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INVERTASE ACTIVITY DURING THE DEVELOPMENT OF CARROT ROOTS

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Abstract—Evidence was obtained that roots of carrot (*Daucus carota* L.) contain an acid invertase (optimum pH 4·5) and an alkaline invertase (optimum pH 7·4). The acid and alkaline invertases were partially purified and K_m for sucrose found to be $5{\cdot}6 \times 10^{-2}$ M and $2{\cdot}4 \times 10^{-2}$ M respectively. The acid invertase was distributed between the cell wall and supernatant fractions of root homogenates. This distribution was readily and reversibly altered by varying the pH of the extraction medium. Almost all of the alkaline invertase activity was found in the supernatant fraction of the homogenates. Sugar content and the activities of both invertases were shown to vary widely during the development of carrot roots. High acid invertase activity was found in rapidly growing tissue in which the sucrose content was low or was declining rapidly. With the exception of young seedlings, alkaline invertase activity was most marked in tissues that were storing sucrose. Possible roles for the two invertases in the control of sucrose metabolism are proposed.

INTRODUCTION

AN ACID and a neutral invertase have been demonstrated in extracts of storage tissue of sugar cane.[1] Marked changes in the activities of these two enzymes occur during the development of sugar cane. These changes are closely associated with changes in sucrose metabolism.[2] These observations have led to the proposal that both acid and neutral invertase play vital roles in the control of sucrose metabolism in sugar cane.[3] Carrot roots resemble the storage tissue of sugar cane in their ability to store large quantities of sucrose. We know little about the control of sucrose metabolism in carrots. Vaughan and MacDonald[4] have shown that extracts of mature carrot roots show very little invertase activity at pH 5·0 and that such activity develops to a considerable extent when disks of the root are incubated in water for 24 hr. We do not know whether this activity represents the only invertase present in carrots or whether it plays any significant role in sucrose metabolism. The work reported in this paper was undertaken to see whether carrots contained acid and neutral invertases and to see whether any evidence of a regulatory function for such enzymes could be found by comparing their activities with sugar content at different stages in the development of carrots.

RESULTS

Evidence for Acid and Alkaline Invertases

Invertase activity in extracts of disks of dormant mature carrot roots was low and was optimum at pH 7·5 (Fig. 1). There was a very slight indication of another peak of activity at

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[1] M. D. HATCH, J. A. SACHER and K. T. GLASZIOU, *Plant Physiol.* **38**, 338 (1963).
[2] M. D. HATCH and K. T. GLASZIOU, *Plant Physiol.* **38**, 344 (1963).
[3] J. A. SACHER, M. D. HATCH and K. T. GLASZIOU, *Plant Physiol.* **38**, 348 (1963).
[4] D. VAUGHAN and I. R. MACDONALD, *Plant Physiol.* **42**, 456 (1967).

pH 4·5. When disks, similar to those used in the above experiment, were aerated in distilled water at 25° for 24 hr (aged) they gave extracts with a very high invertase activity that showed a marked optimum at pH 4·5 (Fig. 1). These results indicated that the invertase activities at the two pH optima varied independently. This view was confirmed by determining the effect of ageing on invertase activity of disks of young carrot roots that showed appreciable activity at pH 4·5 and pH 7·5 (Table 1). The simultaneous presence of invertase activity at

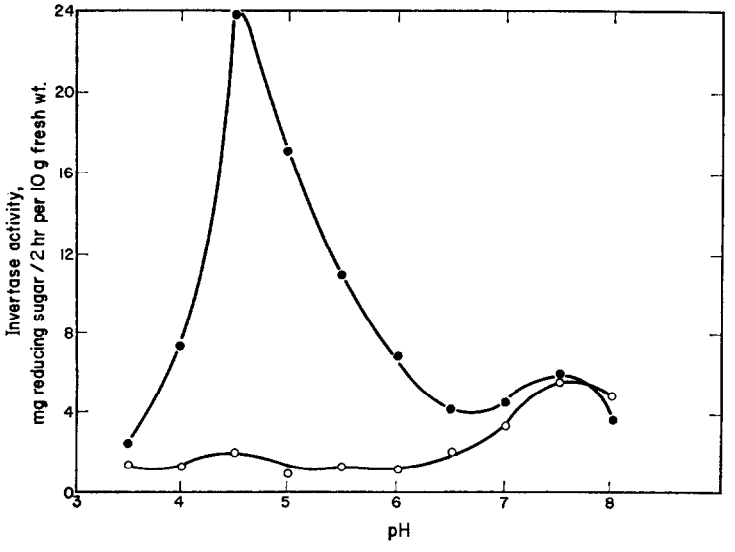

FIG. 1. EFFECT OF pH ON INVERTASE ACTIVITY IN SUPERNATANT FRACTION OF EXTRACTS OF FRESHLY CUT (o——o) AND 24-hr AGED (●——●) DISKS OF ROOTS OF MATURE CARROT.

TABLE 1. INDEPENDENT VARIATION OF INVERTASE ACTIVITY AT pH 4·5 AND pH 7·5 DURING AGEING OF DISKS OF YOUNG CARROT ROOTS

	Invertase activity (mg reducing sugar/2 hr/10 g initial fresh wt.)			
	At pH 4·5		At pH 7·5	
Tissue	Supernatant fraction	Cell-wall fraction	Supernatant fraction	Cell-wall fraction
Freshly cut	6	4	33	2
Aged for 48 hr	112	60	15	6

pH 4·5 and at pH 7·5 made freshly cut disks of young roots suitable material for an attempt to separate the two invertase activities. Fractionation of the supernatant fraction of a tissue homogenate with ammonium sulphate led to a very substantial separation of activity at pH 4·5 from that at pH 7·5 (Fig. 2). These partially purified preparations were used for determining K_m for sucrose. Using the Woolf plot,[5] we obtained values of $5{\cdot}6 \times 10^{-2}$ M for the acid invertase and $2{\cdot}4 \times 10^{-2}$ M for the alkaline invertase.

[5] M. DIXON and E. C. WEBB, *Enzymes*, Longmans, London (1964).

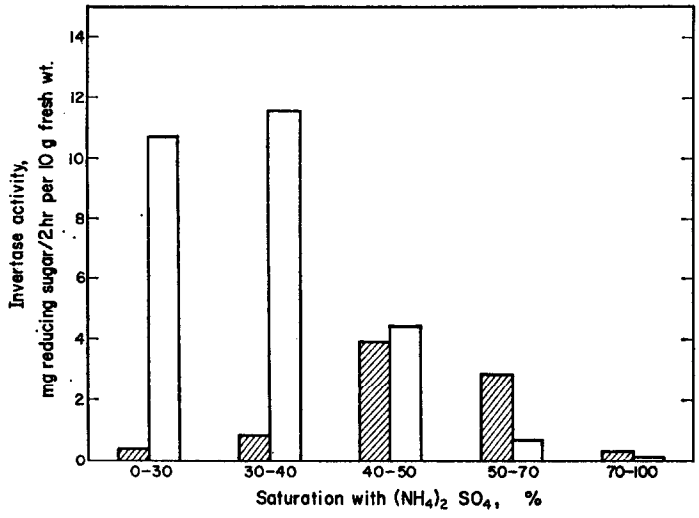

FIG. 2. PARTIAL SEPARATION OF ACID (■) AND ALKALINE (□) INVERTASE BY AMMONIUM SULPHATE FRACTIONATION OF SUPERNATANT FRACTION OF FRESHLY CUT DISKS OF YOUNG CARROT ROOT.

Association of Invertase with Cell-Wall Fraction

Much of the invertase activity of storage tissues remains in the cell-well fraction even after repeated extraction.[4, 6] We found that when freshly cut disks of both young and mature carrot roots were extracted with 0·1 M sodium citrate at pH 7·0, 90% of the total alkaline invertase activity appeared in the supernatant fraction of the extracts (Table 1). In contrast, much of the acid invertase activity was found in the cell-wall fraction when aged disks were extracted in the same way. The acid invertase activities in the cell-wall fraction and in the supernatant fraction showed identical pH optima. During ageing of disks of immature and mature carrots the marked changes in acid invertase activity in the supernatant fraction were very closely paralleled by similar changes in acid invertase activity in the cell-wall fractions.

The similar behaviour of acid invertase activity in the supernatant fraction and in the cell-wall fractions could represent two similar enzymes with different cellular locations, or a single enzyme with two locations, or a single enzyme with a single location *in vivo* but which had been redistributed by our extraction procedure. We investigated the latter possibility in experiments designed to discover whether the distribution of acid invertase activity could be altered by varying the extraction procedure. Jansen, Jang and Bonner[7] showed that enzymes firmly bound to *Avena* coleoptile cell walls could be removed with 0·15 N NaCl at pH 7·5. We found that this treatment did not alter the distribution of acid invertase activity in homogenates of aged disks of mature carrots. Arnold[8] showed that extraction with 0·2 M borate buffer at pH 8·5 solubilized most of the acid invertase found in the cell-wall fraction of grapes. Extraction of aged disks of carrot in borate buffer had the reverse effect (Table 2). None the less, we did find that the distribution of acid invertase activity in homogenates of aged disks could be drastically altered by changing the pH of the extraction medium (Table 2).

[6] J. EDELMAN and M. A. HALL, *Biochem. J.* **95**, 403 (1965).
[7] E. F. JANSEN, R. JANG and J. BONNER, *Plant Physiol.* **35**, 567 (1960).
[8] W. N. ARNOLD, *Biochim. Biophys. Acta* **128**, 124 (1966).

With the exception of borate buffer, an increase in the pH of the extraction medium brought about a marked increase in the proportion of the total acid invertase activity that appeared in the supernatant fraction of the homogenate. In addition we found that total acid invertase

TABLE 2. EFFECT OF COMPOSITION AND pH OF EXTRACTION MEDIUM ON DISTRIBUTION OF ACID INVERTASE ACTIVITY IN HOMOGENATES OF 48-hr AGED DISKS OF MATURE CARROT ROOT

Experiment*	Extraction medium	Invertase activity at pH 4·5 (mg reducing sugar/2 hr/10 g initial fresh wt.)	
		Supernatant fraction	Cell-wall fraction
1	0·1 M Na_2HPO_4–KH_2PO_4, pH 5·8	8	201
	0·1 M Na_2HPO_4–KH_2PO_4, pH 7·0	20	93
2	0·1 M Sodium citrate, pH 4·5	4	114
	0·1 M Sodium citrate, pH 7·0	47	40
	Distilled water	5	105
3	0·2 M Sodium borate, pH 7·5	5	236
	0·2 M Sodium borate, pH 8·0	4	269
	0·2 M Sodium borate, pH 8·5	3	238
4	0·103 M Na_2HPO_4–0·048 M citric acid, pH 5·0	12	249
	0·126 M Na_2HPO_4–0·037 M citric acid, pH 6·0	26	123
	0·165 M Na_2HPO_4–0·018 M citric acid, pH 7·0	78	54
	0·195 M Na_2HPO_4–0·003 M citric acid, pH 8·0	287	60

* Comparisons within each experiment were made with replicate samples of disks.

TABLE 3. EFFECT OF pH ON DISTRIBUTION OF ACID INVERTASE ACTIVITY IN HOMOGENATES OF 48-hr AGED DISKS OF MATURE CARROT ROOT

Treatment of homogenate	Invertase activity at pH 4·5 (mg reducing sugar/2 hr/10 g initial fresh wt.)			
	After homogenization in 0·091 M Na_2HPO_4–0·055 M citric acid at pH 4·5		After homogenization in 0·165 M Na_2HPO_4–0·018 M citric acid at pH 7·0	
	Supernatant fraction	Cell-wall fraction	Supernatant fraction	Cell-wall fraction
pH not changed	5	320	181	98
pH changed from pH 4·5 to pH 7·0 with 0·2 M Na_2HPO_4	225	88	—	—
pH changed from pH 7·0 to pH 4·5 with 1 M citric acid	—	—	14	341

activity fell as the pH of the extraction medium was raised from pH 5·0 to pH 7·0. Total activity rose again when the pH of the extraction medium was increased above pH 7·0.

The effect of pH on the distribution of acid invertase activity was reversible. In the experiment reported in Table 3, a sample of 48-hr aged disks of mature roots was extracted

in Na_2HPO_4–citric acid buffer at pH 4·5 and the homogenate was divided into halves. One half was fractionated into cell wall and supernatant fractions at once. The other half of the homogenate was fractionated after its pH had been altered to pH 7·0. A duplicate sample of disks was treated in the same way except that the extraction was performed at pH 7·0 and the change was from pH 7·0 to pH 4·5.

Invertase Activity and Sugar Content during Development

In assessing the relationship between sugar content and acid invertase activity we do not think it meaningful to report the activity in the cell-wall fraction separately from the activity in the supernatant fraction. This argument is based on the effect of pH on the distribution of the activity in the homogenates, the similar pH optima of the activity in the cell wall and supernatant fractions, and the parallel changes in activity that occur in the two fractions during ageing. Accordingly the activities are given as total acid invertase. The same procedure has been adopted for alkaline invertase activity.

Table 4 shows the relationship between invertase activity and sugar content at different

TABLE 4. INVERTASE ACTIVITY AND SUGAR CONTENT DURING DEVELOPMENT OF CARROT

Stage of development	Invertase activity (mg reducing sugar/2 hr/10 g fresh wt.)		Sugar content (mg/10 g fresh wt.)	
	At pH 4·5	At pH 7·5	Sucrose	Reducing sugar
Dormant seeds	158	71	111	25
2-Day-old seedlings	236	155	47	31
4-Day-old seedlings	372	91	23	38
6-Day-old seedlings	581	84	8	45
Roots of 20-day-old plants	158	4	5	12
Roots of 40-day-old plants	60	5	34	70
Roots of 55-day-old plants	35	20	158	158
Roots, diameter at crown 2 cm	19	35	231	215
Roots, diameter at crown 3 cm	28	49	—	—
Fully developed root from growing plant	5	14	296	147
Fully developed roots, from storage clamps, allowed to sprout in the light for:				
0 days	2	3	241	278
11 days	2	1	286	261
27 days	2	1	242	182
90 days	1	2	468	176
Fully developed roots, from storage clamps, allowed to sprout in the dark for:				
40 days	1	—	82	185
50 days	1	—	60	171
Shoots formed on roots allowed to sprout in the dark for 40 days	239	0	9	72

stages of development. We stress five aspects of these results. Firstly, study of the change in sugar content shows that the seeds contained appreciable amounts of sucrose that was rapidly used during germination. Marked storage of sucrose in the roots did not begin until the roots were about 50 days old. After 50 days there was a substantial accumulation of both sucrose and reducing sugars in the roots. These storage sugars were used when the roots were forced to sprout in the dark but there was no evidence of substantial breakdown when the roots sprouted naturally in the presence of light. Secondly, acid invertase activity changed greatly during the development of the root and these changes were closely and inversely related to sucrose content. Thirdly, alkaline invertase activity varied less than acid invertase activity and, with the exception of young seedlings, was highest in tissues that were storing sucrose. Fourthly, invertase activity at the two pH optima varied independently. Finally, roots that had sprouted in the dark contained little or no invertase activity although

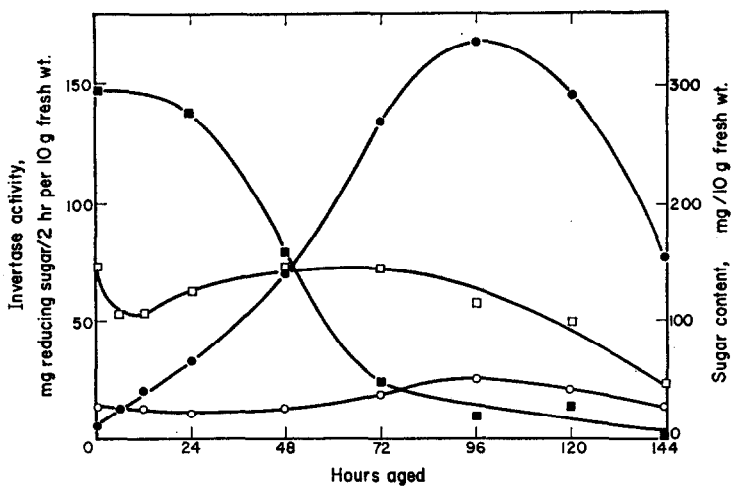

FIG. 3. EFFECT OF AGEING ON ACID INVERTASE ACTIVITY (●——●), ALKALINE INVERTASE ACTIVITY (○——○), AND CONTENT OF SUCROSE (■——■) AND REDUCING SUGAR (□——□) OF DISKS OF ROOT OF MATURE CARROT.

Note the different scales on the ordinate axes.

there was a marked decline in sucrose content. The shoots on such roots showed a very high acid invertase activity.

Data, similar to those shown in Table 4, but relating to the changes that take place during the ageing of disks of mature storage tissue, are given in Fig. 3. The major features of these results that we wish to stress are as follows. Firstly, mature roots with a high content of sucrose yielded extracts that showed some alkaline invertase activity but very little acid invertase activity. Secondly, ageing led to a massive increase in acid invertase activity that corresponded to a precipitate decline in sucrose content. Thirdly, ageing did not lead to a corresponding increase in the activity of the alkaline invertase. There are three reasons why we do not think that the above effects of ageing on sucrose content and invertase activity were due to microbial contamination. Firstly, the magnitude of the changes argues strongly against their being due to the metabolism of contaminants. Secondly, Vaughan and MacDonald[4] have provided evidence that ageing of carrot disks under aseptic conditions leads to a marked increase in acid but not alkaline invertase activity. Thirdly, the changes

in sugar content and acid invertase activity that occurred during the ageing of disks of red beet appear to have been largely unaffected by microbial contamination during ageing.[9]

DISCUSSION

The existence of two pH optima for invertase activity, the independent variation of activity at the two pH optima, and our substantial separation of the two activities, lead us to conclude that carrot roots synthesize at least two different invertases. The failure of Vaughan and MacDonald[4] to detect the alkaline invertase in carrot roots was probably due to the fact that they worked with aged disks. In aged disks alkaline invertase activity is likely to have been low and to have been overshadowed by the very active acid invertase. Thus in respect of invertase, carrot roots have been shown to be similar to sugar cane storage tissue[1] and to the seeds of *Phaseolus vulgaris* and *Vicia faba*.[10] Collectively the above results indicate that the ability to synthesize both an acid and a neutral or alkaline invertase may be a widespread property of higher plants.

It has been suggested that much of the invertase of a number of tissue cultures[11] and storage organs[4, 6] of plants is firmly bound to the cell wall *in vivo*. In our experiments, regardless of the extraction procedure, only a very small fraction of the alkaline invertase activity was found in the cell-wall fraction. Consequently we do not think that this enzyme is located on the cell wall *in vivo*. A similar situation appears to exist in sugar cane storage tissue.[1, 12]

The results of our study of the distribution of the acid invertase are complex. Extraction and fractionation at an acid pH gave high activities in the cell-wall fraction and little activity in the supernatant. These results agree with those obtained for carrot disks by Vaughan and MacDonald[4] and are similar to those reported for aged disks of other storage organs.[4, 6] However, our subsequent experiments leave no doubt that the pH and, to a lesser extent, the chemical composition of the extraction medium can markedly affect not only the relative activities of acid invertase in the cell wall and supernatant fractions but also the total activity of the homogenate. The effect of pH is reversible, and is so marked that we conclude that analysis of the distribution of acid invertase in homogenates of carrot roots cannot, at present, give us decisive information about the location of the enzyme *in vivo*. Our results and those of Arnold[8] weaken some of the evidence that acid invertase is predominantly a cell-wall enzyme. In the studies with disks of storage organs[4, 6] and in the work with tissue cultures[11] there are no reports of attempts to extract at pH 7·0 or above. Thus it is possible that the acid invertase content of the cell-wall fractions in the above work was due, at least in part, to the conditions of extraction.

Our data do not explain why alteration of the pH of the extraction medium affects the relative activities of acid invertase in the cell wall and supernatant fractions. The results strongly indicate that variation in the pH affects the binding of the acid invertase to the cell wall or to some component of the homogenate that sediments with the cell wall. Hawker[13] has recently published evidence that borate frees acid invertase from the cell-wall fraction of grape berries by disrupting either a tannin–protein association or a complex between tannin, protein, and cell wall. The formation of an identical complex is not the cause of the presence of acid invertase in cell-wall fractions of carrot because both Hawker[13] and ourselves have shown that borate is ineffective in releasing invertase from the cell-wall fraction.

[9] J. S. D. Bacon, I. R. MacDonald and A. H. Knight, *Biochem. J.* **94**, 175 (1965).
[10] R. A. Cooper and R. N. Greenshields, *Nature* **191**, 601 (1961).
[11] J. Strauss, *Plant Physiol.* **37**, 342 (1962).
[12] J. S. Hawker and M. D. Hatch, *Physiol. Plantarum* **18**, 444 (1965).
[13] J. S. Hawker, *Phytochem.* **8**, 337 (1969).

The sugar content and the activities of the acid and alkaline invertases in carrots have been shown to vary in a way that suggests that the two enzymes play important but different roles in the control of sucrose metabolism. The activity of acid invertase was shown to be high during the use of storage sucrose. This was so during germination and during the development of the ageing phenomenon in disks of storage tissue. High activities of the enzyme were also found in rapidly growing tissues, namely in young roots and in shoots formed when roots were allowed to sprout in the dark. These high activities of acid invertase were clearly characteristic of tissues of which the sucrose content was either very low or was declining very rapidly. Further, from the results in this and in a previous[14] paper it is clear that the development of acid invertase activity during ageing of disks of carrot storage tissue is accompanied by the loss of the ability to store sugars in the form of sucrose. The above results indicate that the function of the acid invertase of carrots is the hydrolysis of sucrose under conditions where there is a heavy demand for hexose. Thus, by determining the capacity for sucrose hydrolysis, the enzyme could contribute to the control of sucrose storage, and, when present at very high activities, act as a mechanism that prevents sucrose storage and channels the available carbon to hexose. The results of work with other plants are consistent with this view of the function of acid invertase. High activities of acid invertase have been shown to be correlated with rapid growth in roots of *V. faba*,[15] in sugar cane storage tissue[2] and in lentil epicotyls;[16] with the breakdown of storage sucrose in aged disks of red beet;[9] and with hexose formation during the ripening of grape berries.[17] In each of these instances the high activities of acid invertase may be viewed as a means of rapidly hydrolysing exogenous and endogenous sucrose and diverting the available carbon to hexose.

Measurements of alkaline or neutral invertase activity during development appear to have been reported only for carrot and sugar cane.[2] In both plants the available data indicate that the function of the alkaline or neutral invertase is to catalyse sucrose hydrolysis in cells that lack appreciable acid invertase activity. Although we lack enough data to assess the complete significance of the ability of these plants to make two quite different invertases at different stages in development, we think it worthwhile to propose the following working hypothesis. In cells with a high demand for sucrose hydrolysis the demand is met primarily by an acid invertase, at least some of which is located at the tonoplast. High activity of the tonoplast enzyme prevents sucrose storage. Storage is possible only in cells that lack appreciable acid invertase activity at the tonoplast. In such cells the need for sucrose hydrolysis is met primarily by an alkaline or neutral invertase located in the cytoplasm and separated from storage sucrose by the tonoplast. This proposed distribution of the enzymes is consistent with their pH optima as it is very likely that the contents of the vacuole have an acid pH. We suggest, therefore, that the significance of the presence of two different invertases is that, by their spatial separation and their independent variation, they permit independent control of sucrose storage and utilization.

EXPERIMENTAL

Material

Mature carrots of unspecified variety were bought locally and were used at once. Seeds, seedlings, and young carrots were of the variety "Scarlet Intermediate". Seedlings were obtained by germinating seeds

[14] T. AP REES and H. BEEVERS, *Plant Physiol.* **35**, 839 (1960).
[15] E. ROBINSON and R. BROWN, *J. Exp. Botany* **3**, 356 (1952).
[16] K. SEITZ and A. LANG, *Plant Physiol.* **43**, 1075 (1968).
[17] J. S. HAWKER, *Phytochem.* **8**, 9 (1969).

at 25° on moist filter paper in the dark. Roots of increasing age were obtained from seeds sown in vermiculite and watered with Knop's solution.[18] The young plants were grown in a greenhouse at 15–25°. Mature roots were made to sprout by burying them in sand at 15–25°.

Disks (10 × 1 mm) were prepared by mechanical slicing of cylinders of tissue that had been removed with a cork borer. The cylinders were taken parallel to the vertical axis of the root. The disks were aged by gentle circulation in aerated distilled water at 25°. The water was changed twice during the first 4 hr of ageing and at 12hr intervals thereafter.

Extraction and Assay of Invertase

All extracts were prepared at 4°. Unless stated otherwise, the following procedures were used. Tissue (10 g fresh wt.) was homogenized in 10 ml 0·165 M Na_2HPO_4–0·018 M citric acid buffer at pH 7·0. Separation into cell wall and supernatant fractions was achieved by centrifuging the slurry at 35,000 *g* for 20 min. The supernatant was then dialysed against 5 l. 0·017 M Na_2HPO_4–0·002 M citric acid buffer at pH 7·0 at 4° for 18–24 hr and was then taken for assay of invertase. The pellet at 35,000 *g* was re-suspended in 10 ml. of extraction medium and was centrifuged at 1100 *g* for 10 min. The pellet was washed in this way a further three times with extraction medium and then twice with distilled water. The washed pellet is called the cell-wall fraction. For measurement of total invertase activity the unfractionated homogenate was dialysed as described above and then taken for invertase assay. In the separation of the acid and alkaline invertases the supernatant from the initial centrifugation at 35,000 *g* was taken and fractionated at once at pH 7·5–8·0 by successive additions of solid $(NH_4)_2SO_4$. The precipitates obtained with $(NH_4)_2SO_4$ were dissolved in 6·0 ml 0·165 M Na_2HPO_4–0·018 M citric acid buffer (pH 7·0) and were then dialysed for 18 hr against 5 l. of the same buffer at one-tenth the strength.

For the assay of acid invertase the reaction mixture was 1·0 ml of the supernatant or the cell-wall fraction and 4·0 ml 0·091 M Na_2HPO_4–0·055 M citric acid buffer (pH 4·5) that contained 50 mg of sucrose. The same mixture was used for alkaline invertase except that the buffer was 0·187 M Na_2HPO_4–0·007 M citric acid at pH 7·5. Incubation was at 30° for 1–2 hr. The reaction was stopped by the successive addition of about 5·0 ml 6·5% (w/v) $ZnSO_4$ solution and about 5·0 ml saturated $Ba(OH)_2$ solution. The amounts of these solutions were adjusted to give a final pH of 7·0. After removing the $BaSO_4$ by centrifugation the reducing sugars in the supernatant were measured by a modification of the Somogi method.[19] The invertase activities were not dependent upon the presence of inorganic phosphate. The use of citric acid–sodium citrate buffer for extraction and dialysis followed by assay of the acid invertase in 0·2 M citric acid–sodium citrate buffer (pH 4·5), and by assay of the alkaline invertase in 0·2 M Tricine (*N*-Tris-(hydroxymethyl)–methylglycine) buffer at pH 7·6, gave enzyme activities very similar to those obtained when the enzymes were assayed in the presence of phosphate as described above. Thus our invertase activities can not be ascribed to sucrose phosphorylase.

Estimation of Sugar Content

Tissue was extracted as for the invertase assays and the supernatant after centrifugation at 35,000 *g* was taken for analysis. For reducing sugars 0·5–1·0 ml of the supernatant was added to 4·0 ml of 0·091 M Na_2HPO_4–0·055 M citric acid buffer at pH 4·5. $ZnSO_4$ solution (6·5%) and saturated $Ba(OH)_2$ solution were added at once and the reducing sugars determined as described above. For sucrose determinations the procedure was the same except that the mixture of extract and buffer was incubated for 1 hr at 30° with 0·02 ml of commercial invertase preparation (British Drug Houses Ltd., Poole, Dorset; 340 enzyme units/ml) before the addition of the $ZnSO_4$. The sucrose content was estimated from the difference in the amounts of reducing sugar found in the presence and absence of the treatment with invertase.

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[18] E. J. Hewitt, *Sand and Water Culture Methods used in the Study of Plant Nutrition*, Commonwealth Agricultural Bureaux, Farnham Royal, England (1966).

[19] S. Hestrin, D. S. Feingold and M. Schramm, in *Methods in Enzymology* (edited by S. P. Colowick and N. O. Kaplan), Vol. I, p. 231, Academic Press, New York (1955).