A POLARIMETRIC STUDY OF THE SUCROCLASTIC ENZYMES IN *BETA VULGARIS*

By R. A. ROBERTSON, M.A., JAMES COLQUHOUN IRVINE,
D.Sc., Ph.D., AND MILDRED E. DOBSON, M.A., B.Sc.,
Carnegie Scholar.

*From the Chemical and Botanical Research Laboratories, United College,
University of St. Andrews*

(Received April 16th, 1909)

Notwithstanding the large number of exhaustive investigations which have been recently carried out on the sugar beet the actual mechanism of the sugar synthesis seems to be practically unknown. Two alternatives seem possible, the simpler of which is that both glucose and fructose are formed in the leaf region and are there subsequently transformed into sucrose which passes by means of the leaf meristem directly to the root. The alternative explanation, that the disaccharide is a degradation product of starch, seems unlikely. Little or no starch is found under normal conditions in the growing leaf and, moreover, such a change would involve the partial transformation of maltose or glucose into fructose. This would necessitate a fundamental change in the sugar molecule, which seems improbable. Again, according to Grafe, no definite sugars have ever been isolated in the degradation products of cellulose, and thus the latter does not seem to be a likely source of the sucrose.

The first alternative is supported by many arguments. Friedrich Strohmer in his valuable contribution to the Wiesner Fest-Schrift¹ shows conclusively from the result of his own experiments and those of A. Girard that the formation of sugars is restricted to the leaf region and, moreover, that the roots of adult plants contain no reducing sugars save in the early stages of growth.

It must be maintained that the evidence seems to be increasing that formaldehyde is one of the initial substances formed during the natural process of photo-synthesis of sugars, and it would thus appear that a close parallel can, in this case, be drawn between natural and artificial

1. *Über Ausspeicherung und Wanderung des Rohrzuckers in der Zucker-Rübe.*

synthesis. Fenton's recent successful reduction of carbon-dioxide to formaldehyde¹ and the earlier work of Usher and Priestley,² who showed that the same reaction proceeded in plant cells, has strengthened the argument considerably. There seems little doubt that the artificial sugars obtained from formaldehyde contain large quantities of ketoses, and thus it can be readily understood that in the leaves of the beet the necessary constituents for the formation of cane sugar may be formed. The final stage of the process, the condensation of the glucose and fructose, although theoretically a simple reaction, is one which the organic chemist has been unable to duplicate.

Perhaps the most notable example of the comparative failure of ordinary chemical methods to produce naturally-occurring compounds is to be found in the meagre results which have attended attempts to synthesize disaccharides in the laboratory. Moreover the methods followed in the few successful cases must of necessity be widely different in their nature from those of the natural process. In view of these facts Grabe's statement that the fundamental rôle in the carrying out of these reactions must be ascribed to enzymes seems justifiable.³

Assuming that cane-sugar is formed by enzyme action, two alternative theories may be offered. The reaction may be occasioned either by a special enzyme, termed the associating enzyme, capable of condensing glucose and fructose, or the hydrolytic action of invertase may be a reversible change which is capable under suitable conditions of producing sucrose from the constituent sugars. This latter view is upheld by Gonnermann and Stoklasa, and brings the reaction into line with the cases of reversible zymolysis studied by Croft Hill. This idea has also recently received considerable support in the results obtained by Kohl,⁴ who subjected invert sugar solutions to the action of yeast extract rich in invertase. A series of titrimetric estimations showed that an equilibrium point was reached, presumably owing to the partial formation of sucrose. The result is interesting, but is not altogether in agreement with the nature of reversible change, as apparently conditions were realised in which both the fructose and glucose contained in the invert sugar completely disappeared on continued action. Pantanelli,⁵ in a paper on a similar topic, contributes the remarkable statement that

1. *Trans. Chem. Soc.*, Vol. XCVIII, p. 687, 1907.
2. *Proc. Royal Soc.*, Vol. LXXVIII, B, p. 318, 1906.
3. Macchiati, *Comp. Rend.*, Vol. CXXXV, p. 1128.
4. *Beih. Bot. Centralblatt*, Vol. XXIII, 1, p. 64b-64o, 1908.
5. *Rendiconti Accademia Lincei*, 5, Vol. XVI, pp. 419-428, 1907.

concentrated solutions of invert sugar undergo partial reversion when preserved at room temperature, 'particularly when the solution is feebly alkaline.' The reaction was accelerated by the addition of fungus revertase. The first conclusion seems to have been arrived at by the observation that the reducing power of the solution diminished. In view of Lobry de Bruyn and van Ekenstein's work¹ on the interconversion of hexoses in the presence of alkalies the result seems capable of a simpler interpretation.

Although opinion, in the meantime, seems undivided in attributing sucrose formation in plants to enzyme action, it would appear that it is still doubtful if the action is due to a definite specialised enzyme or is merely the result of reversible zymolytic change.

The following research was undertaken in the hope that the biochemical formation of sucrose by enzyme action could be detected polarimetrically, and for evident reasons the sugar beet was selected for experiment. The adoption of the polarimetric method seemed in this case specially advisable as affording a more accurate index of alteration in the composition of sugar mixtures than any method based on the quantitative use of Fehling's solution.

In view of the fact that the chemical activity of many enzymes is increased by the presence of other enzymes, no attempt was made to separate, even approximately, the individual enzymes from the various mixtures obtained. The total product was tested in its action towards suitable optically active substrata, and in view of the well-defined selective action of enzymes, the method seems justifiable. As far as possible, substrata were used which would readily react and would give large polarimetric differences. The optical activities were determined with an instrument displaying a tripartite field and sensitive to $\frac{1}{100}$ of a degree. Throughout the work standard two-decimetre tubes were used; these were provided with a water-jacket and all the determinations were made at 20° C., accurate to $\frac{1}{20}$ °. As a rule the sodium-flame was used, except in special cases where, on account of the unavoidable turbidity of the solutions, the incandescent light was substituted.

Some difficulty was occasionally experienced in filtering the solutions successfully, but this was overcome by shaking with finely-divided ignited silica, a method which we found did not introduce any experimental error.

For the purposes of the present investigation only the examination of the enzymes capable of reacting with carbohydrates and glucosides

1. *Rec. trav. chim. Pays-Bas*, Vol. XIX, p. 1, 1900.

was necessary, and accordingly in the first place the general nature of the soluble enzymes present in the leaves of the adult plant was studied. The plants used weighed on an average about 650 grams. The leaves were detached at the junction of the stem, and after being finely divided, were macerated with water in a sterilised mortar. The pulp thus obtained was mixed with a large excess of water (two litres), and kept in a thermostat at 30° C. for five hours, during which time the mass was kept thoroughly mixed by means of a powerful mechanical stirrer. The liquid was then filtered under pressure and the bright-red filtrate rapidly diluted with four times the volume of alcohol. The mixed enzymes were thus obtained in the form of a grey precipitate, which was filtered off, washed with aqueous alcohol and dried in a vacuum. The dry product gave very little inorganic residue on ignition, and was devoid of any action on Fehling's solution even on prolonged boiling. The solubility in water was slight, one gram of the substance requiring about 700 c.c. of water to effect complete solution at 25° C. The aqueous solution, which became turbid on the addition of traces of alkali, showed practically no activity when examined in a two-decimetre tube. This result was unexpected, but seems to be due to compensation, as enzymes of opposite activity were afterwards shown to be present.

In testing for the presence of probable enzymes, standard solutions of the active substrata were mixed with excess of the solid enzymes, and the mixture sterilised by the addition of a few drops of toluene. The initial specific rotation was determined without delay, after which the liquid was kept in a thermostat at 30° C., polarimetric readings being taken every twelve hours. In each case a control experiment, in which no enzyme was used, was conducted under parallel conditions, and results were only accepted as positive when these control solutions showed no alteration in rotatory power. The following table shows the principal results obtained:—

	Enzyme	Substrate	$(\alpha)_D^{20^\circ}$ initial		$(\alpha)_D^{20^\circ}$ final		Diff.	
1.	Invertase	...Sucrose	...	+ 66·6°	...	+ 64·4°	...	2·2°
*2.	Emulsin	...Amygdalin	...	— 34·4°	...	— 34·4°	...	nil
3.	Diastase	...Starch	...	—	...	+ 52·0°	...	
4.	Maltase	... α -methyl-glucoside	...	+ 157·5°	...	+ 128·4°	...	29·1°
5.	Lactase	...Lactose	...	+ 53·1°	...	53·4°	...	0·3°

*In view of the possibility that the enzyme action might be inhibited by the hydrocyanic acid produced from amygdalin, other substrata were afterwards used but a negative result was also obtained.

In the case of Experiment 3, the dilute starch paste used was rapidly liquefied and gave a dextro-rotatory filtrate. The concentration of

dissolved matter was estimated by evaporation of an aliquot part of the solution and weighing the residue dried at 100° C. In this way it was shown that the specific rotation of the solution gradually decreased from + 108°, when the first reading was made, to the constant value + 52°. This ultimate complete conversion into glucose through the intermediate formation of a more highly rotatory compound is in agreement with the idea put forward by Payn¹ that the hydrolysis of starch in the joint presence of diastase and maltase passes through the following stages:—

The chemical activity of the leaf enzymes used in the above experiments was somewhat disappointing, few positive results being obtained. This seems to be due to the method of preparation and purification which would yield only the more soluble enzymes. A similar result has already been obtained by Brown and Morris² in a study of the enzymes present in foliage leaves. It seems in fact to be a general experience that careful filtration of the original aqueous extract is not desirable in the preparation of enzymes as the product obtained displays very little reactive power. Better results are, as a rule, given by straining the liquid extract of the macerated tissues through fine muslin, and a similar method was therefore adopted in the case of the leaves of the adult beet.

PREPARATION OF MIXED ENZYMES FROM THE LEAF

The leaves were macerated with water and extracted in the thermostat as already described, the resulting liquid being strained through several folds of fine muslin. On adding a large excess of alcohol to the turbid filtrate, a brown sludge separated from which the supernatant liquid was decanted away. After washing well with absolute alcohol, the insoluble matter was filtered under pressure and washed with 50 per cent. alcohol until the washings were inactive and ceased to reduce Fehling's solution. The purified precipitate was then diluted with water so as to give a mixture containing 1 per cent. of the dry enzymes, and the liquid was rendered antiseptic by the addition of a little toluene. A filtered sample was optically inactive and did not reduce Fehling's solution.

This method of obtaining the enzymes suspended in water neces-

1. *Comp. Rend.*, Vol. LIII, p. 127.
2. *Trans. Chem. Soc.*, Vol. LXIII, p. 604, 1893.

sitates a modification in the preparation of the test-solution. Where possible, 20 per cent. aqueous solutions of the substrata were prepared, and 25 c.c. introduced by means of a pipette into a standard 50 c.c. flask, which was then diluted to the mark with the homogeneous enzyme sludge. The control solutions were similarly prepared, the dilution to half the original concentration being of course made with water. The experimental solutions were filtered through several baryta filters and the optical rotations determined without delay. All the solutions were kept in the thermostat at 35° C., the rotations being determined at regular intervals. The following positive results were obtained:—

Substrate used	<i>c</i>	$(\alpha)_D^{20^\circ}$ initial	$(\alpha)_D^{20^\circ}$ final	difference
α -methylglucoside ...	9.995 ...	+ 157.0° ...	+ 130.6° ...	27.4°
Sucrose ...	10.006 ...	+ 66.2° ...	+ 31.4° ...	34.8°
Inulin ...	1.2502 ...	— 34.2° ...	— 38.4° ...	4.2°
Starch ...	— ...	— ...	+ 60.0° ...	—

The results confirmed those already obtained with the purified enzymes, but the actions were much more rapid. Although marked optical differences were observed in a few hours the experiments were in each case continued for twenty days. The joint presence of maltase and diastase was confirmed, while inulase and lactase were again shown to be absent. In this case, however, evidence was obtained that invertase was present, as shown by the following figures:—

Experiment—Concentration of sucrose = 10.0060,	initial $(\alpha)_D^{20^\circ}$ = + 66.2°
	final $(\alpha)_D^{20^\circ}$ = + 31.4°
Control— „ „ = 10.0060,	initial $(\alpha)_D^{20^\circ}$ = + 66.6°
	final $(\alpha)_D^{20^\circ}$ = + 63.2°

The result was verified in a duplicate experiment, but in no case was complete inversion obtained.

EXAMINATION OF THE ENZYMES FROM THE STEM AND ROOT REGIONS

After removal of the leaves, the roots were separated from the short stem region and the enzymes isolated as described above in the form of an aqueous sludge. Prolonged washing with dilute alcohol was, of course, necessary to completely remove sugars and also the red colouring matters. In addition, the bulky solid residue left in each case on the muslin filter after extracting the macerated organs with water was utilised in the following manner:—The material, after being washed with water, was spread on sheets of filter paper and dried in a current of

air at 30° C. After several days' treatment, the brittle residue was powdered finely in an agate mortar, again digested with a large excess of water, filtered and dried. In this way we were able to avoid the extensive decomposition which results when attempts are made to desiccate the tissues without in the first place removing all soluble organic matter. A similar method has been used by Brown and Morris in demonstrating the presence of diastase in foliage leaves¹. We thus obtained four preparations, viz.:—stem-sludge, solid stem, root-sludge, and solid root, all of which were found to be capable of promoting hydrolytic changes. The average yields obtained were:—

Weight of stem used	... 133 grams.	Weight of solid stem obtained	... 6.5 grams.
Weight of root used	... 520 grams.	Weight of solid root obtained	... 19 grams.

As usual each preparation was found to be inactive and to be devoid of any action upon Fehling's solution. In preparing the test solutions the method already described was adopted, that is, an aliquot part of the solution was diluted to half the concentration with the stem or root sludge. In the case of experiments with the solid stem or solid root, half a gram of the powdered tissues was added to 25 c.c. of the test solution, which was then diluted to 50 c.c. with water. The control solutions were subjected to a similar dilution without the addition of any enzymes. Sterilisation was effected by the addition of a little toluene or chloroform, and heating was conducted in the thermostat at 30° to 35° C. The optical changes were complete in a few hours, but the values given below were those obtained after seven days' treatment.

TEST FOR INVERTASE

Substrate = sucrose ('Kahlbaum').				c (initial) = 5.000	c (final) = 2.500
Stem sludge	...	initial $(\alpha)_D^{20^\circ}$	= + 66.10°	...	final $(\alpha)_D^{20^\circ}$ = — 12.8°
Solid stem	...	„ $(\alpha)_D^{20^\circ}$	= + 66.80°	...	„ $(\alpha)_D^{20^\circ}$ = + 36.0°
Root sludge	...	„ $(\alpha)_D^{20^\circ}$	= + 66.10°	...	„ $(\alpha)_D^{20^\circ}$ = + 61.8°
Solid root	...	„ $(\alpha)_D^{20^\circ}$	= + 66.10°	...	„ $(\alpha)_D^{20^\circ}$ = + 62.0°

A well marked difference therefore exists between the results obtained with the root and stem preparations. In the former the optical changes observed were small and did not differ in any marked degree from those shown by the control solutions.

It would thus appear that while invertase is present in the stem region it is absent in the root. The result agrees with the general

1. *Loc. cit.*

observation that this particular enzyme is, as a rule, absent in storage organs during the period in which reserve material is being laid down. Our observation is, however, at variance with those obtained by Gonnermann and Stoklasa.¹

TEST FOR EMULSIN

Substrate = β -methylglucoside		c (initial) = 4.060		c (final) = 2.030
Stem sludge	...	initial $(\alpha)_D^{20^\circ} = -33.5^\circ$	...	final $(\alpha)_D^{20^\circ} = -22.5^\circ$
Solid stem	...	„ $(\alpha)_D^{20^\circ} = -33.5^\circ$	...	„ $(\alpha)_D^{20^\circ} = -22.1^\circ$
Root sludge	...	„ $(\alpha)_D^{20^\circ} = -33.5^\circ$	...	„ $(\alpha)_D^{20^\circ} = -22.7^\circ$
Solid root	...	„ $(\alpha)_D^{20^\circ} = -33.5^\circ$	...	„ $(\alpha)_D^{20^\circ} = -25.1^\circ$

The above results were obtained twenty-four hours after commencing the experiments; meanwhile the control solutions suffered no alteration in rotatory power. As in each case the liquids reduced Fehling's solution actively after treatment with the enzymes, the results are accepted as indicating the presence of emulsin in all the preparations. As a confirmatory test, salicin was substituted for β -methylglucoside as a substrate. In each case the result was the same, the specific rotations of the solutions diminishing about 14° in twenty-four hours, and the presence of both glucose and saligenin was detected in the resulting liquids.

TEST FOR MALTASE

Maltase was found to be present in both the root preparations, but was absent in the stem as shown by the following typical results:--

Substrate = α -methylglucoside		c (initial) = 4.9992		c (final) = 2.5005
Solid root	...	initial $[\alpha]_D^{20^\circ} + 156.0^\circ$	final $[\alpha]_D^{20^\circ} + 140.0^\circ$	diff. = 16.0°
Solid stem	...	„ $+ 156.0^\circ$	„ $+ 155.4^\circ$	„ 0.6°
Substrate = Maltose (Kahlbaum)		c (initial) = 5.0010		c (final) = 2.5005
Solid root	...	initial $[\alpha]_D^{20^\circ} + 132.1^\circ$	final $[\alpha]_D^{20^\circ} + 96.8^\circ$	diff. = 35.3°
Solid stem	...	„ $+ 132.1^\circ$	„ $+ 128.4^\circ$	„ = 3.7°

In using maltose as a substrate we took the precaution of adding a minute trace of caustic soda to the solution in order to promote mutarotation. The optical changes observed are therefore not due to stereochemical alterations in the sugar.

1. Czapek's *Biochemie der Pflanzen*, Bd. I, p. 375.

TEST FOR DIASTASE

Substrate Starch solution c (initial) approx. 0.50

Stem sludge ...	no initial α taken	...	final	$[\alpha]_D^{20^\circ}$	+ 44.0°
Solid stem ...		...	..		+ 32.0°
Root sludge ...		...	..		+ 24.0°
Solid root ...		...	..		+ 28.0°

The final concentrations were determined by evaporation of a measured volume of the solutions and weighing the residue dried at 100° C. All the products reduced Fehling's solution actively even in the cold. We are in the meantime unable to explain the fact that the end values obtained are lower than those calculated for glucose; no β -glucose was present as the rotations remained permanent on adding a trace of alkali. The concentration would of course only be approximate, but any experimental error thus introduced would be insufficient to account for the discrepancy between the calculated value for glucose and those experimentally found. Possibly inactive products may have been formed, or a partial conversion into other hexoses may have taken place. It will be noticed that in the case of enzymes extracted from the leaf the hydrolysis of starch resulted in the formation of a reducing sugar which displayed the correct rotatory power for glucose.

SUMMARY OF THE DISTRIBUTION OF SUCROCLASTIC ENZYMES IN THE ADULT BEET

(1). *Invertase*.—Invertase was not detected in the carefully purified mixture of enzymes obtained from the leaf. When, however, the enzymes are obtained by filtering through muslin and are afterwards washed free from reducing sugars, invertase is then found to be present.

Both the aqueous sludge containing the enzymes of the stem and also the powdered stem contain invertase.

Similar preparations from the root contain no invertase.

(2). *Diastase* was found in all the preparations examined.

(3). *Maltase* is present in the leaf and root regions, but was not detected in the stem preparations.

(4). *Inulase* seems entirely absent in the leaf region. Positive results were obtained with all the preparations from the stem and root, but the optical changes observed were irregular.

(5). *Emulsin*.—The enzymes obtained from the leaf gave negative results using amygdalin as a substrate; the stem and root preparations on the other hand gave positive results with salicin and β -methyl-glucoside.

(6). *Lactase* was absent in every case.

ATTEMPTED REVERSIBLE ZYMOLYSIS BY THE ACTION OF BEET ENZYMES

The methods adopted in the attempted condensation of glucose and fructose by enzyme action were similar to those successfully applied by Croft-Hill¹ in the enzymatic synthesis of maltose from glucose. As sucrose is incapable of forming an osazone, and as no suitable insoluble derivative is known, the separation of which would serve to detect the formation of the sugar in the presence of monosaccharides, recourse was again had to a polarimetric method of following the reaction. The plan adopted was to subject concentrated sterilised solutions of invert sugar to the action of the various enzyme preparations, polarimetric readings being taken at intervals. Change in rotation cannot, of course, in itself be considered evidence of sucrose formation. Other factors may play a part, including the possible self-condensation of both glucose and fructose. The experiments were therefore extended by hydrolysing the products and ascertaining if the optical value for invert sugar was reproduced. This joint evidence of a fall in laevo-rotatory power due to enzyme action, and a subsequent increase to nearly the initial value of hydrolysis, would justify the conclusion that condensation of the hexoses had taken place. In the absence of positive results indicating self-condensation of glucose or of fructose this double change in rotation would be significant of the presence of sucrose, or at all events, of a glucosidic compound containing both glucose and fructose. Before commencing our observations several preliminary experiments were necessary. The preparation of a suitable solution of invert sugar, displaying the maximum rotatory power, presented some difficulty. In the first place pure crystallised glucose and fructose ('Kahlbaum') were dried until constant in weight, the former at 110° C., and the latter in a vacuum. Equal weights of each sugar, accurate to 1 milligram were then dissolved in water in a standard flask and made up to the mark at 20° C. After allowing ample time for mutarotation to take place, the rotations were determined in a two-decimetre tube ($t = 20^{\circ}\text{C.}$). The result was, however, unsatisfactory, as the values obtained were not very uniform and were invariably too low. This is shown in the following table:—

1.	$\left. \begin{array}{l} \text{c. for glucose} = 20\cdot0010 \\ \text{c. for fructose} = 20\cdot0013 \end{array} \right\}$	$(\alpha)_D^{20^{\circ}} = -21\cdot2^{\circ}$
2.	$\left. \begin{array}{l} \text{c. for glucose} = 20\cdot0015 \\ \text{c. for fructose} = 20\cdot0005 \end{array} \right\}$	$(\alpha)_D^{20^{\circ}} = -19\cdot1^{\circ}$
3.	$\left. \begin{array}{l} \text{c. for glucose} = 20\cdot0006 \\ \text{c. for fructose} = 20\cdot0003 \end{array} \right\}$	$(\alpha)_D^{20^{\circ}} = -19\cdot8^{\circ}$

1. *Loc. cit.*

As the correct value for invert sugar is $(\alpha)_D = -24.7^\circ$ it would appear that this method of directly weighing out the sugars is inapplicable, probably owing to the difficulty in obtaining fructose in an anhydrous condition. Although the solutions referred to above were used in preliminary experiments, we prepared our invert sugar solutions by the method recommended by Maumené.¹ Pure sucrose (Kahlbaum) in quantities of 150 grams was dissolved in water (250 c.c.), and heated in sealed flasks at 106° C. for fifty hours. The resulting liquid gave $(\alpha)_D^{20} = -24.1^\circ$ calculating on the complete conversion into glucose and fructose. The effect of hydrolytic agents on this solution was then studied in order to ascertain if any sucrose remained unaltered. A test portion was diluted to half the concentration with water containing varying amounts of hydrochloric acid, and the optical changes on heating observed. It is of course well known that hydrochloric acid, even in dilute solution, readily decomposes fructose, and consequently suitable conditions of acid, concentration, and temperature had to be determined which would suffice to hydrolyse sucrose and yet be without action on fructose. Using solutions containing 5.3, 2.6, and 1.3 per cent. of acid the laevo-rotation of the invert sugar diminished steadily when the liquid was maintained at 50° C. At 20° C., however, although a similar fall in laevo-rotation was recorded in the case of the strongest acid solution, a constant value was ultimately obtained. On the other hand, the solution containing 2.6 per cent. of acid remained unaltered in rotatory power when kept for six days at 20° C. This result proved the complete absence of sucrose, and gave the desired conditions for carrying out a polarimetric test for sucrose formation in the subsequent experiments.

In the first place the precipitated enzymes obtained from adult leaves were used, but, as was expected from the feeble activity of the preparation, no positive results were obtained. Even after an interval of two months the optical activity of an invert sugar solution was found to be quite unaltered by the action of the enzymes. Recourse was therefore had to the more active enzyme preparations previously referred to as the 'stem sludge' and the 'root sludge.' Only small optical changes were, however, observed, but these were in the right direction, and were supported by the evidence of hydrolysis. The results of one typical experiment may be quoted:—

Initial concentration of invert sugar solution	=	40.0100
Concentration when diluted with stem sludge	=	20.0050
Initial specific rotation of the mixture	=	— 24.7°
After 350 hours' action at 35°	=	— 21.2°
Decrease in laevo-rotation due to enzyme action	=	3.5°
Increase in laevo-rotation due to subsequent hydrolysis	=	2.1

1. *Journal des fabricants de sucre*, Vol. XXXI, p. 46.

Control experiments showed that the enzymes were without action on either glucose or fructose solution alone. It would thus appear that the glycolytic enzymes described by Stoklasa are without action on concentrated glucose solutions. The above data represents about the average result obtained in five different experiments, and corresponds with the formation of approximately 4 per cent. of sucrose. The addition of maltose did not effect any appreciable improvement in the process and, moreover, prevented the hydrolysis being studied.

It must be admitted, even if the above results can be correctly ascribed to sucrose formation, that the magnitude of the changes are small. It appeared possible that this might be due to alteration in the nature of the associating enzyme once the storage of sucrose had ceased, and, consequently, in another series of experiments, young plants, which had not begun storing, were used as the source of the enzyme preparations. The isolation of the mixed enzymes was carried out as already described, but no differentiation into leaf and root region was made, the entire plants being macerated and extracted. The sludge finally obtained consisted of a mixture of 445 grams of the mixed enzymes diluted to a litre with water.

The following experiment showed that the sludge possessed associating powers:—

70 c.c. of invert sugar solution giving $(\alpha)_{\text{D}}^{20^\circ} = -23.7^\circ$ were introduced into a standard 100 c.c. flask and made up the mark with the homogeneous sludge. A portion filtered at once gave in a one-decimetre tube the rotation $\alpha_{20} = -6.86^\circ$. The solution was then kept at 35°C . in a thermostat for a week, when it was found that the rotation had diminished to -5.95° . The subsequent optical changes were only small, and the constant value observed was $\alpha = -5.70^\circ$. The total change in α therefore amounted to $+1.16^\circ$. Expressed in specific rotations the change becomes $(\alpha)_{\text{D}}^{20^\circ} = -23.9^\circ \rightarrow (\alpha)_{\text{D}}^{20^\circ} = -19.8^\circ$, and is, moreover, in the right direction. The subsequent hydrolysis was carried out by diluting a test portion to half the original concentration with water containing sufficient hydrochloric acid to give a 2.6 per cent. acid solution. The initial rotation was $(\alpha)_{\text{D}}^{20^\circ} = -19.3^\circ$, and on standing at 20° this gradually altered to the constant value $(\alpha)_{\text{D}}^{20^\circ} = -22.7^\circ$, the actual change in α being -0.99° . This result can only be explained on the assumption that a glucosidic product had been formed possessing a less laevo- or more strongly dextro-rotation than invert sugar, and which is, moreover, capable of undergoing hydrolysis to give an equimolecular mixture of glucose and fructose. Assuming

that sucrose is the only glucosidic product thus formed, the result indicates the production of about 6 per cent. of the disaccharide. The above observation was controlled by a duplicate experiment in which a sample of the invert sugar solution was diluted with water in place of the enzyme sludge and kept in the thermostat for an equal time. No appreciable alteration in rotatory power was, however, observed even after three months. It was also shown that the sludge was without action on glucose or fructose solutions alone; only in the joint presence of both sugars were any optical alterations observable.

These results are summarised below :—

Nature of Experiment	Length of tube in decimetres	Initial α_{20°	Final α_{20°	Δ	Behaviour on hydrolysis
Invert sugar and enzyme sludge	1	— 6.86	— 5.75	16.6	— 5.55° $\rightarrow$ — 6.54°
Control sol. of invert sugar	1	—14.43	—14.37	0.4	No change
Glucose (20 %) and enzyme sludge	2	20.21	20.13	0.4	No change
Fructose (20 %) and enzyme sludge	2	—34.65	—34.80	0.4	No change

The column marked Δ contains the optical changes expressed in percentages, and it will thus be seen that a marked difference exists between the first experiment and the subsequent controls.

The result was verified in a duplicate series of experiments in which the treatment with the enzymes was continued for three months: once more the control solutions gave perfectly negative results, but the invert sugar solution underwent similar alterations to those quoted above :—

$$\begin{aligned} \text{Initial } (\alpha)_D^{20^\circ} &= -24.05^\circ & \text{Final } (\alpha)_D^{20^\circ} &= -18.10^\circ, & \text{Diff.} &= +5.95^\circ. \\ \text{Change of rotation on hydrolysis } (\alpha)_D^{20^\circ} &= -18.1^\circ & \rightarrow & & -27.2^\circ. \end{aligned}$$

A slight irregularity will be noticed in the permanent end value obtained on hydrolysis. This we are in the meantime unable to explain, but nevertheless it is evident that the rotation changes are of the same nature as those discussed above, and are in agreement with the idea that about 6.6 per cent. of sucrose had been produced in the reaction.

It is of course premature to claim the above results as a successful bio-chemical synthesis of sucrose, but it is difficult to find any other explanation of the optical changes other than that already suggested, viz., that the aldose and ketose combine to form a glucosidic derivative, and sucrose is the only compound of this type now known,

DISCUSSION OF THE RESULTS

The considerations of interest to the plant physiologist accruing from this work group themselves around the following heads:—

- (a) The topography and work of the enzymes.
- (b) The synthesis of a glucosidic compound, presumably sucrose, by the action of beet sludge on invert sugar.
- (c) The difference in magnitude of the results obtained *in vivo* as contrasted with that *in vitro*.

(a) 1. The topography of the enzymes here worked out may be tabulated thus:—

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

A significant feature in this enzyme distribution is the absence of invertase from the beet root. Kastle and Clark¹ found that in inulin- and starch-producing plants such as the artichoke and the potato, even in the tubers, when the inulin and starch were undergoing storage, invertase was present in larger quantity than either inulase or diastase. In the beet root, when sucrose is being stored the appropriate enzyme for its formation and hydrolysis is absent. Since in the artichoke and potato respectively the inulin and starch are formed *in loco*, the conclusion to be drawn, on analogy, in regard to the beet seems to favour the hypothesis that sucrose is not formed *in loco* in the root, but is only stored there after undergoing translocation as sucrose from the other organs of the plant.

(a) 2. The varied associations of the enzymes in the different plant organs is a striking point. To take the case of diastase, which occurs in all parts of the beet, it is found that in the leaf it is associated with invertase and maltase, in the stem with emulsin in addition, while in the root a third variation in the environment of activation of diastase is produced by its association with maltase, inulase and emulsin. The questions which naturally suggest themselves are as to whether the diastatic activity is affected by the different environment in each case, and as to whether a given combination of enzymes may not inhibit the appearance of some other enzyme which *a priori* might be expected to be present. This latter bears on the absence of invertase in the root in an

1. *Amer. Chem. Journ.*, Vol. XXX, p. 422, 1903.

environment containing an association of diastase, maltase, inulase and emulsin. That some interaction of the enzymes of an acceleratory, retardatory or inhibitory nature occurs is quite in agreement with the doctrine propounded on the result of experimental research by several investigators¹.

(a) 3. Besides the differences in the environmental spheres of activation resulting from the varied association of the enzymes themselves in the different plant organs, other factors enter to complicate the result. For example, the associated enzymes of the leaf are subjected to different light conditions from those in the root; in the leaf to alternation of light and darkness, in the root to continuous darkness. That light affects the results in the case of invertase action is shown from the recent researches of Kohl,² who has found that in darkness inversion gives place to the opposite process sooner than in light. Girard's discovery³ that the amount of sucrose in the beet leaf is much increased towards evening may bear directly on this point. Other factors affecting the result are, the presence of products of hydrolysis, differences in the osmotic pressure in the cells of various organs, and differences in the reactions of the cell contents. In the last two cases the 'intensity factor'⁴ would be affected, on the one hand by alteration in the concentration of the enzyme and on the other from the acceleration or retardation of the enzyme action due to increased or diminished acidity.

(b) 1. Of the experiments on reversible zymolysis the first series made with pure enzyme extracts of beet leaves on invert-sugar substrates gave no positive results. It is, however, a common experience that pure enzyme extracts are less active than sludges. This was strikingly shown in the negative results obtained in testing for invertase by the action of the leaf extracts on sucrose, whereas, when the sludge was substituted, invertase was proved to be present. The presumption is that by the action of the leaf sludge on invert sugar, reversible zymolysis would have taken place. The second series of experiments with stem sludge on invert sugar gave figures corresponding with the presence of 4 per cent. of sucrose—a result to be expected from the ascertained presence of invertase in the stem. The third series with the sludge of seedling plants gave the higher figure (6 per cent. sucrose), indicating the greater activity of the plant at the beginning of the storage as compared with the diminished activity of

1. Bayliss, *The Nature of Enzyme Action*, p. 657, and references.
2. *Abstr. Bot. Centr.*, Vol. CVIII, p. 137, 1908.
3. *Compt. Rend.*, T. XCVII, p. 1305, 1883.
4. Visser, *Zeit. f. physik. Chem.*, Vol. LII, p. 283, 1905.

the older plant at the end of the storage period. The reasons for regarding the products as sucrose have already been stated.

(b) 2. That the cane-sugar stored in the beet root is formed from antecedent monosaccharides by reversible zymolysis in the organs containing invertase (viz., in the leaf and stem), and thence translocated as such seems highly probable.

The alternative view that the sugars travel downwards as monosaccharides and are subsequently condensed into the disaccharide¹ meets the diffusion difficulty. The absence of invertase from the root as here shown, and the results of Strohmer's researches proving that practically no reducing sugars occur in the root, while, according to Girard², sucrose is present in all parts of the plant in the earliest stages of development, militate against this view.

(b) 3. In relation to the translocatory difficulty in the first view, reference may be made to the researches of Hanstein³ and Puriewitsch⁴ on the diffusion of disaccharides in the maize endosperm, to Peklo's recent discovery⁵ that the sieve tubes of the beet serve as the sugar-conducting channels, and to Pfeffer's dictum on the great regulatory faculty of the cell cytoplasm in relation to the materials to be diffused.

(b) 4. That the metabolism of the sucrose during the second spring to supply the young shoots takes place in the stem⁶ is supported by the fact that invertase is found in that organ.

(c) 1. The apparently relatively unlimited quantity of disaccharides capable of being formed in the living plant is in marked contrast to the small results obtained experimentally *in vitro*. This is not surprising when it is remembered that the reaction is presumably a reversible one. *In vitro* the equilibrium stage attained is permanent owing to the fact that the reaction products are not removed, while in the plant the equilibrium phase is only momentary owing to the continual removal of the products as they are formed.

1. Maquenne, *Compt. Rend.*, T. CXXI, p. 834, 1895.

2. *Compt. Rend.*, T. CII, 1887.

3. *Flora*, p. 419, 1894.

4. *Ber. d. Bot. Ges.*, p. 206, 1896.

5. *Bot. Centr.*, Vol. CVIII, p. 239, 1908.

6. Strohmer, *loc. cit.*