Phytochemistry, 1976, Vol. 15, pp. 619–623. Pergamon Press. Printed in England.

PASSAGE OF SUGARS ACROSS THE PLASMALEMMA OF CARROT CALLUS CELLS

DAVID PARR* and JACK EDELMAN†
Biology Department, Queen Elizabeth College, University of London, London W8 7AH, England

(*Revised received* 14 *November* 1975)

Key Word Index—*Daucus carota N.*; Carrot callus; Umbelliferae; plasmalemma; sugar influx and efflux; active uptake; passive diffusion; apparent free space.

Abstract—In pieces of carrot callus, the regions exterior to the plasmalemma were washed out in running tap-water within 15 min. The effect of temperature on sugar efflux and influx, suggests that the passage of sugars across the plasmalemma occurs mainly, if not entirely, by passive diffusion. The results of other workers are considered, and we conclude that the reported apparent active uptake across the plasmalemma may in reality have been due to active uptake across the tonoplast. Estimates of the apparent free space were unaffected by the sugar used, or the temperature, and we suggest that the apparent free space includes most, if not all of the metabolic compartment.

INTRODUCTION

Dainty [1] has defined the apparent free space (AFS) of a tissue as that part which is in free diffusive connection with the surrounding medium; its extent varies depending on the solute used in its measurement. Some workers using sugars have claimed that the metabolic compartment (MC) or cytoplasm is part of the AFS, i.e. that the plasmalemma is a negligible barrier to the diffusion of sugars [2–4], while others take the opposite view [5, 6].

An associated question is that of the active uptake of sugars into plant cells, and more especially the locus of such uptake. Claims have been made that active systems operate at the plasmalemma [7, 8], whilst other authorities consider that sugars diffuse across this membrane [9, 10]. There are also reports that both active and passive uptake are possible into the MC [2, 11], or that active uptake occurs only at the tonoplast [12]. It may be significant that two leading proponents of obligatory active uptake across the plasmalemma have now conceded that diffusive passage is possible between the region of the AFS outside the plasmalemma (which we term the external compartment, EC) and the storage compartment (SC) or vacuole [13].

In this paper we present evidence which has led us to question the validity of some measurements of uptake into the MC, and to suggest that there is confusion between uptake across the plasmalemma, and uptake across the tonoplast, arising from the extended times used to wash out the free space.

RESULTS

Washout kinetics

Pieces of callus were pre-incubated in ^{14}C-labelled sucrose, blotted and transferred through a succession of aliquots of distilled water and mechanically shaken in each for 2 min. The tissue was blotted between each aliquot, and was extracted at the end of the experiment. The radioactivity in a sample of each aliquot was counted, and the course of washout of activity plotted on a semi-log basis [14] (Fig. 1). A similar experiment was performed in which the tissue was cooled towards the end of the incubation, and all washing was carried out in ice-cold water; the two-phase nature of the washout is

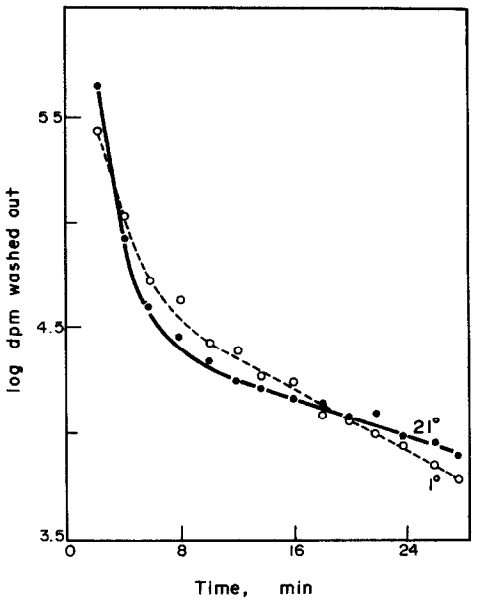

Fig. 1. Semi-log plot of the radioactivity washed from pieces of CRT II, plotted against the time at the end of each two min washing period. Tissue was preincubated for 4 hr in 40 mM-sucrose [U-^{14}C] at 21°, then blotted and shaken for 2 min each in a succession of aliquots of water at 0° or 21°.

* Present address: Biophysics Department, University College London, Gower Street, London WC1E 6BT.
† Present address: The Lord Rank Research Centre, Lincoln Road, High Wycombe, Bucks. HP1E 3QR.

apparent (Fig. 1). The curves obtained at the two temperatures are similar; both are biphasic, and the first phase is complete within 15 min. We consider that the initial phase represents material washed from the EC, whilst the second phase represents material diffusing through the plasmalemma and coming from the MC. On the basis of this, we have washed pieces of tissue for exactly 15 min in order to remove all material from the EC, but a minimum of that from the MC. That this desired effect was achieved was partially confirmed by the recovery of almost exclusively symmetrically labelled sucrose from tissue incubated in asymmetrically-labelled sucrose and extracted directly after a 15 min wash, while largely asymmetrically labelled material was present in the medium (we presume that alteration of the position of the label can only occur in the MC).

Estimating apparent free space and external compartment

We washed pieces of callus tissue in running tap water for 30 min to ensure that the EC was completely filled with water and the MC was fully turgid. Pieces were then incubated for several hr in a radioactive sugar solution of known volume and activity. We assumed that after this time the labelled material in the free space (as defined in [1]) was in equilibrium with that in the medium, and that their concentrations were identical.

To determine the AFS, the tissue was carefully blotted, and the radioactivity in the free space washed out by agitation in a known volume (between 250 and 300 ml) of water for 45 min. Radioactivity in samples of this washing water was estimated, and the total calculated. From the activity of the incubation medium at the conclusion of the experiment, the percentage free space was determined, assuming the tissue has a relative density of one.

The EC was determined as follows. During incubation in a volume (x) of radioactive sugar, the CO_2 evolved was trapped. At the end of the experiment the EC was washed out in running tap water for 15 min, and the tissue extracted to give soluble and insoluble fractions, both of which were assayed for ^{14}C. If 'a' was the total radioactivity recovered in CO_2 plus soluble and insoluble fractions, and 'b' the total activity in the incubation medium at the start of the experiment, then it might have been expected that $b - a = c$, where 'c' was the total activity recovered in the medium at the end of the experiment. However, this was not so, because the effective volume of the incubation medium was increased by the volume of the EC (d), thus: $b - a = c(x + d)/x$. From this equation, the volume of the EC (d) in a known weight of tissue was calculated, and thus the percentage, again assuming that the tissue has unit relative density, and that the CO_2 and cytoplasmic material lost during the 15 min wash was negligible.

The size of the AFS and the EC was similar (Table 1), and when the two values were both estimated with one piece of tissue, they were the same. The values estimated using sucrose were similar to those estimated using monosaccharide (glucose or invert sugar), and there was no significant difference between estimates made at 0° and at room temp. (Table 1).

Effect of temperature on sugar uptake

When pieces of callus were incubated in labelled glucose for 2 hr at 0° or 23°, then washed in running tap water for 15 min and extracted, the amount of radioactivity taken up was similar ($Q_{10} = 0.08$), but the distribution of this activity was very different (Table 2), and had the much greater Q_{10} expected of a metabolic process. In a similar experiment, the pulse of labelled glucose was chased by incubating the tissue in water for a further 2 hr. The pulse and chase were carried out at the same temperature, and the results (Table 3) resemble those of Table 2, but also show that at the lower temperature much more radioactivity passed back into the medium during the chase.

This experiment was also carried out using labelled sucrose, and similar results were obtained, except that the Q_{10} for uptake was marginally higher, perhaps reflecting the higher wall bound acid invertase activity at this temperature, since inversion of sucrose effectively increases the external sugar concentration, and we have also shown that the rate of sugar entry into callus cells is related to their concentration up to external levels of several hundred millimolar (Fig. 2). We have shown that fructose and galactose are taken up to a similar extent

Table 1. Estimates made of external compartment (EC) and apparent free space (AFS) of CRT II callus tissue using various sugars and temperatures

	% AFS		% EC	
Estimate using	Mean	Standard deviation	Mean	Standard deviation
sucrose	23.5	±7.6 (2)	18.5	±5.7 (7)
monosaccharide	16.5	±0.6 (2)	21.0	±3.9 (8)
mannitol	18.4	±2.6 (2)	19.8	±1.6 (2)
0°	20.0	±6.0 (4)	22.1	±5.0 (6)
21–24°	18.4	±2.7 (2)	18.6	±4.0 (11)

Numbers in parenthesis indicate number of estimates.

Table 2. The uptake and subsequent distribution of glucose by CRT II. Groups of pieces were incubated in 200 µl of 40 mM-glucose-[U-^{14}C] for 2 hr at 0° or 23°, then washed in running tap water for 15 min and extracted.

	Total uptake (µmol glucose	% Distribution of recovered radioactivity		
Temperature	/g fr. wt)	CO_2	Soluble	Insoluble
0°	2.84	0.3	98.6	1.1
23°	3.38	7.4	78.9	13.7

Table 3. The uptake and subsequent distribution of glucose by CRT II after chasing. Pieces of tissue were fed with 200 µl 40 mM-glucose-[U-^{14}C] at 0° or 23° for 2 hr, then washed for 15 min in running tap water and chased for a further 2 hr in 200 µl H_2O at the same temperature

	Total uptake (µmol	% Distribution of recovered radioactivity			
Temperature	glucose /g f. wt)	CO_2	Soluble	Insoluble	Chase medium*
0°	2.99	0.3	16.7	1.7	81.3
23°	3.19	12.1	22.8	22.2	42.9

* Corrected to account for 19.7% EC.

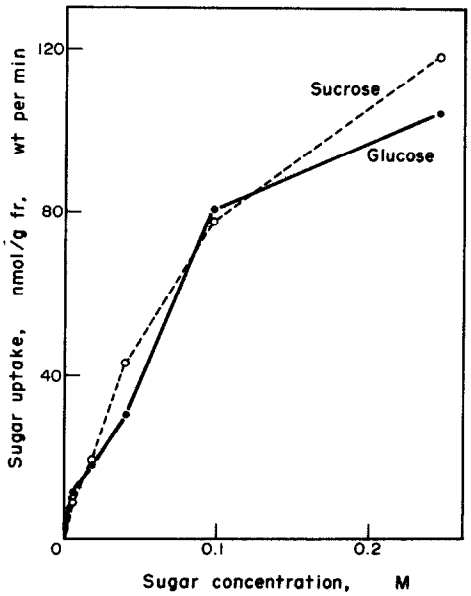

Fig. 2. The total uptake of sucrose and glucose by pieces of CRT II. The tissue was fed with 100 μl of sugar-[U-^{14}C] for 2 hr at 37°, washed in running tap water for 15 min, and extracted.

as glucose and sucrose at 23°, but mannose may behave differently [15].

Analysis, by two-dimensional chromatography followed by autoradiography of the labelled components in the soluble extracts and residual medium, showed that the majority of label in all cases was still in the form in which it was fed.

Effect of concentration on sugar uptake

We found that the relationship between fed sugar concentration and uptake rate was neither linear nor hyperbolic (Fig. 2).

DISCUSSION

We have found that the efflux of sugars from carrot callus has two distinct phases; curves of a similar nature have been obtained by Hawker [16] using sugar cane. He found that a plateau was reached after which no further sucrose was washed out, despite its presence in the tissue (in the SC). We believe that his results must be interpreted in one of two ways; either there is no sugar efflux through the plasmalemma, so that prior to reaching the plateau all of the radioactivity originates from the EC, or the plasmalemma is permeable to sugars, and once the plateau is reached all sugars in the MC have been washed out, leaving only material in the SC.

We claim that the latter alternative must apply, since in our work material washed out during the second phase contained a variety of labelled metabolites which could only have arisen in the MC. The initial phase of washout we equate with material coming from the EC (i.e. exterior to the plasmalemma), and we conclude that within 15 min the contents of the EC are completely removed from pieces of carrot callus of the size used here (about 100 mg); and subsequent washing only further depletes the MC. We believe that similar times apply to tissue discs used by other workers. We thus suggest that whilst sugars pass freely across the plasmalemma, this membrane does present some limited resistance to diffusion. Three phases have been found for the loss of labelled ions [14] and thiourea [6] from plant cells. The extra phase is equated with material coming from the SC, which we believe does not occur with sugars because they are actively retained within the tonoplast [17, 18]; this accounts for the faster rate of the second phase of washout at the lower temperature. If the tonoplast were freely permeable to sugars at normal temperature then a two phase efflux curve would also result, but no soluble labelled material would remain in the tissue after reaching the plateau [16]. It is also possible that organelles may constitute barriers to diffusion, resulting in one or more further phases, but since only two phases were observed, and chloroplast and mitochondrial inner membranes are considered to be freely permeable and impermeable to sucrose respectively, we consider this unlikely.

Using washing times of 15 min the passage of sugars into the tissue across the plasmalemma appeared to be insensitive to temperature, and occurred to a similar extent with all of the sugars investigated, with the possible exeption of mannose. This leads us to propose that this passage is a simple physical process, perhaps due to diffusion through hydrophilic pores in the membrane [6, 19]. This is significant for the results of a number of investigations. Times of up to 2 hr have been considered necessary to remove "free space" material from 3 mm sugar cane discs [9] although after this time no further sugar can be removed [16] and the half-time of washout of 0.5 mm discs is less than 1 min [9]. Some workers using such extended washing times have described them as estimating "active sugar uptake" [13], and a number consider that the active systems operate at the plasmalemma, despite washing for times which must have removed a good deal of the MC (e.g. Gayler and Glasziou—1 hr [20], Hanson—90 min [21], Bowen—over 45 min [8], and others who wash for undisclosed times [22, 23]). Whilst it is possible that there are interspecific differences, we consider it unlikely, and conclude that these workers have washed out the AFS, including the MC, and hence that the active systems which they characterise actually operate at the tonoplast. There is ample evidence that sugar passage across this membrane is an active process (see [15] for references), and the considerably greater efflux of labelled sugars from callus tissue at low temperature found in this work is taken to reflect the greatly reduced active transfer from MC to SC in the cold, together, perhaps, with the release of some sugar from the SC due to the lack of active retention. The greater efflux could be due to active retention within the MC, but experiments in which the pulse and chase were given at different temperatures show that this was not so [15]. If the plasmalemma is freely permeable to sugars, the pool sizes of which in the MC are small [15, 24–26], then even if the MC is not completely washed out, the operation of active systems across the tonoplast may be reflected in the diffusion of sugars across the plasmalemma, a view shared by Glinka [6].

Edelman and Hanson [27] recovered sucrose as the major labelled product after feeding invert sugar to CRT II and washing for 90 min. We found that invert sugar

was still the major component after a 15 min wash. Glasziou and Gayler [13] found that glucose influx into sugar cane at low temperatures was only 2% of that at 30°; this differs radically from the results presented here. We suggest that both of these conflicts are due to measurement of uptake after removal of most, if not all, material from the MC. Kursanov and Brovchenko [28] washed out up to 30% of the sugar from sugar beet discs in 30 min; they claim that this originates from the free space "which is not bounded by membranes", but the appearance of labelled material in their washings within 10 min of the start of photosynthesis in $^{14}CO_2$ shows that some of the material recovered must have come from the MC.

Estimates of uptake by measuring the removal of sugars from the bathing solution are not open to this criticism, but do not allow distinction to be made between tonoplast and plasmalemma. The one major group using this method have concluded that sugar entry into maize scutellum has both active and passive components [11, 19, 29–34], although their proposed pressure flow of sugars from mesophyll via plasmadesmata and phloem into the medium [17] does not operate in callus tissue in which the phloem is non-functional [18].

We assume that if sugar efflux across the plasmalemma is passive, then its influx will be also. Our results do not preclude the operation of active systems at this membrane, especially since those which have been characterized are saturated at very low sugar concentrations [8, 22, 23, 35], but they do preclude such systems being the only means of sugar uptake. We found that the relationship between fed sugar concentration and uptake was not quite the linear one expected of a physical system. Similar curves have been reported by other authorities [11, 19, 34, 36], and Glasziou and Gayler [13] calculated that any carrier would have "an extraordinarily high K_m"—about 70 mM from our results. The curves could be due to the stimultaneous operation of both active and passive uptake, but they could also be the result of diffusion into a MC which becomes physically saturated at high sugar concentrations.

Our results also have a bearing on the extent of the AFS; the presence of 2 phases in the 0° wash out curve and the low Q_{10} of total sugar uptake indicate that sugars diffuse across the plasmalemma, and hence that the AFS as defined by Dainty [1] and measured using sugars will include a large part, if not all, of the cytoplasm (or MC), especially if the estimates are made over an extended time. In this respect it is important to stress that we have taken into account the metabolism of the sugar used to determine the EC and AFS, a precaution omitted by some previous workers. Estimates of the relative extent of the MC cannot be made from our results, but since estimates of AFS and EC do not differ significantly, the MC volume must be negligible when compared with the EC, and of course with the SC. It could alternatively be because much of the MC is washed out during even a 15 min wash, but this is less likely since the radioactivity removed during this wash is almost exclusively in the form in which it is fed, or in a form obtainable without entry into the MC. The AFS estimated using mannitol did not differ from that measured using other sugars, and uptake studies [15] show that this sugar alcohol may penetrate into the MC, albeit more slowly than other sugars. Hence the AFS measured using mannitol may not be simply the EC as claimed by Dainty and others [1, 6].

EXPERIMENTAL

Tissue culture. CRT II carrot callus was grown as described previously [37].

Radioactive chemicals. These were purchased from the Radiochemical Centre, Amersham, Bucks. In some cases sucrose-[G-^{14}C] was prepared from $^{14}CO_2$ generated by the action of lactic acid on $BaCO_3$-[^{14}C], by photosynthesis using *Canna indica* leaves and a method based on that of ref. [38]. *Radioactive counting* was done in liquid scintillation using butyl-PBD in toluene, and including EtOH when aqueous samples were to be counted. Dried insoluble residues were held in suspension in a thixotropic gel of 4% Cab-O-Sil. $^{14}CO_2$ was absorbed in glass fibre filter paper impregnated with NaOH and $Ba(OH)_2$ [15], and the papers dried thoroughly before counting. The counting efficiency of each method was accurately determined in order to compare results [15].

Tissue incubation. Pieces of tissue weighing *ca* 100 mg were incubated in labelled sugars in sample tubes covered with aluminium foil to prevent photosynthesis.

Tissue extraction. Enzyme reactions were stopped by immersing the tissue in boiling EtOH, and the labelled components extracted further by grinding in aq EtOH.

Acknowledgements—DRP gratefully acknowledges the encouragement and advice of Dr. J. S. Hawker and receipt of a Science Research Council Studentship.

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