DUE TO ELECTROLYTES AND TO NON-ELECTROLYTES.

In the following table, compiled from the work of Dixon and Atkins, the freezing-point Δ is shown for the sap of various leaves, pressed after treatment with liquid air. In addition P, the osmotic pressure in atmospheres calculated from Δ, and C, the electrical conductivity in mhos at $0°$, are shown. Furthermore, in order to record the osmotic pressure due to electrolytes, the column under Δ_e shows, as already explained, the depressions of freezing-points of solutions of potassium chloride having the conductivities shown in the column C. The column $\Delta - \Delta_e$ contains the values of depression of freezing-point occasioned by non-electrolytes.

Almost all these determinations were carried out in the late autumn or winter; accordingly but few of the pressures found exceed 20 atmospheres, though in previous work, in which the freezing-point only was measured in each case, values up to 30 atmospheres were found. Those plants marked with an asterisk were growing in greenhouses. In some of the latter very low pressures were met with, and in producing them electrolytes were preponderant as a general rule, whereas in the case of plants under average conditions of assimilation in the open air in Ireland the reverse is the case, most of the pressure being due to non-electrolytes. The increase in the quantity of electrolytes in old leaves as compared with young leaves under similar conditions is well shown by the values of Δ_e for *Ilex aquifolium* and *Populus alba*.

It is remarkable that the only Pteridophytes examined, Equisetum, Polypodium, and Selaginella, have unusually high conductivities, and that the major portion of the osmotic pressure of their cells is due to electrolytes.

TABLE XXXVI.

OSMOTIC PRESSURES AND ELECTRICAL CONDUCTIVITIES OF LEAVES.

Name of Leaf.	Δ.	Δe.	Δ − Δe.	P.	$C \times 10^5$.
*Agave americana,** Jan. 11 ..	0·840	0·180	0·660	10·11	364
*Aloe plicatile,** Jan. 11	0·292	0·185	0·107	3·52	370
*Anthurium andreanum,** Jan. 27	0·623	0·480	0·143	7·49	964
,, *crystallinum,** Jan. 27	0·727	0·670	0·057	8·73	1,349
Apium graveolens (etiolated bases), Dec. 5	1·302	0·570	0·732	15·66	1,141
Cerasus laurocerasus, Nov. 28	1·522	0·160	1·362	18·31	321
Chamærops humilis, mature, Nov. 28	1·424	0·490	0·934	17·13	984
Chamærops humilis, just expanded, Nov. 29	1·598	0·490	1·108	19·22	977
Cordyline australis, Nov. 28 ..	1·116	0·470	0·646	13·43	939
Equisetum telmateia, main stem, Aug. 14	0·801	0·600	0·201	9·64	1,257
Equisetum telmateia, lateral branches, Aug. 14	0·771	0·550	0·221	9·28	1,162
Eucalyptus globulus, Nov. 29..	0·970	0·405	0·565	11·68	814
Fraxinus excelsior, Sept. 30 ..	1·645	—	—	19·52	—
,, *oxyphylla,* Oct. 3 ..	1·252	—	—	15·06	—
Hedera helix, Nov. 29.. ..	1·437	0·255	1·182	17·29	512
Helianthus multiflorus, Oct. 2	0·764	—	—	9·18	—
Ilex aquifolium, new ultimate, Dec. 4	1·218	0·212	1·006	14·65	427
Ilex aquifolium, antepenultimate, Dec. 4	1·259	0·365	0·894	15·14	730
Magnolia acuminata, Sept. 30	1·503	—	—	18·07	—
*Monstera deliciosa,** Dec. 10 ..	0·552	0·287	0·265	6·64	574
*Musa sapientum,** Dec. 10 ..	0·785	0·150	0·635	9·44	303
*Passiflora quadrangularis,** Dec. 10	1·162	0·350	0·812	13·98	706
Pinus laricio, leaves one year old, Nov. 30..	1·289	0·424	0·865	15·50	848
*Platycerium alcicorne,** Jan. 27	0·625	0·445	0·180	7·51	895
*Polypodium irioides,** Jan. 27	0·886	0·515	0·371	10·65	1,034
Populus alba, spring leaves, Aug. 28	1·326	0·450	0·876	15·95	908
Populus alba, summer leaves, Aug. 28	1·487	0·325	1·162	17·88	654
*Saccharum officinarum,** Dec. 10	0·484	0·384	0·100	5·83	772
*Selaginella mertensii,** leaves and aerial stems, Jan. 27 ..	0·845	0·554	0·291	10·16	1,116
Syringa vulgaris, Aug. 22 ..	2·119	0·220	1·899	25·50	446
,, ,, Aug. 13 ..	2·004	0·230	1·774	24·10	461
Ulmus campestris, Oct. 2 ..	1·237	—	—	14·88	—
Vitis Veitchii, Oct. 2 ..	0·764	—	—	9·18	—
Wistaria sinensis, Sept. 30 ..	0·709	—	—	8·52	—

* Growing in greenhouses.

In the bast, wood parenchyma, and medullary rays of woody stems, fairly high pressures may be met with; for on pressing out the sap or on centrifuging the stem after treatment with liquid air, the values found are of considerable magnitude, even though diluted with the contents of the tracheæ of the wood. The latter does not approximate to pure water, as is commonly stated, but, owing to the fact that it contains considerable quantities of sugars, possesses an osmotic pressure of from 0·5 to 3·0 atmospheres in the cases examined up to the present by Dixon and Atkins (1915, 1). This will be explained in detail in Chapter XI.

FRUITS.

The methods of cryoscopy and electrical conductivity measurements together afford a ready means of studying the series of changes which occurs during the ripening of fruits. Isolated determinations carried out by the author (1910) sufficiently illustrate the remarkably high pressures that are attained in sugary fruits. Some of these are recorded below.

That even these are under-estimates is proved by reference to Table XXX., p. 118, where it is shown that treatment with liquid air to obtain the sap results in a much greater depression of freezing-point being found, even in such a soft tissue as the pulp of ripe grapes. Under M are given the values calculated for the mean molecular weights of the sap solutes, by employing the formula $M = \dfrac{s}{l} \times \dfrac{k}{\Delta}$, in which s denotes the weight of the solutes in a weight l of solution, and k is a constant. The figures indicate that part of the osmotic pressure must be due to the salts of organic acids, and to the acids themselves, for the presence of large quantities of glucose or fructose (mol. wt. 180) or of sucrose (mol. wt. 342) would considerably raise the average molecular weight.

TABLE XXXVII.

FRUIT NOT TREATED WITH LIQUID AIR.

	Δ.	P.	M.
Pyrus malus	1·571	18·90	—
,, ,, ...	1·653	19·88	168
,, ,, ...	1·473	17·72	165
Vitis vinifera	2·131	25·64	127
,, ,, ...	2·306	27·73	128
Prunus communis	2·455	29·53	—
,, ,, ...	2·350	28·27	—
,, ,, ...	2·400	28·86	—
Citrus limonum, February	1·029	12·37	159
,, ,, ,, ...	1·171	14·06	145
,, ,, August	0·870	10·46	—
Citrus aurantium, August	1·598	19·22	—
,, ,, ,, ...	1·525	18·34	—
,, ,, February	1·031	12·40	164
Lycopersicum esculentum	0·656	7·89	—
,, ,, ...	0·575	6·92	110
,, ,, ...	0·506	6·09	122

The following results obtained by Dixon and Atkins (1913, 2) from material treated with liquid air make it clear that even in acid fruits by far the larger proportion of the osmotic pressure is still due to non-electrolytes:

TABLE XXXVIII.

OSMOTIC PRESSURE OF FRUIT TREATED WITH LIQUID AIR.

Fruit.	Δ.	Δe.	Δ − Δe.	P.	$C \times 10^5$.
Citrus aurantium, Nov. 22 ..	1·206°	0·104°	1·102°	14·51	208
,, *limonum*, Nov. 25 ..	1·089°	0·170°	0·919°	13·10	345
Lycopersicum esculentum, Nov. 26	0·731°	0·228°	0·503°	8·79	457
Pyrus malus, Nov. 22.. ..	1·919°	0·080°	1·839°	23·18	161
Vaccinium oxycoccus, Nov. 27	1·556°	0·087°	1·469°	18·72	176
Vitis vinifera, Nov. 25 ..	3·185°	0·056°	3·129°	38·32	112

In all cases in which the total depression of freezing-point is apportioned between electrolytes and non-electrolytes, it must be borne in mind that molecular proportions are being considered, and not percentages by weight. Furthermore, since electrolytes dissociate into at least two ions, one molecular proportion (when in a dilute solution) will produce twice as great a depression of freezing-point as will a molecular proportion of a non-electrolyte. Again, as the non-electrolytes consist in the main of mono- or disaccharides, having molecular weights of 180 and 342 respectively, whereas those of the electrolytes rarely exceed the lower value, and as a rule are very much smaller, it is clear that the percentage of electrolytes present by weight in vegetable saps is in the majority of cases small compared with that of non-electrolytes.

CHANGES DURING THE RIPENING OF FRUITS.

For the fruit of *Solanum laciniatum* Nicolosi-Roncati (1907), records a decrease in electrical conductivity, accompanied by a rise in osmotic pressure, according as maturity is approached. In such respects, however, it is probable that there is a great difference between fruits of various species.

TABLE XXXIX.

Solanum laciniatum.

Description of Fruit.		$C_{16} \times 10^4$.	Δ.
Not well developed, pericarp green	..	155	1·23°
Somewhat larger, pericarp yellow	..	117	1·41°
Mature	..	92	1·78°

By far the most exhaustive cryoscopic researches upon the ripening of fruits are those of Cavara. This author
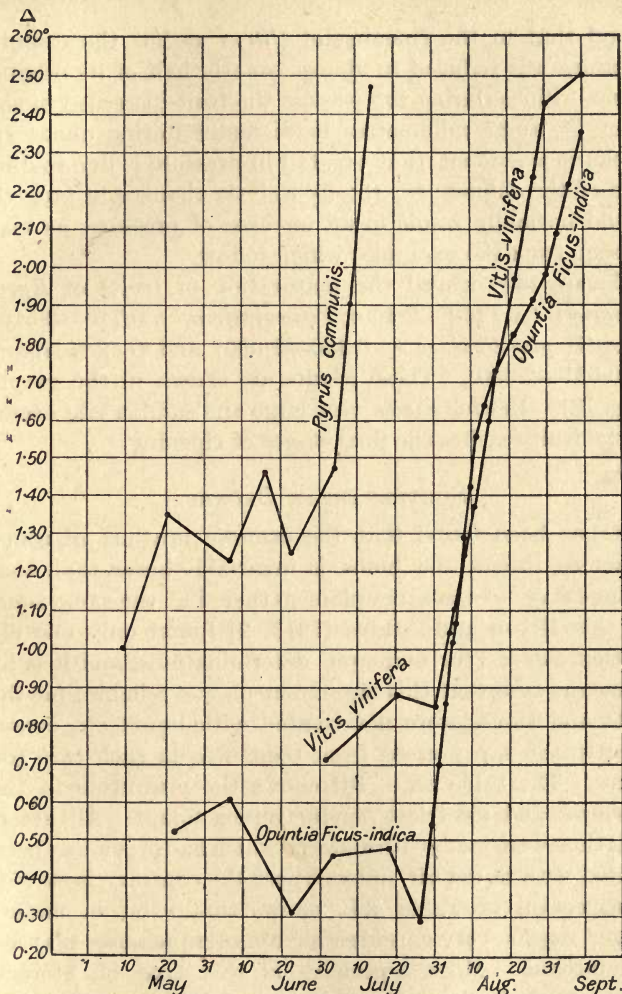
FIG. 18.—To show Cavara's Results on the Changes of Osmotic
Pressure in Fruits during Ripening.

Depressions of freezing-point are plotted as ordinates, and the abscissæ
represent the days of the months named.

found that in the ripening of *Citrus medica* the osmotic pressure was reduced to almost exactly half of its original value. Since during the process the fruit increased in size from 14 to 27 millimetres in diameter during about six weeks, it is evident that this fall in pressure is due to dilution of the solutes; for the hydrolytic changes in progress would normally result in an increase of pressure, as may be seen from the examples which follow.

Cavara also traced the maturation of fruits of *Pyrus communis* ($\triangle$ 1·031—2·460), *Vitis vinifera* ($\triangle$ 0·710—2·516), *Opuntia piccolominea* ($\triangle$ 0·460—2·080), and *O. ficus-indica* ($\triangle$ 0·521—2·360). These results are shown in the graphs (Fig. 18). In each case a very large and sudden rise occurs as the fruit reaches the final stages of ripening.

SUBTERRANEAN ORGANS.

It has been found that the osmotic pressure of roots, rhizomes, tubers, and bulbs, is invariably lower than that of the leaves of the same plant gathered at the same time. To this Dixon and Atkins (1912, 2) found only one exception out of very numerous determinations, and it is by no means sure that this measurement was reliable; for the roots and leaves were not treated with liquid air, consequently the sap pressed from them was in each case too dilute. The table on p. 161 shows the magnitude of the pressures and electrical conductivities found. All determinations were made with material which had previously been treated with liquid air, unless where the contrary is stated.

Inspection of Table XL. shows that even in underground organs very considerable osmotic pressures may be encountered. More especially is this true of storage organs, such as the roots of Beta and Brassica, the tuber of Helianthus, and the bulb of Allium, in all of which soluble carbohydrates accumulate. In these plants it may be

seen that the pressures exerted by the electrolytes are
small compared with those due to the carbohydrates and
other non-electrolytes. In most varieties of roots and
rhizomes, however, the two classes of bodies share in pro-
ducing the pressures fairly evenly, though in some the
influence of the electrolytes is most marked—in Equisetum,
for example. The leaves of Allium, Beta, Brassica, and
Helianthus, were not examined, but by analogy it is likely
that they have even higher pressures than the underground
organs.

TABLE XL.

OSMOTIC PRESSURE OF SUBTERRANEAN ORGANS.

Description of Sample.	Δ.	Δe.	$\Delta - \Delta e$.	P.	$C \times 10^5$.
Allium cepa, bulb, Nov. 27 ..	0·935°	0·105°	0·830°	11·26	214
Beta vulgaris, root, Nov. 26 ..	1·206°	0·191°	1·015°	14·51	383
„ „ Dec. 12 ..	1·761°	0·277°	1·484°	21·18	555
Brassica rapa, white root, Nov. 27	1·127°	0·175°	0·952°	13·55	356
Equisetum telmateia, rhizome, Nov. 16	0·597°	0·410°	0·187°	7·19	822
Eucalyptus globulus, roots 1 to 4 mm. diameter, Dec. 5 ..	0·591°	0·360°	0·231°	8·11	723
Ilex aquifolium, roots less than 3 mm. diameter, Nov. 8 ..	0·682°	0·315°	0·367°	8·21	629
Ilex aquifolium, roots less than 1 mm. diameter, Nov. 19 ..	0·635°	0·300°	0·335°	7·64	596
Ilex aquifolium, roots 1 to 4 mm. diameter, Nov. 19 ..	0·858°	0·305°	0·553°	10·32	613
Ilex aquifolium, roots 1 to 4 mm. diameter, Dec. 4 ..	0·862°	0·300°	0·562°	10·38	603
Pteris aquilina, rhizome, Dec. 5	0·929°	0·403°	0·526°	11·18	807
Solanum tuberosum, tuber, Nov. 22	0·588°	0·290°	0·298°	7·08	583
Helianthus tuberosus, March, untreated	1·153°	—	—	13·87	—
	1·062°	—	—	12·76	—
	1·553°	—	—	18·67	—

Roots functioning mainly as water-absorbing organs do
not usually contain sufficient solutes to cause high pres-

sures. In this connection, the experiments on Ilex (Dixon and Atkins, 1915, 2) are of interest. They show that the pressure increases with the size of the root, whereas the

ILEX AQUIFOLIUM —Roots.

FIG. 19.

The upper curves, A. B, and C, show the depressions of freezing-point and osmotic pressures in atmospheres of thick (over 2 millimetres in diameter), thick and thin, and thin roots respectively. The lowest curve shows the pressures due to electrolytes. In this case the size of the root is immaterial, as the individual determinations of the three kinds lie on the same curve.

electrical conductivity only fluctuates slightly, as may be seen from Fig. 19. The meaning of this appears to be

that the more massive roots function largely as storage organs, for in them the pressure of the non-electrolytes attains considerable dimensions, as can be seen in the table.

It may here be remarked that though the leaves of Ilex, gathered at the same time as the samples of the roots, always possessed higher pressures, yet the conductivity of the young leaves was considerably below that of the roots. The oldest leaves, on the other hand, contained a far greater quantity of electrolytes than did the roots.

This progressive change in the character of the sap on passing from the roots to the leaves may be illustrated by the following figures obtained with *Iris germanica*, the entire plants having been dug up and examined:

TABLE XLI.

Iris germanica, NOVEMBER 15.

			Δ.	Δ_e.	$\Delta - \Delta_e$.	P.	$C \times 10^5$.
Leaves, green tops	..	..	1·085°	0·360°	0·725°	13·05	726
,, bases ..	..	..	1·084°	0·384°	0·700°	13·04	776
Rhizome	..	..	0·829°	0·165°	0·664°	9·97	335
Roots ..	..	..	0·764°	0·390°	0·374°	9·20	786

Here the osmotic pressure decreases from above downwards, but the electrical conductivity increases, with the exception of that of the rhizome. The tops of the leaves are appreciably richer in sugars than the bases, though their osmotic pressure is the same. As is well known, Iris stores no starch in the green portions of the leaves, except a negligible quantity in the guard cells of the stomata. In the white portions of the bases of the leaves starch is, on the contrary, plentiful, as also in the rhizome and roots. It appears that the gradient of sugar concentration from

the assimilating to the storing tissues is maintained by the conversion of sugar into starch in the latter.

It must be pointed out that there is some uncertainty as to the accuracy of conductivity measurements upon sap pressed from small roots, owing to the adhesion to them of particles of clay. Washing is not permissible in such cases, as there is the possibility of dilution of the sap taking place if the cells are not at their maximum degree of turgidity, as well as of the adherence of water.

SEASONAL VARIATIONS IN THE OSMOTIC PRESSURE AND ELECTRICAL CONDUCTIVITY OF PLANT ORGANS.

Several attempts have been made to correlate the variations in pressure with external conditions, such as amount of sunlight, temperature, or rainfall.

Trinchieri (1909) has given both tables and graphs illustrating the behaviour of *Salpichroa rhomboidea* throughout the year. The rhizomes, cortex and entire organ, upper and lower portions of the stems, and the leaves, were all examined. The rainfall, too, was shown in a graph. He took plants both from a shady position in a Coniferetum and from ground formerly used as a vineyard. As a general rule slightly higher values were obtained from the plants growing in the sunny position in the Naples Botanic Gardens. The fluctuations are very considerable in all the organs. Since such a number of factors are concerned, it is not easy to single out the influence of any one as of especial importance.

Dixon and Atkins (1912, 1, 2 and 3) also studied the annual variations in the leaves and roots of *Ilex aquifolium*, and of the leaves of *Hedera helix* and *Syringa vulgaris*.

No correlation could be discovered between the fluctuations in pressure and the rainfall. This is not surprising, as the cells are normally fully distended. The results of

SYRINGA VULGARIS.— Leaves
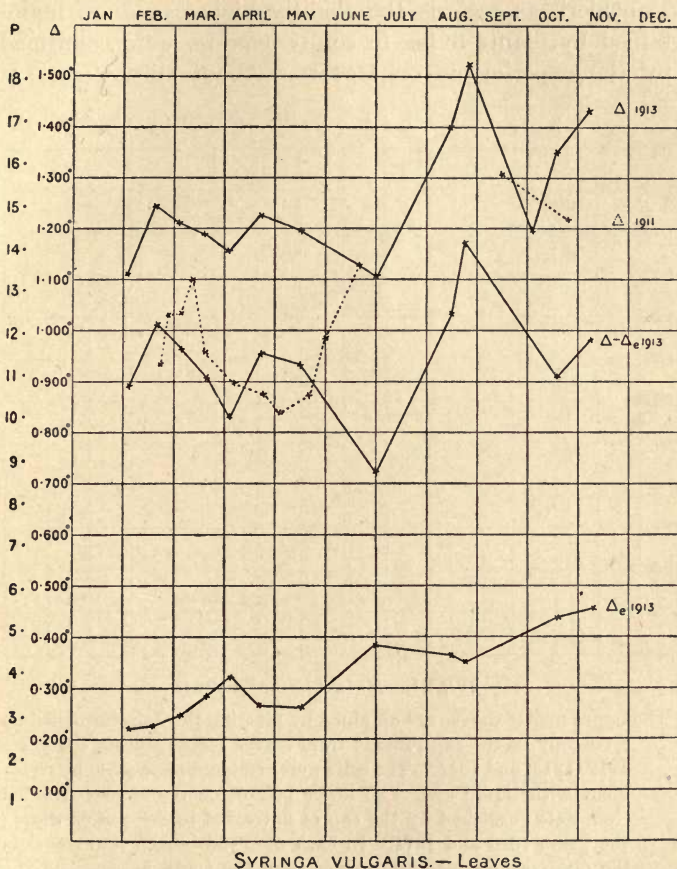
FIG. 20.

The unbroken curves show the depressions of freezing-point and osmotic pressures produced by the total solutes (Δ), carbohydrates ($\Delta - \Delta_e$), and electrolytes (Δ_e) of leaves of a deciduous tree treated with liquid air. These were gathered in 1913. The dotted line shows the values of Δ for untreated leaves gathered in 1911.

Trinchieri, as regards the slightly higher pressure maintained by plants living in sunny aspects, were confirmed by the experiments on Hedera. About thirty pairs of
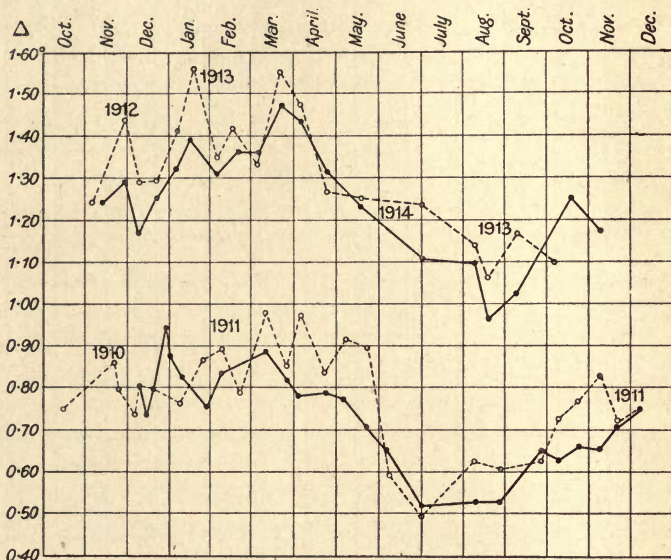
FIG. 21.—*Hedera helix:* LEAVES.

The upper pair of curves are obtained by plotting the data furnished by cryoscopy of the sap pressed from leaves picked during the years 1912, 1913, and 1914. The cells were rendered permeable by treatment with liquid air. The lower pair of curves are constructed from data furnished by the sap of untreated leaves picked during the years 1910 and 1911. In each case the broken line connects the observations made on leaves from the south aspect, and the full line those made on leaves from the north aspect.

determinations made at intervals over nearly two years yielded, as mean values for osmotic pressures, 9·61 atmospheres and 9·00 atmospheres for plants from a southern and northern aspect respectively.

ILEX AQUIFOLIUM. — Mature Leaves.
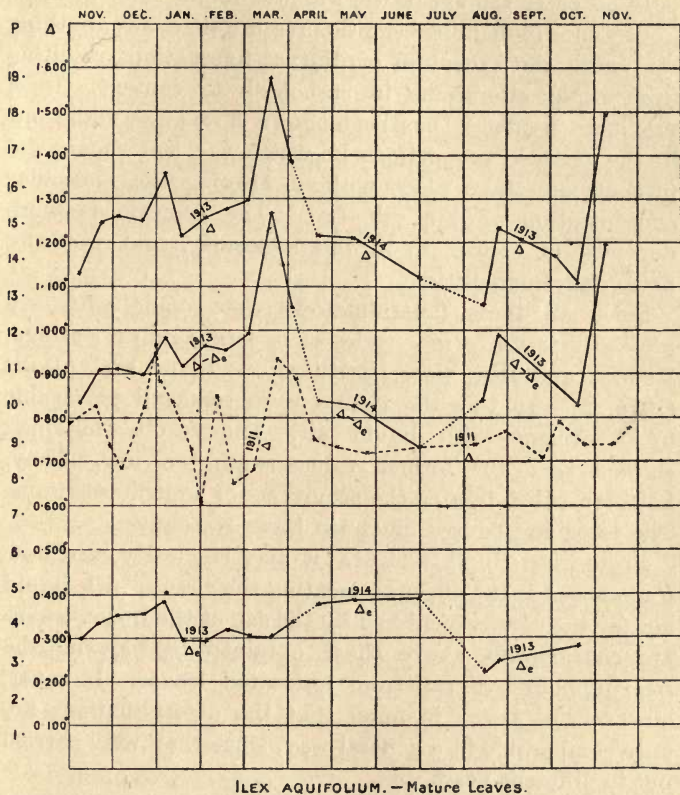
FIG. 21A.

The full-line curves show the depressions of freezing-point and osmotic pressures produced by the total solutes, carbohydrates, and electrolytes of leaves of an evergreen treated with liquid air. The dotted line represents the values of Δ for untreated leaves gathered in a previous year. Since the leaves employed were all of the same age, there is no rise in their electrolyte content as the season advances.

In all cases the age of the leaf was found to be of more importance in defining osmotic pressure than was anything else, with the exception of diurnal fluctuations resulting from rapid alterations in carbohydrate content. In a previous chapter it has been shown how large variations in the relative proportions in which the leaf sugars are present may take place without altering the molecular concentration of the cell sap. The osmotic pressure depends, of course, upon the molecular, and not the percentage composition.

Since all these determinations were made with sap pressed directly, a new series of experiments on Syringa, Hedera, and Ilex, were instituted by Dixon and Atkins (1915, 2). In this the tissues were rendered permeable by treatment with liquid air. The results obtained showed that the former values were too low. They, however, substantiated the occurrence of large fluctuations. The foregoing figures illustrate these researches.

From Figs. 20, 21, and 21A, it may be clearly seen how pronounced is the effect of treating the tissues with liquid air, for the curves furnished by the sap of the frozen leaves are considerably above those obtained by plotting the freezing-points of sap from untreated leaves. It must, however, be borne in mind that the determinations are only comparable in a general way, since they were carried out in different years.

On the whole it may be said that, though the daily fluctuations are most important, yet the osmotic pressure does rise with the age of the leaf. This increase is largely due to the accumulation of electrolytes, as shown by conductivity measurements. Thus, while the leaves of Syringa attain their highest pressures when mature and under favourable conditions for photosynthesis—viz., about the months of August or September—in evergreens two pressure

maxima are shown. Ilex and Hedera put forth their leaves more or less continuously, but the majority of leaves appear to arise at two periods—the beginning of winter and the early summer. At these periods new leaves open out in quantity at intervals during one or two months; consequently the proportion of young leaves is a maximum at such times. For this reason, and on account of the heavy drain upon the sugars of the mature leaves occasioned by the rapid growth of the younger ones, the osmotic pressure is found to be at its lowest during the summer and at the onset of winter.

Fig. 21 also shows that, as previously mentioned, the osmotic pressure of leaves of Hedera is slightly greater in plants from a southern aspect than in those facing north-wards.

Summary.

From the foregoing tables an idea may be gained of the magnitudes of the osmotic pressures commonly met with in plant tissues. The determinations of Dixon and Atkins were primarily undertaken with the object of ascertaining whether the pressures were sufficient for the requirements of the cohesion theory of the ascent of sap. In passing it may be remarked that they were found to be ample.

More generally osmotic pressure measurements serve as a ready means of studying the variations of the molecular concentration of the sap solutes. By combining them with determinations of electrical conductivity it is possible to distinguish between the portion of the pressure caused by electrolytes and that due to non-electrolytes, which are mainly sugars. The former, as a rule, remain remarkably constant, but rise slowly with age in tissues from which evaporation is continually taking place. The latter, on the other hand, are subject to large fluctuations, accord-

ing as the sugars are increased by photosynthesis, by translocation, or by hydrolysis of insoluble carbohydrates, or are reduced by respiration, translocation, or by constructive metabolism.

The pressures found in the leaves almost invariably exceed those of the roots. It must, however, be remembered that the values recorded in the tables are all calculated for 0°, whereas the actual pressures are higher owing to the plant tissues being normally at 10° to 20°. Thus if in a particular instance the sap of the leaves and roots had an identical freezing-point, yet if the former were warmer than the latter they would possess a higher pressure.

Among the highest pressures are those of fruits, in which when ripe all the available polysaccharides have been converted into sugar.

Pressures of considerable magnitude are also met with in subterranean storage organs of various kinds, especially in those in which soluble reserves accumulate.

CHAPTER X

OSMOTIC PRESSURE IN RELATION TO PLANT DISTRIBUTION, MORPHOLOGY, AND CELL DIVISION

SECTION I.—DISTRIBUTION.

DESERT PLANTS.

IN order that a plant may be able to absorb water from its surroundings it is necessary for its total forces of capillarity, imbibition, and osmotic attraction, to exceed those of its medium. Now, in plants growing in arid regions very high osmotic pressures are met with, as has been shown by Fitting's (1911) plasmolytic determinations, some of which are quoted in Table XLII.

TABLE XLII.

FLORA OF ROCKY DESERT IN SAHARA.

Percentage of Total Number of Plants examined (46).	Not plasmolyzed by x Grm. Mol. KNO_3.	Approximate Minor Limit of Osmotic Pressure.
21	3·0	100 atm.
35	1·5	53 ,,
52 (? 32)	1·0	37 ,,
11	0·3 to 0·6	10 to 20 ,,

In the first column it may be noticed that the percentages reach the total of 119. On recalculating them from Fitting's data, the values 21, 39, 26, and 11, were obtained. Thus, allowing for the inclusion of some border-line results

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in the second rather than in the third group, it seems probable that the value 52 is a misprint for 32.

Fitting points out that the lowest osmotic pressures are found in annuals, and the highest in bushes. Of the latter class ten were found to have pressures of about 100 atmospheres, six of them having sap rich in sodium chloride.

He also criticizes the cryoscopic method, and calls attention to the alterations which take place in the expressed sap, as a source of error, mentioning in particular the browning due to oxidation.* But this and other similar changes are, in fact, quantitatively insufficient to cause any appreciable error, as has been shown by direct experiment. Cryoscopic measurements combined with determinations of electrical conductivity would, indeed, have thrown much light upon the proportions in which electrolytes and non-electrolytes were concerned in producing the high pressures met with in desert plants. It is, however, obvious that an investigator can apply the plasmolytic method while travelling under conditions which would render the more elaborate technique of cryoscopy quite impossible.

To the low osmotic pressure of some annuals Fitting attributes the fact that in particularly dry years they do not thrive. The bushes, however, are able to draw water from the soil moisture which exists a few inches below the dry upper layer.

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* Diese und alle ähnlichen ausschliesslich an ausgepressten Zellsäften gewonnenen Resultate dürften aber nur selten Rückschlüsse auf die osmotischen Drucke in den unverletzten Zellen erlauben: Autolytische und andere Prozesse sind ja während und nach der Zertrümmerung der Zellen ganz unvermeidlich. Geben doch die Verf. selbst an, dass die Zellsäfte häufig braun wurden. Es ist zu bedauern, dass in zahlreichen neueren ausländischen Arbeiten die kryoskopische Methode zur Ermittlung osmotischer Drucke ohne Grund so sehr bevorzugt wird.

The behaviour of plants growing in salt swamps in the desert was also examined by Fitting. In a comparison between the pressures found in the same plants growing on cultivated land, on salt marshes, and on rocky or sandy desert, he clearly shows how closely the two latter habitats resemble one another as regards a physiological scarcity of water.

It has been supposed that the diminution of loss by evaporation is the principal benefit derived by such plants from their high pressure. This effect, while undoubtedly of some importance, does not, however, seem to be the principal one. It appears more probable that such high pressures enable the roots to abstract water from the soil against the action of its forces of imbibition and capillarity. Pringsheim, too (1906), has drawn attention to this rôle of osmotic pressure.

FUNCTION OF ESSENTIAL OILS.

Many desert plants are highly odoriferous, and it has been pointed out by Dixon (1898) that the accumulation in the intercellular spaces of the vapour of essential oils of high molecular weight forms a very effective check to evaporation. His experiments on " specific evaporation " confirm the above suggestion.

Giglioli (1911) has sought an explanation of the function of these oils in the alterations produced by them in the permeability of the protoplasm, and Osterhout (1913) has shown that the first action of such substances as anæsthetics is to lower the permeability of the protoplasm to salts. Thus it is quite possible that evaporation may be hindered both by a physiological alteration in the protoplasmic surface and by a purely physical retardation of the water molecules by the heavy vapours.

Accordingly, the power to prevent excessive loss of

water by transpiration, and that of elaborating or permitting the penetration of substances suitable for maintaining high osmotic pressures, should be considered as factors governing the distribution of desert plants.

SALT-MARSH, SAND-DUNE, AND ESTUARINE PLANTS.

Much precision has been lent to the ecological studies of Harshberger (1911) by his use of a hydrometer, with thermometer attached, in mapping out the distribution of plants in marshes and estuaries. The instrument is portable and readily used, and in this respect is greatly superior to chloride titrations, freezing-point, or electrical conductivity measurements, as a means of making comparative measurements of salinity.

The hydrometer can also be used in rough estimations of the salinity of soil water, being very suitable for noting the progress of attempts to reclaim salt marshes.

Harshberger has proved that the degree of salinity is the determining factor in the distribution of salt-marsh plants, although the texture of the soil, its aeration, and the lines of drainage, are also of importance.

By means of conductivity determinations, Scofield (1905) traced the salt-water limits of wild-rice, a grass of much economic importance. The maximum salinity which it was capable of resisting in the field was that represented by an approximately 0·03 normal solution of sodium chloride, the water of the sea in the same region having a conductivity equal to that of a 0·28 normal solution. Thus it was shown to be mere waste of time to attempt to sow the wild-rice in many tracts of land which were, to all appearances, quite suitable.

It was demonstrated by Drabble and Lake (1905, 1907) that salt-marsh and sand-dune plants are physiologically xerophytes, and possess relatively high osmotic pressures.

Their investigations were carried out by the method of plasmolysis, as was also the more recent work of Falck (1913) on the " alfvar " vegetation of the island of Oeland. This type of plant association consists of stunted and xerophytic species, and is similar to that found on the steppes. Falck considers that the average normal value for the osmotic pressure of plants is between 11 and 14 atmospheres, since they are isotonic with 0·3 to 0·35 gramme-molecular potassium nitrate. There are, however, but few of the alfvar plants which do not exceed this value considerably. The highest value found by him was in epidermal cells of *Helianthemum oelandicum* and amounted to over 55 atmospheres, since 1·6 gramme-molecular potassium nitrate was isotonic with them.

References to many of the recent researches in this line may be found in Falck's paper, but it has been impossible to mention more than a few of these here.

A number of interesting examples of plants accommodating themselves to an increase in the salinity of their surroundings were studied by Cavara (1905). In the salt lagoons of Cagliari evaporation proceeds vigorously from the spring till early autumn. This leads to a corresponding rise in osmotic pressure, as shown by Table XLIII.

It is very probable that in these plants the increase in pressure is due to the slow penetration of the salts of the soil-water. Electrical conductivity measurements would have decided this question, but they were not carried out.

This power of adaptation, it may be added, is shown to a very marked degree by the cells of Bacteria and Cyanophyceæ, and in these groups the protoplasm appears to be extremely permeable. Raciborski (1905) succeeded in obtaining the germination of Torula sp. in a saturated solution of lithium chloride. This possesses the highest osmotic pressure of any known solution, namely, 965·3 atmospheres,

according to the method of calculation adopted by Diete-rici, which makes use of data derived from vapour pressure measurements.

TABLE XLIII.

OSMOTIC PRESSURES OF PLANTS IN SALT LAGOONS.

	March, April.		August, September.	
	Δ.	P.	Δ.	P.
Atriplex crassifolia	—	—	5·52°	66·3
Halochnemum strobilaceum ..	3·76°	45·1	7·25°	87·0
	4·89°	58·7	7·26°	87·1
	5·72°	68·6	8·50°	102·0
Obione partulacoides	2·75°	33·0	7·25°	87·0
Salicornia herbacea	2·32°	27·8	4·20°	50·4
	3·26°	39·1	6·55°	78·6

Other interesting examples of adaptation to high external osmotic pressures are furnished by the mangroves and many tropical plants of the strand flora. According to Von Faber (1913), transpiration of water from mangrove leaves is very considerable whether in shade or in direct sunlight. In view of the fact that many mangrove-trees reach a height of 8 metres, and are exposed to direct sunlight, to a temperature of 40° to 45°, and to a sea-breeze, the passage of large quantities of water vapour from the leaves is to be expected. Schimper (1890, 1891) and Karsten (1891) considered that life was possible for such trees owing to their having certain xerophytic characters. Von Faber, however, believes that the high osmotic pressure of the leaves quite satisfactorily explains the possibility of their deriving a sufficient water-supply from the sea, or even in some cases from pools concentrated by evaporation to such an extent that salt crystals had

separated. In the roots the pressures found are very considerably smaller than in the leaves, and may only be half as great.

The following table contains some of Von Faber's results, which were obtained by the plasmolytic method:

TABLE XLIV.

Osmotic Pressure of the Epidermal Cells of the Leaves of Tropical Strand Flora.

					Atmospheres.
Rhizophora mucronata	..	..	..	..	72
,, *conjugata*	..	..	..	..	58
Avicennia alba ..	..	..	..	..	68
,, *officinalis*	..	..	..	..	52
Sonneratia alba ..	..	..	..	..	64
Bruguiera gymnorhiza	..	..	..	..	34
Ceriops Candolleana ·	..	..	..	..	32
Ægiceras majus ..	..	..	..	..	29
Acanthus ilicifolius	..	..	..	..	24
Lumnitzera racemosa	..	..	..	..	30

The salt taste of the leaves points to the presence of electrolytes as being responsible for a very considerable portion of the high pressures.

These plants have a very great power of adapting themselves to the medium surrounding their roots, and are found both on coral islands and in the almost fresh water of estuaries. Furthermore, it was found possible to plant *Rhizophora mucronata*, taken directly from the sea, in fresh water without causing any serious damage.

ALGÆ.

A fundamental distinction between marine and freshwater algæ is that they are adapted to growth in solutions of different osmotic pressure. That it is not owing to a poisonous effect of salt water that fresh-water algæ cannot live in the sea is sufficiently shown by Osterhout's researches

on antagonism of ions, for he finds sea-water to be a perfectly balanced solution in this respect.

Quite similarly, the destructive action of fresh water upon seaweeds is very noticeable. Yendo (1914) has recently drawn attention more particularly to this aspect on account of the serious losses to Japanese seaweed industry resulting from it.

Some algæ, such as species of Enteromorpha, can withstand sudden alternations of fresh and salt water. These are usually found, in the British Isles as well as in Japan, growing on those portions of the beach which are bathed in a stream of fresh water at low-tide, but by the sea during high-tide. Others, normally growing in the open sea, can accommodate themselves to a lesser salinity. Yendo illustrates this by the behaviour of *Fucus vesiculosus*, which may be found in a brackish lagoon and in the sea, but not in the connecting channel, where fluctuations of salinity occur.

With a view to testing the nature of the equilibrium between the osmotic pressure of a seaweed and the surrounding salt water, the following experiment was carried out by Dixon and Atkins : A quantity of *Ascophyllum nodosum* was collected near Kingstown, Co. Dublin, and was immediately brought to the laboratory and pressed. It was not treated with liquid air, as at the time the authors had not discovered the necessity for this. However, as the results show, the error introduced in this case cannot have been of any considerable importance. A portion was kept in darkness in sea-water for twenty-four hours, and was then pressed; while a portion of the same sample was placed in sea-water to which slightly more than an equal volume of fresh water had been added, for three and a half hours. Table XLV. shows the values of Δ, P, and M (the mean molecular weight), so obtained.

TABLE XLV.

OSMOTIC PRESSURE AND ITS ADJUSTMENT TO EXTERNAL MEDIUM.

Ascophyllum nodosum.	Δ.	P.	M.
Thallus from sea, April 6, 1910	1·988	23·91	35·5
Surrounding sea-water	1·870	22·62	—
Thallus kept 24 hours in sea-water in darkness at the laboratory, April 7	1·963	23·62	43·7
Thallus as in previous experiment, after 3½ hours in diluted sea-water	1·115	13·41	44·5
Diluted sea-water	0·887	10·67	—

The figures recorded in the table show that normally the alga has a slightly higher osmotic pressure than the sea-water, and that this is practically entirely due to electrolytes is demonstrated by the low value of M. When kept in the dark for one day, there is scarcely any change in the pressure, but the value of M appears to rise slightly. Alteration of the medium, however, leads to a very rapid readjustment of the pressure, through the diffusion outwards of the salts. As might be expected, the mean molecular weight is practically unaffected by this change. These experiments support those of Osterhout upon the electrical resistance of Laminaria, from which he concluded that the composition of the cell sap was almost identical with that of the surrounding sea as regards its content of electrolytes, and that the living protoplasm occasioned the extra resistance which he found. The researches of Osterhout have already been discussed in Chapter VII. Since diluted sea-water is a physiologically balanced solution as regards its ionic composition, it is evident that the injurious effects noticed on the large scale in Japan are occasioned by fluctuations which are too rapid for the proper adjustment to be effected. Accordingly, patho-

logical results follow in the constitution of the surface layer of the protoplasm, and it is possible that cells may in some cases be even ruptured by excessive intake of water.

Rapid changes in the sea-water brought about by the increased volume of fresh water delivered by the rivers during flood have been proved by Yendo to be the cause of the serious injury to the algal flora known in Japan as "reef-burning." This often occasions losses in the sea-weed industry of as much as £1,200 a mile per annum, and the evil effects continue for several years. In addition to the destruction of algæ, there is the loss due to the migration of molluscs and pelagic fishes. The presence of strong coastal currents deflects the river-water from its seaward course, so a region of brackish water along the coast results. It is by the extension of this zone in time of flood that the phenomenon of "reef-burning" is occasioned.

EPIPHYTES AND PARASITES.

The relationship between the osmotic pressures of epiphytes and parasites, and those of the plants upon which they grow, has been investigated by Senn (1913) in a number of instances. By means of the plasmolytic method it was found that the pressures of the former were the greater, so that the passage of water from the conducting tracts of the host is easily understood.

SECTION II.—OSMOTIC PRESSURE AND MORPHOLOGICAL VARIATIONS.

NORMAL AND ABNORMAL FRUITS.

Realizing that all morphological differences are ultimately based upon chemical and physical differences of a qualitative or quantitative nature, Gortner and Harris

(1913) attempted to discover a connection between the depressions of freezing-point, densities, and mean molecular weights, of normal and abnormal fruits of *Passiflora gracilis*.

The abnormalities consisted in the occurrence of external sutures and placentæ in excess of the usual number.

These authors distinguished between the effects of heavy and light pressing upon the nature of the sap obtained, and in other ways took care that the abnormal fruits and the controls were subjected to exactly similar treatment.

As the result of the examination of twenty-three samples of abnormal fruits and their controls, it was found that a very small excess ⳙpressure and diminution in mean molecular weight was found in favour of the abnormals. These are shown below.

TABLE XLVI.

DIFFERENCES IN OSMOTIC PRESSURES OF NORMAL AND ABNORMAL FRUITS.

	Number of Experiments showing Positive Difference.	Number of Experiments showing Negative Difference.	Mean Value of Differences.
Δ.	15	8	$+0.0107°$
M.	7	14	$-2.091°$
P.	15	6	$+0.2275$ atm.

In view, however, of the impossibility of applying the same degree of pressure and of the magnitude of individual variations, it does not seem that any importance whatever can be attached to the results of this interesting application of physico-chemical methods, and, indeed, Gortner and Harris themselves recognize this fully.

POLYMORPHISM OF ALGÆ.

Klebs and other workers found that many green algæ were capable of existing in two or more forms, but the stimulus inducing the change was unknown. It remained for Livingston (1900) to show that this was of a simple nature, being none other than the osmotic pressure of the solution in which the algæ grew.

Livingston demonstrated that the responses of *Stigeoclonium* (*tenue* ?), both in form and in reproductive activity, which accompany a change in concentration of the Knop's solution in which it is growing, are due to changes in the osmotic pressure of the medium, and are in no way functions of its chemical composition. Further work (1901) established the fact that solutions of non-electrolytes produce the same result as those of electrolytes, for in them, too, osmotic pressure is the controlling factor in determining the form of the plant. This is effective through changes in the water content of the cells.

It should be mentioned that this species of Stigeoclonium grows on moist bark, and there exhibits the palmella form. The spherical cells multiply by division in vertical planes at right angles to each other, the daughter cells separating more or less completely after division. In the filamentous form the cells are cylindrical and remain in contact; division takes place in transverse planes. Both forms effect reproduction by means of comparatively large asexual biciliate zoospores.

In addition, Livingston found that a high osmotic pressure* affects the plant in four ways: (*a*) It decreases vegetative activity; (*b*) it inhibits the production of zoospores; (*c*) it causes cylindrical cells to become spherical;

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* The highest osmotic pressures employed by Livingston in these researches only slightly exceeded 8 atmospheres at 0°.

(*d*) it frees the alga from certain limitations as to the orientation of the planes of cell division. Conversely, a low pressure reverses these effects. It was also ascertained that there were certain quantitative differences between the concentrations required to inhibit zoospores in the palmella and in the filamentous stage, those required for the former being the greater. Between the behaviour in this respect of electrolytes and of non-electrolytes, such as sucrose, quantitative differences were found. These are all explicable on the assumption that the permeability of the cells is greater towards the latter. Furthermore, it was proved that prolonged darkness does not induce a change in the form of the alga.

That all these phenomena are due merely to variations in the amount of water in the vacuoles appears very improbable. It seems more reasonable to connect them with alterations in the state of imbibition of the protoplasmic colloids; also with changes in the rate of oxidations which normally occur in the cell, owing to increases or decreases in the area of external surface, and consequent disturbance of the usual rate of intake of atmospheric oxygen. The loss of their motility experienced by many bacteria when brought into solutions of a high osmotic pressure appears to be a quite similar instance of disturbance caused by the withdrawal of water from the protoplasm, for bacteria are very freely permeable to most salts. Wladimiroff (1891) has employed this arrest of the activity of motile bacteria to measure osmotic pressures.

POLYMORPHISM OF FUNGI AND STRUCTURAL CHANGES IN ANGIOSPERMS.

Beauverie (1900, 1911) has studied the growth of various fungi, such as the Mucorinæ and the conidial forms of the higher groups, in solutions of different concentrations.

Working with such forms as Aspergillus, Sterigmatocystis, Penicillium, Clonostachys, etc., he found that increase in the osmotic pressure of the liquid medium results in— (a) Reduction in height of the aerial portion of the fungus and the lateral dilatation of the cells constituting this region; (b) the predominance of the submerged portion of the mycelium relative to the aerial portion. In a certain number of cases a great concentration of the medium determines the complete immersion of the plant. There is then manifested a profound alteration in the form of the reproductive organs, which usually remain sterile.

The results obtained with seedlings of Phaseolus, Pisum, Lupinus, Zea, and Triticum, were also of interest. As a rule the roots became more or less abnormal in their macroscopic appearance and branching. Microscopical examination also showed profound modifications in the histology of the root. For example, haricot beans (Phaseolus sp.) were grown in water and in Knop's solution to which sodium chloride had been added to make a 1 per cent. solution. The former developed normally, with a large portion of the centre occupied by pith. The latter, however, contained no pith, the centre being filled up with the proto- and metaxylem, outside which was a thick layer of secondary xylem. Furthermore, the central cylinder was protected from the action of the medium by the early development of abundant cork arising from the pericycle.

EFFECT OF INCREASE AND DECREASE OF OSMOTIC PRESSURE UPON GROWTH AND IRRITABILITY.

A great number of researches have been carried out upon the effect of increase or decrease of osmotic pressure upon growth and irritability. These have been treated of by Livingston (1903), also by Loeb (1906, 1913), and consequently they will be considered here very briefly.

In a general way it may be said that increase of osmotic pressure lessens the rate of growth of cells and the rate of regeneration of lost organs in the lower animals. Loeb has found that the effect of a hypertonic solution may be such as to prevent the division of a cell while permitting the mitotic division of the nucleus. Furthermore, exposure for a short period to a hypertonic solution forms an essential part of one of Loeb's methods for bringing about artificial parthenogenesis.

Another interesting result obtained by Loeb is that the larvæ of certain Copepods are negatively phototropic above 25°, but react positively below 10°. The addition of sodium chloride to the sea-water in which they are living has, however, the result of causing them to react positively, whereas dilution with distilled water has the opposite effect. These phenomena all appear to be related to the withdrawal of water from the protoplasm, with consequent changes in its viscosity and in the concentration of the reacting substances within it. The subject is one of great interest, and to deal with it superficially would be misleading. It has been discussed in detail in the books by Loeb and by Livingston referred to previously.

CHAPTER XI

THE FUNCTIONS OF THE WOOD

Two of the main functions of wood appear to be to transmit water upwards from the roots to the branches and leaves, and to furnish mechanical support. Of these, the latter has been dealt with at length in the well-known researches of Schwendener, which are summarized by Haberlandt in his "Physiological Plant Anatomy," and the former has been discussed fully by Dixon in "Transpiration and the Ascent of Sap."

It must, however, be conceded that the wood has a third function, one of nutrition in a narrow sense; for, to be accurate, water is a nutritive substance. Accordingly, it is with the transpiration stream considered as a medium for the distribution of food materials that the author proposes to deal in the present chapter.

So long ago as 1858 Th. Hartig recognized that the soluble products of the reserve materials found in the wood parenchyma and the medullary rays must utilize the tracheæ as their channels of transport to the higher regions of plants. This he demonstrated by the depletion of these stores in ringed branches. He concluded that the materials assimilated in the leaves are passed down in the bark and stored in the wood parenchyma and medullary rays. In spring these store materials are brought into solution, and passed into the tracheæ, where they rise with the upward moving current of water from the roots.

In 1888 A. Fisher demonstrated by chemical means the presence of reducing sugars in the tracheæ of a large number of trees at various times of the year. He does not appear to have tested for sucrose.

From this it might be inferred that the conveyance of carbohydrates in the wood described by Hartig, and supposed to occur noticeably only in spring, in reality takes place all the year round, but in spring most markedly.

Notwithstanding this, it is surprising to find how Sachs's (1887) statement that the water in the tracheæ is " an exceedingly dilute solution of these (nutritive) salts, which may be compared at once to ordinary drinking-water," seems to have won the ear of writers; so that the function of the tracheæ in conveying organic substances upward is either ignored in text-books and omitted from the consideration of plant physiologists, or its continuance throughout the year is discredited or left doubtful; as, for example, by Jost (1907), and in the cautious statement of Haberlandt (1914).

Leclerc du Sablon (1902), however, investigated the seasonal variations in the reserve materials of the stems and roots of trees, and has recently (1911) summarized his researches. His analyses deal with the total contents of the wood, both of cells and tracheæ. He clearly points out the function of the wood parenchyma cells in mobilizing reserve carbohydrates, which are then passed upwards by the elements of the wood, and to some extent by the bast also. Since these investigations are already in an accessible form, they will not be considered here at any length. Nicoloff (1911) has also given a very good account of the functions of the medullary rays and of the tracheæ.

RESEARCHES ON WOOD SAP.

In connection with investigations on the osmotic pressures and conductivity of solutions of vegetable origin, Dixon and Atkins (1915, 1) found it desirable to make observations on the sap drawn from the conducting tracts of trees.

With this end in view, wood taken from freshly cut branches and roots of *Acer pseudoplatanus* in the month of August was subjected to such pressure that sufficient sap was yielded for the determinations. The results are given in Table XLVII.

TABLE XLVII.

SAP PRESSED FROM WOOD OF *Acer pseudoplatanus*, AUGUST, 1913.

	Δ.	Δe.	$\Delta - \Delta e$.	P.	$C \times 10^5$.
Roots	0·468°	0·163°	0·305°	5·63	350
2 - metre level, small branches	0·370°	0·136°	0·234°	4·45	292
9 - metre level, small branches	0·226°	0·103°	0·159°	3·15	222

It may be mentioned here for the sake of comparison that a 1 per cent. solution of glucose gives a depression of 0·106°, a 1 per cent. solution of sucrose gives a depression of 0·054°, and a 1 per cent. solution of potassium chloride gives a depression of 0·459°.

Accordingly the sap must contain 1·5 to 3 per cent. of sugar at least, on the assumption that the non-electrolytes are all glucose; or from 3 to 6 per cent. approximately if sucrose forms the preponderating part.

A little consideration made it clear that the liquid issuing from the crushed wood must contain some sap pressed from the cells of the medullary rays and of the

wood parenchyma. If these cells were burst by the pressure, a more or less concentrated solution liberated from their vacuoles would be set free to contaminate the sap in the tracheæ. If, on the other hand, the cells are unbroken, their semi-permeable membranes will filter the solutions of the vacuoles, and the escaping liquid will dilute the wood sap with nearly pure water, as previously shown by the authors (1913, 1). Evidently, then, the true concentration of the sap in the tracheæ may be very different from that pressed from the wood.

The possibility of centrifuging the sap from the tracheæ of pieces of freshly cut wood subsequently suggested itself, and this method was found very successful.

The solution obtained thus was found to be much less concentrated than that obtained by pressure. In the following table are given measurements made on sap derived by centrifuging pieces of the same branches and roots as those which supplied the sap for the determinations recorded in Table XLVII.

TABLE XLVIII.

SAP FROM *Acer pseudoplatanus*, AUGUST, 1913.

	Δ.	Δe.	$\Delta - \Delta e$.	P.	$C \times 10^5$.
Centrifuged from tracheæ of root	0·070°	0·032°	0·038°	0·84	69
Centrifuged from tracheæ of branch, 9-m. level ..	0·049°	0·019°	0·030°	0·59	41
Pressed from leaf cells treated with liquid air ..	1·207°	0·646°	0·561°	14·52	1,341

Comparison of the depressions of freezing-point and the conductivities of the liquids obtained from the wood by the two methods shows conclusively that pressure, by

bursting the cells, had contaminated the sap of the tracheæ both with electrolytes and non-electrolytes. This contamination was further shown by the fact that, while the sap *pressed* from the wood became more or less deeply coloured brown owing to the presence of a chromogen and an oxidase, that *centrifuged* from it remained almost colourless, indicating that either one or both of these bodies were retained in the substance of the wood, or, more precisely, in the living cells. Moreover, the centrifuged sap is neutral to litmus, whereas that obtained by pressure is acid.

At the same time the interesting fact was made clear that even in the month of August the carbohydrates present in the tracheæ are sufficiently concentrated to produce a depression of freezing-point amounting to 0·030° and 0·038° in stem and root respectively. It thus appears that the solution of carbohydrates passing up through the tracheæ in the transpiration stream has the same freezing-point as a 0·25 per cent. solution of glucose, or a 0·50 per cent. solution of sucrose. The concentrations of the carbohydrates of the root approximated to those of 0·3 per cent. solution of glucose, or of 0·7 per cent. of sucrose.

These observations strikingly negative Sachs's view that the stream rising from the roots to the leaves during transpiration is to be regarded as a very dilute solution of salts only.

It is interesting to note how much more concentrated the sap of the vacuoles of the cells of the leaves was at the same time. This was extracted by pressure from leaves which had been treated with liquid air to render their protoplasm permeable.

Contemporaneously with the experiments on *Acer pseudoplatanus* similar measurements were made on the sap of *Populus alba*, and are recorded in Table XLIX.

TABLE XLIX.

Sap of *Populus alba*, August 28, 1913.

	Δ.	Δe.	Δ − Δe.	P.	$C \times 10^5$.
Centrifuged from tracheæ of stem at 12-m. level ..	0·047°	0·016°	0·031°	0·57	34
Centrifuged from tracheæ of root	0·072ª	0·024°	0·048°	0·87	52
Pressed after treatment with liquid air from bark of stem, 12-m. level..	1·215°	0·218°	0·997°	14·62	453
Pressed after treatment with liquid air from bark of root	1·101°	0·179°	0·922°	13·23	383
Pressed after treatment with liquid air from spring leaves	1·326°	0·438°	0·888°	15·95	909
Pressed after treatment with liquid air from summer leaves	1·487°	0·315°	1·172°	17·88	654

In this case the sap centrifuged from the tracheæ of the stem and the root gave no direct reaction with Fehling's solution. Evidently neither glucose nor maltose was present. On inversion, however, a noticeable reduction took place, indicating the presence of sucrose to the amount of about 0·5 per cent. and 1 per cent. respectively in the stem and root.

The sap from the cells of the bark of stem and root, and from the leaves examined at the same time, showed, as appears above, much higher concentrations.

It was then determined to investigate the concentration and constituents of the wood sap at different seasons of the year, and to see if variations of any considerable magnitude occurred in it. With this end in view, pieces were cut from the branches, at the same level on each occasion, and roots of the following trees, and examined cyroscopically, electrically, and chemically: *Acer pseudoplatanus*,

Cotoneaster frigida, Fagus silvatica, Ilex aquifolium, Populus alba, and *Salix babylonica.*

Under hexose and sucrose is given an approximate estimate of the amount of these sugars present; when 5 drops of the sap decolorized 10 drops of Fehling's solution, × × × × is set in the hexose column, when equal volumes were required × ×, and when 20 drops of the sap had to be used for 10 drops of the solution × is put down. Under sucrose a similar notation is used, indicating the volume of inverted sap required to decolorize the copper solution. Allowance is made for hexose, if any was found previous to inversion. In the same way a barely perceptible reduction is indicated by +, and a more marked trace by + +. It may be mentioned that the values of these signs approximately correspond, × × × × to 1 per cent., × × × to 0·75 per cent., × × to 0·50 per cent., and × to 0·25 per cent.; + to 0·01 per cent., and + + to 0·1 per cent.

TABLE L.

Acer pseudoplatanus : WOOD SAP FROM STEM AT 8-METRE LEVEL, AND FROM ROOT.

Date.		$\Delta.$	$\Delta_e.$	$\Delta - \Delta_e.$	$P.$	$C \times 10^5.$	Hexose.	Sucrose.
March 3, stem		0·120°	0·022°	0·098°	1·44	47·0	× ×	× × × *m*
Apr. 10*	stem	0·223°	—	—	2·68	—	× ×	× × × × *m*
	root	0·076°	—	—	0·91	—	0	× × ×
Aug. 25	stem	0·049°	0·019°	0·030°	0·59	41·0	—	—
	root	0·070°	0·032°	0·038°	0·84	69·3	—	—
Oct. 7	stem	0·041°	0·021°	0·020°	0·50	45·7	+	+ +
	root	0·080°	0·027°	0·053°	0·96	57·7	0	× ×
Dec. 19	stem	0·091°	0·032°	0·059°	1·09	69·0	—	—
	root	0·059°	0·035°	0·024°	0·71	74·9	—	—

Where *m* is written in the sucrose column, the presence of maltose was detected by phenyl hydrazine. Of course,

maltose, being a reducing sugar, contributes to the precipitate observed before inversion. It is somewhat hydrolyzed by boiling with acid for a short time, and consequently adds to the precipitate occurring after inversion.
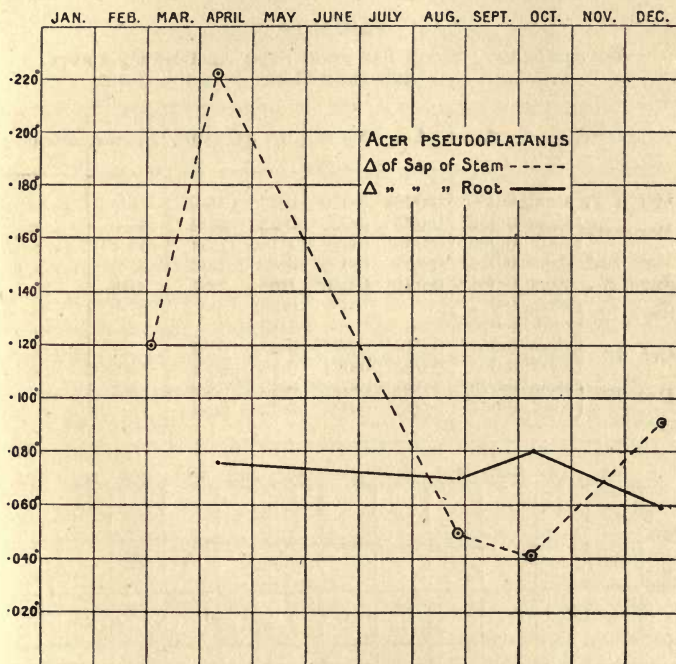
Fig. 22.

Its presence renders the identification of a hexose by the reduction test rather doubtful.

An asterisk is placed on the date at which the leaves of the buds began to expand.

These observations are graphically recorded in Fig. 22, in which the ordinates represent the depressions of freezing-point and the abscissæ the months of the year. The broken

line is the graph for the concentration of the sap of the wood of the stem; the full line that of the root. The figure shown on p. 193 is fairly typical of the behaviour of Populus, Fagus, and Salix.

TABLE LI.

Ilex aquifolium : Wood Sap from Stem at 1-Metre Level, and from Root.

Date.		Δ.	Δe.	$\Delta - \Delta e$.	P.	$C \times 10^5$.	Hexose.	Sucrose.
March 2,	stem	0·056°	0·026°	0·030°	0·67	55·9	—	—
March 25	stem	0·052°	0·025°	0·027°	0·63	54·4	+ +	× ×
	root	0·096°	0·056°	0·040°	1·16	119·4	× ×	× ×
May 14,*	stem	0·074°	0·035°	0·039°	0·89	76·6	× ×	× × *m*
June 27,	stem	0·084°	0·034°	0·050°	1·01	72·4	+ +	×
Sept. 12	stem	0·082°	—	—	0·98	—	+ +	—
	root	0·099°	—	—	1·19	—	×	—
Oct. 8	stem	0·051°	0·029°	0·022°	0·62	61·7	×	× ×
	root	0·063°	0·042°	0·021°	0·78	90·4	×	× ×
Dec. 23*	stem	0·072°	0·030°	0·042°	0·86	63·9	×	×
	root	0·061°	0·037°	0·024°	0·73	80·3	×	+
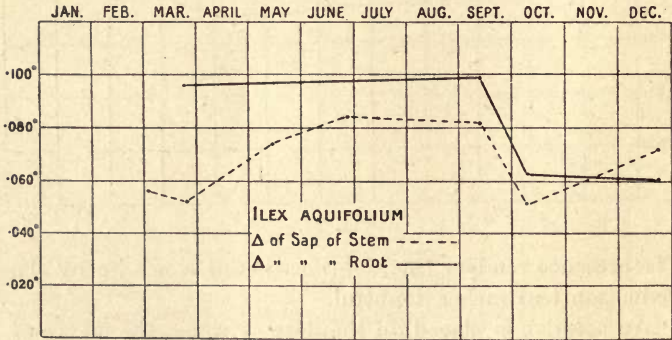
FIG. 23.

The results obtained with the evergreen Ilex differed very considerably from those afforded by deciduous trees.

They are shown in Table LI. and in Fig. 23. The sub-evergreen Cotoneaster behaved much in the same way as did Ilex.

CONCENTRATION OF TOTAL SAP SOLUTES.

The general form of the curves for the concentration of the wood sap in the stem of the deciduous trees examined is similar—viz.: There is a cusp in the early spring, followed by a rapid fall.* Then a period of low concentration in the summer and early autumn,† followed by a rise, which is gradual at first, and then becomes steeper as it approaches the spring maximum. So far as the investigation goes, it shows that the concentration of the wood sap in the root follows that of the stem, and is generally lower than it; but there does not appear to be such a pronounced rise in the spring, and the succeeding fall is not so rapid. Accordingly, during the vernal decline in concentration it is often found that the solutes of the wood sap of the root exceed those of the stem. Sometimes this difference persists until the concentration of the latter begins to rise again—e.g., Acer, Populus, and possibly Fagus.

The few observations made upon Salix indicate that the concentrations in the root and the stem are closely similar.

As before mentioned, *Ilex aquifolium* behaves quite differently from the deciduous trees with regard to the seasonal variations in its wood sap.

* This sudden rise and fall during the spring in the concentration of the carbohydrates of the sap was detected by Schröder (1869) in his investigation of the bleeding of trees.

† A. Fischer (1888) also found that there was less glucose in the vessels of several trees in the summer and autumn than in the spring.

Here the graph has two cusps—one in summer and one in winter—and corresponding depressions in spring and autumn.* The relation of the concentration found in the root to that of the stem is reversed, for while in deciduous trees the sap solutes of the wood are generally greater in the stem than in the root, the converse was found to hold in the case of Ilex. Only in winter was it less in the root than in the stem.

CONCENTRATION OF NON-ELECTROLYTES.

The form of these curves is not greatly altered if we deduct from each observation the amount of depression due to the electrolytes. Hence they convey a fair impression of the variations in the concentration of the soluble carbohydrates in the tracheæ, and it has not been thought worth while to plot special graphs for the latter.

From inspection of the tables it is quite clear that, except occasionally in the summer and autumn, the molecular concentration of the carbohydrates in the transpiration stream in deciduous trees is greater than that of the salts. Furthermore, since the sugars have high molecular weights (glucose 180, sucrose and maltose 342), the actual percentage weights of these substances in the sap must be far greater than that of the electrolytes, which have low molecular weights and are largely ionized. Hence we must admit that the translocation of carbohydrates is

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* It is difficult to correlate the variations in concentration in the sap of Ilex with the periods of bud expansion. The period for bud expansion appears uncertain, especially in pruned or lopped trees; thus, in the season 1912-13 buds expanded from November to January. In 1914 the summer buds were opening as early as April 22, and expansion was complete about the beginning of June. On the whole, however, most buds open during the winter and summer months.

at least as important a function of the transpiration current as the transference upwards of nutritive mineral salts.

CONCENTRATION OF ELECTROLYTES.

With the exception of two observations made in June on *Cotoneaster frigida* and in October on *Populus alba*, it appeared that the concentration of electrolytes was greater in the wood of the roots than in that of the stems This would seem to suggest that, while the quantity of dissolved carbohydrates in the transpiration stream may be added to on its upward passage, the amount of dissolved electrolytes is not thus reinforced, but usually is diminished as the stream rises.   Doubtless some of the dissolved salts are abstracted and used in various processes of metabolism. The observation on Cotoneaster, however, showed that this does not always hold good, for in it the concentration of electrolytes in the stem was slightly greater than in the roots.   Furthermore, in a number of observations made on *Acer macrophyllum* (see Table LII.) no steady gradient from below upwards is revealed.

VARIATION IN CONCENTRATION AT DIFFERENT LEVELS IN THE CONDUCTING TRACTS.

The pieces of the stems yielding the sap examined in the foregoing experiments were always taken from the same plant and from the same levels above the ground.   These were as follows:

Acer pseudoplatanus, 8 m.	*Ilex aquifolium*, 1 m.
Cotoneaster frigida, 6 m.	*Populus alba*, 12 m.
Fagus silvatica, 12 m.	*Salix babylonica*, 5 m.

It seemed desirable to compare the sap from the same level on the different occasions, in case a difference in

level in the tree is associated with a change in concentration.*

The subject for the investigation was an old tree which had been cut across near the ground many years previously. From the level of the soil three similar branches about 30 centimetres in diameter took their origin, and rose to a height of about 10 metres. One of these was cut down in the middle of October, and samples of the wood excised at various levels—viz.: (1) ground-level, at (2) 2 metres, (3) 4 metres, (4) 6 metres, and at (5) 8 metres above ground-level, and finally (6) from the small branches about 10 metres above the ground. The following table gives the results of determinations made on the sap centrifuged from the wood, and shows how the concentration varies from below upwards at that time of year:

TABLE LII.

Acer macrophyllum : Wood Sap, October.

	Δ.	Δe.	Δ − Δe.	P.	$C \times 10^5$.	Hexose.	Sucrose.
Stem, 10-m. level	0·068°	0·037°	0·031°	0·81	79·0	+ +	× ×
,, 8-m. ,,	0·048°	0·033°	0·015°	0·57	70·9	+	×
,, 6-m. ,,	0·040°	0·030°	0·010°	0·49	64·8	0	0
,, 4-m. ,,	0·035°	0·025°	0·010°	0·42	54·2	0	0
,, 2-m. ,,	0·046°	0·028°	0·018°	0·56	61·0	0	×
,, 0-m. ,,	0·053°	0·039°	0·014°	0·63	84·6	0	×
Root	0·060°	0·035°	0·025°	0·72	76·4	0	× ×

* Schröder (1869) found this to be the case in bleeding sap of *Acer platanoides* and Betula. The sugar concentration in Acer was found to be greater in the root and in the upper parts of the stem than in the lower parts of the stem. In Betula, on the contrary, the concentration of the bleeding-sap is less in the top of the stem and in the root than it is in the base of the stem.

DISTRIBUTION OF CARBOHYDRATE RESERVES IN THE WOOD.

Starch was present in large quantities in the wood at the time these determinations were made. The cells of the medullary rays, the last few layers of elements formed in each year-ring and the first layer of the next, and the elements in contact with the vessels, were densely crowded with starch grains. The sheath of starch-containing elements round the vessels was continuous, and often many-layered, in the root and in the stem at the ground-level. Higher up only some of the elements in contact with the vessels contained starch, but those which did so were densely packed. Also it was noticed that in this region the first layer of the spring wood was without starch, except where it was in contact with vessels. Generally there appeared fewer starch-containing elements at the higher levels. In the root the number of starch-containing cells is still further augmented by the fact that the vessels are much more numerous, hence the number of elements constituting the starch-bearing sheaths is more considerable. A quantitative estimation of the cross-section of the various elements of the wood in some trees will be given farther on.

Leclerc du Sablon (1902) has quantitatively analyzed the wood of the stem and roots of trees at various periods of the year. He examined both deciduous trees and evergreens. As typical of the behaviour of the former, his results with the chestnut are quoted in the following table. The variations in the carbohydrate content are very great.

From these figures it may be seen that the quantities of stable reserves, polysaccharides, far exceed those of the sugars. During the winter there is a diminution in storage products, due mainly to respiration. The sugars increase in the spring, and the polysaccharides (starch, dextrin,

and the more easily hydrolyzable portion of the cellulose) during the late summer and autumn.   During the autumn and winter carbohydrates are present in the root in greater quantities than in the stem.   In the summer the two are more nearly equal.

TABLE LIII.

SEASONAL VARIATIONS IN THE SUGARS AND POLYSACCHARIDES OF THE CHESTNUT TREE, STATED AS PERCENTAGES OF THE DRY WEIGHT.

Date.	Sugars.		Polysaccharides.		Total.	
	Stem.	Root.	Stem.	Root.	Stem.	Root.
Jan. 11	4·0	1·9	20·7	25·3	24·7	27·2
Feb. 26	4·3	4·7	20·4	21·0	24·7	25·9
Mar. 28	2·7	3·3	18·8	21·4	21·5	24·7
May 20	2·3	3·1	17·6	16·7	19·9	19·8
June 22	2·1	3·6	18·3	18·2	20·4	21·8
July 27	2·6	3·6	18·5	20·7	21·1	24·3
Sept. 12	2·2	1·8	23·7	28·5	25·9	30·3
Oct. 19	2·2	1·6	24·2	27·5	26·4	29·1
Nov. 22	3·2	1·1	21·5	27·8	24·7	28·9
Dec. 26	3·7	1·9	19·3	25·4	23·0	27·3

It appears very probable that the process of " seasoning " timber by soaking it in water, salt or fresh, may owe its preservative effects to the fact that during it polysaccharides are hydrolyzed, and the sugars of the cells and tracheæ diffuse away rapidly.   Thus by the elimination of these products and of the disintegrated protoplasm the timber is freed from material which would otherwise provide a suitable medium for the growth of fungi or bacteria.

FUNCTION OF THE LIVING ELEMENTS OF THE WOOD.

Examination of sections for the estimation of the starch content of the wood cannot fail to force on one the remarkably regular and close connection existing between the starch-containing elements and the vessels. Further, when we take into account that the transference of carbohydrates can no longer be regarded as an occasional and accessory function of the vessels, but is certainly a continual and principal function, the starch layer round each of them becomes evidently a glandular sheath to the vessel for the secretion into it of the sugars to be transmitted upwards. The location of starch in the elements on the borders of the year-rings is clearly connected with the sudden transmission upwards of immense quantities of sugars in the spring. The depletion of the glandular layer of the spring vessels will be made good from the stores massed close by in the outer margin of the year-ring.

We may imagine these carbohydrate glands forming a sheath round the vessels to act somewhat as follows: In spring their stored starch is rapidly brought into solution, and the resulting sugars secreted into the vessels. The solution in the tracheæ acting osmotically through the semi-permeable membrane formed by the outer tissues of the root determines a flow of water from the soil to the tracheæ, and the resulting hydrostatic pressure is responsible for the exhibition of bleeding and root pressure characteristic of the spring. This simultaneously forces much of the air in the tracheæ into solution, and raises the sugars towards the buds. The observations recorded show that the maximum concentration of the sugars in the tracheæ is simultaneous with, or just previous to, the expansion of the leaves. The activity in transpiration of the developing leaves is forwarded not only by the opening

up of the water-channels on the removal of air-bubbles, but also by the growth of the leaves themselves, rendered possible by the accession of sugars, etc., carried up in the sap. The increase in the volume of the transpiration stream from these causes, and more favourable external conditions, leads to a dilution of the sugars, and is responsible largely for the rapid decrease in concentration at the time of the expansion of the leaves, as shown in all the curves. Possibly somewhat earlier there is a diminution in the enzymic activity of the cells round the vessels, and this initiates, and later contributes to, the dilution of the sugars. The secretion, however, does not cease at any time of the year; and consequently even in summer we find the enormous transpiration stream possessing a very noticeable concentration of sugars, which is, indeed, greater than that in which the same substances are present in the human blood. To make good this expenditure the glandular cells must be constantly replenished by supplies forwarded from the organs of carbon assimilation through the bark, medullary rays, and wood parenchyma. The rise in concentration of the sap in the tracheæ towards the end of the year is to be ascribed, in all probability, to the more or less uniform continuance of this secretion, coupled with the reduction of transpiration, entailing a diminution in the rate of the current past the secreting cells prior to leaf fall. For it is evident that, if the rate of the secretion of sugars remains approximately constant, the concentration of the sap will depend upon the volume of the transpiration stream. In winter the complete cessation of transpiration allows of a further concentration.*

* In view of this continued secretion of carbohydrates into the tracheæ, it is advisable to centrifuge the sap from the wood immediately on its removal from the tree; otherwise the concentration observed may be greater than that actually obtaining during transpiration.

It must be remembered that the inflow of water from the ground to the elements of the wood of the roots takes place *across* the cortical cells of the root. For, though the latter have a much higher osmotic pressure than have the tracheæ, they function merely as a complex semi-permeable membrane, as they are already fully distended. Leclerc du Sablon (1911), in the absence of measurements of the osmotic pressure of the tracheæ, imagined it to be greater than that of the cortical cells. Consequently he explains root pressure as a flow occasioned by the osmotic gradient, from ground, through cortex to tracheæ. By implication this view requires the cortical cells to secrete a more concentrated sugar solution than they contain, even though pure water is available continually to dilute it. The facts experimentally ascertained, however, show that such a secretion does not take place, for the sugar in the tracheæ is far less concentrated than in the cells. Thus the view put forward here differs considerably from that of Leclerc du Sablon.

Where the wood parenchyma is specially concentrated round the vessels, while the tracheids form comparatively large tracts without living cells intermingled, we may with probability assume that a certain amount of division of labour has come about, and that the vessels function as the principal channels for the transmission of carbohydrates. The tracheids, on the other hand, are chiefly concerned with the upward conveyance of the water. Of course, as long as no bubbles are developed in the vessels they will transmit the sap in them, with all its constituents, more rapidly than will the tracheids, when both are under the tension generated by the leaves. But if a bubble arises the stream will be deflected into the tracheidal columns. There, owing to the smaller size of the compartments composing the channels, the tensile column will acquire greater

stability. Hence, when the wood is rich in water the most rapid transit upwards will be effected in the vessels, which at the same time will be comparatively rich in carbohydrates, while a slower and more dilute stream will pass up in the tracheids. If drought, by causing an increase in tension, produces bubbles in the conducting channels, the major part of the stream, with its solutes, will have to pass up through those tracheids in which no ruptures (bubbles) have developed, thus securing a stable, if slow, supply to the leaves. According to Strasburger's observations (1891), during transpiration the majority of the vessels of the spring wood in the conducting zone are without bubbles.

The recognition of the glandular function of the wood parenchyma, the translocatory activity of the medullary rays, and the transmission in the tracheæ of the circulating carbohydrates, affords a satisfactory explanation of the presence of living elements among the otherwise lifeless tissue of the wood.

THE MECHANISM OF THE SECRETION OF THE WOOD PARENCHYMA CELLS.

As mentioned in previous chapters, there is a fall in osmotic pressure as one proceeds from the leaves to the roots. It has been shown by Dixon and Atkins (1915, 2), that the interpretation of this appears to be that there is, during the period of active assimilation, a progressive decrease in the sugars in the direction named. These substances, accordingly, either diffuse from regions of high to those of lower concentration, or their motion in this direction is facilitated by the cells of the bast. That such a motion need not always be downwards is indicated by results obtained by Dixon and Atkins (1910), in which the concentration of sugars in leaves remained very large

when they were maintained for several days in almost total darkness, being still attached to the tree the other leaves of which were assimilating actively. When the weather conditions interfered with the formation of sugars by the exposed leaves, the concentration of these substances in the covered ones decreased very markedly.

Under normal summer conditions in, for example, Syringa there is a decrease in concentration from the leaf cells, through the bast, to the cells of the medullary rays. Transformation of sugars into starch takes place in the latter, hence the low pressure is maintained in this region.

It was pointed out, when dealing with the permeability of the plasmatic membrane, that sugars are frequently found to pass through the protoplasm at a quite appreciable rate. Since the wood parenchyma cells are in contact with a very dilute solution of mineral salts, it is quite possible that the " secretion " of sugars into the stream may in reality be accounted for by simple diffusion, through the plasmatic membrane. Furthermore, as Osterhout has shown, the permeability of the latter may undergo repeated increases or decreases within certain limits quite unaccompanied by any injurious effects. From such considerations as these one is led to the view that this " secretion " is not necessarily an instance of the intervention of the protoplasm as an active agent, but that it is, perhaps preferably, to be thought of as due to diffusion. Though the writer advances this view, he does not wish in any way to throw doubt on the fact that living cells can secrete concentrated solutions when supplied with dilute ones, and in other ways act in a manner that, when looked at with the comparatively superficial knowledge of the present day, appears to be in opposition to physico-chemical laws.

THE SUGARS OF THE TRANSPIRATION STREAM.

A survey of the roughly quantitative estimations of the sugars as recorded in the foregoing tables makes it clear that, though hexoses and maltose may be present, sucrose is by far the most important sugar of transport. Thus, in Populus, Acer, Cotoneaster, and Salix, it far exceeds the hexoses and maltose combined. In Ilex and Fagus the proportions appear to be more evenly balanced, though on two occasions sucrose was found to be entirely absent from the wood of the latter. This preponderance of sucrose is decidedly remarkable. Its presence in such quantity cannot, it seems, be explained by supposing it to have been stored as such in the parenchymatous cells of the wood, for these are loaded with starch in quantities sufficient to produce maltose and glucose in amounts far in excess of the sucrose found; yet the proportion of the latter preponderates. Since maltose, by the action of maltase, gives rise to glucose only, one is led to suppose that a part of this hexose undergoes transformation into d-fructose (lævulose), and that these two are then united to form sucrose. The possibility of such changes taking place has been discussed in Chapter I. The alternative, that sucrose is here directly derived from starch, just as starch is condensed from sucrose, must also be considered.

In this connection it is of interest to note that Hasselbring and Hawkins (1915) have shown that, when the roots of the sweet-potato are stored, the starch present in them is rapidly transformed into sucrose and hexose, of which the former is in large excess. These authors give a bibliography of earlier work on similar changes in other plants. Gore (1914) has also shown that sucrose increases as starch decreases in ripening bananas.

The very general absence of any appreciable quantities

of maltose in the sap appears to indicate that this sugar, arising from the action of diastase on starch, is itself further hydrolyzed to glucose to a great extent. This is to be regarded as evidence of the presence of maltase in the cells.

The whole subject is one of great interest, and further and more accurate analyses of the sugars are necessary before any degree of certainty can be reached.

Before closing it may be profitable to consider the distribution of the elements of the wood with regard to its bearing on the co-ordinated functions of the living cells and of the tracheæ.

THE LIVING AND NON-LIVING ELEMENTS OF THE WOOD.

As is well known, all woody stems are traversed by medullary rays, which place the wood and the bast in close communication. In conifers the wood consists of spindle-shaped tracheids with bordered pits, and with (e.g., Taxus) or without (e.g., Pinus) internal spiral thickenings. In other types of wood there are, in addition, wood parenchyma cells, and vessels formed by the union of elongated tubular cells, the end walls of which are absorbed in many places. In such stems a great part of the transpiration stream appears to travel in the vessels, though a portion may traverse the wood fibres, which are thick-walled tracheids. Thickenings, spiral, reticular, scalariform, or annular, are to be found in the conducting elements, and pits are situated on the thinner portions of the cell walls.

The medullary rays consist mainly of living cells, though in some, especially in the larger types of ray, there are tracheids. Many of the cells are seen to be loaded with starch grains and other reserve materials. These may frequently be observed in the wood parenchyma cells also.

The immediate source of supply for the medullary ray

cells is the bast. For through it the surplus assimilates pass downwards from the leaves to stems, rhizomes, roots, or other storage organs. From the medullary rays the reserves pass to the wood parenchyma cells when these are present.

The distribution of the parenchymatous cells in the wood shows an orderly arrangement forming vertical plates in connection with the medullary rays, and very regular sheaths surrounding the vessels. The cells abutting on the spring wood are also found to be very rich in starch in many cases. The importance of this arrangement has recently been emphasized by Dixon and Atkins (1915, 1), and in the winter these sheathing cells are differentiated in a striking manner when treated with iodine solutions, owing to their being crowded with starch grains.

In connection with researches on the ascent of sap, an investigation was carried out by Dixon and Marshall (1915) upon the relative areas occupied in the cross-section of a woody stem by the walls, by the living cells, and by the lumina of the non-living conducting tracts. Since the results have a direct bearing on the present subject, they will be quoted here at some length.

A QUANTITATIVE STUDY OF THE AREAS OCCUPIED BY ELEMENTS OF THE WOOD.

The method adopted was to obtain photomicrographs of transverse sections of the wood of various trees at such a magnification that, whilst the individual cells were of sufficient size to permit of their lumina being accurately cut out with a sharp-pointed knife, yet a fairly large portion of the section was in the field, so as to minimize irregularities owing to the inclusion or exclusion of medullary ray tissue, which would tend to distort the true proportions. To further avoid this source of error, since the results obtained

were in some cases very variable, it was deemed advisable to make a number of photographs of each type of wood, and to cut them up as before described. The portions representing the lumina of the water-conducting elements and of the living cells were preserved separately, as were also those occupied by the cell walls. The relative areas of each were then found by weighing the pieces of paper, and expressing the individual values as percentages of the total. In this manner the following tables were obtained, illustrating the composition of the wood of dicotyledonous trees. A preliminary experiment upon the wood of a conifer, *Pinus silvestris*, gave the figures here recorded:*

Lumina of medullary ray-cells ..	..	6·9 per cent.
„ „ tracheids ..	..	61·2 „
Walls of tracheids and cells ..	..	31·9 „

TABLE LIV.

Ilex aquifolium: Areas of the Elements of the Wood in Cross-Sections.

Section.	Cells.	Tracheids.	Vessels.	Tracheœ.	Walls.
A	13·1	11·7	15·9	27·6	59·2
B	6·5	17·8	29·1	46·9	46·6
C	11·4	21·3	10·8	32·1	56·4
D	9·9	20·5	14·0	34·5	55·5
Mean ..	10·2	17·9	17·4	35·3	54·4

The results obtained with Ilex may be taken as illustrating the variations met with in the individual determinations. In trees, such as Acer, in which the medullary rays are very large, a wider divergence is found between separate measurements.

In Table LV. are given the mean values for the wood

* These were obtained by cutting up a *camera lucida* drawing instead of a photomicrograph.

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of other trees, taken from tables similar to that just quoted.

Thus it may be seen that the area occupied by the lumina of the living cells varies from about one-tenth (Salix) of, to almost equality (Acer) with, the area of the lumina of the tracheæ, the mean being about one-quarter. The latter may fairly be taken as a representative value, for it is based on twenty-two observations on seven kinds of trees.

TABLE LV.

MEAN VALUES FOR AREAS OF THE CELLS, TRACHEÆ, AND WALLS, IN A
CROSS-SECTION OF THE WOOD OF A DICOTYLEDONOUS STEM.

Name of Tree.	No. of Observations.	Lumina of Cells.	Lumina of Tracheids.	Lumina of Vessels.	Total of Tracheæ.	Walls.
Acer pseudoplatanus	6	28·2	12·8	20·7	33·5	38·3
Fagus sylvatica..	3	13·1	25·0	15·8	41·0	45·9
Ilex aquifolium..	4	10·2	17·9	17·4	35·3	54·4
Syringa vulgaris	2	10·0	10·1	30·1	40·3	49·7
Populus alba ..	2	9·8	25·8	28·0	53·8	36·4
Cotoneaster frigida	3	7·4	12·6	37·2	49·8	42·8
Salix babylonica	2	5·7	18·9	38·4	57·4	37·0
Mean of seven kinds of trees	—	12·1	17·6	26·8	44·4	43·5

Quantitative experiments of this type serve to clarify one's ideas on the anatomy of woody stems, and further data of this nature would no doubt prove very useful. The labour, however, necessary to obtain them is very considerable.

The foregoing study of the distribution of the storage cells and tracheæ of the wood clearly shows how the view as to the nutritive character of the transpiration stream is in accord with the histology of the stem. The rôle assigned

to the living cells also makes intelligible their presence in such large quantity—about one-fourth of the area of the lumina of the tracheæ.

Functional Differentiation of Types of Medullary Ray Cells.

The views advanced by Dixon and Atkins as to the relationship of the medullary ray cells to those of the other elements of the wood are in close agreement with those put forward by Nicoloff (1911); and since the latter investigator arrived at them by an entirely different method—the microscopic examination of the tissues at intervals during the year—it may not be devoid of interest to quote from his work *in extenso*. Nicoloff, moreover, draws attention to specialization of the cells of the rays:

" When the physiological rôle of the medullary rays is being considered, it must be studied with due regard to the relation of this tissue to its surroundings. It has been usual to regard the medullary rays as vertical plates of cells running in a radial direction in the stems, and facilitating the transport of nutritive materials from the exterior to the interior, and *vice versa*. Such a definition of their functions is incomplete. It is true that they do in fact transmit nutritive materials in a radial direction from the exterior—the sieve tubes—to the elements of the wood. The importance of this rôle has been much more apparent since Fischer's researches, which showed how great a part the vessels play in the upward conveyance of reserves. Now, it is known that the vessels themselves draw these reserves from the rays, or else from the other elements of the wood, which in turn draw them from the rays. Thus it is possible to establish a cycle of nutritive substances, in which the starting-point is the centres of assimilation taken collectively. The assimilates enter the sieve tubes

and penetrate into the medullary rays at different levels for transmission to the wood, which is the principal store-house. After this they travel through the vessels leading to the regions in which growth is taking place.

" However, if the rôle of the medullary rays as a tissue for the transference of reserves has been sufficiently insisted upon, their great importance as the storehouse of the stem has not been adequately emphasized. This function may be easily understood when one remembers that in certain trees, as already mentioned, the rays represent a very large proportion of the total mass (one-fifth or even one-fourth), and that all this tissue, at certain times of the year, is closely packed with foodstuffs (starch). It is true that the wood parenchyma and wood fibres (Salix, Acer, etc.) also at times contain for their part large quantities of these substances, but for the major portion of the year the quantity contained in the rays is much superior. This rôle of the medullary rays becomes even more important in trees in which the wood parenchyma is poorly repre-sented.

" As for the division of physiological labour between the two elements composing the medullary rays, we can now be more explicit. In fact, we see that, when starch reappears in spring, it fills the two kinds of ray cells, with the exception sometimes of those of the upright approximately cubical cells abutting on the vessels. When the starch is dissolved, it ordinarily disappears first of all in the radially elongated cells. The order in which this solution is effected becomes more marked according as the morphological differentiation of the two types of element becomes more complete (Salix, Corylus, Liquidambar, Viburnum, etc.). The order just described is the usual one, although it so happens that certain trees show another order for the solution of the starch, in spite of the pro-

nounced differentiation of their medullary rays (Æsculus, Tilia, etc.). . . . If the solution of starch takes place in the order indicated, the conclusion can be drawn that the upright cells, other than those in contact with the vessels, are to be regarded as forming a storage tissue, whereas the radially elongated cells serve principally as channels for the conduction of foodstuffs."

Nicoloff further remarks upon the scarcity of glucose in the medullary ray cells, as shown by the copper-reducing test, although it may be found in the wood parenchyma and fibres, and especially in the vessels. This absence of sugar he thinks remarkable in view of the high osmotic pressures found by Kny (1909) in the ray cells. To the writer it appears that the presence of sucrose sufficiently accounts for the pressures. Curiously enough, Fischer makes no mention of testing for this sugar by inversion, and Nicoloff in this respect followed faithfully in his steps. As already pointed out, sucrose is by far the most abundant of all the sugars in many woody stems.

The researches of the earlier physiologists (Th. Hartig, Reichard, Schröder, N. J. C. Müller, Russow, Grebnitzky, Baranetzky, and A. Fischer) upon the starch of the wood and its transformations have been considered in detail by Strasburger (1891).

SUMMARY.

1. Sugars (monosaccharides and disaccharides, or both) are found at all times in the sap in the tracheæ of the trees examined, and usually in greater quantities than electrolytes.

2. The greatest concentration of the sugars occurs in the early spring; this is followed by a rapid dilution in spring and early summer, so that a minimum occurs in the summer or autumn. A rise in concentration, slow at first,

then takes place through the winter, culminating in the vernal maximum.

3. The vernal maximum coincides with the period of greatest root pressure, and is simultaneous with or just prior to the opening of the leaf buds.

4. The rise in transpiration, initiated by the expanding leaves and facilitated by the opening of the conducting channels by root pressure, is largely responsible for the dilution of the carbohydrates. The falling off and cessation of the transpiration stream in the autumn allows the concentration again to rise.

5. The conveyance upwards of sugars, of which sucrose appears to be the most important, is a continual and primary function of the tracheæ.

6. The sheath of wood parenchyma round the vessels functions as a store from which sugars pass into the rising transpiration stream.

7. The relation of the medullary rays to these sheaths supports the view that they convey the carbohydrates from the bark to the glandular sheaths.

8. The presence of large quantities of soluble carbohydrates in the wood sap of roots is probably responsible for root pressure and bleeding, by producing an osmotic flow across the root cortex.

9. The curves for the concentration of solutes in the stem of the evergreen Ilex, and of the sub-evergreen Cotoneaster, show smaller fluctuations than do those of deciduous trees; they have two cusps—one about January, and the other about August, in Ilex, and in February and October in Cotoneaster. It is to be noted that in the case of Ilex the buds expanded during the rise preceding each of these cusps.

10. The concentration of the carbohydrates is generally greater in the tracheæ of the stem than in those of the root

except during the summer. This rule is broken by Ilex,* where the concentration in the root is the greater throughout the year, except in winter. The electrolytes, however, are present as a rule in greater quantity in the root.

11. In general the vessels function, in times when water is abundant, to convey rapidly solutions of organic and inorganic substances to the leaves. The columns of tracheids may be supposed to afford a permanent channel for the water and salts, and to a less degree for the organic substances. This is never put out of action, even in times of greatest drought.

* The Ilex stem was examined at 1 metre above ground-level; the other stems were cut across much higher up.

CHAPTER XII

THE PLANT OXIDASES

IN the last ten years the attention of physiologists has been largely directed to the study of respiration and oxidations which take place in living and dead cells.

Numerous researches have been directed to the unravelling of the complicated interrelations of the mechanism whereby the fundamental need of oxygen is supplied, and it has been shown that enzymes termed "oxidases" are concerned in the utilization of this gas.

It is with some of the more recent results of the study of oxidases that this chapter is intended to deal. The subject as it was known up to 1910 has been exhaustively treated of by Kastle (1910), also by Czapek (1910) and Clark (1910), to whose publications the author is much indebted.*

SECTION I.—THE NATURE OF PLANT OXIDASES.

ENZYMIC NATURE OF OXIDASES.

On the whole, the substances which effect oxidations in plants have the properties of enzymes, though their behaviour is in some ways peculiar. The usual routine

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* I have followed the custom of the American authors, and of Fowler (1911), Moore and Whitley (1909), in writing "oxidase" rather than "oxydase," to denote the enzyme that splits up a (per)oxide. The spelling "oxydase" has been taken directly from the French, in which both "oxygen" and "oxydant" retain the letter "y." It seems an undesirable anomaly to spell "peroxide" with "i" and "peroxydase" with "y," as many do at present.

adopted in deciding whether a given reaction is enzymic or an ordinary chemical change is to boil the solution. If the reaction is no longer brought about, it may be provisionally concluded that it is enzymic, its cessation being due to the destruction of a thermolabile substance. But enzymes have two other very important characteristics: firstly, that a small quantity of the enzyme brings about a relatively enormous transformation of the substrate; and, secondly, that the rate of this change is proportional to the amount of enzyme present (provided the substrate is in large excess), though the total amount transformed is independent of it if a sufficient time be allowed to elapse. It may be added that the enzymes are colloidal, and the reactions they bring about or catalyze are in some cases reversible, the point of equilibrium being usually very near that of complete change in one direction. Furthermore, their action is as a rule specific, one enzyme only acting on one substrate, or on one class of substrates, and may in many instances be inhibited entirely, or reduced in velocity by very minute quantities of paralyzors.

EFFECT OF HEAT ON OXIDASES.

Now, the oxidizing substances of plants are destroyed by heat, though in some cases peroxidases resist total inactivation even when their aqueous solutions are boiled for short periods. Bach and Chodat (1903) have shown that with laccase peroxidase the time required for complete destruction by boiling depends upon the concentration of the enzyme. Thus a solution which necessitated boiling for eighteen minutes to effect inactivation was found to be quite inert after three minutes when diluted with twenty times its volume of water. Alcoholic solutions are, however, inactivated by raising them to the temperature of boiling water.

It has been stated by Woods (1902) that the peroxidase from tobacco leaves may be formed afresh within two hours when a boiled solution is allowed to stand. This he attributes to the presence of thermostable zymogens, which yield a new supply of enzyme. It is remarkable that no other workers have as yet been able to find this zymogen, though organic catalysts of oxidation which are moderately thermostable have been shown to exist.

CLASSIFICATION OF OXIDASES.

How far the oxidases are specific in their action seems to be in doubt. Five different oxidases at least have been described as occurring, or rather five different classes of oxidases—viz., laccases, tyrosinases, alcoholases, purine oxidases, and aldehydases. The laccases or phenolases act on many phenols, and are very widely distributed in plants. The tyrosinases act on tyrosin, or polypeptides from which tyrosin can be split off, to produce a body which further reacts with amino-acids, yielding dark-coloured pigments termed " melanins," as shown by Abderhalden and Guggenheim (1907, 1908). An example of the alcoholases is furnished by the enzyme found in certain bacteria, which converts ethyl alcohol into acetic acid. The purine oxidases have been extracted so far from animal tissues only, and the existence of specific aldehydases is still hypothetical.

Researches in plant physiology deal almost entirely with the two classes of enzyme at the head of the list; there is at present no proof that the laccase or tyrosinase from one species is identical with that from another, though they may produce certain colour reactions in common. The part played by inhibitors in bringing about apparent specific action by oxidases will be treated of later on.

Component Parts of a Complete Oxidase.

Before going farther a distinction must be made between the terms " oxidase " and " peroxidase." Originally those tissues which could catalyze oxidations of natural chromogens, or of added ones, such as guaiacum resin, benzidine, α-naphthol, pyrogallol, or tyrosine, were said to contain an oxidase; whilst those requiring the addition of a peroxide, such as hydrogen peroxide, or a spontaneously oxidized essential oil, in order to effect oxidations, were described as containing a peroxidase. The view that an oxidase consisted of a peroxidase and a naturally occurring peroxide was put forward by Kastle and Loevenhart (1901), and has gained very general acceptance. Keeble and Armstrong (1912, 3) record that in certain flowers the organic peroxide accumulates during darkness, so that apparently the tissues contain oxidase at one time and peroxidase at another. It has been the author's experience also that the occurrence of peroxide, in a tissue which at times contains it, is extremely variable. Certain species of plants never have any peroxide normally, though even in some of them it is possible to induce the formation of small quantities by prolonged deprivation of light. At present it is usual to refer to the " direct " oxidase action, or to the " indirect," according as the addition of a peroxide is not or is required to bring about oxidation. Strictly speaking it would be more correct to refer to both as peroxidase actions, for the essential is that a peroxide is split up and oxygen deprived from it is transferred to an easily oxidizable substance.

Throughout this book the term " oxidase " will be reserved as a general term, as it is undoubtedly both expressive and comprehensive. When dealing, however, with reactions involving laccase or tyrosinase, both of

which have been shown to consist of a peroxidase plus a peroxide, use will be made of the following terminology—viz., " complete peroxidase system " as equivalent to the enzyme plus peroxide, and " incomplete peroxidase system " or simply " peroxidase " to denote the enzyme.

The addition of hydrogen peroxide has been mentioned above. That this substance is not identical with the naturally occurring peroxide is proved by the almost universal presence of catalase in plant tissues. This enzyme, for the existence of which as an individual there seems now to be quite sufficient evidence, splits up hydrogen peroxide in such a way that the oxygen is liberated in the molecular condition, as is shown by its inability to effect oxidation. When the peroxide is decomposed by platinum black, oxidations may result if suitable compounds are to hand.

COMPOSITION AND PROPERTIES OF LACCASES OR PHENOLASES.

For a full account of the methods of preparation of accase, tyrosinase, and artificial oxidases, the reader is referred to the monographs by Kastle (1910) and by Clark (1910), and to Euler's " General Chemistry of the Enzymes." The effects of small quantities of acids, alkalies, and manganese salts upon the activity of these enzymes is also considered there as well as the identity of Medicago-oxidase with a mixture of calcium salts of organic hydroxy acids, including glycollic, citric, malic, and mesoxalic. A short account will, however, be given of other features of interest in connection with these enzymes.

As previously mentioned, laccase acts upon many phenols. It derives its name from its presence in the latex of the lac-tree, *Rhus vermicifera*, the white juice of which it converts into a smooth dark varnish or lacquer,

as was originally shown by Yoshida (1883). Without asserting the identity of laccase with the phenolases met with in other plants, it may be stated that its action upon a number of reagents is qualitatively similar, hence there is no objection to the use of the term provisionally.

It has been pointed out by Bertrand (1896) that the aromatic monophenols and monamines are not easily oxidized by laccase, but that substances which it readily attacks are all members of the benzene series containing hydroxyl or amino groups in the ortho or para positions.

Towards both heat and alcohol laccase is more stable than is tyrosinase. Bertrand (1896, 2) was able to separate these two enzymes, which exist together in *Russula delica*, by precipitating a chloroform-water extract of the fungus, in which equal weights of tissue and water had been employed, by the addition of one and a half times its volume of 95 per cent. alcohol. An aqueous extract of the precipitate obtained from the alcoholic solution was found to oxidize tyrosin, but had very little action on hydroquinone or pyrogallol. The filtrate, however, when concentrated at 50°, still gave the laccase reactions, but not those of tyrosinase.

As was mentioned before, the phenolase so widely distributed in plant tissues is considered to consist of a peroxidase and an organic peroxide. A great number of experimentally ascertained facts can be satisfactorily explained on this hypothesis. For instance, sap pressed from the leaves of *Sambucus niger* was found by the author (1913) to turn a dilute guaiacum solution blue when tested at once. On standing for two hours in diffuse light, this power was lost, but the addition of a few drops of a neutral four-volume solution of hydrogen peroxide resulted in the appearance of the blue colour on testing. Again, the fresh sap when boiled for two minutes failed to restore the

activity of sap which had stood. Consequently both con-
stituents of the oxidizing system must have been destroyed
by heat. Other experiments showed that the peroxide
is less stable than peroxidase towards heat.

THE INFLUENCE OF LIGHT UPON THE AMOUNT OF OXIDASE IN PLANT TISSUES CONSIDERED IN RELATION TO THE COMPOSITION OF THE ENZYME.

The effect of light upon the oxidase content of plants has
been studied by Keeble and Armstrong (1912, 3). By
means of the laccase reagents, α-naphthol and benzidine,
they satisfied themselves that the tissue of a normally
illuminated plant contains less peroxidase than does the
corresponding tissue of a plant kept in darkness; also that
the organic peroxide constituent of the complete oxidase,
though it may be absent from the normal plant, frequently
makes its appearance after that plant has been maintained
for some time in darkness. Thus it is clear that the
diurnal alternations of light and darkness must in this
respect alone have a very marked influence upon the
metabolism of plants.

While engaged in testing the influence of light upon the
distribution of peroxidase in the flowers of Iris spp., in the
course of an investigation upon the correlation of enzyme
and anthocyan pigment, the author (1915, 1) found that
Iris flowers never contain organic peroxide normally, and,
owing most probably to the presence of an inhibitor,
many varieties show but little peroxidase. Some species
show a marked increase of oxidizing power when placed
in the dark for twenty-four hours, and indeed, prolonged
darkness leads to the appearance of peroxidase in flowers
from which it had been absent. In a few cases, however,
deeply pigmented Irises gave no peroxidase action either
before or after enclosure in a dark chamber, even when the

treatment was prolonged for four days. Again, contrary to the experience of Keeble and Armstrong, with other flowers, in two cases only was organic peroxide formed in darkness.

Thus, since the occurrence of the peroxide constituent of the oxidizing system is so variable, being influenced by external conditions, Keeble and Armstrong regard it of relative unimportance so far as the distribution of oxidases is concerned, whether a tissue is or is not found to contain the complete peroxidase system. This opinion seems to the writer to be fully justified.

For a discussion of Traube's (1882) theory of oxidation by means of peroxides, and its modifications by Bach (1897), Engler (1904), and others, reference should be made to the monographs and textbook previously named. The very interesting monograph by Dakin on " Oxidations and Reductions in the Animal Body " should also be consulted.

Chemical Composition of Laccase.

The chemical constitution of laccase is not known, for, as is the case with many enzymes, its purification is very difficult. A pure specimen prepared by Slowtzoff (1900) gave all the reactions for protein, and contained 12·8 per cent. nitrogen, 0·53 per cent. sulphur, and but little ash. Neither manganese nor phosphorus could be detected in it. Slowtzoff considers it to belong to the group of albumins. Its activity is greatest in faintly alkaline solutions, but is not destroyed by weak acids or by peptic or pancreatic digestion. It must be pointed out, however, that it is only to the negative results of such analyses that much attention should be given; thus, for instance, manganese was not found in the ash, therefore this metal cannot be an essential constituent of the enzyme. Again, it cannot

be a proteid, since neither peptic nor tryptic digestion destroys it. Numerous "pure" preparations of peroxidase have been made since Slowtzoff's, but without any definite conclusion having been drawn. Among these were those of Bach and Chodat (1903), Tschirch and Stevens (1905), Bach (1908), Bach and Tscherniack (1908), Deleanò (1909), Euler and Bolen (1909). The last-named investigators found that the longer their peroxidase from horse-radish was dialyzed, the more active it became, and that its nitrogen content increased, whereas the ash decreased. Bach and Chodat also used horse-radish as the source of their peroxidase. In its purest form this was an amorphous brownish substance. It contains nitrogen and reducing substances which are not sugars, but gives none of the reactions of protein. On heating with caustic soda ammonia is evolved, also substances having the odour of pyridin bases. Both ammonia and pyrrol are given off when it is heated with metallic potassium. It leaves but little ash, which consists of phosphates of calcium, magnesium, sodium, and potassium. Iron and manganese are absent from it.

COMPOSITION AND PROPERTIES OF TYROSINASE.

The enzyme tyrosinase occurs in many fungi and in some of the flowering plants. Its action is not specific for tyrosin alone, but includes the oxidation of many related substances. It is differentiated from laccase by the type of substrate upon which it acts, as well as by its greater susceptibility to destruction by heat, alcohol, acids, and alkalies.

Abderhalden and Guggenheim (1907) showed that $\frac{N}{100}$ hydrochloric acid entirely stops the action of tyrosinase, but the same concentration of sodium hydroxide exerts only a retarding influence. Since neutralization fails to

restore the former activity of the enzyme, it is clear that it has been destroyed by the reagent, and not merely inactivated.

As a general rule, tyrosinase is found wherever laccase is, but the converse of this is not true.

Bertrand and Mutermilch (1907) prepared it from bran by precipitation of an aqueous extract with alcohol in certain proportions. The substance so obtained was free from laccase. It is soluble in water, and may be filtered through a Chamberland filter to yield a clear solution which does not darken when exposed to the air. The addition of tyrosin results in the production of a rose colour, altering to red and then brownish-black.

It was ascertained by Bach (1908, 1) that tyrosinase, like laccase, consists of a peroxidase and a peroxide. For whereas the addition of hydrogen peroxide to an active solution prepared from sound fungal tissue did not increase its activity, yet when a decomposing tissue was the source of enzyme, and the tyrosinase action was not vigorous, a marked increase resulted from the addition of a little very dilute hydrogen peroxide. That the naturally occurring peroxide can thus be replaced shows that the specific action of the enzyme is due to the peroxidase.

In order to illustrate the effect of added hydrogen peroxide just mentioned, it may not be amiss to quote the figures obtained by Bach in his experiment. Aqueous extracts of tyrosinase were made from young unblemished, older slightly damaged, and from putrid specimens of *Russula delica*. These are solutions I., II., and III., respectively. Of each extract, diluted ten times with water, 10 c.c. was taken and mixed with 10 c.c. of a solution containing 0·05 per cent. tyrosin and 0·04 per cent. sodium carbonate. To this 30 c.c. of water was added, and after standing for twenty-four hours it was titrated

with 0·002 N potassium permanganate in presence of dilute sulphuric acid till completely colourless.

The results are recorded in the table which follows. In the lower row of permanganate numbers are the figures obtained after the addition of 1 c.c. of 0·05 per cent. hydrogen peroxide solution to each tube at the same time as the tyrosin was poured in.

TABLE LVI.

EFFECT OF ADDITION OF HYDROGEN PEROXIDE.

	Number of Extract.		
	I.	II.	III.
Appearance before titration	Deep black; black sediment	Violet-black	Dark brown
Permanganate required ..	37·8 c.c.	13·6 c.c.	8·3 c.c.
Permanganate required after addition of hydrogen peroxide	37·3 „	26·7 „	23·2 „

It may be remarked that the amount of hydrogen peroxide added is all, or almost all, used up in the reaction with the enzyme; consequently, as, indeed, is shown by Column I., the increase in permanganate required is not due to interaction of the latter with peroxide.

Further evidence of the complex nature of tyrosinase was obtained by Bach (1908, 1), for on shaking a solution of the enzyme with magnesium carbonate, it was without action on tyrosin till after the addition of peroxide. Thus treatment with the carbonate must have removed the peroxide constituent of the oxidizing system.

It is still uncertain, however, whether the action of

tyrosinase is similar to that of other oxidases. Bach (1909) has suggested that it may be quite different, and have effect only on substances which contain a labile atom of hydrogen.

INHIBITORS OR PARALYZORS OF OXIDASE ACTION.

It has already been mentioned that many substances lessen or stop the activity of oxidases, as, for example, the majority of acids when in anything more than a very dilute condition. Such a result may be due to a reversible process, the original activity being restored by removal of the harmful substance, or it may be occasioned by the complete destruction of the peroxidase, peroxide, or of both. If only the peroxide is put out of action, it can easily be replaced by hydrogen peroxide, and so the presence of the uninjured peroxidase may be detected.

In the living cell, however, the activity of oxidases is regulated and co-ordinated with the functions of the protoplasm in general. This is, in some cases at least, brought about by the production of inhibitors. The nature and mode of action of these substances is not fully understood at present, but it has been shown by Keeble and Armstrong (1912, 3) that the effect of the inhibitor is to paralyze the peroxidase, for when it is removed by suitable means the enzyme resumes its activity. Thus treatment of certain white flowers, which showed no oxidase reactions in the epidermis, with 0·4 per cent. hydrogen cyanide for twenty-four hours, followed by thorough washing with water, resulted in the benzidine being acted upon strongly in the epidermis after the addition of peroxide. These workers also found that a saturated solution of carbon dioxide served to remove inhibitor, but was not so effective.

It has been stated above that the peroxidase is paralyzed by the inhibitor. It is, however, quite possible that its

action may proceed as before, and that the inhibitor itself may be oxidized instead of the natural or artificial chromogen. It was found by the author (1914, 1) that the tissues of the leaf of *Iris germanica* contain certain areas which fail to give indications of the presence of peroxidases When the whole leaf is pressed, the sap gives no blue colour with guaiacum and peroxide, but sap from the tip of the leaf gives the reaction. Thus the lower portion of the leaf contains an inhibitor, especially in the mesophyll cells, as shown by examination of sections treated with benzidine and α-naphthol. The leaf sap has a powerful reducing action, for potassium permanganate solutions are rapidly decolorized by it even in the cold. This is still effected with cold sap, previously boiled, so is not due to a reducing enzyme. Furthermore, a small quantity of the leaf sap quickly renders the purple sap of the petals colourless, and the addition of hydrogen peroxide does not restore the colour. This is not remarkable, as the peroxide itself decolorizes the anthocyan of the flower, due, it seems, to the reducing action which it so often exhibits in presence of an easily reducible substance.

That this action of Iris leaf sap is a reducing one is made still further clear by the fact that, not only does it prevent the production of guaiacum blue when added to leaf sap of *Hedera helix*, but it destroys the blue when the reaction has been brought about before its addition. When, however, Hedera sap is added to Iris sap and guaiacum, it is found that, after a certain quantity has been poured in, the further addition of hydrogen peroxide causes the blue colour to appear. Previous to the addition of Iris sap, the Hedera sap was capable of giving the reaction directly, without the added peroxide. Hence its supply of organic peroxide must have been used up by the reducing agent of Iris.

Separation of Enzyme and Inhibitor.

Dialysis of the Iris leaf sap (Atkins, 1913) in presence of toluene was found to remove the reducing agent, for after several days it gave the peroxidase reaction with guaiacum. The catalase reaction was also brought about by the dialyzed sap. As dialysis proceeded, the effect of the sap upon the purple colouring matter of the petals was tried daily. That which afforded the peroxidase reaction with guaiacum was found to have altogether lost the power of decolorizing the petal extract. Decolorization was, however, effected by the complete peroxidase system of Hedera leaf. So it appears that either reduction or further oxidation converts the purple pigment into a colourless substance, but the possibility is not excluded that the Hedera peroxidase system obtains oxygen at the expense of the anthocyan.

The separation of peroxidase and inhibitor can most rapidly be carried out by pouring the leaf sap into strong alcohol, when the enzymes are precipitated. After washing with spirit, they may be dissolved in water, and the peroxidase then gives a blue with guaiacum and peroxide, the colour being destroyed by addition of the filtrate.

An inhibitor was also removed by dialysis from the sap of mature leaves of *Aspidium filix-mas*.

These reducing agents are active in aqueous solution, and accordingly are not similar to those studied by Keeble, Armstrong, and Jones (1913), which become most active in alcoholic solution, and are thought by those authors to be concerned in the destruction of anthocyan pigments when flowers containing them are immersed in alcohol. Wheldale (1914, 1) and Tswett (1914), while not denying the presence of reducing substances, do not think this

rôle can be assigned to them.   This point will be discussed later on.

Again, it is well known that certain tannins act as inhibitors of oxidase action.   Thus it was found by the author that sap from the young red leaves of *Vitis Veitchii* shows the direct reaction with guaiacum, whereas sap of the more mature green leaves, which contain tannin, give no reaction.   Furthermore, Aso (1890) succeeded in separating oxidases from tannin by precipitating them with alcohol.   It is also possible that the failure of extracts of the Fucaceæ to show oxidase reactions may be connected with the presence in them of fucosan, which has recently been shown by Kylin (1913) to be a tannin-like substance.   These extracts are efficient reducing agents, as they almost decolorize dilute methylene blue and the guaiacum blue produced by leaf sap of Hedera.   All the author's attempts to obtain an active oxidase from these algæ by dialysis or precipitation were quite unsuccessful.

MODE OF ACTION OF INHIBITORS.

Enough has been said to demonstrate the presence of reducing agents which act as inhibitors.   All reducing agents are, of course, bodies which are themselves very prone to oxidation.   In fact, it is possible to arrange such unstable substances in a scale, in which each would yield oxygen to the one above it and take it from the one below it.   By this transmission of oxygen from one compound to another considerable changes may take place in a cell without any absorption of gaseous oxygen, as Bunzel (1912) has pointed out.   Whether naturally occurring inhibitors actually do lessen oxidase activity has not yet been completely investigated.   Measurements with Bunzel's apparatus would seem the most likely way in which to obtain decisive evidence.   At present there appears to

be some ground for the view that inhibitors are very easily oxidizable substances occurring in cells in which there is a physiological lack of oxygen. It must be admitted, however, that it is difficult to reconcile such a theory with their presence in the petals of flowers, such as dominant white varieties of *Primula sinensis*, where their function has been supposed to be to suppress the formation of anthocyan. In this connection, Molisch (1914) has shown that the heat evolution of the leaves of many species of plants is very considerable, but that of the thallus of Fucus is relatively small. Since, with the exception of traces in the mucilage, oxidases have not been found in this plant, it appears that there may be such a thing as inhibition amounting to the slowing down of oxidation changes to a very marked extent. As the evidence is incomplete and conflicting, it would be unwise to attempt to form any conclusion at the present.

With regard to the chemical nature of the inhibitors there is no very definite information. Kastle and Loevenhart (1901) studied the effect of a number of substances in inhibiting or destroying the activity of laccase from the potato. The list includes hydrogen cyanide, phenyl hydrazine, hydroxylamine, sodium thiosulphate, and decinormal solutions of certain acids. Weak acids, such as carbonic, boric, and phosphoric, have been shown by Bertrand to be inactive at all concentrations. The action of the strong acids and other inhibitors in the same list is probably in a large measure due to their effect upon the oxidases merely on account of the colloidal condition of the latter, for their chemical nature is extremely varied. They give no clue to the possible constitution of the naturally occurring inhibitors. The action of hydrogen cyanide in removing the inhibitor of petals might be considered as pointing to the aldehydic struc-

ture of the latter, which would be changed by cyan-hydrin formation. But this leaves the action of carbonic acid quite unexplained. Also on one occasion it was found that such an inhibitor appeared to have been partly removed by keeping Iris flowers in water saturated with toluene for about a day. Since toluene, hydrogen cyanide, and carbon dioxide, all render protoplasm permeable without precipitating colloids, as does treatment with alcohol, it is likely that such apparent destruction of inhibitor is really due to its elimination by diffusion. Further experiments on this point are desirable. It may be added that the artificial ripening of persimmons sub-jected to high pressures of carbon dioxide has recently been studied by Lloyd (1911), and may be cited as an example of the effect of this gas in rendering the proto-plasm more permeable, so that enzymes and their sub-strates can come into contact readily. [See note, p. 302.]

SECTION II.—DISTRIBUTION OF OXIDASES IN PLANT FAMILIES.

PLANTS WITH COMPLETE AND INCOMPLETE PEROXIDASE SYSTEMS.

There is considerable evidence for the belief that oxi-dases are present in every vegetable cell, though their amount and degree of activity varies greatly.

Their detection may be effected in many cases by simply bruising or cutting the tissue, which is then seen to darken, owing to the action of the enzyme upon a naturally occur-ring chromogen. The correctness of this explanation is shown by the failure of boiled tissues to darken, and by the fact that, in those which darken naturally, oxidation of added artificial chromogens is also brought about. The darkening of the sap pressed from some plants is very

remarkable, that of the sunflower (*Helianthus multiflorus*) becoming a very intense dark brown within a few minutes. Among other saps which, in the author's (1913) experience, darken rapidly may be mentioned those from the leaves of *Hedera helix, Syringa vulgaris, Magnolia acuminata, Catalpa bignonioides, Fraxinus oxyphylla,* and *F. excelsior.*

A great many plants, however, give a light-coloured sap which does not darken on standing. In this group are included *Wistaria sinensis, Eucalyptus globulus, Chamærops humilis, Cordyline australis, Equisetum telmateia.* These also fail to colour the artificial chromogens till after the addition of hydrogen peroxide, or of an old sample of an essential oil, such as turpentine, in which peroxide formation has taken place. In this class the organic peroxide constituent of the peroxidase system may be supposed to be lacking.

Plants containing Inhibitors.

Yet again in a third group of plants no peroxidase can be detected in the sap even after the addition of peroxide. This includes the leaf saps of *Iris germanica, Aspidium filix-mas,* and *Pteris aquilina.* Only the mature leaves of the last-named behave in this manner, for the young ones give the indirect action. In all these cases, however, it has been found possible to demonstrate the presence of peroxidases by dialysis of the sap, from which an inhibitor diffuses out, or by precipitation of the enzyme with alcohol, as described in a previous section.   [See note, p. 250.]

Occurrence of Phenolases and Tyrosinases.

The presence of oxidases of the phenolase (laccase) type has been demonstrated in every land plant thoroughly examined; certain tissues may fail to give the usual tests, owing to inhibitors having been produced, or possibly a

whole plant may be under their influence. Bourquelot and Bertrand (1895, 1896), Zellner (1907), Pringsheim (1909), Kastle (1906), and others, have shown that pheno-lases are of almost universal occurrence in fungi, whereas the distribution of tyrosinases is not so general. Clark (1910) tested a large number of groups of phanerogams and pteridophytes, and has found phenolases to be of very general occurrence, the peroxidase without peroxide being most frequently encountered. The absence of oxidase in certain acid saps was noted. Moore and Whitley (1909) had previously drawn attention to the fact that the pulp of lemons, limes, and oranges, was free from oxidase, though it occurs in the seeds. It has recently been shown by Reed (1914) that peroxidase can be located in the tissue between the compartments of the fruit of the orange by means of microchemical reagents.

Passerini (1899), too, studied the distribution of oxidase, but restricted his investigation to determining the presence of organic peroxide. The complete system was found in eighty-one species from forty-nine families. This wide-spread occurrence of oxidases is demanded by Palladin's theory of the respiration of plants, and there seems no reason to doubt that they occur, in various degrees of activity, in every living cell. Owing to the possibility of treatment with reagents liberating inhibitors from vacuoles or specialized regions of the protoplasm, negative results must always be regarded as an incentive to further research in the direction of effecting a separation of inhibitor and oxidase.

In this connection the behaviour of the marine algæ alluded to in a former section urgently calls for further investigation. It was found by the writer (1914, 2) that, out of twenty-nine members of the Chlorophyceæ, Phæo-phyceæ, and Rhodophyceæ examined, only *Furcellaria*

fastigiata gave the direct reaction, strongly with guaiacum and benzidine, and feebly with α-naphthol. *Delesseria sanguinea* also acted on all three reagents, but only after the addition of peroxide. Of the others, only *Laminaria saccharina* and *L. digitata* gave a well-marked blue colour with guaiacum, upon the addition of peroxide, while slight reactions were given by *Cystoclonium purpurascens*, *Gracilaria confervoides*, and *Polysiphonia fastigiata*. All, however, oxidized benzidine in presence of peroxide. It is as yet uncertain whether this is in every instance due to an enzyme, and it is especially produced in the walls and mucilage—for example, in the walls and cells of *Sphacelaria cirrhosa*, and in the mucilage connecting the cells of the diatoms Tabellaria and Pinnularia epiphytic on Sphacellaria. In Ulva it was found that the blue oxidation product of benzidine appeared in cell walls of the decolorized thallus quite as rapidly in boiled as in unboiled tissue.

The presence of an inhibitor in these algæ has already been mentioned. Segers-Laureys (1914) too found a peroxidase in the mucilage of various brown algæ. Duggar and Davis (1914) also carried out an investigation as to the absence of oxidizing enzymes in the cells of *Fucus vesiculosus*. [See note, p. 250.]

Oxidase Reagents and their Limitations.

In the course of this chapter, mention has been made of the use of certain reagents for the detection of oxidases. These are usually dissolved in alcohol, and the solution is then diluted with water to such an extent that the solute, present only in small quantities, is still held in solution. This dilution is usually accomplished just before applying the reagent, as oxidation by dissolved oxygen takes place to a smaller extent in alcohol than in

water. The retardation of oxidase action occasioned by strong alcohol is well known.

A great number of substances have been used thus for the detection of oxidases macroscopically and microscopically. For the latter purpose those which easily penetrate tissues and give crystalline oxidation products, such as benzidine, are as a rule to be preferred to compounds, such as guaiacum resin, which do not diffuse readily. But as the behaviour of tissues towards different reagents frequently presents one with a series of reactions of varying intensity, it is advisable to employ several of them in each investigation.

A list of about forty oxidase reagents is given by Kastle in his monograph. Of these, many have been but little used, on account of their too great susceptibility to oxidation by atmospheric oxygen, which renders it hard to decide whether the colour produced in any tissue is really due to enzyme action. Thus, according as the reagent employed by an investigator is relatively stable or unstable, so will the number of tissues remaining colourless increase or decrease; since it really is a question of whether the reducing action of the cell contents is sufficiently feeble to permit of the oxidation of the artificial chromogen which has been added. [See note, p. 302.]

For example, when examining the tissues of *Rosa rugosa*, the writer (1913) found that the petals of white flowers contained tannin, and fail to react with α-naphthol. A very faint darkening of benzidine is, however, produced in the veins, whilst *p*-phenylenediamine blackens the veins and causes a slight general discoloration. The above changes only take place after the addition of a few drops of very dilute hydrogen peroxide. Red petals, on the contrary, are free from tannin, and when decolorized in alcohol give a faint reaction in the veins with α-naphthol

and peroxide. Benzidine alone renders the petal slightly brown and darkens the veins; whilst p-phenylenediamine gives a brown, changing to dark green, the distribution being the same as is that of the benzidine reaction. These experiments illustrate the graded action of the inhibitor tannin upon the different reagents, and also, as some think, upon the production of red anthocyanin, if it be assumed that its chromogen is present. The coloured flowers contain a complete peroxidase system, but in them, too, the actions on the reagents differ in intensity.

From among these numerous reagents, Keeble and Armstrong (1912, 1) selected a-naphthol and benzidine as being the most suitable for general use in microchemical work. Other authors also have employed these, and, in addition, guaiacum for use with sections and tissue extracts.

The above-mentioned reagents are only for the detection of phenolase, and for tyrosinase the best substrate is, according to Bertrand and Bourquelot (1896), undoubtedly tyrosin. For the detection of the latter enzyme, Chodat and Staub (1907) advocate the use of p-cresol, with the addition of glycin or another amino-acid. The action is greatly increased in rapidity by this group of acids, and results in the production of a violet colour, changing to blue with a reddish fluorescence. [See note, p. 250.]

While it is necessary to bear in mind the different degrees of sensitiveness of the various oxidase reagents, and the very striking influence of inhibitors, it is nevertheless obvious that those cells which act strongly upon all the reagents employed are certainly those in which oxidation processes are most active in the plant, and so their distribution must be of physiological significance.

Since histological studies on oxidases are so intimately connected with questions as to their physiological functions, an account of this branch will be deferred till a later section.

SECTION III.—THE QUANTITATIVE ESTIMATION OF OXIDASE.

COLORIMETRIC METHODS FOR PHENOLASES.

The estimation of oxidases and of their rate of action presents many difficulties. The following colorimetric methods have been tried. Laborde (1898), and later on Brunn (1909) and Euler and Bolin (1909), used an alcoholic solution of guaiacum resin, which becomes blue on oxidation. Slowtzoff (1900) estimated laccase by the rate of coloration of an aqueous solution of p-phenylenediamine and m-toluidene. The oxidation of phenolphthalin to phenolphthalein was followed by Kastle and Shedd (1901), the latter substance giving an intense red upon the addition of a trace of alkali. Von Czyhlarz and Von Fürth (1907) made use of the leucobase of malachite green. By determining the extinction coefficient of the solution from time to time, the rate of the reaction was studied. None of these proved reliable or of general application, both because further oxidation of the coloured oxidation products of the chromogens results in the formation of colourless substances, and on account of the natural condition of the sap pressed from plant tissues, which is rarely colourless or free from turbidity after filtration.

VOLUMETRIC METHODS FOR PHENOLASES.

Estimations of an aldehydase were carried out by Medvedew (1897) by titrating the acid formed by the oxidation of salicylaldehyde. Bach (1904) introduced the method of titrating the iodine liberated by the oxidation of hydriodic acid, the latter being derived from potassium iodide and acetic acid.

Gravimetric Method of Chodat and Bach.

Among the most successful attempts to examine quantitatively the action of oxidases of the laccase class are those of Chodat and Bach (1904), who weighed the purpurogallin formed under standard conditions from the interaction of pyrogallol and hydrogen peroxide in the presence of a peroxidase.

The following table illustrates the precision of their determinations, as well as the effect of varying the quantities of the substances which interact:

TABLE LVII.

Effect of Varying Quantities of Enzyme, Peroxide and Pyrogallol.

I. Pyrogallol, 1 gramme; hydrogen peroxide, 0·1 gramme; peroxidase, from 0·01 to 0·1 gramme in 50 c.c.

Weight of peroxidase:

0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08

Weight of purpurogallin formed:

0·021	0·042	0·066	0·083	0·102	0·123	0·145	0·166

II. Pyrogallol, 1 gramme; peroxidase, 0·1 gramme; hydrogen peroxide, from 0·01 to 0·1 gramme.

Weight of peroxide:

0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08

Weight of purpurogallin formed:

0·020	0·042	0·060	0·078	0·099	0·121	0·142	0·168

III. Peroxidase, 0·1 gramme; hydrogen peroxide, 0·1 gramme; pyrogallol, 1 to 4 grammes.

Weight of pyrogallol:

1·0	1·5	2·0	3·0	4·0

Weight of purpurogallin formed:

0·168	0·205	0·203	0·208	0·202

The results obtained by Chodat and Bach make it clear that the weight of pyrogallol oxidized is proportional both

to the quantity of peroxidase and of peroxide. It is, however, independent of the quantity of substrate, provided the latter is present in sufficient excess. The actual weight of substrate must be far greater than the maximum amount that could be oxidized in the time. It seems probable that what is needed is sufficient pyrogallol to keep the surface of the peroxidase saturated with it by adsorption.

METHODS FOR ESTIMATING TYROSINASE.

Von Fürth and Jerusalem (1907) investigated the action of tyrosinase by determining the quantity of melanin resulting from its action on tyrosin. This they effected by a spectro-photometric method, and by the hæmatokrit. The sedimentation of the pigment was brought about by boiling with calcium chloride, and the volume of the deposit measured in a graduated centrifuge tube. Bach (1908), however, found that the brown pigment produced by the action of the enzyme upon tyrosin can be oxidized further to a colourless compound by a dilute acid solution of permanganate. This process can be rendered quantitative by the use of 0·002 N permanganate, and is both the simplest and the most accurate method for the measurement of tyrosinase activity.

MANOMETRIC METHODS OF MEASURING OXIDASE ACTIVITY.

However, the most fundamental quantity to measure seems to be the amount of oxygen absorbed. This also permits of investigations being made upon the action of an enzyme on various substrates and under diverse conditions in a strictly comparable manner.

The method was adopted by Foà (1908), and by Mathews (1909) in his work on the oxidation of the sugars. More

recently Bunzel (1912) has perfected it, and introduced several necessary precautions previously omitted.

Bunzel, too, finds that the amount of chemical change is directly proportional to the concentration of the oxidase present, and concludes that the typical plant oxidase with which he worked " is not an enzyme in the customary sense of the word, but rather a substance entering directly into the reaction, and being destroyed in the course of the same." It must not, however, be forgotten that Bunzel is only measuring the organic peroxide present, assuming the correctness of this view of the nature of the complete oxidase.

He proposes as a unit, to express the oxidase content of a plant juice, " a solution of such a strength that 1 litre of it will be capable of bringing about the consumption by pyrogallol of the equivalent of 1 gramme of hydrogen— *i.e.*, a unit of 8 grammes of oxygen." Since it seems likely that much valuable knowledge will be gained from the systematic application of Bunzel's method to problems of plant physiology, his apparatus will be described here.

REQUIREMENTS AND LIMITATIONS OF MANOMETRIC METHODS OF ESTIMATING OXYGEN.

Since the rate of oxidase action is to be measured by the oxygen absorption, numerous precautions in estimating this gas by pressure changes must be observed. Of these Bunzel mentions the following:

1. The temperature of the reacting substances must be maintained constant to within $0.1°$. This limit is fixed, as a variation of $0.1°$ corresponds to a pressure alteration of 0.025 centimetre of mercury, which is the limit of accuracy of the manometer readings. In addition, constancy of temperature is desirable, on account of the large temperature coefficient of the rate of chemical reactions.

2. Since, in all oxidations effected at the expense of oxygen derived from the air, it is only the amount of the gas in solution which can take part, the necessity for maintaining this quantity constant is obvious. To insure this the following conditions must be observed: (a) The partial pressure of the gaseous oxygen in the reaction flask should be constant throughout any experiment, and the same in any series of experiments. (b) The reaction of the solution should be the same throughout the work. (c) The temperature within the flasks should always be the same. (d) The liquid should be kept saturated with oxygen under the above conditions.

3. The standard conditions should prevail before the reaction begins. This involves the complete saturation of the gases in the apparatus with water vapour.

4. The change in pressure indicated should be entirely due to oxygen absorption. To secure this, all carbon dioxide produced must be removed as fast as it is formed.

5. The carbon dioxide formed should be measured, to serve as a check upon the oxygen absorbed.

6. The rate of oxidation should always be observed. If the reaction is completed in a few hours, or even in a few days, the total quantity of oxygen absorbed should be measured.

All manometric methods in which the contents of living cells are concerned are of necessity liable to the error that part of the oxygen required may be derived, not from the air at all, but from easily reducible substances in the solution. Supplies from this source do not occasion any change in oxygen pressure, and so escape measurement.

The possibility of the injury of the enzyme by shaking must also be borne in mind, as this is known to cause some enzymes to become less active. This point was investigated specially.

Bunzel's Oxidase Apparatus.

For detailed descriptions of the electrically heated thermostat and of the shaking apparatus used, reference should be made to the original papers. Since the principle of the method can most readily be grasped by one acquainted with the oxygen absorption flask, it is figured here.
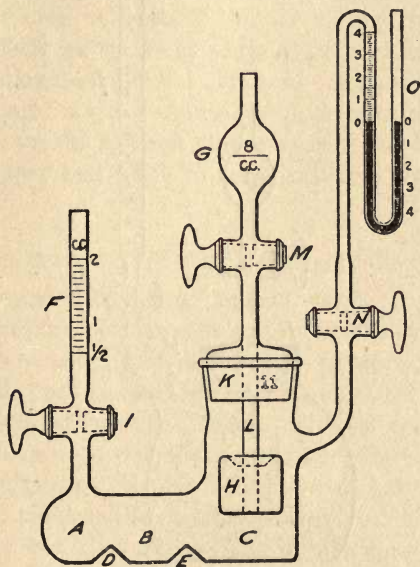
Fig. 24.—Bunzel's Oxidase Apparatus.

The volume of the interior of the apparatus is about 150 c.c., and in the latest form is divided into three compartments, A, B, C, by the grooves D and E. The earlier apparatus had only two compartments, but the risk of accidental and premature mixing of the liquids is minimized by the extra division. Opening into A is a burette,

F, used for the delivery of the plant sap to be investigated. A stopcock, I, disconnects the two. G is another burette used for 1 per cent. pyrogallol solution. By opening the cock M this is allowed to flow into C, through L.
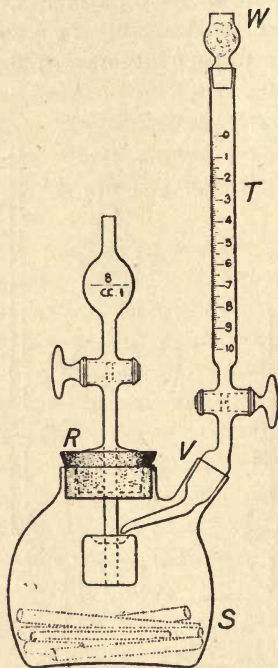
Fig. 25.—Airtight Titration Vessel for Use with Bunzel's Oxidase Apparatus.

The basket H contains 1 c.c. normal sodium hydroxide solution, and is suspended from K, the ground-glass stopper which carries the burette. The edges of H are bent inwards to prevent splashing out of its alkaline contents, and it is suspended at a sufficient height above C to insure

that no pyrogallol enters it. For, as is well known, alkaline solutions of pyrogallol rapidly absorb oxygen. The apparatus is completed by the manometer O, which may be disconnected by the stopcock N.

For titrating the contents of the basket H at the end of each experiment, the whole piece GH is removed, and the ground-glass joint K inserted into a large-bore rubber stopper, R, after pulling out a rubber bung. The stopper R serves to close a flask, S, shown in Fig. 25. In the bottom of this are sticks of sodium hydroxide to remove all carbon dioxide from the air in it. A burette, T, filled with dilute acid is inserted into the flask by a ground-glass aperture, V, in which it can rotate, so as to be placed above H. The upper end of V is kept dust-free by a plug of cotton in W.

MATERIALS USED IN BUNZEL'S OXIDASE ESTIMATIONS.

As a source of oxidase, Bunzel employed the juice pressed from potatoes through a piece of silk cloth. Fresh juice only was used, and previous to pressing the tubers were peeled and passed through a meat-chopper. In experiments upon beets the leaves and roots were similarly treated. In no case was the sap further cleared by filtration through paper. It may be remarked that such treatment would yield sap of a truly comparable nature from each source, but that the values obtained would in no case be absolute, nor would the results from different tissues have any accurate comparative value. Preparation of the sap after treatment with liquid air would entirely do away with this source of error.

Pyrogallol was used as the substance to be oxidized, for several reasons. It is obtainable in a high degree of purity, is very soluble in water, and its solutions are very nearly neutral. Again, the best of the known existing

methods for the measurement of peroxidases was also based upon the oxidation of pyrogallol in presence of hydrogen peroxide.

The most important reason of all is considered by Bunzel to be that the action of the oxidase upon pyrogallol can be assumed, on the analogy of the behaviour of other enzymes, to be purely catalytic; for pyrogallol is slowly oxidized in presence of atmospheric oxygen, with the production of colours passing from yellow through orange to deep red. Thus, the action is not initiated by the enzyme, but merely accelerated.

THE METHOD OF CARRYING OUT ESTIMATIONS WITH BUNZEL'S APPARATUS.

The oxidase apparatus (Fig. 24) is placed in the thermostat, being clamped to the shaking machine. At this stage 8 c.c. of 1 per cent. pyrogallol and 2 c.c. of plant-juice have been run out from the pipettes into the compartments C and A respectively, while H contains 1 c.c. normal sodium hydroxide solution. Of the stopcocks, M is closed, but N and I remain open. The heating current is then switched on, and about half an hour suffices to reach the constant temperature and to allow the air in the apparatus to become nearly saturated at that temperature. The thermostat is now opened sufficiently to permit of the stopcock I being closed. When this is done, shaking is begun at the rate of five complete excursions in 3·3 seconds. Readings are taken at intervals of ten or twenty minutes, the shaking being interrupted for the purpose. After about two hours no further oxygen absorption takes place.

As a check on the quantity of oxygen used up, the carbon dioxide produced is estimated by removing the piece GH, and placing it in position in the carbon dioxide-

free titration flask S. The contents of H are then titrated by running in $\frac{N}{10}$ sulphuric acid from T. As indicator, phenolphthalein, followed by Congo-red, is used, and from the difference in their end points the carbon dioxide absorbed may be calculated.

Since the volume of the apparatus, the temperature, and the initial and final pressures, are known, the actual weight of oxygen used up can be calculated when desired.

The most considerable source of error was found to be that occasioned by an initial rise of pressure on closing the stopcock and beginning to shake. This appears to be due to an incomplete state of saturation of the air in the flask. It can be allowed for by blank experiments.

The Effect of Variable Factors on the Total Oxygen Absorption.

Before arriving at the quantities set forth previously, Bunzel tested directly the results of using widely divergent amounts of the reagents, and of otherwise altering the conditions. He ascertained the limits of concentration of the pyrogallol for accurate work to lie between about 0·25 and 10 per cent. The age of the solution was found to be immaterial. Absorption of oxygen was shown to be at least approximately proportional to the amount of oxidase solution used. The effects of altering the concentration of alkali, rate of shaking, and of small temperature variations, were also tested. Shaking of the potato-juice for fifteen to thirty minutes before the addition of the oxidizable substance reduces its oxidizing power to about half its original value, but when prolonged further little additional alteration can be detected.

TYPICAL ESTIMATION BY BUNZEL'S METHOD.

As an illustration of the method, the following table may be quoted. It contains the results obtained with concentrations of pyrogallol varying from 2·5 to 10 per cent. This range was extended in another table. The numbers at the top of the columns refer to the various flasks, which differed somewhat in volume.

TABLE LVIII.

MANOMETER READINGS EXPRESSED IN CENTIMETRES OF MERCURY IN APPARATUS.

Time in Minutes.	Temp.	No. 1.	No. 4.	No. 5.	No. 7.	No. 11.	No. 12.
0	36·4°	0·00	0·00	0·00	0·00	0·00	0·00
15	36·4°	0·50	0·60	0·52	0·55	0·70	0·65
30	36·4°	0·62	0·80	0·90	0·80	1·00	1·05
45	36·4°	0·85	0·95	0·98	0·80	1·20	1·10
105	36·4°	1·25	1·40	1·32	1·60	1·70	1·30
120	36·4°	1·40	1·60	1·40	1·60	2·20	1·40
135	36·5°	1·50	1·58	1·45	1·65	3·20	1·50
150	36·4°	1·58	1·80	1·60	1·80	*	1·60
165	36·5°	1·70	1·80	1·70	1·80	—	1·60
Final readings corrected to a volume of 150 c.c. ..	—	1·62	1·83	1·72	1·87	—	1·42
Pyrogallol per cent.	—	10	10	5	5	2·5	2·5

* Pyrogallol solution splashed into bulb.

Bunzel applied his method to the examination of oxidase content in pathological conditions of beet leaves. This subject will be discussed later.

OXIDATION BY CATALYSTS OF INORGANIC ORIGIN.

Since the foregoing chapter was written, a lengthy paper by Ewart (1914) has appeared, in which the action of oxidases is compared with that of inorganic catalysts. Ewart emphasizes the importance of the action of other salts upon the activity of such oxidizing systems. These may act as inhibitors or sensitizers. He also believes that "there is no justification for the use of such terms as 'peroxidase,' 'katalase,' 'œnoxydase,' or 'tyrosinase,' to indicate specific substances, ferments, or groups of ferments. The 'tyrosinase' of the potato is also a 'katalase,' a 'peroxidase,' a 'pyrogallase,' a 'hydroquinonase,' and a 'paraphenylendiaminase.' It is, however, permissible to use such terms as 'katalase action' or 'peroxidase action,' and such names as 'laccase,' 'russulase,' 'potatase,' 'carrotase,' etc., as temporary names to indicate the origin of the substances whose chemical nature is yet unknown. Comparison with metallic oxidases shows that we are not even on safe ground in assuming the existence of specifically distinct classes of plant oxidases, such as phenolases, aminoxidases, and iodoxidases."

With much of the above sweeping criticism of previous work the present writer is in disagreement. Moreover, it appears to him that a number of Ewart's observations are open to other interpretations.

The action of oxidizing salts upon guaiacum resin and other oxidase reagents has formed the subject of a large number of papers, and, in addition to a few mentioned by Ewart, the following may be noticed: Bourquelot and Bougault (1897), Breteau (1898), Wolff (1908), Cushny (1908), and Alsberg (1908), as well as Kastle's (1910) monograph. The extraordinary sensitiveness of guaiacum to the presence of copper is such that the water distilled

from copper boilers can, as first pointed out by Bourquelot and Bougault, be readily distinguished from tap-water and from that distilled from glass. The writer (1914, 3) has suggested the use of this reaction as a test for copper to be used in water analysis, in the absence of certain other salts whose presence can readily be detected by the usual tests. In the presence of hydrogen peroxide under standard conditions, 1 part of copper can be found when present in 100,000,000 parts of water.

ADDENDA.—Chodat and Schweizer (1915) have shown that when tyrosinase, p-cresol and glycerin are mixed and kept for one hour in a solution saturated with hydrogen, a red colour is produced five minutes after the admission of oxygen. When oxygen was admitted directly, thirty minutes elapsed before the production of the colour. This furnished evidence of the union of the enzyme and its substrate at a measurable rate.

Bunzel (1915) has recently studied alfalfa laccase, and has proved that the salts of strong bases with weak acids accelerate oxidation by means of the hydroxyl ions liberated from them. There is no quinol oxidizing enzyme in *Medicago sativa*, as supposed by Euler and Bolin.

Wolff and Rouchelmann (1915) have failed to find oxidizable chromogens in certain plants by extracting with acid aqueous ether, though these are present as a rule. It appears, however, that their list, which includes the leaves of the oak, acacia and iris, really shows the presence of inhibitors rather than the absence of chromogens.

Reed (1915, 1) has succeeded in showing that enzymes able to oxidize p-phenylenediamine are present in all the algæ he tested.

CHAPTER XIII

THE OXIDASES IN RELATION TO PIGMENTATION, AND THE ANTHOCYAN PIGMENTS

It has been known for a considerable time that there is a connection between the occurrence of oxidases and sap pigments in stems and the veins of leaves. Reinke investigated the chromogens as far back as 1882, and he was by no means the first in the field. More recently they have been studied by Wheldale (1910), Molisch (1905), Willstätter (1913), Combes (1913), Keeble, Armstrong and Jones (1913), Bartlett (1913), Everest (1914), Tswett (1914), and others.

SECTION I.—WHELDALE'S THEORY OF ANTHOCYANIN FORMATION.

The sap-soluble anthocyan pigments are regarded by Wheldale as oxidation and condensation products of colourless or light-coloured chromogens which are present in living cells as part of a glucoside molecule. The hydrolysis of the glucoside is considered to be a reversible enzyme action, effected possibly by an emulsin, and only the chromogen thus liberated (which may itself be still a glucoside owing to the retention of one or more sugar groups) can be attacked by the oxidase. The chromogens are by this author supposed to be either flavones or xanthones, which are yellow colouring matters known to

be widely distributed. On this view the anthocyanins are a group of related substances, differing individually according to the flavone from which each one has arisen. Palladin's view that the anthocyanins are related to the respiratory chromogens does not appear to differ fundamentally from Wheldale's, but the latter is far more precise.

The hypothesis may be summarized in the following scheme:

glucoside (of flavone) + water $\rightleftharpoons$ chromogen (flavone) + sugar (by action of a glucoside-splitting enzyme possibly).

x (flavone) + oxygen = anthocyanin (the oxygen being supplied from an organic peroxide by means of a peroxidase).

The amorphous nature of some of the anthocyanins is considered by Wheldale and Bassett (1914, 1) to point to their high molecular weight, and direct determinations confirm the supposition. The high molecular weight involves the condensation as well as the oxidation of the relatively simple flavones, and in this respect the hypothesis has sometimes been misquoted. The importance of the condensation is seen from the fact that the production of anthocyanins from flavones has only recently been successfully accomplished, though if it were a matter of simple oxidation or reduction the change should be readily effected. Willstätter has, however, obtained by the reduction of quercetin considerable quantities of allocyanidin, accompanied by a small amount of cyanidin. The latter he believes to be identical with the compound obtained by hydrolysis of cyanin, the diglucoside anthocyanin of the cornflower. Combes (1913), Tswett (1913), and Everest (1914, 1 and 2), have also obtained substances by reduction

of flavones which resemble anthocyanins and antho-cyanidins in many of their qualitative reactions. Wheldale (1915), on the other hand, points out that in the absence of analytical data it is not possible to accept their evidence as conclusive, and also that the body obtained by reducing apigenin, which occurs in Antirrhinum, with nascent hydrogen, in no way resembles the Antirrhinum anthocyanin. Furthermore, the latter contains a higher percentage of oxygen than does apigenin.

The researches of Wheldale being primarily concerned with problems of genetics, her further work was directed to the study of the anthocyanins rather than to that of the oxidase system, for the universal presence of some enzyme of this type at once excludes it from acting as a Mendelian unit character. These sap-soluble pigments will be referred to in a subsequent section.

Microchemical and Genetic Researches on Oxidase Distribution.

Though the above reasoning, based on the widespread occurrence of oxidases, is undoubtedly true, researches by microchemical methods upon the distribution of oxidases and inhibitors in tissues have not been without fruit, as is shown by the results obtained by Keeble and Armstrong (1912). These authors employed α-naphthol and benzidine as reagents, and found that whereas in flowers the epidermis and veins oxidized the latter to a brown substance, frequently deposited as crystals, yet α-naphthol was only oxidized to a lavender tint in the veins, the slight shade of colour sometimes occurring elsewhere being negligible. (In passing it may be pointed out that very feeble oxidation of benzidine causes the appearance of a blue colour.)   Accordingly, in their earlier papers they refer to " epidermal " and " bundle " oxidases.   This distinction appears in their

later work to be regarded as only a convenient method of describing the locality of the enzyme. It is the writer's view (1914, 1) that all the differences in reaction can satisfactorily be explained on the supposition that α-naphthol is more sensitive to the inhibitors of the petal than is benzidine. In support of this is the fact that in certain Iris flowers treatment with hydrogen cyanide resulted in the removal of inhibitors, after which the benzidine reaction was given with greater intensity, and α-naphthol, previously oxidized only in the veins, afforded a dark blue colour in the epidermis also. In other varieties of Iris the two reagents were vigorously acted upon in both epidermis and veins, when the reagents, including hydrogen peroxide, were applied directly to the freshly gathered flowers.

Keeble and Armstrong (1912) found that tissues containing anthocyan pigments always afforded peroxidase reactions, and very frequently contained organic peroxide also. Since the latter (and to a lesser degree the former also) varies in quantity according to the amount of light to which the tissue containing it has been subjected, they do not regard its absence at a given time as of any great importance. As the distribution of peroxidase greatly exceeds that of sap pigment, they concluded that the presence or absence of chromogen, not of peroxidase, is a Mendelian unit. They point out that, if two factors were involved in the production of pigment in a red-stemmed variety of *Primula sinensis* investigated by them, it ought to be possible to obtain two types of green-stemmed plants—viz., one lacking chromogen, and another lacking the oxidase system. Only one kind has, however, been found. The results of crosses are shown here, adopting the usual symbols, C for chromogen, and O for oxidase:

$$\text{Green stem} \times \text{reddish stem} = Oc \times OC,$$
$$\text{and } F_1 = OOCc;$$

and it is found that the F_1 plants are pigmented, as both O and C are present.

Such F_1 plants produce gametes OC and Oc; hence, when self-fertilized, F_2 consists of 3 OC : 1 Oc—namely, 3 reddish-stemmed plants : 1 green-stemmed.

Now, although chromogen is the factor limiting the production of anthocyan in a cell, the oxidases are in their opinion part of the mechanism, as is further illustrated by the fact that it is precisely those tissues which are rich in oxidase in unpigmented varieties that are rich in anthocyan in the pigmented.

Further research showed that among the red-stemmed there were two varieties—dark red and reddish. These differed in that the former contained the pigment in epidermis, cortex, and stele; whereas in the latter it is confined to the epidermis and adjacent cells. As the result of crossing, it was seen that F_1 plants were reddish, and F_2 consisted of 3 reddish : 1 dark-red stemmed. This dominance of the light-coloured variety is evidently due to the presence of an inhibitor sufficiently strong to suppress pigment formation in the deeper tissues.

With regard to the production of colour in flowers, the results of genetic experiments revealed the presence of two varieties of white flowers in *Primula sinensis*. These are known as "recessive" and "dominant" whites. The former evidently lack the factor for colour, for when crossed with a coloured variety they yield a coloured F_1, which when self-fertilized, gives 3 coloured : 1 white in the F_2 generation. The dominant whites, however, yield a white F_1 when similarly mated, and afford 3 white : 1 coloured in F_2. Keeble and Armstrong have shown that in the dominants an inhibitor of oxidase action is present, whereas the recessives contain no such substance. Again, in flaked or ever-sporting varieties of this plant

they established a close parallel between depth of coloration and intensity of oxidase action.   Very similar results were obtained with *Dianthus barbatus* and *Geranium sanguineum,* for the details of which the original papers should be consulted.   They also proved that the white patches in coloured flowers and the unmasked appearance of the yellow eye of the flower were due to inhibitor, for addition of oxidase reagents failed to result in the coloration of such regions till after treatment with hydrogen cyanide, whereby the inhibitor was removed.

THE OXIDASES OF CYTISUS ADAMI.

By application of α-naphthol and benzidine as microchemical tests for oxidases, Keeble and Armstrong (1912, 2) obtained striking confirmation of the correctness of Baur's (1909) brilliant researches on the constitution of *Cytisus Adami*.   These have recently been summarized both by the above authors and by Skene (1914), so a very brief account will here suffice.

Baur regards *C. Adami* as a periclinal chimæra, an organism the outer parts of which are composed of one species, and the inner of another.   As is well known, this interesting plant was obtained many years ago by the French gardener Adam, supposedly by grafting *C. purpureus* on *C. laburnum*.   To this chimæra *C. purpureus* has given only the single-layered epidermis, whilst the other tissues are formed from *C. laburnum ;* for while *C. purpureus* has purple flowers, and those of *C. laburnum* are yellow, *C. Adami* bears yellow and purple flowers, and in addition buff ones.   The latter arise from the combination of a purple epidermis with hypodermal cells containing yellow plastids.   Keeble and Armstrong have summarized their results in the accompanying scheme, which needs no ex-

planation if the facts concerning colour production in Primula be borne in mind. The arrows indicate the reactions given by the various types of flower:

OXIDASES OF *Cytisus Adami*.

C. Adami.

Epidermal direct oxidase, as in *C. purpureus* ⟵ {Purple flowers} ⟶ {Bundle direct oxidase, as in *C. purpureus*

⟻———————— Buff flowers ————————

No epidermal oxidase, as in *C. laburnum* ⟵ {Yellow flowers} ⟶ {Bundle peroxidase, as in *C. laburnum*
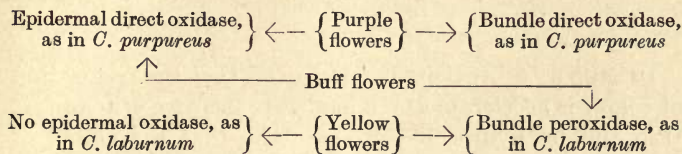
THE RESTORATION OF ANTHOCYAN PIGMENT IN DECOLORIZED FLOWERS.

Owing to the accumulation of a large amount of experimental evidence, the salient features of which have been mentioned in the preceding pages, there was a general consensus of opinion that oxidases were concerned in the production of anthocyan pigments. It was also contended by Keeble, Armstrong, and Jones (1913, 1), that the return of colour which takes place when a flower decolorized in alcohol is transferred to water, is explicable in terms of oxidase action. This view has recently been opposed by Wheldale and Bassett (1914, 1), and by Tswett (1914), while Willstätter (1913) has brought forward yet another explanation of the phenomenon.

Keeble and Armstrong attributed the decoloration to the action of the reducing agents of the tissue containing the anthocyanin. They conceived the normal colour to be due to a balanced reaction between the oxidase and the reducing substance, but, owing to the lessening of enzyme activity occasioned by the application of strong alcohol, equilibrium is displaced in favour of the transformation

back to the chromogen. The relations were by these writers expressed in the following scheme:

$$\text{Chromogen} \left\{ \begin{array}{l} \xrightarrow{\hspace{2cm}} \\ \text{Oxidase} \\ \text{+ Water} \end{array} \right\} \text{Anthocyan pigment}$$

$$\left\{ \begin{array}{l} -\text{Water} \\ \text{Reducing agent} \end{array} \right\}$$
$$\xleftarrow{\hspace{2cm}}$$
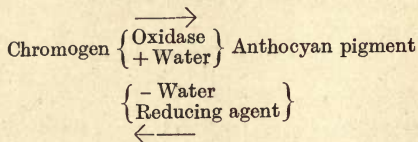
In support of it, they demonstrated that the restoration of colour is accelerated by a moderate increase of temperature and by hydrogen peroxide; also that the action of peroxidase is very feeble in strong spirit, though it can produce a slight browning of benzidine in the tissues (but not *in vitro*), even when the solution contains as much as 95 per cent. alcohol. The possibility of the anthocyan colour change being of the indicator type was examined by Keeble, Armstrong, and Jones, and dismissed as unsatisfactory.

Wheldale and Bassett (1914, 1) have recently pointed out that this fading and restoration of colour is exhibited by most pigments of the anthocyanin class, and has been recorded by Hansen (1884), Molisch (1905), and Grafe (1911), in various plants. Furthermore, restoration of colour takes place in boiled solutions on evaporation, and so cannot be due to oxidase action. This criticism seems to the author to be quite unanswerable. In addition it has been shown by Wheldale that the acceleration of the return of colour is due, not to hydrogen peroxide, but to the acid contained in this reagent as usually prepared, and that when dry hydrochloric or hydriodic acid is passed into absolute alcohol containing decolorized petals, the usual red colour is produced. This is also the case when hydrogen cyanide gas is passed in. Thus, in spite of the fact that the latter substance is an inhibitor of oxidase action, the reaction still takes place.

Turning now to the question of what causes the decoloration of the petals in the first instance, Wheldale and Bassett have brought forward an ingenious analogy between this and the decolorization of phenolphthalein solution, rendered red by ammonia, which takes place in strong alcohol, but is reversed on diluting with water. In this case also evaporation to dryness leaves a coloured residue Thus it seems reasonable to consider the observations with anthocyanin as explicable in a similar manner—namely, by ionization changes, resulting in the production of a colourless undissociated compound which gives coloured ions. There is, however, no direct proof of the correctness of this view.

It is nevertheless indisputable that plant tissues do contain reducing agents. These are capable of causing the disappearance of the colour of anthocyanin, as shown by Keeble and Armstrong, and of both anthocyanin and the blue oxidation product of guaiacum, as found by the author (1913) for the leaf sap of Iris. In addition, the above-mentioned collaborators (1913, 3) have drawn attention to decolorization of anthocyanin by nascent hydrogen. It appears that such naturally occurring reducing substances must be responsible for some, at least, of the destruction of anthocyanin which takes place in strong alcohol. If it is thus easily reduced, it ought to be possible to oxidize it back again to the coloured condition, as has been done by Bartlett with the pigment of Dioscorea sp., though, so far as the writer is aware, there is no proof that coloured products so obtained are identical with anthocyanin. Undoubtedly the whole question has now been settled once for all by Willstätter, who tried the effect of alcohol upon pure crystallized anthocyanin, in which case reducing agents were of necessity absent. Furthermore, since it has been shown—quite conclusively, as the writer

believes—that the restoration of pigment in the tissues is not connected with oxidase action, and since there is much evidence that it is not an oxidation change at all, it is hard to see how its converse, the disappearance of colour, can be due to reduction.

The suggestion advanced by Willstätter (1913) to explain the behaviour of anthocyanin is that in presence of strong alcohol intramolecular change takes place, with the production of a colourless isomer. This can be paralleled by the transformations of the triphenylmethane series of dyes.

PEROXIDASE REACTIONS OF RELATED SPECIES OF IRIS.

Between the distribution of anthocyan pigments and oxidases there is in many cases so close an agreement as to amount almost to a definite proof that there is some causal connection; but though there appears to be reason to suppose that the production of colour necessitates the presence of a complete peroxidase system during that process, yet a number of facts ascertained by the writer (1914, 1 and 3; 1915, 1) do not support such a conclusion. These, however, are being investigated further with a view to ascertaining the causes of such discrepancy.

The examination of numerous species and varieties of Iris was undertaken much on the lines of Keeble and Armstrong's work, employing benzidine and α-naphthol as microchemical reagents. In the course of the work, which extended through two years, well over a hundred flowers were tested, including members of about sixty or seventy varieties. It was found that, adopting the classification advocated by Dykes (1913) in " The Genus Iris," the most striking feature of the results was that related varieties had closely similar peroxidase distribution, irrespective, in

TABLE LIX.

XIPHION GROUP.

No.	I. xiphioides (Ehrb.)—English Iris.	Restoration of Colour.	Untreated.				Treated with Hydrogen Cyanide.			
			Benzidine.		α-Naphthol.		Benzidine.		α-Naphthol.	
			Veins.	Epidermis.	Veins.	Epidermis.	Veins.	Epidermis.	Veins.	Epidermis.
1	Pale purple, falls	0	++	++	++	++	−	−	−	−
2	Pale red purple, mottled with more intense patches, falls and standards	+ slight	++	++	++	traces	−	−	−	−
3	Dark purple, falls and standards	+ in patches	++	++	+	0	−	−	−	−
4	White with blue specks, yellow central patch, falls	0	++	++	++	++	+++	+++	+++	+++
5	White, pale blue patches, falls. (Pale blue standards)	0	+	0	+	0	+++	+++	+++	+++
6	Blue, yellow central patch surrounded by white, falls. (Blue standards)	+ slight	−	−	+	0	+++	+++	++	+
7	Purple spots on pale blue, yellow central patch surrounded by white, falls. (Standard more intense purple)	+ slight	+	0	+	0	+++	+++	++	+
8	Deep purple spots on purple, yellow central patch surrounded by white, falls. (Deep purple standards)	+	+	+ partly	+ slight	0	−	−	++	0
9	Deep purple red, yellow in central patch surrounded by white. (Purple red standard)	+	+	+ partly	+ slight	0	+++	+++	++	0

many cases, of pigmentation. Owing to the considerable alterations produced in the peroxidase by keeping in the dark, it is essential for a truly comparable examination that all the flowers should have been exposed to equal illumination. Since some of the plants flowered in February, and others in succeeding months up to July, it was not possible to secure uniformity in this condition. Nevertheless, as the results obtained with members of one variety picked in the gardens and examined in the laboratory under the weather conditions existing one day agreed well with those given by the same variety on succeeding days, one is led to the conclusion that this is not a serious source of error. Direct experiment has also confirmed this, as will be shown farther on.

In a general way it may be said that the power of oxidizing benzidine is more widespread and of greater intensity than that of oxidizing α-naphthol, yet in many cases the latter reagent is strongly acted on in the epidermis. The failure of the flowers to react with α-naphthol is due to an inhibitor, which is apparently able to stop the action on it to a greater extent than that on benzidine. Its removal by the cyanide method is illustrated by the following table. Hydrogen peroxide was added in every case, as no reaction takes place without it.

With regard to the restoration of the original anthocyan pigment, when the decolorized falls are placed in water, the chief factor seems to be the intensity of colour in the untreated tissues. For when this is not fairly deep, the losses sustained through diffusion, and destruction by reducing agents too, in all likelihood, are sufficient to prevent any reappearance of anthocyanin.

In the table (No. LIX.) it is not easy to correlate peroxidase distribution with that of anthocyanin. It appears, however, that in Nos. 1 to 4 the supply of chromogen is the

limiting factor, whereas in Nos. 5 to 9 the presence of an inhibitor is indicated, and this is borne out by the results obtained after treatment with hydrogen cyanide.

Very striking are the colours developed on applying the reagents to the Spanish Iris—*I. xiphium*. The group is characterized by the intensity of its peroxidase actions, as may be seen from Table LX. There is, however, a very definite inhibition area, which remains quite uncoloured. This coincides with the distribution of the deep yellow plastid pigment on the haft of the falls. Prolonged treatment with the reagents in part obliterated this white area, apparently because the inhibitor slowly diffused away. All this is quite similar to the behaviour of Primulas, as shown by Keeble and Armstrong.

TABLE LX.

XIPHION GROUP.

No.	*I. xiphium* (Linn.).	Benzidine.		a-Naphthol.	
		Veins.	Epi-dermis.	Veins.	Epi-dermis.
75	Var. *lusitanica*, Ker., form Thunderbolt, falls	+ + +	+ + +	+ + +	+ + +
	Var. *lusitanica*, Ker., form Thunderbolt, standards	+	0	+ +	0
76	Var. *chrysolora*, Hort., falls	+ + +	+ + +	+ + +	+ + +
	Var. *chrysolora*, Hort., standards	+ + +	+	+ + +	+
77	Form yellow fall, blue standard	+ + +	+ + +	+ + +	+ + +
78	Form blue fall, blue standard	+ + +	+ + +	+ + +	+ + +

In the Pogoniris group, however, one is faced by a most puzzling series of reactions, as shown in Table LXI. With remarkable uniformity, the peroxidase reactions of this

group are very slight, yet many of the flowers are deeply pigmented with anthocyanin; furthermore, attempts to find more marked reactions in unopened buds, which are, of course, shielded from intense sunlight, only resulted in further confirmation of the behaviour of the mature flowers.

EFFECT OF ABSENCE OF LIGHT UPON THE PEROXIDASE OF IRIS FLOWERS.

As already mentioned, Keeble and Armstrong (1912, 3) found the quantity of peroxidase and of organic peroxide to have increased in flowers kept in the dark. Before concluding definitely that the reactions of Iris flowers had the meaning attributed to them—viz., that they were genuine expressions of the properties of particular species and varieties—it was necessary to inquire into the possibility that they might have been in part due to differences in illumination. With a view to testing this, the Irises mentioned in Table LXII. were picked at 5 p.m. on a hot sunny day in July. Of each flower, one of the falls was removed and examined immediately. The reactions afforded by these are given in column No. 1. The flowers were then placed in total darkness, with their stalks in water. After twenty-one hours the second of the falls was removed, and the third after sixty-six hours. The behaviour of these is shown in Nos. 2 and 3 respectively. In the table, + refers to the whole of the fall, unless a portion such as the claw is mentioned. Thus, " + claw " indicates a less general distribution than does + alone. The reagent employed was α-naphthol, as it is more selective in its action than is benzidine

It is at once evident that the absence of light permits of the accumulation of peroxidase in active condition, and even leads to the formation of organic peroxide in the

TABLE LXI.

POGONIRIS GROUP.

No.		Benzidine.		a-Naphthol.	
		Veins.	Epidermis.	Veins.	Epidermis.
32	I. pumila, Linn., purple ..	+ +	+	+ claw	0
33	,, ,, ,, ..	+ +	+	0	0
34	,, ,, ,, ..	+ +	+	+ +	+
35	I. pumila, yellow (I. attica) ..	0	0	0	0
36	,, ,, ...	+	0	+	0
37	,, ,, bud.. ..	+	0	+ claw	0
38	I. germanica, Linn., young fl...	0	0	0	0
39	,, ,, ,, ..	0	0	0	0
40	I. germanica major	+ traces	0	0	0
41	I. germanica purpurea ..	+ + in traces	0	0	0
42	I. germanica, var. florentina ..	+ in parts	0	+ tr'ces	0
43	,, ,, ,, bud	+ traces	0	0	0
44	I. germanica, var. florentina, form albicans (Lange), viz., Princess of Wales	+	0	+	0
45	Ditto, bud	+ traces	0	0	0
46	I. flavescens, D.C., var. Munito	+	0	+	0
47	I. flavescens, var. Redouté ..	+ traces	0	+ tr'ces	0
48	I. flavescens var.	0	0	0	0
49	I. pallida, Linn.	+ +	+	+	0
50	,, ,, ...	+	+	+ trace	0
51	I. pallida, var. dalmatica ..	+ +	0	+	0
52	I. pallida, var. Queen of May	+ +	0	+ +	0
53	I. Victorine, Hort.	+ traces	0	0	0
54	I. variegata, {falls	+	0	+	0
	Linn., var. {standards ..	0	0	0	0
55	I. variegata var. {standards ..	0	0	0	0
	{falls ..	+	0	+	0
56	I. variegata var. {falls ..	+ traces	0	0	0
	{standards ..	0	0	0	0
57	I. variegata var., falls and standards	0	0	0	0
58	I. Kochii	0	0	0	0
59	I. Mrs. Horace Darwin, hybrid	+ +	+	+ +	+
60	I. Mrs. Langtry ,,	+ +	+	+ +	0

TABLE LXII.

Effect of keeping in Darkness upon the Peroxidase Reactions of Iris Flowers.

Number of Hours in Darkness:	No. I. (0) Epidermis	No. I. (0) Veins	No. II. (21) Epidermis	No. II. (21) Veins	No. III. (66) Epidermis	No. III. (66) Veins
I. Monnieri, deep yellow: unopened bud	0	+ claw	+ + claw	+ * (+ + claw)	+ + claw	+ +
,, slightly withered flower	0	+ claw	+ + claw	+ (+ + claw)	+ (+ + claw)	+ + + *
,, more withered	0	+ claw	+ + claw	+ +	+ (+ + claw)	+ + + *
I. xiphioides, dark blue: mature young flower	0	+ trace claw	0	+ +	+ claw	+ +
,, withered	0	+ +	0	+ +	+ claw	+ claw
,, intense claret colour	0	0	0	+ traces	0	+ +
,, light lavender: bud	0	0	0	+ traces	+ +	+ +
,, young mature	0	+ claw and edges	+ traces	+ +		+ +
,, older	0	+ trace	+	+ +	+	+ +
,, withered	0	+ + blade (+ claw)	0	+	0	+

* These gave a colour without the addition of hydrogen peroxide, and so contained organic peroxide.

veins of *Iris Monnieri*. The effect, apparently, is only brought about slowly, for, although it is noticeable after twenty-one hours, it only becomes well marked after sixty-six. It may also be seen that the age of the flower is without influence on the intensity of the reactions, except that in the buds the latter are not as strong as in the more mature flowers. Since these buds opened in the dark, it is clear that the absence of active peroxidase cannot be due to its destruction by light.

The almost complete absence of peroxidase activity, as judged by the α-naphthol reaction, in certain deep-claret-coloured varieties of *I. xiphioides*, is hard to reconcile with the view that the pigment is produced as the result of the action of the enzyme. Treatment with hydrogen cyanide or with toluene, as previously described, resulted in the production of the α-naphthol purple in the veins of the falls when the reagent was subsequently added. Thus it appears that in this case the presence of a powerful inhibitor, which is removed by the above methods, renders the enzyme inert. If the production of inhibitor occurred after pigment formation or in definitely localized portions of the cells, the discrepancy might be explained. It is, however, quite likely that the inhibitor is a reducing agent sufficiently strong to check the oxidation of α-naphthol. The subject is one which leaves much room for further work, and the author hopes to be able to investigate the matter more fully.

SECTION II.—THE ANTHOCYAN PIGMENTS.

ANTHOCYANINS AND THEIR PARENT SUBSTANCES.

The chemical constitution of this group of substances is now fairly completely known through the researches of Willstätter and his pupils, which have shed much light on

the subject. A summary of this work up to a recent date has been given by Stewart (1915), so it will only be briefly discussed here, from the standpoint of the physiologist rather than of the pure chemist.

As was previously mentioned, Wheldale (1909, 1; 1910, 2; 1911) considers the anthocyanins to be derivatives of flavones and xanthones, and has isolated in a pure condition both red and magenta anthocyanin from varieties of *Antirrhinum majus* containing the pale yellow hydroxyflavone apigenin, and the deeper yellow luteolin, both of which are probably combined as glucosides in the living cell. The structure of these bodies, originally isolated by Perkin, is shown below:

Apigenin.

Luteolin.
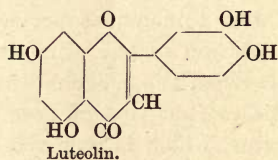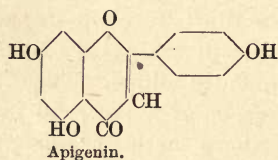
For the red and magenta anthocyanins and the above two flavones, Wheldale (1913, 2) found the following percentage compositions:

TABLE LXIII.

ANALYSES OF ANTHOCYANINS AND FLAVONES.

					C.	H.	O (by Difference).
					Per Cent.	Per Cent.	Per Cent.
Red ..	..	..	..	..	51·81	5·01	43·18
Magenta	..	..	..	..	50·50	5·11	44·39
Apigenin	..	..	..	..	66·66	3·70	29·64
Luteolin	..	..	..	..	62·90	3·49	33·61

The molecular weights of the red and magenta anthocyanins were found to be 572 and 717 respectively by means of ebullioscopic determinations in ethyl alcohol. Since the results of the combustions may most simply be expressed by the formulæ, $C_8H_9O_5$ for the red, and $C_{15}H_{18}O_{10}$ for the magenta, the boiling-point determinations indicate their formulæ to be respectively 3 $(C_8H_9O_5)$—i.e., $C_{24}H_{27}O_{15}$, mol. wt. 555—and 2 $(C_{15}H_{18}O_{10})$—i.e., $C_{30}H_{36}O_{20}$, mol. wt. 716. Evidence derived from analyses of the lead salts supported these values. It was also ascertained that the red contained twelve hydroxyl groups, and the magenta anthocyanin fifteen. These figures and the high values for the percentage of oxygen in anthocyanins led Wheldale to conclude that the production of the latter in the plant was due both to condensation and oxidation of the flavones.

Assuming such flavones to be the parent substances of the anthocyanins of Antirrhinum—and on this point the chemical and genetic evidence is decisive—it may be regarded as well established that the anthocyanins examined by Wheldale are more complex than those studied by Willstätter, and are richer in oxygen. They cannot, therefore, be reduction products, though other anthocyanins may quite conceivably be so.

MENDELIAN EXPERIMENTS BEARING ON ANTHOCYANIN FORMATION.

These researches illustrate the fruitfulness of the combination of chemical methods with those of Mendelian analysis. By the latter it had been shown that the varieties of colour occurring in *Antirrhinum majus* necessitated for their elucidation the grouping of at least six factors. It remained for chemical research to show that

these were of a tangible nature. Thus the albino or true white contains neither apigenin nor luteolin. The ivory contains apigenin and a factor, absent from yellow, which inhibits the formation of luteolin, for ivory is dominant to yellow. In addition to these there are eight varieties, of which four are combinations of red anthocyanin with one or other of the two flavones, and in four others the red is replaced by the magenta. The complete list is given below:

1. White.
2. Yellow.
3. Ivory.
4. Yellow tinged with bronze.
5. Ivory tinged with rose-doré.
6. Bronze.
7. Rose doré.
8. Yellow tinged with crimson.
9. Ivory tinged with magenta.
10. Crimson.
11. Magenta.

Of these, rose-doré is due to the combination of apigenin with red anthocyanin, whereas the latter and luteolin yield bronze. Crimson results from the presence of magenta anthocyanin with luteolin, whereas with apigenin the colour of the magenta is not appreciably altered. The Mendelian relationship of the above varieties, from 4 to 11, is concisely shown in the following scheme drawn up by Wheldale. In it, those which are dominant have arrows pointing in their direction.
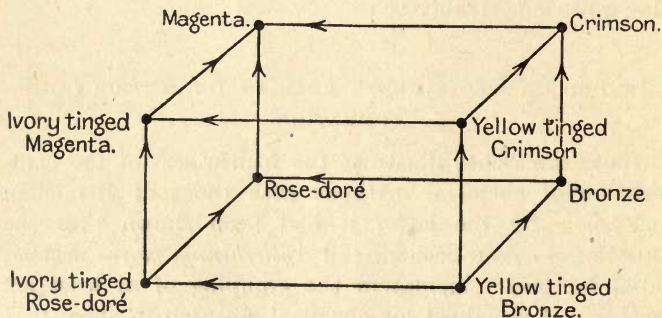
FIG. 26.

Some Sources and Properties of Pure Anthocyanins.

As a source of anthocyanin, Willstätter and Everest (1913) have recently employed the cornflower.

The blue pigment was by them proved to be the potassium salt of an acid (cyanin), which is violet in the free state, whereas the red pigments are combinations of cyanin with simple organic acids to form oxonium salts. These workers attributed the various shades of colour in the flower to such substances. Willstätter and Everest are of the opinion that cyanin is a flavone derivative, and that all anthocyanins are present in the living cell as glucosides.

Willstätter (1914), in conjunction with various collaborators, distinguishes anthocyanins, which are glucosides, from anthocyanidins in which the carbohydrate side-chain has been split off. The former are decolorized by cold alcohol, whereas the latter are stable in the cold, but decolorized on warming. In both the colour is restored by acids, though Everest (1914, 2) states that this is not so easily effected with the anthocyanidins. Futhermore, anthocyanins are not extracted by amyl alcohol from dilute aqueous sulphuric acid, whereas anthocyanidins are quantitatively taken up by the alcohol. These can be distinguished *inter se* by their colours, their solubilities, and the reactions they give with ferric chloride. Anthocyanins are most readily identified by their specific rotations, which are very high—from 200 to 1,400.

Cyanin on hydrolysis gives cyanidin (the hydrochloride of which has the formula $C_{15}H_{11}O_6Cl$) and two molecules of glucose. The pigment of the cranberry also gives cyanidin, and one molecule of galactose, whereas *Rosa gallica* gives cyanidin and two molecules of glucose. When heated with alkali to a higher temperature, cyanidin gives phloroglucinol and protocatechuic acid.

Pelargonin, obtained from Pelargonium, affords two molecules of glucose and one of pelargonidin, the hydrochloride of which has the following formula: $C_{15}H_{11}O_6Cl$. Heating with alkali in this case gives rise to phloroglucinol and p-hydroxybenzoic acid.

Delphinin, from Delphinium, when hydrolyzed, gives two molecules of glucose, two of p-hydroxybenzoic acid, and delphinidin. The hydrochloride of this body has the composition $C_{15}H_{11}O_7Cl$. Treatment of the latter with alkali breaks it up into phloroglucinol and gallic acid.

Another crystalline anthocyanin has been isolated from grapes. It is known as œnin, and forms a picrate which crystallizes in red prisms. Œnin is a monoglucoside of œnidin, prepared as the hydrochloride $C_{15}H_{15}O_7Cl$.

The pigment of the bilberry, too, myrtillin, has been isolated in the pure state. It is a glucoside of an anthocyanidin which is also present in the anthocyanin of *Althea rosea*.

In the same paper Willstätter and Mallison deny that the coloured products obtained by the reduction of morin, luteolin, quercetin, and other flavones, or of the yellow pigment of *Ampelopsis hederacea*, can be considered as anthocyanins. In a later paper (1914, 2), however, they describe the production of allocyanidin, and cyanidin in small quantity, by the reduction of quercetin. [See p. 292.]

The relationships of the various anthocyanins may perhaps be rendered plainer by the following scheme:

Source of Anthocyanin.	*Anthocyanidin and Other Products.*
Cornflower, flower (cyanin)	Cyanidin and glucose, two molecules.
Rosa gallica, flower	Cyanidin and glucose, two molecules.
Cranberry, fruit	Cyanidin and galactose, one molecule.
Pelargonium, flower (pelargonin)	Pelargonidin and glucose, two molecules.
Delphinium, flower (delphinin)	Delphinidin, glucose two molecules, and p-hydroxybenzoic acid, two molecules.
Grape-skins (œnidin)	Œnin and glucose, one molecule.
Bilberry, fruit (myrtillin)	Myrtillidin and glucose.

When heated with alkali the anthocyanidins are split up as follows:

Anthocyanidin.		Decomposition Products.
Pelargonidin, $C_{15}H_{10}O_5$	..	Phloroglucinol and p-hydroxybenzoic acid.
Cyanidin, $C_{15}H_{10}O_6$ ..	..	Phloroglucinol and protocatechuic acid.
Delphinidin, $C_{15}H_{10}O_7$..	..	Phloroglucinol and gallic acid.

Of the above, cyanidin is isomeric with the flavones luteolin and fisetin, pelargonidin with apigenin; and delphinidin, quercetin and morin are represented by the same molecular formula.

Willstätter and Everest (1913) have adduced reasons for the belief that anthocyanins unite with alkalies in virtue of the possession of phenolic hydroxyl groups, and form oxonium salts with acids, as in the following scheme, in which I. represents the hydrochloride, and II. represents the free colouring matter, with a pyrone ring. Glucose and other substances may be attached to the free valencies as side-chains.
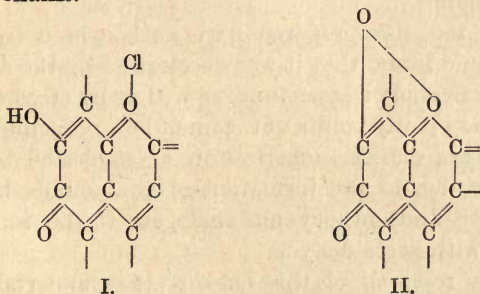
Among the earlier workers on pure anthocyanin was Grafe (1911). Crystalline anthocyanin was obtained by him from *Pelargonium zonale* in rosettes of needles, which melt at 270°. It has the empirical formula $C_{18}H_{26}O_{13}$, and

contains two hydroxyl, three carboxyl, and two aldehyde groups. It may be dialyzed, and gives no reducing sugar when hydrolyzed. An amorphous deep brown pigment was also obtained from the same flowers. This may be represented by the formula $C_{24}H_{44}O_{20}$, is non-dialyzable, and gives glucose when hydrolyzed. The analyses of the products obtained by Willstätter and by Grafe from Pelargonium are not in good agreement. Possibly they were dealing with different substances. In any case, the complete removal of water of crystallization constitutes a difficulty. Grafe ascertained that a colourless addition product of anthocyanin may be isolated by means of sodium hydrogen sulphite, whilst Tswett (1913, 1914) found phenylhydrazine and hydrogen cyanide to behave similarly. This shows that the free carbonyl group forms part of the chromophoric complex, or possibly the compounds may resemble the colourless isomer in structure. The addition of a mineral acid decomposes the addition product; consequently the red colour of the anthocyanin in acid solution again appears.

Tswett, too, has prepared pure anthocyanin from red cabbage, and found that it was decolorized by the addition of methyl alcohol or of acetone, as well as by ethyl alcohol. This, he very justly points out, cannot be a reducing action, but is rather one of isomerization, as suggested by Willstätter, or due to the formation of compounds between the alcohols and anthocyanin analogous to the formation of acetal with acetaldehyde.

Another research of this class is that undertaken by Bartlett (1913) upon the chromogen isolated from the aerial tubers of the Hawaiian bitter yam, a species of Dioscorea. This was considered by its investigator to have possible chemical and physiological relationships with the ammonia-greening anthocyanin of that plant, as it forms

green salts and oxidizes to a red compound which would itself pass for an anthocyanin if it were not insoluble in water. This oxidation is effected by plant oxidases, or spontaneously in water or solutions of halogen salts. Bartlett expresses the opinion that in the cell it is probable that the chromogen, in the form of a glucoside, becomes oxidized to an anthocyanin.

THE ARTIFICIAL PRODUCTION OF COLOURED SUBSTANCES SUPPOSED TO BE ANTHOCYANINS.

It has been thought by some that anthocyanins are derived from tannins, and, indeed, in the writer's (1913) own experience the mature leaves of *Vitis Veitchii* contain tannin and lack anthocyanin, whereas when the autumnal red anthocyanin appears in them they are free from tannin.

Wissemann (1912) has studied the distribution of tannin and anthocyanin in a very great number of plants belonging to the most diverse orders. He could not, however, find any precise relation between the occurrence of the two substances, but ascertained that, in a general way, when they varied with the age of the plant they varied in the same sense.

However this may be, Malvezin (1908) obtained a wine-red colour by heating green grapes with 2 per cent. hydrochloric acid under pressure at 120° for half an hour. Similar results were obtained with the tannins from hops, from *Prunus domestica*, *P. avium*, Ampelopsis sp., but not with those from the oak. He also records that gallotannin gives a red-violet precipitate when allowed to stand in sunlight with hydrochloric acid and formaldehyde. This is soluble in dilute aqueous alcohol, giving a red-violet solution which becomes blue-green with ammonia.

Similar coloured compounds have also been obtained in this manner by Laborde (1908), by Dezani (1910), and by Keegan (1913). In the course of a chemical investigation upon the red-coloured substances obtained by warming tannins with dilute sulphuric acid, Kunz-Krause (1898, 1899) came to the conclusion that they were dehydration products of aromatic hydroxy-acids. Very probably the derivatives described by the later workers are of a like nature, and have no relationship to anthocyanin.

Nierenstein and Wheldale (1911, 1912) also obtained substances bearing a resemblance to anthocyanin as far as colour is concerned, by means of oxidizing quercetin and chrysin. These again, as the authors point out, are far simpler in constitution than are the naturally occurring. anthocyanins.

A reaction somewhat similar to that described by Malvezin has been investigated by Peche (1913) as a microchemical test for tannin. Sections of leaves or stems which contain iron-greening tannins are placed in 20 per cent. caustic potash, mixed with about an equal volume of formalin, and heated quickly over a strong flame till the cells in which tannin is located become blue-green. When acid is added the colour changes to a cinnabar-red; this pigment in its solubilities and colour somewhat resembles anthocyanin.

Peche suggests that the function of the formaldehyde is to prevent the unlimited oxidation of the phenolic hydroxyl groups. He also gives a figure illustrating the similarity in the distribution of tannin cells and of those containing natural anthocyanin as shown in cross-sections of the leaf of *Prunus padus*. This he believes to be evidence of the origin of anthocyanin from tannin.

It was furthermore found by Peche that an alteration in colour took place in his artificial anthocyanin upon the

addition of salts of alum, barium, or zinc; this is analogous to the formation of aluminium salts or lakes by anthocyanin as obtained by Miyoshi (1900) and Grafe (1911). The colour of Peche's pigment was destroyed by the addition of sodium hydrogen sulphite, and restored by acidifying in a manner similar to natural anthocyanin as shown by Grafe.

Tswett (1913, 1914) states that in apples, pears, white grapes, pulp of red grapes, bananas, petals of Rosa and Cyclamen, alcohol-soluble tannin-like substances are present which yield colouring matters similar to anthocyanin when treated with strong mineral acids in presence of formaldehyde or acetaldehyde. In other tissues, such as the leaves of white cabbage, mesophyll of red cabbage, Pelargonium leaves, carrots, and potatoes, these tannin-like substances are wanting, and no coloration of this type was produced. Artificial colouring substances of this class agree with natural anthocyanins, not only in their absorption spectra, but also in their chemical reactions, being either decolorized or altered in colour by alkalies and acids, and being decolorized by the aldehyde reagents, sodium hydrogen sulphite, phenylhydrazine, and hydrogen cyanide.

In this connection, the researches of Combes (1911, 1 and 2; 1914) are of interest.. He comments on the facts that though, as shown by Mirande (1907), the quantities of tannins, of glucose, and of oxidase, are greater in coloured tissues than in those free from anthocyanin, and though he had himself pointed out the influence which abundance of carbohydrates has upon the production of anthocyanin, yet no direct relationship has, in his opinion, been proved between the presence of these substances and the contemporary formation of the pigment. Combes considers that if anthocyanin formation takes place in a tissue as the fina result of an uninterrupted series of changes, it should be

possible to obtain from an anthocyanin-yielding tissue, before the colour had actually appeared, a substance which could be made to afford anthocyanin. Experiments with *Ampelopsis hederacea* resulted in his obtaining from the red leaves a substance which crystallized in rosettes of violet-red needles, and gave an insoluble green lead salt upon the addition of neutral lead acetate. From the green leaves, on the other hand, a compound crystallizing in rosettes of needles was also found, but it was of a yellowish-brown colour and gave a yellower lead salt. By treating an alcoholic solution of the yellow crystals with hydrochloric acid and sodium amalgam, he succeeded in obtaining a purple-red-coloured solution which gave rosettes of needles of the same hue. A number of reactions studied by Combes gave identical results with both the artificial red substance and the natural anthocyanin, to such an extent that Combes believes the former to be nothing else than the natural pigment. Since this has resulted by the use of a reducing agent, sodium amalgam, he points out that all previous theories as to the formation of anthocyanin involving an oxidation must be erroneous.

Everest (1914, 1 and 2) also has obtained coloured compounds, which he considers to be anthocyanins, by the reduction of the flavones quercetin, morin, and luteolin, and of extracts from the following flowers—viz., daffodil, primrose, viola (yellow), and wallflower (lemon-yellow). The method found to be most satisfactory was to reduce the compound dissolved in a mixture of 5 volumes of absolute alcohol and 1 volume of concentrated hydrochloric acid by means of magnesium ribbon or turnings. As reasons for his statement that these bodies are identical with natural anthocyanins, Everest adduces the similarity of their behaviour in dissolving in amyl alcohol, in giving

certain colour changes with acids, alkalies, hydrogen peroxide and nascent hydrogen, in becoming decolorized in neutral solutions of alcohol (owing to isomerization), and in being restored in colour by acids. The absorption bands given by the natural anthocyanin and by Everest's compounds were also found to be similar. However, Wheldale and Bassett (1914, 3), are of the opinion that such reduction products are of quite different structure from the natural anthocyanins. The writer does not feel competent to decide between the conflicting results, but he is of opinion, after a survey of their evidence, that neither Combes nor Everest have definitely established the identity of their products with natural anthocyanin.

Wheldale and Bassett have also obtained a coloured substance by the reduction of quercetin. Analyses of this showed it to be a reduced derivative of quercetin, from which its molecular weight differed but little when determined by the raising of the boiling-point of alcohol. It does not, however, in their opinion resemble any natural anthocyanin.

Factors influencing the Production of Anthocyan Pigments.

From the time of Senebier (1791), numerous physiologists have studied the anthocyan pigments and the influence of various factors upon their production. Evidence as to the effect of exposure to light and to high and low temperatures was slowly accumulated, and the researches of Overton (1899) appeared to prove that in certain plants exposure to light was necessary for the production of anthocyanin. Working with *Hydrocharis morsus-ranœ*, he further showed that, other conditions being maintained constant, low temperatures favoured the production of

pigment, whilst disappearance of the red colour followed upon an increase in temperature.

It was later demonstrated by Eberhardt (1903) that greater quantities of the red colouring matter were present in plants exposed to dry air than in similar ones in air of a normal degree of humidity.

The effect of the addition of various sugars to the culture medium was investigated by Overton. When in sufficient quantities, glucose, fructose, and sucrose, all induced the formation of anthocyanin in plants in which the pigment was normally located in the mesophyll, but failed to do so in those in which it was in the epidermis. This effect was due to the chemical action of the sugars, not to their osmotic pressure, since salt solutions isotonic with them did not lead to the development of a red colour.

In the course of his researches on the respiratory chromogens, Palladin (1908), too, found that exposure to light resulted in the production of more anthocyanin in fragments of the leaves of *Rumex patientia* placed in 20 per cent. sucrose solution than was formed in similar portions kept in the dark. The latter, however, did contain some red pigment, whereas controls in water were entirely devoid of anthocyanin. The respiratory chromogens also were present in large quantity in the illuminated cells, in smaller quantity in those in the sucrose solution which were maintained in the dark. In the control they could still be detected by the addition of a peroxidase and peroxide, but the amount was much reduced.

That the presence of light is not absolutely necessary for the formation of anthocyanin was further illustrated by the work of Chartier and Colin (1911), for they found that the red root-tip of the seedlings of certain Crassulaceæ still showed the colour when grown in complete absence of light. Various experiments of Moreau quoted by these

authors also demonstrated that anthocyanin appears in red cabbage even in the dark.

An increase in the carbohydrate content of many leaves, leading to the production of anthocyanin, was ingeniously effected by Combes (1909). The method adopted was to insolate them, and to prevent the translocation of the assimilates by ringing the stem.

Molliard (1909) allowed the development of radishes to take place in sugar solutions, and found that a red cell sap was only formed in those portions which were near the surface. This he explains as being due to the lack of sufficient oxygen in the deeper portions of the culture medium.

The action of insects upon vegetable tissues, giving rise to hypertrophy and red pigmentation, has been investigated by Mirande (1907). In such pathological conditions as arise from these lesions he was able to demonstrate an accumulation of sugars, tannins, and oxidases.

All the above researches seem to point to one conclusion —that in plants which can produce anthocyan pigments the presence of a sufficient supply of sugar is a necessity. Whether this condition is realized by the mobilization of the reserves of seeds, by photosynthesis, by employing sugary culture media and increasing the permeability of the cells by strong illumination, or by decreasing the intensity of respiration by exposure to cold, is after all immaterial. The careful quantitative study by Combes (1909) of the relation between the carbohydrates of various leaves, such as *Ampelopsis hederacea*, and the production of red pigment, has finally proved the great importance of an abundance of sugars. A good illustration of this has been pointed out by Dixon, who observed that small branches of *Ribes rubrum*, removed from the bush before the opening of the flowers in early spring, bear only white

blossoms. These look very beautiful against the otherwise black branches. The flowers of this plant are, of course, normally of a red colour. Probably the removal of the branch results in the cutting off of the sugars which ascend in the vessels of the wood, and are derived from the carbohydrate reserves of the root and stem.'

In Table LXIV. are quoted Combes's analyses of red and green foliage leaves. Those of Ampelopsis were gathered on August 5. The leaves which were red had matured more rapidly than the others on the plant, owing to the fact that they were in a very well illuminated position.

In Spiræa the red leaves were borne on branches from which a ring of cortex had been removed, the green on the untreated portions. Both were gathered on the same day.

TABLE LXIV.

CARBOHYDRATES OF RED AND GREEN LEAVES: PERCENTAGES OF DRY WEIGHT.

	Soluble Carbohydrates.			Insoluble Carbohydrates.	Total Carbohydrates.
	Sugars.	Dextrins.	Glucosides.		
Ampelopsis hederacea :					
Green leaves	0·74	2·78	2·43	2·42	8·37
Red leaves ..	0·98	1·88	2·79	5·02	10·67
Rosa canina :					
Green leaves	2·42	1·30	8·22	9·72	21·66
Red leaves ..	2·64	1·23	8·24	5·33	17·44
Sorbus latifolia :					
Green leaves	0·71	1·15	2·20	11·99	16·05
Red leaves ..	0·80	1·07	2·52	1·20	5·59
Mahonia aquifolium :					
Green leaves	0·57	0·80	3·41	2·38	7·16
Red leaves ..	1·30	0·60	4·30	8·78	14·98
Spiræa paniculata :					
Green leaves	2·21	1·01	1·64	10·75	15·62
Red leaves ..	4·26	0·92	6·15	26·58	37·91

The green leaves of Rosa, Sorbus, and Mahonia, were gathered on October 19, the red on October 29. In the interval the occurrence of a sharp frost had induced the formation of the pigment.

From these figures it may be seen that the leaves in which the red colour appeared were richer in sugars and glucosides, but poorer in dextrins, than were the green. In Ampelopsis, which was examined in August, the red leaves, which were brightly illuminated, had a larger total of carbohydrates than the green. In Spiræa the red leaves on the ringed branches were of necessity richer in carbohydrates than those on the untreated stems, which remained green. The other deciduous plants, however, Rosa and Sorbus, contained less carbohydrate in the red than in the green leaves. The inferiority was due exclusively to the insoluble types of carbohydrate, the soluble being somewhat more abundant in the red. Thus, before the fall of the leaf a portion of the carbohydrate was translocated.

These figures throw light upon the high osmotic pressures found by Dixon and Atkins in leaves about to fall. The carbohydrates are all mobilized, and diffuse or are conveyed from regions of high concentration in the leaf to those of lower concentration in the petioles, branches, main stem, and roots. In Mahonia, on the other hand, the leaves are persistent, and the red contain more carbohydrates, both soluble and insoluble, than do the green.

Combes deduces from analyses that his anthocyanin must be a glucoside, and points out that this is in agreement with the researches of Von Portheim and Scholl (1908). The work of Willstätter has, of course, now placed this conclusion beyond doubt. Another view advanced by Combes does not seem to be so certainly established, and, indeed, he himself admits that there are many exceptions to it. It is that, since there is an increase in the total

glucosides during the formation of anthocyanin, the latter cannot be formed at the expense of pre-existing glucosidic chromogens. It arises rather by direct synthesis (*de toutes pièces*) in the presence of considerable quantities of sugars.

As pointed out by Combes (1914), in certain cases— *Ampelopsis hederacea,* for example—a yellow pigment may be obtained from the green leaves, which he claims to have converted into anthocyanin by reduction. But even in this case he urges that the amount of yellow pigment present does not seem to be sufficient to justify the conclusion that all the anthocyanin of the red leaves has arisen from it. The view held by Combes as to the production of anthocyanin is, clearly, very different from those of Palladin and Wheldale, both of which have already been mentioned. Its consideration has been deferred to this stage, as, though it may seem more logical to discuss all the theories of anthocyanin formation in the same place, it is far more convenient to consider each in relation to the evidence on which it is based.

Combes (1910) also studied the gaseous exchanges of green leaves during the formation of anthocyanin in them, and a similar investigation upon the flowers of *Cobœa scandens* (which are green when they open, and then become red) has recently been completed by Rosé (1914). In the two researches the same conclusion was reached— that there was no relation between the variation in the intensity of respiration due to the red pigmentation. In both floral and foliage leaves assimilation was less in the red than in the green stage. Whilst in these and other respects the red and green agreed closely, yet *during* the formation of the anthocyanin it was found, by examination of the diurnal gaseous exchanges, that oxidations are more active than in the normal conditions of vegetation.

From a study of the total sugars and glucosides of the

corollas of *Cobœa scandens*, Rosé was led to adopt Combes's view of the formation of anthocyanin by direct synthesis. In Table LXV. are quoted Rosé's interesting analyses of the corollas gathered at the four stages in the development of the flower.

TABLE LXV.

CORRELATION OF SUGARS AND ANTHOCYANIN IN FLOWERS.

Stage of Flower of Cobœa scandens.	Reducing and Non-Reducing Sugars in Corolla.		Glucosides in Corolla.	
	Fresh Weight.	Dry Weight.	Fresh Weight.	Dry Weight.
	Per Cent.	Per Cent.	Per Cent.	Per Cent.
1. A green closed bud ..	0·862	7·619	0	0
2. Opened, greenish-yellow	0·996	10·056	0	0
3. Becoming pigmented, rose colour	2·790	28·880	0	0
4. With violet pigment ..	1·298	14·104	0·239	2·613

From these figures Rosé concludes that the sugars increase up to the stage at which the anthocyan pigment is formed, and then diminish, whereas it is only in this last stage that glucosides are found in the corollas of *Cobœa scandens*.

As previously mentioned, Overton noted the destructive effect of high temperatures upon anthocyanin in the living cell. This subject has been studied by Fitting (1912) more recently. He showed that flowers of *Erodium gruinum*, which have an intense blue colour when plucked on a cool morning, change through light wine red to a clear rose within a few seconds of their being placed under a glass shade at a temperature of 40° to 42°. This process is reversible, but the return to the original colour takes place more slowly. Other genera, such as Geranium, Iris, Viola,

Agrostemna, exhibit similar changes, though these reactions are not given by all flowers. Fitting attributes them to dissociation changes, since they are also given in tissues killed with chloroform, as well as in aqueous and alcoholic extracts. It is very probable that they are due to isomeric transformations of the anthocyanin, as suggested by Willstätter in the case of decolorization by strong alcohol.

The relation of oxidases to the occurrence of anthocyanin has already been discussed at length, so need not be treated of here.

THE ORIGIN AND DISTRIBUTION OF ANTHOCYANINS IN THE CELL.

The recent work of Guilliermond (1911, 1913, 1914) upon mitochondries has given much precision to the study of the cytology of the plant cell in relation to its physiology. At the outset it is advisable to consider his terminology.

Mitochondries (μίτος, a thread; χόνδρος, a grain) was the name given by Benda to filaments or grains occurring in the cell which could be differentiated by special treatment, though they could also be observed in the living cell. They are very minute organoids, a few thousandths of a millimetre in length, existing as isolated grains—mitochondries in the strict sense—or as rows of grains like streptococci: chondriomites. They may also have the form of more or less elongated sinuous filaments, which are termed *chondriocontes*, and resemble bacilli. Any one of these forms may change into any other. The various types occurring in a cell are collectively designated the *chondriome*.

Guilliermond has shown that not only do these bodies give rise to chloro-, chromo-, and amylo-plasts, but they are also the seat of the elaboration of phenolic substances and of anthocyanin. He has traced the successive stages

of this process in a number of tissues, such as the epidermis of the floral leaves of *Iris germanica*, in the leaves of the rose, of the walnut, in the seedling of Ricinus, and in the tuber of the potato. No description can be as illuminating as a glance at Guilliermond's (1914, 1) coloured plates. In the rose leaf, however, the sequence is much as follows: In very young cells mitochondries of elongated form are visible, and are grouped irregularly round the nucleus. In slightly older cells these filaments have become more highly refractive, and are considered to contain a phenolic substance, which is colourless in the rose, but is sometimes of a faint yellow shade in other plants.

Cells which are still further developed contain a pale pink anthocyanin in the mitochondries, and in the next stage the colour is intensified, the filaments are thicker, and their ends have become club-shaped. This enlargement of the extremities continues, until a constriction in the middle severs the connection, as is usual when a drop of liquid possesses a length which exceeds the limit of stability for the ratio of length to diameter. The droplets formed in this manner then coalesce, so that the cell is largely occupied by one or more deep red vacuoles. The rapid increase in volume of the latter results in the dilution of the anthocyanin, so that individually they are of a lighter shade, though the macroscopic appearance is unaltered, as the total amount of pigment has not varied.

There is some evidence that the mitochondries themselves possess a lipoid membrane supported by an albuminoid framework. Various hypotheses have been put forward as to their mode of action. Regaud (1911), for example, considers that they have a selective action upon certain substances elaborated by the protoplasm. This is, after all, only another way of stating the fact that these substances are observed to accumulate within the chondriome.

A second hypothesis as to their function has been advanced by Mayer and Schoeffer (1913)—namely, that these bodies are the seat of the oxidation processes of the cell. These authors appear to regard the mitochondries as myelin forms—namely, granules or droplets of a lipoid nature—and this view is also held by Löwschin (1913). Guilliermond, however, considers them to be organoids of the cell which are of universal occurrence. It would be foreign to the purpose of the present chapter to discuss this vexed question fully.

REVIEW OF THEORIES OF ANTHOCYANIN FORMATION.

The numerous researches upon the formation of the anthocyanins may be roughly divided into three groups:

1. The purely chemical investigations of Willstätter and his school. These have shown that Wheldale was correct in regarding the anthocyanins as related to the flavones.

2. The researches of a biochemical nature upon the relationship between the distribution of complete per-oxidase systems and of anthocyanins.

3. Physiological investigations upon the factors influencing the production of anthocyanin.

(a) Willtsätter has shown that a number of the anthocyanins are glucosides of various bodies isomeric with the flavones. Hydrolysis removes the sugar, giving rise to anthocyanidins. One of these—cyanin—he has obtained in small quantity by the reduction of quercetin. The main product, however, is allocyanidin, isomeric with cyanidin, but differing in the fact that in it the pyrone ring is opened.

Wheldale has investigated the red and magenta anthocyanins of Antirrhinum, which arise from apigenin or luteolin. These have higher molecular weights and a higher percentage of oxygen than have the parent flavones. They are not glucosides, and appear to be more complex than

the pigments of this class studied by Willstätter. They are accordingly regarded as oxidation and condensation products.

(b) The production of dark-coloured substances in injured plant-tissues or in expressed sap is undoubtedly due to the action of an enzyme-system upon more or less colourless chromogens. Though this is so, there appears to be no direct proof that oxidases are concerned in the production of the anthocyan pigments, inasmuch as a precursor of anthocyanin has not as yet been converted into this pigment by the action of added enzyme. Of indirect evidence there is, on the other hand, a considerable amount. Thus, the very general parallel between intensity of oxidase action and depth of pigmentation may be considered to be a weighty argument for this view, even though the distribution of chromogen is the main factor limiting the occurrence of anthocyanin. To this parallel there are exceptions, notably in the genus Iris, so it cannot be taken as conclusive evidence.

The presence of areas free from anthocyanin in tissues, in which it is elsewhere abundant, has often been described. The localization of an inhibitor of oxidase action in such areas furnishes to the writer's mind the most convincing of the proofs as yet available that oxidases are concerned in the processes leading to the formation of this pigment.

(c) The physiological and biochemical investigations upon the occurrence of anthocyanin and large quantities of sugar in the same tissues point to the importance of the latter in the synthesis of the former. This may be partly explained by the fact that the pigment is a glucoside, but the quantities of sugar required appear to be far in excess of the proportion necessary to unite with the modified flavone to produce the amount of anthocyanin actually found.

Now, since direct chemical evidence shows that in some cases, at least, a flavone when reduced gives an anthocyanidin, and this unites with a sugar to yield an anthocyanin, it appears that the peroxidase-system can have nothing to do with the origin of the pigment. Such a view cannot, however, be accepted without careful consideration, for the relation between the absence of anthocyanin and the presence of an inhibitor of oxidase action is very striking. The anthocyanin of Antirrhinum, also, is too complex a body to be accounted for by simple reduction.

The third series of researches, those upon the abundance of sugar in cells which form anthocyanin, seems, however, to indicate what appears to the writer to be an explanation of the conflicting evidence. Palladin has pointed out that tissues richly supplied with sugar become richer both in oxidizing enzyme and in chromogen. Thus, since anthocyanins have been shown to arise in tissues with a similar abundance of sugars, it may well be that the coexistence of oxidase and of anthocyanin in the same cell is due to the fact that abundance of sugar favours the formation of both, and need not involve the production of the pigment by the enzyme-system. Indeed, if the latter is concerned in the respiration of sugars, the production of the enzyme in quantity when its substrate is present in large amount is quite in keeping with the behaviour of cells with regard to other enzymes, and offers a sufficient explanation of the function of the oxidase. Such a view, however, leaves out of account the known facts with regard to the distribution of anthocyanin and inhibitor.

The following suggestion is here offered in a tentative manner as an endeavour to correlate the evidence furnished by the two lines of research. Palladin (1908, 1, 2) in his earlier papers conceived of plant respiration as a taking in of oxygen by a readily oxidizable substance,

a chromogen or "phytohæmatin," to form a peroxide. The latter is then split up by peroxidase, and its oxygen utilized for the oxidation of reducing substance elaborated by the protoplasm. More recently Palladin (1914) has brought forward the view that the respiration of a substance such as glucose is a hydrolytic oxidation, whereby the carbon is oxidized anaerobically to carbon dioxide, and the hydrogen thus set free combines with a respiratory pigment, reducing it to a colourless chromogen. In the following aerobic stage oxygen is absorbed, with the production of water and the pigment. These processes are shown in the following equations:

1. Anaerobic stage:

$$C_6H_{12}O_6 + 6H_2O + 12R = 6CO_2 + 12RH_2.$$

Glucose.   Respiratory pigment.   Chromogen.

2. Aerobic stage:

$$12RH_2 + 6O_2 = 12H_2O + 12R.$$
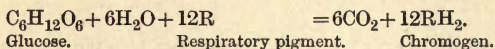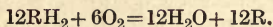
According to this scheme, alcohol, if formed by a zymase, is oxidized further. As shown by Weevers (1911), the action of respiratory enzymes need not involve the production of alcohol, for in the spadix of an Aroid he found that, even in an atmosphere of hydrogen, respiration of glucose gave rise to carbonic and citric acids.

Now, if for respiratory pigment in the above equation a flavone, quercetin for example, be substituted, the following is obtained:

(a) $C_6H_{12}O_6 + 6H_2O + 6C_{15}H_{10}O_7 = 6CO_2 + 6C_{15}H_{14}O_7.$

Quercetin.

(b) $C_{15}H_{14}O_7 - H_2O = C_{15}H_{10}O_6.$
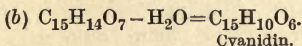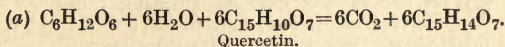
Cyanidin.

When the change shown in (a) takes place in presence of glucose, the aerobic oxidation may be forestalled by the union of the reduced flavone with the sugar to afford cyanin, the production of cyanidin by elimination of water

being possibly a stage in the reaction. Since equation 1 is probably brought about by a respiratory enzyme, it may be seen that the same process involves a reduction of one substance and an oxidation of another. Accordingly the result is obtained that an oxidizing enzyme is concerned in the reducing processes leading to the production of anthocyanin. The writer is of course aware that the peroxidase-system may have nothing to do with the appearance of the pigment, and that, even if it is concerned in its formation, the scheme outlined here may be quite erroneous. It is put forward merely as appearing to combine into a coherent whole the results obtained by diverse lines of research.

PROVISIONAL CLASSIFICATION OF PLANT PIGMENTS.

Before leaving the very complex subject of plant pigments, it may be of interest to quote an outline classification recently suggested by Keeble, Armstrong, and Jones (1913, 3). Detailed accounts of the plastid pigments may be found in Czapek's " Biochemie der Pflanzen," and in Willstätter and Stoll's " Untersuchungen über Chlorophyll."

1. Plastid pigments:
    (a) Chlorophyll pigments contain (Mg and) .. C, H, O, N.
    (b) Carrotin contains .. .. C, H.
    (c) Xanthophyll (oxidized carrotin) contains   C, H, O.
2. Sap-soluble pigments:
    (a) Yellow. Hydroxyflavone glucosides, or derivatives thereof, contain .. .. C, H, O.
    (b) Red—e.g., of wallflower. Hydroxyflavone glucoside derivatives contain .. .. C, H, O.
    (c) Red and brown—e.g., of plum. Substances produced by the oxidation of phenols in presence of amino-acids contain .. .. C, H, O, N.

NOTE.—Willstätter and Zechmeister (1914) have since succeeded in synthesizing pelargonidin.

CHAPTER XIV

THE OXIDASES IN RELATION TO PLANT PATHOLOGY AND TO TECHNOLOGY.

SECTION I.—PLANT PATHOLOGY

DISEASE OF THE MULBERRY LEAF.

SINCE upon the death of the protoplasm oxidases act without the restraints to which they are subjected during its life, it might well be supposed that conditions unfavourable to the normal metabolism of the cell might result in increased oxidase activity. This has been found true in a number of instances. For example, it had been observed that when mulberry-trees were cut back too frequently, an abnormal yellow colour and crinkled appearance resulted in the leaves. Suzuki (1900), investigating this, found that an excessive production of oxidases had taken place in such areas. He attributed this to the lack of proper nutrition of the rapidly growing tissues.

THE MOSAIC DISEASE OF TOBACCO PLANTS AND THE POTATO LEAF-ROLL DISEASE.

Much the same phenomena were observed by Woods (1902) in the " mosaic disease " of tobacco plants which had been cut back. He also demonstrated that the condition was rendered more acute by the application of certain manures which increase the rate of growth, and adduced reasons for the belief that the excess of

oxidases interfered with the action of diastases in the plant. With this view as to the cause of the injurious symptoms, Pozzi-Escot (1905) expressed agreement, and Doby (1912), in the course of an investigation on the potato leaf-roll disease, found that oxidases, both of the laccase and tyrosinase classes, were present in abnormal quantities in the diseased tissues.

Allard (1915) has, however, shown that it is possible to induce the disease in healthy plants by inoculating them with the sap pressed from those which are affected. Furthermore, dilution of the sap up to 1 part in 10,000 of water did not entirely stop transmission of the disease. Thus it appears certain that the excess of oxidases is a pathological symptom of the mosaic disease of tobacco— and by analogy of the mulberry leaf disease—rather than a primary cause. An organism causing the infection has not been discovered as yet.

The " Curly-Top " Disease of the Sugar Beet.

Recently Bunzel also (1913) has investigated the oxidase content of normal leaves of the sugar beet, and of those affected with the " curly-top " disease. This had been studied by Townsend (1908) and by Shaw (1910), and has been described by the latter as resulting in " an inward curling of the leaves, a distortion of the veins of the affected leaves, hairy roots, and checked growth." Heavy losses are occasioned by it both by reason of the stunting of the growth and of the prevention of seed production. Its cause was and has remained quite unknown, though it was shown by Ball (1911) that it develops after the bite of an insect, the curly-top leaf hopper (*Eutettix tenella*).

Bunzel's careful investigation into its symptoms, as revealed by a very elaborate series of measurements upon

the oxidases present in the various organs both of healthy and diseased plants, has brought to light the remarkable fact that oxidases are present in the leaves of the latter in from two to three times as great quantities as they are in the former. The measurements were carried out upon the sap pressed from the tissues through a piece of raw silk. They are therefore open to error owing to the fact that the protoplasm of the cells acts as a semi-permeable membrane during pressing, as shown by Dixon and Atkins (1913, 1). The errors thus introduced are, however, well within the range of the large variations found by Bunzel, and could be completely avoided by previous freezing of the tissues in liquid air.

The oxidase determinations were carried out with the apparatus previously described (see p. 243), and the results are expressed in terms of the unit adopted in testing the method—viz., an oxidase solution of such a strength that 1 litre of it could effect the oxidation of the equivalent of 1 gramme of hydrogen.

Bunzel first of all investigated the oxidase content of normal organs, and the variations in it produced by alterations in external conditions, such as the illumination. Some of his results are quoted in the following tables. The experiments were carried out during the month of August.

TABLE LXVI.

Juice pressed from Healthy Beet Plants.

	Oxidase Units.
Small mature leaves, up to 15 cm.	0·499
Large mature leaves, 30 to 40 cm.	0·184
Very young leaves, 2 to 7 cm.	0·180

From this it may be seen that those leaves which are apparently normal, but fail to develop to full size, contain nearly three times as much oxidase as either large mature leaves or very young leaves.

TABLE LXVII.

JUICE PRESSED FROM LARGE LEAVES OF HEALTHY BEET PLANTS AT VARIOUS TIMES.

			Oxidase Units.		
			Plant A.	Plant B.	$\dfrac{R \times 100}{J}$.
Collected at sunrise, 6.10 a.m.	..	..	0·259	0·256	—
„ sunset, 7.15 p.m.	..	..	0·468	0·453	—
„ 6 a.m.	..	..	0·256	0·266	2·59
„ 9.30 a.m.	..	..	0·259	0·266	2·22
„ 2.45 p.m.	..	..	0·319	0·288	2·00

These results apparently point to a considerable increase in the amount of oxidase during the period of illumination.

Bunzel, however, has pointed out that, when calculated on a basis of the total solids in the tissue yielding the sap, the oxidases are seen to fall off to about four-fifths of their former value, as shown in the column under $\dfrac{R \times 100}{J}$, where R denotes oxidase units and J represents the total solids in the samples. This, it must be remarked, seems to the writer to give an exaggerated idea of the actual decrease; for though Bunzel intends the ratio to give a measure of the amount of the net loss of water by transpiration, in reality it points to an erroneous value, since the total solids are altered not only by this, but by storage of the sucrose assimilated as shown in Bunzel's own analyses. Nevertheless, there is no doubt that this does not invalidate Bunzel's conclusion. In the first two experiments in the preceding table, chemical analyses of the tissue were not performed, but it seems likely that the apparent increase in oxidase is to be accounted for by excessive transpiration in their case also.

TABLE LXVIII.

JUICE PRESSED FROM DIFFERENT PARTS OF A SINGLE HEALTHY PLANT.

Oxidase Units.

Roots, lower half..	0·237
Roots, upper half	0·144
Stem	0·022
Pedicels and midribs of leaves..	0·173
Leaves	0·230
Seeds (groups of calyxes, each containing several seeds)	0·590

On the whole these results seem to indicate that oxidase activity is greatest in those portions of the plant in which the cells are young, and the vacuoles, as a consequence, are small.

Having quantitatively examined the oxidase content of normal beet plants, Bunzel turned his attention to plants suffering from curly-top, and to those which were, from drought or unknown causes, stunted in their growth. Some of his results are shown in the tables on p. 298.

From the following tables it is clear that plants which are small because of retarded growth have much larger quantities of oxidase in their leaves than have normal plants in either their fully developed or immature leaves. Diseased plants resemble stunted and " trotzer " plants in having an increased oxidase content. The term " trotzer " is applied to those plants which fail to develop a seed stem and fail entirely to flower. Between the roots of the various types no well-marked difference in oxidase content appears to exist, as the values found for each sort vary within fairly wide limits.

Though Bunzel's experiments have not thrown any direct light upon the cause of the disease, they are none the less of great interest, and bring out in a very striking manner the relationship between morphology and chemical

TABLE LXIX.

JUICE FROM PLANTS SUFFERING FROM CURLY-TOP.

	Oxidase Units.	
	August 21.	August 22.
Diseased curly-top leaves, insect-injured.. ..	0·381	0·388
Large healthy control leaves, from same plants ..	0·191	0·144
Roots from same plants	0·252	0·187

TABLE LXX.

JUICE FROM STUNTED PLANTS, NOT INFECTED, AND FROM NORMAL CONTROL PLANTS.

	Oxidase Units.	
	Stunted.	Normal.
Leaves	0·367	0·201
Upper half of root	0·086	0·172
Lower half of root	0·158	0·288

TABLE LXXI.

JUICE FROM CURLY-TOP PLANTS, BOTH NORMAL AND "TROTZER," AND FROM HEALTHY "TROTZER" PLANTS.

	Oxidase Units.			
	Diseased "Trotzer."		Healthy "Trotzer."	Diseased Normal.
	August 3, 8 a.m.	August 4, 8 a.m.	August 5, 8 a.m.	August 7, Noon.
Leaves ·..	0·496	0·446	0·446	0·403
Roots	0·216	0·183	0·237	0·324

composition. It has been suggested by Bunzel that plants with an excess of oxidase are in a condition of "fever." It must, however, be borne in mind that Bunzel's determinations are in reality measurements of the peroxide constituent of the complete oxidase system, since they are based upon the absorption of oxygen through the direct action of the juice on pyrogallol without the addition of hydrogen peroxide. Whether they really indicate differences in the absolute amount of enzyme, or are explicable by variations in the amount of peroxide or even of inhibitor, is undoubtedly of great interest, but must be considered as being only of secondary importance as compared with the fact that the absorption of oxygen is far greater in the diseased and stunted plants than in the normal.

OXIDASES IN HEALTHY AND CURLY-DWARF POTATOES.

Under the above heading Bunzel (1914) has published another elaborate comparative study of plant tissues, normal and pathological. The plan of the investigation was very similar to that on the beet, but a great variety of oxidizable materials, eighteen in all, was employed. It was found that the oxidase activity of the sap pressed from the foliage of normally developing potato plants is greatest in the early stages of development. It falls off with growth of the plants, and rises again when growth has about attained to its maximum. Curly-dwarf potatoes were shown to possess a greater degree of oxidase activity than healthy ones of the same age, both in the juice of tubers and of foliage. The numerical results of the original paper are clearly shown in graphs, but need not be considered in detail here, as the method has already been sufficiently illustrated.

The significance of such changes in oxidase activity is most probably due to the importance of this class of enzyme

in the respiration of the cell. Palladin's theory of the mechanism of respiration has already been briefly alluded to in a previous section. Though a discussion of the recent work on this subject would be of great interest, it is omitted here, both on account of the large proportions which it has assumed, involving as it does many questions of a chemical nature, and because the writer's acquaintance with it is not based on laboratory experience.

SECTION II.—THE BEARING OF OXIDASE INVESTIGATIONS ON TECHNOLOGY.

LACQUER INDUSTRY.

In addition to the interest of oxidase study for the silk and sugar industries mentioned in the last section, there are several other more direct applications. The researches of Yoshida in 1883 showed that an oxidase was concerned in the production of the well-known black varnish obtained from the milk-like sap of the lac-tree, *Rhus vernicifera*, and allied species. He obtained from the sap an acid—urushic acid—which, when oxidized by the enzyme, becomes black and forms the basis of the varnish which is so much used in China and Japan. Eleven years later Bertrand confirmed and extended Yoshida's work, giving the name " laccase " to the enzyme, and pointing out the relationship of urushic acid to the hydroxy-derivatives of the benzene series.

TEA AND COCOA TRADE.

In the preparation of tea, also, an oxidase has been proved by Mann to play an important part. In green tea the leaf is roasted immediately after picking, before any appreciable oxidation takes place. In black tea, on the other hand, an oxidation of the tannin is effected by an oxidase of the laccase class, resulting in the production of a soluble brown

substance, to which the colour of the infusion is due. The pungency, on the contrary, is dependent on the amount of unoxidized tannin, while the flavour is caused mainly by an essential oil. By carefully regulating the different processes of withering, rolling, and oxidation, the qualities of the tea may be altered within limits. All the operations involved must be carried out with the utmost cleanliness to avoid bacterial contamination as far as possible, as such gives rise to sourness, rendering the tea unfit for consumption.

In the procuring of cocoa beans, too, fermentation processes are involved which loosen the seeds in the fruit. In these stages yeasts and acetic-acid-producing bacteria are active. Oxidases are also at work both during the fermentation and subsequent drying, as shown by Loew, the change from the violet colour of the fresh bean to a deep brown being due to their agency.

TOBACCO TRADE.

The curing of tobacco again involves a fermentation. The leaves, after a preliminary withering, are " sweated " in moderate-sized heaps, and fermented in very large heaps containing many tons. It was at one time thought that this was a bacterial process, but, owing to the work of Loew and other American chemists, it has been shown to be mainly one of respiration of starch, sugars, and tannin, brought about by oxidases in conjunction with hydrolytic enzymes. Boekhout and De Vries (1909) are, however, of the opinion that some of these changes are due to oxidation without the intervention of an enzyme.

TIMBER TRADE.

Another instance of the importance of oxidases in commerce is that of the blemish known as " sap stain " in

lumber, by which considerable portions of the wood of certain trees, when exposed to the air by sawing into planks or beams, were discoloured, and so depreciated in value considerably. It has been demonstrated by Bailey (1910) that this alteration is brought about by an oxidase.

GENERAL AGRICULTURE.

The preparation of ensilage has been investigated by Russell within the last few years. He studied the changes taking place in green maize stems packed closely together to form a " silo." Bacterial action sets in, and also action of the respiratory oxidases and other enzymes of the plant cells. So large an amount of heat is generated by the oxidases that the temperature rises to such a degree as to inhibit further bacterial growth. The hydrolytic and proteolytic enzymes still retain their activity, however, and a complicated series of changes ensues, in which organic acids appear among the products.

NOTE.—Rose (1915) has investigated the oxidizing power of healthy apple bark, and of that injured by Illinois canker, and has found the latter to be the more active. It also has a smaller amount of acid. He considers that oxidation takes place in approximately the inverse ratio of the acidity. The slowing down of oxidation in Bunzel's apparatus is due to accumulation of acids.

In the writer's opinion the effect of the acidity or alkalinity of the medium is to alter the configuration of the molecule of the sugar, chromogen or other substrate. Mathews and Walker (1909) have shown how the oxidation of cystein is affected by the acidity of the medium, and in conjunction with others Mathews has studied the subject extensively. E. A. Werner, too, has proved that the configuration of the thiocarbamide molecule is altered by the acidity or alkalinity of the medium; a number of his papers on this subject have appeared in the *Journal of the Chemical Society* during the last three years.

In one configuration a molecule is often more reactive than in another.

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CHAPTERS XII.—XIV.

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