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The Location of Acid Invertase Activity and Sucrose in the Vacuoles of Storage Roots of Beetroot (*Beta vulgaris*)

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Vacuoles were isolated from freshly cut slices of the storage roots of beetroot (*Beta vulgaris*), and from slices that had been washed in aerated water for 1–3 days. The unique vacuolar location of betanin permitted the use of a correlative method to determine whether sucrose and acid invertase were located in the vacuoles. The specific content (the activity of the enzyme or amount of substrate per mg of protein) and the percentage recoveries for betanin, sucrose and acid invertase were determined for the different fractions obtained during the isolation of the vacuoles. For each fraction the specific content of betanin was plotted against those of sucrose and acid invertase. Similar correlative plots were drawn for the percentage recoveries. For both specific contents and percentage recoveries the correlation coefficients for sucrose and for acid invertase versus betanin were close to unity, and the lines passed near the origins. It is concluded that, in beetroot, most of the sucrose and much of the acid invertase are in the vacuoles. Measurements of vacuolar sucrose and acid invertase in beetroot slices washed for 1–3 days demonstrated an inverse relationship between sucrose content and acid invertase activity.

Although the enzyme has yet to be completely characterized, there is adequate evidence that most plants contain an acid invertase that is distinguished by its ability to hydrolyse sucrose optimally in the range pH 4.0–5.3. Estimates of the maximum catalytic activity of acid invertase in a range of plant tissues show it to be highest where the demand for hexose from incoming or stored sucrose is greatest, and to be inversely proportional to the sucrose content of the tissue. These observations, and the assumption that the vacuole is the site of sucrose storage, prompted the suggestion that some of the acid invertase of plant cells is in the vacuole, where it plays a major role in regulating sucrose storage. In cells with a high demand for hexose, vacuolar acid invertase was seen as a mechanism that prevented sucrose storage and ensured that any sucrose stored in or entering the vacuole was converted into hexose, which was generally respired. Storage of sucrose was envisaged as occurring only when the activity of vacuolar acid invertase was low (Ricardo & ap Rees, 1970; ap Rees, 1974). In the different regions of the root of pea (*Pisum sativum*) the relationship between acid invertase activity and the manner in which sucrose is metabolized has been shown to be consistent with this hypothesis (Dick & ap Rees, 1976). Further, invertase has been shown to be in the vacuole in yeast (Meyer & Matile, 1974). The aim of the work described in the present paper was to use a mechanical technique for the isolation of vacuoles (Leigh & Branton, 1976) to determine directly whether acid invertase and sucrose are located in the vacuoles of the storage roots of beetroot (*Beta vulgaris*).

Mature beetroots contain large amounts of sucrose and little detectable acid invertase. If thin slices of beetroot are washed by continuous circulation in aerated water, there is, over a period of 4 days, a marked increase in acid invertase activity and a corresponding fall in sucrose content (Bacon *et al.*, 1965; Vaughan & MacDonald, 1967). Thus, by examining vacuoles isolated from freshly sliced beetroot and from slices that had been washed for various periods, we hoped to establish whether acid invertase and sucrose were located in vacuoles, and whether an increase in vacuolar acid invertase was accompanied by a decrease in vacuolar sucrose.

As the yield of intact vacuoles was small relative to the number of cells disrupted (Leigh & Branton, 1976), it was not practicable to decide whether enzymes or substrates were located in the vacuole by using the conventional method of comparing the amounts present in the initial and final preparations. Therefore we used a correlative method based on de Duve's (1964) postulate of biochemical homogeneity, which states that members of a given population of organelles, separated by centrifugation, are enzymically homogeneous. In beetroot, the red pigment betanin appears to be confined to the vacuole (Mabry *et al.*, 1972). From the above postulate it follows that in any given population of vacuoles there

will be a constant relationship between the amount of betanin and the amounts of other vacuolar components. Thus any enzyme or substrate that is entirely or predominantly located in the vacuole should satisfy the following criteria during cell fractionation. Firstly, the ratio of the specific content of the enzyme or substrate to the specific content of betanin should be the same in all fractions obtained during vacuole isolation. Specific content is defined as the activity of an enzyme or the amount of a substrate in a fraction per mg of protein in that fraction. Thus if the specific contents of the enzyme or substrate and those of betanin are determined for each fraction, and are plotted as a correlation, the result should be a straight line that passes through the origin and gives a high value for the correlation coefficient. Secondly, in any one fraction, the percentage recovery of any constituent of the vacuole should be the same as that of betanin. Correlative plots of these percentage recoveries should yield a straight line with a slope of unity that passes through the origin and gives a high value for the correlation coefficient. An approach similar to that described above has been used successfully in studies of enzyme location in subcellular components of rat liver (de Duve *et al.*, 1955) and in membrane fractions from plants (VanDerWoude *et al.*, 1974; Williamson *et al.*, 1975; Leonard & VanDerWoude, 1976).

Experimental

Reagents

Dextran sulphate, 4-nitrophenyl disodium orthophosphate, poly(ethylene glycol) 4000, invertase concentrate, and common laboratory reagents, which were of the highest purity available, were obtained from BDH Chemicals, Poole, Dorset, U.K. [^{3}H]-Inulin (sp. radioactivity over 300mCi/mmol, [U-^{14}C]-glucose (sp. radioactivity 230mCi/mmol) and n-[1,2(n)-^{3}H]hexadecane (certified sp. radioactivity 2.17μCi/g) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Glucose oxidase (grade III) was from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K.; peroxidase (type I), glutamate–oxaloacetate transaminase (aspartate aminotransferase) assay kit no. 55 UV and *o*-dianisidine hydrochloride (3,3′-dimethoxybenzidine dihydrochloride) were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.; Metrizamide {2-[3-acetamido-2,4,6-tri-iodo-5-(*N*-methylacetamido)benzamido]-2-deoxy-D-glucose} was from Vestric Ltd., Stonefield Way, Ruislip, Middx., U.K.; sodium diatrizoate [3,5-bis(acetylamino)-2,4,6-tri-iodobenzoic acid sodium salt] was from Uniscience, Jesus Lane, Cambridge, U.K. Miracloth was obtained from Calbiochem, Bishops Stortford, Herts., U.K.

Plants

Storage roots of beetroot (*Beta vulgaris* L.) were obtained locally and were either used at once or after storage for up to 6 months in moist vermiculite in covered bins that were kept on the roof of the Botany School. Tissue slices for washing were prepared with the apparatus described by Leigh & Branton (1976), which was set to cut slices about 1mm thick and 6–16cm^2 surface area. The slices were washed in vigorously aerated water at 25°C (250g fresh wt. in 5 litres); the water was changed thrice during the first 12h of the washing period and twice daily thereafter.

Isolation of vacuoles

Vacuoles were isolated by a modification of the method of Leigh & Branton (1976). For preparations from beetroot that had not been subjected to the washing procedure, 450g of beetroot was sliced into 1 litre of collection medium (1M-sorbitol, 5mM-EDTA, 25mM-2-mercaptoethanol, 50mM-Tris/HCl, pH8.0) at 17–20°C. The slicing was done with the apparatus described by Leigh & Branton (1976), which was operated at 530rev./min and was set to cut slices approx. 0.1mm thick. The suspension of sliced tissue and extracted cell contents was then passed through a stainless-steel sieve and through Miracloth. The residue from this filtration was then sliced, as described above, into the filtrate, which was then filtered a second time. After this second filtration, the residue was discarded, and the filtrate, which is called the unfractionated homogenate, was retained. Four 220ml samples of this unfractionated homogenate were centrifuged in round-bottomed polycarbonate bottles for 20min at 1300g (rotor JA-14; $r_{av.}$ 8.7cm; Beckman J-21C centrifuge). The centrifugation was carried out at 4°C, as were all subsequent steps in the preparation of vacuoles. The supernatant was removed by aspiration and the pellets were resuspended in a total of 20ml of 15% (w/v) Metrizamide in resuspension medium [1.2M-sorbitol, 1mM-EDTA, 25mM-2-mercaptoethanol, 25mM-Tris/Mes (4-morpholine-ethanesulphonic acid, pH8.0] to give the fraction called 1300g pellet. Samples of this fraction were then put in 15ml polycarbonate tubes (4ml of the fraction in each of four tubes) and each sample was covered with 5ml of 10% (w/v) Metrizamide in resuspension medium and then with 2ml of resuspension medium. The resulting discontinuous density gradients were then centrifuged for 10min at 430g (15ml buckets; $r_{av.}$ 17cm, MSE Magnum refrigerated centrifuge). The gradients were divided into fractions as shown in Fig. 1 below. The gradient pellets were resuspended in a total of 4ml of resuspension medium.

Except for the following modifications, the above method was also used to isolate vacuoles from washed slices of beetroot. At the end of the washing period about 1kg of slices was plasmolysed by incubation in

1.0M-sorbitol at 17°C for 1h, and was then used for the preparation of vacuoles. The collection medium was 1.0M-sorbitol, 5mM-EDTA, 20mM-potassium metabisulphite, 0.1% (w/v) dextran sulphate, 50mM-Tris/HCl, pH7.6. The resuspension medium was 1.2M-sorbitol, 10mM-EDTA, 20mM-potassium metabisulphite, 25mM-Tris/HCl, pH7.6. The potassium metabisulphite was added to the above media immediately before use, and the pH was adjusted with NaOH. In experiments in which the sucrose content of the fractions was measured, Metrizamide was replaced with sodium diatrizoate, because, although both compounds interfered with the assay for sucrose, the latter could be removed with a cation-exchange resin.

Enzyme assays

For the assay of acid invertase in the fractions obtained during the isolation of vacuoles, the fractions were dialysed overnight at 4°C against 15 litres of 10mM-Na_2HPO_4/5mM-citric acid adjusted to pH5.0 with NaOH. The volume of the non-diffusible material was decreased by dialysis against powdered poly(ethylene glycol) 4000 at 4°C. A sample was then assayed for invertase, as described by Ricardo & ap Rees (1970), in a reaction mixture of 1.2ml that contained 58mM-sucrose and 20mM-Na_2HPO_4/10mM-citric acid adjusted to pH5.0 with NaOH. Samples were incubated at 30°C for up to 2h. The reaction was stopped by adding 2.0ml of 0.23M-$ZnSO_4$ followed by 3.0ml of saturated $Ba(OH)_2$ solution to give a final pH of 7.0. Glucose was assayed with glucose oxidase as described by Kilburn & Taylor (1969), except that the buffer was that described by Lloyd & Whelan (1969). For measurement of the acid invertase activity of tissue slices, samples (approx. 2g fresh wt.) were homogenized thoroughly, with a pestle and mortar, in 5ml of 50mM-Na_2HPO_4 adjusted to pH7.6 with citric acid. The homogenate was centrifuged at 35000g for 10min, and the resulting supernatant was then dialysed and assayed as described above.

The activity of acid phosphatase was measured in a reaction mixture of 1ml that contained 3mM-4-nitrophenyl disodium orthophosphate and 50mM-acetic acid/NaOH at pH4.5. The reaction was started by adding 0.1ml of the appropriate subcellular fraction; incubation was for 1h at 37°C and the reaction was stopped by the addition of 2ml of 1M-NaOH. The release of 4-nitrophenol was determined from measurements of A_{410}. Previously described methods were used for the assay of the following enzymes in 0.1ml samples of the different subcellular fractions: cytochrome oxidase (EC 1.9.3.1), Hodges & Leonard (1974); glucose 6-phosphate dehydrogenase (EC 1.1.1.49), Kornberg & Horecker (1955); 6-phosphogluconate dehydrogenase (EC 1.1.1.44), ap Rees *et al.* (1976); NAD–malate dehydrogenase (EC 1.1.1.37), Greenway & Sims (1974). Aspartate aminotransferase (EC 2.6.1.1) was assayed with a kit available from Sigma.

Analytical methods

For measurement of the sucrose content of beetroot tissue, samples (approx. 1g fresh wt.) were extracted successively, at 90°C, with 5ml volumes of aq. 80% (w/v) ethanol, aq. 50% (w/v) ethanol and water. The extracts were combined, made up to 25ml with water, and a sample was removed for determination of glucose by the method described under 'Enzyme assays'. The ethanol was removed from the remainder of the extract by rotary evaporation at 25°C and the residue was then made up to 25ml with water. Samples (1.0ml) of this were then incubated at 17–20°C overnight in a reaction mixture of 5.0ml that contained 0.1ml of invertase concentrate and 20mM-Na_2HPO_4/10mM-citric acid adjusted to pH4.5 with NaOH. At the end of the incubation, samples were removed for the determination of glucose as described above. The sucrose content was calculated from the difference between the amounts of glucose present before and after treatment with invertase. The sucrose present in the different fractions obtained during the isolation of vacuoles was measured as described above, but after the removal of substances that interfered with the assay. Protein was removed by adding 0.2ml of 0.23M-$ZnSO_4$ and 0.3ml of saturated $Ba(OH)_2$ solution to 1.0ml samples of the fractions. Then sodium diatrizoate was precipitated by adding 200mg of Dowex-50 resin (H^+ form) to the sample, which was left for 10min before being filtered through a glass microfibre prefilter (Whatman GF/C) and a 0.8μm Millipore membrane filter; the filtrate was then assayed for sugars.

Betanin was assayed as described by Leigh & Branton (1976) except that 1ml of 1mM-EDTA/50mM-Tris/HCl, pH7.5, was added to the sample instead of 1ml of water. The A_{537} was measured, and betanin concentration was calculated from ε_{537} = 62000 litre·mol^{-1}·cm^{-1} (Wyler *et al.*, 1959). DNA was measured as described by Leigh & Branton (1976). Protein, after precipitation with 7% (w/v) trichloroacetic acid, was assayed as described by Lowry *et al.* (1951), with bovine serum albumin as a standard. Samples that contained sodium diatrizoate were dialysed for 17h at 4°C against 10 litres of 50mM-KH_2PO_4/NaOH (pH7.5) before the addition of the trichloroacetic acid to the non-diffusible material to precipitate the protein. Radioactivity was measured by liquid-scintillation spectrometry with the scintillant described by Turner (1969). Corrections for quenching were made by the channels-ratio method. The quench curve was prepared by adding beetroot sap to samples of n-[1,2(n)-^{3}H]hexadecane of known

radioactivity. Vacuoles were counted with a Levy ultraplane Neubauer haemocytometer.

Results and Discussion

Measurements on freshly cut slices of beetroot showed that they contained large amounts of sucrose (91–146 μmol/g fresh wt.) and very little acid invertase activity (0.05 μmol of sucrose hydrolysed/h per g fresh wt.). Washing the slices for 3 days led to a 100–200-fold increase in acid invertase activity, and to the disappearance of 95% of the stored sucrose.

During fractionation, the vacuoles behaved essentially as described by Leigh & Branton (1976). Intact vacuoles were recovered in fraction 2 (Fig. 1); considerable betanin was also recovered in fraction 4, but this was derived from broken vacuoles. The integrity and purity of intact vacuoles isolated from freshly sliced beetroot have been described in detail (Leigh & Branton, 1976). The same tests of contamination and integrity were applied in the present work. The results showed that, in these respects, the vacuoles that we obtained from freshly sliced beetroots compared favourably with those described previously. In particular, we stress that the vacuoles recovered in fraction 2 of the density gradient were not significantly contaminated by nuclei, mitochondria and plastids (Fig. 1). Yields of vacuoles from slices that had been washed for 44–71 h were lower than those from freshly sliced beetroots: only 5–10% of the betanin placed on the density gradients was recovered in fraction 2 compared with 20–30% when freshly cut slices were used. We attribute this decrease to the presence in the extracts of secondary products formed as a result of the metabolic changes caused by the washing of the slices. The addition of dextran sulphate and potassium metabisulphite to the isolation media alleviated these deleterious effects, but did not eliminate them. Vacuoles isolated from washed slices were examined for contamination as described for those from freshly sliced beetroots (Leigh & Branton, 1976). The recoveries, in fraction 2, of the markers for contaminants were similar to those obtained with freshly cut slices (Leigh & Branton, 1976). Thus, although the vacuolar preparations from washed slices were not appreciably contaminated, the ratio contaminants/(intact vacuoles) was higher than in preparations from freshly cut slices because the latter contained more intact vacuoles. The specific activities of betanin (μmol/mg of protein) in fraction 2 from fresh and washed slices were, respectively, 0.40 ± 0.03 (mean ± S.E.M. from 19 fractionations)

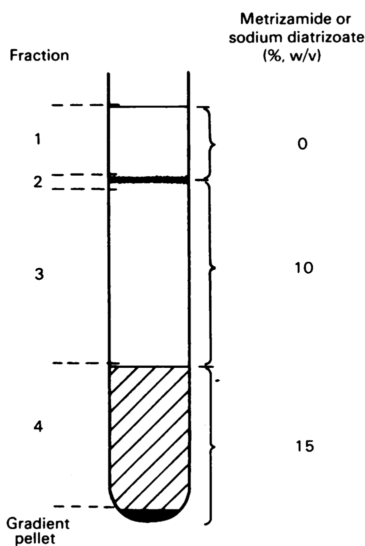

Fig. 1. *Fractions obtained during purification of vacuoles by density-gradient centrifugation*

The 1300 *g* pellet, obtained by centrifuging the unfractionated homogenate, was subjected to density-gradient centrifugation as described in the text. The gradient was divided into fractions as shown. The shading represents the red pigment: intact vacuoles were concentrated in fraction 2. Markers for other organelles, e.g. cytochrome oxidase (mitochondria), aspartate aminotransferase (plastids), DNA (nuclei) and glucose 6-phosphate dehydrogenase (cytoplasm), were concentrated in fraction 4 and the gradient pellet (Leigh & Branton, 1976).

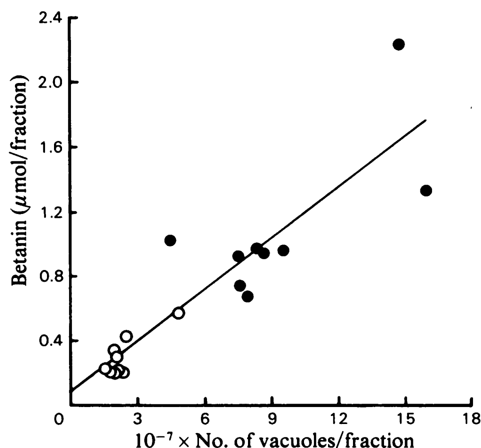

Fig. 2. *Relationship between the number of vacuoles and the betanin content in vacuole-enriched fractions isolated from freshly sliced beetroot*

The number of vacuoles and the betanin content of the 1300 *g* pellet (●) and fraction 2 (○) of the gradients obtained from nine different homogenates were determined. The line was fitted to the points by the least-squares method: the correlation coefficient was 0.90.

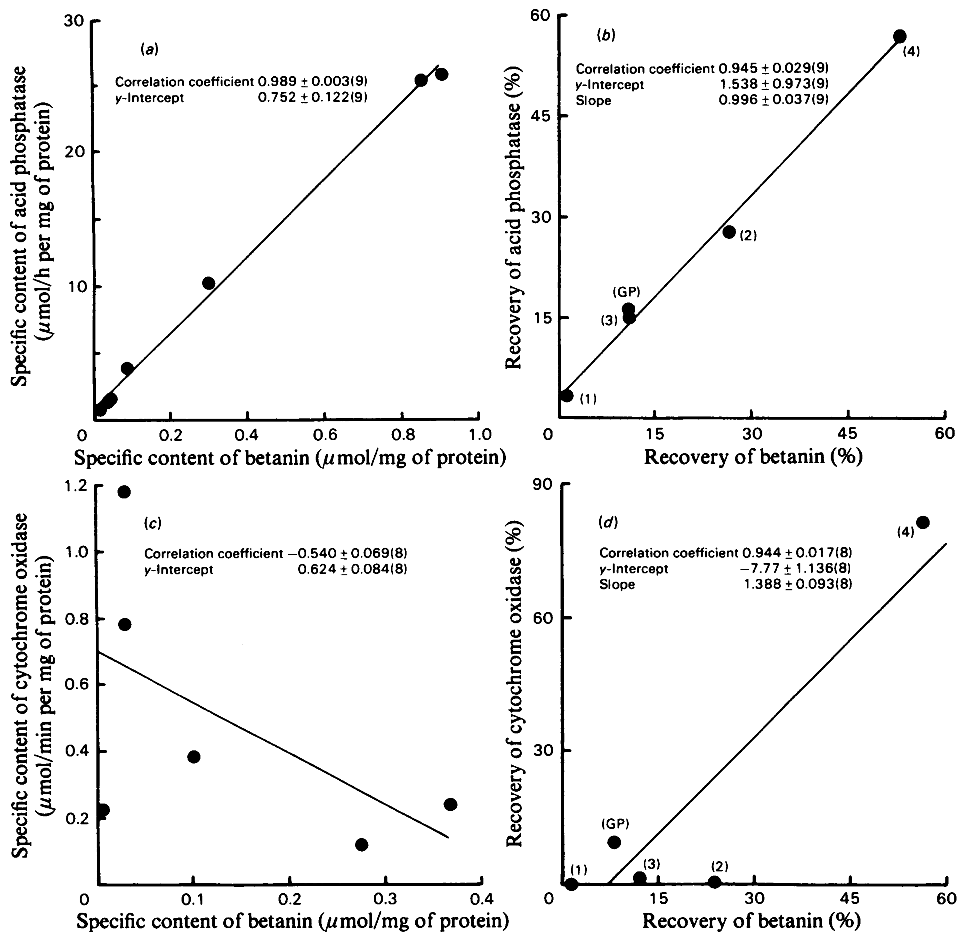

Fig. 3. *Relationships between specific contents and percentage recoveries of betanin and those of acid phosphatase (a, b) and cytochrome oxidase (c, d) in fractions produced during the isolation of vacuoles from freshly sliced beetroot*

The graphs for acid phosphatase are of data from fractions obtained from a single homogenate; those for cytochrome oxidase are from another homogenate. Each point represents a single fraction produced during the isolation of vacuoles. Each graph is typical of results obtained with a number of different homogenates. Above each graph are given, for the different homogenates, the mean values (±S.E.M.) for the correlation coefficient, the intercept on the ordinate, and, for the percentage recoveries, the slope of the line. The numbers of homogenates fractionated are given in parentheses. Lines were fitted to the points by the least-squares method. The graphs of specific contents include all the fractions obtained during the isolation of vacuoles. The graphs for percentage recoveries include only the fractions from the density gradient and the recoveries are expressed as percentages of the amounts in the 1300*g* pellets. (*a*) Specific contents of betanin and acid phosphatase; (*b*) percentage recoveries of betanin and acid phosphatase; (*c*) specific contents of betanin and cytochrome oxidase; (*d*) percentage recoveries of betanin and cytochrome oxidase. In (*b*) and (*d*) the number in parentheses beside each point refers to the fraction of the gradient from which the data are derived; GP is the gradient pellet.

and 0.14 ± 0.02 (mean ± S.E.M. from seven fractionations). These values are, respectively, ten and five times those of the 1300*g* pellets placed on the gradients.

Successful application of the correlative method described in the present paper requires good recovery of enzymes and substrates during the fractionations. In our experiments the activities of enzymes and the amounts of substrates recovered from the different fractions of the gradients (Fig. 1) ranged from 85 to 115% of those in the 1300*g* pellets. For the investigation of the relationship between the specific content

of betanin and those of enzymes or substrates, we used data from all fractions obtained during vacuole isolation, namely the unfractionated homogenate, the 1300*g* supernatant, the 1300*g* pellet and the five fractions from the density gradient (Fig. 1). For the study of the percentage recoveries we used only the fractions from the density gradient. The recoveries are expressed as percentages of the amounts in the 1300*g* pellet rather than the amounts in the unfractionated homogenates. We did this because only a small percentage of the vacuolar components in the unfractionated homogenates are within intact vacuoles (Leigh & Branton, 1976). Apart from Fig. 2, each correlative plot is of data from a single experiment and each point represents a single fraction. For any one enzyme or substrate, the graphs for specific content and percentage recovery are of data from fractions obtained from a single extract. The data are presented in this way rather than as mean values from all the different extracts because the absolute yields and recoveries varied from experiment to experiment. This gave a large spread of data for individual fractions, but did not appreciably alter the relationship between the distribution of betanin and those of the enzymes or substrates. The constancy of these relationships may be judged from the S.E.M. values for the correlation coefficients that are given above each graph.

Our experimental approach rests on the assumption that, in beetroot, betanin is confined to the vacuoles. Examination of plasmolysed tissue with a microscope revealed the cytoplasm as a colourless region surrounding the highly pigmented vacuole. Further evidence was obtained by determining the relationship between betanin content and the number of vacuoles present in the vacuole-enriched fractions (Fig. 2). The betanin content of the fractions correlated with the number of vacuoles present (correlation coefficient 0.90). The scattering of the points is attributed to breakage of vacuoles during the handling of the fractions. We suggest that in beetroots the betanin is confined to the vacuoles.

We tested the validity of our experimental approach by determining the relationship between the betanin content of the different fractions and the activities of one enzyme known to be associated with vacuoles and another known to be confined to some other organelle. For the latter we chose cytochrome oxidase. For the former we chose acid phosphatase, as there is independent histochemical and biochemical evidence that it is a vacuolar enzyme (Matile, 1975; Walker-Simmons & Ryan, 1977; Boller & Kende, 1978). The specific contents and recoveries of betanin and acid phosphatase correlated closely (Figs. 3*a* and 3*b*). This shows that acid phosphatase in beetroots is predominantly vacuolar. If the initial homogenate was not filtered through Miracloth, the activity recovered in the gradient pellet was greater than expected from its betanin content, although the activity recovered in the other fractions was not altered. We attribute this extra activity in the gradient pellet to acid phosphatase associated with cell-wall fragments that are recovered in this fraction unless the homogenate is filtered through Miracloth. There was not a close correlation between the betanin contents of the different fractions and their cytochrome oxidase activities (Figs. 3*c* and 3*d*). Similar experiments showed that the distribution of none of the following correlated with that of betanin: DNA, aspartate aminotransferase, 6-phosphogluconate dehydrogenase, glucose 6-phosphate dehydrogenase and NAD–malate dehydrogenase.

The above experiments support our argument that

Table 1. *Distribution of ^{3}H, betanin, sucrose and acid phosphatase in fractions obtained after slicing beetroot into collection medium labelled with [^{3}H]inulin*

Beetroots were sliced into 1 litre of collection medium that contained 10μCi of 0.033mM-[^{3}H]inulin (>300mCi/mmol). Vacuoles were isolated from this homogenate and each fraction was assayed for ^{3}H, betanin, sucrose and acid phosphatase as described in the Experimental section. For each fraction the amounts of ^{3}H, sucrose, and acid phosphatase are related to the betanin content; the amounts in fraction 1 were not detectable (N.D.).

	Ratio		
Fraction	^{3}H (d.p.m.)/ betanin (pmol)	Acid phosphatase (μmol/h)/ betanin (μmol)	Sucrose (μmol)/ betanin (μmol)
Unfractionated homogenate	127	29.5	189
1300*g* supernatant	120	30.7	185
1300*g* pellet	242	30.9	214
Gradient fraction 1	N.D.	N.D.	N.D.
2	13	33.0	199
3	34	27.4	124
4	236	32.2	199
Gradient pellet	1321	34.1	195

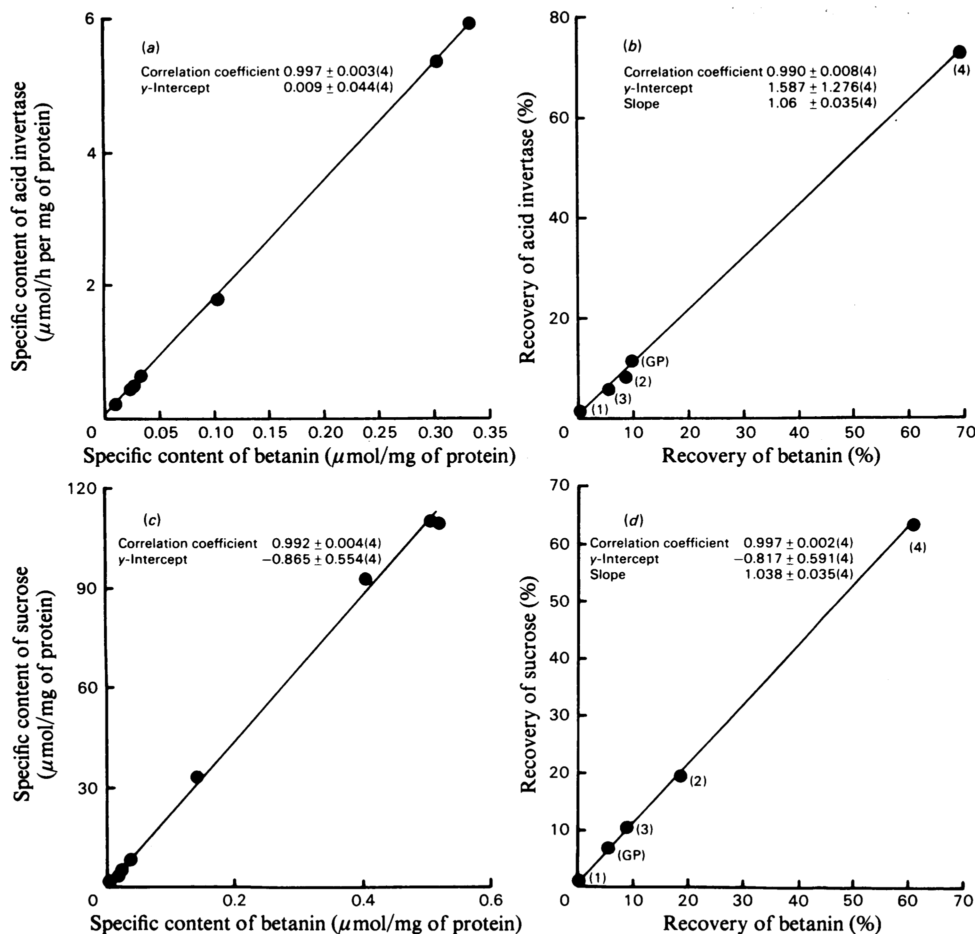

Fig. 4. *Relationships between specific contents and percentage recoveries of betanin and those of acid invertase* (*a*, *b*) *and sucrose* (*c*, *d*) *in fractions produced during the isolation of vacuoles from beetroot*

The graphs for acid invertase are of data from fractions of a single homogenate made from beetroot slices that had been washed for 64h; those for sucrose are from a single homogenate of freshly sliced beetroot. Each graph is typical of results obtained with a number of different homogenates. Above each graph are given, for the different homogenates, the mean values (±S.E.M.) for the correlation coefficient, the intercept on the ordinate and, for the recoveries, the slope of the line. The numbers of homogenates fractionated are given in parentheses. The data are presented as described in Fig. 3. (*a*) Specific contents of betanin and acid invertase; (*b*) percentage recoveries of betanin and acid invertase; (*c*) specific contents of betanin and sucrose; (*d*) percentage recoveries of betanin and sucrose.

only substances that are entirely or predominantly located in the vacuole show the expected correlation with betanin. An exception to this conclusion might arise if the vacuole-enriched fractions contained significant numbers of vacuoles or vesicles that had broken and resealed during disruption of the tissue and had trapped some of the initial homogenate within them. Such vesicles would contain betanin in a constant ratio to the other components of the homogenate and, if they were isolated and mistakenly identified as vacuoles, would give data similar to those in Figs. 3(*a*) and 3(*b*). The fact that a number of enzymes did not show a constant relationship to betanin (Figs. 3*c* and 3*d*) is evidence against this possibility. More direct evidence was obtained by adding [^{3}H]inulin to the collection medium before slicing the beetroot. Vacuoles were then isolated by the standard procedure and the ^{3}H/betanin ratio in each fraction was determined (Table 1). A similar value for this ratio in all fractions would indicate significant entrapment of homogenate. In fact the ^{3}H/betanin ratio was not constant and was lowest in the fraction

that contained the purified vacuoles. In contrast the ratios sucrose/betanin and acid phosphatase/betanin were more or less constant. In similar experiments we showed that added catalase (EC 1.11.1.6) and added [U-^{14}C]glucose behaved in the same way as [^{3}H]inulin. By assuming that all of the ^{3}H in fraction 2 represents entrapment, and by comparing the amounts of ^{3}H and betanin in the unfractionated homogenate and fraction 2, it can be calculated that a maximum of 10% of the betanin in fraction 2 could have been due to entrapment. We conclude that our procedure is adequate to reveal whether an enzyme or substrate is located predominantly in beetroot vacuoles.

The extent to which acid invertase activity is located in beetroot vacuoles was investigated with slices that had been washed for periods varying from 44 to 71 h. The total activity of the enzyme detected in the 1300*g* pellets ranged, according to the length of the washing period, from 1.3 to 10.6 μmol of sucrose hydrolysed/h, and 5–12% of this activity was recovered in fraction 2. We found that there was a strong correlation between betanin and the activity of acid invertase, in respect of both specific content and percentage recovery (Figs. 4*a* and 4*b*). We conclude that, in washed slices of beetroot, a significant proportion of the acid invertase is in the vacuoles. As with acid phosphatase, we found that failure to filter through Miracloth led to higher than expected activity in the gradient pellet. We attribute this to acid invertase associated with fragments of the cell wall.

In order to determine whether sucrose is located in the vacuoles of beetroot we isolated vacuoles from freshly sliced tissue. The distribution of sucrose among the different fractions correlated closely with that of betanin (Figs. 4*c* and 4*d*). We conclude that, in beetroot, sucrose is stored in the vacuoles. This conclusion is expected on general grounds, but until now has lacked direct evidence. The relationship between the amount of sucrose in the vacuole and the activity of vacuolar acid invertase was determined by measuring both in preparations of vacuoles isolated from slices of beetroot that had been washed for various periods. The ratios sucrose/betanin and acid invertase/betanin during this time are shown in Fig. 5, and establish an inverse relationship between sucrose content and acid invertase activity of the vacuoles. Over the same time there was no consistent change in the ratio acid phosphatase/betanin. This indicates that there was not a general increase in the activities of vacuolar enzymes.

The present paper provides adequate evidence that, in the storage roots of beetroot, sucrose and much of the acid invertase and acid phosphatase are located in the vacuole. Our work also shows that high sucrose content is associated with low activity of acid invertase in the vacuoles, and that an increase in the

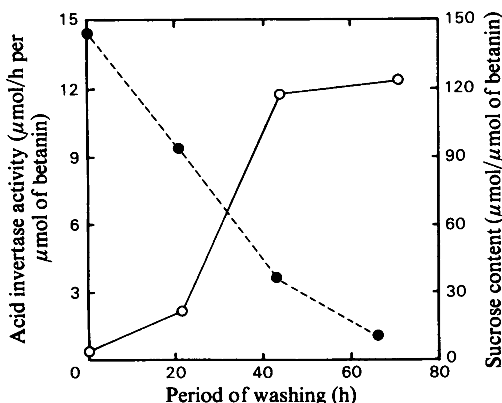

Fig. 5. *Sucrose content and acid invertase activity of vacuoles isolated from beetroot slices that had been washed for different times*

Vacuoles were isolated by density-gradient centrifugation from beetroot slices that had been washed at 25°C for periods ranging from 0 to 71 h. The amounts of betanin and sucrose and the activity of acid invertase in the isolated vacuoles (fraction 2, Fig. 1) were determined. Sucrose content and enzyme activity are related to betanin content.

latter is accompanied by a decline in the former. Goldschmidt & Branton (1977) have presented conclusions similar to ours in a preliminary report of a study of beetroot vacuoles, and there is also a preliminary report that sucrose is located in the vacuoles of castor-bean endosperm (Beevers & Nishimura, 1978). The present work and the above-mentioned reports provide further support for the view that vacuolar acid invertase plays a major role in determining the fate of sucrose in plant tissues. Our work also suggests that some of the acid invertase and acid phosphatase in beetroots may be located on the cell wall. This evidence is not conclusive, because of the possibility that the enzymes became associated with the cell wall during the preparation of the homogenates (ap Rees, 1974).

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