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EFFECT OF CATIONS ON INVERTASE ACTIVITY AND SUGAR LEAKAGE OF CARROT SLICES

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Key Word Index—*Daucus carota*; Umbelliferae; carrot; Na^+; K^+; Mg^{2+}; Ca^{2+} (Sr^{2+}); cycloheximide; membrane leakiness; acid invertase activity.

Abstract—The presence of Na^+, K^+, Mg^{2+}, Ca^{2+} or Sr^{2+} in the ageing solutions of slices freshly excised from storage roots produces an immediate decrease in sugar loss when compared with water. Although this seems to be a general salt effect, specific differences between the cations could be detected as early as 10 min from the beginning of incubation. *Ca* 4 hr after excision the rate of sugar reabsorption by the slices equals that of sugar loss. This recovery process is prevented by cycloheximide (1 μM) and by Mg^{2+} at concentrations higher than 2–4 mM. Besides decreasing leakage the cations stimulate the rise in invertase activity. At concentrations higher than 2 mM this stimulation seems to be, essentially, of an osmotic nature. At concentrations lower than 2 mM the effect is probably related to increased invertase secretion to some extracytoplasmic compartment, e.g. the cell wall.

INTRODUCTION

The excision of tissues from storage organs and their subsequent incubation in an aqueous environment (washing or ageing) brings about numerous biochemical and fine structural changes [1–5] as reflected in the activity of several enzymes. For instance, the activity of acid invertase (EC3.2.1.26), which is almost undetectable in the mature carrot root, rises enormously in excised slices as they are aged [6]. Although ageing is often carried out in distilled water, sometimes running tap water or solutions containing low concentrations of calcium salts are used instead. Interactions of the minerals present in the incubation media with the tissues would be expected and it is in fact known that inorganic salts alter the permeability and the integrity of the excised tissues [7–11]. But while the monovalent cations appear to produce a 'shock' on the membranes, Ca^{2+} is considered to be a membrane stabilizer [12–14], hence the assumption that the presence of Ca^{2+} in the ageing solutions helps to maintain healthy excised tissues and so achieve 'optimum' biochemical responses [2, 3, 12]. However, not much is known about the effects exerted by the salts, themselves, on the biochemical changes triggered by tissue excision and ageing.

While studying the development of invertase activity during ageing of carrot slices we were concerned about the effect that Ca^{2+} (used for slice integrity) might have on enzyme development. We decided, therefore, to examine the effect of this cation as well as of the three other major cations (Na^+, K^+ and Mg^{2+}) on the rise in invertase activity. We also studied the effect of the four cations on sugar leakage.

RESULTS AND DISCUSSION

Sugar leakage

As a simple test of membrane integrity, we measured the amount of sugar lost by slices of storage roots to the incubation media. In order to decrease the contamination of solutions with debris of broken cells, the slices were thoroughly rinsed before the experiments. Despite this precaution, freshly-cut slices from any of the storage roots we studied lost sugars to the incubation media for several hr. The proportion of sugars released varied, however, with the species. For instance, turnip slices were much leakier than carrot or sugar beet slices; we observed this species difference with roots both bought at the market and grown by ourselves under identical conditions (Fig. 1).

Although the leakiness of the membranes can be, in part, a consequence of the mechanical shock of slicing, it must also reflect the peculiar nature of the storage tissue. It has been shown that in the intact storage organs metabolism is very low and cellular membranes are in a rudimentary state [3, 5, 15]; however, upon slicing and ageing, marked alterations in metabolism and membrane organization do occur. Our results suggest that during the initial 3–4 hr of ageing important membrane 'restoring processes' must have taken place since, by that time, sugar reabsorption equalled or exceeded sugar loss, even in turnip slices which had lost 70–80% of their sugars (Figs. 1–3).

Sugar leakage is a complex process not only because it involves several tissue compartments (free space, cytoplasm, vacuole) each with distinct kinetic parameters [11] but also because the organization of the cellular membranes becomes progressively more complex as a consequence of ageing. This could imply that the nature of the interactions between external factors (e.g. cations present in the medium) and the tissues also became more complex with time. This aspect was investigated next.

In freshly-cut slices, Ca^{2+} produced an immediate and marked reduction in sugar leakage (Figs. 1–3). This well documented property of Ca^{2+} [2, 3, 7–14] is termed the Viets effect. However, it can be brought about by other cations. Thus, despite indications that monovalent cations might accentuate the shock due to tissue excision [8, 9,

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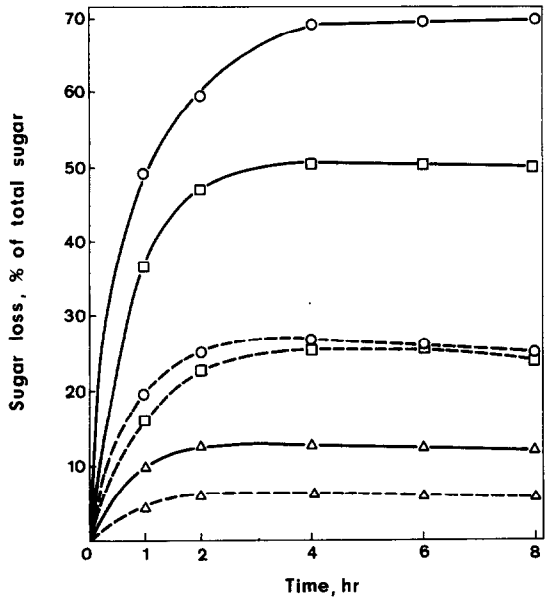

Fig. 1. Loss of total sugar by freshly-cut slices of turnip (○), sugar beet (□) and carrot (△) when incubated for up to 8 hr in water (——) or in 20 mM calcium chloride (.....). For each sampling time the sugar present in the media is given as the percent of the total amount of sugar in the slices at the start of incubation. The values are averages of duplicate experiments; the differences between replicate samples were low ($\leqslant \pm 0.8\%$) for all calcium chloride-incubated slices and for water-incubated carrot slices and high for the water-incubated slices of turnip ($\leqslant \pm 7.5\%$) and sugar beet ($\leqslant \pm 3.0\%$).

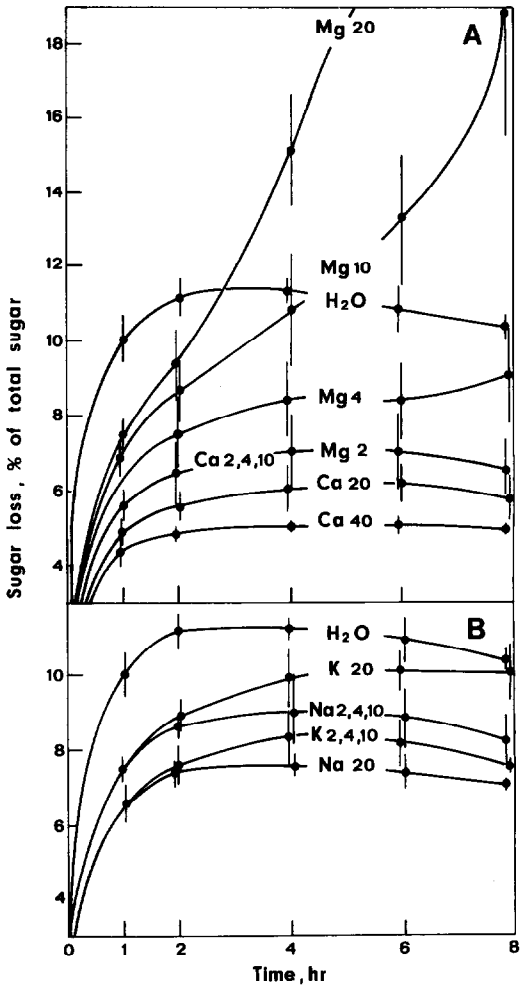

Fig. 2. Loss of total sugar by freshly-cut slices of carrot when incubated for up to 8 hr in water (A and B) or in solutions of calcium chloride, magnesium chloride (A), sodium chloride or potassium chloride (B). The sugar present in the media at each time is given as the percent of the total amount of sugar in the slices at the start of incubation. The slices were incubated in single salt solutions of a certain concentration (mM) as represented on the curves by the cation symbol and by the figures. Note that the similarity of effects for several solutions led to superimposed curves (e.g. the effects of calcium chloride at 2, 4 and 10 mM and of magnesium chloride at 2 mM were identical). Experimental points and vertical bars indicate mean ± s.d. for 3–5 experiments.

14], Na^+ and K^+, in addition to Mg^{2+} and Sr^{2+} (a Ca^{2+} analogue), also caused a very rapid decrease in leakage, relative to water (Figs. 2 and 3). The identical behaviour of these cations could possibly be attributed, at least partly, to a relief of the osmotic shock produced by water, as postulated by Ehwald *et al.* [11]. But the fact that we could already detect differential effects of the cations on leakage as early as 10 min from the beginning of incubation suggests that other cation-specific interactions were also involved. For instance, it is known that cations bind rapidly and reversibly to proteins and other membrane constituents, exerting a cross-linking action; but while Ca^{2+} is a good cross-linker Mg^{2+} is a poor one [16].

When the slices were incubated in the cation-containing solutions for rather long periods (up to 8 hr), we observed an amplification of the cation-specific effects on leakage that we had detected in the early phase of the process. But, in addition, we observed a shift in the behaviour of some cations (Fig. 2). Although Ca^{2+} (2–40 mM) and Na^+ or K^+ (2–20 mM) still restricted leakage to a lower level than that induced by water, K^+ at 20 mM was progressively less effective than at 10 mM in restricting leakage. The most remarkable change with time was that observed with Mg^{2+} at concentrations higher than 2 mM. Its initial protective effect against leakage shifted rather abruptly to a stimulating one after 1–2 hr from the start of the incubation (Fig. 2); this led to an extensive loss of solutes, the slices having a flaccid appearance by the end of the 8 hr incubation period.

These results indicate that the cations could be influencing efflux, influx or both to a different extent, but which we do not know since we only measured the net process (leakage). Regarding their long-term effect on this process the four cations may be grouped into two classes. Ca^{2+} and Na^+ (the extracellular cations) can be in contact with the slices at relatively high concentrations and for long periods without disturbing the membrane properties or interfering with their repair. On the other hand, Mg^{2+} and, to a smaller extent, K^+ (the intracellular cations) do alter the membrane properties, or some process of their repair, at concentrations at which Ca^{2+} and Na^+ are harmless. The fact that Sr^{2+} mimics Ca^{2+} in its capacity

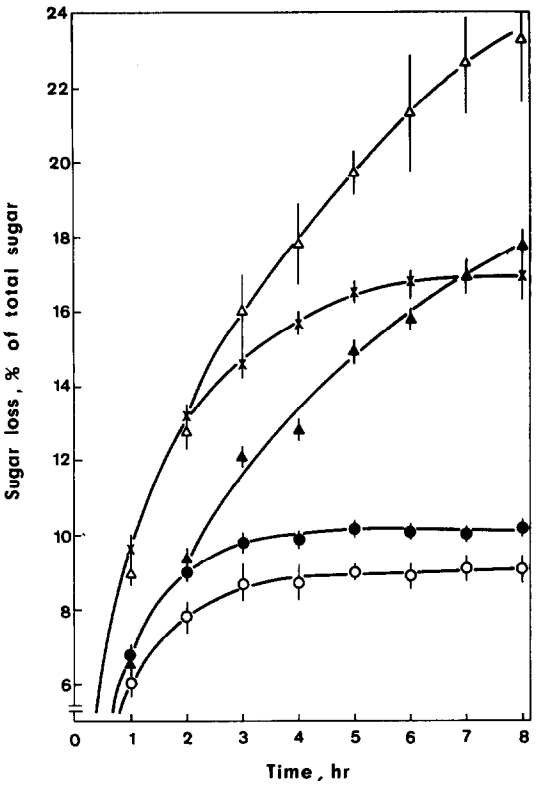

Fig. 3. Loss of total sugar by freshly-cut slices of carrot when incubated for up to 8 hr in water (X), 2 mM strontium chloride (○), 2 mM calcium chloride (●), 1 μM cycloheximide (Δ) or 1 μM cycloheximide plus 2 mM calcium chloride (▲). The sugar present in the media at each time is given as the percent of the total amount of sugar in the slices at the start of incubation. Experimental points and vertical bars indicate mean ± s.d. for two experiments. The carrots used in these experiments were 8-months-old.

to decrease leakage (Fig. 3), indicates that the action of Ca^{2+} is essentially of a non-metabolic nature.

The enhancement of leakage induced by cycloheximide (Fig. 3) suggests that the synthesis of new proteins is needed for the membranes to restore their normal selective permeabilities. It is well-known that protein synthesis is initiated as soon as the tissues are excised [2, 3, 15]. However, it is not clear why 1–2 hr are required between cycloheximide application and enhancement of leakage. During this time the inhibitor did not even interfere with the capacity of Ca^{2+} to reduce leakage (Fig. 3). It could be that the initial 1–2 hr leakage is almost independent of cell metabolism and that the inhibitor did not reach its cellular target.

It is of interest that the Mg^{2+} enhancement of leakage also showed a lag-time of 1–2 hr. However, it is not possible to know from our experiments whether Mg^{2+} could be affecting the synthesis of some membrane constituent. Protein synthesis is not likely to have been inhibited by the Mg^{2+} concentrations that increased leakage. The optimum Mg^{2+} concentration for the *in vitro* synthesis of proteins was reported to be 10–15 mM for liver microsomes [17] and 16–22 mM for ribosomes obtained from different plant tissues [18]. As the calcium content of plant storage organs is known to be very low [19], we suggest that Mg^{2+} above a certain concentration may be antagonistic to Ca^{2+} in some cellular process of relevance for the maintenance of cell integrity.

The above considerations refer to leakage of total sugar. Table 1 shows the behaviours of sucrose and reducing sugars for slices incubated in cycloheximide and 2 mM chloride solutions. In order to interpret these results, the invertase activity of the slices must be taken into account. This activity is barely detectable after 1 hr of incubation but reaches appreciable values by 8 hr, except when cycloheximide is present (see Table 2 and Fig. 4). So only in the cycloheximide-aged slices would we expect no hydrolysis of sucrose; indeed, *ca* 100% of the initial sucrose is found after 8 hr of incubation, in spite of leakage to the medium (Table 1). Since the highest rise in invertase was produced in the presence of calcium chloride (Fig. 4), it was not expected to find that incubation in this salt did not lead to sucrose disappearance (Table 1). Thus, calcium chloride seems to reduce both the leaking out of sugars and the contact of sucrose with invertase. Conversely, sucrose disappearance was evident when the slices were incubated in water or in the other salts. The difference in the percentage of sucrose and reducing sugar found in the media by 1 hr of incubation, when invertase activity is very low, suggests that sucrose leaks out from the slices more slowly than monosaccharides. Calcium chloride seems to affect this property.

Rise in invertase activity

Invertase activity, which is almost undetectable in freshly-cut carrot slices, reaches an appreciable level in slices aged for 8 hr in water (Table 2). Cycloheximide (1 μM) inhibits this increase in activity by 80–100%; this agrees with the assertion that the enzyme is synthesized *de novo* during ageing [20]. Ca^{2+} and Sr^{2+} (2 mM) stimulated the rise in invertase activity by 50–100%. We further studied the effect of cations (Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) on the rise in activity for a wide range of concentrations (Fig. 4). Only Mg^{2+}, at concentrations higher than 2–4 mM, prevented invertase activity from rising. Considering that the same range of Mg^{2+} concentrations stimulated leakage (Fig. 2), we think that their negative effect on invertase is the result of some sort of damage to the cells.

The stimulation of invertase produced by the three other cations (and by Mg^{2+} below 2–4 mM) seems to be the result of two distinct situations. Thus, K^{+} over the whole range of concentrations used and Ca^{2+} and Na^{+} in the range 2–10 mM may just have stimulated invertase through an osmotic effect. Support for this proposal is provided by the fact that mannitol solutions (6–30 mM) of osmotic potentials equivalent to those of the K^{+}, Ca^{2+} and Na^{+} chloride solutions produced a similar stimulatory effect (Fig. 5). We used mannitol as an osmoticum since it was unlikely to be appreciably absorbed or metabolized by the carrot slices [21, 22].

There remains to be explained the stimulation of invertase induced by Ca^{2+}, Na^{+} and Mg^{2+} at concentrations lower than 2 mM which, apparently, cannot be attributed to an osmotic effect. In this context it will be useful to discuss first the molecular nature of invertase and its probable location in the cell. It has been reported that invertase from both yeast and higher plant cells is a

Table 1. Effect of water, of the chlorides of sodium, potassium, calcium and magnesium (2 mM) and of cycloheximide (1 μM) on the amounts of reducing sugars and sucrose lost to the ageing media (after 1, 4 and 8 hr of incubation) or retained in the carrot slices (at the end of the 8 hr incubation period)

Ageing media	Type of sugar	Present in the ageing media at			Retained in the slices at 8 hr	Recovered at 8 hr*
		1 hr	4 hr	8 hr		
Water	Reducing sugars	5.5 ± 0.4	9.3 ± 0.7	12.0 ± 0.7	110.0 ± 2.9	122.0
	Sucrose	4.2 ± 0.0	5.7 ± 0.1	3.8 ± 0.0	80.6 ± 0.5	84.4
NaCl	Reducing sugars	4.6 ± 0.5	8.9 ± 0.3	9.4 ± 0.7	101.2 ± 4.0	110.6
	Sucrose	3.6 ± 0.1	4.3 ± 0.1	3.2 ± 0.0	83.3 ± 8.4	86.5
KCl	Reducing sugars	5.0 ± 0.1	8.1 ± 0.1	10.6 ± 0.1	110.3 ± 6.4	120.9
	Sucrose	3.7 ± 0.1	5.0 ± 0.2	3.3 ± 0.1	81.9 ± 4.1	85.2
$CaCl_2$	Reducing sugars	2.6 ± 0.0	4.5 ± 0.1	4.7 ± 0.0	100.7 ± 3.0	105.4
	Sucrose	2.6 ± 0.1	3.5 ± 0.1	2.8 ± 0.1	95.3 ± 8.3	98.1
$MgCl_2$	Reducing sugars	3.8 ± 0.0	5.9 ± 0.2	5.6 ± 0.3	108.7 ± 5.6	114.3
	Sucrose	3.1 ± 0.1	3.9 ± 0.1	3.2 ± 0.1	84.7 ± 1.9	90.3
Cycloheximide	Reducing sugars	6.1 ± 0.1	14.8 ± 0.7	18.7 ± 0.7	85.1 ± 0.1	103.8
	Sucrose	4.5 ± 0.1	8.1 ± 0.1	11.8 ± 0.3	92.5 ± 8.5	104.3
Cycloheximide + $CaCl_2$	Reducing sugars	3.0 ± 0.2	7.8 ± 0.2	10.0 ± 0.1	93.2 ± 6.5	103.2
	Sucrose	2.6 ± 0.1	4.3 ± 0.3	7.1 ± 0.3	94.8 ± 6.2	101.9

The figures represent, for each type of sugar, the percentage (± s.d.) of the amount present in the freshly-cut slices. The amounts of sugars present in 50 freshly-cut slices were: reducing sugars, 228.4 ± 10.6 μmol (taken as 100%); sucrose, 351.5 ± 8.4 μmol (taken as 100%). Values obtained from three samples of carrot slices.

*Obtained by adding up the two preceding columns.

Table 2. Effect of cycloheximide (1 μM), calcium chloride (2 mM) and strontium chloride (2 mM) on the development of invertase activity (nkat/g fr. wt) of carrot slices aged for 8 hr in different solutions

Freshly-cut slices	Aged slices				
	H_2O	Cyclo heximide	Cyclo heximide + $CaCl_2$	$CaCl_2$	$SrCl_2$
< 0.01	2.30 ± 0.50	< 0.01	< 0.01	4.18 ± 0.18	4.52 ± 0.16

Mean values ± s.d. for two experiments.

glycoprotein [15, 23]. At least part of the invertase of aged slices is synthesized *de novo* [20]. Most of the enzyme is secreted to the cell wall [24–27] and/or into the vacuole [27]. In sycamore cells, the secretion of glycoproteins and polysaccharides to the external medium is stimulated when cations are present [28]. The cations appear to exert their action ($Ca^{2+} > Na^+ > K^+ > Mg^{2+}$) at the outer surface of the plasma membrane without entering the cells.

If carrot invertase is a glycoprotein and is partially located in the cell wall, the stimulation of its development by Ca^{2+}, Na^+ and Mg^{2+} (at concentrations lower than 2 mM) could be similar to the stimulation of glycoprotein secretion by cations as observed in sycamore cells [28]. The role of the cations in invertase development would thus be essentially on the process of secretion, and not on the synthesis itself, which is supported by the fact that Sr^{2+} mimics Ca^{2+} in its stimulation effect. However, we still do not exactly know the relative distribution of invertase between cell wall and vacuole and whether the cations of the external medium have any influence on the secretion of the enzyme to the vacuole. For suspension cultured cells and for callus tissues of carrot, it was reported that at least 60% of the invertase seemed to be located in the cell wall [24, 25].

Invertase stability

Invertase activity is sometimes measured by incubating the unbroken slices pretreated with ethyl acetate in a sucrose-containing solution [29, 30]. On comparing this procedure with the usual slice disintegration, we consistently found higher invertase activities in the tissue homogenates than in the ethyl acetate- or ethanol-treated slices. Apparently, some of the enzyme was lost during the pretreatment. Although the proportion of enzyme lost varied with the batch of carrots, we noticed that higher recoveries were obtained in slices aged in 2 mM solutions of calcium chloride, sodium chloride and magnesium

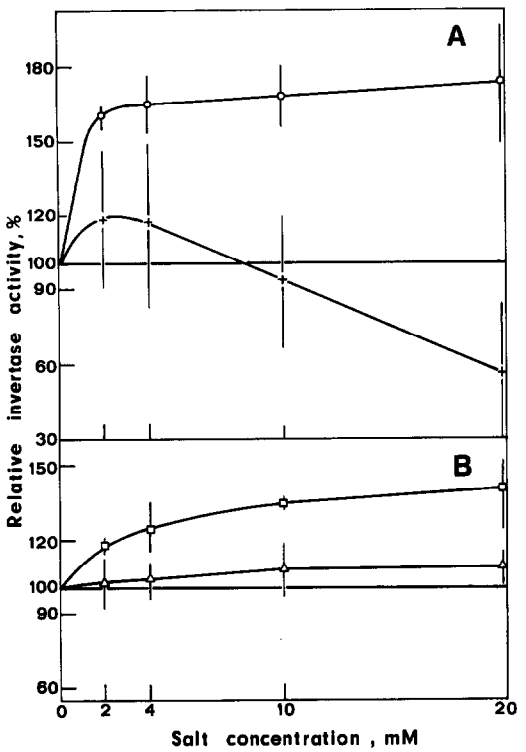

Fig. 4. Relative invertase activities of 8 hr salt-aged carrot slices. (A) Slices aged in calcium chloride (○) or in magnesium chloride (+); (B) slices aged in sodium chloride (□) or in potassium chloride (△). The invertase activities are expressed as relative values taking the activity of 8 hr water-aged slices as 100%. Experimental points and vertical bars indicate mean ± s.d. for 3–5 experiments.

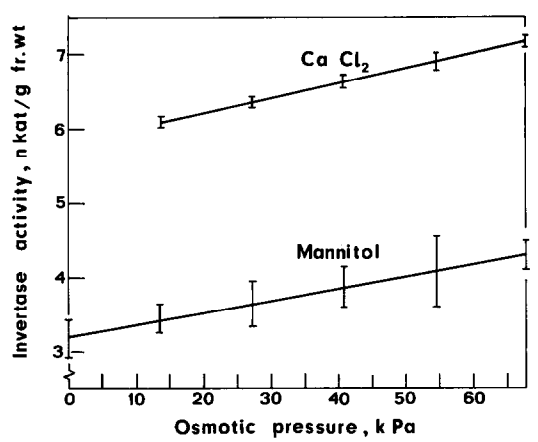

Fig. 5. Invertase activity developed by carrot slices aged for 8 hr in solutions of calcium chloride or mannitol of equivalent osmotic pressure. Experimental points and vertical bars indicate mean ± s.d. for two experiments.

chloride than in those aged in water or potassium chloride (Table 3). These results suggest that low concentrations of Ca^{2+}, Na^+ and Mg^{2+} could, in addition to stimulating invertase secretion, favour the stability of the enzyme or

Table 3. Effect of the presence of salts in the ageing media of carrot slices on the ratio of invertase activities determined by two different procedures (unfractionated homogenates/unbroken slices pretreated with ethanol)

Ageing media	Invertase ratio (homogenate/ethanol-treated slices)
H_2O	3.1 ± 0.2
KCl (2 mM)	2.7 ± 0.3
$MgCl_2$ (2 mM)	2.0 ± 0.4
NaCl (2 mM)	2.0 ± 0.2
$CaCl_2$ (2 mM)	1.9 ± 0.2

The slices were aged for 8 hr in the solutions.
Mean values ± s.d. for two experiments.

promote its attachment to some cellular constituent. Observations with peroxidase (a glycoprotein) have shown that, in tissue homogenates, its distribution between the supernatant and the pellet depended on the presence of Ca^{2+} which acts as a cross-linker between the enzyme and some cellular component [31].

In conclusion, the two cation effects reported, i.e. the decrease in leakage and the stimulation of invertase development, appear to be mediated by some interaction with the membranous structures of the cell. Cations may have exerted a cross-linking action on membranes and, at the same time, stimulated the fusion of secretory vesicles with the membranes. It is known, for instance that Ca^{2+} is a good cross-linker [16] and also a good inducer of the fusion of phospholipid vesicles [32]. However, we would like to stress the fact that the high invertase activity detected in the homogenates of calcium chloride-aged slices did not have its counterpart in the amount of sucrose hydrolysed by the slices during ageing (Table 1). At this moment, we are unable to explain these results since we do not know enough about the location of invertase in the cell, its activity *in vivo*, the path of sucrose during efflux and the effect Ca^{2+} exerts on these processes.

EXPERIMENTAL

Plant material and growth conditions. Seeds of carrot (*Daucus carota* L. cv Nantes), and of turnip (*Brassica rapa* L. cv Bola de neve), were purchased from Jerónimo Pereira Mendes, Lisbon; seeds of sugar beet (*Beta vulgaris* L. cv Kawepoly) were obtained from K.W.S., Einbeck, Germany. The plants were grown in a greenhouse under natural climatic conditions in a mixture of peat and white sand (1:1) and watered twice a week with a nutrient soln [33]. Previously we had observed that the results varied greatly when we used carrots bought in the market.

Tissue incubation. Unless otherwise stated, mature carrots of 5–7 months were used since invertase development in the slices increases with root age for maturing carrots [34] but starts to decrease for very old carrots (older than 8 months). Plants were collected at 9 a.m. and the roots used at once. The slices, 10 mm in diameter, 1 mm thick, were rinsed × 6 with H_2O before incubation. Samples of 66 slices (vol. = 4 ml) were incubated at 30° in 150 ml of the appropriate soln contained in a 300-ml Erlenmeyer flask; these were gently shaken at 100 strokes per min. For the assay of sugars released from the slices, 0.5–1.0 ml of the incubation solns were withdrawn at the appropriate time. At the end of the 8 hr incubation period the slices were rinsed twice with

H_2O and frozen until assayed for invertase activity (at the most 1 week later).

Sugar assays. Total sugar was determined by the PhOH–H_2SO_4 method [35] and reducing sugars either by Summer's method [36] or by the Somogyi–Nelson method [37]. Sucrose was determined by the Somogyi–Nelson method after hydrolysis with yeast invertase.

Invertase assay. Of the two invertases present in carrots [6], only the acid invertase (pH optimum 4.5) was measured. The frozen slices were disrupted by grinding in a mortar with 0.4 ionic strength Na_2HPO_4–citric acid buffer, pH 7.5 [33]. The resultant homogenate was (a) used for the measurement of total invertase activity and (b) centrifuged at 35000*g* for 10 min. The supernatant and the pellet from the latter treatment were then assayed for 'soluble' and 'cell-wall' invertase. When 8 hr H_2O-aged slices were extracted with 0.4 ionic strength Na_2HPO_4–citric acid, pH 7.5, less than 30% of the invertase activity was soluble. For this reason, we routinely determined invertase activity in the unfractionated extracts (total invertase), which were incubated at pH 4.5 and 30° with 50 mM sucrose, as described previously [33]. For comparison, total invertase was also determined in intact frozen slices treated with EtOAc or EtOH and incubated at 30° and pH 4.5 in 50 mM sucrose, following the procedure of ref. [30].

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