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Doctor's Dissertation

Sucrose Synthetase from Triploid

Quaking Aspen Callus Tissue

Larry Lester Graham

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SUCROSE SYNTHETASE FROM TRIPLOID
QUAKING ASPEN CALLUS TISSUE

A thesis submitted by

Harry Lester Graham

B.S.Ch.E. 1964, Northwestern University

M.S.Ch.E. 1968, University of Toledo

M.S. 1971, Lawrence University

in partial fulfillment of the requirements
of The Institute of Paper Chemistry
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Appleton, Wisconsin

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After years of relentless study, laborious research, and
innumerable calculations, I have hereby ascertained that,
no two snowflakes...are alike....

Johnny Hart

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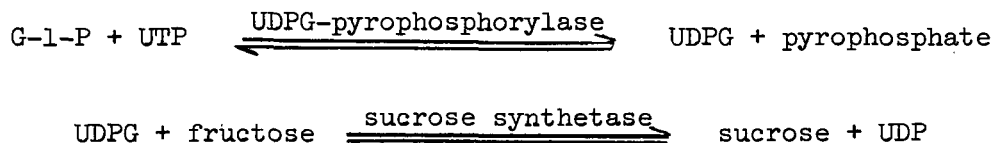
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ABSTRACT

Incubation of uridine triphosphate and ^{14}C -labeled glucose-1-phosphate with crude extracts of dark-grown triploid quaking aspen callus tissue led to the formation of two labeled products, uridine diphosphate glucose (UDPG) and sucrose. The following reaction scheme was suggested for their formation.



Both enzymes, UDPG-pyrophosphorylase (E. C. 2.7.7.9) and sucrose synthetase (E. C. 2.4.1.13) are known to occur in plants; however, this thesis contains the first evidence for the presence of sucrose synthetase in any tree tissue, specifically in cultured tree callus tissue. Radioactive tracer studies showed that label could be incorporated into sucrose from uridine diphosphate- ^{14}C -glucose and ^{14}C -fructose in the presence of the crude callus extract. There was some evidence that the formation of UDPG from sucrose and UDP was promoted by the crude callus extract.

Both the UDPG-pyrophosphorylase and the sucrose synthetase activities in the crude extract obtained from the dark-grown triploid aspen callus sedimented at $100,000 \times g$, suggesting that these enzymes were membrane-bound. The sucrose synthetase from this tissue was purified by ultracentrifugation, solubilization with digitonin, salt fractionation, and gel filtration. Specific activities for the purified sucrose synthetase ranged from 0.143 to 0.243 micromole sucrose formed per milligram protein per minute. The purified sucrose synthetase was free of UDPG-pyrophosphorylase activity. A sedimentation coefficient of $11.13 \times 10^{-13} \text{ sec}^{-1}$ was obtained for the purified sucrose synthetase. It had a molecular weight between 350,000 and 415,000 as determined by gel filtration on an agarose column. An optimum for the sucrose synthesis reaction was observed at pH 8.

The Michaelis constants for the purified sucrose synthetase in the sucrose synthesis direction were found. The value of K_{-m} (UDPG) was 0.11 mM ([fructose] = 100 mM) and the value of K_{-m} (fructose) was 5 mM ([UDPG] = 2 mM). High concentrations of UDPG inhibited the sucrose synthetase in its catalysis of the sucrose synthesis reaction. ADPG and GDPG could be utilized also as substrates by the enzyme; however, the effectiveness of these two substrates was much less than that of UDPG. The properties of the sucrose synthetase from aspen callus confirmed that it was a similar enzyme to that prepared from other plant sources.

Sucrose synthetase activity was found also in crude extracts prepared from dark-grown loblolly pine, shortleaf pine, western hemlock, and eucalyptus callus tissues in addition to crude extracts prepared from light-grown Douglas-fir and triploid quaking aspen callus tissues.

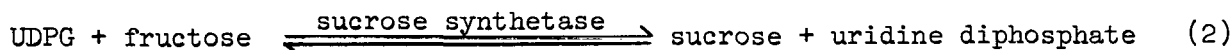
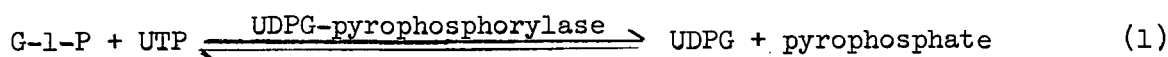
The importance of sucrose synthetase probably is not as a sucrose synthesizing enzyme. The current consensus is that it functions in vivo in the sucrose-cleavage or UDPG-formation direction. This enzyme was very abundant in dark-grown quaking aspen callus tissue, accounting for nearly 5% of the protein in the tissue. Sucrose was the primary carbon source supplied to the callus tissue in the medium upon which it was grown. Because of its abundance in the tissue, sucrose synthetase must be very important to its growth and metabolism. Sucrose synthetase would appear to be the primary route for utilization of sucrose by callus tissue via the formation of nucleoside diphosphate sugars. These compounds are, in turn, precursors in important biosynthetic processes within the tissue, such as cell-wall synthesis.

INTRODUCTION

Undifferentiated triploid quaking aspen (Populus tremuloides Michx.) callus tissue has been maintained in the dark on defined media at The Institute of Paper Chemistry for several years. The growth requirements for this tissue and the effects of different media upon its initiation and physical nature have been studied as a part of the aspen improvement program of the Institute (1-4). The production of individual trees from this undifferentiated tissue was a notable achievement (5-7). More recently, the occurrence of cyclic AMP within this tissue has been studied (8). Tree improvement by means of callus, cell, and protoplast culture is a continuing area of research at The Institute of Paper Chemistry (9).

The original objective of this thesis research was to isolate and study a cellulose synthesizing system from the undifferentiated triploid aspen callus tissue. Incubation of crude extracts from the callus with uridine triphosphate (UTP) and ^{14}C -glucose-1-phosphate (^{14}C -G-1-P) resulted in at least two ^{14}C -containing products; however, there was no clear evidence for cell-free cellulose synthesis.

One of these two radioactive products had the paper chromatographic mobility of a disaccharide and was identified as sucrose. The other product was identified as uridine diphosphate glucose (UDPG). The following reaction scheme is suggested for the formation of sucrose when UTP and G-1-P were incubated with the crude callus extract:



Both enzymes, sucrose synthetase and UDPG-pyrophosphorylase, are found in many plant tissues. It is currently thought that the physiological role of sucrose synthetase is actually in the reverse direction to that shown in Reaction (2). If this were the case, the sucrose synthetase could be responsible for the synthesis of UDPG and possibly other nucleoside diphosphate glucoses, for use by the plant cell in cell wall synthesis, manufacture of other sugars, and production of storage polysaccharides from sucrose. Sucrose, resulting from the process of photosynthesis, is the primary transport sugar supplied to the non-photosynthetic portions of a plant or plant cell (10). When this disaccharide is broken into its constituent monosaccharides, energy may be provided to the cell by means of catabolism, or the monosaccharides may be utilized in biosynthetic processes within the cell. In addition to being the primary transport sugar in differentiated plants, sucrose has been the commonly used carbon source for cultured plant tissues grown on defined media (11,12). The nucleoside diphosphate sugars are known to be extremely important in plant cell metabolism. Of the two pathways for formation of UDPG from sucrose shown in Fig. 1, the sucrose synthetase route would appear to be the more direct.

OBJECTIVES AND SCOPE OF THE THESIS

Preliminary experimental results suggested that the crude extract from aspen callus tissue contained an activity capable of synthesizing sucrose. The study of sucrose synthetase in tree callus tissues was of interest since sucrose is the most commonly used carbon source for any cultured plant tissue (11,12).

The objectives of the thesis were to determine:

1. If the crude extracts obtained from quaking aspen callus tissue contain sucrose synthetase;

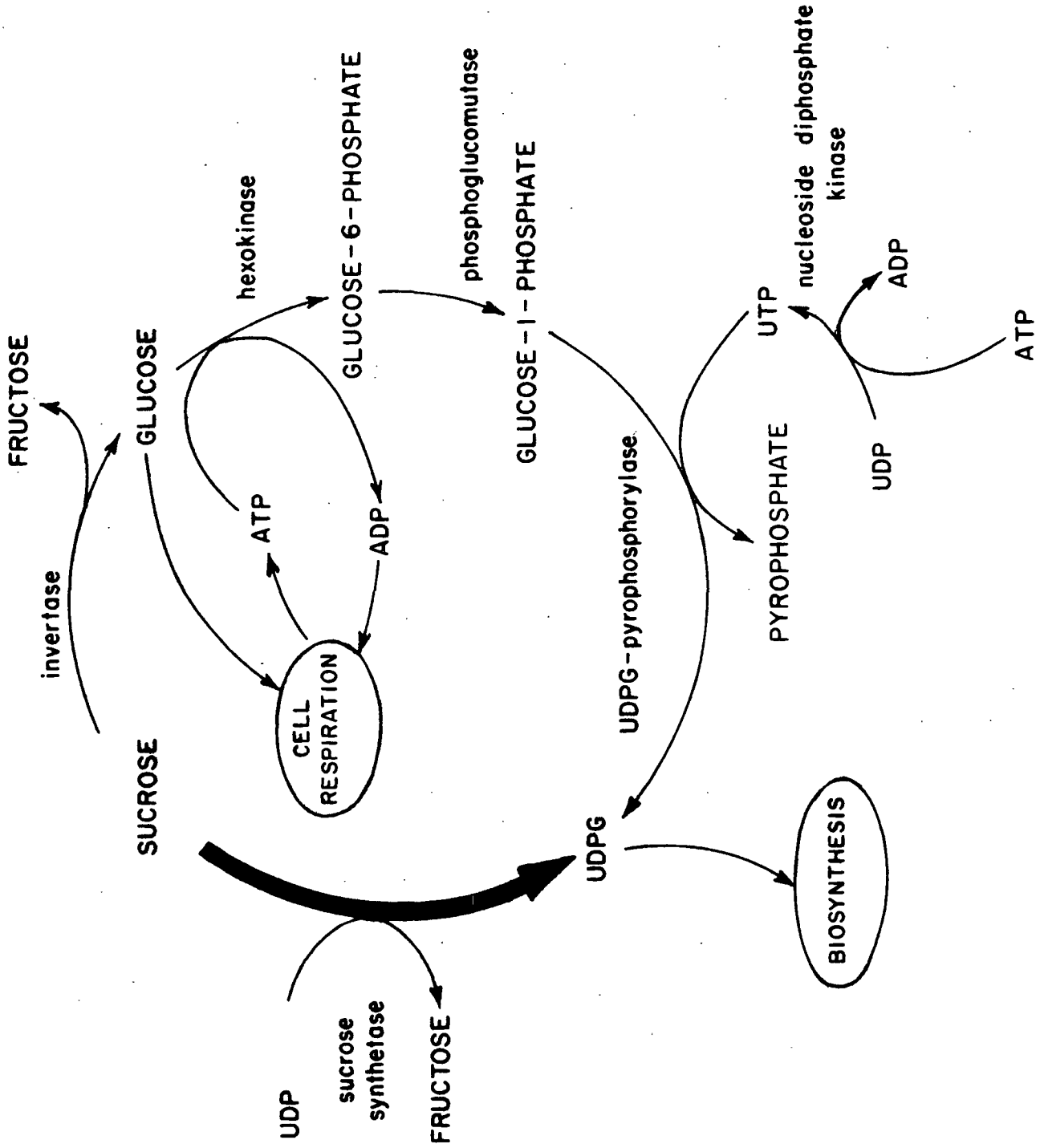


Figure 1. Two Possible Routes to UDPG from Sucrose

2. Whether or not the sucrose synthetase found in the aspen callus tissue is similar to the sucrose synthetase found in other plant tissues; and,
3. If other cultured tree tissues contain sucrose synthetase activity.

The prime function of sucrose synthetase in dark-grown cultured tissues is not logically the promotion of sucrose synthesis. The importance of this enzyme is that it could provide a route for utilization of sucrose via the sucrose cleavage reaction [reverse of Reaction (2)].

The first objective was met by incubating crude callus extracts with radioactive fructose or radioactive glucose-containing substrates and various other reagents and observing the pattern of the radioactive products. The two principal products were identified as sucrose and UDPG.

In order to satisfy the second objective, the sucrose synthetase had to be purified from the crude callus extract. Once this was accomplished, some of the physical properties of the enzyme and the kinetics of the sucrose synthesis reaction were investigated. The properties and kinetics were compared with those reported in the literature for sucrose synthetase preparations from other plant sources.

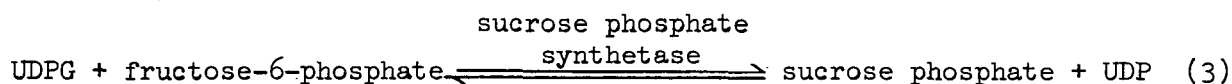
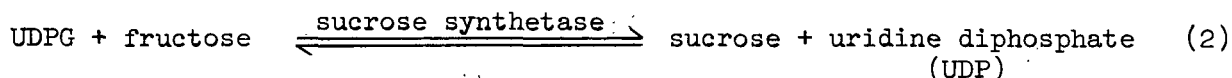
Crude extracts from cultured tissues from other tree species were assayed for sucrose synthetase activity to demonstrate that this activity was not unique to the dark-grown triploid quaking aspen callus tissue.

This is the second report of sucrose synthetase activity in cultured plant tissues and the first report of this enzyme from any tree species.

SUCROSE SYNTHETASE: A REVIEW OF THE LITERATURE

The first nucleoside diphosphate sugar reported in the literature was uridine diphosphate glucose (UDPG) which was discovered to be an intermediate in the enzymatic conversion of galactose-1-phosphate to glucose-1-phosphate (13). The structure of UDPG, as well as structures for other important compounds discussed in this thesis, is presented in Fig. 2. Since the discovery of UDPG, this compound and other nucleoside diphosphate sugars have been found in many different tissues. Many of these compounds have been shown to be important in the biochemistry of carbohydrates. They are involved in the enzymatic interconversions of monosaccharides and act as glycosyl donors in the biosynthesis of oligo- and polysaccharides. This area of carbohydrate biochemistry has been more than adequately reviewed (10,14-19).

Soon after the discovery of UDPG, two important enzymes were found in wheat germ which utilized this compound in the biosynthesis of sucrose and sucrose phosphate (20-22). The reactions promoted by these enzymes are:



UDP-glucose:D-fructose 2-glycosyltransferase (E. C. 2.4.1.13), which is more commonly known as sucrose synthetase, catalyzed Reaction (2). UDP-glucose:D-fructose-6-phosphate 2-glycosyltransferase (E. C. 2.4.1.14), which is involved in Reaction (3), has the common name sucrose phosphate synthetase.

Frequently, another enzyme, sucrose phosphatase (E. C. 3.1.3.1) is found associated with sucrose phosphate synthetase. This enzyme catalyzes the following irreversible reaction:

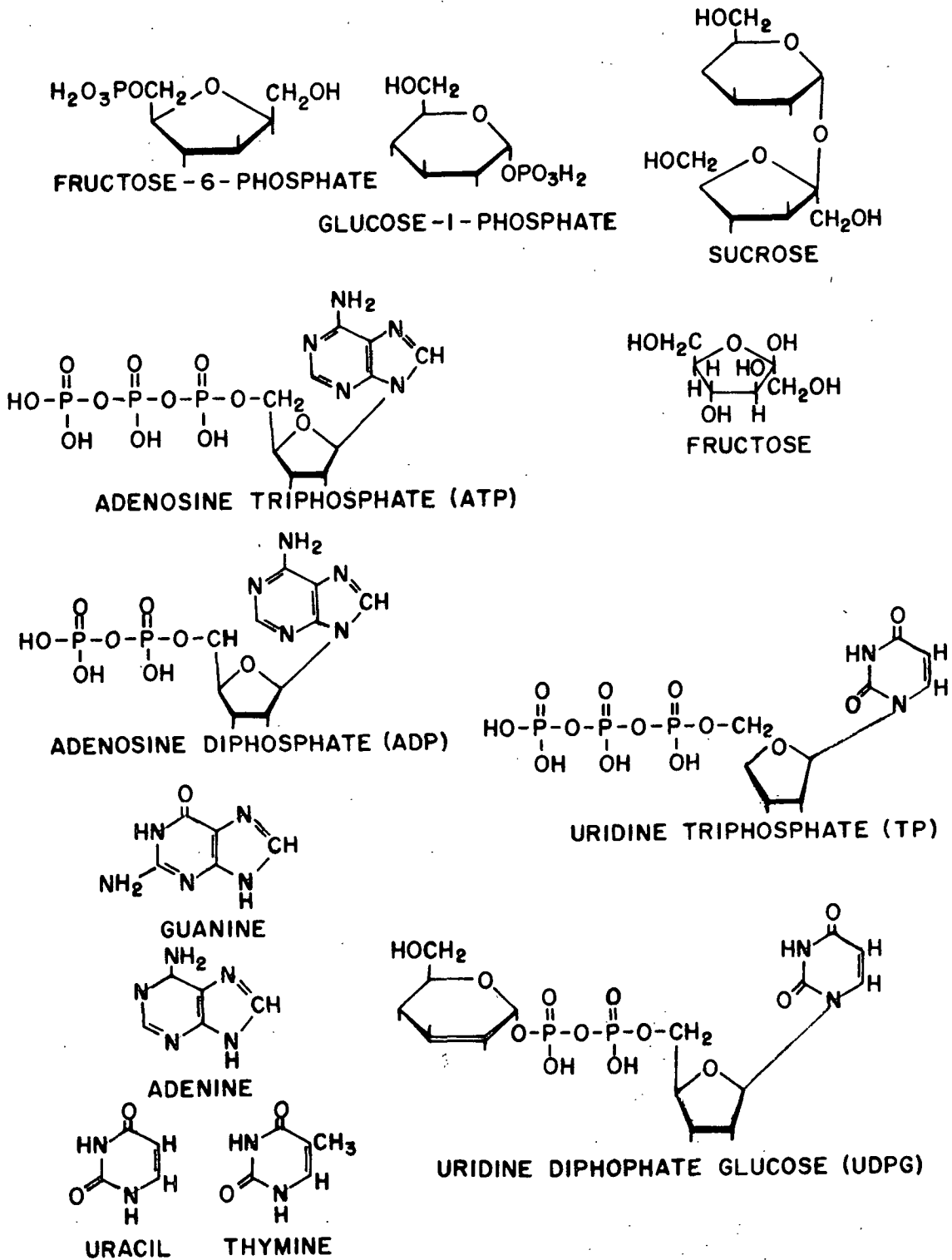
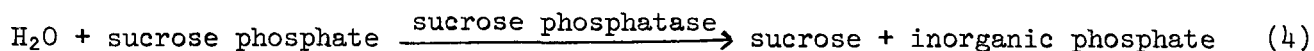


Figure 2. Structures of Important Compounds



Therefore, both the sucrose synthetase and the sucrose phosphate synthetase, in conjunction with the sucrose phosphatase, are capable of producing sucrose with the UDPG substrate. It is currently thought, however, that the sucrose phosphate synthetase-sucrose phosphatase complex is primarily responsible for production of sucrose from photosynthetically fixed carbon whereas the sucrose synthetase is responsible for the synthesis of UDPG, and possibly other nucleoside diphosphate sugars, from sucrose. These two separate roles are illustrated in Fig. 3.

Sucrose synthetase has been found not only in wheat germ but also in many different plant tissues including oranges and lemons (23), Jerusalem artichoke tubers (24), tobacco leaves (25), tapioca tuber (26), corn endosperm (27,28), broad and castor beans (28), peas (29,30), pea seedlings (31,32), sugarcane leaf (33) and stem (34), sugar beet leaf (35) and root (36,37), sweet potato root (38-40), potato tuber (41-44), ripening rice grains (44,45), mung bean seedlings (46-49), tomato, pea and maize roots (50), and cultured tobacco and carrot callus tissue (12).

Various methods have been employed in the isolation and purification of this enzyme including salt fractionation, DEAE-cellulose chromatography, gel filtration, and sucrose density gradient centrifugation. The enzyme from crude extracts has been found to be soluble, except in the case of lemons (23) where it was found in particulate fractions. Sucrose synthetase has generally been found to precipitate from solutions which were 40 to 60% saturated with ammonium sulfate.

Data for the enzyme obtained from different sources are in Table I. It has been purified up to 250-fold; the degree of purification depends, of course,

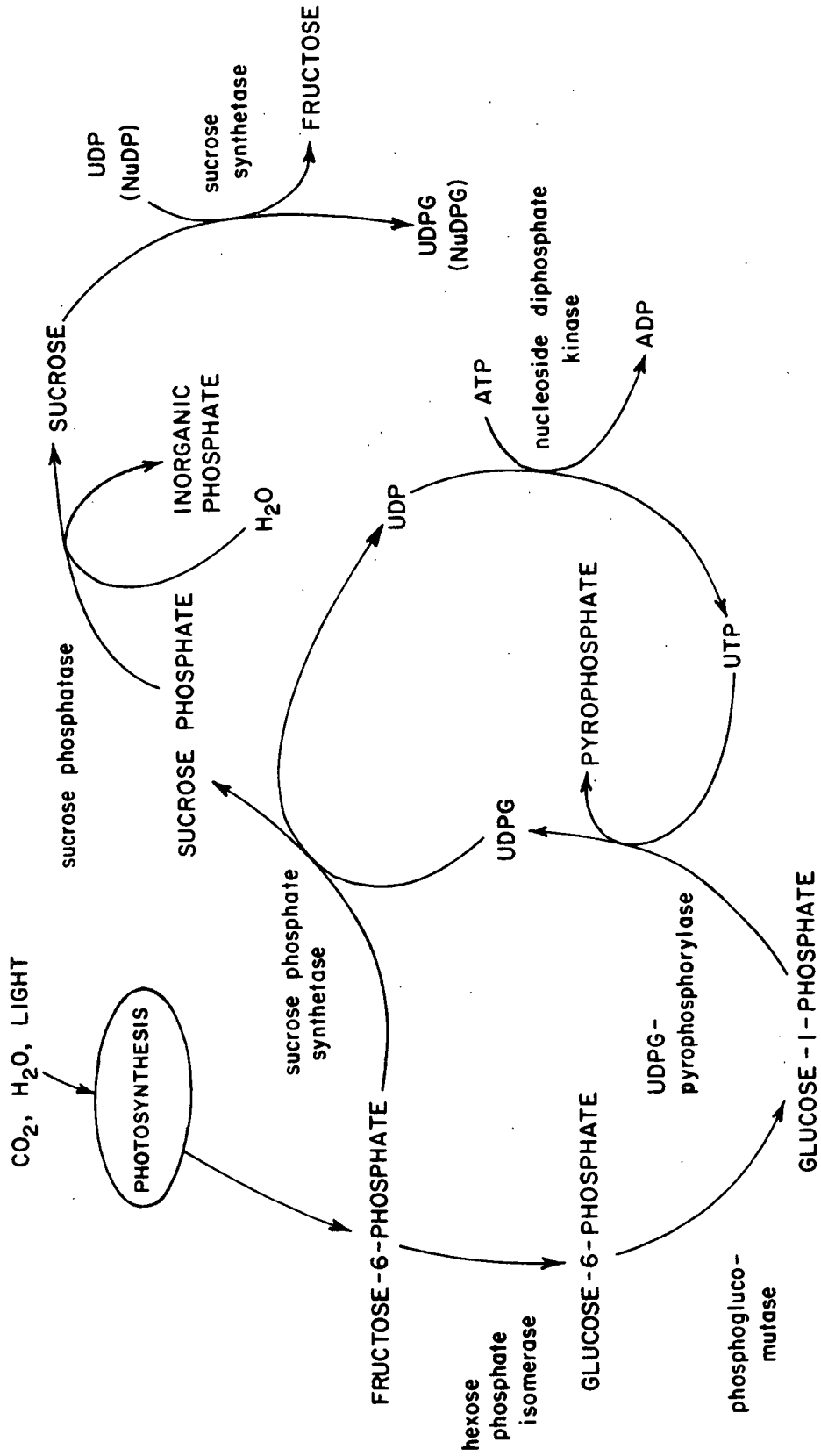


Figure 3. Roles of Sucrose Phosphate Synthetase and Sucrose Synthetase

TABLE I

CHARACTERISTICS OF SUCROSE SYNTHETASE PREPARATIONS FROM VARIOUS SOURCES

Source	Reference	Purifi- cation	Specific Activity ^a	Molecular Weight ^c	UDPG + Fructose			Sucrose + UDP			UDPG + Fructose			Temp., °C	pH	K _{eq} ^f
					Temp., °C	K _m ^b	V _{max} ^c	Temp., °C	K _m ^b	V _{max} ^c	Temp., °C	K _m ^b	V _{max} ^c			
Wheat germ	(21), 1955	48	0.08		7.2	37	2.3							7.4	37	2-3
Jerusalem artichoke	(24), 1964				8.0	22	0.64	1.6	8.3	22	0.32	33		7.6	30	1.4-1.8
Sweet corn	(27), 1964				7.4		1.1		7.4		0.06					
Sugar beet root	(36), 1964	>80			6.0		0.3	3.1	6.0		0.061	110		7.2		1.3
Sugar beet root	(37), 1965				7.2		0.27		6.0		0.077					
Potato tuber	(42), 1968				7.5	37	1.65	11.8								
Potato tuber	(43), 1969	34	6.33													
Mung bean seedling	(46), 1970	78.5	0.018	1,000,000	7.8		0.21	2.0	6.0	37	0.13	31				
Sweet potato root	(32), 1971	40	3.27	540,000	7.5	37	1.67 ^d 0.71 ^e	1.54 ^d 1.0 ^e								
Tapioca tuber	(26), 1971	97	2.78	375,000- 405,000	7.5	37	8.3	2.6	6.16	1.47	7.5	37	6.6	10	3.70	3.07
Mung bean seedling	(48), 1972	252	0.097						7.5	25	17	0.19		7.5	25	6.66
Pea seedling	(31), 1972				8.0				8.0		0.06	63				
Rice grains	(44), 1972		3.54		7.5	37	2.7	2.9	6.0	37	31	0.13				
Potato tuber	(44), 1972		2.79		7.5	37	2.0	1.4	6.0	37	30	0.11				
Pea seedling	(32), 1972	100	80-90													
Rice grains	(45), 1973	11.4	5.7	400,000	8.0	37	5.3	6.9	6.0	37	0.8	290				

^a μmole sucrose produced/mg protein/min.^b mM.^c μmole/mg protein/min.^d Without magnesium chloride.^e With magnesium chloride.^f Equilibrium constant in sucrose synthesis direction.

upon the relative amount of the enzyme found in the crude extract as well as upon the purity achieved. The specific activities for the various preparations summarized in Table I range from 2.4 to 190 μ mole sucrose produced/mg protein/30 min. One much higher specific activity has been reported (32).

The more recent work with purified sucrose synthetase indicates that it has a molecular weight of approximately 400,000 and is a tetramer consisting of four identical subunits (45,48). Sedimentation coefficients, $S_{20,w}$, have been reported at $12.4 \times 10^{-13} \text{ sec}^{-1}$ for the enzyme from mung bean seedlings (48) and $13.3 \times 10^{-13} \text{ sec}^{-1}$ for the enzyme from ripening rice grains (45). The enzyme from mung bean seedlings was also reported to have a diffusion constant, $D_{20,w}$, of $3.03 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ and a f/f_0 value (frictional ratio) of 1.49 (48).

Kinetic data from the literature for both the sucrose synthesis reaction (utilizing UDPG) and the sucrose cleavage reaction (forming UDPG) are presented also in Table I. Various pH optima for the sucrose synthesis reaction have been reported. The optimum appears to lie between pH 7 and 9 (21,24,26,42,45). The enzyme from tapioca tuber was strongly inhibited by tris-HCl buffer in the sucrose cleavage direction but was active in this direction when a phosphate buffer was used (26).

The active site of this enzyme probably involves sulfhydryl groups (42). The preparation from sugarcane stem tissue was inhibited by phenol oxidation products, but the inhibition was reversed by thiol compounds (34).

Sucrose synthetase has been found capable of utilizing substrates other than UDPG and UDP. Kinetic data for the enzyme from various sources using these other substrates are presented in Table II. Thus, sucrose synthetase, acting in the proposed physiological direction, may play an even larger role

TABLE II

KINETIC DATA FOR VARIOUS SUCROSE SYNTHETASES
USING DIFFERENT SUBSTRATES

Source	Reference	Substrate	K_{-m}^a	Substrate	K_{-m}^a
Tapioca tuber	(26)	UDPG	8.3	UDP	6.6
		ADPG	6.7	ADP	8.3
Corn endosperm	(27)	UDPG	1.1	UDP	0.06
		ADPG	2	ADP	2.1
Sugar beet root	(36)	UDPG	0.3	UDP	0.061
				TDP	0.094
Sugar beet root	(37)	UDPG	0.27	UDP	0.077
		TDPG	0.095	TDP	0.094
Sweet potato root	(39)	UDPG	1.67	UDP	0.13
		ADPG	5.0	ADP	0.44
Potato tuber	(42)	UDPG	1.65		
		ADPG	8.6		
		TDPG	1.0		
		GDPG	2.77		
Ripening rice grains	(45)	UDPG	5.3	UDP	0.8
		ADPG	3.8	ADP	3.3
Mung bean seedling	(46,48)	UDPG	0.21	UDP	0.80
		ADPG	1.8	ADP	0.19
		TDPG	1.7	TDP	0.30
		CDPG	2.5	CDP	0.44
		GDPG	2.5	GDP	0.77

^a
mM.

in utilization of sucrose by the plant cell than has been suggested. The various nucleoside diphosphate glucoses listed in the table are known to be substrates for important syntheses and conversions. For example, ADPG is involved in starch synthesis. TDPG is a precursor of TDP-D-galactose and TDP-L-rhamnose which in turn are precursors for other more complex saccharides. Both UDPG and GDPG have been proposed as substrates for the enzymes involved in cellulose synthesis (51).

Delmer and Albersheim (47), working with extracts from mung bean seedlings, found sucrose phosphate synthetase and sucrose phosphatase only in photosynthetic tissue extracts. Although UDPG-pyrophosphorylase activity was low in these extracts, it was high in extracts from nonphotosynthetic tissues. On the other hand, ADP-, TDP-, CDP-, and GDP-glucose-pyrophosphorylase activities were generally higher in extracts from photosynthetically active tissues than in those from nonphotosynthetically active tissues. The levels of sucrose synthetase activity were higher in nonphotosynthetic tissues than in photosynthetic tissues. These authors suggested that:

1. Sucrose phosphate synthetase and sucrose phosphatase are the enzymes responsible for the biosynthesis of sucrose from photosynthetically fixed CO₂;
2. The major function of sucrose synthetase is to catalyze the synthesis of the various nucleoside diphosphate glucoses from translocated sucrose in nonphotosynthetic tissues;
3. The various nucleoside diphosphate glucose pyrophosphorylases, other than UDPG-pyrophosphorylase, play the major role in production of the nucleoside diphosphate glucoses in photosynthetic tissues; and,
4. Sucrose synthetase and UDPG-pyrophosphorylase play a major role in the uptake and metabolism of sucrose in nonphotosynthetic tissues.

Rollit and Maclachlan (31) studied the synthesis of cell wall glucan from sucrose by enzyme preparations from etiolated pea seedlings. Enzyme extracts from the apical tissue contained a freely reversible sucrose synthetase activity. Particulate fractions from the same tissue contained β -glucan synthetase which utilized UDPG to produce both alkali-soluble and alkali-insoluble products. When both synthetic activities were incubated with UDP and ^{14}C -sucrose, the radioactive glucose moiety was moved from sucrose to glucan in vitro. The authors suggested that these two synthetic activities cooperate in vivo to produce β -glucan for expanding cell walls.

Vieweg (50) found sucrose synthetase activities in tomato, pea and maize roots. The high levels of this activity in regions where active cell wall synthesis from glycosyl nucleotide occurred suggested that this enzymatic activity is important in providing nucleoside diphosphate sugars for polysaccharide synthesis whereas invertase activities provide initial substrates for other metabolic pathways.

Recently, several authors have suggested the possibility of a regulatory role for sucrose synthetase in plant cell metabolism (38,49,52).

Both dark- and light-grown cultures of plant tissues require an exogenous source of carbon and energy (11). This is normally supplied in the growth medium as sucrose. Despite the extensive use of this sugar for over twenty-five years, there has been no comprehensive study, until recently, of the enzymes in the cultured plant tissue responsible for the breakdown of sucrose for use by the tissue. Thorpe and Meier (12) studied the levels of sucrose synthetase, soluble invertase and insoluble invertase in tobacco callus. These authors were the first to demonstrate the presence of sucrose synthetase in

any cultured plant tissue. They found a degree of correspondence, particularly for dark-grown tissue, between the activity of sucrose synthetase in the tobacco callus tissue and the growth rate of the tissue. They concluded that this enzyme is probably more important in the utilization of sucrose by the cultured tissue than is either of the invertases.

EXPERIMENTAL

The experimental results presented and discussed in this section are organized into three major areas corresponding to the three objectives of the thesis.

Demonstration of the presence of sucrose synthetase activity in crude callus extract involved several areas of investigation. The first indications that crude extract might contain sucrose synthetase activity came from the incubation of the extract with UTP, ^{14}C -G-1-P, and fructose. Incubation of these three reagents with the extract produced two radioactive products, one of which was thought to be sucrose. The reaction scheme represented by Reactions (1) and (2) was suggested by the formation of these two labeled products. The effects of fructose concentration and fructose-6-phosphate concentration upon formation of the two labeled products were investigated and added support to the contention that the suggested reaction scheme was responsible for formation of the two products. It was found that the product, which was thought to be sucrose, could also be formed when UTP, nonradioactive G-1-P, and ^{14}C -fructose were incubated with callus extract. More direct evidence for the presence of sucrose synthetase in the crude extract was obtained by incubation of UDPG- ^{14}C with fructose in the presence of the callus extract. The reaction of nonradioactive UDPG with ^{14}C -fructose in the presence of the crude extract also produced the radioactive product which was thought to be sucrose. The two labeled products were identified as sucrose and UDPG. All of these results were consistent with the hypothesis that sucrose synthetase and UDPG-pyrophosphorylase are found in the crude callus extract.

Sucrose synthetase had to be purified from the crude callus extract in order to investigate its similarities or dissimilarities with the enzyme found in other plant sources. A purification procedure was developed and the physical characteristics of the purified enzyme were examined. The kinetic nature of the sucrose synthesis reaction catalyzed by the purified enzyme was also investigated.

Finally, crude extracts were prepared from a number of cultured tissues from other tree species and were assayed for sucrose synthetase activity.

Before presenting and discussing the results outlined above in detail, the general experimental techniques used throughout the research effort will be discussed.

GENERAL EXPERIMENTAL TECHNIQUES

Crude callus extracts were prepared and used directly in the early phases of the research. Later, the crude extract was used as the starting material for purification of the sucrose synthetase.

PREPARATION OF CRUDE EXTRACTS

Tissue was removed from the agar medium upon which it had been grown. The details of the origin and method of subculture of the callus tissue are discussed in Appendix I. The dark centers of the callus pieces were removed and discarded. The remaining tissue was weighed before being homogenized in a Ten Broeck tissue grinder with 1 mM dithiothreitol (DTT) in 0.1M 3-(morpholino)-propanesulfonic acid (MOPS buffer) which had been adjusted to pH 8.0. This homogenate was centrifuged twice at $1000 \times g$ for 10 minutes to remove whole cells and debris. The supernatant from this centrifugation was used as the

crude callus extract. All work was done at 4-5°C in the cold room or in a refrigerated centrifuge.

PROTEIN ANALYSIS

Protein contents of the crude extracts and various purification stages were determined using the microbiuret technique of Koch and Putnam (53) as discussed in Appendix II.

LIQUID SCINTILLATION COUNTING

Counting was performed in a Beckman DPM-100 liquid scintillation counter using Cocktail D [10 g (PPO) 2,5-diphenyloxazole and 200 g naphthalene made up to 2 liters with dioxane]. PPO was obtained from either Beckman or Interex Corp. The dioxane was either Baker AR or liquid scintillation grade obtained from International Chemical and Nuclear Corp.

Since liquid scintillation vials were reused, only the middle energy range ($^{14}\text{C}/^3\text{H}$ window) of the instrument was used.

OTHER MATERIALS AND EQUIPMENT

All radioactive compounds were purchased from International Chemical and Nuclear Corp. These are discussed in Appendix III. All other compounds were purchased from either Calbiochem or P-L Biochemicals except as noted. DE-81 anion exchange paper was purchased from Reeve Angell.

A Beckman Model L-2 preparative ultracentrifuge and a Sorvall RC2-B refrigerated centrifuge were used in the preparation of extracts and fractions. Column chromatography was monitored using a LKB Uvicord Type 4701A.

INCUBATION SAMPLES

The reactions catalyzed by components of the crude callus extract or purified enzyme were studied by incubating aliquots of the particular preparations in prepared samples. These samples were incubated in a Dubnoff incubator for the desired period of time before being frozen in an acetone-dry ice bath to terminate the reactions. The samples were then concentrated for analysis by freeze-drying and reconstituting them with measured volumes of double-distilled water (ddH₂O). Two different standard sample compositions were frequently used. Sample concentrations different from those presented below will be noted where necessary.

Standard G-1-P Samples

Standard G-1-P samples were 2 mM in MgCl₂, 230 μM in UTP and contained 0.33 μCi ¹⁴C-G-1-P (225 μCi/μmole) per ml of sample. These samples were generally incubated at 30°C for 2 hours and analyzed by paper chromatography.

Standard UDPG Samples

Standard UDPG samples were 2 mM in MgCl₂, 100 mM in fructose, 33 μM in unlabeled UDPG and contained 0.08 μCi UDPG-¹⁴C (155 μCi/μmole) per ml of sample. These samples were incubated at 30°C and analyzed by either paper chromatography or anion exchange paper binding.

ANALYSIS OF INCUBATION SAMPLES

Two methods, paper chromatography and anion exchange paper binding, were used to analyze the freeze-dried incubation samples for ¹⁴C-containing products. In either case, the freeze-dried sample was first reconstituted with a measured volume of double distilled water (ddH₂O) and aliquots of the reconstituted sample were analyzed.

Paper Chromatography

When paper chromatography was used, aliquots of the reconstituted samples were spotted along with appropriate standards on Whatman No. 1 paper. After development with an appropriate solvent system, each paper chromatogram was marked into 1-inch wide horizontal strips (spotting origin centered in Strip No. 2). The chromatogram was cut vertically to remove the sample portion. The standard portion was sprayed to reveal the standard spots. The sample portion (or section of it of interest) was cut into the 1-inch horizontal strips which were individually counted in Cocktail D in the liquid scintillation counter. In the earlier work the entire length of the chromatogram was usually counted and Strip No. 1 immediately above the spotting origin was used as a measure of background count. In later work, however, only the portion of the chromatogram corresponding to sucrose was counted, and background was measured on a similar group of strips.

Details of the paper chromatography development and detection methods are presented in Appendix IV.

Anion Exchange Paper Binding

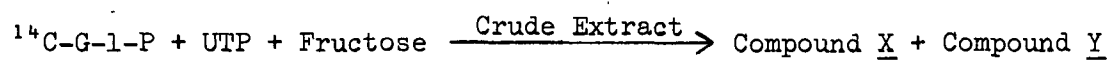
Aliquots of the reconstituted sample were spotted and dried on circles of Whatman DE-81 paper. The paper circles were then washed with 30 ml of ddH₂O on a filter funnel before being counted in Cocktail D in the liquid scintillation counter. The anion exchange paper binds UDPG, therefore this analytical technique was used only for samples in which UDPG-¹⁴C was the labeled substrate. Because sucrose, fructose, and glucose are washed from the paper, the technique can be used to monitor the disappearance or appearance of UDPG-¹⁴C. Delmer (49) used this technique in studying the regulatory properties of sucrose synthetase from mung bean seedlings. The binding by the DE-81 paper of radioactivity

from the various labeled substrates used in this research is discussed in Appendix III.

SUCROSE SYNTHETASE FOUND IN CRUDE CALLUS EXTRACTS

REACTIONS INVOLVING G-1-P, FRUCTOSE, AND UTP IN THE PRESENCE OF CRUDE EXTRACT

The presence of sucrose synthetase in the crude callus extract was first indicated when UTP, ^{14}C -G-1-P, and fructose were incubated with the extract.



Paper chromatography of aliquots, from samples in which ^{14}C -G-1-P and UTP were incubated with crude callus extract, revealed four concentrations of radioactivity as illustrated in Fig. 4(b). The first of these, which occurred at or near the spotting origin, was considered to be due at least partially to unreacted ^{14}C -G-1-P. G-1-P did not move in the solvent system used to develop this chromatogram. The concentration of count farthest from the spotting origin had a mobility identical to that of glucose and was accounted for by either ^{14}C -glucose contamination of the labeled G-1-P or by release of ^{14}C -glucose during the incubation. Another count peak was found which had a chromatographic mobility similar to that of a disaccharide and was referred to as Compound X. For a while, Compound X was thought to be either cellobiose or laminaribiose, and its presence was taken as evidence for either cellulose- or callose-related synthesis during the incubation. Extensive investigation of Compound X showed that it could be neither of these two compounds and it was positively identified as sucrose. The evidence for this identification is presented in a later section. The fourth area of radioactivity concentration lay between the spotting origin and the Compound X peak on chromatograms developed with Solvent IA and was

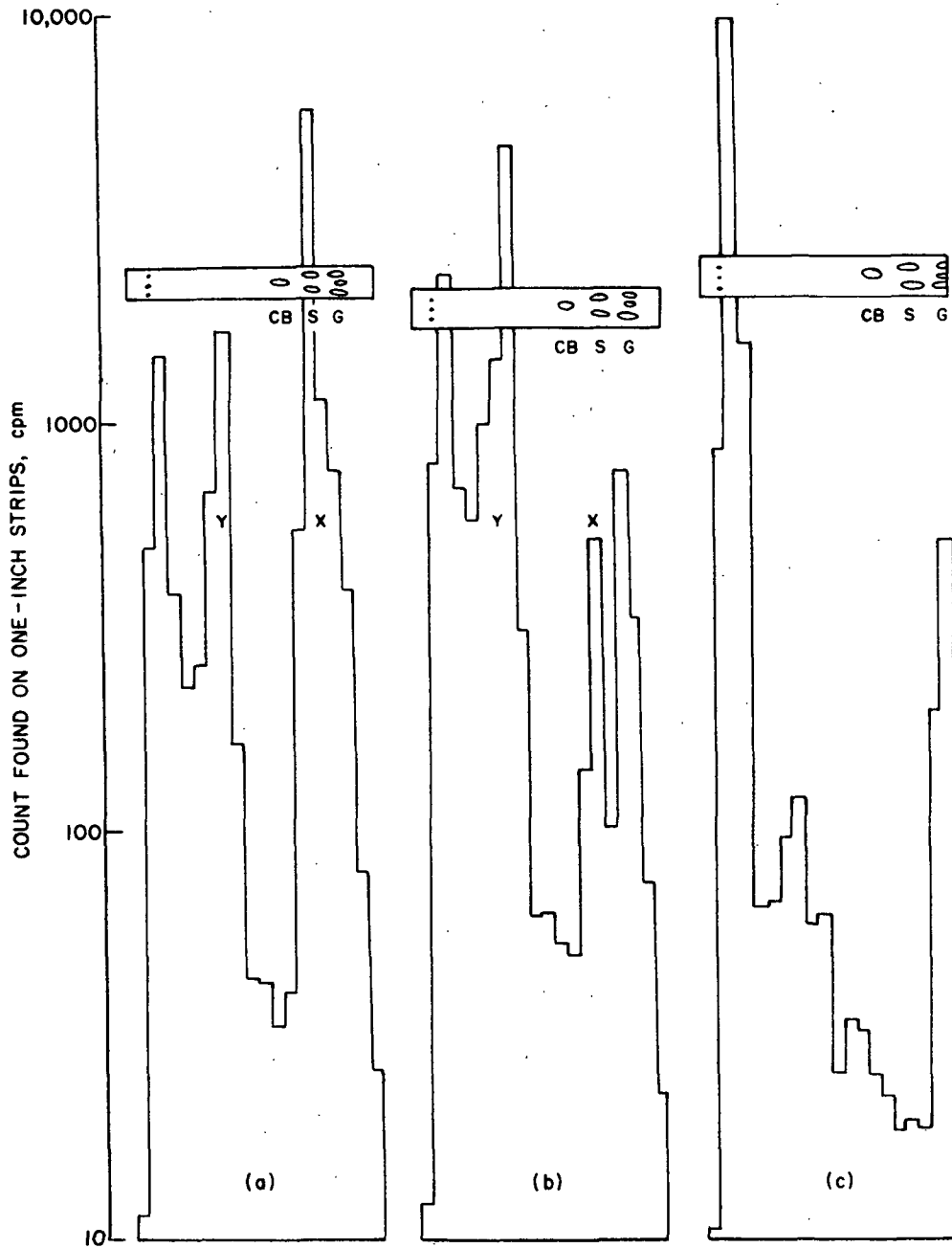


Figure 4. Paper Chromatography Results for Samples in Which ^{14}C -G-1-P and UTP Were Incubated with Crude Callus Extract

Standard G-1-P samples were used. Samples (a) and (c) were also 100 mM in fructose whereas Sample (b) contained no exogenous fructose. Samples (a) and (b) each contained 190- μg protein/ml of sample. Sample (c) was a no-extract control. Paper chromatograms were developed for 50 hours with Solvent IA.

Y = Compound Y; X = Compound X; CB, S, and G designate standard cellobiose, sucrose and glucose spots.

referred to as Compound Y. This material was identified as UDPG as discussed later.

Each histogram in Fig. 4 represents the counting data for the paper chromatogram for an aliquot of the particular reconstituted incubation sample. Development of the chromatograms was from left to right as presented in the figure. Each vertical bar in an individual histogram represents the count measured on a 1-inch strip from the chromatogram. The spotting origin for each chromatogram was located in the center of the second strip from the top of the chromatogram which is represented in each histogram by the second vertical bar from the left. Pictorial representations of the detected standard portions of each chromatogram are superimposed upon the histograms to show the mobility of the standards as compared to the mobility of the various count peaks detected. The same format for presenting counting data from paper chromatographic analysis of incubation samples is used throughout the presentation of experimental results.

Fructose was added to the incubation sample represented by Fig. 4(a), but there was no exogenous fructose added to the sample represented by Fig. 4(b). The sample represented by Fig. 4(c) was a no-extract control which contained an equivalent volume of buffer in place of the extract used in the other two samples. It should be noted that when fructose was added to the incubation sample, the production of Compound X was enhanced at the expense of the production of Compound Y.

Specific activities of 485 and 157 Compound X cpm/ μ g protein/2-hr incubation period were obtained for Samples (a) and (b), respectively. The specific activities for Compound Y in these two samples were 44 and 413 Compound Y cpm/ μ g protein/2-hr incubation period. During the early phases of the research, 2-hour incubation times were commonly used. The specific activities based upon

the 2-hour incubation time should not be taken as rates since later work indicated that the reactions catalyzed by purified enzyme preparations proceeded quite rapidly and were complete within a matter of minutes. The specific activities presented here were useful, however, in comparing the results obtained with crude extracts using various levels of fructose in incubation mixtures.

The results presented in Fig. 4 were obtained with extract which had been prepared by grinding the tissue in 0.1M tris-HCl buffer, pH 8.0, instead of the MOPS buffer which was more commonly used. The use of this buffer did not, however, change the overall pattern of the results obtained as can be seen from the results presented in Fig. 5, which were obtained using extract from tissue ground in MOPS buffer.

Effect of Fructose Concentration on Formation of Compound X and Compound Y

The effect of fructose concentration upon the formation of Compound X and Compound Y from ^{14}C -G-1-P and UTP by the crude callus extract was explored further. Samples containing varying amounts of fructose were prepared and incubated with crude extract as described in Fig. 5. Specific activities obtained from these results and the protein analysis of the crude extract are presented in Table III: It was observed from the results presented in the figure and the specific activities presented in the table that, as fructose concentration in the incubation samples increased, the production of Compound X was enhanced at the expense of the production of Compound Y.

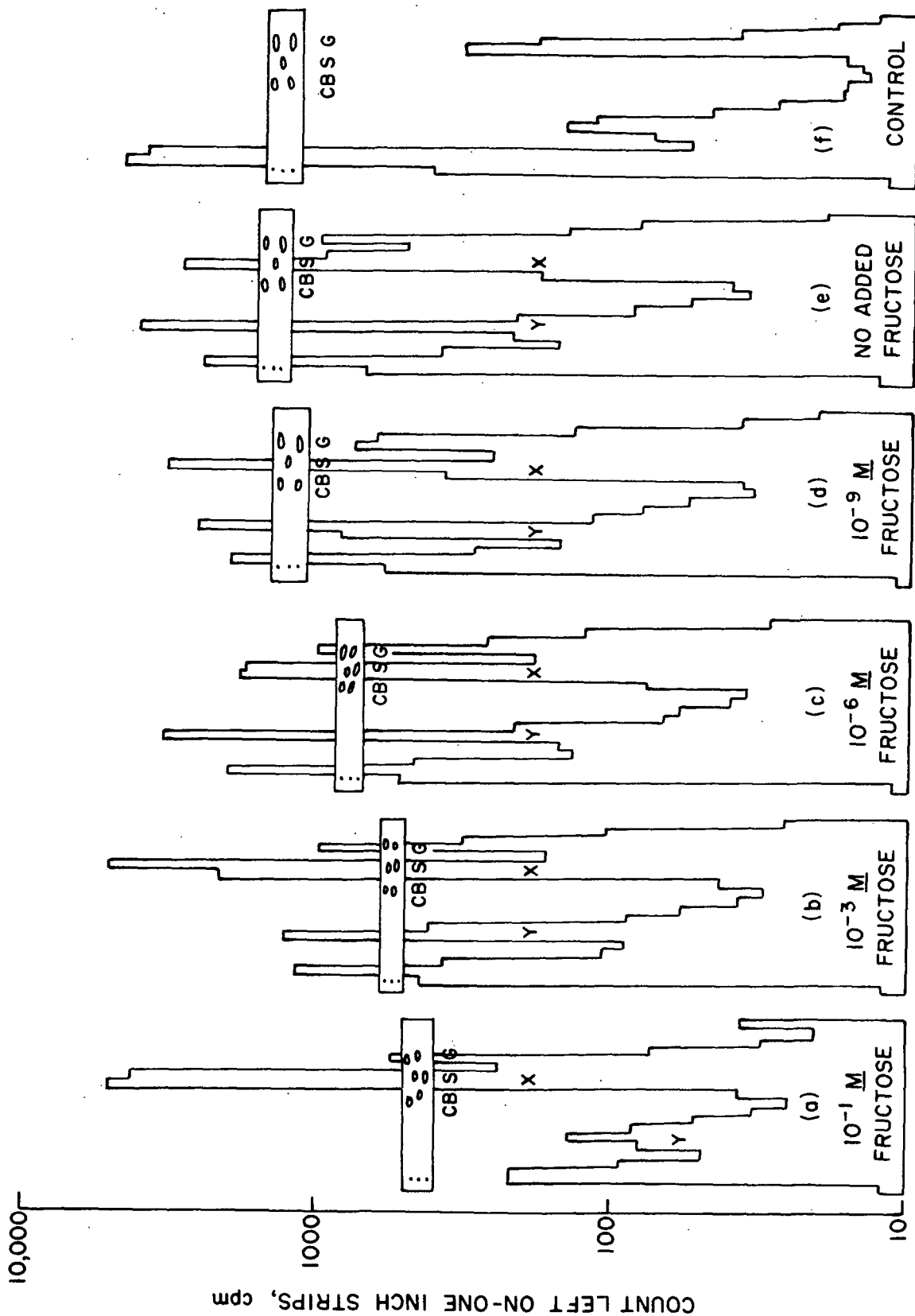


Figure 5. Paper Chromatography Results Showing Effect of Fructose upon Formation of Compound X and Compound Y

Standard G-1-P samples were used except that each sample contained fructose as indicated. Sample (f) was a no-extract control whereas the others each contained 380- μ g protein/ml of sample. Incubation and analysis were similar to that described in Fig. 4.

Y = Compound Y; X = Compound X; CB, S, and G designate cellobiose, sucrose, and glucose standard spots.

TABLE III
EFFECT OF FRUCTOSE CONCENTRATION

Fructose Concentration ^a	Compound <u>Y</u> Specific Activity ^b	Compound <u>X</u> Specific Activity ^b
10 ⁻¹	4	350
10 ⁻³	57	255
10 ⁻⁶	126	125
10 ⁻⁹	118	120

^aMolar.

^bCpm/ μ g protein/2-hr incubation.

Effect of Fructose-6-phosphate Concentration on Formation of Compound X and Compound Y

Standard G-1-P samples containing different fructose-6-phosphate concentrations and 530 μ g protein per ml of sample were incubated and analyzed as in Fig. 4.

Specific activities for the formation of Compound X and Compound Y versus the fructose-6-phosphate (F-6-P) concentration are presented in Table IV.

TABLE IV
EFFECT OF F-6-P CONCENTRATION

F-6-P Concentration ^a	Compound <u>Y</u> Specific Activity ^b	Compound <u>X</u> Specific Activity ^b
10 ⁻¹	19	45
10 ⁻³	18	59
10 ⁻⁶	32	80
10 ⁻⁹	30	80

^aMolar.

^bCpm/ μ g protein/2-hr incubation.

These data show that F-6-P had no stimulating effect upon the production of either Compound X or Compound Y from UTP and ¹⁴C-G-1-P in the presence of crude callus extract. In fact, F-6-P had an inhibiting effect.

Effect of Mg⁺⁺ Ion and Substitution of GTP for UTP

Standard G-1-P samples, except as noted in Table V, contained 530 µg protein per ml of sample and were incubated and analyzed as discussed in Fig. 4. The results of this experiment are presented in Table V.

TABLE V
EFFECT OF Mg⁺⁺ ION AND GTP

MgCl ₂ ^a Concn.	UTP Concn. ^a	GTP Concn. ^a	Fructose Concn. ^a	Compound <u>Y</u> Specific Activity ^b	Compound <u>X</u> Specific Activity ^b
2	0.23	--	100	7	278
--	0.23	--	--	36	106
--	0.23	--	100	6	165
2	--	0.24	100		37
2	--	0.24	--		5

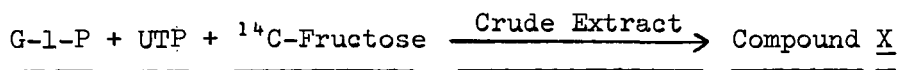
^aMillimolar, mM.

^bCpm/µg protein/2-hr incubation.

The specific activities for Compound X and Compound Y production presented in Table V were calculated by subtracting the cpm obtained in the Compound X or Compound Y regions of control chromatograms from the cpm found in these same regions on sample chromatograms and dividing the result from this calculation by the amount of protein contained in the spotting aliquot. The stimulation of Compound X synthesis when fructose was added to the incubation samples was again demonstrated by the results presented in Table V. It could be seen also

from the data in the table that magnesium ion aided this synthesis. GTP could be substituted for UTP when incubating ^{14}C -G-1-P with crude extract, but it was not nearly so effective in promoting synthesis of Compound X as was UTP.

The results presented so far indicated that Compound X could be sucrose. The results given in Fig. 4 and 5 showed that Compound X had a paper chromatographic mobility identical to that of authentic sucrose. Addition of fructose to incubations of ^{14}C -G-1-P and UTP with crude callus extract enhanced the production of Compound X. If Compound X was indeed sucrose and was produced by the reaction scheme presented in the Introduction [Reactions (1) and (2)], it should be possible to produce Compound X using ^{14}C -fructose as the labeled substrate.



An attempt was made to produce Compound X by incubating nonradioactive G-1-P, UTP and ^{14}C -fructose with crude callus extract. The results of this effort are presented in Fig. 6. Figure 6(a) shows the counting data for the sample containing the extract. Figure 6(b) shows the counting data for a no-extract control. A peak of radioactivity corresponding to Compound X was found in the chromatogram of the extract-containing sample; such a peak did not appear in the control sample. This indicated that radioactivity was incorporated into Compound X from radioactive fructose. Comparison of the results for the two samples also showed a peak of radioactivity at the spotting origin of the chromatogram for the extract-containing sample; this peak did not appear in the control. This product may have been one of the fructose phosphates resulting from an activity other than sucrose synthetase in the crude extract; however, the identity of this product was not pursued further.

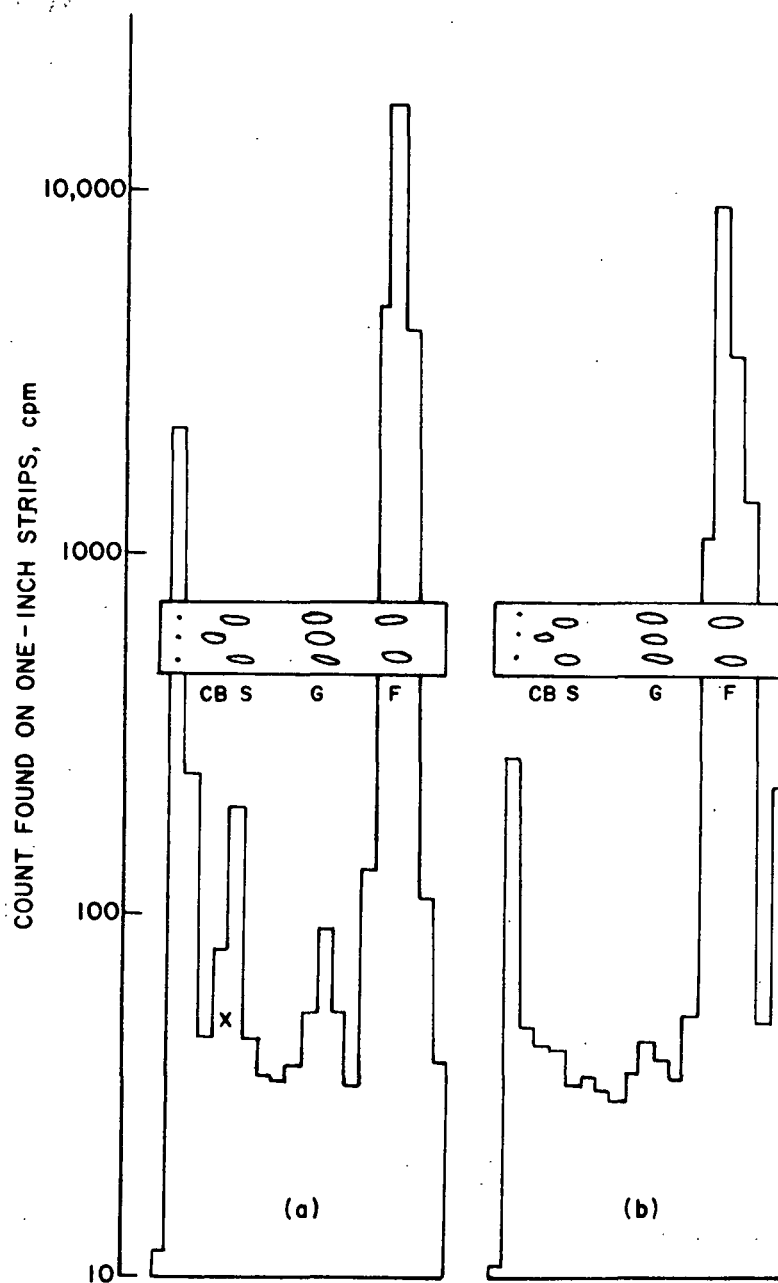
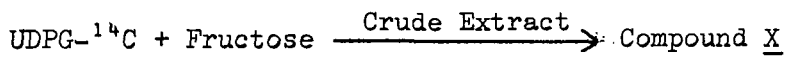


Figure 6. Paper Chromatographic Results for the Incubation of ^{14}C -Fructose with G-1-P and UTP

Samples were 2 mM in MgCl_2 , 1.8 mM in G-1-P, and contained 0.33 μCi ^{14}C -fructose (185 $\mu\text{Ci}/\mu\text{mole}$)/ml of sample. Sample (a) contained 400- μg protein/ml of sample. Sample (b) was a no-extract control. Samples were incubated for 2 hours at 30°C. Paper chromatograms were developed with Solvent III for 72 hours.

X = Compound X; CB, S, G, and F represent cellobiose, sucrose, glucose, and fructose standard spots.

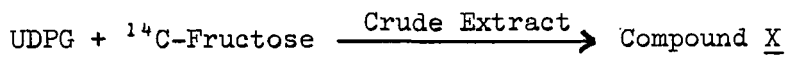
DIRECT EVIDENCE FOR SUCROSE SYNTHETASE IN
CRUDE CALLUS EXTRACT



If Compound X were actually sucrose and were produced by Reaction (2), it should be possible to introduce radioactivity into Compound X from UDPG-¹⁴C when this is incubated with fructose in the presence of crude callus extract. This was done as can be seen from the assay of radioactivity presented in Fig. 7.

The DE-81 anion exchange paper binding technique was also used in this experiment. Aliquots of the reconstituted samples were spotted on DE-81 circles which were then washed with ddH₂O on filter funnels. The washed paper discs were counted in Cocktail D in the liquid scintillation counter. Averages of 316 cpm for Sample (a), 568 cpm for Sample (b), and 1060 cpm for Sample (c) were found on the paper circles after washing. Since the paper binds UDPG, the lower the count remaining on the paper, the more labeled UDPG was consumed during the incubation period. Thus, more labeled UDPG was consumed during the incubation period by the sample which had exogenous fructose than by the sample to which no fructose was added.

The specific activity calculated for Sample (a) from the chromatographic results was 42.5 Compound X cpm/μg protein/2-hr incubation period.



In order to lend more support to the case for synthesis of Compound X (or sucrose) from UDPG and fructose by an activity in the crude callus extract, non-radioactive UDPG was incubated with radioactive fructose in the presence of callus extract as described in Fig. 8. The results presented in this figure show that the product was formed from the two substrates in the presence of the extract.

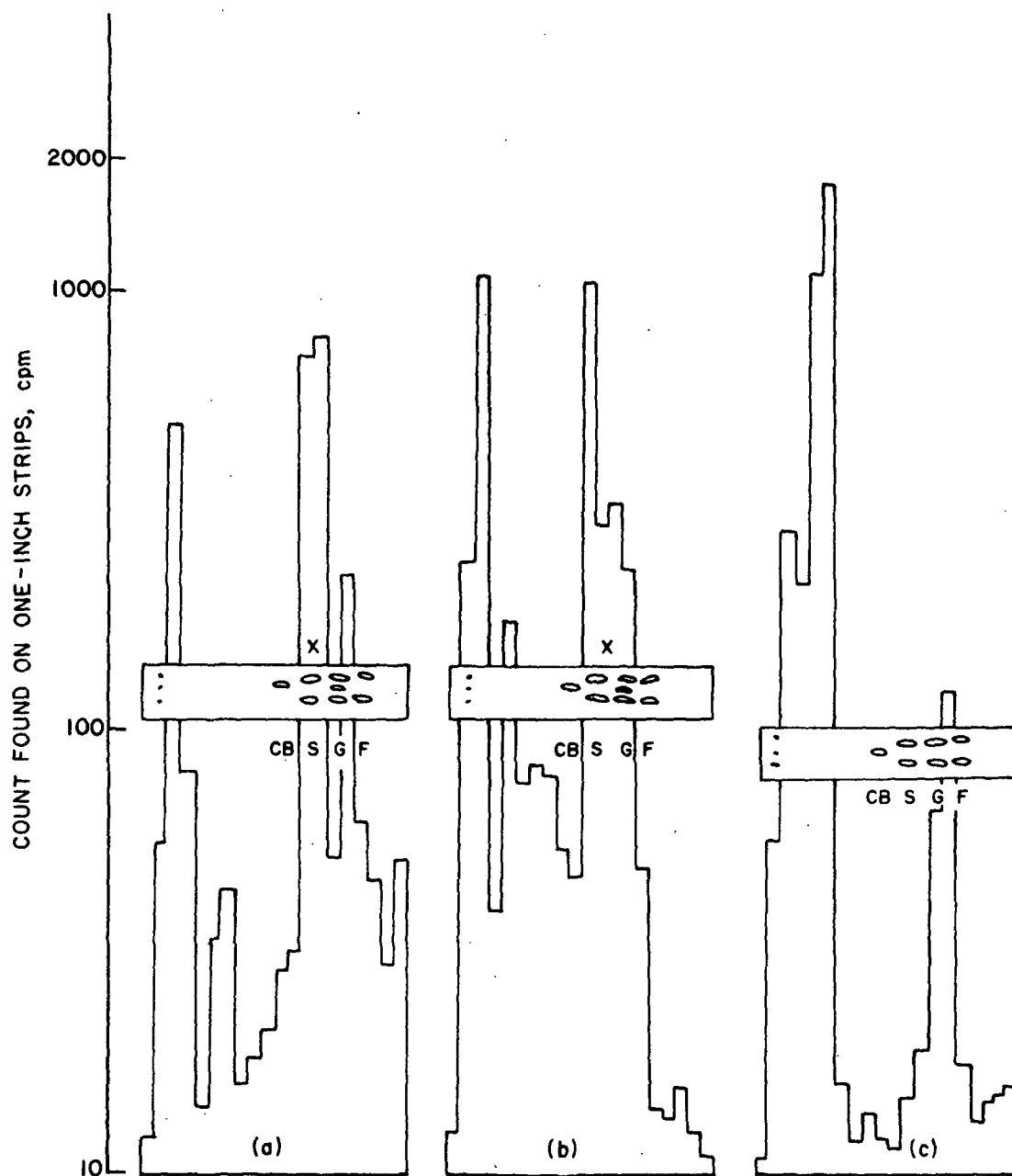


Figure 7. Paper Chromatographic Results for the Incubation of Labeled UDPG and Fructose with Crude Callus Extract

Standard UDPG samples were used except that the fructose concentration in Samples (a) and (c) was 1 mM and there was no exogenous fructose in Sample (b). Samples (a) and (b) each contained 450- μ g protein/ml of sample. Incubation and analysis were similar to that discussed in Fig. 4.

X = Compound X; CB, S, G, and F designate cellobiose, sucrose, glucose, and fructose standard spots.

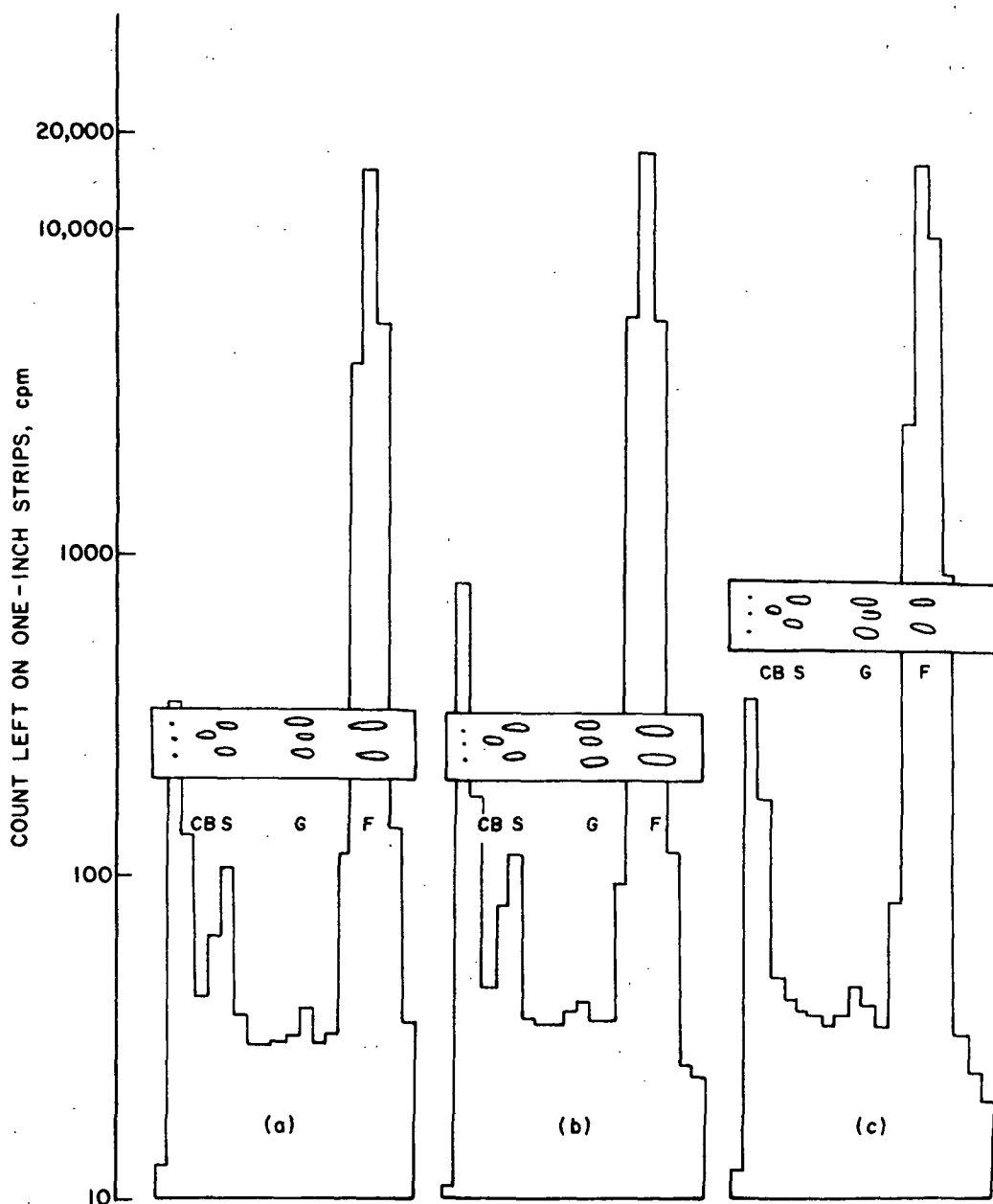


Figure 8. Paper Chromatographic Results for Incubation of Unlabeled UDPG and ^{14}C -Fructose with Crude Callus Extract

Samples were 2 mM in MgCl_2 and 26.6 μM in UDPG and contained 0.4 μCi ^{14}C -fructose (185 $\mu\text{Ci}/\mu\text{mole}$)/ml of sample. Samples (a) and (c) were also 1 μM in unlabeled fructose. Samples (a) and (b) contained 400- μg protein/ml of sample. Sample (c) was a no-extract control. Incubation and analysis were identical to that discussed in Fig. 6.

X = Compound X; CB, S, G, and F designate cellobiose, sucrose, glucose, and fructose standard spots.

Incubation of ^{14}C -Sucrose and UDP with Crude Callus Extract

In order to demonstrate the actual existence of sucrose synthetase in the crude callus extract, it should be shown that the reverse of Reaction (2) can also be promoted by the extract. This was not demonstrated easily. The data for various sucrose synthetases from different sources given in Table I indicated that the equilibrium for this reaction in vitro is favored in the direction of sucrose synthesis rather than in the direction of sucrose cleavage to form UDPG and fructose. A number of workers (26,31,42,45) have had difficulty initiating the cleavage reaction with purified sucrose synthetase. The data presented in this section, however, show that a small amount of radioactivity was transferred by the crude callus extract from uniformly labeled sucrose to material which remained at the spotting origin of paper chromatograms, developed with Solvent III. Amounts of radioactivity equivalent to that at the chromatogram origins were bound to DE-81 paper. More radioactive fructose and glucose were found in the incubation samples which contained extract than were found in the no-extract control samples.

Samples containing 4 μCi of ^{14}C -sucrose (360 $\mu\text{Ci}/\mu\text{mole}$) were 0.66 mM in UDP and were adjusted to pH 6.0. Samples (a) and (c) were also 2 mM in MgCl_2 . Samples (a) and (b) also contained callus extract whereas Sample (c) was a no-extract control. Samples were incubated for 2 hours at 30°C before being freeze-dried and reconstituted. Aliquots were spotted on paper strips which were then developed with Solvent III for 72 hours before being detected and counted in the usual manner. Aliquots were used also for the DE-81 binding assay method of analysis for sucrose synthetase activity. The results for both paper chromatography and DE-81 analysis are summarized in Table VI. A tabular summary of the counting data for the paper chromatograms is presented in Appendix V.

TABLE VI
EVIDENCE FOR SUCROSE CLEAVAGE REACTION

Sample	Count Left at Origin of Paper Chromatograms, cpm	Count Left on DE-81 Anion Exchange Paper, cpm	Apparent UDPG, cpm ^a	Apparent, UDPG, cpm ^b
(a) With MgCl ₂	635	835	189	198
(b) Without MgCl ₂	660	883	214	247
(c) Control	446	636		

^aFrom paper chromatography.

^bFrom anion exchange paper binding.

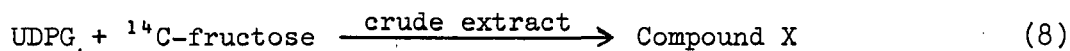
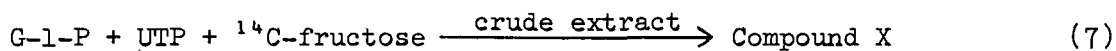
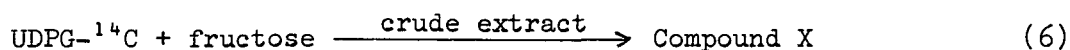
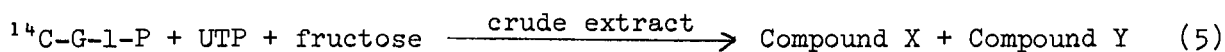
The results presented in Table VI show some apparent radioactive UDPG in the samples which contained extract (radioactivity remaining at spotting origin of chromatograms or not washed from DE-81) in excess of that found in the no-extract control sample. As will be shown later (Fig. 15) UDPG remains at the spotting origin of chromatograms developed with Solvent III. The binding of UDPG to the anion exchange paper is substantiated in Appendix III. Although the apparent UDPG counts are quite small compared to the total ¹⁴C-sucrose count contained in the samples, it was considered that they provided evidence for the synthesis of UDPG from sucrose and UDP by the crude callus extract.

The counting data presented in Appendix V showed that the extract-containing samples contained approximately 1.6 times as much ¹⁴C-glucose radioactivity as the no-extract control; in addition, the extract-containing samples contained approximately 2.8 times as much ¹⁴C-fructose as the no-extract control. Since the relative amounts of these two labeled sugars were nearly equal in the original uniformly labeled ¹⁴C-sucrose (Appendix III), these results also indicated

that some cleavage of the sucrose to form UDPG by the callus extract occurred during the incubation period.

DISCUSSION OF CRUDE EXTRACT INCUBATION RESULTS

The results presented in this section show that the following reactions were catalyzed by the crude extract obtained from triploid aspen callus tissue:



The radioactive reaction products, Compound X and Compound Y, were defined functionally in terms of their paper chromatographic mobilities. Compound X had a mobility similar to that of a disaccharide whereas the mobility of Compound Y was between the spotting origin and that of Compound X when Solvent IA was used to develop paper chromatograms which had been spotted with aliquots of the incubation samples.

Increasing fructose concentrations enhanced the production of Compound X at the expense of the production of Compound Y. Fructose-6-phosphate, on the other hand, inhibited the formation of both products. Since Compound X had a paper chromatographic mobility identical to that of sucrose and radioactivity was introduced into Compound X from either radioactive fructose or a substrate containing radioactive glucose, it seemed certain that Compound X was sucrose produced by a sucrose synthetase activity in the callus extract. If the reaction scheme proposed earlier [Reactions (1) and (2)] was responsible for the synthesis

of sucrose from ^{14}C -G-1-P and UTP, it seemed very likely that Compound Y was UDPG. The work discussed in the next section gave added support to these identifications.

The addition of magnesium ion to the incubation mixtures enhanced the formation of both products; however, the results do not indicate whether the effect was upon only one of the two enzymes, sucrose synthetase or UDPG-pyrophosphorylase, or upon them both. The same difficulty occurred in localizing the possible inhibitory effect of F-6-P upon the synthesis of both products.

UTP could be replaced to a limited extent by GTP in incubation mixtures in which crude callus extract was used. The reduced effectiveness of GTP, compared to that of UTP, could be due to low GDPG-pyrophosphorylase activity in the extract or a lower affinity for the guanine moiety than for the uracil moiety at the active site of sucrose synthetase.

The sucrose-cleavage or UDPG-synthesis reaction [reverse of Reaction (2)] was more difficult to demonstrate using crude callus extract. The equilibrium constant for Reaction (2) has been reported to be in favor of sucrose synthesis in vitro (21,24,36,48). However, some evidence for the promotion of sucrose cleavage to form UDPG by the crude extract was found in the results presented in Table VI and Appendix V.

IDENTIFICATION OF PRODUCTS

The results presented in the last section showed that two radioactive products resulted from the incubation of ^{14}C -G-1-P and UTP with the crude extract obtained from triploid aspen callus tissue. The identification of these two products by various means is discussed in this section.

Larger quantities of the two products, Compound X and Compound Y, were obtained by paper chromatography from samples such as those for which data were presented in Fig. 4 and 5. Several such samples were combined and spotted (several spots per chromatogram). The chromatograms were developed and one vertical strip from each chromatogram was counted to locate the horizontal portions of each chromatogram corresponding to the two products. The uncounted portions of the chromatograms, containing the two products, were then eluted with ddH₂O and the washings were freeze-dried. These freeze-dried samples of Compound X and Compound Y were used for identification of the products by various means including paper chromatography, incubation with enzymes, and crystallization as discussed in the following subsections. Compound X was identified as sucrose and Compound Y was identified as UDPG.

Paper Chromatography of Compound X

A sample of Compound X obtained by paper chromatography and containing 75 cpm/10 μ l was used to spot chromatograms which were developed using six different solvent systems. This sample of Compound X contained some ¹⁴C-glucose contamination; however, this contamination did not invalidate the results which are presented in Fig. 9. The counting data for the six different chromatograms are presented in this figure. The six different solvent systems used were as follows:

- IA n-butanol:pyridine:water (6:4:1)
- II ethyl acetate:acetic acid:water (3:3:1)
- III ethyl acetate:pyridine:water (8:2:1)
- IV n-butanol:ethanol:water (40:11:19)
- V ethyl acetate:acetic acid:formic acid (88%):water (18:3:1:2)
- VI n-butanol:acetic acid:water (4:1:5, organic phase)

The chromatograms were sprayed and counted in the usual manner.

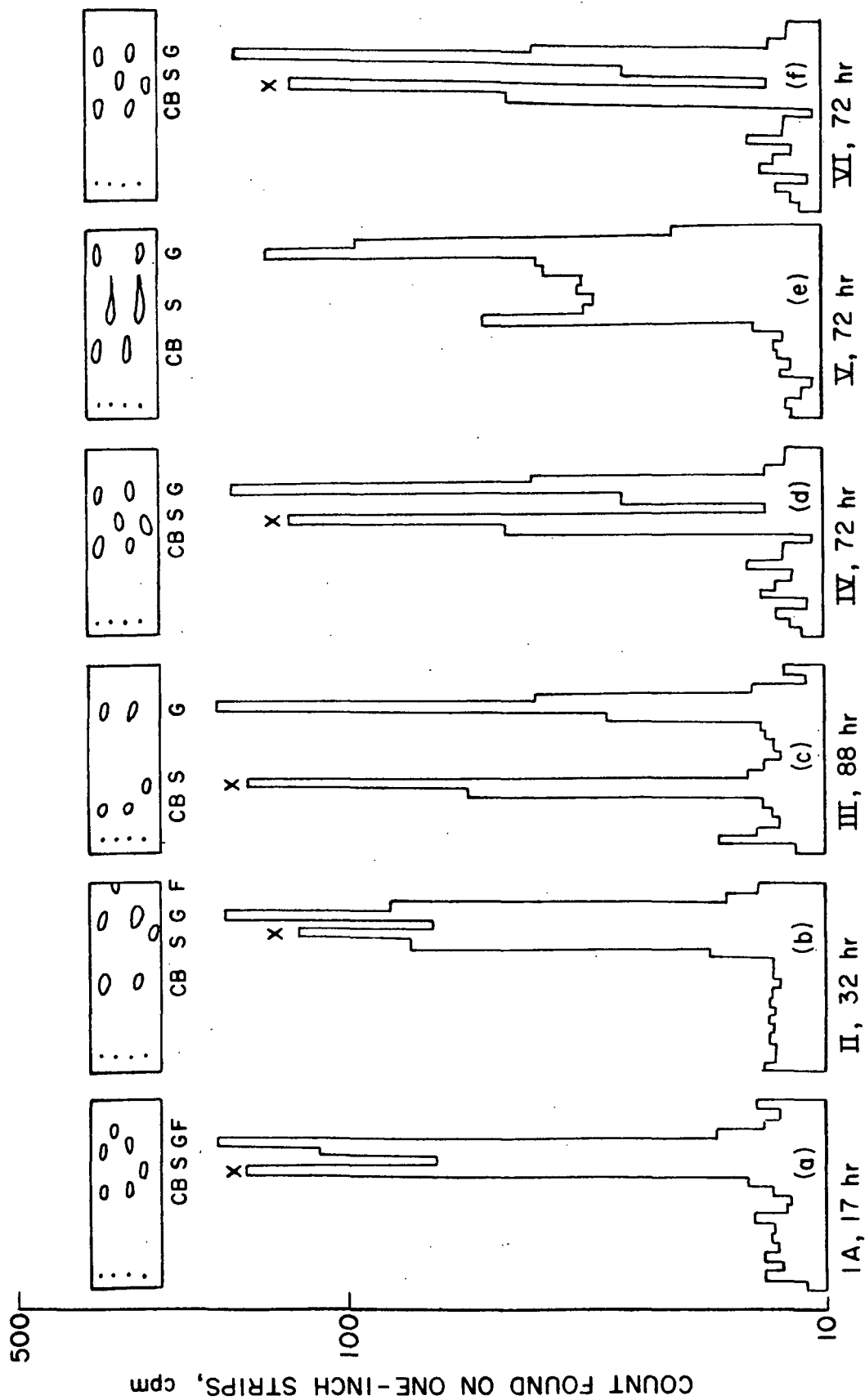


Figure 9. Results for Paper Chromatography of Compound X Using Six Different Solvent Systems

Paper chromatograms were developed as indicated in the figure. X identifies the Compound X peak. CB, S, G, and F designate the cellobiose, sucrose, glucose, and fructose standard spots.

Solvent system II was shown to separate trehalose, cellobiose, maltose, and laminaribiose from sucrose particularly well. It can be seen from the data presented in the figure that the count peak which had been referred to as Compound X corresponded to the standard sucrose on chromatograms developed using all six solvents.

Incubation of Compound X with Invertase

Invertase (β -D-fructofuranoside fructohydrolase, E. C. 3.2.1.26) is an enzyme capable of hydrolyzing sucrose to fructose and glucose. The invertase obtained from yeast catalyzes the hydrolysis of sugars possessing a terminal unsubstituted β -D-fructofuranosyl residue. The enzyme is much less specific as far as the afructone is concerned. It also has some transferase capability in that it will, to a limited extent, transfer the fructose removed to another alcoholic group such as an -OH group on sucrose or other sugars (54).

A sample of Compound X, prepared by paper chromatography, was not contaminated with ^{14}C -glucose and gave 165 cpm/10 μl . Aliquots (0.3 ml) of this sample of Compound X were freeze-dried in 25-ml Erlenmeyer flasks. An invertase solution containing 2-mg invertase/ml was prepared in 0.05M sodium acetate buffer (pH 5.0). The invertase was a highly purified preparation from Candida utilis yeast purchased from Sigma Chemical Co. Invertase solution (4.5 ml) was added to the flask containing freeze-dried Compound X labeled (A) while 4.5 ml of the sodium acetate buffer was added to the flask containing Compound X which was labeled (B). Both flasks were incubated in the shaking water bath at 50°C. After 15 minutes, 1.5-ml aliquots were removed from each flask and freeze-dried.

The freeze-dried aliquots from the samples were then each reconstituted with 0.1 ml ddH₂O. A 5- μ l aliquot of (A) gave 57 cpm whereas a 5- μ l aliquot of (B) (no-invertase control) gave 64 cpm. Paper strips were spotted with 30- μ l aliquots and developed using Solvent III for 72 hours before detection and counting. Counting results for the chromatograms for the sample and the control are presented in Fig. 10.

Examination of the data presented in Fig. 10 showed that the count peaks for sucrose and glucose trailed the standard sucrose and glucose spots. The paper chromatography results in Fig. 11 should justify the identification of the count peaks as sucrose and glucose, however. Sample 151-1 was a mixture of sucrose, glucose, and fructose dissolved in the acetate buffer. Sample 151-2 was a mixture of the same three sugars dissolved in the acetate buffer and heated at 50°C for one hour. Sample 151-3 was a mixture of the same three sugars dissolved in distilled water. These samples of standard sugars were spotted on paper strips which were then developed with Solvent III for 72 hours before being detected with the benzidine-trichloroacetic acid spray reagent. It can be seen from the pictorial representation of the detected chromatograms presented in Fig. 11, that the sugars dissolved in the acetate buffer trailed the standard spots whereas the sugars dissolved in distilled water did not.

Taking into account the reduced mobility of the sugars when the sodium acetate buffer was present, the data in Fig. 10 showed that the invertase enzyme removed radioactive glucose from Compound X. No significant amounts of radioactive fructose appeared; therefore, sucrose was synthesized, when ¹⁴C-G-1-P and UTP were incubated with crude callus extract, from nonradioactive fructose carried into the incubation samples with the crude callus extract itself or nonradioactive fructose added intentionally to the sample. The count peak at the origin of the chromatogram

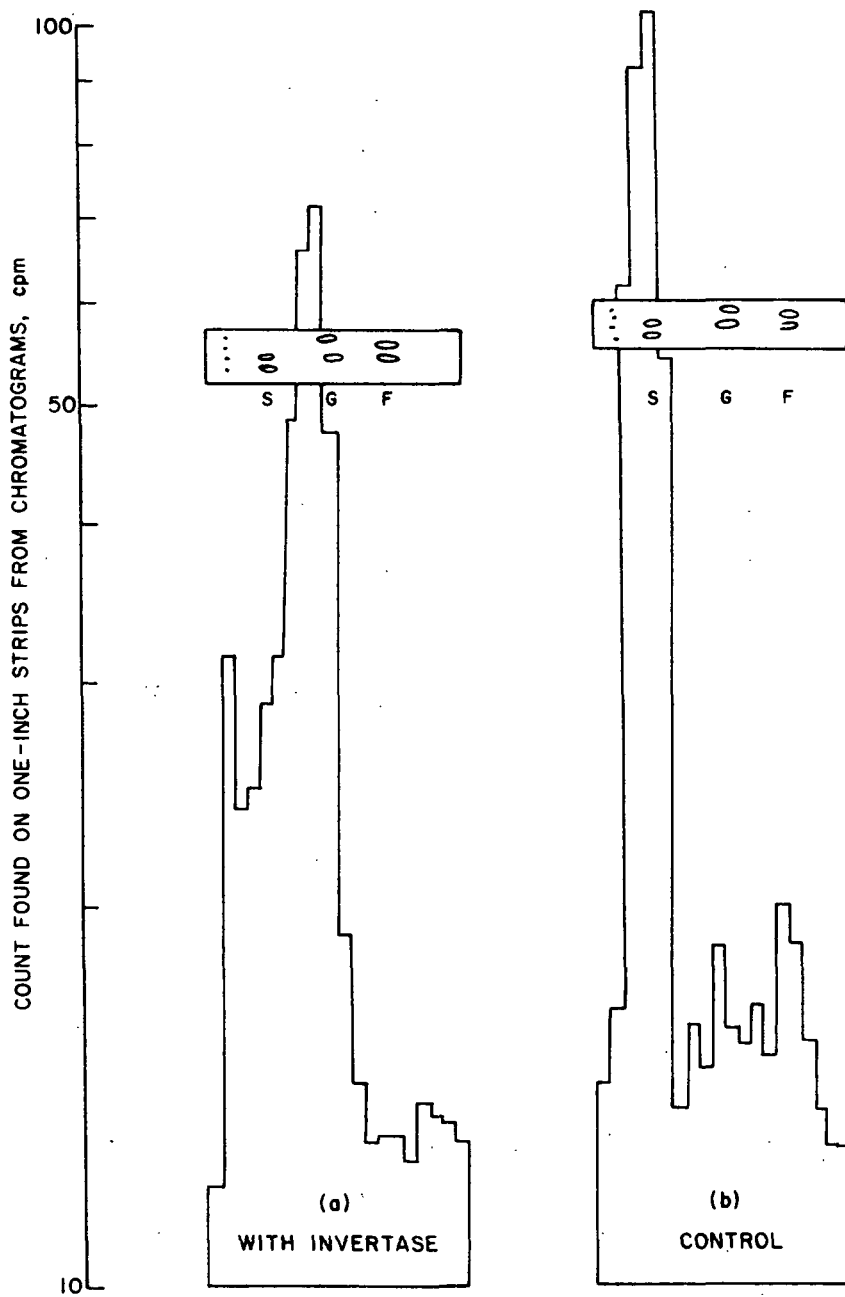


Figure 10. Paper Chromatographic Results for Treatment of Compound X with Invertase

S, G, and F designate the sucrose, glucose, and fructose standard spots. The experiment was conducted as described in the text.

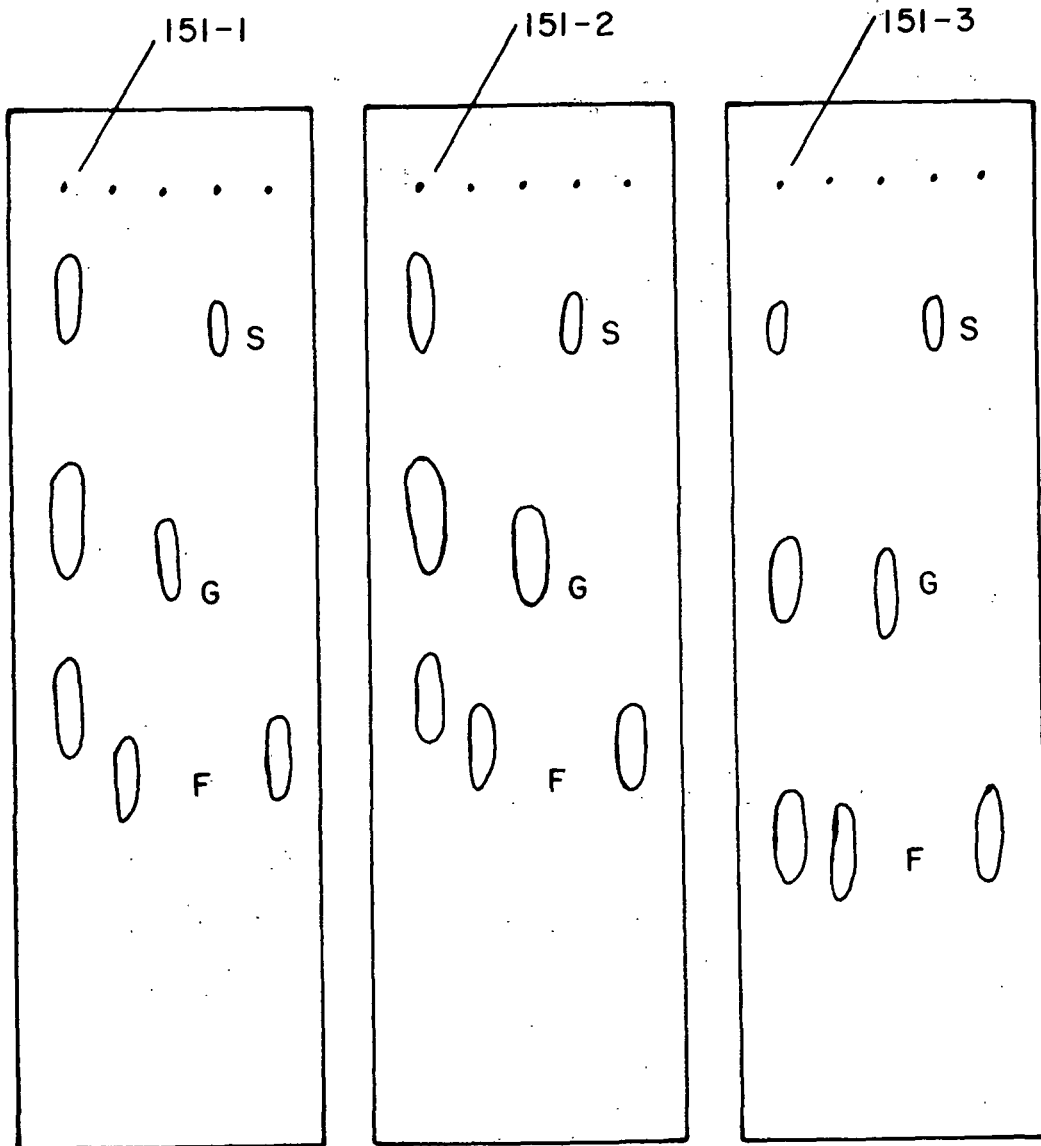


Figure 11. Effect of Acetate Buffer upon the Mobilities of Mixed Sugars

Experiment was conducted as described in text. S, G, and F designate sucrose, glucose and fructose standard spots.

for Sample (A) might have been a result of the transferase activity found in even highly purified invertase preparations from yeast.

The action of yeast invertase is quite specific for unsubstituted-fructose; therefore, the invertase must have removed a fructose residue from Compound X to give the radioactive glucose. This confirmed the hypothesis that Compound X was radioactive.sucrose.

Crystallization of Compound X with Nonradioactive Sucrose

A sample of Compound X contained 230 cpm/10- μ l aliquot when counted in 15 ml of Cocktail D and 2 ml ddH₂O. One gram of nonradioactive sucrose was added to 0.5 ml of this Compound X. This sample was then freeze-dried and subsequently dissolved in a minimum of hot water. Crystallization was induced by addition of ethanol. The resulting crystals were dried, and weighed portions of the crystals were counted in 15 ml Cocktail D and 2 ml ddH₂O. The dissolution and crystallization were repeated two more times. Weighed portions of crystals from each crystallization were counted as before. The data for this experiment, which confirmed the identity of Compound X as sucrose, are presented in Table VII.

TABLE VII

CRYSTALLIZATION OF COMPOUND X WITH SUCROSE

Crystallization	I	II	III
Yield, g	0.76	0.5	0.25
Cpm/mg crystals ^a	10.79	10.83	10.62

^aCorrected for background.

The average specific activity for the three crystallizations was 10.75 cpm/mg crystals whereas the value expected based upon the amount of Compound X count and nonradioactive sucrose used was 11 cpm/mg crystals. These results confirmed that Compound X was sucrose. Other miscellaneous results which also supported this conclusion are presented in the next section.

Miscellaneous Product Identification

Acid hydrolysis of Compound X showed, as was shown using invertase, that the only radioactive component of Compound X was ^{14}C -glucose. Anion exchange chromatography showed that although Compound Y was bound to the ion exchange resin used, Compound X was not bound, confirming the nonionic nature of Compound X.

Acid Hydrolysis of Compound X

A 0.2-ml aliquot of a sample of Compound X obtained by paper chromatography and containing 76 cpm/5 μl was immersed with 3.0 ml of 0.1M HCl in a boiling water bath for 1.5 hours before being freeze-dried. The freeze-dried sample was then reconstituted with 0.2 ml ddH₂O and still gave 75 cpm/5 μl aliquot. A 25- μl aliquot of this reconstituted sample was spotted against standards on Whatman No. 1 paper and developed for 46 hours with Solvent III before detection and counting. The counting data for this chromatogram are presented in Fig. 12. Paper chromatographic results for the original sample of Compound X can be found in Fig. 9(c). The hydrolysis results showed that ^{14}C -glucose was the only radioactive component of Compound X. This conclusion was identical to that reached after Compound X was hydrolyzed by highly purified invertase.

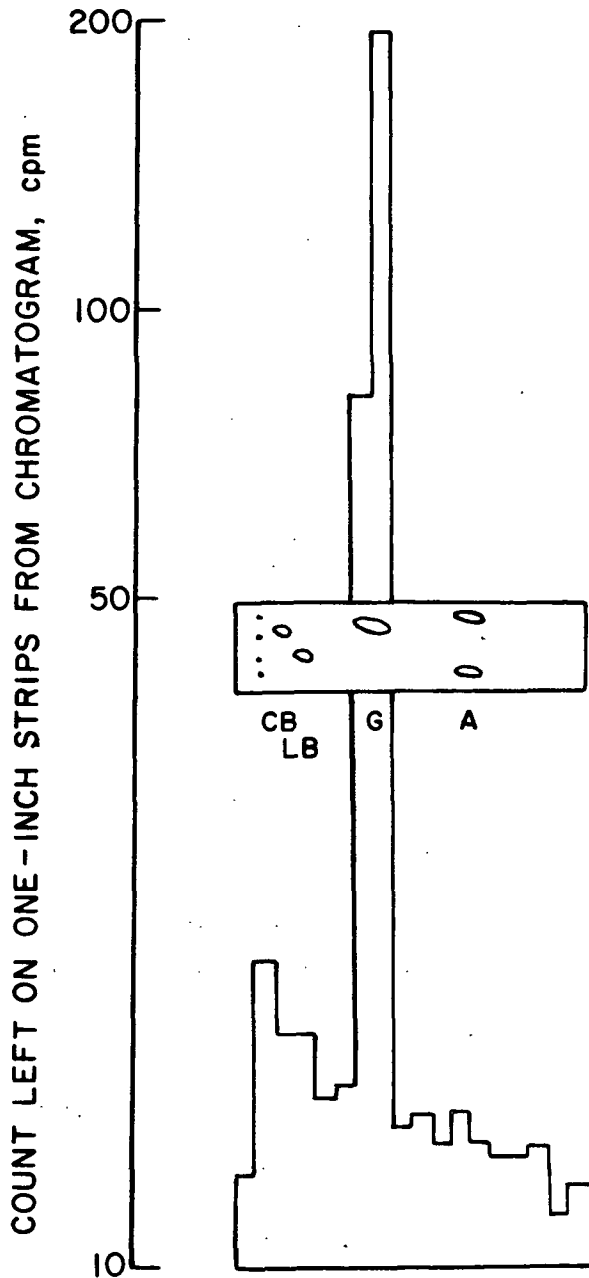


Figure 12. Paper Chromatogram for Hydrolyzate of Compound X

CB, LB, G, and A designate cellobiose, laminaribiose, glucose, and arabinose standard spots.

Sucrose or Compound X would have appeared between cellobiose and glucose.

Anion Exchange Resin Chromatography

Anion exchange resin chromatography was used to investigate the ionic nature of both Compound X and Compound Y. Columns were prepared in Pasteur pipets using Bio-Rad AG 1-X2 anion exchange resin (chloride form). Samples in which ^{14}C -G-1-P and UTP had been incubated with crude callus extract were combined and freeze-dried as were no-enzyme control samples in which the same substrates had been used. These two freeze-dried samples were reconstituted with ddH₂O and were referred to as composite sample and composite control, respectively. The composite sample gave 13,400 cpm/5- μ l aliquots whereas the value found for the composite control was 7,080 cpm/5- μ l aliquot. Counting data for paper chromatograms of these two composites are presented in Fig. 13(a) and (b). One of the prepared columns was loaded with 0.5 ml of the composite sample while the other was loaded with 1.0 ml of the composite control. Both columns were eluted with ddH₂O and the eluate was collected in 5-ml fractions. The fractions were freeze-dried and reconstituted with 0.1 ml ddH₂O. Paper chromatograms were prepared using the reconstituted fractions as indicated in Fig. 13. The counting data for the chromatograms for the first two 5-ml fractions eluted from the composite sample column are presented in Fig. 13(c) and (d) whereas the counting data for the first 5-ml fraction from the composite control column are presented in Fig. 13(e).

These results showed that although Compound X and glucose were eluted from the anion exchange column, Compound Y was retained when the column was eluted with water. Thus, Compound X was shown to be nonionic whereas Compound Y was ionic in nature, lending support to the conclusion that Compound Y was UDPG.

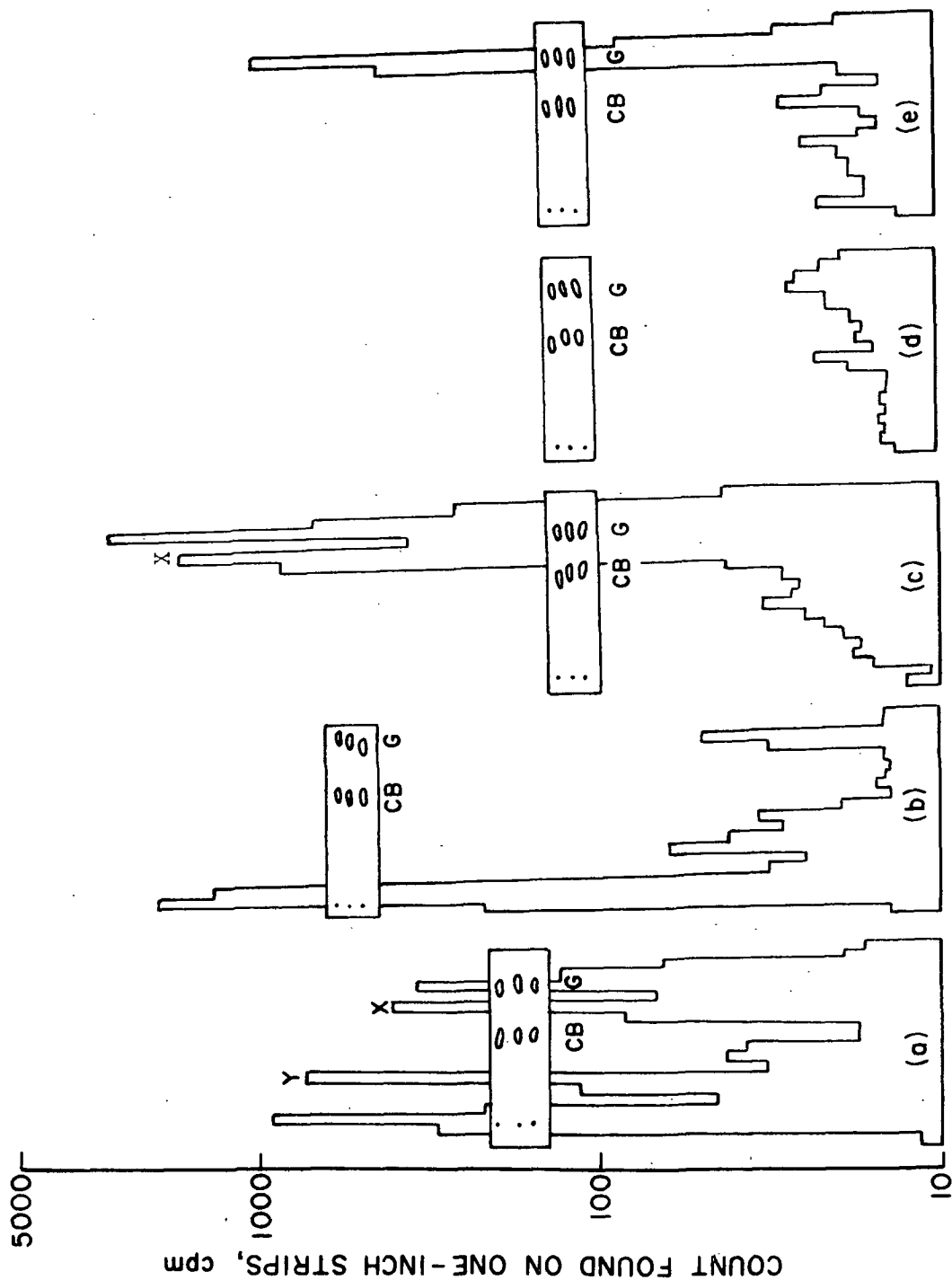


Figure 13. Paper Chromatographic Results for Ion Exchange Resin Chromatography

Sample (a) was the composite sample before column chromatography. Sample (b) was the composite control before column chromatography. Sample (c) was the first 5-ml fraction of eluate from the composite sample column. Sample (d) was the second 5-ml fraction from the composite sample column. Sample (e) was the first 5-ml fraction from the control column. Chromatograms were developed using Solvent IA for 50 hours.

Y = Compound Y; X = Compound X; CB and G designate cellobiose and glucose standard spots.

Acid Hydrolysis of Compound Y

In order to ascertain the nature of the radioactive moiety in Compound Y, a sample of this product which contained 190 cpm/5- μ l aliquot, obtained by paper chromatography, was submitted to acid hydrolysis. An 0.1-ml aliquot was immersed in a boiling water bath with 3.0 ml of 0.1M HCl for 1 hour before freeze-drying. Another 0.1-ml aliquot was freeze-dried as a control. Both were reconstituted with 0.1 ml ddH₂O. The reconstituted hydrolyzate contained 112 cpm/5- μ l aliquot whereas the control contained 174 cpm/5- μ l aliquot. Twenty-five- μ l aliquots of each were spotted on paper. The chromatograms were developed with Solvents IA, II, and III as indicated in Fig. 14. It can be seen from the results in Fig. 14 that the count in Compound Y was found primarily in a radioactive glucose moiety after acid hydrolysis. The two count peaks found when Compound Y was chromatographed with Solvent IA were typical of Compound Y even when a sample obtained from only one of the two peaks was eluted and rechromatographed with this solvent. This behavior, two peaks with Solvent IA, was also typical of UDPG in this solvent system, as will be seen in the next section.

Comparison of the Paper Chromatographic Behavior of Compound Y and UDPG

The counting data for chromatograms of Compound Y developed with Solvents IA, II, and III, which were presented in Fig. 14, are presented again in Fig. 15 along with counting data for similar chromatograms of authentic UDPG-¹⁴C. The similarity of the results obtained for Compound Y and UDPG-¹⁴C was striking, and this led to the conclusion that Compound Y was UDPG. Although the proof of this identification of Compound Y was not as rigorous as was the proof of the identity of Compound X as sucrose, the evidence for this identification was adequate to substantiate the claim that sucrose was synthesized from

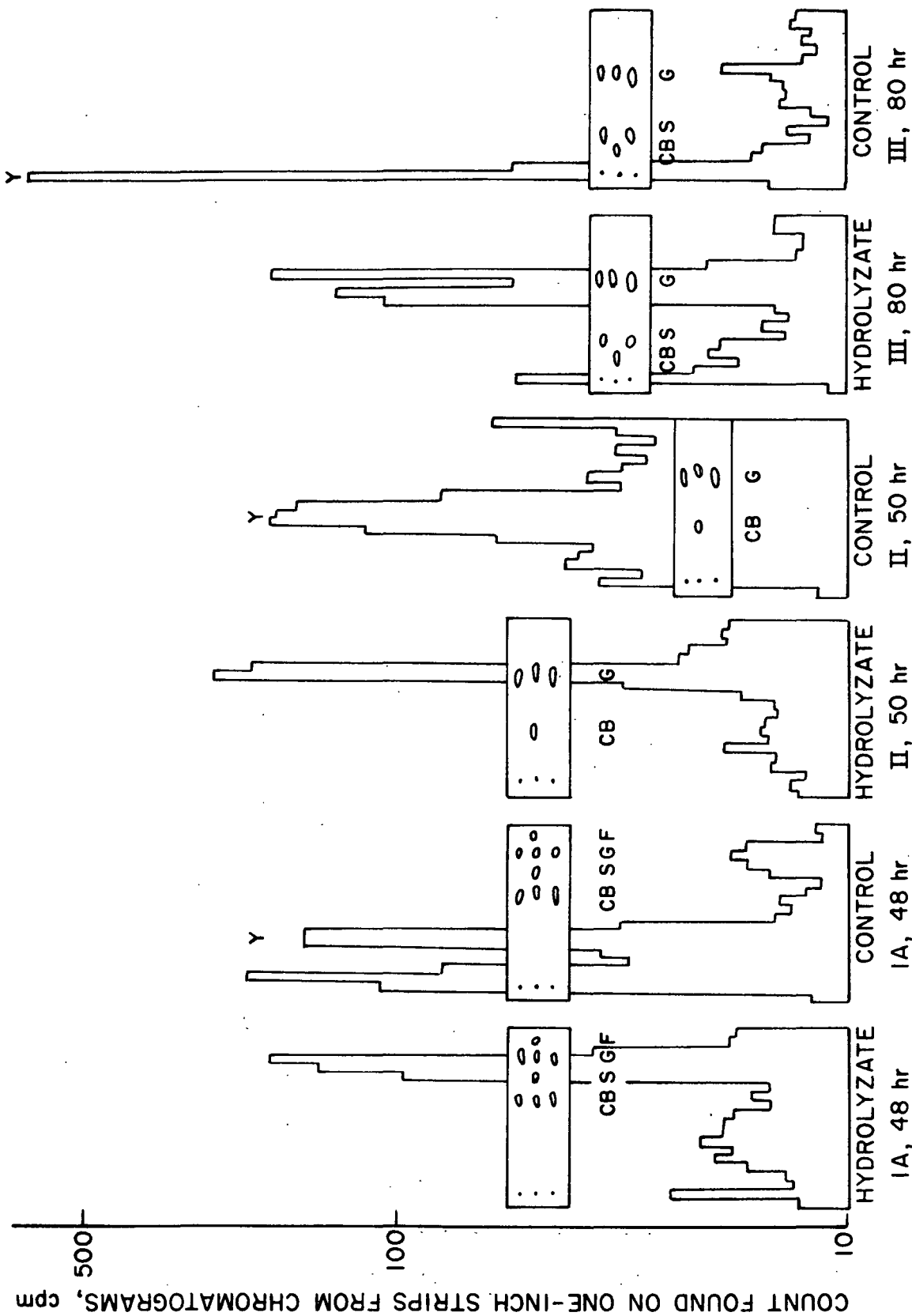


Figure 14. Paper Chromatographic Results for Hydrolysis of Compound Y

The experiment was conducted as indicated in the text. Paper chromatograms for the hydrolyzate and control were developed as indicated in the figure.

Y = Compound Y; CB, S, G, and F designate the cellobiose, sucrose, glucose, and fructose standard spots.

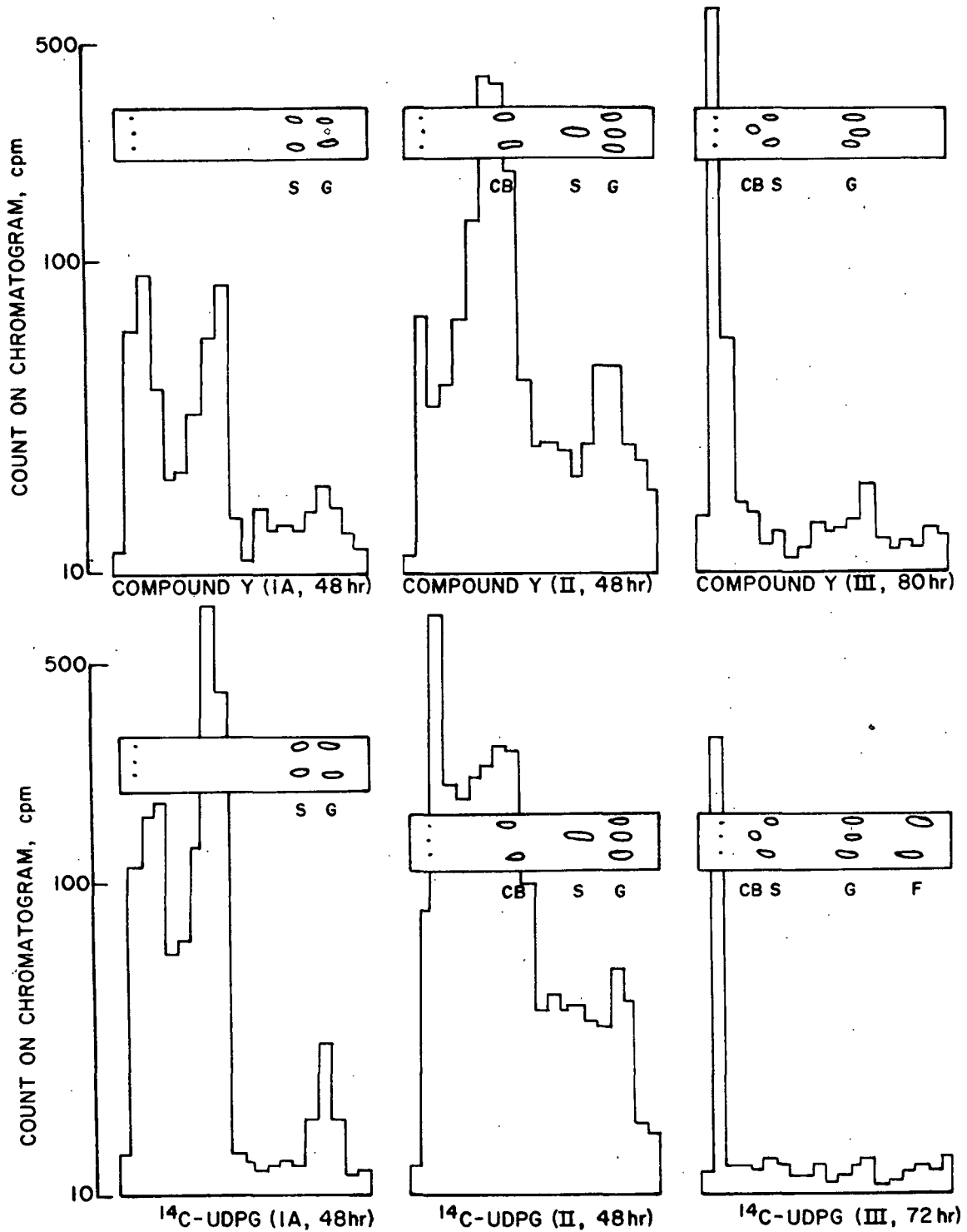


Figure 15. Paper Chromatographic Behavior of Compound Y and UDPG-¹⁴C

Paper chromatograms for Compound Y and labeled UDPG were developed as indicated in the figure. CB, S, G, and F designate cellobiose, sucrose, glucose, and fructose standard spots.

^{14}C -G-1-P and UTP in the presence of crude callus extract by the scheme [Reactions (1) and (2)] suggested earlier.

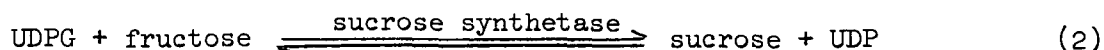
Note that the known UDPG- ^{14}C gave two radioactive peaks when chromatographed on paper using Solvent IA.

Summary of Product Identification

When ^{14}C -G-1-P and UTP were incubated with crude callus extract, two radioactive products, Compound X and Compound Y, were detected by means of paper chromatography and liquid scintillation counting of the paper chromatograms. Samples of Compound X and Compound Y were eluted from preparative paper chromatograms and subjected to various tests in order to identify Compound X as sucrose and Compound Y as UDPG. It previously was shown that the crude extract could incorporate radioactivity into Compound X from any of the radioactive substrates G-1-P (with UTP), UDPG, or fructose. When the concentration of fructose was increased in the crude extract incubation mixtures, the production of Compound X was enhanced at the expense of Compound Y. Paper chromatography of Compound X in six different solvent systems showed this product to have mobilities identical to those of authentic sucrose. Hydrolysis of Compound X, which had been produced from radioactive G-1-P and UTP by the crude extract, gave only radioactive glucose when the hydrolysis was conducted with either acid or invertase. The degradation of Compound X by the invertase enzyme showed that it had to contain an unsubstituted fructosyl moiety. The fact that Compound X could be crystallized to constant specific activity with authentic nonradioactive sucrose confirmed its identity as sucrose.

Anion exchange chromatography of Compounds X and Y showed that Compound X was not bound to the anion exchange resin whereas Compound Y was bound. Acid hydrolysis of Compound Y showed that the labeled moiety in this product was

¹⁴C-glucose. Paper chromatography of Compound Y in three different solvent systems showed that it had mobilities identical to those of authentic UDPG. This fact, coupled with the positive identification of Compound X as sucrose led to the conclusion that Compound Y was UDPG and that the two products were formed from ¹⁴C-G-1-P and UTP by the crude callus extract according to the following reaction scheme:



Since the tissue from which the extract was obtained was grown in the dark on a medium whose primary carbon source was sucrose, sucrose synthetase, functioning in the reverse direction to that shown in Reaction (2), would be a very important enzyme within the tissue for utilization of sucrose. Since sucrose is the primary carbon source in the various media upon which most cultured plant tissues are grown (12), the results suggest further, that sucrose synthetase is an important enzymatic constituent in any cultured plant tissue. Thorpe and Meier (12) found that this was the case for carrot and tobacco callus tissue.

THE SUCROSE SYNTHETASE FROM TRIPLOID QUAKING ASPEN CALLUS TISSUE

Before the properties of the sucrose synthetase from aspen callus tissue could be investigated or the reaction which it catalyzes studied, it was necessary to isolate and purify the enzyme.

PURIFICATION OF SUCROSE SYNTHETASE FROM ASPEN CALLUS

The procedure used for isolation of the sucrose synthetase from crude callus extract relied upon centrifugation, treatment with digitonin, salt fractionation and gel filtration chromatography. The details of the development of this procedure are discussed in the following subsections.

Enzymes Bound to Membranes

Crude extracts from the callus tissue were shown to catalyze the synthesis of sucrose from G-1-P, UTP, and fructose as well as from UDPG and fructose. The first attempt at isolation of the sucrose synthetase involved centrifugation at $100,000 \times g$.

Crude extract was prepared as previously described. A portion of this crude extract was centrifuged at $100,000 \times g$ for two hours at 5°C . The pellet obtained from the centrifugation was resuspended in 1 mM DTT in MOPS buffer (0.1M, pH 8.0) and the three different preparations (crude extract, $100,000 \times g$ supernatant, and resuspended pellet) were assayed for sucrose synthetase activity.

One set of assay samples (G-1-P substrate) were 2 mM in MgCl_2 , 2 mM in UTP and 1 mM in fructose and contained $0.33 \mu\text{Ci } ^{14}\text{C-G-1-P}$ ($262 \mu\text{Ci}/\mu\text{mole}$) per ml of sample. The other set of samples (UDPG substrate) were 2 mM in MgCl_2 , 1.67 mM in fructose, and $33 \mu\text{M}$ in nonradioactive UDPG and contained $0.67 \mu\text{Ci UDPG-}^{14}\text{C}$ ($275 \mu\text{Ci}/\mu\text{mole}$) per ml of sample. Each extract or centrifugal fraction was incubated in both types of samples for 2 hours at 30°C . Aliquots of the reconstituted samples were analyzed for labeled sucrose by paper chromatography. The results of this experiment are presented in Table VIII. These sample concentrations apply only to results in this subsection (Tables VIII and IX).

The results presented in Table VIII were surprising in that it had been assumed, based upon the experience of other workers using different sources for the enzyme, that the sucrose synthetase would be soluble. The only case found in the literature for an insoluble or particulate sucrose synthetase enzyme was for the preparation from lemon fruit (23). As can be seen from the data in Table VIII, the "insoluble" nature of the activity from aspen callus could be an aid to isolation of the enzyme in that nearly 90% of the protein

was removed by centrifugation at $100,000 \times g$ with relatively little loss of activity. These results also suggested that both the UDPG-pyrophosphorylase and the sucrose synthetase activities were bound to membrane fragments after preparation of the crude extract. This hypothesis was tested further by means of another experiment.

TABLE VIII

RESULTS FROM $100,000 \times g$ CENTRIFUGATION OF CRUDE EXTRACT

	Crude Extract	$100,000 \times g$ Supernatant	Redispersed $100,000 \times g$ Pellet
Protein content of fraction, mg/ml	1.75	1.3	0.13
Fraction volume, ml	15.8	15.4	10.0
G-1-P substrate samples			
Sucrose cpm/aliquot of reconstituted sample	1696	1059	999
Sucrose cpm/ μg protein/2-hour incubation	59	49	460
Yield, %	100	60	38
UDPG substrate samples			
Sucrose cpm/aliquot of reconstituted sample	835	435	923
Sucrose cpm/ μg protein/2-hour incubation	29	49	426
Yield, %	100	49	69

A crude extract was again prepared from the callus tissue in the usual manner. A portion of this extract was centrifuged at $12,000 \times g$ for 20 minutes at $5^{\circ}C$. The pellet from this centrifugation was resuspended in 1 mM DTT in MOPS buffer. A portion of the supernatant from this centrifugation was recentrifuged at $100,000 \times g$ for 2 hours at $5^{\circ}C$. The pellet from this centrifugation was also

TABLE IX

DISTRIBUTION OF ENZYME ACTIVITIES IN CENTRIFUGAL FRACTIONS

	Crude Extract	12,000 × g Supernatant	Redispersed 12,000 × g Pellet	100,000 × g Supernatant	Redispersed 100,000 × g Pellet
Protein content, mg/ml	1.65	0.75	0.25	1.46	0.23
G-1-P substrate samples					
Sucrose cpm/aliquot of reconstituted sample	3369	2044	3747	1488	4158
Sucrose cpm/μg protein/ 2-hour incubation	123	169	926	61	1160
UDPG substrate samples					
Sucrose cpm/aliquot of reconstituted sample	1949	1598	2162	1038	2382
Sucrose cpm/μg protein/ 2-hour incubation	71	132	533	42	650

resuspended in the MOPS buffer containing 1 mM DTT. The crude extract and various centrifugal fractions were assayed for protein and enzymatic activity in a manner identical to that used to obtain the results presented in Table VIII. The counting results for the sucrose portions of the paper chromatograms are presented in Table IX.

These data confirmed that both the UDPG-pyrophosphorylase and the sucrose synthetase activities sedimented together at the two different g forces. This supports the hypothesis that the enzymes are bound to membrane fragments of different sizes which resulted from the tissue grinding procedure. Although the removal of a large portion of the nonactive protein by high-speed centrifugation was advantageous, a means of solubilizing the sucrose synthetase in the resuspended $100,000 \times g$ pellet was necessary for further purification of this activity.

Liberation of Sucrose Synthetase from Membrane Fragments

Attempts to release the sucrose synthetase from membrane fragments by means of *n*-butanol extraction or extraction of acetone powders were unsuccessful. Treatments with digitonin was shown to be effective for this purpose, however. Digitonin treatment is frequently used to solubilize particulate enzyme preparations and its effectiveness is often taken as evidence for a membrane-bound activity (55). Residual digitonin in the treated preparations was found to interfere with the microbiuret protein determinations; therefore, digitonin-treated material was routinely passed through a Bio-Gel P-6 column to remove residual digitonin prior to use. The solubility of digitonin is limited and heating is required to put it into aqueous solution. During digitonin treatment of an extract, a large amount of digitonin came out of solution; therefore, before the treated material was passed over the Bio-Gel P-6 column, undissolved digitonin was removed by centrifugation.

A 100,000 × g pellet was prepared from crude extract and resuspended in the MOPS buffer (0.1M, pH 8.0) containing 1 mM DTT as described in the preceding section. This material was slowly agitated on a magnetic stirrer with an equal volume of 1.5% digitonin in the MOPS buffer for two hours at 5°C. This digitonin-treated extract was then centrifuged at 1000 × g for 15 minutes at 5°C to remove undissolved digitonin and the supernatant was loaded on a Bio-Gel P-6 column which had been equilibrated with the 1 mM DTT solution in MOPS buffer. The column was eluted with the DTT solution and the excluded volume was collected for assay. The column was monitored using a LKB Uvicord Type 4701A instrument which measured the ultraviolet absorbance of the column eluate at 254 nm. The excluded volume which was collected from the P-6 column showed strong absorbance at this wavelength.

The eluate from the P-6 column and the resuspended 100,000 × g pellet were assayed for sucrose synthetase activity by incubating aliquots in standard UDPG samples for 45 minutes. The reconstituted samples were analyzed by paper chromatography for radioactive sucrose. Results are presented in Table X.

TABLE X

SUCROSE SYNTHETASE ACTIVITY BEFORE AND AFTER DIGITONIN TREATMENT

	Redispersed 100,000 × g Pellet	Eluate from Bio-Gel P-6 Column
Protein content of fraction, mg/ml	0.82	0.69
Sucrose cpm/aliquot of reconstituted sample	926	942
Sucrose cpm/μg protein/45-min incubation	1130	1360

These results showed that sucrose synthetase activity equivalent to that found in the 100,000 × g pellet was recovered in the digitonin-solubilized material.

Salt Fractionation of Digitonin-Solubilized Material

In earlier experiments with the crude callus extracts, sucrose synthetase activity was precipitated by ammonium sulfate between 20 and 60% saturation; however, the bulk of the activity came down between 40 and 60% saturation. This corresponded with the experience of other workers with sucrose synthetase from other plant sources. In the isolation of sucrose synthetase from aspen callus tissue, the purpose of salt fractionation of digitonin-solubilized material was primarily to concentrate the activity for subsequent column chromatography; however, there was also some improvement in specific activity.

Salt fractionation was carried out by adding to the protein solution in the cold room, an amount of saturated ammonium sulfate solution adequate to achieve the desired percentage of saturation. After complete mixing of the two solutions, the resultant solution was allowed to stand for one-half hour before it was centrifuged at 12,000 × g for 10 minutes to remove the precipitated fraction. The pellet for each salt fraction was redissolved in the MOPS buffer which was 1 mM in DTT.

Salt fractions were prepared from digitonin-solubilized material which had been freed from digitonin by use of the Bio-Gel P-6 column. The P-6 eluate and the salt fractions were assayed for sucrose synthetase by incubation in standard UDPG samples for 4 minutes. Reconstituted samples were analyzed for radioactive sucrose by paper chromatography. Results are presented in Table XI.

TABLE XI

SALT FRACTIONATION OF DIGITONIN SOLUBILIZED MATERIAL

Fraction ^a	Protein Content, mg/ml	Sucrose Cpm/ Aliquot of Sample	Sucrose Cpm/ μ g. Protein/ 4-Min Incubation
Eluate from Bio-Gel P-6	0.19	888	936
0-20% (NH ₄) ₂ SO ₄ saturated fraction	0.053	165	623
20-60% (NH ₄) ₂ SO ₄ saturated fraction	0.087	860	1970
60-80% (NH ₄) ₂ SO ₄ saturated fraction	0.012	16	266
80-100% (NH ₄) ₂ SO ₄ saturated fraction	nil		

^aNone of the salt fractions were desalted prior to use.

These results showed a significant increase in specific activity in the 20-60% saturation of ammonium sulfate-precipitated material over that found in the eluate from the Bio-Gel P-6 column. The primary purpose of the salt fractionation, however, was to concentrate the preparation to a smaller volume than that obtained from the P-6 column, for loading onto the agarose column which was the final step in the sucrose synthetase purification procedure.

Gel Filtration Chromatography

Little success was obtained in attempting to purify the digitonin-solubilized P-6 eluate by DEAE-cellulose chromatography. Gel filtration using Bio-Gel A-5m was found to give a higher specific activity sucrose synthetase preparation. The sucrose synthetase was found to be too large for fractionation on Bio-Gel P-200 which has a fractionation range of from 40,000 to approximately 200,000 for globular proteins. The Bio-Gel A-5m fractionates globular proteins having

molecular weights ranging from 80,000 to 5,000,000. An example of the use of the Bio-Gel A-5m in this purification is summarized below.

A 20-60% saturation of ammonium sulfate fraction was prepared from the eluate obtained from the Bio-Gel P-6 column in the manner which has been discussed previously. This salt fraction was loaded on a Bio-Gel A-5m column which had been equilibrated with 1 mM DTT in MOPS (0.1M, pH 8.0). The column was eluted with the same DTT-containing MOPS buffer at a flow rate of 12.3 ml/hour. The column had a void volume of approximately 33 ml.

Both the agarose (Bio-Gel A-5m) and P-6 columns were monitored using the LKB Uvicord instrument to measure the ultraviolet absorption of the column eluates at 254 nm. In the case of the P-6 column, the sucrose synthetase activity was found to elute from the column in conjunction with a large amount of ultraviolet light-absorbing material; however, in the case of the agarose column, the large amount of ultraviolet light-absorbing material actually eluted from the column prior to the sucrose synthetase activity. The trace of ultraviolet absorption versus time for the agarose column is presented in Fig. 16. There was little or no noticeable ultraviolet absorption associated with the sucrose synthetase activity as can be seen from the results presented in Fig. 16; this might be due to either the relatively low concentration of this activity in the eluate or the low sensitivity of the monitoring instrument to protein, or both. The location of the sucrose synthetase activity in the fractions obtained from the agarose column required assay of the individual fractions by incubation of aliquots in standard samples. An example follows:

Six fractions were collected from the agarose column as indicated in Fig. 16. Fractions A and E contained 16.4 ml of eluate whereas the other four fractions each contained 12.3 ml of eluate. The fractions were assayed for

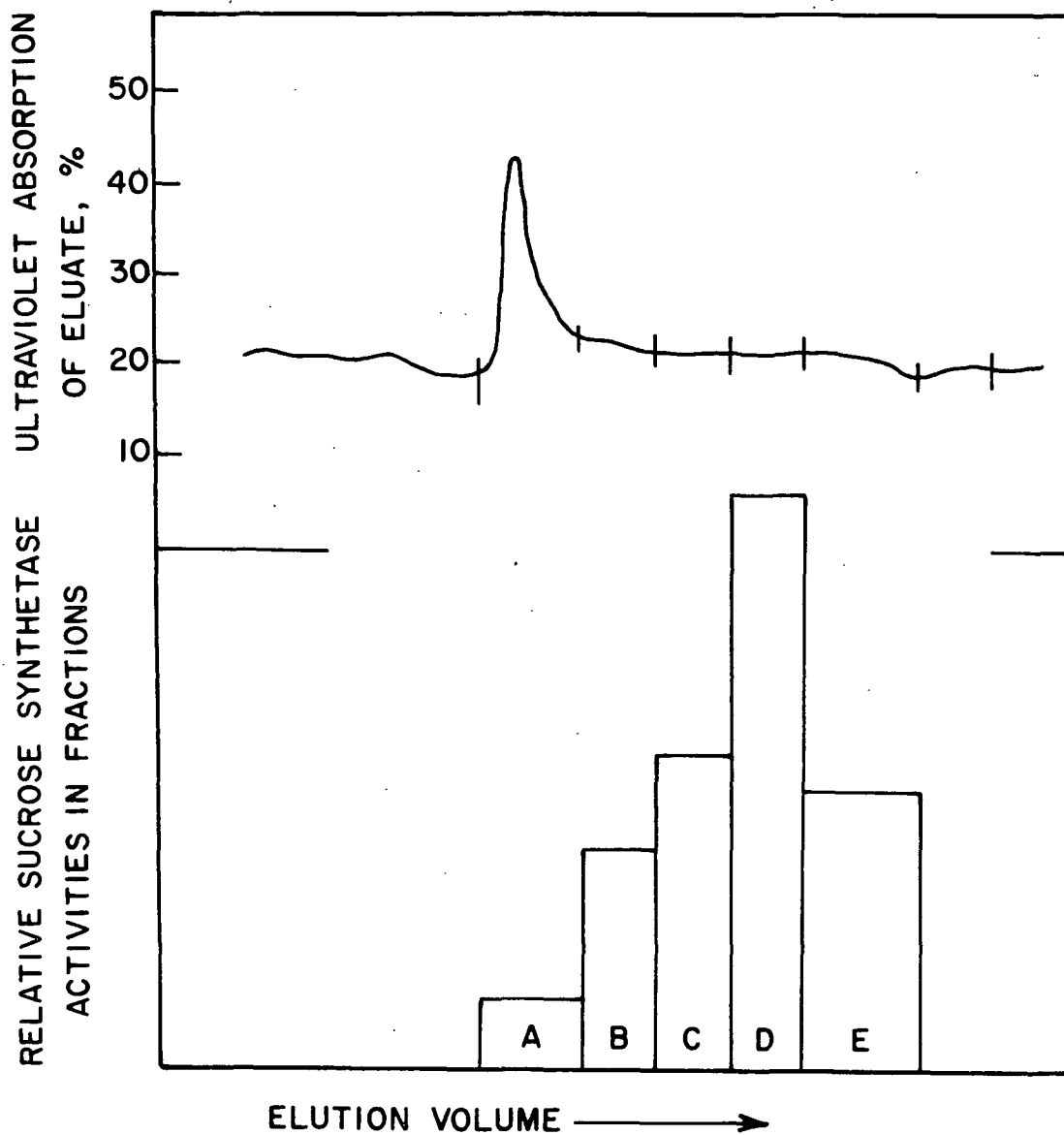


Figure 16. Agarose Column Chromatography of Sucrose Synthetase from Triploid Quaking Aspen Callus Tissue

Fractions A through E are described in the text. Ultraviolet absorption of the eluate was measured with the Uvicord instrument. Activities in fractions were measured using standard UDPG samples. Column void volume was 33 ml.

sucrose synthetase by incubating aliquots in standard UDPG samples for 5 minutes. Aliquots of the reconstituted samples were analyzed for sucrose production by the anion exchange paper binding technique. The apparent sucrose cpm reported in Fig. 16 and in Table XII were obtained by subtracting the count retained on the DE-81 paper circles for the particular fraction from that retained for a control sample in which boiled crude extract had been used in place of the agarose column eluate fractions.

The data in the table show the capability of the agarose column to further purify the salt fraction. Fraction A had high ultraviolet absorption, whereas there was no noticeable ultraviolet peak for Fraction D which contained the highest specific activity of sucrose synthetase. The Bio-Gel A-5m column had a void volume of approximately 33 ml. In order to eliminate a dilution problem, a smaller column was used for the rest of the sucrose synthetase preparations discussed below. This smaller column was used to obtain the results in the purification table presented in the next section.

It should be noted that the sucrose synthetase activity does not elute from the column as a sharp peak. Sucrose synthetase is thought to be a tetramer made up of four identical subunits. The breadth of the activity peak shown in Fig. 16 was probably due to breakdown of the tetramer on the agarose column. The activity peak was well within the included portion of the eluate.

Sucrose Synthetase Purification Procedure

The procedure for isolation of sucrose synthetase from dark-grown triploid aspen callus tissue developed during this research was relatively simple and straightforward. The fact that the enzymatic activity in the crude extracts obtained from the callus tissue was particulate and could be removed from a large portion of the inactive protein in the crude extract by centrifugation

TABLE XII

RESULTS FROM AGAROSE CHROMATOGRAPHY

	Protein Content, mg/ml	Count Left on DE-81 from Aliquot of Reconstituted Sample, cpm	Apparent Sucrose Cpm/Aliquot of Reconstituted Sample	Apparent Sucrose Cpm/ μ g Protein/5-Min Incubation
20-60% $(\text{NH}_4)_2\text{SO}_4$ saturation fraction	0.163	270	662	810
Agarose chromatography fractions				
A	0.114	842	90	154
B	0.059	793	139	471
C	0.049	773	159	648
D	0.048	644	288	1200
E	0.094	799	133	283
F	0.050	965	--	--
Boiled crude extract control		932		

helped simplify the overall procedure. The entire purification procedure is outlined in Fig. 17 and discussed in the following paragraphs.

Dark-grown triploid aspen callus tissue of an appropriate age (since its last subculture) was allowed to come to cold-room temperature before proceeding with the isolation procedure which was carried out at 5°C except for the actual incubations and analysis. The tissue was removed from the agar medium upon which it was grown and the dark centers of the callus pieces were removed with a cork borer of appropriate size. The remaining tissue was weighed and homogenized with 1 mM DTT in MOPS buffer (0.1M, pH 8.0) using a Ten Broeck tissue grinder. The resulting homogenate was centrifuged at 1000 × g to remove whole cells and debris. The pellet resulting from this centrifugation was discarded and the supernatant (crude callus extract) was centrifuged at 100,000 × g for 2 hours. The pellet from this centrifugation was resuspended in the DTT-containing MOPS buffer. The resuspended pellet was stirred slowly on a magnetic stirrer for 2 hours with an equal volume of 1.5% digitonin in MOPS buffer. The digitonin-treated material was centrifuged at 1000 × g to remove undissolved digitonin before being loaded on a Bio-Gel P-6 column to remove residual digitonin. The P-6 column was eluted with the DTT containing MOPS buffer. The eluate from this column was monitored with the Uvicord instrument. The excluded volume, which showed high ultraviolet absorption, was collected for further treatment.

An appropriate amount of saturated ammonium sulfate solution was added to the eluate from the P-6 column to bring the total solution to 20% saturation with ammonium sulfate. This solution was allowed to stand for 1/2 hour before being centrifuged at 12,000 × g for 15 minutes. The pellet from this centrifugation was discarded and the supernatant was mixed with enough saturated ammonium

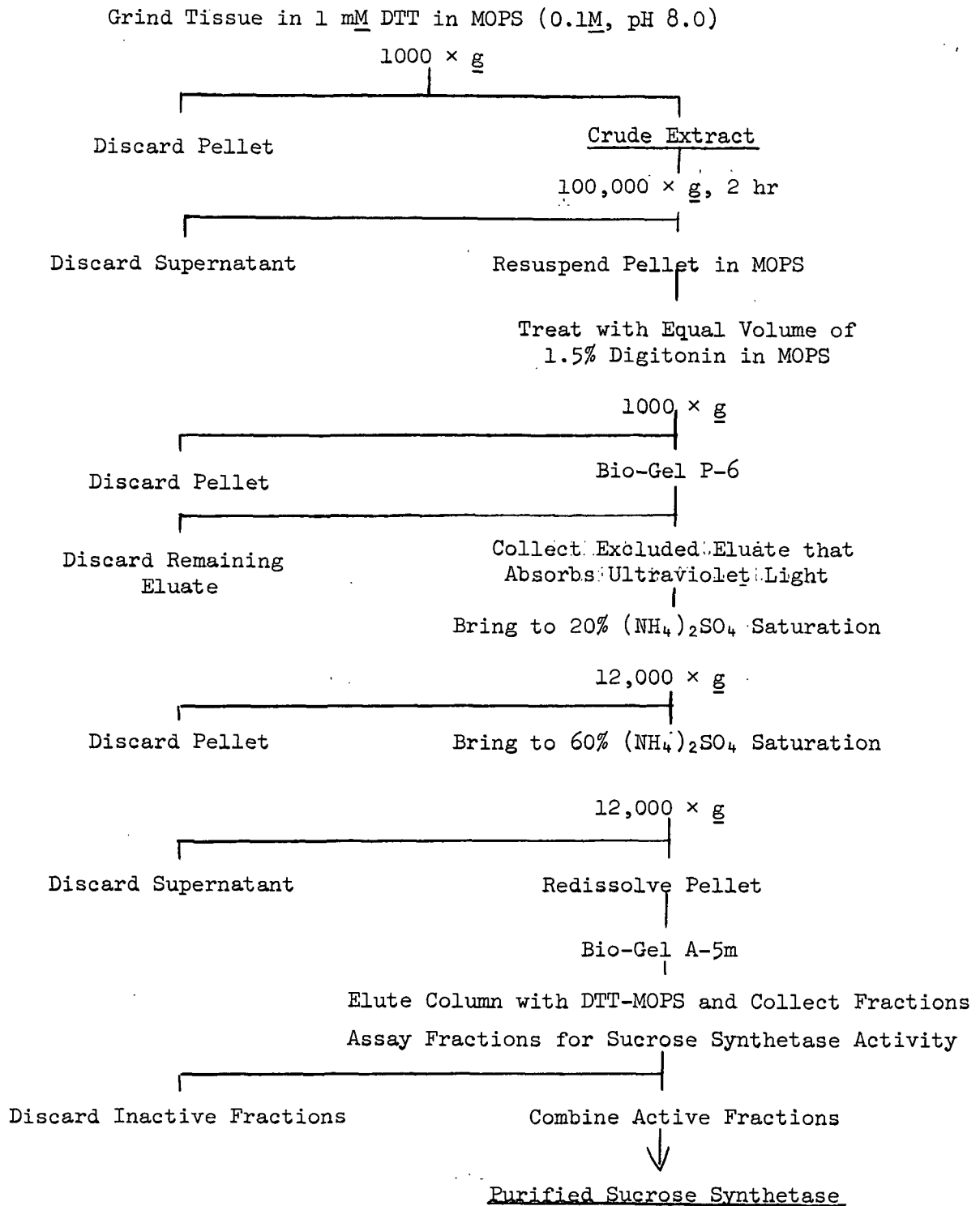


Figure 17. Purification of Sucrose Synthetase from Aspen Callus

Procedure carried out at 5°C. DTT-MOPS = 1 mM DTT in 0.1M MOPS, pH 8.0.

sulfate solution to bring the concentration in the solution to 60% saturation. After standing for 1/2 hour, the mixture was centrifuged at $12,000 \times g$ for 15 minutes. The pellet from this centrifugation was redissolved in the DTT-containing MOPS buffer. The redissolved fraction precipitated by 20-60% ammonium sulfate was loaded on a Bio-Gel A-5m (agarose) column which was approximately 1.6 cm in diameter and 14 cm in length. This column was eluted with the DTT-containing MOPS buffer and the eluate from the column was collected in fractions by means of an equal-time fraction collector and monitored with ultraviolet absorption.

The fractions of eluate collected from the agarose column were assayed for sucrose synthetase activity by incubating aliquots in standard UDPG samples for 4 minutes. Assay samples were analyzed for disappearance of UDPG using the DE-81 anion exchange paper binding technique. The fraction(s) which showed the highest sucrose synthetase activity by this assay method was used to study properties of sucrose synthetase.

Results of one such purification (2.43-fold) of the sucrose synthetase from aspen callus are presented in Table XIII. Purifications of the preparations discussed in the following sections ranged from 1.73- to 4.4-fold with an average for five different preparations of 2.89-fold. This degree of purification was low compared with many of the preparations from different tissues presented in Table I. The specific activity of the sucrose synthetase preparation summarized in Table XIII was 0.186- μ mole sucrose formed per mg protein per minute. Although this value was quite low compared with values for many of the preparations presented in Table I, it compared favorably with the activities of the preparations from wheat germ and mung bean seedlings which had much higher degrees of purification. If this enzyme were a very predominant portion

TABLE XIII

PURIFICATION OF SUCROSE SYNTHETASE FROM TRIPLOID ASPEN CALLUS TISSUE

	Specific Activity, μ mole sucrose produced/ mg protein/ min	Recovery of Protein from Crude Extract, %	Purification	Recovery of Enzyme Activity, %
Crude extract	0.076	100	1.0	100
Eluate from P-6 column	0.105	37	1.37	51
20-60% $(\text{NH}_4)_2\text{SO}_4$ saturation fraction	0.150	9	1.96	17
Active agarose column eluate fractions	0.186	5	2.43	13

108

^aFour-minute incubations.

of the total protein content of the callus tissue, the relatively high specific activity combined with the relatively low degree of purification would be reasonable. The calculations of specific activity and degree of purification are summarized in Appendix VI. These results indicated nearly 5% of the protein in the aspen callus tissue could be sucrose synthetase.

Disk Electrophoresis

An attempt was made to follow the purification of sucrose synthetase from aspen callus tissue using disk electrophoresis by a technique similar to that of Davis (56). With this technique a single protein was found in the 20-60% salt fraction; it may have been sucrose synthetase. However, there was a considerable amount of staining at the top of all of the gels for the various fractions tested. The gels for the eluate from the agarose column showed four protein bands which indicated breakdown of the tetrameric enzyme when eluted from Bio-Gel A-5m. Because of the staining of the gels at the top, the electrophoresis results were not used as a criterion of purity for the enzyme obtained from agarose chromatography. Pictures of representative gels are presented in Appendix X.

Separation of Sucrose Synthetase from UDPG-Pyrophosphorylase

A sample of the enzyme obtained from the agarose chromatography procedure, which gave 0.243- μ mole sucrose/mg protein/minute with radioactive UDPG, was used to check for the presence of UDPG-pyrophosphorylase activity in this preparation. Standard G-1-P samples which were also 100 mM in fructose were incubated and analyzed for radioactive sucrose by the paper chromatography method. Crude extract incubation samples gave 155-sucrose cpm/ μ g protein/2 hours; however, samples in which the active agarose column fractions had been assayed gave only 8 sucrose cpm/ μ g protein/2 hours. This demonstrated that

the majority of the UDPG-pyrophosphorylase activity found in the crude extract was removed by the purification procedure.

PROPERTIES OF THE SUCROSE SYNTHETASE FROM ASPEN CALLUS TISSUE

Once the sucrose synthetase was isolated from dark-grown aspen callus, it was possible to examine some of its characteristics and investigate the sucrose synthesis reaction.

Stability

It was observed that there was a rapid loss of activity in crude extracts with time even when they were kept in the cold room. This loss of activity could be minimized by incorporation of DTT in the MOPS buffer used for tissue homogenization. DTT is used widely to stabilize sulfhydryl enzymes against oxidation (57). DTT-containing buffer was used in all subsequent work, and it was not determined if the purified enzyme required protection from sulfhydryl oxidation.

However, the effects on the purified enzyme of storage in the cold room were evaluated. Three 2-ml aliquots of purified enzyme containing 0.104-mg protein/ml were each mixed with 3 ml of saturated ammonium sulfate solution and allowed to stand in the cold room for 1/2 hour before centrifugation at 12,000 × g for 15 minutes. The supernatants were discarded. One pellet was immediately redissolved in 2.0 ml of the DTT-containing MOPS buffer and assayed for sucrose synthetase activity using standard UDPG samples. After one week another pellet was redissolved and assayed; at the same time, the redissolved pellet from the previous week was reassayed. After two weeks, the third pellet was redissolved and the redissolved pellets were assayed for sucrose synthetase activity. All

pellets and redissolved pellets were stored in the cold room in closed containers during the entire period.

The results of this experiment are presented in Table XIV. These results showed that it was preferable to store the enzyme as an ammonium sulfate-precipitated pellet than as a redissolved enzyme. Storage of the enzyme in the freezer was not investigated because freezing and thawing destroyed the activity.

TABLE XIV
EFFECT OF STORAGE ON SUCROSE SYNTHETASE ENZYME ACTIVITY

Storage Condition	Sucrose Cpm/Aliquot of Reconstituted Sample	Activity Retained, %
Enzyme before precipitation with salt	728	
Salt-precipitated pellet, redissolved immediately after centrifugation	552	
After one week	366	66
After two weeks	75	14
Salt-precipitated pellet, redissolved after storage for one week	442	80
Salt-precipitated pellet, redissolved after storage for two weeks	174	32

Sedimentation Coefficient

A sample of the purified sucrose synthetase was obtained from aspen callus tissue in the usual manner. This sample, occupying a volume of 6.2 ml, was treated with 1.1-gm Lyphogel (purchased from Gelman Instrument Co.) overnight in the cold room to concentrate the sample. After removal of the gel, 0.6 ml of concentrated enzyme solution was obtained and used to determine the sedimentation coefficient of the enzyme at 9°C.

Only one peak was observed during the centrifugation indicating that the preparation was homogeneous; however, this single peak was quite small due to the dilute nature of the enzyme solution (even after concentration), and it diffused rapidly.

A sedimentation coefficient of $11.13 \times 10^{-13} \text{ sec}^{-1}$ at 9°C was obtained. This compares favorably with the values of $12.4 \times 10^{-13} \text{ sec}^{-1}$ for sucrose synthetase from mung bean seedlings (48) and $13.3 \times 10^{-13} \text{ sec}^{-1}$ for the enzyme from ripening rice grains (26) (both corrected to water at 20°C). Conversion of the value obtained for the enzyme from aspen callus tissue to water at 20°C would increase the value obtained slightly.

After centrifugation, the concentrated sample was diluted and assayed for sucrose synthetase activity in the usual manner. It gave a specific activity of 470 sucrose cpm/ μg protein/minute.

The data from which the sedimentation coefficient was obtained are presented in Appendix VII.

Molecular Weight

The molecular weight of the purified sucrose synthetase from aspen callus tissue was estimated by comparing the elution volumes of the activity peak from the Bio-Gel A-5m column for several preparations with the elution volumes of proteins of known molecular weights from the same column. The molecular weight standards used to obtain the standard curve were equine ferritin (Mann Research Laboratories), mushroom phenol oxidase (P-L Biochemicals, Inc.) and Micrococcus lysodeikticus polynucleotide phosphorylase (P-L Biochemicals, Inc.).

Bio-Gel A-5m gives a linear relation between the logarithm of molecular weight and elution volume for globular proteins having molecular weights from

80,000 to 5,000,000. The results plotted in Fig. 18 for protein standards gave a linear relationship and a molecular weight estimate for the sucrose synthetase from aspen callus of 350,000 to 415,000. These values represented elution volumes between 22.9 and 23.5 ml, the range for four different sucrose synthetase preparations. The more recent molecular weight values for sucrose synthetase from other tissues given in Table I range from 375,000 to 540,000.

Bio-Gel A-1.5m, which has a linear range for proteins of molecular weight from 45,000 to 1,500,000, might have been a better choice for the last step of the sucrose synthetase isolation procedure.

THE SUCROSE SYNTHESIS REACTION

Effect of Time

The effect of incubation time upon the formation of sucrose from UDPG and fructose by the purified enzyme was investigated.

Figure 19 shows the radioactivity bound to DE-81 anion exchange paper from equal aliquots of standard UDPG samples as a function of incubation time. Samples contained 60 μ g protein per ml of sample. The sucrose cpm for equal aliquots of the reconstituted samples, as measured by the paper chromatography technique, are presented, again as a function of incubation time, in Fig. 20.

It was observed when these two plots were compared that the amount of radioactive UDPG (measured by the anion exchange paper technique) decreased as the amount of radioactive sucrose (measured by the paper chromatography technique) increased. This was expected. However, the amount of radioactive sucrose decreased slightly at longer incubation times; this reduction was also observed when crude extracts were used. One explanation could be loss of radioactive

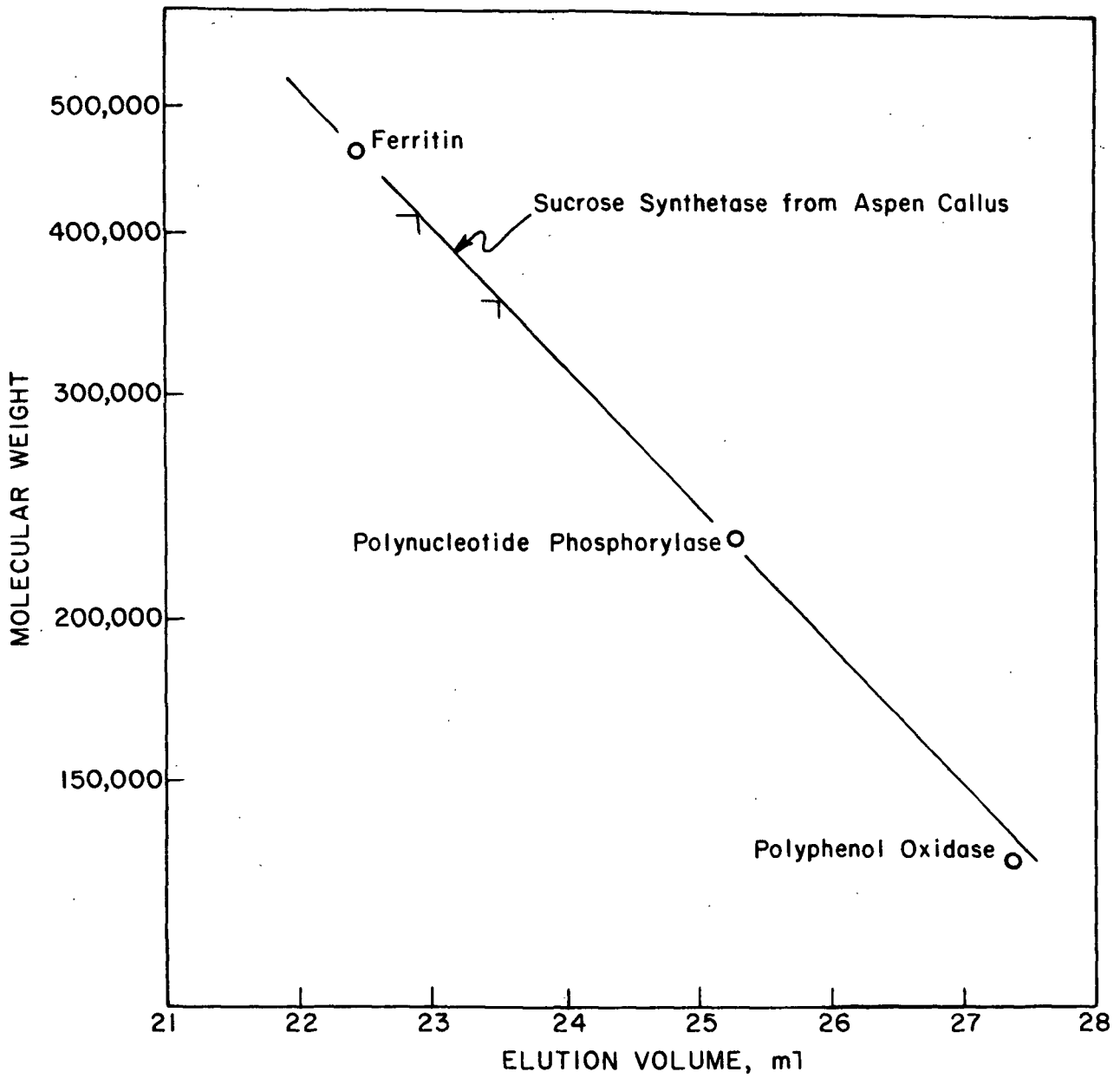


Figure 18. Determination of Molecular Weight

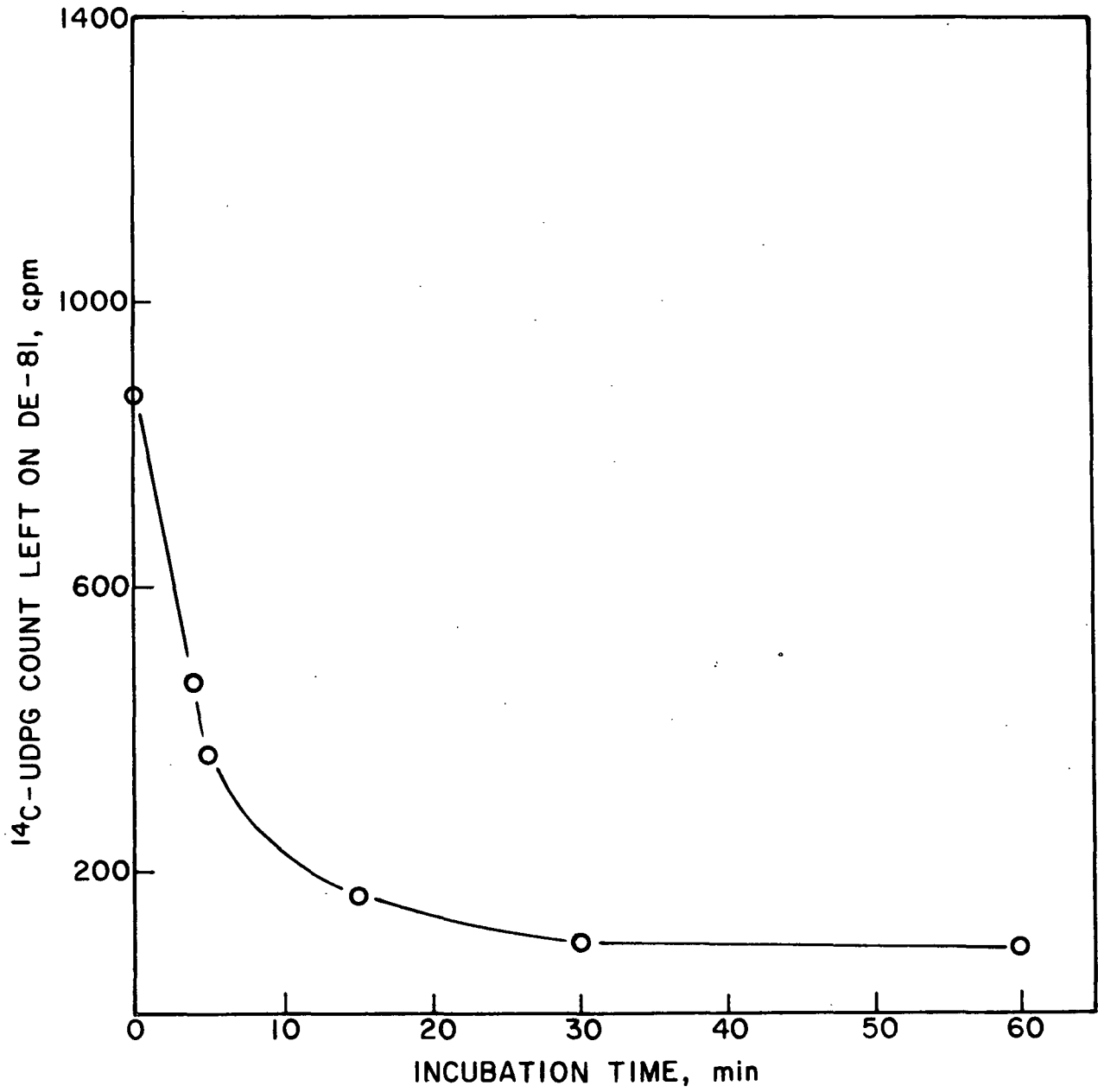


Figure 19. Disappearance of Labeled UDPG with Time

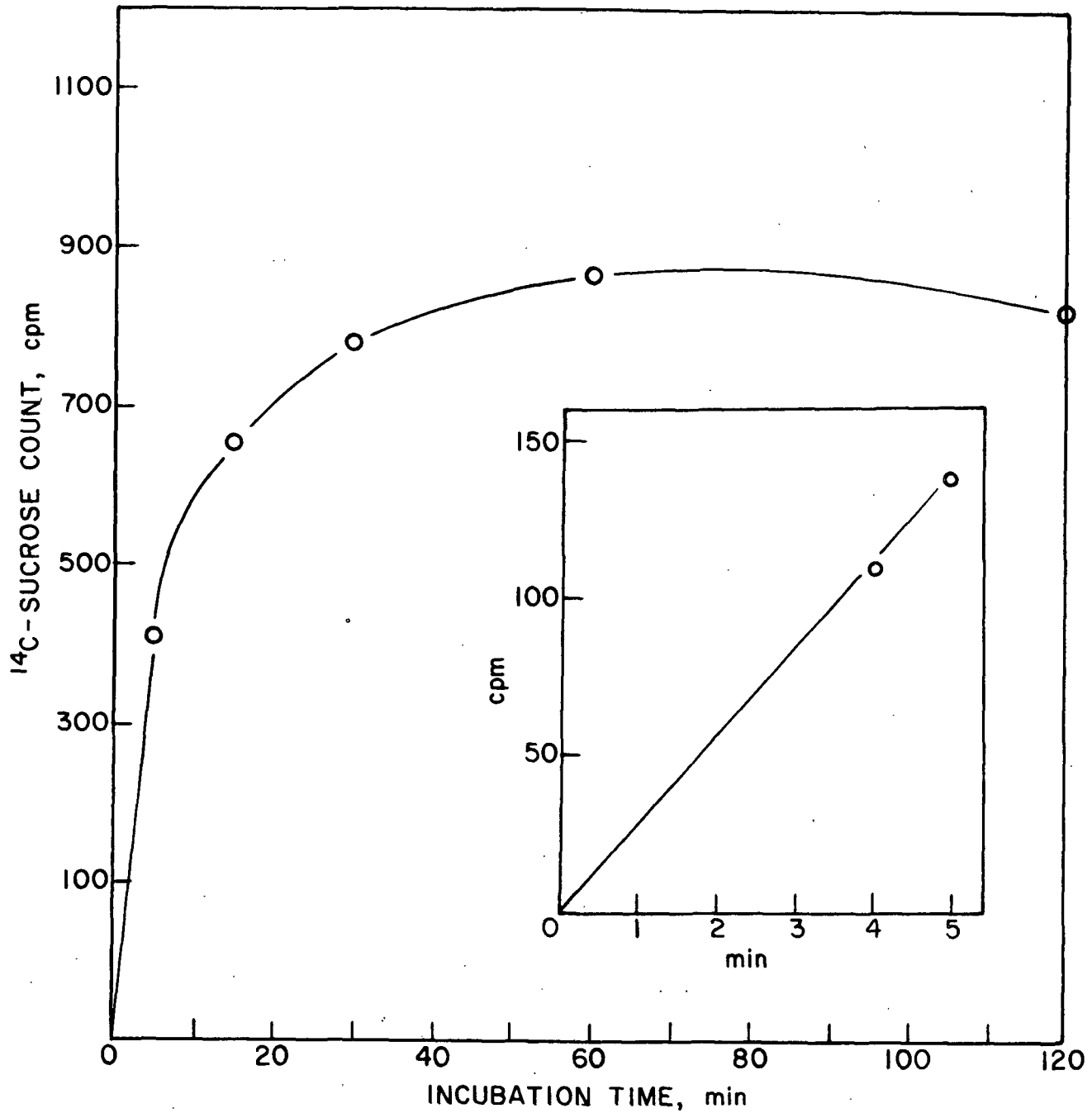


Figure 20. Formation of Labeled Sucrose with Time

Inset in figure shows linearity up to 5 min with data from another experiment.

glucose during the sucrose cleavage reaction when the UDPG substrate had been consumed and the driving force was away from sucrose synthesis.

The data in Fig. 19 and 20 indicated that the reaction could be considered linear with time during the first 5 minutes of incubation. This was confirmed in another experiment using purified enzyme in standard UDPG samples (34- μ g protein per ml of sample). The results (inset in Fig. 20) showed that linearity did exist up to 5 minutes; therefore, a standard incubation time of 4 minutes was chosen for subsequent characterization experiments. Reproducible results were difficult to obtain with incubation times much less than 4 minutes.

Effect of Enzyme Concentration

The effect of enzyme concentration upon the sucrose synthesis reaction was also investigated. A sample of purified enzyme containing 0.144-mg protein/ml was diluted to various concentrations and used in standard UDPG samples incubated for 4 minutes. Aliquots of the reconstituted samples were analyzed for radioactive sucrose by paper chromatography. The results in Fig. 21 showed that the effect of enzyme concentration upon the sucrose synthesis reaction was linear, at least up to the level of 48- μ g protein (purified enzyme)/ml in the incubation samples.

Effect of Temperature

Standard UDPG samples were incubated with purified enzyme (20- μ g protein per ml of sample) for 4 minutes at three different temperatures. Aliquots of the samples were analyzed for radioactive sucrose by paper chromatography. The results presented in Table XV showed increasing activity with increasing temperature up to 45°C. Since all previous work had been done at 30°C, this incubation temperature was used in subsequent work.

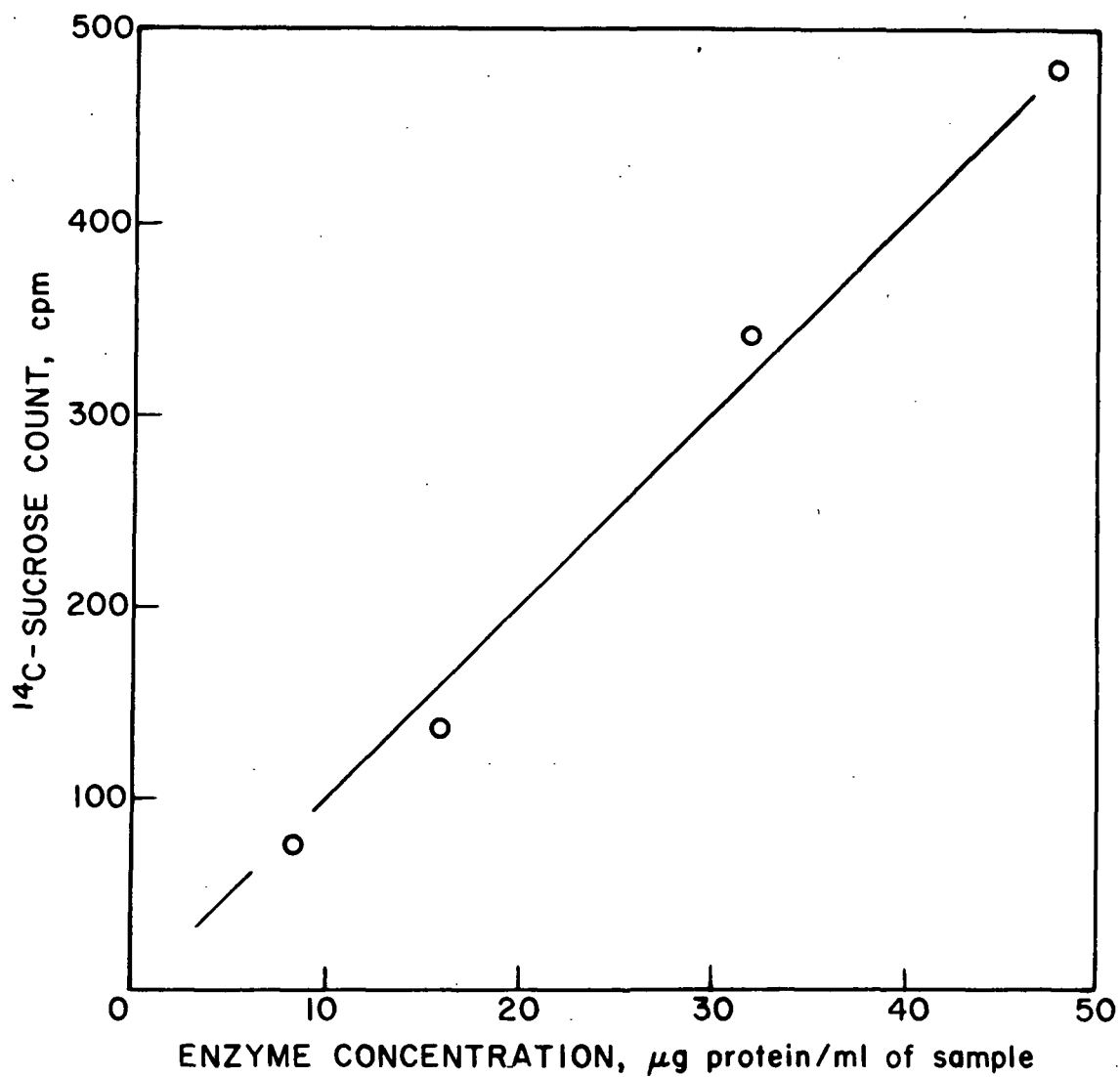


Figure 21. Effect of Enzyme Concentration upon Formation of Sucrose

TABLE XV

EFFECT OF TEMPERATURE UPON SUCROSE SYNTHESIS REACTION

Incubation Temperatures, °C	Sucrose Cpm/Aliquot of Reconstituted Sample
30	480
37	638
45	756

Effect of pH

The effect of pH upon the sucrose synthesis reaction was determined using samples which contained the standard UDPG sample concentrations of reagents in a total volume of 0.3 ml. The pH of each sample was adjusted using 0.05 ml of 0.1M MOPS buffer whose pH had been adjusted to give the desired value in the final sample. Each sample contained 24- μ g protein/ml of sample. Samples were incubated at 30°C for 4 minutes. The samples were analyzed for radioactive sucrose by means of paper chromatography. Duplicate samples were run for each pH value, and duplicate paper chromatograms were used for each of these samples. Average results for each pH are presented in Fig. 22. These results showed a pronounced pH optimum for the sucrose synthesis reaction at pH 8; it is not necessarily the pH optimum for the sucrose-cleavage reaction.

Effect of Substrate Concentration

The kinetic properties of the purified enzyme in the direction of sucrose synthesis were also examined.

 K_m (UDPG)

The effect of UDPG concentration on the reaction was studied. Samples contained 2 mM MgCl₂, 0.24 μ Ci UDPG-¹⁴C (155 μ Ci/ μ mole), 100 mM fructose, and varying concentrations of nonradioactive UDPG. Two different experiments were

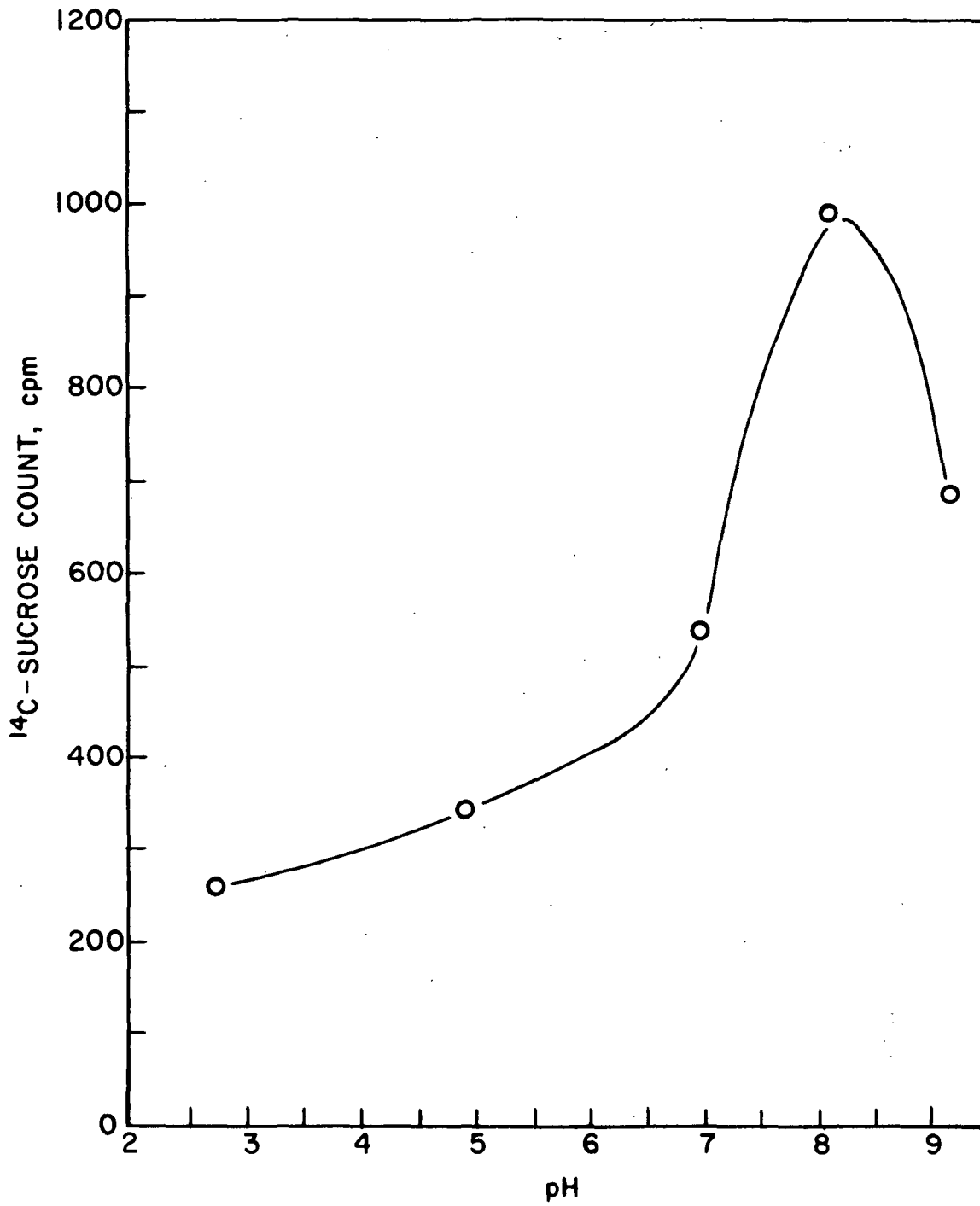


Figure 22. Effect of pH upon Sucrose Synthesis Reaction

run with different enzyme preparations. Each sample contained 0.1-ml enzyme in a total sample volume of 0.3 ml. Samples were incubated at 30°C for 4 minutes before freeze-drying. Aliquots of the reconstituted samples were analyzed for radioactive sucrose and the amount of sucrose produced in each sample was calculated based upon the specific activity of UDPG-¹⁴C in the particular sample. A Lineweaver-Burk plot of the experimental results is presented in Fig. 23 showing the reciprocal substrate concentration ($1/\underline{s}$) versus the reciprocal reaction velocity ($1/\underline{v}$). The substrate concentration (\underline{s}) has units of mmole UDPG/liter, whereas the units for the reaction velocity (\underline{v}) are μ mole sucrose produced/mg protein/minute. Results from the two different experiments are plotted in Fig. 23 as indicated. The calculations upon which this plot is based are tabulated in Appendix VIII.

A linear Lineweaver-Burk plot indicates that the enzyme-catalyzed reaction follows Michaelis-Menten enzyme kinetics. The experimental results showed that for lower UDPG concentrations (higher $1/\underline{s}$) the plot was linear. At higher UDPG concentrations (lower $1/\underline{s}$), however, the experimental points deviated from the straight-line relationship. This deviation indicated that the sucrose synthesis reaction was inhibited by UDPG at higher UDPG concentrations (58). The results obtained using two different enzyme preparations agreed quite well with each other.

The two intercepts of a Lineweaver-Burk plot can be used to obtain constants important in enzyme kinetics; the Michaelis constant, K_m (UDPG), obtained from Fig. 23 was 0.11 mM and the maximum velocity, V_{max} , was 0.8- μ mole sucrose produced/mg protein/min. K_m (UDPG) values of 0.64, 0.27, and 0.21 mM UDPG have been reported for the enzymes from Jerusalem artichoke (24), sugar beet root (37), and mung bean seedlings (46), respectively. Substrate inhibition of the

enzyme by UDPG has not been reported. If substrate inhibition occurred with these enzymes and was overlooked, the K_{-m} (UDPG) values reported could be inaccurate.

K_{-m} (Fructose)

The effect of fructose concentration upon the sucrose synthesis reaction was investigated at two different UDPG concentrations. Because higher specific activity UDPG- ^{14}C was not available, it was not possible to study the effect of fructose concentration on the sucrose synthesis reaction at UDPG concentrations higher than 5 mM , however, this UDPG level was still 45 times the K_{-m} (UDPG).

Samples were 2 mM in MgCl_2 , either 2 mM or 5 mM in UDPG, and contained 0.36 μCi of UDPG- ^{14}C (155 $\mu\text{Ci}/\mu\text{mole}$) and varying amounts of fructose. Samples also contained purified sucrose synthetase (35 $\mu\text{g}/\text{ml}$ of sample). Incubation was at 30°C for 4 minutes. Freeze-dried samples were reconstituted with ddH_2O and analyzed for radioactive sucrose by paper chromatography.

Two Lineweaver-Burk plots are presented in Fig. 24 corresponding to the two UDPG concentrations used. The calculations upon which this figure is based are presented in Appendix IX. The Michaelis constants obtained from the two plots were 2.94- mM fructose ($[\text{UDPG}] = 5 \text{ mM}$) and 5- mM fructose ($[\text{UDPG}] = 2 \text{ mM}$). Both of these values were within the range reported in Table I; however, the value for a UDPG concentration of 2 mM was more reliable due to the higher specific activities of UDPG- ^{14}C used in this portion of the experiment.

Effect of Additives

The effect of various additives on the sucrose synthesis reaction was also investigated. Samples containing the standard concentrations of reagents, in

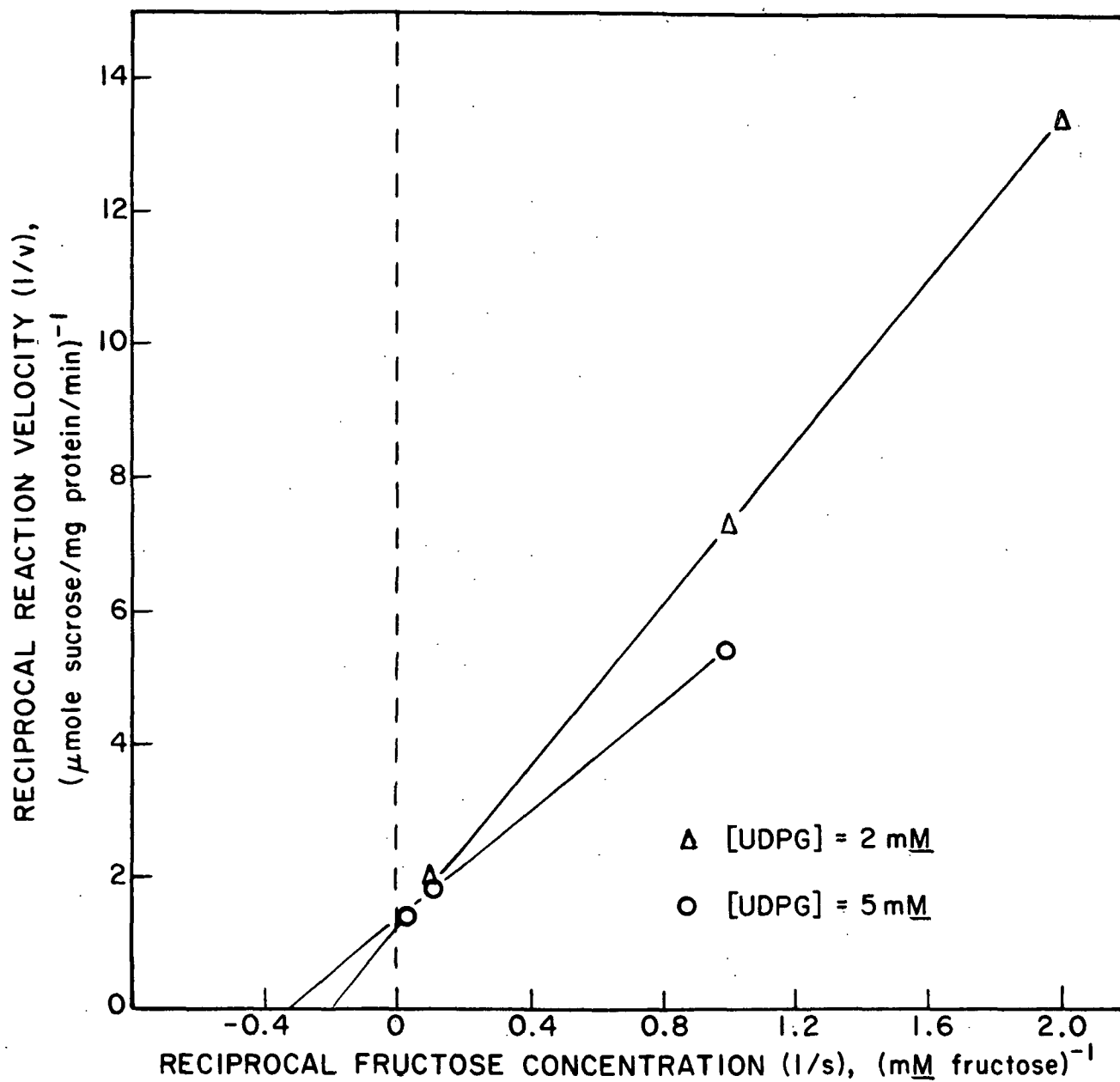


Figure 24. Lineweaver-Burk Plot for Fructose

addition to the reagents indicated in Table XVI, were incubated under the standard conditions and analyzed for sucrose synthesis by means of the DE-81 anion exchange paper technique (disappearance of radioactive UDPG). The results of this portion of the study are presented in Table XVI.

TABLE XVI
EFFECT OF ADDITIVES UPON SUCROSE SYNTHESIS REACTION

	Concentration in Sample, μM	Apparent Sucrose, cpm^a	Relative Activity, %
2,4-Dichlorophenoxyacetic acid (2,4-D)	25	513	105
3-Indoleacetic acid (IAA)	25	520	106
Gibberellic acid (GA)	25	485	99
Kinetin	25	500	102
UTP	25	446	91
GTP	25	431	88
ATP	25	427	87
GDPG	25	366	75
ADPG	25	463	95
ADP	250	503	103
CDP	250	383	78
GDP	250	393	80
UDP	250	105	21
No reagent added		489	100

^a Difference between radioactivity left on DE-81 from 10 μl of reconstituted sample and radioactivity left on DE-81 from 10 μl of reconstituted control.

The first group of additives in the table represented growth hormones such as the ones used in callus growth media. These additives had little effect upon the amount of sucrose produced. UTP, GTP, and ATP appeared to have a slight inhibiting effect upon the sucrose synthesis reaction. GDPG and GDP each inhibited the reaction whereas ADP and ADPG exerted only slight effects. UDP exerted a sizable effect which was expected from a product of the reaction.

Other Substrates

The effectiveness of other substrates, ADPG and UDPG, was also investigated. Samples were 2 mM in MgCl₂, and 133 μM in the particular nucleoside diphosphate glucose used. Each sample contained 2 μCi (185 μCi/μmole) ¹⁴C-fructose and purified enzyme (48-μg protein/ml of sample). Incubation was for one hour at 30°C. Reconstituted samples were analyzed for radioactive sucrose by paper chromatography. Results are presented in Table XVII.

TABLE XVII

OTHER SUCROSE SYNTHESIS SUBSTRATES

Substrate Used	Specific Activity ^a
UDPG	121
ADPG	11.1
GDPG	1.3

^a μMole sucrose/mg protein/1-hr incubation.

It was difficult to compare these results directly with literature K_{-m} values as presented in Table II; however, the general trend followed that which was predicted from the results obtained with sucrose synthetase from mung bean seedlings (46,48).

SUCROSE SYNTHETASE ACTIVITY IN OTHER CULTURED TREE TISSUES

Since the sucrose synthetase activity discussed in the preceding sections appeared to be an important component of extract obtained from dark-grown triploid aspen callus tissue, other tree callus tissues were assayed for this activity. Crude extracts were prepared from different tissues which were cultured as previously described in the Tissue Culture Laboratory at The Institute of Paper Chemistry. These crude extracts were assayed for protein and diluted with 1 mM DTT in MOPS buffer (0.1M, pH 8.0) to give extracts which contained 0.1 to 0.3-mg protein/ml of extract. The diluted extracts were reassayed for protein and then were incubated in standard UDPG samples using standard conditions. Reconstituted samples were analyzed for radioactive sucrose by paper chromatography. The percentage freeze-dry weights of the tissues used to prepare the extracts were also determined.

The data obtained for the various tissues are presented in Table XVIII. All of these tissues were grown in the dark on the media specified in the table, except for a Douglas-fir tissue and a triploid aspen tissue which were grown in the light. The formulae for the various growth media are given in Appendix I. The tissue ages reported in the table were the age since the last subculture of the particular sample of tissue used for the determination of sucrose synthetase activity.

The specific activities for the tissues in the table ranged from 0.004 to 0.148- μ mole sucrose produced/mg protein/minute. Specific activities (μ mole sucrose/mg protein/min) in crude extracts have been reported at 0.082 for sweet potato root (39), 0.5 for ripening rice grains (45), and 0.0004 for mung bean seedlings (48).

TABLE XVIII

ASSAY OF CULTURED TREE TISSUES FOR SUCROSE SYNTHETASE ACTIVITY

	Medium	Dry Weight, %	Age Since Last Sub-culture, weeks	Protein Content of Fresh Tissue ^a	Protein Content of Dry Tissue ^b	Sucrose Cpm/ µg Protein/ 4-Min Incubation	Specific Activity ^c
Loblolly pine	IPA2	3.70	4	3.41	92.1	467	0.057
Loblolly pine	10	5.92	1-1/2	24.39	412.1	326	0.040
Douglas-fir ^d	DF10	6.14	4	18.35	298.9	310	0.038
Shortleaf pine	10	6.74	2	7.88	116.8	1216	0.148
Western hemlock	10	6.25	2	11.66	185.5	687	0.084
Eucalyptus	CO4	6.84	3-1/2	12.82	187.4	30	0.004
Diploid quaking aspen	107	7.67	7	15.58	203.1	30	0.004
Tetraploid quaking aspen	107	3.89	4	13.08	336.3	34	0.004
Triploid quaking aspen	107	7.05	6-1/2	15.04	213.3	1146	0.018
Triploid quaking aspen	107	6.37	5-1/2	6.79	106.6	326	0.040
Triploid quaking aspen	107	7.44	3-1/2	7.13	99.6	507	0.062
Triploid quaking aspen	107	6.07	3	9.38	154.7	558	0.068
Triploid quaking aspen	107	7.04	2-1/2	10.09	143.3	310	0.038
Triploid quaking aspen	10	7.45	5-1/2	7.50	100.7	1029	0.126
Triploid quaking aspen ^d	10	4.40	9	4.96	112.8	538	0.066

^aMg protein/g fresh weight.^bMg protein/g dry weight.^cµMole sucrose/mg protein/min (4-min incubations).^dLight-grown tissue.

The data reported in the table could have been expressed in terms of μ mole sucrose/g fresh weight of tissue/minute. The specific activities for the tissues represented in Table XVIII in these units ranged from 0.056 to 1.18. Thorpe and Meier (12) found sucrose synthetase activities of 0.136- μ mole sucrose/g fresh weight/minute for tobacco callus and 0.499- μ mole sucrose/g fresh weight/minute for carrot callus tissue.

Thus, the results presented in the table showed that the various tree callus tissues tested contained sucrose synthetase activity. This result was expected since this enzyme appears to be an important component of cultured plant tissues for the utilization of sucrose in the culture medium. The fact that significant amounts of activity were found in different tree tissues makes these results more useful.

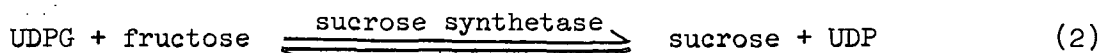
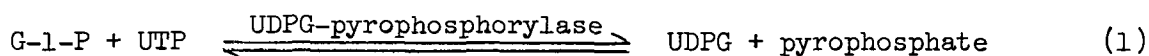
The data presented in Table XVIII suggested that shortleaf pine callus or triploid aspen grown on Medium 10 may have been better choices for this research based upon their apparent abundance of sucrose synthetase activity. The results for diploid and tetraploid quaking aspen may have been influenced more by the particular samples of tissue than by ploidy.

The results presented in this thesis are only the second report of sucrose synthetase in cultured plant tissues and the first report of this enzymatic activity from any tree tissue.

CONCLUSIONS

It was shown that sucrose synthetase is present in dark-grown triploid quaking aspen callus tissue. Although this enzyme has been found in many different plants, this thesis is only the second report of it in cultured plant tissues. This is the first time that the enzyme has been demonstrated in any tissue from a tree. Furthermore, the enzyme was found to be very abundant in the aspen callus, accounting for as much as 5% of the protein in crude extracts prepared from the tissue.

Sucrose synthetase would not have been found using ^{14}C -G-1-P as the labeled substrate in the callus tissue if the tissue had not contained UDPG-pyrophosphorylase activity as well. The following reaction scheme for the formation of two labeled products when UTP and ^{14}C -G-1-P were incubated with crude callus extract was supported by all the experimental work:



Since sucrose synthetase is abundant in the callus tissue, it must be a very important enzyme in callus tissue metabolism. Its importance probably is not as a sucrose synthesizing enzyme, but as a sucrose utilizing enzyme. Cultured plant tissues are grown on media containing large amounts of sucrose as the primary carbon source (11,12,62). The sucrose cleavage reaction catalyzed by sucrose synthetase [reverse of Reaction (2)] could provide a direct and efficient route for utilization of sucrose by the cultured tissue. It was difficult to demonstrate the sucrose cleavage reaction in vitro. However, many other workers (26,31,42,45) have had this same difficulty using sucrose synthetase preparations from other plant sources.

The properties of the sucrose synthetase purified from aspen callus are similar to those of the enzyme from meristematic or rapidly-growing plant tissues. The specific activities of the preparations used to obtain these properties ranged from 0.186 to 0.243- μ mole sucrose formed/mg protein/minute.

The sucrose synthetase from aspen callus had a molecular weight between 350,000 and 415,000. The enzyme from sweet potato root has a molecular weight of 540,000 (39), that from ripening rice grains has a molecular weight of 400,000 (45), and that from mung bean seedlings has a molecular weight between 375,000 and 405,000 (48). There was some evidence that the enzyme from aspen callus may be a tetramer as are the enzymes from ripening rice grains and mung bean seedlings. Sedimentation coefficients of 12.4×10^{-13} and 13.3×10^{-13} sec⁻¹ (corrected to water at 20°C) were reported for the enzymes from mung bean seedlings and ripening rice grains, respectively. A similar value of 11.13×10^{-13} sec⁻¹ (in MOPS buffer at 9°C) was found for the aspen callus sucrose synthetase.

A Michaelis constant [K_{-m} (UDPG)] of 0.11 mM UDPG was found for the sucrose synthetase from aspen callus operating in the sucrose synthesis direction. Values of 0.64, 0.27, and 0.21 mM UDPG were reported for the enzymes from Jerusalem artichoke tuber (24), sugar beet root (37), and mung bean seedlings (46), respectively. The enzyme from aspen callus was inhibited by higher concentrations of UDPG. This has not been reported for sucrose synthetase preparations from other plant sources. If substrate inhibition existed for these preparations, but was overlooked, the K_{-m} (UDPG) values reported in the literature may have been inaccurate. The other Michaelis constant [K_{-m} (fructose)] for the sucrose synthetase from aspen callus was 5-mM fructose. This value was within the range of values reported for enzymes prepared from different plant sources presented in Table I.

Many of the sucrose synthetase preparations reported in the literature could catalyze the synthesis of sucrose from nucleoside diphosphate glucoses other than UDPG. UDPG, however, was generally the best substrate for the enzymes prepared from meristematic tissues. The enzyme from aspen callus could catalyze the synthesis of sucrose from UDPG, ADPG, and GDPG. The effectiveness of GDPG was an order of magnitude lower than that of ADPG, which was, in turn, an order of magnitude lower than that of UDPG. This pattern is the same as that observed with the sucrose synthetase from mung bean seedlings (46).

The sucrose synthetase found in aspen callus tissue appeared to be the same enzyme as found in a number of other plant tissues. However, it was notably different in one respect. The enzyme from aspen callus was associated with, or bound to, membrane fragments in the crude extract prepared from the tissue. Only one other sucrose synthetase preparation, that from lemons, (23), has been reported to be particulate.

The sucrose cleavage reaction was difficult to demonstrate using crude callus extracts. In the case of aspen callus sucrose synthetase, the intact membranous structure with which the enzyme is associated in the tissue may be necessary for the sucrose cleavage reaction. This could account for the difficulty in demonstrating this reaction in vitro. The association of the enzyme with a membrane could indicate also that the enzyme is involved in the uptake of glucose by the cell from the sucrose supplied in the culture medium. These are promising areas for future investigation.

The size of the sucrose synthetase, the indication that it is tetrameric, and its association with membranes are all compatible with the possibility that it plays a key regulatory role in callus metabolism. Recent work with sucrose synthetase from other plants has focused upon the regulatory properties

of the enzyme (38,49,52). This is another promising area for future investigation.

Sucrose synthetase activity was found in crude extracts of other cultured tree tissues. These included dark-grown loblolly pine, shortleaf pine, western hemlock, and eucalyptus, and light-grown Douglas-fir and triploid quaking aspen. The list of cultured tissues in which sucrose synthetase has been found was increased significantly during this investigation.

Because of its abundance in the dark-grown aspen tissue and its presence in other cultured tree tissues, sucrose synthetase would appear to have an important role in cultured plant tissue metabolism. This conclusion should be investigated further by studying the growth of cultured tree tissues on media containing other sugars, comparing the sucrose synthetase from different cultured tree tissues, and studying the regulatory properties of the enzyme.

GLOSSARY

A	Arabinose
ADP	Adenosine diphosphate
ADPG	Adenosine diphosphate glucose
Agarose	Bio-Gel A-5m
ATP	Adenosine triphosphate
CB	Cellobiose
CDP	Cytidine diphosphate
CDPG	Cytidine diphosphate glucose
cpm	Counts per minute, measured with $^{14}\text{C}/^3\text{H}$ window
$D_{20,w}$	Diffusion constant corrected to water at 20°C
ddH ₂ O	Double-distilled water
DE-81	Whatman DE-81 filter paper, or the analysis method using this paper
DTT	Dithiothreitol
F	Fructose
F-6-P	Fructose-6-phosphate
G	Glucose
G-1-P	Glucose-1-phosphate
G-1-P substrate sample	Defined on page 54
GDP	Guanosine diphosphate
GDPG	Guanosine diphosphate glucose
GTP	Guanosine triphosphate
K_{-eq}	Equilibrium constant for sucrose synthesis direction of reaction catalyzed by sucrose synthetase
K_{-m}	Michaelis constant, substrate concentration at which $\underline{v} = \frac{1}{2} v_{-max}$
LB	Laminaribiose

MOPS	3-(Morpholino)-propanesulfonic acid, a good buffer
NuDP	Nucleoside diphosphate
NuDPG	Nucleoside diphosphate glucose
P-6	Bio-Gel P-6
Pi	Inorganic phosphate, PO_4^{---}
PPi	Pyrophosphate
PPO	2,5-Diphenyloxazole
S	Sucrose
Standard G-1-P sample	Defined on page 20
Standard UDPG sample	Defined on page 20
TDP	Thymidine diphosphate
TDPG	Thymidine diphosphate glucose
UDP	Uridine diphosphate
UDPG	Uridine diphosphate glucose
UDPG- ^{14}C	UDPG with ^{14}C in the glucose moiety
UDPG substrate sample	Defined on page 54
UTP	Uridine triphosphate
\underline{v}	Reaction velocity, $\mu\text{mole product formed/mg protein/min}$
$\underline{v}_{\text{-max}}$	Maximum reaction velocity, occurring when enzyme is saturated with substrate
\underline{X}	Compound \underline{X} , defined on page 22, identified as radioactive sucrose
\underline{Y}	Compound \underline{Y} , defined on page 22, identified as UDPG radioactive in the glucose moiety

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APPENDIX I

TRIPLOID ASPEN CALLUS TISSUE

With the exception of the examination of different tree callus tissues for sucrose synthetase activity, all work involved triploid aspen callus tissue grown in the dark on Medium 107. The techniques for initiation and subculture of this tissue have been discussed by Winton (4,6); however, a brief discussion of the history of this particular tissue will be presented in this Appendix.

The original source of the tissue used for the investigation was a triploid clone of normally diploid quaking aspen (Populus tremuloides Michx.) which has been designated T-2-56 (59).

On October 6, 1966, sections of lateral roots about 1 inch in diameter were collected from locally grown ramets of the triploid quaking aspen clone T-2-56, originally discovered near Bruce Crossing, Upper Michigan. Root sections 15-cm long were dipped in wax and planted in a 1:1 mixture of sand and vermiculite. Root sprouts appeared after 4-5 weeks and were excised when 2-3-cm tall and rooted in the same mixture under plastic bags in the greenhouse. Rooted cuttings were transplanted to individual pots and were 20-30-cm tall in January. On January 23, 1967, six stems were cut off 5-7-cm above the soil surface and leaves were removed. Sections 2-3 mm in diameter and 15-20-cm long were green and succulent; they were sterilized in Hilex bleach (5.25% NaOCl) plus a few drops of Tween 20 to break surface tension. After three rinses in sterile water, the stems were cut into internodal segments 5-8-mm long and placed base up, five per plate in three plates each of several media. After four weeks of incubation at 27°C in the dark, selected pieces of callus tissue were removed from the stem segments and were weighed and subcultured to fresh medium. Cloning was continued

for three more subcultures with selected pieces until the best firm white callus was growing on Medium 1. This callus was maintained on the same medium, being subcultured every 4-6 weeks depending on seasonal variation in growth. After one year and 13 subcultures, some callus was transferred to Media 100 and 107.

The callus which had been maintained on Medium 107 was used for this thesis research beginning in the winter of 1971-72. The tissue was subcultured every 4 to 8 weeks by selecting the best pieces and preparing sterile inocula 3 mm on a side from the firm white edges of the callus pieces. Inocula were then placed on Medium 107 and returned to the incubator.

Other tissues which were assayed for the presence of sucrose synthetase activity were initiated and maintained in a similar manner in the Tissue Culture Laboratory at The Institute of Paper Chemistry. The composition of the various media is presented in Table XIX.

TABLE XIX

TISSUE CULTURE MEDIA

Component	Medium 1, mg/liter	Basal DF Medium, mg/liter
MgSO ₄ ·7H ₂ O	764	370
Na ₂ SO ₄	425	
KNO ₃	425	1,900
KH ₂ PO ₄		170
Ca(NO ₃) ₂ ·4H ₂ O	170	
CaCl ₂ ·2H ₂ O		440
KCl	140	
NaH ₂ PO ₄ ·H ₂ O	35	
NH ₄ NO ₃		1,650
Fe (as EDTA)	5.6	5.6
MnSO ₄ ·H ₂ O	9	16.9
ZnSO ₄ ·7H ₂ O	3.2	10.6
H ₃ BO ₃	3.2	6.2
KI	1.6	0.83
Na ₂ MoO ₄ ·2H ₂ O		0.25
CuSO ₄ ·5H ₂ O		0.025
CoCl ₂ ·6H ₂ O		0.025
Inositol	100	100
Nicotinic acid	0.05	0.5
Thiamine-HCl	0.01	0.1
Pyridoxine-HCl	0.01	0.1
Asparagine		100
Sucrose	20,000	30,000
Agar	8,000	8,000
2,4-D	0.04	
Kinetin	1	
pH adjusted to 5.7 ± 0.1 with 2-3 ml 1% NH ₄ OH		

Medium 100 is identical to Medium 1 except that it contains 10 mg/liter of each of the following components:

Alanine	Histidine	Methionine	Tryptophan
Asparagine	Hydroxyproline	Phenylalanine	Folic acid
Aspartic acid	Isoleucine	Threonine	Adenylic acid ^a
Citrulline	Proline	Tyrosine	Cytidylic acid ^a
Cysteine	Serine	Valine	Guanylic acid ^a
Glutamic acid	Leucine	Arginine	Uridylic acid ^a
Glycine	Lysine	Ornithine	Thymine

^aSupplied as -3'-(2')-phosphoric acid.

Medium 107 is identical to Medium 100 except that it contains no tryptophan.

Medium CO₄ is identical to Medium 1 except that it contains 150 mg/liter of coconut milk.

Media 10 and DF-10 are identical and contain in addition to the Basal DF components, 5 mg/liter β-naphthoxyacetic acid (NOAA) and 0.1 mg/liter benzylaminopurine (BAP).

Medium IPA-2 is identical to the Basal DF medium except that it also contains 5 mg/liter NOAA, 0.01 mg/liter isopentenylaminopurine, 2 mg/liter glycine, 3 mg/liter thiamine, 340 mg/liter KH₂PO₄, 1 mg/liter ascorbic acid, 100 mg/liter adenine sulfate, and 0.1 mg/liter each of biotin, riboflavin and folic acid.

APPENDIX II

PROTEIN DETERMINATIONS

All protein determinations were based upon the microbiuret technique of Koch and Putnam (53). This technique is based upon the absorption of ultraviolet light at 330 nm by a complex formed between peptide linkages and Cu^{++} ion in an alkaline medium. The ultraviolet absorption at 392 nm can be used to eliminate effects of turbidity.

The reagent contains 1.25-g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 230 ml of 10M NaOH, and 25 ml 28.5-30% NH_3 made up to 500 ml with ddH_2O .

Two variations of the technique have been used depending upon the protein concentration in the particular sample being assayed. The first variation, suitable for protein concentrations greater than 0.5 mg/ml, involved the addition of 4.0 ml of the copper reagent to 1.0 ml of the sample to be assayed. After one-half hour at room temperature the absorbance of the sample was measured at 330 nm and 392 nm with the Beckman DU spectrophotometer. The absorbance at 330 nm minus twice the absorbance at 392 nm gives the protein concentration from a standard curve based on crystalline bovine serum albumin dissolved in the same buffer solution as the sample. Such a standard curve using this variation of the technique is presented in Fig. 25. This figure shows that the plot is linear with protein concentration. Due to possible variations with aging of the copper reagent, new standard curves were generated each time protein analyses were made.

In the second variation, suitable for protein concentrations greater than about 0.1-mg protein/ml, 2.0 ml of the copper reagent were added to 1.0 ml of sample. A separate standard curve was necessary when this variation of the technique was used.

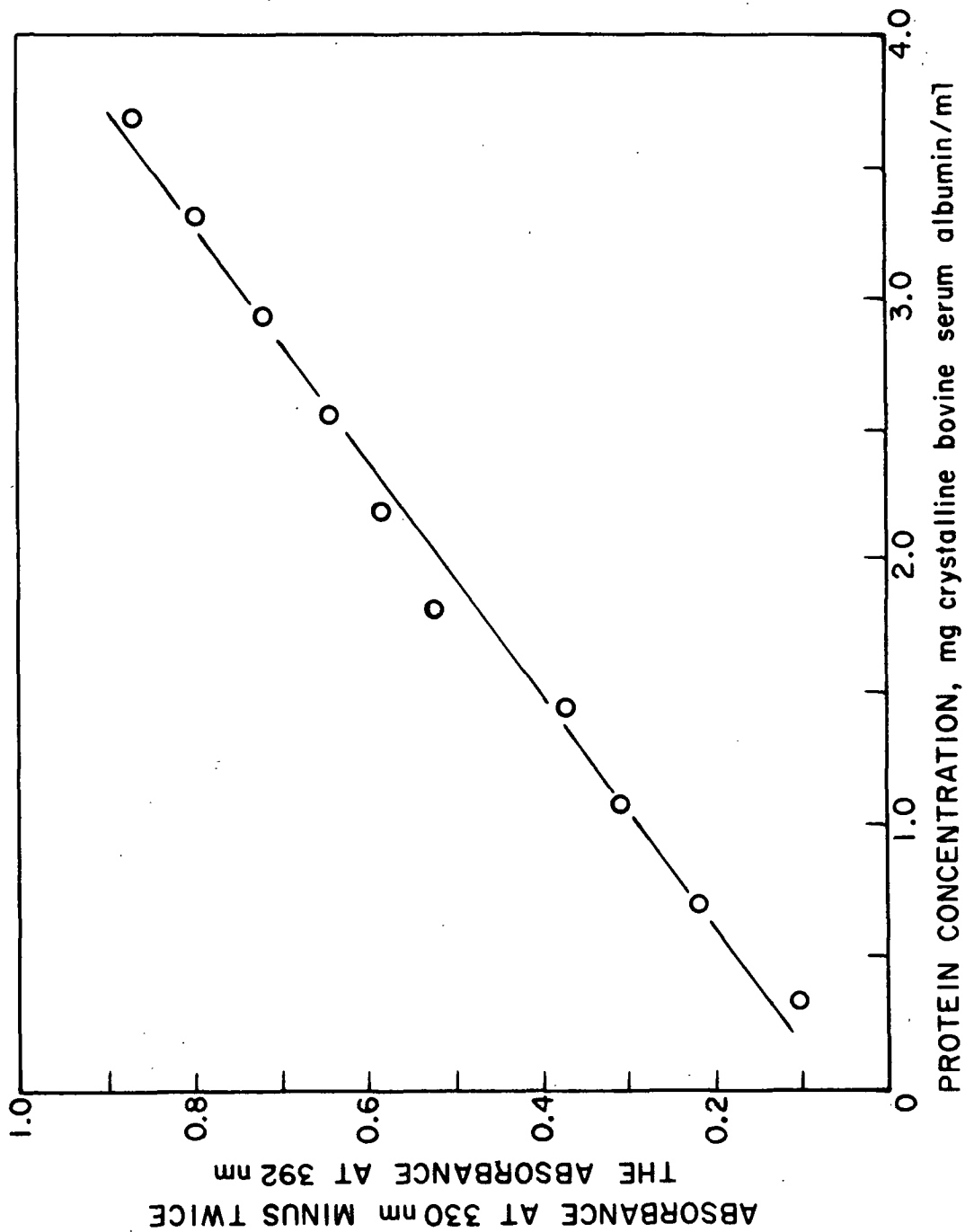


Figure 25. Microbiuret Protein Determination Standard Curve

The protein concentrations obtained using this technique are actually in equivalent mg BSA/ml; however, since the method measures the number of peptide linkages present, it should give representative mg protein/ml for other proteins. The components of the buffer used in this research did not interfere with the method; however, digitonin was found to interfere and had to be removed from any sample prior to assay for protein.

APPENDIX III

RADIOACTIVE SUBSTRATES

Four different ^{14}C -containing substrates were used during the course of this research. All were obtained from the Isotope and Nuclear Division of ICN Pharmaceuticals, Inc., Cleveland, Ohio. Each is discussed separately below.

D-GLUCOSE- ^{14}C (UL)-1-PHOSPHATE, DIPOTASSIUM SALT

This compound is supplied by ICN with specific activities between 150 and 210 $\mu\text{Ci}/\mu\text{mole}$ and was referred to commonly as ^{14}C -G-1-P. Generally, 50 μCi of this compound were ordered and diluted with 50 ml of ddH₂O. One such sample (225 $\mu\text{Ci}/\mu\text{mole}$) gave 815 cpm/ μl after dilution.

Figure 26 gives the counting results for a paper chromatographic examination of a sample of ^{14}C -G-1-P. The chromatogram was developed for 48 hours with Solvent IA before it was counted. These results show that the ^{14}C -G-1-P moved only slightly from the spotting origin and contained a fair amount of ^{14}C -glucose contamination. Similar chromatographic results can be seen in Fig. 4(c).

URIDINE-DIPHOSPHO-GLUCOSE [D-GLUCOSE- ^{14}C (UL)]

This compound is supplied by ICN with specific activities ranging from 50 to 150 $\mu\text{Ci}/\mu\text{mole}$ and was referred to commonly as UDPG- ^{14}C . Generally, 10 μCi of this compound were ordered and diluted with 25 ml ddH₂O. One such sample (155 $\mu\text{Ci}/\mu\text{mole}$) gave 466 cpm/ μl . Results of assaying radioactivity of paper-chromatographed UDPG- ^{14}C can be found in Fig. 14 and 15.

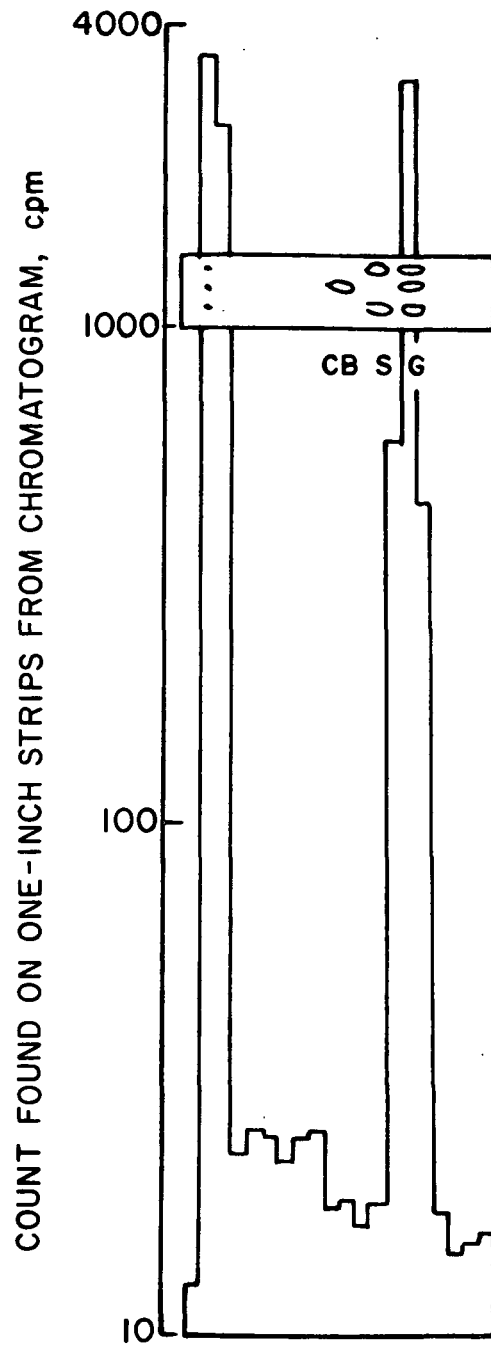


Figure 26. Paper Chromatography of $^{14}\text{C-G-1-P}$

The chromatogram was developed with Solvent IA for 48 hours.

CB, S, and G designate cellobiose, sucrose, and glucose standard spots.

D-FRUCTOSE-¹⁴C (UL)

¹⁴C-fructose is supplied by ICN with specific activities ranging from 50 to 250 $\mu\text{Ci}/\mu\text{mole}$. A 100 μCi sample of this compound was ordered and diluted with 50 ml of ddH₂O. One μl of this diluted sample gave 2300 cpm. The paper chromatographic behavior of this compound can be seen in Fig. 6(b) and Fig. 8(c).

SUCROSE-¹⁴C (UL)

¹⁴C-sucrose is supplied by ICN with specific activities ranging from 200 to 480 $\mu\text{Ci}/\mu\text{mole}$. One 100 μCi sample was ordered and diluted with 50 ml of ddH₂O; this diluted sample gave 2130 cpm/ μl . A sample of this radioactive compound was checked by paper chromatography using Solvent III for 72 hours. Results of the assay of radioactivity are presented in Fig. 27(a). These results show that the radioactive sucrose contained some radioactive glucose and fructose contamination. Another sample of the labeled sucrose was hydrolyzed with acid and chromatographed; results of radioactivity for this chromatogram (same conditions) are presented in Fig. 27(b).

BINDING OF LABELED COMPOUNDS TO DE-81 ANION EXCHANGE PAPER

The binding of the labeled compounds to DE-81 anion exchange paper was investigated. Five- μl aliquots of each compound were spotted on circles of the DE-81 paper. Some of the circles were counted while others were washed with ddH₂O on filter funnels before counting. Data are reported in Table XX.

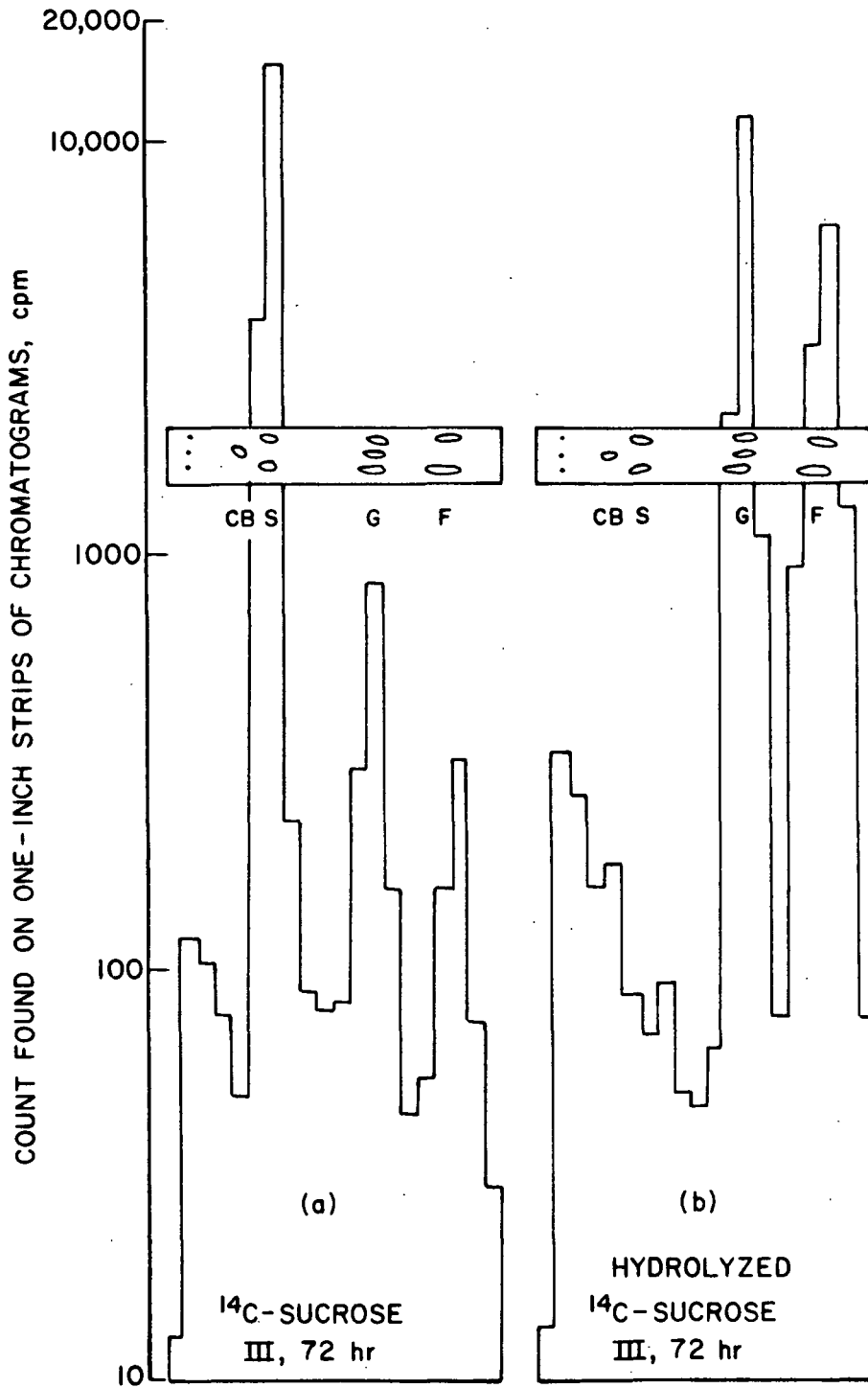


Figure 27. Paper Chromatography of ^{14}C -Sucrose

CB, S, G, and F designate cellobiose, sucrose, glucose, and fructose standard spots.

TABLE XX

DE-81 BEHAVIOR OF LABELED COMPOUNDS

Compound	Count on DE-81 Before Washing, cpm	Count on DE-81 After Washing, cpm
^{14}C -UDPG	1330	1355
^{14}C -fructose	7188	100
^{14}C -sucrose	6945	74
^{14}C -G-1-P	2801	2960

These results confirm that the radioactive G-1-P and UDPG are bound to the DE-81 paper whereas the nonionic compounds are washed from it.

APPENDIX IV

PAPER CHROMATOGRAPHY

Descending paper chromatography was used extensively for identification of products, isolation of radioactive compounds for counting, and preparation of larger quantities of radioactive products for other analyses (e.g., isolation of labeled sucrose for crystallization to constant specific activity with authentic sucrose).

Whatman No. 1 paper was used exclusively.

Six different solvent systems were used at different times to achieve particular separations. These solvent systems were as follows:

- IA n-butanol:pyridine:water (6:4:1)
- II ethyl acetate:acetic acid:water (3:3:1)
- III ethyl acetate:pyridine:water (8:2:1)
- IV n-butanol:ethanol:water (40:11:19)
- V ethyl acetate:acetic acid:formic acid (88%):water (18:3:1:2)
- VI n-butanol:acetic acid:water (4:1:5, organic phase)

Typically, a paper chromatogram would be spotted with an aliquot of the particular sample and authentic sugar standards as indicated in Fig. 28(a). After being spotted, the chromatogram would be allowed to equilibrate in the developing tank with the particular solvent to be used. The chromatogram would then be developed with the solvent system; generally, the solvent front would run off the end of the chromatogram during the period of time required for the desired separation.

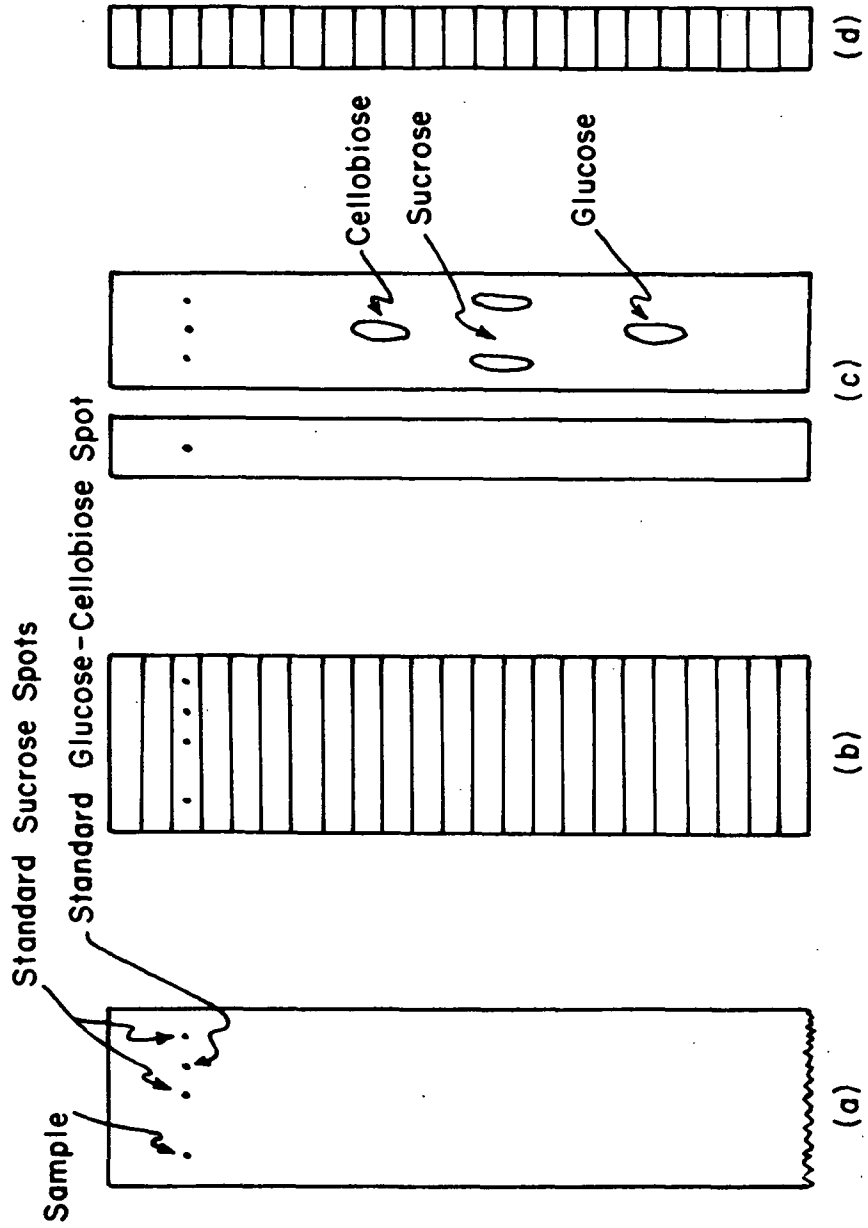


Figure 28. Paper Chromatography

After the desired development time had elapsed, the chromatogram would be hung up to dry and then marked with pencil into one-inch horizontal strips using a template as indicated in Fig. 28(b). The sample portion would then be cut away from the standard portion of the chromatogram as indicated in Fig. 28(c). The standard portion would then be sprayed with a benzidine-TCA reagent (60) consisting of 0.5-g benzidine dissolved in 10-ml acetic acid, 10-ml 40% (w/v) trichloroacetic acid, and 80-ml ethanol. After drying, the sprayed portion of the chromatogram would be placed in an oven at approximately 100°C until spots of the standard compound became visibly brown as indicated in Fig. 28(c).

The sample portion could then be cut into 1-inch horizontal strips as indicated in Fig. 28(d). All of the strips, or only those strips corresponding to a compound of interest in the particular sample, could be counted.

Counting was done by placing the paper strip in a liquid scintillation vial with enough Cocktail D (10 g PPO and 200 g naphthalene made up to 2 liters with scintillation grade 1,4-dioxane) to cover the strip. The ^{14}C -only ($^{14}\text{C}/^3\text{H}$, window) portion of the counting spectrum was generally used for counting, as counting vials were reused after careful washing. This portion of the counting spectrum gave the most reproducible and reliable results.

Counting data for entire chromatograms were represented in histogram form as was the practice in the body of the thesis. When only the sucrose counts were of interest, the portions of the chromatogram corresponding to the sucrose standard spots were counted and corrected for background using similar strips from a control sample chromatogram.

When larger quantities of a radioactive compound were desired, several sample spots would be run and the strips corresponding to the desired compound would then be eluted with ddH₂O.

APPENDIX V

PAPER CHROMATOGRAPHY DATA FOR SUCROSE CLEAVAGE REACTION

The paper chromatography results used for Table VI are presented in Table XXI. Samples A and B contained crude extract whereas Sample C was a no extract control. Chromatograms were developed with Solvent III for 72 hours. The composition and incubation of the samples were discussed in the main body of the thesis (p. 34).

TABLE XXI

PAPER CHROMATOGRAPHY RESULTS USED FOR TABLE VI

Sample A			Sample B		
Strip	Cpm	Standard Spots	Strip	cpm	Standard Spots
1	13		1	15	
2	635	Spotting origin	2	650	Spotting origin
3	495		3	456	
4	637	Cellobiose	4	433	Cellobiose
5	55,849	Sucrose	5	41,622	Sucrose
6	38,832	Sucrose	6	54,903	Sucrose
7	413		7	675	
8	142		8	193	
9	179		9	167	
10	3,250	Glucose	10	501	Glucose
11	7,283	Glucose	11	7,536	Glucose
12	899	Glucose	12	3,559	Glucose
13	102		13	126	
14	603		14	102	
15	3,424	Fructose	15	1,507	Fructose
16	3,598	Fructose	16	4,811	Fructose
17	488		17	1,280	
18	62		18	69	
19	38		19	42	
20	30		20	27	

Sample C		
Strip	Cpm	Standard Spots
1	19	
2	446	Spotting origin
3	467	
4	290	Cellobiose
5	10,456	Sucrose
6	87,612	Sucrose
7	13,329	Sucrose
8	392	
9	170	
10	184	
11	664	Glucose
12	4,919	Glucose
13	1,856	Glucose
14	126	
15	621	Fructose
16	604	Fructose
17	1,529	Fructose
18	250	Fructose
19	71	
20	42	

APPENDIX VI

PURIFICATION OF SUCROSE SYNTHETASE

In this appendix are discussed the calculations upon which the results presented in Table XIII are based. The purification data for four other sucrose synthetase preparations are also presented.

ASSAY SAMPLES

The standard samples used for assay of sucrose synthetase activity at the various stages in the enzyme purification were made by mixing 1.0 ml 0.06M MgCl₂, 5.0 ml 0.6M fructose, 3.0 ml ddH₂O, 5.0 ml 0.2 mM UDPG, and 6.0 ml UDPG-¹⁴C and dividing the resulting 20 ml of solution into 40 0.5-ml portions. The UDPG-¹⁴C came from 10 μCi of UDPG-¹⁴C which had been diluted with 25 ml of ddH₂O; this labeled UDPG (155 μCi/μmole) gave 343.6 cpm/μl. The 0.5-ml samples were stored in 10-ml Erlenmeyer flasks in the freezer prior to use. When used, each sample was thawed, and 0.25 ml of the extract or fraction being assayed was added to it giving a total volume of 0.75 ml for each sample. The concentrations in each sample after addition of extract or fraction were 2 mM MgCl₂, 100 mM fructose, and 33 μM UDPG. Each sample contained 0.06 μCi of UDPG-¹⁴C. Samples were incubated with the particular extract or fraction at 30°C for 4 minutes prior to freeze-drying. Freeze-dried samples were reconstituted with 0.5 ml ddH₂O and 10-μl aliquots were analyzed for radioactive sucrose production using the DE-81 anion exchange paper technique.

MICROMOLE UDPG PER CPM

The μmole of UDPG (or sucrose) in the assay samples can be calculated as follows:

Each sample was 33 μM in UDPG;

$$(33 \mu\text{mole UDPG/liter}) \times (0.75 \text{ ml/sample}) \times (1 \text{ liter/1000 ml}) = 0.02475 \mu\text{mole UDPG/sample};$$

$$(6.0 \text{ ml UDPG-}^{14}\text{C/40 samples}) \times (1000 \mu\text{l/ml}) \times (343.6 \text{ UDPG-}^{14}\text{C cpm}/\mu\text{l}) = 51,540 \text{ UDPG-}^{14}\text{C cpm/sample}; \text{ and}$$

$$0.02475 \mu\text{mole UDPG/sample}/(51,540 \text{ cpm/sample}) = 4.8 \times 10^{-7} \mu\text{mole/cpm}.$$

CALCULATIONS FOR TABLE XIII

The calculations for the results presented in Table XIII are summarized in Table XXII. An example based upon the results for the crude callus extract follows:

The crude extract contained 0.19-mg protein/ml; 0.25 ml of crude was used in a standard sample; the reconstitution volume for the sample after incubation and freeze-drying was 0.5 ml; 10- μl aliquots of the reconstituted sample were analyzed for labeled sucrose production by the DE-81 technique;

$$(0.19\text{-mg protein/ml extract}) \times (0.25\text{-ml extract/sample}) = 0.0475\text{-mg protein/sample};$$

$$(0.0475\text{-mg protein/sample}) \times (10 \mu\text{l spot}/0.5 \text{ ml/sample}) \times (1 \text{ ml}/1000 \mu\text{l}) = 9.5 \times 10^{-4} \text{ mg protein}/10 \mu\text{l reconstituted sample};$$

$$(918 \text{ cpm left on DE-81 from } 10 \mu\text{l of reconstituted control}) - (320 \text{ cpm left on DE-81 from } 10 \mu\text{l of reconstituted sample}) = 598 \text{ apparent sucrose cpm}/10 \mu\text{l of reconstituted sample}/4 \text{ min};$$

$$(598 \text{ sucrose cpm}/10 \mu\text{l})/(9.5 \times 10^{-4} \text{ mg protein}/10 \mu\text{l}) = 6.3 \times 10^5 \text{ sucrose cpm/mg protein}/4 \text{ min}; \text{ and}$$

$$(6.3 \times 10^5 \text{ sucrose cpm/mg protein}/4 \text{ min}) \times (4.8 \times 10^{-7} \mu\text{mole/cpm}) = 0.076 \mu\text{mole sucrose produced/mg protein/min}.$$

The results of similar calculations for the other steps in the purification procedure are presented in Table XXII.

TABLE XXII

SUMMARY OF CALCULATIONS FOR TABLE XIII

	Crude Extract	P-6 Column Eluate	20-60% Ammonium Sulfate Fraction	Purified Enzyme
Mg protein/ml of fraction	0.19	0.19	0.087	0.054
Mg protein/10- μ l aliquots of reconstituted sample ($\times 10^4$)	9.5	9.5	4.35	2.95
Cpm left on DE-81 from 10- μ l aliquots of reconstituted sample	320	97	427	464
Apparent sucrose cpm/10- μ l aliquots of reconstituted sample	598	821	491	454
Sucrose cpm/mg protein/4-min incubation ($\times 10^{-5}$)	6.32	8.64	12.4	15.4
μ Mole sucrose produced/mg protein/ 4-min incubation	0.306	0.419	0.601	0.746
μ Mole sucrose produced/mg protein/ 30 min	2.3	3.1	4.5	5.6

A summary of the results obtained for several different sucrose synthetase preparations is presented in Table XXIII. These preparations were used for determining properties of the sucrose synthetase from aspen callus tissue.

TABLE XXIII
ENZYME PURIFICATION CALCULATIONS FOR DIFFERENT EXPERIMENTS

Experiment	61	62	63	65	66
Protein concentrations, mg protein/ml					
crude extract	0.19	0.25	0.456	0.274	0.251
purified enzyme	0.059	0.109	0.104	0.0853	0.144
Count left on DE-81 from 10- μ l aliquots of reconstituted sample					
control	918	901		849	851
crude extract	320	284		347	245
purified enzyme	464	292		240	176
Sucrose cpm/10- μ l aliquots of reconstituted sample					
crude extract	598	717	877 ^a	502	606
purified enzyme	454	709	726 ^a	608	675
Milligram protein/10- μ l aliquots of reconstituted sample ($\times 10^4$)					
crude extract	9.5	12.5	22.8	11.0	10.4
purified enzyme	2.95	5.45	5.2	3.4	5.76
Sucrose cpm/mg protein/4-min incubation ($\times 10^5$)					
crude extract	6.32	5.72	3.84	4.56	6.82
purified enzyme	15.4	13.05	13.09	20.0	11.72
Purification	2.43	2.28	3.63	4.4	1.73
Specific activity, μ mole _b sucrose/mg protein/min	0.186	0.159	0.159	0.243	0.143

^aFrom paper chromatography.

^bFour minute incubations.

APPENDIX VII

SEDIMENTATION COEFFICIENT

The sedimentation coefficient (at 9°C in MOPS buffer) for sucrose synthetase from aspen callus was determined by John Carlson. The log sheet for this determination is on file at The Institute of Paper Chemistry (Beckman Model E, Log Sheet No. 1608). The plot from which the sedimentation coefficient was obtained is presented in Fig. 29.

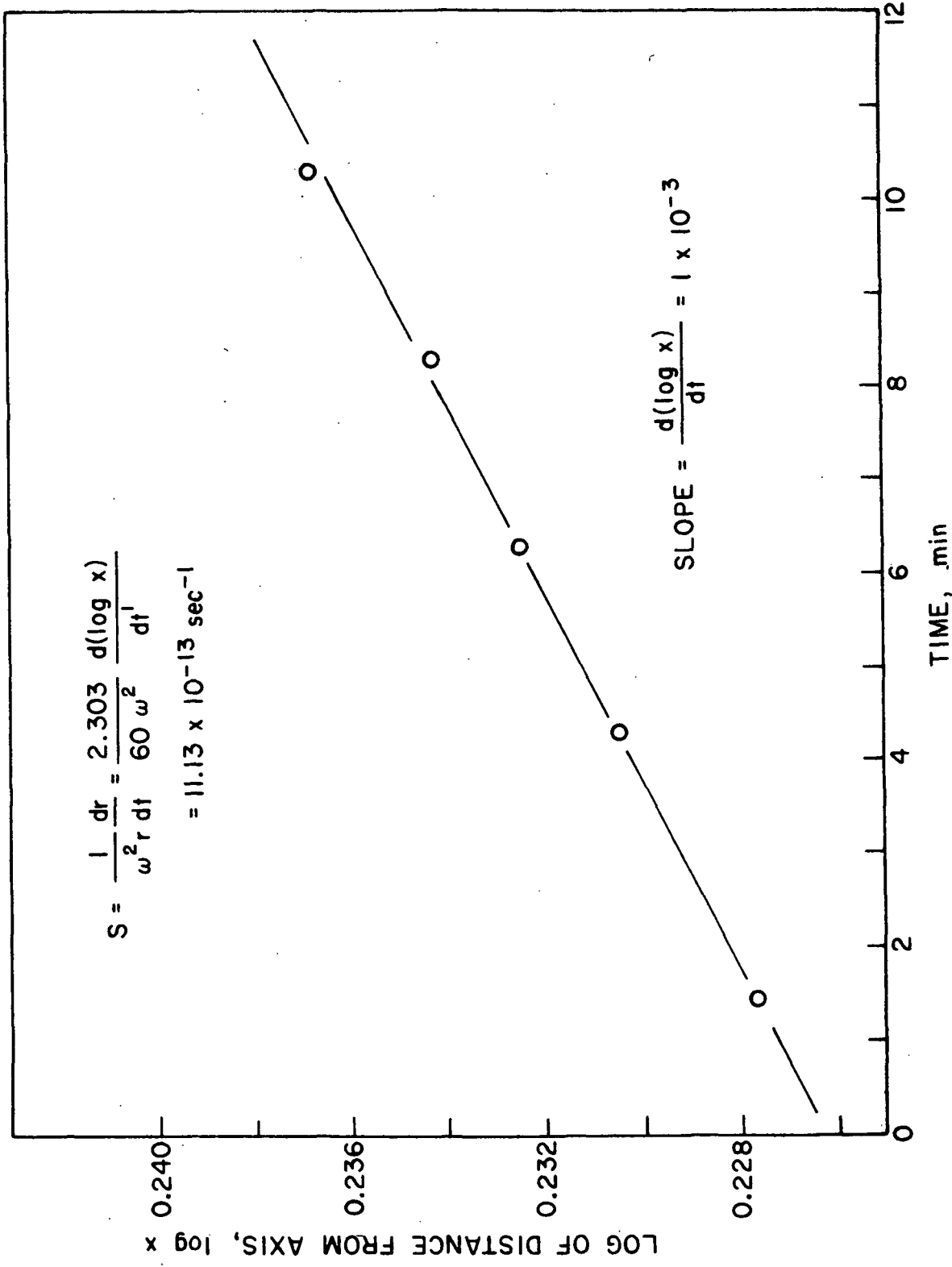


Figure 29. Determination of Sedimentation Coefficient

APPENDIX VIII

DETERMINATION OF K_m (UDPG)

The effect of UDPG concentration upon the sucrose synthesis reaction was investigated as discussed in the main body of the thesis. Two different enzyme preparations were used. That from Experiment 62 contained 0.109-mg protein/ml and that from Experiment 63 contained 0.104-mg protein/ml. Samples had a total volume of 0.3 ml, of which 0.1 ml was the sucrose synthetase preparation. Samples were 100 mM in fructose and 2 mM in $MgCl_2$. Each sample contained 20,600 cpm of UDPG- ^{14}C . The UDPG concentration varied from sample to sample as indicated in Table XXIV. Samples were incubated at 30°C for 4 minutes and reconstituted with 0.25 ml ddH₂O after freeze-drying. Aliquots of the reconstituted samples were analyzed for labeled sucrose by paper chromatography. The total extrapolated sucrose counts for each sample are reported in Table XXIV. Calculations similar to those discussed in Appendix VI were used to generate the data points for Fig. 23. The results of these calculations are summarized in Table XXIV.

TABLE XXIV

CALCULATIONS FOR DETERMINATION OF K_m (UDPG)

Sample	A	B	C	D	E	F	G	H	I
62-U3	109	4.5	21.8×10^{-5}	0.0238	2.18	0.545	15	0.066	1.83
62-U4	151	3.0	14.6×10^{-5}	0.0221	2.02	0.505	10	0.1	1.98
62-U5	295	1.5	7.3×10^{-5}	0.0215	1.97	0.492	5	0.2	2.04
62-U6	2,050	0.3	1.46×10^{-5}	0.0300	2.76	0.690	1	1.0	1.45
62-U7	3,580	0.15	0.73×10^{-5}	0.0261	2.39	0.597	0.5	2.0	1.675
62-U8	15,700	0.015	0.073×10^{-5}	0.0111	1.02	0.255	0.05	20.0	3.92
63-UA	210	1.5	4.36×10^{-5}	0.0091	0.88	0.220	5.0	0.2	4.55
63-UB	1,086	0.6	1.74×10^{-5}	0.0189	1.82	0.455	2.0	0.5	2.19
63-UC	11,900	0.075	0.218×10^{-5}	0.0260	2.50	0.625	0.25	4.0	1.60
63-UD	13,104	0.060	0.174×10^{-5}	0.0228	2.19	0.548	0.20	5.0	1.82
63-UE	14,386	0.045	0.131×10^{-5}	0.0188	1.805	0.457	0.15	6.66	2.22
63-UF	16,000	0.030	0.087×10^{-5}	0.0154	1.48	0.370	0.10	10.0	2.71
63-UG	25,546	0.015	0.044×10^{-5}	0.0112	1.08	0.270	0.05	20.0	3.70

A - Sucrose cpm/sample.

B - μ Mole nonradioactive UDPG in sample.C - μ Mole nonradioactive UDPG/cpm in initial sample.D - Sucrose produced, μ mole/4 min.E - μ Mole sucrose/mg protein/4 min.F - μ Mole sucrose/mg protein/min.

G - UDPG concentration in sample, mM.

H - $1/s$, $1/mM$ UDPG.I - $1/y$, (μ mole sucrose/mg protein/min) $^{-1}$.

APPENDIX IX

DETERMINATION OF K_{-m} (FRUCTOSE)

The effect of fructose concentration upon the sucrose synthesis reaction was investigated as discussed in the body of the thesis. Two different UDPG concentrations were used, 2 mM and 5 mM. The purified sucrose synthetase contained 0.104-mg protein/ml; 0.1 ml of enzyme was used in each sample, which had a total volume of 0.3 ml. Samples were 2 mM in $MgCl_2$, either 2 or 5 mM in UDPG, and contained varying concentrations of fructose as indicated in Table XXV. Each sample contained 30,900 cpm of UDPG- ^{14}C , which was equivalent to 4.85×10^{-5} μ mole UDPG (or sucrose)/cpm.

TABLE XXV

SUMMARY OF CALCULATIONS FOR DETERMINATION OF K_{-m} (FRUCTOSE)

Sample	A	B	C	D	E	F	G	H
F51	646	0.0313	3.01	0.752	5.0	50.0	1.335	0.02
F52	479	0.0233	2.24	0.560	5.0	10.0	1.79	0.10
F53	153	0.0077	0.074	0.186	5.0	1.0	5.39	1.0
F22	1083	0.0210	2.02	0.505	2.0	10.0	1.98	0.10
F23	299	0.0059	0.556	0.139	2.0	1.0	7.22	1.0
F24	160	0.0031	0.299	0.075	2.0	0.5	13.35	2.0

A - Total sucrose radioactivity in sample, cpm.

B - μ Mole sucrose in sample.

C - μ Mole sucrose/mg protein/4 min.

D - μ Mole sucrose/mg protein/min.

E - Concentration of UDPG, mM.

F - Concentration of fructose, mM.

G - $1/v$, (μ mole sucrose/mg protein/min) $^{-1}$.

H - $1/s$, (mM fructose) $^{-1}$.

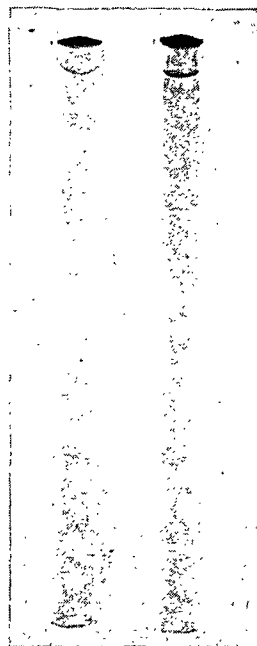
Samples were incubated at 30°C for 4 minutes and reconstituted with 0.25 ml ddH₂O after freeze-drying. Aliquots of the reconstituted samples were analyzed for radioactive sucrose by paper chromatography. The total cpm of sucrose for each sample are reported in Table XXV. Calculations similar to those discussed in Appendix VI were used to generate the data points for Fig. 24. The results of these calculations are summarized in Table XXV.

APPENDIX X

DISK ELECTROPHORESIS

Attempts were made to follow the purification of the sucrose synthetase from aspen callus tissue using a disk electrophoresis technique similar to that of Davis (56). The various fractions (in sucrose solution) were loaded on the top of gels. After electrophoresis, the gels were fixed and stained using a standard method (61). Heavy staining occurred at the top of all gels as can be seen in Fig. 30 for two representative gels. This staining at the tops may have been due to degraded enzyme, to retardation of gel penetration by the sucrose present during loading or to the size of the sucrose synthetase relative to the porosity of the gel used. The staining made it impossible to use disk electrophoresis as a criterion of purity.

Gel (a) has the fraction from 20-60% saturated ammonium sulfate obtained from the P-6 column eluate. This gel shows one protein band in addition to the heavy staining at the top of the gel. On gel (b) is the purified enzyme from agarose chromatography. Four protein bands are visible in this gel in addition to the heavy staining at the top. These bands may indicate breakdown of the tetrameric sucrose synthetase on the agarose column used as the final step in enzyme purification.



(a) (b)

Figure 30. Electrophoresis Gels.

- (a) Gel for Redissolved Fraction from 20-60% Saturated $(\text{NH}_4)_2\text{SO}_4$;
- (b) Gel for Agarose Column Eluate