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STUDIES ON THE LOCATION OF SUCROSE PHOSPHATASE IN PLANT TISSUES

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Abstract—More than 90 per cent of the sucrose phosphatase activity present in homogenates of immature stem tissue of sugar cane remains in the supernatant fraction after centrifugation at 100,000 *g*. In all properties studied the partially purified specific sucrose phosphatase from the supernatant was similar to the enzyme previously purified from the mitochondrial fraction. A specific sucrose phosphatase occurs in all tissues of sugar cane and other plants examined. Most of the enzyme appears in the supernatant fraction and attempts to isolate particles retaining more of the enzyme were unsuccessful. There is sufficient enzyme in both mature and immature stem tissue of sugar cane for sucrose accumulation to proceed by the proposed pathway which involves the synthesis and hydrolysis of sucrose phosphate. Sugar cane leaves contain enough enzyme to allow sucrose phosphate to be an intermediate in the synthesis of sucrose during photosynthesis.

INTRODUCTION

THE PARTIAL purification and properties of a specific sucrose phosphate phosphohydrolase (sucrose phosphatase) isolated from mitochondria of immature stem tissue of sugar cane and carrot roots has been described.[1] The release of sucrose phosphatase activity from the mitochondrial pellet by freezing and thawing suggested that the enzyme is located within a mitochondrial membrane. Although the supernatant remaining after the precipitation of mitochondria from homogenates of the above tissues also hydrolysed sucrose phosphate it was not known whether the hydrolysis was due to a specific sucrose phosphatase or to non-specific phosphatases. Attempts to separate a specific sucrose phosphatase from the phosphatases in the supernatant were not successful.

The present paper establishes that there is a relatively large amount of a specific sucrose phosphatase in the supernatant fraction of plant homogenates and that this enzyme is similar in all properties studied to that from the mitochondrial fraction. Further studies on the location of sucrose phosphatase in tissues and cells of sugar cane and other plants are reported.

RESULTS

Sucrose Phosphatase Activity in Homogenates

Sucrose phosphatase from sugar cane requires magnesium ions for maximum activity and in systems with no added magnesium it is completely inactive in the presence of EDTA.[1] In view of this property of the enzyme, homogenates of sugar cane were assayed for sucrose phosphatase activity in the presence of magnesium ions or EDTA. With EDTA the rate of hydrolysis of 61 μM sucrose phosphate was only a few per cent of the rate in the presence of $MgCl_2$. However, the rate of hydrolysis of 12 mM fructose 6-phosphate was virtually

[1] J. S. HAWKER and M. D. HATCH, *Biochem. J.* In press (1966).

unchanged by the addition of EDTA (Table 1). The very low activity towards sucrose phosphate of the phosphatase which hydrolyses fructose 6-phosphate can be explained in terms of the difference in concentration of sucrose phosphate and fructose 6-phosphate supplied. The Michaelis constant of sucrose phosphatase for sucrose phosphate is about 0·15 mM[1] whereas for the hydrolysis of fructose 6-phosphate by soluble sugar cane stem extracts the Michaelis constant is about 1 mM. Additional evidence for the presence of a specific sucrose phosphatase in carrot homogenates was obtained by studies on the inhibition of activity by sucrose and heat inactivation of the enzyme. The hydrolysis of 61 μM sucrose phosphate by enzyme preparations from carrot was inhibited 68 per cent by 50 mM sucrose whereas the hydrolysis of 12 mM fructose 6-phosphate was unaffected. Heating carrot preparations at 50° for 10 min reduced the rate of sucrose phosphate hydrolysis by 60 per cent while the rate of fructose 6-phosphate hydrolysis dropped by only 25 per cent.

TABLE 1. THE EFFECT OF EDTA ON THE RATE OF HYDROLYSIS OF SUCROSE PHOSPHATE AND FRUCTOSE 6-PHOSPHATE BY SUGAR CANE ENZYME PREPARATIONS

	Sucrose phosphate hydrolysis (mμmoles/hr/g tissue)		Fructose 6-phosphate hydrolysis (mμmoles/hr/g tissue)	
Enzyme source	+$MgCl_2$	+EDTA	+$MgCl_2$	+EDTA
Immature tissue (super.)	8,900	252	12,200	9,550
Immature tissue (20,000 *g* ppt)	410	—	856	- -
Mature tissue	480	0	1,500	1,410
Parenchyma	91	1	600	610
Dissected conducting tissue	211	8	850	990
Leaf laminae	37,700	1,800	5,700	6,000

Enzyme preparations were made as described in the Methods section. Assays were carried out in the presence of 10 mM $MgCl_2$ or 30 mM EDTA. The rates of hydrolysis shown are those observed with 61 μm [fructosyl-^{14}C] sucrose phosphate or 12 mM fructose 6-phosphate.

Purification of sucrose phosphatase from immature tissue of sugar cane. Since the unwashed 20,000 *g* precipitate from immature tissue homogenates contained only 4·4 per cent of the total sucrose phosphatase activity (Table 1), it was not necessary to remove this fraction of the activity before purifying sucrose phosphatase from immature tissue. Thus the dilution required when separating particles from supernatant was avoided. The results for the purification of sucrose phosphatase are shown in Table 2. Twenty per cent of the original sucrose phosphatase activity was recovered in the final fraction. This purification procedure, as compared to many previous attempts, was successful probably because the enzyme was maintained at as high a concentration as possible and also because the operations were completed in less than a day. The enzyme was specific for sucrose phosphate and the striking changes in relative activity towards several phosphorylated compounds achieved by purification are shown in Table 3.

TABLE 2. PURIFICATION OF SUCROSE PHOSPHATASE FROM IMMATURE TISSUE OF SUGAR CANE

Fraction	Sucrose phosphate hydrolysis (mμmoles/g tissue/hr)	Fructose 6-phosphate hydrolysis (mμmoles/g tissue/hr)	Ratio of activity, (sucrose phosphate) / (fructose 6-phosphate)
Filtrate through muslin	9900	6500	1·5
33–42% satd. $(NH_4)_2SO_4$	2900	1160	2·5
Sephadex G-100 fractions	3800	170	22
Calcium phosphate gel fraction	1920	40	48

The rates of hydrolysis of sucrose phosphate and fructose 6-phosphate were determined as described in the Methods section.

TABLE 3. SUBSTRATE SPECIFICITY OF SUGAR CANE SUCROSE PHOSPHATASE

Substrate	Relative activity: Filtered homogenate	Relative activity: Partially purified enzyme
Sucrose phosphate	100	100
Fructose 6-phosphate	67	2·1
Fructose 1-phosphate	55	1·4
Fructose 1,6-diphosphate	85	0
Glucose 6-phosphate	59	1·1
Glucose 1-phosphate	38	0·5
UDP	>289	3·8
α-Glycerophosphoric acid	114	2·8
p-Nitrophenyl phosphate	199	10·2

The rates of hydrolysis by filtered homogenate (filtrate through muslin of Table 2) and partially purified enzyme of sugar cane of the compounds listed were determined as described in the Methods section.

Properties of sucrose phosphatase. The partially purified enzyme had similar properties to those reported for the sucrose phosphatase purified from sugar cane mitochondria.[1] The Michaelis constant for sucrose phosphate was between 0·11 and 0·15 mM and the pH activity curve was similar in shape with a pH optimum between pH 6·4 and 6·7. Manganese chloride, potassium fluoride, inorganic phosphate and sucrose all inhibited activity. Small quantitative differences between the two enzyme preparations in the stimulation by different concentrations of added magnesium chloride were probably due to different levels of magnesium ions present in the preparations. The addition of EDTA (10 mM) completely inhibited the activity of both preparations. Storage of the soluble enzyme at −15° for 8 days resulted in loss of 50 per cent of the activity. After a further 16 days storage the activity was still 50 per cent of the original. Thirty-five per cent of the original activity remained after storage for a total of 80 days.

Intra-cellular distribution of sucrose phosphatase. Although some of the sucrose phosphatase in sugar cane homogenates is associated with particles which behave like mitochondria during centrifugation,[1] the majority of the enzyme remains in the supernatant (Table 1). In an attempt to determine whether the sucrose phosphatase remaining in supernatants after centrifugation at 20,000 *g* had been released from particles during homogenization of plant

tissues several modifications to the extracting medium and technique were made. Tissues of plants were ground in a mortar, filtered through muslin and the filtrate centrifuged usually only once. Precipitates were not washed to avoid possible particle disruption. In spite of the precautions taken very little of the total sucrose phosphatase activity was precipitated (Table 4). The unwashed 100,000 *g* precipitate of sugar cane extracts contained very little sucrose phosphatase. The relatively higher value for spinach leaf precipitate was probably due to the fact that the ratio of precipitate to supernatant in this preparation was high and consequently higher contamination of the precipitate could be expected. Although only 3 per cent of the sucrose phosphatase activity remained associated with particles in sugar cane leaf homogenates, 38 per cent of the total UDPG : fructose glucosyltransferase was present in the 20,000 *g* precipitate.

TABLE 4. INTRACELLULAR DISTRIBUTION OF SUCROSE PHOSPHATASE

Tissue	Sucrose phosphate hydrolysis (mμmoles/g of tissue/hr)		Activity in ppt as percentage of total
	Supernatant	20,000 *g* ppt	
Sugar cane			
Immature tissue			
0·3 M-mannitol	11,000	775*	7·1
0·3 M-mannitol	12,100	758	6·5
0·6 M-mannitol	10,300	750	7·5
1 M-mannitol	10,400	800	6·8
Mature stem tissue	2,210	141	6
Leaf laminae	113,000	3,580	3·1
Carrot root	7,370	730	9
S. robustum	28,100	1,280	4·4
Spinach leaf (method (*a*))	1,188	114†‡	13·5
Spinach leaf (method (*b*))	10,800	980†	8·3

Tissues were homogenized and fractionated and the rate of sucrose phosphate hydrolysis was determined as described in the Methods section.

*180 in the precipitate after 100,000 *g*; † at 15,000 *g*; ‡ 71 after 1000 *g*.

When sugar cane and tobacco leaves were extracted by the non-aqueous technique the various resulting fractions hydrolysed fructose 6-phosphate. Ribulose diphosphate carboxylase activity was mainly located in the tobacco chloroplast preparation (C. R. Slack, personal communication). However no sucrose phosphatase could be detected in any of the fractions from either tissue.

Sucrose phosphatase content of tissues. The rates of sucrose phosphate hydrolysis by extracts from different tissues of sugar cane (Pindar), other species of sugar cane, and carrot are shown in Table 5. Although these figures are for unpurified extracts, the rates in the presence of EDTA were, by comparison, very low suggesting that most of the hydrolysis was due to sucrose phosphatase. On a fresh weight basis, sugar cane leaves clearly contained far more sucrose phosphatase than any of the other tissues studied.

Distribution of sucrose phosphatase in mature tissue of sugar cane. The structure of mature tissue is such that parenchyma and conducting tissue can be separated by dissecting longitudinal slices. The results in Table 5 show that sucrose phosphatase is present in parenchyma.

Although dissected conducting tissue contains up to 70 per cent parenchyma (as compared to 90–95 per cent for whole tissue) the results strongly suggest that sucrose phosphatase is also present in conducting tissue.

TABLE 5. SUCROSE PHOSPHATASE FROM SUGAR CANE AND OTHER PLANT TISSUES

Source of enzyme	Sucrose phosphate hydrolysis (mμmoles/g tissue/hr)	
	+$MgCl_2$	+EDTA
Sugar cane var. Pindar		
Immature stem	27,000	750
Mature stem	1,440	0
Parenchyma	360	4
Conducting tissue	630	25
Leaf laminae	113,000	5,400
S. robustum		
Immature stem	28,000	1,500
S. spontaneum		
Immature stem	15,200	435
Carrot supernatant	12,400	0
Carrot precipitate (washed)	540	0

Supernatants were prepared and assayed for activity as described in the Methods section except for mature tissue and *S. spontaneum*. In these cases filtrates were assayed without centrifugation. Either 10 mM $MgCl_2$ or 20 mM EDTA was present in reaction mixtures. The carrot precipitate was washed in 10 ml of medium and recentrifuged.

DISCUSSION

Differences in the effect of EDTA, sucrose and heating on the rates of hydrolysis of different concentrations of sucrose phosphate and fructose 6-phosphate by unpurified tissue homogenates are almost certainly due to the presence of two or more phosphatases with different Michaelis constant values. The rate of sucrose phosphate hydrolysis by sucrose phosphatase at saturation is only three times that at 61 μM but the rate for fructose 6-phosphate hydrolysis by the other phosphatase would be 30–40 times greater. Similar increases could be expected for sucrose phosphate hydrolysis by the non-specific phosphatase. The results in Table 1 substantiate this assumption. The phosphatase which hydrolyses 61 μM sucrose phosphate is most likely a specific sucrose phosphatase. It was shown conclusively that this was the case for immature tissue of sugar cane (Tables 2 and 3). In the other tissues studied it is reasonable to assume that the increase in the rate of hydrolysis of sucrose phosphate in the presence of magnesium chloride over that in the presence of EDTA was due to a specific sucrose phosphatase. Therefore quantitative comparisons could be made between different tissues as regards their sucrose phosphatase content. In no tissue studied did the rate of sucrose phosphate hydrolysis in the presence of EDTA exceed 5 per cent of the rate in the presence of magnesium ions. Due to the instability of sucrose phosphatase, purification of the enzyme from extracts before assay would have made quantitative comparisons meaningless.

Most of the sucrose phosphatase partially purified from immature stem tissue of sugar

cane (Table 2) would be that fraction which appears in the supernatant of homogenates prepared in media containing mannitol. From a consideration of the properties studied, it would appear that this sucrose phosphatase and the sucrose phosphatase isolated from mitochondria are similar. Even though only a small percentage of the enzyme is associated with particles, it appears to be tightly bound within a membrane.[1] Attempts to separate particles retaining more of the sucrose phosphatase proved unsuccessful (Table 4). As pointed out previously[1] the presence of sucrose phosphatase in sugar cane tissues provides support for the concept that sucrose phosphate is involved in sucrose accumulation in the manner previously proposed.[2–5] Briefly this scheme involves hydrolysis of sucrose in the free space, phosphorylation of glucose and fructose, synthesis of sucrose phosphate and hydrolysis of sucrose phosphate.

From the results of Sacher *et al.*[3] it can be calculated that the accumulation of sucrose from glucose by immature stem tissue of sugar cane was about 12 μmoles/g/hr at 30 . Slack[6] reported the hydrolysis of between 3 and 16 μmoles of sucrose in the free space of similar tissue at 30°. From figures given for mature stem tissue[5] it can be calculated that the rate of sucrose accumulation from glucose at 30° would be about 2 μmoles/g/hr. The values for sucrose phosphate hydrolysis reported here (Tables 4 and 5) are between 10 and 27 μmoles/g/hr for immature tissue and 1·4 μmoles/g/hr for mature tissue at 22°. Allowing for the temperature differences there is ample sucrose phosphatase present to allow sucrose accumulation to proceed in both tissues by the pathway proposed.

One of the two sucrose synthesizing enzymes in mature stem tissue of sugar cane (UDPG : fructose glucosyltransferase) is situated almost entirely in the vascular tissue.[5] The location of the other enzyme (UDPG:fructose 6-phosphate glucosyltransferase) is not known.[4] However parenchyma tissue is capable of storing sucrose when glucose is supplied in the external medium (Hawker and Hatch, unpublished results). Thus it is probable that at least some of the UDPG:fructose 6-phosphate glucosyltransferase is situated in the parenchyma. The presence of a specific sucrose phosphatase in parenchyma (Table 5) supports this hypothesis.

The functional significance of the sucrose phosphatase associated with particles is not apparent. It is reasonable to expect that if the bulk of the sucrose phosphatase is involved in sucrose storage it would be situated at or near the tonoplast membrane. It is not associated with membrane fragments in tissue homogenates (Table 4) although it may be freed during homogenization. *In vitro* the enzyme is rapidly denatured below pH 6·0[1] and the pH of extracted sugar cane juice (which is mainly vacuolar) is about pH 5·5. However the enzyme could still be in the vacuole as its behaviour *in vivo* may be different or the pH at its site of action may be higher than the overall pH of the vacuole. If the phosphatase were in the vacuole a gradient in concentration of sucrose could be maintained between vacuole and cytoplasm by the movement across the tonoplast and subsequent hydrolysis of sucrose phosphate. Hence the present results provide further support for the hypothesis that sucrose phosphate is involved in sucrose accumulation in sugar cane tissue.[4]

Sucrose phosphatase is possibly also concerned with sucrose movement and accumulation in leaves. However the presence of much higher concentrations of sucrose phosphatase in leaves as compared with stems (Table 5) suggests that the enzyme may have a further role.

[2] K. T. GLASZIOU, *Plant Physiol.* **36**, 175 (1961).
[3] J. A. SACHER, M. D. HATCH and K. T. GLASZIOU, *Plant Physiol.* **38**, 348 (1963).
[4] M. D. HATCH, *Biochem. J.* **93**, 521 (1964).
[5] J. S. HAWKER and M. D. HATCH, *Physiol. Plant.* **18**, 444 (1965).
[6] C. R. SLACK, *Australian J. Biol. Sci.* **18**, 781 (1965).

the hydrolysis of sucrose phosphate produced in chloroplasts during photosynthesis. UDPG:fructose 6-phosphate glucosyltransferase has been found in leaves by several workers and recently in chloroplasts.[7,8] Sucrose phosphate was hydrolysed by their chloroplast preparations but no conclusions about the specificity of the enzymes were possible. Bird *et al.*[7] suggested that synthesis of sucrose as a result of photosynthesis in leaves proceeds mainly, if not solely, via sucrose phosphate in chloroplasts. During photosynthesis the hexose precursors for sucrose synthesis appear initially as their phosphorylated derivatives. Hence no more energy is required for the synthesis of sucrose phosphate than for sucrose. The energy loss from the system is very nearly the same whether fructose 6-phosphate or sucrose phosphate is dephosphorylated. UDPG is needed for sucrose and sucrose phosphate synthesis. Bird *et al.*[7] have found in chloroplasts all the enzymes necessary to synthesize UDPG from fructose 6-phosphate. The fructose 6-phosphate is almost certainly produced by dephosphorylation of fructose-1,6-diphosphate by an alkaline C1 fructose-1,6-diphosphatase.[9] If UDPG: fructose 6-phosphate glucosyltransferase is the enzyme operative in chloroplasts, no fructose is needed and the possibility of shortage of fructose 6-phosphate for UDPG synthesis by excess hydrolysis of fructose 6-phosphate by a phosphatase would be avoided. Furthermore a consideration of the equilibrium constants for the two transferases shows that sucrose phosphate rather than sucrose synthesis is favoured.[10]

If all the carbon dioxide fixed during photosynthesis by sugar cane leaves was converted to sucrose, maximum rates of synthesis of the order of 25 μmoles/g/hr could be achieved (J. C. Waldron, unpublished results). There is sufficient sucrose phosphatase in sugar cane leaves to hydrolyse this amount of sucrose phosphate (Table 5). The amount of fructose 6-phosphate hydrolysis under the conditions employed (Table 1) would not be sufficient to maintain the supply of fructose for sucrose synthesis by UDPG : fructose glucosyltransferase.

If sucrose phosphate is an intermediate in sucrose synthesis the site of its hydrolysis is not known. Isolation procedures designed to maintain the integrity of chloroplasts did not raise the amount of sucrose phosphatase retained by the 20,000 *g* precipitate (Table 4). Most of the small amount precipitated could have been present in the leaf mitochondria or as contamination of the pellet. However no conclusions can be reached as to the location of sucrose phosphatase in leaves since isolated chloroplasts are noted for their leakiness and non-aqueous isolation resulted in complete loss of sucrose phosphatase activity. However it makes no difference to the above argument whether sucrose phosphate is hydrolysed inside or outside the chloroplasts.

MATERIALS AND METHODS

Stem tissue from expanding internodes of sugar cane (*Saccharum officinarum* L., var. Pindar) was classified as immature and tissue from fully expanded internodes as mature. These tissues and leaves were obtained from field-grown cane. Immature tissue (expanding internodes) of *S. robustum* Brandes and Jeswiet ex Grassl (var. Mol 4943) and of *S. spontaneum* L. (var. Mandalay) was also obtained from field-grown plants. Carrot roots and spinach leaves were obtained from the local market.

Sucrose phosphate (phosphorylated at the 6-position of fructose and containing radioactivity only in the fructose moiety) was prepared enzymically (Hatch, 1964).

[7] I. F. Bird, H. K. Porter and C. R. Stocking, *Biochim. Biophys. Acta* **100**, 366 (1965).
[8] S. Haq and W. Z. Hassid, *Plant Physiol.* **40**, 591 (1965).
[9] R. Smillie, *Nature* **187**, 1024 (1960).
[10] J. Mendicino, *J. Biol. Chem.* **235**, 3347 (1960).

Measurement of enzyme activity. The rates of hydrolysis of [fructosyl-^{14}C] sucrose phosphate and of other phosphorylated compounds by enzyme preparations were determined as previously described[1] except that the tris-maleate buffer was adjusted to pH 6·7. Unless otherwise stated the values for sucrose phosphate hydrolysis are maximum velocities calculated as previously described. UDPG:fructose glucosyl transferase activity was determined as described by Hatch *et al.*[11]

Preparation of Sucrose Phosphatase

From immature stem tissue of sugar cane. Tissue (40 g) was cooled to 2°, sliced and ground in a mortar with 15 ml of 0·4 M tris-HCl buffer, pH 8·0, containing 0·06 M cysteine hydrochloride, 0·06 M sodium diethyldithiocarbamate and 0·01 M EDTA. All operations were carried out at 2°. After the homogenate had been squeezed through muslin, the protein fraction precipitating between 33 and 42 per cent saturation with $(NH_4)_2SO_4$ was collected by centrifugation and suspended in 2·5 ml of 2 mM tris-HCl buffer, pH 7·0. The suspension was centrifuged at 10,000 *g* for 15 min and the supernatant was applied to the top of a column of Sephadex G-100 (35 cm × 2·5 cm) which had been washed with 2 mM tris-HCl buffer, pH 7·0. The same buffer was passed through the column and 2 ml fractions were collected. Fractions 8–14 after the emergence of one void volume of eluent contained the bulk of the activity and were pooled and poured onto a calcium phosphate gel column (Hypatite C, volume 2 ml) which had been previously washed with 5 mM potassium phosphate buffer, pH 6·7. The column was washed with 5 ml of 20 mM potassium phosphate buffer, pH 6·7. Sucrose phosphatase was eluted with 4 ml of 80 mM potassium phosphate buffer, pH 6·7. After the addition of 20 mg of bovine serum albumin this solution was applied to a column (15 ml) of Sephadex G-25. After the emergence of one void volume of 2 mM tris-HCl buffer, pH 7·0, the next 4·2 ml containing the activity were collected. This fraction is referred to as "partially purified enzyme of sugar cane".

From particulate and soluble fractions of sugar cane and other plants. Cooled tissue (5 g) was ground in 20 ml of medium containing 0·1 M tris-HCl buffer, pH 7·6, 0·3 M mannitol, 0·01 M EDTA, 6 mM cysteine hydrochloride and 6 mM sodium diethyldithiocarbamate. The filtrate obtained by squeezing the homogenate through muslin was centrifuged for 15 min at 20,000 *g*. The unwashed precipitate was suspended in 5 ml of 2 mM tris-HCl buffer, pH 7·0, and frozen and thawed. In one experiment immature tissue of sugar cane was also ground in media in which the mannitol concentration was raised to 0·6 M and 1 M. Both the supernatants and precipitates were assayed for phosphatase activity.

From mature stem tissue of sugar cane. Longitudinal slices (0·5 mm) were cut and parenchyma and vascular tissue sectioned out and washed in running tap-water for 30 min.[5,12] These tissues and whole tissue were frozen and then ground with twice their weights of tris-maleate buffer, pH 7·5.[13] The homogenates were filtered through muslin and dialysed for 5 hr against 2 l. of diluted tris-maleate buffer (1 : 100), pH 6·5.[13]

From Spinach Leaves

(*a*) Enzyme preparations were made using a modification of the method described by Gee *et al.*[14] Cooled spinach leaf laminae (50 g) were ground in a mortar with 5 ml of 50 mM tris-

[11] M. D. HATCH, J. A. SACHER and K. T. GLASZIOU, *Plant Physiol.* **38**, 338 (1963).
[12] R. L. BIELESKI, Ph.D. Thesis, University of Sydney, Australia (1958).
[13] G. GOMORI, In *Methods in Enzymology* (Edited by S. P. COLOWICK and N. O. KAPLAN), Vol. I, p. 143. Academic Press, New York (1955).
[14] R. GEE, G. JOSHI, R. F. BILS and P. SALTMAN, *Plant Physiol.* **40**, 89 (1965).

HCl buffer, pH 7·5. The homogenate was squeezed through muslin and centrifuged at 1000 *g* for 10 min and 15,000 *g* for 15 min. The precipitates were suspended in 5 ml of 2 mM tris-HCl buffer, pH 7·0, and frozen and thawed before assay.

(*b*) Alternatively a modification of the method of Leech[15] was used. Leaf laminae (10 g) were cut into small pieces and stirred in 50 ml of a medium containing 50 mM tris-HCl buffer, pH 7·5, and 0·6 M mannitol. The suspension was squeezed through muslin and the filtrate was centrifuged at 15,000 *g* for 15 min. The precipitate was suspended in 5 ml of 2 mM tris-HCl buffer, pH 7·0, and frozen and thawed.

From leaves—non-aqueous extraction. Sugar cane leaf tissue was extracted using a modification of the non-aqueous extraction procedure used by Smillie.[16]

Tobacco leaf preparations, prepared by the non-aqueous technique of Bird *et al.*,[7] were a gift from Dr. C. R. Slack.

[15] R. M. Leech, *Biochim. Biophys. Acta* **79**, 637 (1964).
[16] R. M. Smillie, *Can. J. Bot.* **41**, 123 (1963).