The Development of Invertase Activity in Slices of the Root of *Beta vulgaris* L. Washed under Aseptic Conditions

By J. S. D. BACON, I. R. MacDONALD AND A. H. KNIGHT

Departments of Biochemistry and Plant Physiology, Macaulay Institute for Soil Research, Aberdeen

(Received 25 May 1964)

1. When disks of root tissue from sugar or red beet (*Beta vulgaris* L.) are washed in running aerated tap water the sucrose contained in them disappears and glucose and fructose are formed. 2. Invertase activity in the disks has been measured by a polarimetric method. Freshly cut tissue has a very low activity, but a considerable increase occurs during the first 3–4 days of washing, the final activity being sufficient to hydrolyse the sucrose contained in the disk within a few hours. 3. Disks of red beet have been cut and shaken in water under aseptic conditions. Sucrose breakdown and invertase development still took place. Microbial contamination is therefore not responsible. 4. Trisaccharides that appear in sugar-beet disks during the washing process have been isolated and identified; their formation also suggests that a higher-plant invertase is acting. 5. The significance of these results is discussed in relation to protein synthesis in washed storage-tissue slices, and the occurrence of high invertase activity in growing plant cells.

The extensive use of disks cut from roots and tubers in studies of ion uptake by plant cells has revealed certain peculiarities of the metabolism of these tissues. Thus to induce a high capacity for ion uptake it was found necessary to wash the disks in aerated water for 1 or 2 days, longer periods often being adopted as a standard treatment (Steward, 1932; Asprey, 1937; Turner, 1940; Stiles & Dent, 1946; Robertson, Turner & Wilkins, 1947; Sutcliffe, 1952). Under these conditions it was claimed that the tissue could be kept 'fresh...for several weeks' (Briggs & Robertson, 1948), even when exposed to bacterial infection (Turner, 1940). The degree of contamination was apparently judged by the gross appearance of the disks (e.g. turgidity, pigment retention; cf. Sutcliffe, 1952) rather than by a direct examination of their microbial populations.

After an observation by MacDonald & DeKock (1958) that sucrose was rapidly hydrolysed in disks cut from the roots of beet, it was discovered that the invertase activity of this material increased considerably when it was washed in aerated tap water (Bacon, 1961). Although we had reasons for believing that the invertase activity originated in the plant tissue we wished to assure ourselves that microbial infection made no contribution; this was particularly necessary because a bacterial count (kindly performed for us by Dr D. M. Webley) showed the presence of about 3×10^7 organisms/disk after 4 days' washing. We have therefore devised a method by which disks of red beet of fixed dimensions may be prepared aseptically in sufficient

numbers to overcome sampling errors (MacDonald, Webley & Bacon, 1963). As far as we know the only other systematic attempt to prepare aseptic material for such purposes is that of Ingram & Riches (1951), which was made in 1940–41 and was never followed up.

There is no reason to believe that the extent of infection in our tissue was exceptional. Dr R. J. Ellis (personal communication, after our own discovery) found comparable values for red-beet disks washed in double-distilled water. It must therefore be recognized that many studies on the physiology and biochemistry of storage tissue must have been made with heavily contaminated material, and each should be carefully re-examined to see whether the effects recorded may properly be ascribed to the plant tissue.

In our own case we were unable to devise an aseptic washing procedure that incorporated all the features of the original, but we are satisfied that the development of invertase activity was a property of the plant tissue and was not influenced by the presence of microbial infection. The following is an account of some features of this process, which has been the subject of preliminary communications by Bacon (1961) and also by Edelman & Hall (1963, 1964).

MATERIALS AND METHODS

Sugar beet. Mature beets were obtained through the courtesy of the manager of the Cupar (Fife) factory of the

British Sugar Corp., usually in late autumn, and were stored in a cool but frost-free place during the winter. A few roots that had been left in the ground during the winter and had begun to grow again were kindly provided by Dr A. Carruthers of the British Sugar Corp. Research Laboratories, Bramcote, Nottinghamshire.

Other plant material. This was usually purchased locally as required, or grown in the gardens of the Institute, in which case the variety of red beet chosen was Detroit Dark Red.

Preparation and washing of disks. (a) Without aseptic precautions. This is described in detail by MacDonald & Knight (1958). A bacon slicer was used to cut large slices, from which the disks were punched with a cork borer; the freshly cut disks were about 11 mm. in diameter and 1 mm. thick, and each weighed about 0.1 g. They were washed in tap water that was renewed continuously about five times each hour, and so stirred by a stream of air that they were in continuous movement.

(b) With aseptic precautions. Red beet were scrubbed lightly but thoroughly in running tap water and immersed in 0.1% HgCl_2 for 1 hr. The top and bottom were cut off and the middle portion was cut into blocks of cross-section about 2.5 cm. $\times$ 3.5 cm. with a knife wetted with 0.1% HgCl_2 solution. These blocks were immersed in fresh HgCl_2 solution and left for at least 30 min. The remaining operations were performed in a room in which the air was sterilized by ultraviolet lamps. All instruments were sterilized by dipping them in ethanol and burning it off.

Each block was transferred to a dish of sterile water for 10–15 min., pared with a razor to remove tissue contaminated with HgCl_2 and placed in the clamp of a table microtome (American Optical Co., Buffalo, N.Y., U.S.A.). A number of incisions were made through the top surface with a sterile no. 6 cork borer. The incisions, which were parallel to the original axis of the root, did not reach the lower surface of the block, so that the borer could be removed leaving cylindrical plugs *in situ*. The top layer was removed with a razor, and the microtome then adjusted between razor cuts to give slices 1 mm. thick. The slice adhered to the razor and the disks lying within it were picked up with forceps.

It was essential to detect infection as early as possible; single disks were therefore taken systematically during the cutting, and also during the sampling of experimental material, and shaken mechanically (Webley & Duff, 1955) at 29° in a 1 oz. screw-cap bottle containing 2.5 ml. of a medium (Paton, 1959) made by dissolving 5 g. of sucrose, 0.5 g. of Lab-Lemco (Oxoid; Oxo Ltd., London, S.E. 1) and 0.5 g. of Difco Bactopeptone in 100 ml. of water. Bacterial growth was visible within 20 hr. if contamination of freshly cut disks was heavy; contaminated experimental material sometimes showed growth in 5–6 hr. The record of infected bottles and flasks from an experiment sometimes showed that only one block of tissue was infected, or even, occasionally, a single cork-borer incision. Sometimes all the blocks from one beet were infected, and all those from another beet were clear. It was evident that the most important source of infection was the beet itself. Sometimes gross invasion of the inner tissue was visible; in other cases infection had probably reached the middle along conducting tissues. All the sugar beet we examined showed signs of this, and we were unable to obtain uncontaminated disks from them. [Tervet & Hollis (1948) have provided evidence for

infection of the internal tissues of many roots, tubers and fruits.] By rejecting beet that have internal cavities, or that show any signs of mechanical damage, and using roots rather larger than the usual commercial size, we have managed so to improve the procedure that if infection does occur it does not affect more than one flask in 20.

Aseptic disks (12) were placed in a 150 ml. conical flask (Monax glassware; John Moncrieff Ltd., Perth) fitted with an Oxoid aluminium cap (Oxo Ltd.) and containing 30 ml. of sterile water or other medium. A single red beet (about 500 g.) usually provided sufficient disks to fill 24 flasks. Each disk, after being blotted on filter paper, weighed about 75 mg.

Measurement of invertase activity. The following procedure was used unless otherwise stated:

(a) Pre-treatment. The disks were blotted on filter paper and dropped one by one into a beaker cooled in ice and containing enough ethyl acetate to cover them; the beaker was occasionally shaken by hand, during a period of at least 15 min. The disks were then removed and transferred to a flask containing water and crushed ice, and shaken by a wrist-action shaker for 20 min., more ice being added if necessary. The aqueous extract was decanted, fresh ice-water added and the flask again shaken for 5 min.; this last process was then repeated.

(b) Enzyme assay. The ethyl acetate-treated disks were blotted on filter paper and 20 counted into a 100 ml. conical flask containing 1.2 g. of sucrose, 1 ml. of M-sodium acetate buffer, pH 5.0, 21 ml. of water and a few drops of chloroform. The flask was shaken gently in a water bath (metabolic shaking incubator; A. Gallenkamp and Co. Ltd.) at 35°. After equilibration for 30 min., 10 ml. of the medium was withdrawn and mixed with 10 ml. of 5% (w/v) Na_2CO_3 ; exactly 2 hr. after this a further 10 ml. sample was treated similarly. The optical rotation of each was read (sodium lamp) in a 2 dm. tube within 1 hr. of adding the alkali. The invertase activity was calculated from the change in optical rotation (cf. Burger, Bacon & Bacon, 1961), with the assumption that 20 disks increased the total volume of the system by 2 ml.

Qualitative examination of enzymic activities. On many occasions freshly cut disks were tested for invertase activity. Ten ethyl acetate-treated disks were incubated at 35° with 2 ml. of 20% (w/v) sucrose in 0.1 M-sodium acetate buffer, pH 5.0, in stoppered test tubes in the presence of chloroform. Samples were examined by paper chromatography at intervals over a period of 1 week or more, further chloroform being added if necessary when the incubations were prolonged. Tests at other pH values for other enzymes and with other types of tissue preparation were carried out in a similar way.

Paper chromatography. To determine the stage of sucrose breakdown in the washed disks, 12 disks removed from ethyl acetate were pressed on the starting line of a paper chromatogram previously treated with HgCl_2 (Bealing & Bacon, 1953). Small samples (5 $\mu\text{l.}$) were also withdrawn from the invertase assay mixtures, at the beginning and when the two 10 ml. samples were taken, to confirm that changes in polarimetric readings always corresponded to sucrose hydrolysis. These chromatograms and most of the others used were developed by the descending technique in butan-1-ol-acetic acid-water (4:1:5, by vol.; Partridge, 1948) and sprayed with benzidine-trichloroacetic acid (Bacon & Edelman, 1951).

Preparative chromatography. Chromatographic separations on charcoal-Celite with aqueous ethanol, or on Celite with butan-1-ol-water, were carried out as described by Bacon (1959).

A slight but reproducible hydrolysis of sucrose was noticed on columns containing activated charcoal (decolorizing powder) (British Drug Houses Ltd.). This seemed to be due to its acidic properties, and was seen only when the column was over-loaded with sucrose. After washing with alkali followed by large volumes of water had proved unsuccessful, columns containing 40 g. of charcoal and 40 g. of Celite were washed first with 1 l. of water and then with 100 ml. of 0.2 M-sodium acetate immediately before the application of the plant extract; under these conditions hydrolysis was negligible. To reduce the possibility of microbial contamination the suspension of charcoal and Celite was boiled and packed in glass tubes that had been previously filled with boiling water.

RESULTS

Justification of the method of invertase assay. The durations of the ethyl acetate treatment and the subsequent washing were decided by experiments with red beet, assuming that the loss of pigment indicates the effectiveness of the extraction. Although rather lengthy the assay method has the advantage of simplicity, and avoids the difficulty presented by the 'insolubility' of at least part of the invertase.

The possibility that part of the invertase activity is lost from the tissue during ethyl acetate treatment was tested repeatedly by taking ten disks out of ethyl acetate and shaking them in 4 ml. of 0.1 M-sodium acetate buffer, pH 5.0, for 1 hr. The extract was then transferred to a tube containing 0.8 g. of sucrose. Freshly cut disks yielded little but sucrose, but disks washed for 3-4 days contributed also glucose and fructose to the extract. On incubation at 35° in the presence of chloroform there were no signs of further sucrose hydrolysis.

To test whether any invertase activity is destroyed during pretreatment one must compare the activities, before and after treatment, of disks with an appreciable invertase content, namely those that have been washed for 3-4 days at 25° and consequently contain glucose and fructose (MacDonald & DeKock, 1958). Some of this reducing sugar and sucrose escapes from the untreated disks and introduces errors into the invertase assay. Another unknown and possibly variable factor in the use of untreated disks is the penetration of sucrose into the disks. Probably for these reasons the untreated disks gave somewhat more variable results than treated ones, but the level of activity was essentially the same in both (see Table 1).

When disks that had been assayed were washed and replaced in a fresh assay solution, essentially the same result was obtained. Treated disks had the same activity when tested in acetate, citrate or

Table 1. *Invertase activities of sugar-beet disks, before and after treatment with ethyl acetate*

Sugar-beet disks were cut and washed, and their invertase activities measured as described in the Materials and Methods section, with acetate, phosphate or citrate solution of final concentration about 0.04 M, each adjusted to pH 5.0 with NaOH. The activities are expressed as mg. of sucrose hydrolysed/ten disks/hr.; negative values are discussed in the text.

Duration of washing (days)	Ethyl acetate treatment	Invertase activity		
		Sodium acetate buffer	Sodium phosphate buffer	Sodium citrate buffer
0	+	1	-2	-1
	-	-1	-3	-4
3	+	21	18	16
	-	15	15	14
5	+	21	22	22
	-	18	20	17
7	+	20	18	21
	-	17	18	18

phosphate solution at pH 5 (Table 1). Hydrolysis remained linear during the removal of 20% of the substrate.

It was concluded that the assay procedure was a sufficiently reliable guide to changes in the invertase activity of beet disks.

Changes in the soluble sugars during the washing of disks of storage tissue. Freshly cut disks of beet contained large amounts of sucrose, and usually a little glucose and fructose; the amounts of the latter varied considerably, some roots having practically none. Within a single root there was usually more free hexose in the crown (cf. Fort & Stout, 1948), and particularly at the point of insertion of the leaves. The pigment of red beet obscured the trisaccharide region of paper chromatograms, but in sugar beet raffinose could always be seen.

When disks were washed at 25° there was some increase in glucose and fructose after 1 day, and after 4-5 days very little sucrose was left. Table 2 shows the changes in these sugars in red-beet disks washed under aseptic conditions. The sugar shed from broken cells into the suspending medium was reabsorbed, except for some trisaccharide, which persisted as a weak spot on chromatograms throughout the experiment.

When sugar-beet disks were washed without aseptic precautions in aerated running tap water raffinose disappeared and its place was taken by two other trisaccharides. These were isolated and identified as *O*-β-D-fructofuranosyl-(2→1)-*O*-β-D-fructofuranosyl-(2→1) α-D-glucopyranoside and

Table 2. *Changes in the content of soluble sugars during shaking of red-beet disks in water under aseptic conditions*

Disks were cut and washed as described in the text. At intervals samples (ten disks) were removed and each was ground in an all-glass Potter-Elvehjem homogenizer with 2.5 ml. of 0.02 M-HgCl₂. Each homogenate was centrifuged and 100 μ l. of the supernatant fluid applied to a large sheet of Whatman no. 1 paper for separation and determination of sucrose, glucose and fructose by quantitative paper chromatography (Bealing & Bacon, 1953), sucrose and fructose being measured by the Seliwanoff reaction (Bacon & Bell, 1948) and glucose with glucose oxidase (Huggett & Nixon, 1957). The medium in certain flasks was sampled (20 ml.), evaporated to dryness and dissolved in 0.2 ml. of water, and 100 μ l. was applied to a large sheet of paper for analysis of its constituent sugars by the same procedure as for the disk extracts. The results are in each case given in terms of the total flask contents, namely mg./12 disks and mg./30 ml. of medium. The beet used for this experiment was divided into four blocks (a, b, c and d) for preparation of the disks; the origin of the disks in each flask is shown at the head of the column.

Duration of shaking (days)		Sugar content																		
		... 0		1		2		3		4		5		7		10		15		
		a	c	b	d	a	c	b	d	a	c	b	d*	a	c*	a	c	d	d*	
Disks	Block																			
	Sucrose	60	51	64	73	36	35	21	31	11	12	6	11	3	4	3	3	2	2	
	Glucose	3	1	1	1	11	6	17	20	24	15	22	35	24	21	25	25	31	21	
	Fructose	0.4	0	0.7	0.5	3	2	5	4	3	2	3	2	1	1	1	1	3	2	
	Total ...	63.4	52	65.7	74.5	50	43	43	55	38	29	31	48	28	26	29	29	36	25	
Medium†	Sucrose	8.6	10.8	8.9	7.9	0.1	0	0	0	0	0	—	—	—	—	—	—	—	—	
	Glucose	0.2	0.4	1.3	1.3	1.6	1.9	0.3	0.1	0	0	—	—	—	—	—	—	—	—	
	Fructose	0	0	1.4	1.6	2.6	2.7	1.4	1.1	0.3	0.2	—	—	—	—	—	—	—	—	
	Total ...	8.8	11.2	11.6	10.8	4.3	4.6	1.7	1.2	0.3	0.2	—	—	—	—	—	—	—	—	
Total in disks and medium ...		...	72.2	63.2	77.3	85.3	54.3	47.6	44.7	56.2	38.3	29.2	31	48	28	26	29	29	36	25

* After incubation for 1 week in nutrient medium a disk from this flask showed infection with a basidiomycete.

† Values given as two significant figures to indicate the excess of fructose over glucose from day 2; after 5 days the amounts present in the medium were too small to be estimated by this procedure.

O- β -D-fructofuranosyl-(2 $\rightarrow$ 6)-O- α -D-glucopyranosyl-(1 $\rightarrow$ 2) β -D-fructofuranoside by their chromatographic behaviour and infrared-absorption spectra (cf. Bacon, 1959). The same two substances were formed when washed disks were treated with ethyl acetate and incubated with sucrose *in vitro*. These are the characteristic products of fructose transfer by higher-plant invertases (Allen & Bacon, 1956).

A similar disappearance of sucrose and then of hexose occurred from carrot and turnip disks, but disks of tissue from certain composites gave a rather different picture. Thus disks of chicory root still contained appreciable amounts of sucrose after washing for 5 days, despite the presence of a high invertase activity.

The disk extracts and concentrated media of Table 2 were examined qualitatively for the presence of sugar alcohols; no substances with the properties of sorbitol or mannitol were detected (solvent: that of Rees & Reynolds, 1958; spraying reagent: that of Cifonelli & Smith, 1954). The hexitol contents of 12 disks on day 3(d), day 4(a) and day 5(d) therefore could not exceed 0.5 mg., and the hexitol in 30 ml. of medium on day 2(a) and day 4(c) was less than 0.3 mg.

Invertase activity in freshly cut beet disks. Freshly cut beet disks had no invertase activity measurable by the polarimetric assay, but prolonged incubation with sucrose always showed a little breakdown; ten disks incubated at 35° with 2 ml. of 20% sucrose in acetate buffer, pH 5.0, produced overnight glucose and fructose detectable in a 5 μ l. spot on a paper chromatogram.

After incubation for several weeks, or even months, a faint trisaccharide spot was detectable on chromatograms, but breakdown was never complete.

At pH 8 in phosphate buffer the action on sucrose was so slight that it could not be detected with any certainty.

The invertase activity was little affected by the physiological state of the tissue. Disks cut from different regions of sugar-beet roots showed that activity was somewhat higher in the crown. A large root was kept in a pot in the greenhouse until June, when it carried a heavy growth of leaves, but the invertase activity of disks freshly cut from it remained within the experimental error of the polarimetric method.

The invertase activity of ethyl acetate-treated

disks was not increased by exposing them to sucrose solution, to dilute acetic acid or to 0.02 M-disodium hydrogen phosphate, or by prolonged washing.

Effects of washing on the invertase activity of beet disks. When disks from red beet were shaken in flasks in distilled water at 25° under aseptic conditions there was a rapid development of invertase activity, reaching a maximum of about 12 mg. of sucrose hydrolysed/ten disks/hr. after 3 days (Fig. 1). A distinctly higher value (16–27 mg./ten disks/hr.) was reached by contaminated disks washed in the apparatus of MacDonald & Knight (1958); this did not appear to be a consequence of infection, but of the vigorous aeration and perhaps the presence of tap-water constituents. When infection occurred in the flask system the invertase activity was never increased, and sometimes decreased. The activity that developed in distilled water under aseptic conditions was much less when the flasks were not shaken. Disks aerated under aseptic conditions gave higher values than shaken ones.

No change in the very low basal invertase activity could be detected after 5 hr., but after 8 hr. a small increase was always detectable. Extrapolation from the period of rapid increase in activity (10–40 hr.) suggested a lag period of about 5 hr., and this was confirmed by measurements of the reducing sugar produced by disks taken at hourly intervals, treated with ethyl acetate and incubated for a long period with sucrose (Fig. 2). The final sample (11 hr.) has an activity of about 0.5 mg. of sucrose hydrolysed/ten disks/hr., which would be barely detectable in the polarimetric assay.

Fig. 1. Development of invertase activity in disks of red-beet root washed under aseptic conditions. Disks were cut from four blocks (a, b, c and d) from a single stored root in May 1964, and washed as described in the text. At each sampling ten disks were taken from each block and combined to give two 20-disk samples for polarimetric assay. O, Disks from blocks a and b; Δ, disks from blocks c and d.

The newly formed invertase was most active between pH 4.8 and 5.8; there was no evidence for the presence of a second enzyme with a pH optimum at pH 7 or 8. Trisaccharide formation was seen at all pH values between 3.6 and 9.0.

Sugar-beet disks washed under non-sterile conditions developed about the same invertase activity as red beet (20–25 mg./g. fresh wt./hr.; range 10–35 mg./g./hr.). Similar increases were shown by disks from carrot roots, turnip roots, dahlia tubers, Jerusalem-artichoke tubers and chicory roots, the last-named developing a particularly high activity (50 mg./g. fresh wt./hr.).

Other enzymes in beet disks. Since raffinose is the other principal soluble sugar present, the action of disks upon it was examined. Freshly cut disks had an appreciable α-galactosidase activity at pH 5, so much greater than the invertase activity that in the early stages of incubation the products were almost exclusively galactose and sucrose. Melibiose was also hydrolysed by freshly cut disks.

Observations on the effect of washing were complicated by the appearance of invertase, and comparisons of untreated with treated disks by the presence of sucrose and its breakdown products.

Fig. 2. Early stages of invertase development. A single root was cut as described in the text, to fill 24 flasks. Two flasks were taken from the shaking apparatus each hour and 20 disks treated with ethyl acetate. The treated disks were incubated as for polarimetric assay, except that toluene was used instead of chloroform. Samples of the medium were withdrawn at intervals for estimation of reducing sugars by the method of Nelson (1944) and Somogyi (1945). O, Initial value; Δ, after 27 hr. incubation at 35°.

Galactose formation from raffinose was followed in freshly cut material, where sucrose breakdown was slight: little change in the α -galactosidase activity took place during ethyl acetate treatment. The action on melibiose at pH 5 of disks washed for 5 days was not appreciably different from that of freshly cut disks.

No α -galactosidase activity (on raffinose or melibiose) was detected at pH 8 in freshly cut or washed disks, before or after ethyl acetate treatment.

DISCUSSION

There can be little doubt that the invertase that appears during the washing of beet disks is responsible for the rapid breakdown of sucrose that takes place. Assuming sucrose contents of about 150 mg./g. fresh wt. of tissue, the 'basal' invertase in freshly cut disks would require several weeks to bring about complete hydrolysis, whereas the final invertase activity of 20–25 mg. of sucrose hydrolysed/g./hr. (measured at 35°) would require only a few hours; even the activity (1–2 mg./g./hr.) developed during the first day would be enough to produce changes easily detectable on paper chromatograms. The fact that the same mixture of trisaccharides is formed by transfructosylation *in vivo* as *in vitro* provides a qualitative confirmation that invertase is responsible. The nature of the trisaccharides formed suggests that a higher-plant invertase is acting, even in contaminated tissue.

Comparisons with other plant tissues. Is this invertase an entirely new feature, or does it represent a great increase of an activity already present? The activity of freshly cut disks was very feeble; the production of trisaccharides was so slight that their identification was uncertain, but there were indications that they differed from those produced by the much greater activity that develops on washing.

The development of invertase in germinating legume seeds (Pridham, 1958; Cooper & Greenshields, 1961) presents an interesting parallel, particularly as both tissues have an α -galactosidase activity that undergoes little change. Cooper & Greenshields (1961) have produced evidence that suggests that the weak basal invertase activity of ungerminated seeds of *Phaseolus vulgaris* is an 'alkaline sucrose', more specific for sucrose than the typical β -fructofuranosidase that develops on germination, having a pH optimum of 7.7 and no transferring action (but see Pridham & Walter, 1964). We have tested freshly cut beet disks on many occasions but could never detect sucrose hydrolysis with certainty at pH 8, although it was always detectable at pH 5. The basal invertase of our fresh tissue is thus not an 'alkaline sucrose', and we are sure there cannot be an appreciable amount

of such a component in the invertase that develops on washing.

Though the invertase activity of washed disks is rather greater than that recorded for green tissues (see values quoted by Schwimmer, Makower & Rorem, 1961), it is comparable with that of growing non-photosynthetic tissue: Robinson & Brown (1952) found in the region of the broad-bean root tip, where cell growth in length and breadth was taking place, an increase in invertase activity to a maximum of about 150 mg. of sucrose hydrolysed/g. fresh wt./hr. (? at 37°). Hellebust & Forward (1962) reported similar activities (100 mg./g. fresh wt./hr.) for a comparable region of the maize root. Straus (1962) found activities up to a maximum of about 140 mg./g./hr. at 30° for cell-wall preparations from actively growing tissue cultures of various plants. Burström (1957), employing a much lower sucrose concentration (1 mM; well below the K_m), found activities of about 5 mg./g./hr. for wheat-root segments at 22°. All these values are of the same order as our own (10–50 mg./g./hr. at 35°).

Location of invertase in the cell. The invertase is not lost from disks on ethyl acetate treatment, which might suggest that it is bound to insoluble cell structures, although the case of yeast invertase shows that this is not necessarily the correct deduction (Burger *et al.* 1961). Edelman & Hall (1963) reported that the invertase that develops in washed disks cut from tubers of *Helianthus tuberosus* is firmly attached to the cell debris, and could find no means of removing it (see also Kivilaan, Cabrera Beaman & Bandurski, 1961; Straus, 1962). The close correspondence between invertase activity measured in intact disks and in those previously treated with ethyl acetate (Table 1) recalls experiments with root tissue from wheat (Burström, 1957) and maize (Hellebust & Forward, 1962), as well as more numerous studies with yeast, which all suggest that a high proportion of the invertase in living plant cells is immediately accessible to sucrose in the surrounding medium.

In the present case we have to explain how the invertase comes in contact with sucrose already stored within the cell. If the invertase were all in the cell wall the sucrose would have to migrate to this region, which is effectively 'outside' the cell. Subsequent reabsorption of hexose seems feasible, but it is more difficult to believe that trisaccharides formed here could be retained, as they evidently are, even in the exacting conditions of washing with tap water. For this reason we consider that part of the invertase must be situated within the cell, as suggested by Glasziou (1961), and in a region of comparatively high sucrose concentration.

Connexion between invertase and cell growth. If a significant part of the invertase is present in the cell wall this may help to explain connexions between

enhanced invertase activity and cell growth. Straus (1962) argues that, because there is no quantitative correlation between invertase activity in the cell walls and the rate of growth of his tissue cultures, the presence of the enzyme 'is purely fortuitous, and probably serves no function'. (One of his cultures had no invertase activity at all, and this might seem to prove his point, but tissue cultures may occasionally lose biochemical properties that are no longer essential for their continuance in an artificial environment.) From the association of invertase activity with a phase of rapid growth in experiments like those of Robinson & Brown (1952), or its appearance during the germination of seeds, we are inclined to argue that the production of invertase is yet one more sign that growth is taking place in washed disks (Knight, Crooke, MacDonald & Shepherd, 1961; MacDonald, Knight & DeKock, 1961). Hellebust & Forward (1962) suggested that invertase might assist the growth of the root tip by supplying osmotically active substances, substrates for synthetic processes or substrates for energy production. It is not clear, though, why the tissue should need to hydrolyse sucrose when other means exist for introducing the constituent hexoses into the pathways of carbohydrate metabolism; possibly these do not permit a rapid enough supply. It may be significant that the disks accumulate glucose rather than fructose. Hellebust & Forward (1962) found an excess of glucose over fructose throughout the length (3 cm.) of the maize-root segments that they examined. This might mean that growing tissue has a specific need for fructose and that the function of invertase is to provide this, either by direct transfer or by hydrolysis. Alternatively fructose may be converted into glucose almost as fast as it is liberated, glucose being the preferred substrate for growth.

Cook (1959) has considered the possibility that the increases in activity of enzymes in growing root cells may be explained by increases in particular elements of the cell structure. Thus the increase in some enzymes (e.g. phosphatase, transaminase) is of the same order as the increase in cell-wall area or of volume of cytoplasm. He suggests that a structure whose increase might account for the much greater augmentation of invertase activity is the tonoplast, and that the enzyme may exert some influence over the passage of sugars into the vacuole. In the tissues that we have examined, increases in cell size are small but the invertase activity increases at least 100-fold. Although striking changes occur in the distribution of cytoplasm within the cells (MacDonald, 1955) it is unlikely that the tonoplast area could increase to this extent, and, if the basal invertase of the disks should prove to be qualitatively different from that which develops, this argument could not be used at all. For the present

we assume that the increase in invertase activity is a consequence of a functional response of the tissue, and not simply of changes in the relative dimensions of cell structures.

Glasziou and his colleagues have recently discussed possible physiological roles for acid (pH 5.4) and neutral invertases in sugar-cane. Hatch & Glasziou (1963) find *in vivo* a very close connexion between growth of internodes and their acid-invertase content. Sacher, Hatch & Glasziou (1963) and also Sacher & Glasziou (1962) describe experiments *in vitro* in which disks cut from immature internodes were incubated in water for up to 18 hr.; invertase activity sometimes increased, but apparently often changed little or even decreased. It is perhaps not to be expected that a growing tissue already containing an active invertase would react like dormant root tissue, but this variability of response weakens the conclusions that these authors draw from experiments in which water was replaced by solutions of sugars, auxins, nitrogenous substances etc., e.g. they reported a stimulation of invertase synthesis by indol-3-ylacetic acid. Edelman & Hall (1964) find this substance to have an inhibitory effect on disks of artichoke and carrot tissue; invertase synthesis in these two tissues is nevertheless capable of being stimulated, but by gibberellic acid.

It is thus evident that the development of invertase activity is subject to physiological controls, although the nature of the initial stimulus produced by transferring storage-tissue slices to water is still obscure. Inhibitors of protein synthesis have been shown to inhibit the development of enhanced salt uptake (Sutcliffe, 1960) and of enhanced respiratory activity (Click & Hackett, 1963), but invertase is the only specific protein so far shown to appear under these conditions. It also is under the control of inhibitors of protein synthesis (Edelman & Hall, 1963), and its formation seems to be part of extensive changes in the metabolic apparatus of the cell. If, as the work of Nooden & Thimann (1963) suggests, the response of plant cells to auxins also depends on protein synthesis, all these changes could be connected with a resumption of cell growth, and the context of invertase development could be the same in tissue slices as it appears to be in intact organs.

A different aspect is its possible connexion with a problem of sugar-beet technology. The rise in the content of invert sugar that follows exposure to frost may be due to an increased endogenous invertase activity stimulated by cell damage.

This work was done with the technical assistance of Miss D. M. Stewart, Miss K. A. Gilmore and Miss G. W. Coulthard. The infrared-absorption spectra were measured by Dr V. C. Farmer of the Department of Spectrochemistry of the Institute. We are grateful to a number of people

mentioned in the text, but particularly to Dr J. Edelman of Imperial College, for discussions and correspondence, and to our colleague Dr D. M. Webley for his invaluable help in the preparation of disks under aseptic conditions.

REFERENCES

- Allen, P. J. & Bacon, J. S. D. (1956). *Biochem. J.* **63**, 200.
- Asprey, G. F. (1937). *Protoplasma*, **27**, 153.
- Bacon, J. S. D. (1959). *Biochem. J.* **73**, 507.
- Bacon, J. S. D. (1961). *Biochem. J.* **79**, 20 p.
- Bacon, J. S. D. & Bell, D. J. (1948). *Biochem. J.* **42**, 397.
- Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
- Bealing, F. J. & Bacon, J. S. D. (1953). *Biochem. J.* **53**, 277.
- Briggs, G. E. & Robertson, R. N. (1948). *New Phytol.* **47**, 265.
- Burger, M., Bacon, E. E. & Bacon, J. S. D. (1961). *Biochem. J.* **78**, 504.
- Burström, H. (1957). *Physiol. Plant.* **10**, 741.
- Cifonelli, J. A. & Smith, F. (1954). *Analyt. Chem.* **26**, 1132.
- Click, R. E. & Hackett, D. P. (1963). *Proc. nat. Acad. Sci., Wash.*, **50**, 243.
- Cook, F. S. (1959). *Canad. J. Bot.* **37**, 621.
- Cooper, R. A. & Greenshields, R. N. (1961). *Nature, Lond.*, **191**, 601.
- Edelman, J. & Hall, M. A. (1963). *Biochem. J.* **88**, 36 p.
- Edelman, J. & Hall, M. A. (1964). *Nature, Lond.*, **201**, 296.
- Fort, C. A. & Stout, M. (1948). *Proc. Amer. Soc. Sug. Beet Tech.* **5**, 651.
- Glasziou, K. T. (1961). *Plant Physiol.* **36**, 175.
- Hatch, M. D. & Glasziou, K. T. (1963). *Plant Physiol.* **38**, 344.
- Hellebust, J. A. & Forward, D. F. (1962). *Canad. J. Bot.* **40**, 113.
- Huggett, A. St G. & Nixon, D. A. (1957). *Lancet*, **273**, 368.
- Ingram, M. & Riches, J. P. R. (1951). *New Phytol.* **50**, 76.
- Kivilaan, A., Cabrera Beaman, T. & Bandurski, R. S. (1961). *Plant Physiol.* **36**, 605.
- Knight, A. H., Crooke, W. M., MacDonald, I. R. & Shepherd, H. (1961). *J. exp. Bot.* **12**, 13.
- MacDonald, I. R. (1955). Ph.D. Thesis: University of Aberdeen.
- MacDonald, I. R. & DeKock, P. C. (1958). *Ann. Bot., Lond., N.S.*, **22**, 429.
- MacDonald, I. R. & Knight, A. H. (1958). *Ann. Bot., Lond., N.S.*, **22**, 423.
- MacDonald, I. R. & Knight, A. H. & DeKock, P. C. (1961). *Physiol. Plant.* **14**, 7.
- MacDonald, I. R., Webley, D. M. & Bacon, J. S. D. (1963). *Biochem. J.* **89**, 104 p.
- Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
- Nooden, L. D. & Thimann, K. V. (1963). *Proc. nat. Acad. Sci., Wash.*, **50**, 194.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Paton, A. M. (1959). *Nature, Lond.*, **184**, 1254.
- Pridham, J. B. (1958). *Nature, Lond.*, **182**, 1687.
- Pridham, J. B. & Walter, M. W. (1964). *Biochem. J.* **92**, 20 p.
- Rees, W. R. & Reynolds, T. (1958). *Nature, Lond.*, **181**, 767.
- Robertson, R. N., Turner, J. S. & Wilkins, M. J. (1947). *Aust. J. exp. Biol. med. Sci.* **25**, 1.
- Robinson, E. & Brown, R. (1952). *J. exp. Bot.* **3**, 356.
- Sacher, J. A. & Glasziou, K. T. (1962). *Biochem. biophys. Res. Commun.* **8**, 280.
- Sacher, J. A., Hatch, M. D. & Glasziou, K. T. (1963). *Physiol. Plant.* **6**, 836.
- Schwimmer, S., Makower, R. N. & Rorem, E. S. (1961). *Plant Physiol.* **36**, 313.
- Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.
- Steward, F. C. (1932). *Protoplasma*, **15**, 497.
- Stiles, W. & Dent, K. W. (1946). *Ann. Bot., Lond., N.S.*, **10**, 203.
- Straus, J. (1962). *Plant Physiol.* **37**, 342.
- Sutcliffe, J. F. (1952). *J. exp. Bot.* **3**, 60.
- Sutcliffe, J. F. (1960). *Nature, Lond.*, **188**, 294.
- Tervet, I. W. & Hollis, J. P. (1948). *Phytopathology*, **38**, 960.
- Turner, J. S. (1940). *Aust. J. exp. Biol. med. Sci.* **18**, 273.
- Webley, D. M. & Duff, R. B. (1955). *J. appl. Bact.* **18**, 122