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LOCATION AND DENSITY LABELLING OF ACID INVERTASE IN AGING DISCS OF *DAUCUS CAROTA* STORAGE TISSUE

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Abstract—Cell walls prepared from aged discs by extraction in 0 1 M acetate buffer, pH 4 8, possess ionically bound acid invertase which can be removed from the wall by incubation in 1 M sodium chloride in 0 1 M acetate buffer, pH 4 8, and more firmly attached enzyme which is not removed Cell walls prepared in 0 195 M phosphate-0 003 M citrate-buffer, pH 8 0, do not possess ionically bound enzyme Ionically bound invertase is density labelled when discs are aged in 90% deuterium oxide suggesting that at least part of the increase in activity observed during aging is due to *de novo* protein synthesis

INTRODUCTION

WOUNDING of plant tissue is followed by an increase in respiration,[1] nucleic acid,[2,3] polysome[3] and protein[4] synthesis, and the activity of a number of enzymes [5–10] Of these enzymes, invertase (β-fructofuranosidase, E C 3 2 1 26) has probably received the most attention since Bacon[9] showed in 1961 that washed, aerated slices of sugar beet tissue progressively develop invertase activity This observation has since been extended to red beet,[10] Jerusalem artichoke,[6] potato[11] and carrot [11] Invertase appears to be located both on the wall and in the cytoplasm, although there is some dispute about this [12]

Actinomycin-*D*, *p*-fluorophenylalanine and thiouracil prevent the increase in activity after wounding,[6] indicating that protein and nucleic acid synthesis are involved This however does not prove that the increase in activity is due to *de novo* synthesis, since an increase in activity could be due to removal or destruction of inhibitor, or to activation of a performed protein precursor, processes which themselves may depend on a functional protein synthetic apparatus

We have utilized the density labelling technique, first introduced by Hu, Bock and

[1] STILES, W and DENT, K W (1947) *Ann Botany* **11**, 1
[2] CLICK, R E and HACKETT, D P (1963) *Proc Natl Acad Sci U S* **50**, 243
[3] LEAVER, C J and KEY, J L (1967) *Proc Natl Acad Sci U S* **57**, 1338
[4] SAMPSON, M J and LATIES, G G (1968) *Plant Physiol* **43**, 1011
[5] URITANI, I and MINAMKAWA, T (1964) *Arch Biochem Biophys* **108**, 573
[6] EDELMAN, J and HALL, M A (1965) *Biochem J* **95**, 403
[7] KANAZAWA, Y, SHICHI, H and URITANI, I (1965) *Agr Biol Chem* **29**, 840
[8] SACHER, J A, TOWERS, G H. N and DAVIES, D D (1972) *Phytochemistry* **11**, 2383
[9] BACON, J S D (1961) *Biochem J* **79**, 20P
[10] BACON, J S D, MACDONALD, I R. and KNIGHT, A H (1965) *Biochem J* **94**, 174
[11] VAUGHN, D and MACDONALD, I R (1967) *Plant Physiol* **42**, 456
[12] RICARDO, C P P and AP REES, T (1970) *Phytochemistry* **9**, 239

Halvorson[13] and successfully employed by subsequent workers,[8 14 15] to determine whether acid invertase is synthesized *de novo* after wounding This technique involves supplying the system under study with a substance containing a heavy isotope which can be incorporated into the amino acids used for the synthesis of new protein The incorporation of the isotope results in an increase in mass which can be detected by isopycnic equilibrium centrifugation In this way it is possible to decide whether the protein is synthesized after the addition of the labelled compound or whether it had existed in the cells before isotope addition The results presented in this paper show that the aging of discs in deuterium oxide leads to the formation of density labelled invertase We conclude that at least part of the increase in acid invertase activity observed during aging is due to *de novo* protein synthesis

RESULTS AND DISCUSSION

Although the pronounced increase in invertase activity which occurs in aging discs of various storage tissues has been demonstrated by several workers, the intracellular location of the enzyme is still in dispute Two main methods have been used to determine the intracellular distribution of acid invertase The first method, in which intact discs are assayed, involves treating aged discs with ethyl acetate [10] This destroys cell membranes and allows sucrose in the assay medium to come into contact with soluble (cytoplasmic) invertase in the cell The difference in the amount of reducing sugar produced by ethyl acetate-treated discs and untreated discs is then a measure of soluble acid invertase

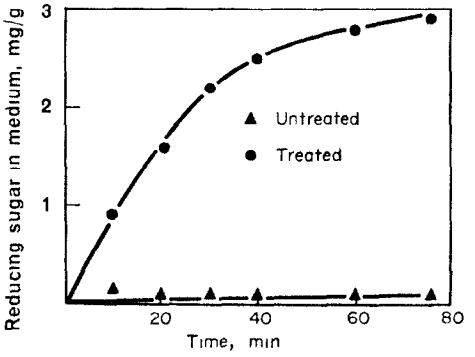

FIG 1 LOSS OF REDUCING SUGAR FROM UNTREATED AND ETHYL ACETATE TREATED CARROT DISCS

Care must be taken however, since although the discs are washed thoroughly after treatment, we have consistently observed a leakage of reducing sugar from the discs for at least a further hour No reducing sugar was lost from untreated discs (Fig 1) These observations indicate that in experiments involving the assay of invertase in ethyl acetate treated discs it is necessary to perform control incubations in which discs are incubated in buffer rather than sucrose If no control incubations are performed, the increased appearance of reducing sugar from ethyl acetate-treated discs will appear to be due to invertase activity, whereas it may be due to the loss of reducing sugar from the damaged cells Many workers make no mention of performing such control incubations

[13] HU, A S L, BOCK, R M and HALVORSON, H O (1962) *Anal Biochem* **4**, 489
[14] FILNER, P and VARNER, J E (1967) *Proc Natl Acad Sci U S* **58**, 1520
[15] LONGO, C (1968) *Plant Physiol* **43**, 660

The alternative method of determining the intracellular distribution of the enzyme is to fractionate the aged discs into cell wall and soluble fractions by homogenization and differential centrifugation. By this method artichoke invertase appears to be located on the cell wall, and cannot be removed by treatment with detergent, organic solvents or proteolytic enzymes [6] Vaughn and MacDonald,[11] however, showed that in carrot, potato and beet discs extracted at pH 5·0, both walls and supernatant possessed invertase activity Twice as much invertase was associated with carrot cell walls as with the supernatant This suggested that invertase was associated with both the walls and the cytoplasm

However, recent work by Ricardo and ap Rees[12] shows that the apparent subcellular distribution of acid invertase is markedly dependent on the pH of the extraction buffer Extraction and fractionation at pH 8 0 caused more invertase to be associated with the soluble fraction than with the walls Extraction and fractionation at pH 5 0 gave high activities in the cell wall fraction and little activity in the supernatant This latter result agrees with the observation of Vaughn and MacDonald,[11] and is similar to that reported for aged discs of artichoke [6] Results presented in Table 1 of this paper agree with the results of Ricardo and ap Rees [12] Extraction of aged discs in 0 1 M acetate buffer, pH 4 8 (acid buffer) resulted in 5 times as much acid invertase being associated with the cell-wall than when extraction was performed in 0 195 M phosphate–0 003 M citrate-buffer, pH 8 0 (alkaline buffer)

TABLE 1 EFFECT OF EXTRACTION pH ON DISTRIBUTION OF ACID INVERTASE BETWEEN CELL WALL AND SUPERNATANT FRACTION

Cellular fraction	Extraction pH	Acid invertase activity (mg reducing sugar/hr/g initial fr wt)	Cellular fraction	Extraction pH	Acid invertase activity (mg reducing sugar/hr/g initial fr wt)
Supernatant	4 8	0 32	NaCl washed	4 8	1 50
	8 0	2 21	cell walls	8 0	1 02
Unwashed cell	4 8	5 10	NaCl wash from	4 8	2 62
walls	8 0	1 08	cell walls	8 0	0 15

Lamport[16] has suggested that cell walls can bind enzymes either ionically or covalently. Enzyme activities removed from the wall by 1 M sodium chloride are apparently ionically bound In the present investigation, carrot cell walls prepared in either acid or alkaline buffer were washed with 1 M sodium chloride Invertase was released only from walls prepared in acid buffer The activity associated with the washed walls was found to be the same as that associated with unwashed walls prepared in alkaline buffer (Table 1). Walls prepared in pH 4 8 buffer possess ionically bound acid invertase which is removed by sodium chloride and firmly bound enzyme that is not removed, while cell walls prepared in pH 8 0 buffer possess only firmly bound enzyme * The *in vivo* location of the ionically bound enzyme cannot yet be decided

We have used the density labelling technique of Hu, Bock and Halvorson[13] to determine whether the ionically bound acid invertase is synthesized *de novo* Aging of discs in 90%

[16] LAMPORT, D T A (1965) *Adv Bot Res* **2**, 151

deuterium oxide for 60 hr causes a 30–40% decrease in the formation of the ionically bound invertase activity The equilibrium distribution in the caesium chloride gradients of invertase prepared from discs ages in water or deuterium oxide is shown in Fig 2(a) and (b) respectively The buoyant densities observed were 1 295 for the normal enzyme and 1 312 for the deuterated enzyme The shift in buoyant density is 1 4% The results suggest that the ionically bound invertase is synthesized *de novo* Although several plant invertases are known to be glycoproteins, e g grape berry invertase contains 25% carbohydrate,[17] there are no reports that carrot invertase is a glycoprotein, and the fact that the buoyant density of the normal enzyme is 1 295 suggests that the enzyme does not contain a carbohydrate moeity

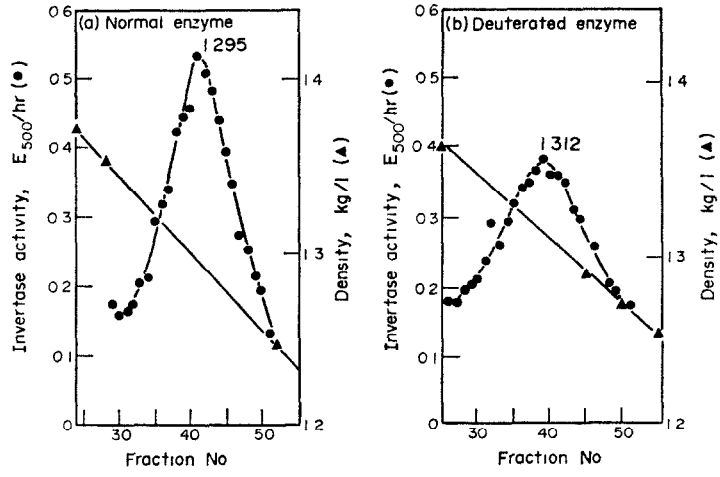

FIG 2 EQUILIBRIUM DISTRIBUTION OF ACID INVERTASE ACTIVITY AFTER CENTRIFUGATION FOR 65 hr AT 39000 rpm

The mechanism by which invertase synthesis is initiated is uncertain although there is evidence that gibberellic acid may be involved [18] The component(s) of the protein-synthesizing machinery which is rate limiting is also unknown, although it has been suggested that on cutting, an enhanced synthesis of *m*RNA leads to the observed increase in polyribosomes [3] The activity of the aminoacyl *t*RNA synthetases does increase to a low maximum between 12 and 24 hr after cutting [19] But the activity then falls to a level equal to or lower than the activity of freshly cut discs by 48 hr, at which time invertase activity is still increasing

EXPERIMENTAL

Preparation and aging of carrot discs Fresh carrots (*Daucus carota*) were obtained as required from local shops Intact, undamaged carrots were always used Discs of phloem parenchyma (1 × 8 mm), prepared with the aid of a cork borer were aged for 60 hr at 28° on a shaker 1 g samples of discs were shaken in 25 ml Erlenmeyer flasks containing 5 ml of 40 μg/ml chloramphenicol in H_2O

* Evidence that the soluble fraction of invertase associated with cell walls of aged discs of mature carrot root is ionically bound was obtained by re-examination of data of RICARDO and ap REES[12] by LITTLE, G and EDELMAN, J (1973) *Phytochemistry* **12,** 67, in a paper published after submission of this manuscript

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[18] EDELMAN, J and BRADSHAW, M J (1969) *Planta* **84,** 94
[19] GORE, N R and WRAY, J L , unpublished observations

Preparation of cell wall and soluble invertase 1 g of aged discs were ground in a mortar with either 0 1 M acetate buffer, pH 4 8 or 0 195 M Na_2HPO_4–0 003 M citric acid buffer, pH 8 0 The homogenate was centrifuged at 1000 *g* for 15 min, and the supernatant collected The supernatant was centrifuged for 1 hr at 152000 *g* and the supernatant was dialysed overnight against 4 l of 0 1 M Tris-HCl buffer, pH 8 0 After dialysis the sac contents were used for the assay of soluble acid invertase The walls were washed 3 × with either acid or alkaline buffer, suspended in extraction buffer and sedimented by centrifugation at 26000 *g*. The supernatant was discarded and an aliquot of the walls was assayed for invertase activity The remainder of the walls were suspended in 5 ml of 1 M NaCl in 0 1 M acetate buffer, pH 4 8 After stirring for 1 hr the suspension was centrifuged at 26000 *g* and the supernatant collected The NaCl washed walls were re-suspended in 0 1 M acetate buffer, pH 8 0, and an aliquot taken for invertase assay The sodium chloride wash was brought to 75% saturation by the addition of a saturated solution of ammonium sulphate, pH 7 5 After stirring for 1 hr the precipitated protein was collected by centrifugation, dissolved in 0 1 M acetate buffer, pH 4 8, and assayed for acid invertase activity

Invertase assay The reaction mixture contained 0 5 ml 50 mM sucrose in 0 1 M acetate buffer pH 4 8, 0 1 M acetate buffer, pH 4 8, and soluble enzyme or cell wall suspension in a final vol of 1 0 ml After the desired incubation period at 28°, reducing sugar was estimated by the method of Nelson [20] Extinction readings were converted to μg reducing sugar by performing glucose standards each time the assay was carried out 0 5 ml of 0 1 M acetate buffer, pH 4 8, replaced sucrose in control incubations Assay tubes were centrifuged on a bench centrifuge before measuring extinctions if this was found to be necessary.

Preparation of invertase for isopycnic equilibrium centrifugation Discs prepared as above were aged for 60 hr at 28° on an orbital shaker 10 g of discs were shaken with either 25 ml H_2O or 25 ml 90% deuterium oxide, containing 40 μg/ml chloramphenicol, in 250 ml Erlenmeyer flasks The aged discs were rinsed with dist H_2O, blotted dry, and ground in a pestle and mortar with 0 1 M acetate buffer, pH 4 8 The brei was centrifuged at 1000 *g* to sediment the walls The supernatant was discarded and the walls were washed 3 × with 0 1 M acetate buffer, pH 4 8 The walls were then suspended in 25 ml 1 M NaCl in 0 1 M acetate buffer, pH 4 8, stirred for 1 hr and then sedimented by centrifugation at 26 000 *g* The supernatant was brought to 75% saturation with saturated ammonium sulphate, pH 7 0, and after stirring for 1 hr, precipitated protein was sedimented, dissolved in 0 1 M acetate buffer, pH 4 8, and used for isopycnic equilibrium centrifugation

Isopycnic equilibrium centrifugation Isopycnic equilibrium centrifugation was performed in a M S E. Superspeed 65 preparative ultracentrifuge using a 3 × 5 ml swing out rotor at 39000 rpm for 65 hr at 4° The gradient mixture had the following composition 1 ml saturated solution CsCl (20°), 1 ml 0 1 M acetate buffer, pH 4 8, and 1 ml enzyme After centrifugation, the tubes were punctured through the bottom and *ca* 60 fractions, of 4 drops each, were collected Six fractions across the gradient were used for refractive index determinations using a Bellingham and Stanley Abbe type refractometer A standard curve relating buoyant density to refractive index was prepared using the equation Q^{25} = (10 8601 × refractive index) −13 4974 given in the *Handbook of Biochemistry* [21] Invertase activity was assayed by adding 0 4 ml of 0 1 M acetate buffer, pH 4 8, and 0 5 ml 50 mM sucrose to the other fractions After incubation at 28° reducing sugar was determined as described above

Treatment of discs with ethyl acetate Discs were treated with ethyl acetate essentially as described by Bacon, MacDonald and Knight [10]

Acknowledgements—This research was supported in part by a grant from the Science Research Council We thank Mr G Johnson for performing some preliminary experiments.

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[21] ANON (1970) *Handbook of Biochemistry* (SOBER, H A , ed), p J-296, Chemical Rubber Co , New York.