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The Biosynthesis of Sucrose in Sugarcane

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INTRODUCTION

Although sucrose is one of the most commonly occurring sugars throughout the plant kingdom, the mechanism of its formation has been obscured by such problems as alternate pathways of synthesis and pronounced difficulty in reproducing the reactions with purified plant preparations.

Working with solutions of either glucose or fructose, early investigators found that intact leaves formed sucrose when their petioles or blades were placed in contact with the hexose solutions (*13, 14, 25*)[2]. Nelson and Auchincloss (*15*) reported that in the potato tuber both fructose and glucose play a dominant role during the conversion of starch to sucrose, and that the process requires oxygen. This view had not been held by previous workers who felt that starch could be converted directly to sucrose. Quite curiously, early efforts to produce sucrose from monosaccharides *in vitro* met with little success. Bonner (*3*) suggested that the grinding or general disruption of plant tissues inactivates the sucrose-synthesizing systems.

At least two mechanisms appear to be involved in uniting glucose and fructose to form a molecule of sucrose. These include the enzyme sucrose phosphorylase and a series of reactions involving uridine compounds. The enzyme invertase has been considered in this respect and Hartt (*8*) reported evidence that invertase was responsible for sucrose formation in sugarcane. Nevertheless, the invertase equilibrium lies far toward hydrolysis and the reverse reaction is not seriously regarded as a mechanism of sucrose formation (*3*). As early as 1934 Burkard and Neuberg (*4*) recognized the existence of phosphate esters of both glucose and fructose in leaves of sugar beet. Hassid and coworkers (*9*) demonstrated the need for a phosphorylative mechanism during sucrose formation while studying the microorganism *Pseudomonas saccharophila*. An enzyme, sucrose phosphorylase, was extracted from the organism which catalyzed the formation of sucrose and inorganic phosphate from glucose-1-phosphate and fructose:

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[2] Italic numbers in parentheses refer to Literature Cited, pp. 282–3.

$$\text{Glucose-1-phosphate} + \text{fructose} \rightleftharpoons \text{Sucrose} + H_3PO_4$$ [3]

According to Hatch, *et al.* (*10*) the enzyme has been reported in juice of sugarcane (*21*). Panda and Ramakrishnan (*18*) described an enzyme in sugarcane-leaf homogenates, with sucrose phosphorylase properties, which was precipitated by a 30- to 60-percent saturation with ammonium sulfate. However, the presence of sucrose phosphorylase in higher plants is not conclusively accepted.

More recently, Leloir and coworkers (*5,12,17*) have developed a series of reactions involving UDP, UTP, and UDPG as essential components of the sucrose-biosynthesis mechanism. The reactions may be summarized as follows:

1, UTP + G-1-P $\rightleftharpoons$ UDPG + PP
2, UDPG + fructose $\rightleftharpoons$ Sucrose + UDP
3, UDPG + fructose-6-P $\rightleftharpoons$ Sucrose phosphate + UDP

Turner (*23*) found that an extract of dried pea seeds formed sucrose from a mixture of G-1-P and fructose, and further experiments indicated that UDPG took part in the synthesis. In later studies (*24*) Turner observed that Mg and DPN was required for sucrose synthesis and that the reaction was stimulated by added adenosine phosphates. He concluded that the glycolytic pathway is possibly involved, as well as hexose phosphorylation, during the formation of sucrose.

Dugger and Humphreys (*7*) observed that boron enhanced the formation of sucrose by sugarcane and pea-seedling homogenates. Boron inhibited sucrose synthesis by enzyme preparations from pea seed. ATP was found to stimulate the overall synthesis of sucrose from UTP, G-1-P, and fructose, but the enzymes UDPG pyrophosphorylase and UDPG-fructose transglycosylase were inhibited by ATP when assayed individually. DPN was not found to be essential for maximum sucrose synthesis.

Hatch and coworkers (*10*) studied a number of enzymes involved in sucrose formation and breakdown in storage tissue of sugarcane. Attempts to demonstrate sucrose phosphorylase failed repeatedly, although UDPG-fructose transglycosylase was active. In a subsequent publication (*20*) these investigators proposed a cyclic scheme by which sugars are actively incorporated into storage space after having passed through an outer space

[3] For convenience the following abbreviations are used throughout this paper: Glucose-1-phosphate (G-1-P), uridine triphosphate (UTP), uridine diphosphate glucose (UDPG), uridine monophosphate (UMP), adenosine triphosphate (ATP), flavin adenine dinucleotide (FAD), diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), magnesium (Mg), pyrophosphate (PP), and the term "molar" is designated by the letter M.

and metabolic compartment. Sucrose is inverted in passing from the medium into the storage area where it reappears as sucrose.

A series of physiological experiments with sugarcane is contemplated at this Station, among which it will be desirable to measure the formation of sucrose in response to various treatments. Thus it is essential to clarify the predominant mechanism of sucrose formation in this crop. The present paper deals with properties and characteristics of the sucrose-forming mechanism which we have encountered in sugarcane.

MATERIALS AND METHODS

All plant materials were obtained from the variety M.336. The basal 14 to 16 inches of leaves +1 to +4[4] were frozen in a mixture of Dry Ice (solidified carbon dioxide) and acetone, and lyophilized at 0 to 2°C. with the aid of a Virtis Roto Freeze drying assembly. The dried tissues were ground to a fine powder in the cold and stored at −20°C. in sealed sample jars. In like manner a series of samples was prepared with meristematic tissue, cane tissue representing internode numbers 8 to 10, 8 to 10 node tissue, leaves +1 and +2, sheaths +1 and +2, and leaves +6 and +7.

Sucrose-synthesis experiments were conducted with homogenates, undialyzed crude extracts, dialyzed crude extracts, and dialyzed protein precipitated by 80-percent saturation with ammonium sulfate. A typical homogenate was prepared by adding 1.7 ml. of water to 300 mg. of dried powder. This approximates 2 gm. of fresh tissue and represents 0.25 to 0.35 mg. of protein. Crude extracts were prepared by placing 15 gm. of the dried tissue in flasks containing 150 ml. of distilled water or 0.1 M $NaHCO_3$, and rotating the mixture mechanically for 1 hour at room temperature. Most of the tissue debris was removed by passing the mixture through four layers of cheesecloth. The suspensions were then chilled to 2°C. and further clarified by centrifuge at 3,500 r.p.m. Aliquots of the crude extracts were dialyzed at 2°C. for varying periods against 0.025-M phosphate buffer (pH 7.0), or against distilled water.

Enzyme preparations were obtained by adding sufficient ammonium sulfate to the chilled crude extracts to bring the saturation to 80 percent. The salt was added slowly with constant stirring. The precipitate-containing samples were allowed to stand at 2°C. for 4 hours in order to increase protein yield. The protein was removed by centrifuge, taken up in a minimum of distilled water and dialyzed 2 hours against distilled water or phosphate buffer. A small amount of precipitate appeared during dialysis which was readily removed by centrifuge at 3,000 r.p.m. Portions of the supernatant liquid were stored at 2°C., while others were first frozen and

[4] The leaf nomenclature employed here is that of Kuijper (*11*) in which the uppermost leaf bearing a visible dewlap is designated +1.

then stored at −20°C. Protein was measured colorimetrically by the micro-method of Sutherland, *et al.* (*22*).

Initial attempts to form sucrose with homogenates by the technique of Dugger and Humphreys (*7*) were unsatisfactory, since the hot ethanol extracted prohibitive quantities of chlorophyll. The following procedure was adopted: Duplicate test tubes received 300 mg. of dried leaf tissue, 1.7 ml. of distilled water, 0.5 ml. of 2-M tris (trishydroxymethylamino methane) buffer (pH 7.2), 50 μmoles of G-1-P, 50 μmoles of fructose, and distilled water or additives to bring the final volume to 5.0 ml. The materials were thoroughly mixed and the reaction was allowed to proceed for 4 hours at 30°C. in a constant-temperature water bath. The reaction was stopped by letting the tubes stand in boiling water for 5 minutes. The reaction mixtures were passed through Whatman No. 1 filter paper and the residues washed with 10 ml. of near-boiling distilled water. The filtrates were cooled and subsequently clarified by the $Ba(OH)_2$ and $ZnSO_4$ method of Nelson (*16*). Sucrose was determined by the resorcinol method of Roe (*19*) as modified by Cardini, *et al.* (*5*).

Undialyzed extracts yielded virtually the same sucrose-forming capacity as the homogenates, but were considerably easier to handle since the filtering and washing steps were eliminated. Two milliliters of the extract were usually employed in a given reaction, which was approximately equivalent to 200 mg. of dried tissue in 2 ml. of distilled water or $NaHCO_3$. When dialyzed enzyme preparations were used they were added at the rate of 0.60 to 0.65 mg. of protein per milliliter of digest.

Reactions involving crude extracts, dialyzed crude extracts, and dialyzed protein were stopped by letting the vessels stand in boiling water for 5 minutes. The mixtures were clarified immediately after cooling and sucrose-assay procedures were conducted thereafter as described for homogenates.

RESULTS AND DISCUSSION

SUCROSE SYNTHESIS BY HOMOGENATES AND CRUDE EXTRACTS

A strong sucrose-forming capability was observed when leaf homogenates or crude extracts were incubated with a combination of G-1-P and fructose. Under optimum conditions of hexose supply, the equivalent of 1 mg. of protein would catalyze the formation of 13 to 16 μmoles of sucrose in 4 hours.

A series of experiments were conducted to determine optimum conditions of time, pH, and hexose concentration for the sucrose-forming mechanism. Figure 1 illustrates that under the specified conditions of the assay at least 4 hours are required for maximum sucrose production. The decline of sucrose after 8 and 12 hours was presumably due to microbial action. Optimum pH was 8.0 although little variation was recorded from pH 7.0

to 9.0 (fig. 2). It is also evident that the reaction can proceed well at pH values as low as 4.0, and as high as 10.0. Hatch, *et al.* (*10*) employed potassium citrate buffer (pH 6.8) for sucrose-synthesis mixtures and tris-HCl buffer (pH 8.2) for the UDPG-fructose transglucosylase reaction. Dugger and Humphreys (*7*) ran their experiments with pea seed homogenates at pH 7.2.

Optimum levels for both G-1-P and fructose lay in the area of 6 μmoles per milliliter of digest (figs. 3 and 4). For G-1-P the response was almost

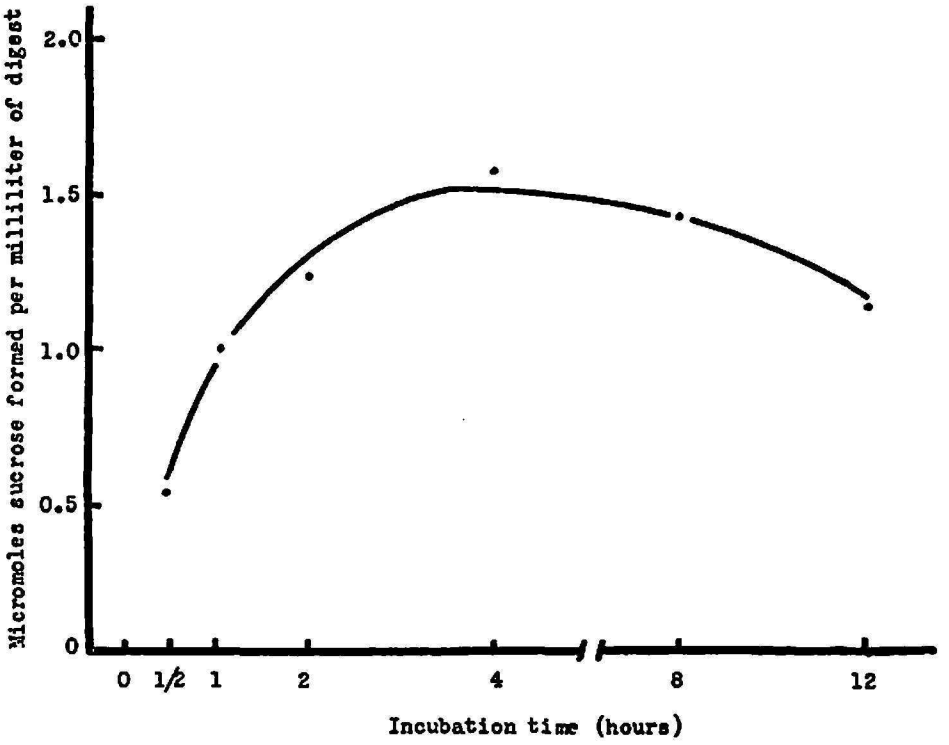

FIG. 1—Sucrose synthesis by crude leaf extracts during varying reaction periods Each milliliter of digest included 12 μmoles of G-1-P, 12 μmoles of fructose, 0.07 to 0.08 mg. of protein. Reaction temperature was 30°C. Tris buffer, pH 7.5, was employed for all reactions.

linear up to 6 μmoles (fig. 3). As little as 0.2 μmoles of fructose increased the reaction markedly, while additions greater than 2 μmoles had but little effect (fig. 4).

Up to this point fructose appeared to be the single most limiting constituent of the reaction. It should also be noted that considerable sucrose was formed when either G-1-P or fructose alone was added to the homogenate while the other hexose was withheld. So long as the reaction is conducted with crude extracts, one would assume that small amounts of endogenous hexose phosphates are present, and that one or the other of these should react with the added hexose to form sucrose. It is also likely that in the crude preparations there is sufficient hexokinase, phosphohexoisomerase,

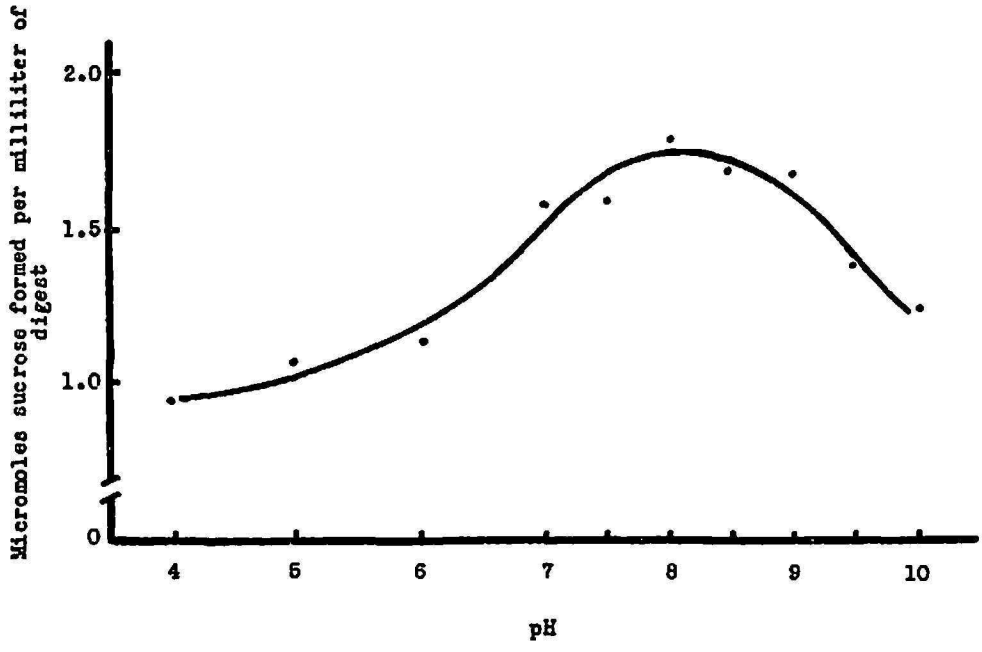

FIG. 2—Effects of varying pH levels upon the formation of sucrose by undialyzed cane-leaf extracts. Each milliliter of digest contained 12 μmoles of G-1-P, 12 μmoles of fructose, 0.07 to 0.08 mg. of protein. Reactions proceeded 4 hours at 30°C.

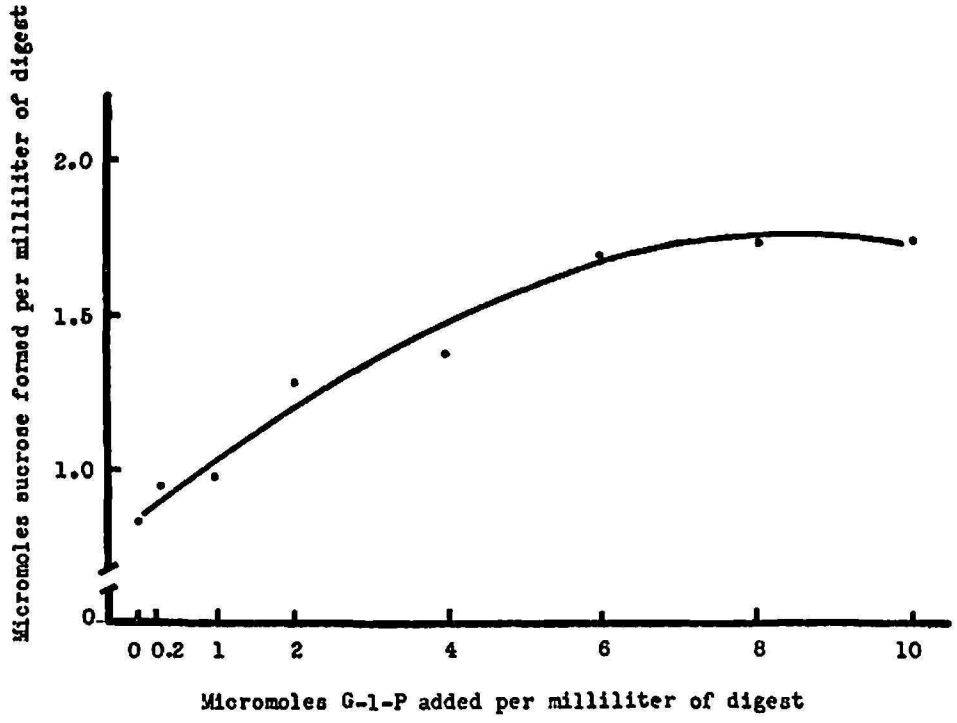

FIG. 3—Effects of added glucose-1-phosphate upon the sucrose-forming capacity of crude extracts from sugarcane leaves. Each milliliter of digest contained 12 μmoles of fructose, 0.07 to 0.08 mg. of protein, variable G-1-P. The reactions proceeded 4 hours at 30°C. in tris buffer (pH 7.5).

phosphoglucomutase, and phosphatase to convert a portion of the added hexose to the aldose or ketose complement needed for sucrose synthesis.

SUCROSE SYNTHESIS IN THE PRESENCE OF ADDED COFACTORS

Turner (*24*) reported that Mg and a combination of DPN, UMP, and ATP brought about maximum sucrose synthesis by extracts from pea seed. Dugger and Humphreys (*7*) used Mg, ATP, and UTP as essential

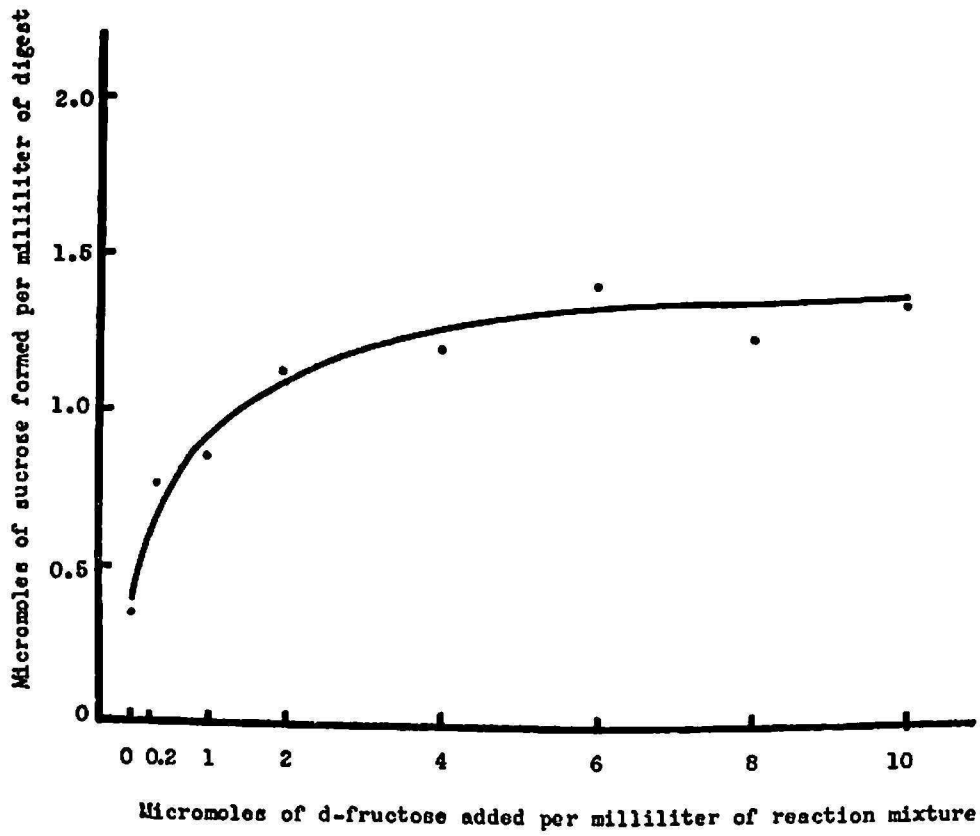

FIG. 4—Effects of added fructose upon the sucrose-forming capacity of cane-leaf extracts. Glucose-1-phosphate was supplied as a constant at 12 μmoles per milliliter of digest. The reactions proceeded 4 hours at 30°C. in tris buffer (pH 7.5).

components of their pea-seedling homogenate experiments and further demonstrated that boron affected the formation of sucrose. A series of experiments was conducted to determine the effects of these materials upon the sucrose-forming mechanism of sugarcane.

Initial experiments with leaf homogenates indicated that a combination of ATP plus UTP, and ATP plus DPN, caused a slightly greater production of sucrose (table 1) than was realized by the G-1-P plus fructose reaction alone. Further experiments with crude extracts (table 2) revealed that a number of factors increased the reaction slightly, including Mg, ATP, UDPG, UTP, and possibly cystein-DPN when added with ATP and

TABLE 1.—*Effects of added ATP, DPN, and UTP on the sucrose-forming capacity of leaf homogenates from sugarcane*

Treatment	Reaction composition	Sucrose formed (μmoles/ml. digest)
1	Control (homogenate + H_2O to volume)	0
2	Standard[1]	1.00
3	Standard + ATP	1.14
4	Standard + DPN	1.11
5	Standard + UTP	1.17
6	Standard + ATP, DPN	1.25
7	Standard + ATP, UTP	1.36
8	Standard + ATP, UTP, DPN	1.00

[1] Standard digest composition: 150 mg. leaf tissue plus 1.85 ml. distilled water; 0.5 ml. tris buffer, pH 7.5; 50 μmoles G-1-P; 50 μmoles fructose; 10 μmoles Mg. Ten μmoles each of ATP, UTP, and DPN were used as additives. Total volume was 5 ml. The reactions proceeded 4 hours at 30°C.

TABLE 2.—*Sucrose synthesis by cane-leaf extracts in the presence of glucose-1-phosphate, fructose, and various added compounds*

Treatment	Reaction composition[1]	Sucrose formed (μmoles/ml. digest)
1	Crude extract[2] + H_2O	0
2	Crude extract + G-1-P and fructose	.78
3	Crude extract + G-1-P and fructose + Mg	.93
4	Crude extract + G-1-P and fructose + ATP	.93
5	Crude extract + G-1-P and fructose + Mg, ATP	1.04
6	Crude extract + G-1-P and fructose + cystein-DPN	.86
7	Crude extract + G-1-P and fructose + UDPG	1.04
8	Crude extract + G-1-P and fructose + UMP	.86
9	Crude extract + G-1-P and fructose + UTP	1.14
10	Crude extract + G-1-P and fructose + ATP, cystein-DPN	1.07
11	Crude extract + G-1-P and fructose + ATP, UMP	1.53
12	Crude extract + G-1-P and fructose + ATP, UTP	1.12
13	Crude extract + G-1-P and fructose + ATP, UDPG, cystein-DPN	1.29
14	Crude extract + G-1-P and fructose + ATP, UTP, cystein-DPN	.78

[1] Reagents listed in table 2 were supplied in the following quantities per milliliter of digest: G-1-P, 10 μmoles; fructose, 10 μmoles; 2.5 μmoles each of Mg, ATP, cystein-HCl, DPN, UTP, UMP, and UDPG. The reactions proceeded 4 hours at 30°C. in tris buffer, pH 7.5.

[2] See Materials and Methods section for details of extraction procedures.

UDPG. The greatest single increase occurred when ATP and UMP were added simultaneously. It was interesting to note that when UTP and UMP were supplied individually, UTP easily caused the greatest increase of sucrose, while UMP had virtually no effect at all. When each was added with ATP, however, UTP showed no further response, while the effect of UMP was vastly increased. Two points are thus apparent: 1, A sucrose-forming mechanism is operative in sugarcane leaves which employs UTP, presumably in the reaction accepted by previous workers (*5,7,12,17,24*):

$$\text{G-1-P} + \text{UTP} \rightleftharpoons \text{UDPG} + \text{PP}$$

2, A phosphorylative mechanism must be present to convert UMP (and

TABLE 3.—*Effect of a composite of added compounds upon the sucrose-forming capacity of cane-leaf homogenates, and results obtained by withholding individual items*

Treatment	Reaction composition	Sucrose formed (μmoles/ml digest)
1	Control (homogenate + H_2O to volume)	0
2	Complete[1]	.07
3	−Fructose	.36
4	−G-1-P	.21
5	−ATP	1.14
6	−Mg	.80
7	−UTP	.67
8	−UDPG	.65
9	−DPN	.56

[1] Complete composition: 150 mg. leaf powder; 1.85 ml. distilled water; 0.5 ml. tris buffer, pH 7.5; 50 μmoles G-1-P; 50 μmoles fructose; 10 μmoles ATP; 10 μmoles Mg; 10 μmoles UTP; 5 μmoles UDPG; and 10 μmoles DPN.

very likely UDP) to UTP by utilizing ATP as the source of phosphate. This also is in agrement with Dugger and Humphreys (*7*), who reported that ATP was essential for the production of sucrose from G-1-P, fructose, and UTP by an enzyme preparation from pea seed.

The addition of UDPG alone had no striking effect on sucrose synthesis, as might have been expected if the UDPG-fructose transglycosylase enzyme were active. However, a combination of ATP, UDPG, and cystein-DPN further stimulated the reaction.

Again working with cane-leaf homogenates, experiments were conducted in which controls contained most of the factors believed to take part in the G-1-P, fructose, UTP reactions. Individual components were withheld and the subsequent results are recorded in table 3. The data indicate that the complete system was almost as well adapted for sugar metabolism as for synthesis. In the "complete" reaction a phosphorylative mechanism favor-

ing metabolism (presumably the glycolytic pathway) must have been operative, as evidenced by the increase of sucrose when ATP and Mg were omitted.

LOSS OF SUCROSE-FORMING CAPACITY BY DIALYSIS

It was soon apparent that the ability to form sucrose by crude extracts was rapidly lost during dialysis. Turner (*24*) reported that dialysis of pea-seed extracts against tapwater, distilled water, and 1-percent KCl caused a complete loss of sucrose-synthesizing ability, but that after a 5-hour dialysis against phosphate buffer, pH 7.0, some activity remained. Dugger and Humphreys (*7*) claimed that 24-hour dialysis of pea-seed enzyme preparations against phosphate buffer did not retard the sucrose-forming activity. Our own preparations lost all appreciable activity by dialysis against both distilled water and phosphate buffer. Figure 5 illustrates the loss of activity when crude extracts were dialyzed against distilled water. It is apparent that about 80 percent of the activity was gone after 30 minutes, and that the remainder was very slowly lost over a period of 24 hours.

Turner had alleviated a similar problem by adding DPN to the dialyzed material, and best results were obtained with a combination of ATP and DPN. We also felt that from the work of Dugger and Humphreys boron may have been an essential factor lost during dialysis. These materials plus a number of metal cofactors were added to the dialyzed extracts in attempts to revive the sucrose-forming capacity. Initial efforts with crude extracts showed that a small fraction of the original sucrose-forming activity could be recovered with boron, DPN, FAD, or a composite of metallic factors (table 4, experiment 1).

Recalling the work of Hatch, *et al.* (*10*) where as much as 3 to 7 mg. of protein was employed in 0.2 ml. of digest[5], we felt that the relative effectiveness of the added cofactors might be increased if protein were not limiting. Consequently we supplied an additional 0.5 mg. of dialyzed protein per milliliter of digest in the next series of tests. Sucrose was also supplied at the rate of 0.75 μmoles per ml. of digest to test the possibility that a small amount of sucrose might be essential to initiate the sucrose-forming reactions. Sucrose was one of the factors known to be rapidly lost from extracts during dialysis. Two levels of FAD were also tested on the basis that a flavoprotein related to sugar synthesis may have been inactivated by dialysis. The results of these tests appear in table 4, experiment 2.

One of the first things observed while running this set of tests was a greatly increased sucrose formation due to the added protein. It should

[5] The quantity of protein used by Hatch and coworkers was 125 to 300 times the amount present in an equivalent volume of the cane-leaf digests.

be pointed out that, in the absence of crude extract, this amount of protein produced very little sucrose. Small activity was restored by added UTP and UDPG. Sucrose had no effect. Boron restored approximately ⅓ of the original sucrose-forming potential but there was no difference between the effects of 0.4 and 2.0 μmoles boron. FAD at the rate of 2.5 μmoles per milliliter restored 60 percent of the activity, which is the closest we have come

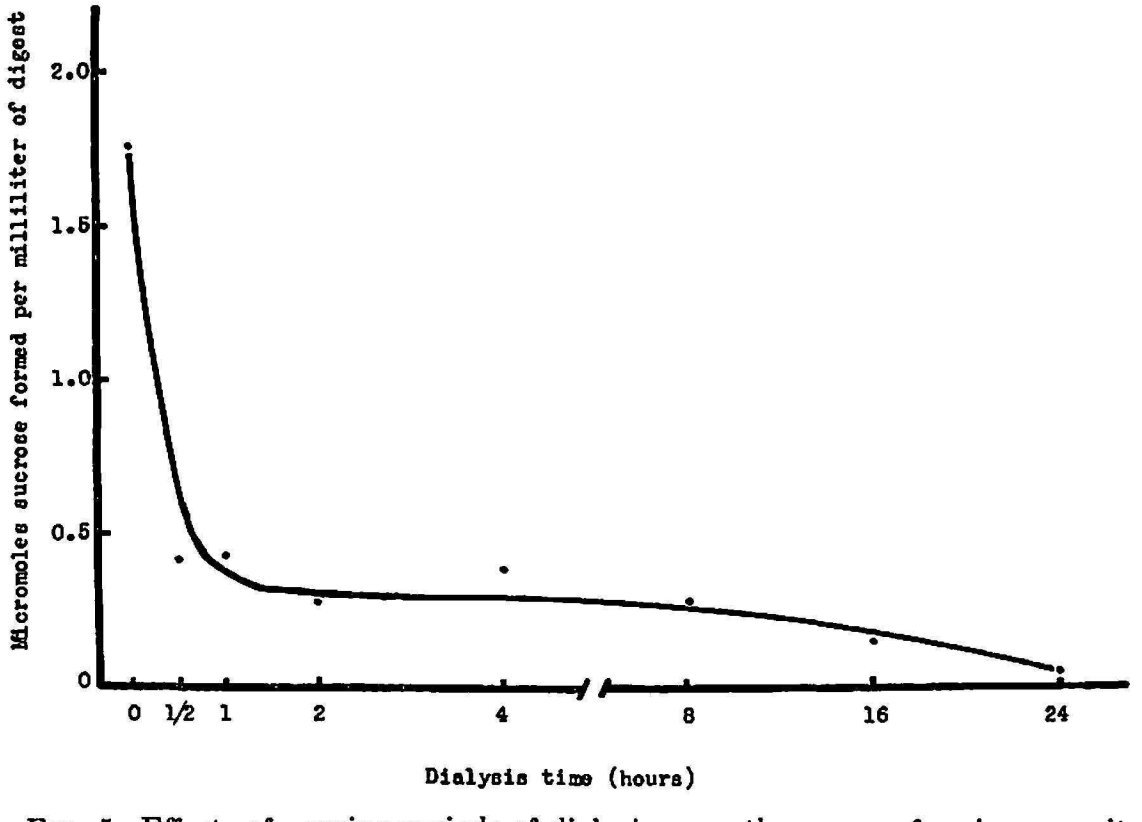

FIG. 5—Effects of varying periods of dialysis upon the sucrose-forming capacity of crude cane-leaf extracts. Each sample was of 10 ml. volume and was dialyzed against 1,000 ml. of distilled water. Each milliliter of test digest contained 12 μmoles of G-1-P, 12 μmoles of fructose, 0.07 to 0.08 mg. of protein. Reactions proceeded 4 hours at 30°C. in tris buffer, pH 7.5.

to reviving the sucrose-synthesis mechanism with additives. Later attempts were made to restore activity with a combination of FAD, boron, and DPN, without added protein, but little more than 40 percent of the activity was restored (table 4, experiment 3).

Nevertheless the possibility that a flavoprotein and DPN might be involved in sucrose formation cannot be ruled out. During previous studies we encountered an enzyme in cane-leaf extracts which catalyzed the oxidation of *d*-glucose (*2*). Glucose oxidase of molds is one of the "yellow enzymes", containing two molecules of FAD per molecule of enzyme (*6*). The sugarcane enzyme is apparently a true dehydrogenase, after the enzyme de-

TABLE 4.—*Sucrose formation by crude extracts and dialyzed protein from leaves of sugarcane*

Experiment	Reaction composition[1]	Sucrose formed (μmoles/ml. digest)
1	a, Undialyzed extract + G-1-P and fructose	1.22
	b, Dialyzed extract + G-1-P and fructose	.11
	c, Dialyzed extract + G-1-P and fructose + composite	.52
	d, Dialyzed extract + G-1-P and fructose + FAD	.50
	e, Dialyzed extract + G-1-P and fructose + cystein-DPN	.43
	f, Dialyzed extract + G-1-P and fructose + B	.44
2	a, Undialyzed extract, G-1-P, fructose, + dialyzed protein	3.83
	b, Dialyzed extract, G-1-P, fructose, + dialyzed protein	.13
	c, Dialyzed extract, G-1-P, fructose, + dialyzed protein+ UTP	.98
	d, Dialyzed extract, G-1-P, fructose, + dialyzed protein + UDPG	.78
	e, Dialyzed extract, G-1-P, fructose, + dialyzed protein + sucrose	.16
	f, Dialyzed extract, G-1-P, fructose, + dialyzed protein + 0.5 μmole FAD	.96
	g, Dialyzed extract, G-1-P, fructose, + dialyzed protein + 2.5 μmoles FAD	2.25
	h, Dialyzed extract, G-1-P, fructose, + dialyzed protein + 0.4 μmole B	1.21
	i, Dialyzed extract, G-1-P, fructose, + dialyzed protein + 2 μmoles B	1.25
3	a, Undialyzed extract + G-1-P, and fructose	2.58
	b, Dialyzed extract, G-1-P and fructose	.08
	c, Dialyzed extract, G-1-P and fructose + 2.5 μmoles FAD	1.00
	d, Dialyzed extract, G-1-P and fructose + 10 μmoles B	.33
	e, Dialyzed extract, G-1-P and fructose + FAD, B, DPN	1.12
	f, Dialyzed extract, G-1-P and fructose + FAD, composite	1.29

[1] Unless otherwise indicated in the table the chemicals employed for each experiment were supplied in the following quantities per milliliter of digest: G-1-P, 10 μmoles; fructose, 10 μmoles; ATP, 5 μmoles; Mg, 5 μmoles; DPN, 1.2 μmoles; TPN, 1.2 μmoles; FAD, 0.5 μmole. The composite used in experiment 1 contained the following in each milliliter of digest: B, Cu, Zn, Mn, Mg, and Mo each at the rate of 1.2 μmoles; DPN and TPN at 1.2 μmoles each, and 2.5 μmoles ATP. Reaction time was 4 hours for experiments 1 and 2, 6 hours for experiment 3. Tris buffer (pH 7.5) was used in all tests.

scribed by Whistler, *et al.* (*28*), in that DPN is required for maximum activity. Previous investigations at this laboratory (*1*) have also revealed the presence of phosphatases in cane leaves which readily attack ATP and the hexose phosphates. Phosphohexoisomerase was also present, as

well as a hexokinase which catalyzes the transfer of phosphate from ATP to glucose.

We would thus visualize the specific mechanism of cane-sucrose formation to be bound in dynamic equilibrium with other pathways, such as glycolysis or the hexose monophosphate shunt and with enzyme systems capable of limiting the supply of free and phosphorylated hexoses. Our present thinking is that, while glucose oxidase itself is somewhat removed from sugar formation, it nevertheless bears heavily enough upon glucose supply to retard sucrose production when inactivated. More specifically, a system capable of removing excessive free glucose should tend to retard glycolysis and permit more of the ATP reserves to enter into the formation of UTP. This is in agreement with Turner's conclusion that the sucrose-forming mechanism of peas is coupled with glycolysis.

SUCROSE SYNTHESIS BY ENZYME PREPARATIONS

Dugger and Humphreys (*7*) precipitated protein from extracts of defatted pea seed by 80-percent ammonium sulfate saturation. This preparation contained sucrose-forming activity which was retained after 6-hour dialysis. The same preparation by Turner (*24*) lost activity after 2-hour dialysis, and possessed no sucrose-forming power when used without dialysis. Hatch and coworkers (*10*) apparently succeeded in maintaining sucrose-forming activity in an 80-percent ammonium sulfate precipitate from extracts of rind-free storage tissue of sugarcane.

Our own enzyme preparations did not retain satisfactory sucrose-forming potential. Extracts were prepared from leaf tissue with distilled water, 0.1 M $NaHCO_3$ solution, and with both solvents using leaf material defatted by the method of Turner. The 80-percent ammonium sulfate precipitates were tested without dialysis and after varying periods of dialysis against distilled water and phosphate buffer (pH 7.0). Some of the preparations possessed sucrose-forming activity which, on a basis of μmoles sucrose per milligram of protein, was very nearly comparable to that obtained by other workers. However, this represented only a small fraction of the activity present in the plant material extracted.

Table 5, experiment 1, reviews the formation of sucrose from G-1-P and fructose, by two concentrations of dialyzed enzyme protein in the presence of the additives UTP, UDPG, and sucrose. It is evident that very little sucrose was formed by any treatment, but that best results were obtained in the presence of added UTP and sucrose. Increasing the protein content by 2.5 times, from 0.62 to 1.55 mg./ml. digest, caused a general increase of sucrose, except where UDPG had been added. The implication was that traces of some essential cofactor lost during dialysis was still bound within the protein, and that, if enough protein was added, a satisfactory sucrose-

forming activity could be achieved. For example, 1.55 mg. of protein per milliliter of digest would be equivalent to 0.31 mg./0.2 ml., or approximately $\frac{1}{10}$ of the protein concentration employed by Hatch and coworkers. By using 10 times more protein and taking into account the 30-minute reaction period used by these investigators, we would have expected a sucrose-forming reaction with G-1-P, fructose, and UTP which was roughly equivalent to the standard UDPG-fructose transglycosylase reaction reported by the Hatch group.

TABLE 5.—*Sucrose formation by dialyzed enzyme preparations from leaves of sugarcane*

Experiment	Digest composition[1]	Sucrose formed (μmoles/ml. digest)	
		0.62 mg. protein	1.55 mg. protein
1	a, Dialyzed protein + G-1-P and fructose	0.021	0.073
	b, Dialyzed protein + G-1-P and fructose + UTP	.031	.125
	c, Dialyzed protein + G-1-P and fructose + UDPG	.019	.021
	d, Dialyzed protein + G-1-P and fructose + sucrose	.052	.120
2	a, Dialyzed protein, 1 μmole UDPG, fructose	0.139	
	b, Dialyzed protein, 5 μmoles UDPG, fructose	.000	
	c, Dialyzed protein, 1 μmole UDPG, fructose + 2.5 μmoles FAD	.014	
	d, Dialyzed protein, 1 μmole UDPG, fructose + 2.5 μmoles FAD, + 2 μmoles boron	.013	
	e, Dialyzed protein, 1 μmole UDPG, fructose + 2.5 μmoles FAD, + 1.2 μmoles DPN	.055	

[1] Unless otherwise indicated in the table, reagents used in experiments 1 and 2 were supplied in the following quantities per milliliter of digest: G-1-P, 12 μmoles; fructose, 12 μmoles; UDPG, 1 μmole; sucrose, 2 μmoles; protein, 1.55 mg. The reactions proceeded 4 hours at 30°C. in tris buffer (pH 7.5).

However, a few simple calculations discouraged us from using greater quantities of protein in the assay. Our protein supply of 1.55 mg./ml. digest represents the water-soluble protein from a minimum of 500 mg. of dried leaf tissue. Using 500 mg. of tissue in a homogenate such as that described earlier, with optimum amounts of G-1-P and fructose, the digest would form 18 to 22 μmoles of sucrose in 4 hours, or approximately 50 to 60 times the sucrose produced by an equivalent amount of protein after partial purification. In other words, the enzyme preparations were synthesizing little more than 2 percent of their theoretical potential. We also know from previous studies (*1*) that 1.55 mg. of cane-leaf protein contains sufficient phosphatase to interfere with the supply of ATP and

G-1-P, even at pH 7.5. A quantity of 3 mg. of cane-leaf protein per 0.2 ml. of digest should hydrolyze the equivalent G-1-P supply (2.0 μmoles) in 1½ to 2 hours. It should be added, however, that we have not been able to dissolve 3 mg. of leaf protein in 0.2 ml. of distilled water.

Our work thus far indicates that some factor or group of factors necessary for sucrose production in cane is not being retained during the dialysis of crude extracts or during the purification of protein fractions. Until these factors are known the study of sucrose-forming mechanisms with highly concentrated protein would seem to have little more than philosophical value. This is particularly true in instances such as those reported by Dugger and Humphreys (7) where several enzymes behaved differently when measured individually than when coupled to the overall sucrose-forming mechanism. There is need among sugarcane physiologists of a means for measuring the sucrose-forming capacity of cane. The reactions measured should approximate those actually taking place within the intact tissue, with all essential factors present, and with coupled pathways such as glycolysis, the hexose monophosphate shunt, or starch biosynthesis having the potential to exert effects common to the living tissue.

Efforts were made to demonstrate the presence of UDPG-fructose transglycosylase, and to increase the sucrose-forming capacity of the enzyme preparations with added FAD, boron, and DPN (table 5, experiment 2). A weak transglycosylase activity was evident which accounts for all of the sucrose-forming potential of the enzyme preparations. Curiously enough this activity was lost when UDPG was increased from 1 to 5 μmoles. Tests with added FAD, boron, and DPN were negative. This suggests that whatever effects these factors may have on sucrose synthesis, they are not related to the UDPG-fructose transglucosylase system, and this, in turn, supports the theory that FAD and DPN are involved in the glycolytic area.

Homogenates of tissues from various parts of the sugarcane plant were tested in order to compare their sucrose-forming capacities. The results are shown in the following tabulation:

Tissue tested[1]	*Sucrose formed (μmoles/ml. digest)*
Meristem	2.42
Leaves +1 and +2	1.36
Leaves +6 and +7	.08
Sheaths +1 and +2	.27
8–10 internodes	1.46
8–10 nodes	1.21

[1] Each sample was composited from 3 canes of the M.336 variety and prepared as described under Materials and Methods. Each digest was composed by 300 mg. of dried tissue; 1.7 ml. of distilled water; 0.5 ml. of 2 M tris buffer, pH 7.5; 50 μmoles of G-1-P; and 50 μmoles of fructose. The reactions proceeded 4 hours at 30°C.

Meristematic tissue was easily the most active while the sheaths and older leaves revealed only traces of activity. Nodes and internodes from 8 to 10 cane tissue, which included the rinds, produced sucrose on a level roughly comparable to leaves +1 and +2.

SUMMARY AND CONCLUSIONS

Sucrose is formed from glucose-1-phosphate and fructose in the presence of tissue homogenates or undialyzed crude extracts from leaves of sugarcane. Optimum pH for the reaction is 8, although sucrose is formed readily from pH 7 to 9. Optimum concentrations of both glucose-1-phosphate and fructose lie in the area of 6 μmoles per milliliter of digest. Small quantities of sucrose are formed when either of the hexoses is added in the absence of the other.

The reaction is stimulated by UTP, indicating that the formation of UDPG, and consequently the enzyme UDPG-fructose transglycosylase, is involved in sucrose production. UMP likewise stimulates the production of sucrose when added with ATP. DPN in cystein solution promotes the reaction in the presence of UDPG and ATP. There is no conclusive evidence that Mg, ATP, and DPN are required for sucrose production when added individually. ATP appears to promote glycolysis and retard sucrose formation under certain conditions, and promote sucrose production at other times by phosphorylative reactions.

The sucrose-forming capacity of crude extracts is lost during dialysis against phosphate buffer or distilled water, primarily within the first 30 minutes of dialysis. A small amount of the activity can be returned by adding UTP, UDPG, and cystein-DPN. Boron, at the rate of 0.4 μmoles per milliliter of digest, was more effective and returned up to 30 percent of the activity.

Most effective in reviving the lost activity was FAD at the rate of 2.5 μmoles per milliliter of digest, which returned 60 percent of the sucrose-forming potential. The possibility is discussed that a flavoprotein with a specific requirement for DPN is involved in sucrose formation.

Enzyme preparations failed to retain satisfactory sucrose-forming activity. Protein precipitated by 80-percent ammonium sulfate saturation was dialyzed for short periods against distilled water or phosphate buffer, according to published procedures which have been found satisfactory for pea-seed and pea-seedling extracts. Less than 3 percent of the potential activity was retained by the partly purified protein. Increasing the protein content of the digests promoted activity, indicating that a small quantity of some essential factor must have been bound with the dialyzed protein. A weak UDPG-fructose transglycosylase activity was present in the en-

zyme preparations. The reaction was retarded by FAD, and by FAD in combination with boron and DPN.

A comparison of various cane tissues indicates that the meristem is most active in sucrose synthesis. Homogenates from leaves +6 and +7, as well as sheaths +1 and +2, were only slightly active. Internode numbers 8 to 10 and 8- to 10-node tissues were about comparable with leaves +1 and +2.

At the moment our data indicate that both sucrose phosphorylase and a UDPG-fructose transglycosylase system are active in sugarcane. Sucrose phosphorylase appears to be the dominant system.

RESUMEN Y CONCLUSIONES

La sacarosa se forma de fructuosa y de glucosa-fosfatada-1, en la presencia de tejidos homogenados o de extractos crudos sin dializar de las hojas de caña de azúcar. El pH óptimo para la reacción es 8, aunque la sacarosa se forma fácilmente con pH de 7 a 9. Las concentraciones óptimas de la glucosafosfatada-1 y de glucosa se producen en el área de "6 μmoles" por mililitro de digesto. También se forman pequeñas cantidades de sacarosa cuando se añade cualquiera de las hexosas en ausencia de la otra.

La reacción es estimulada por UTP, indicando que la formación de UDPG, y consecuentemente la enzima UDPG- y fructuosa transglucosilasa, está presente en la producción de sacarosa. Asímismo, la UMP estimula la producción de sacarosa cuando se le agrega ATP. La DPN en solución cisteínica promueve la reacción en la presencia de UDPG y ATP. No hay evidencia categórica de que Mg, ATP, y DPN sean necesarias para la producción de sacarosa cuando se añaden separadamente. El ATP propicia la glicólisis y retarda la formación de sacarosa bajo ciertas condiciones, y en otras ocasiones promueve la producción de sacarosa por reacciones fosforilativas.

La capacidad de formación de sacarosa de extractos crudos se pierde durante el proceso de diálisis contra *phosphate buffer* o agua destilada, principalmente dentro de los primeros 30 minutos de diálisis. Puede revertirse una pequeña cantidad de la actividad, agregándole UTP, UDPG, y DPN cisteínico. El boro, a una proporción de 0.4 μmole por mililitro del digesto fue más efectivo y revirtió hasta el 30 por ciento de la actividad. Más efectivo en revivir la actividad perdida fue el FAD, a una proporción de 2.5 "μmoles" por mililitro de digesto, el cual revirtió el 60 por ciento del potencial en la formación de sacarosa. También se discute la posibilidad de que una flavoproteína, con requisito específico de DPN, esté envuelta en la formación de sacarosa.

Las preparaciones de enzimas no retuvieron satisfactoriamente la actividad formadora de sacarosa. La proteína, precipitada por un 80 por ciento de saturación de sulfato amónico, fue dializada por períodos cortos

contra agua destilada o *phosphate buffer*, según procedimientos publicados, en los cuales se dice que resulta satisfactorio para la semilla de guisantes y extractos de plántulas de guisantes. Menos del 3 por ciento de la actividad potencial fue retenida por la proteína parcialmente purificada. Aumentando el contenido proteínico de los digestos se promueve la actividad, indicando que una pequeña cantidad de algún factor esencial ha tenido que estar ligado con la proteína dializada. Una débil actividad UDPG-fructuosa transglucosilasa estuvo presente en las preparaciones enzimáticas. La reacción fue retardada por FAD, y por FAD en combinación con boro y DPN.

Una comparación de varios tejidos de caña indica que el meristemo es más activo en la síntesis de sacarosa. Homogenados de hojas +6 y +7, así como las yaguas +1 y +2, resultaron ligeramente activos. Los entrenudos del 8 al 10 y los tejidos de nudos del 8 al 10 eran casi comparables a las hojas +1 y +2.

Por el momento, nuestra información demuestra que tanto la sacarosa fosforilasa como el sistema UDPG-de fructuosa transglucosilasa, están activos en la caña de azúcar. La sacarosa fosforilasa parece ser el sistema preponderante.

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