Biochimica et Biophysica Acta, 1152 (1993) 61–68

BBAMEM 76087

The α-D-glucosyl C-2 hydroxyl is required for binding to the H^+-sucrose transporter in phloem

Simon D.J. Griffin, Karen D. Buxton and Iain A. Donaldson

Department of Biochemistry, University of Oxford, Oxford (UK)

(Received 16 March 1993)

Key words: Sucrose transport; Proton co-transport; Phloem; α-Glucoside; 4-Hydroxyphenyl β-D-fructofuranoside; (*A. graveolens*)

The specificity of uptake of sucrose into isolated phloem tissue from *Apium graveolens* has been investigated using a number of analogues of sucrose. The presence of a single saturable transport system for sucrose was confirmed using the double isotope method of Inui and Christensen (J. Gen. Physiol. 50 (1966) 203–224). 4-Hydroxyphenyl β-D-fructofuranoside showed no inhibition of sucrose uptake, whereas 4-hydroxyphenyl α-D-glucopyranoside showed competitive inhibition with a K_i of 6.7 mM. 4-Methoxyphenyl α-D-glucopyranoside also inhibited sucrose uptake competitively ($K_i = 6.0$ mM). This compound was also synthesised radioactively labelled with ^{14}C and its uptake into phloem tissue was conclusively demonstrated to occur by active transport on the same carrier as sucrose. Contrastingly, 4-methoxyphenyl α-D-2-deoxyglucopyranoside displayed non-competitive inhibition of sucrose influx ($K_i = 2.5$ mM) and uptake of the ^{14}C-labelled compound was insensitive to FCCP, PCMBS and sucrose. We conclude that the hydroxyl group at the C-2 position on the glucopyranosyl moiety is essential for binding to the sucrose carrier in this tissue.

Introduction

Uptake of sucrose across the plasma membranes of higher plants is accomplished by active transport, mediated by a proton-dependent trans-membrane carrier [1]. A variety of systems have been used to characterise the substrate specificity and kinetic activity of this carrier. These include mature plants and cotyledons of *Ricinis communis* [2], cotyledons of *Sinapis alba* [3], excised phloem tissue from petioles of *Apium graveolens* [4], plasma membrane vesicles isolated from *Beta vulgaris* [5–8], *Spinacia oleracea* [9] and *Ricinis communis* [10,11], leaf discs of *Vicia faba* [12,13] and protoplasts prepared from *Glycine max* [14–17].

Many of the above investigations into the specificity of the sucrose transporter employed techniques involving mesophyll cells [12–17], which display 'biphasic' unsaturable kinetics for the uptake of sucrose, resulting from a complex mixture of uptake, metabolism and vacuolar sequestration [18]. Since any one or a combination of these processes may be influenced by sucrose analogues, under such a regime, the exact effects that such compounds have on the cell membrane sucrose transporter can be difficult to evaluate. Nevertheless, the most extensive investigation of the effects of various sucrose analogues upon the rate of sucrose uptake employed soybean cotyledon protoplasts [14–17]. These studies concluded that increasing the hydrophobicity of the fructosyl residue, by the replacement of hydroxyl groups with fluorine [19,20], increased the affinity of these cells for the analogues above that of sucrose [14]. It was proposed that the β-D-fructofuranosyl moiety presents a hydrophobic surface, which is augmented by these substitutions and which is required to strengthen attachment to the carrier protein. It was further concluded [17], from studies of the effects of aryl α-D-thioglucopyranosides on uptake of sucrose, that it is the hydroxyl groups at positions C-3, C-4 and C-6 of the glucopyranosyl moiety that confer specificity of recognition by the transport protein, with no significant contribution by the hydroxyl at C-2.

We report an investigation of the specificity of sucrose transport into 'isolated phloem' of celery, which represents a mixture of phloem parenchyma and sieve element-companion cell complexes, from which the xylem and the surrounding collenchyma have been removed. This tissue has been shown to display saturable kinetics for the uptake of sucrose, a feature which appears to be attended by the absence of signifi-

Correspondence to: I.A. Donaldson, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK.
Abbreviations: FCCP, carbonylcyanide *m*-fluorophenylhydrazone; PCMBS, *p*-chloromercuribenzenesulphonic acid; PEG, poly(ethylene glycol); Mes, 2-(*N*-morpholino)ethanesulphonic acid.

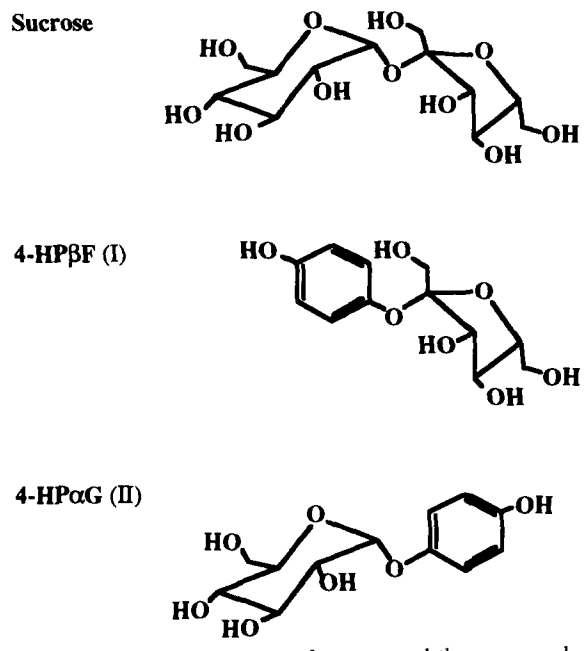

Fig. 1. Representative structures of sucrose and the monosaccharide analogues 4-HPβF and 4-HPαG.

cant invertase, sucrose synthase and large vacuoles [4]. These characteristics have allowed us to perform an accurate kinetic analysis of the interaction of sucrose analogues with the sucrose carrier, using this system.

In order to compare directly the contributions of the β-D-fructofuranoside and α-D-glucopyranoside residues to binding to the sucrose carrier, we chose two analogues bearing the same aglycone and differing solely in the glycosyl component. These were (4′-hydroxyphenyl) β-D-fructofuranoside (4-HPβF), and (4′-hydroxyphenyl) α-D-glucopyranoside (4-HPαG) (Fig. 1). Based on our findings we have expanded our studies to investigate the uptake into phloem tissue of the labelled analogues [*methyl*-^{14}C](4′-methoxyphenyl) α-D-glucopyranoside and [*methyl*-^{14}C]2-deoxy(4′-methoxyphenyl) α-D-glucopyranoside, and also the effects of unlabelled preparations of these compounds when used as inhibitors of the uptake of sucrose.

Materials and Methods

Plant material

Celery plants (*Apium graveolens* L. cv. Sutton's Solid White) were grown from seed (Suttons, Torquay, UK) in John Innes No. 2 potting compost, under glass, approx. 30 weeks before use. Glasshouse temperature was maintained at not less than 15°C with a 14 h photoperiod, supplementary illumination being provided by high-pressure sodium vapour lamps giving a resultant photon density of not less than 150 μmol m^{-2} s^{-1} in the 350–700 nm range. 8-week-old seedlings were transplanted into 10 litre pots. The plants were watered daily and supplied weekly with Fison's *Liquinure*®.

Uptake experiments

Celery phloem tissue was isolated and treated according to the method of Daie [4]. For characterisation of inhibition by sucrose analogues, approx. 30 mg (fresh weight) of excised phloem tissue was incubated in 1 ml aerated medium containing a range of sucrose concentrations around its K_m (40, 20, 10, 5 and 2.5 mM), in the presence or absence of one of the analogues. After 1 h of treatment in this way, when sucrose uptake was demonstrated to be linear, the tissue was washed three times in 3 ml of pre-incubation medium. Following this, the tissue was extracted overnight at 50°C in 1 ml 80% (v/v) ethanol and the resultant ethanolic solution added to 4 ml of scintillation cocktail (1:4 (v/v) methanol/Scintillator 299™ (Packard, Pangbourne, UK)). Total radioactivity was measured on a Beckman LS 1701 scintillation counter. Each measurement was repeated five times per experiment and the mean of the values calculated. This protocol was also followed for the study of the uptake of 4-MPαG; however, since the uptake of 4-MPα2dG was only linear up to 45 min, incubations using this substrate were shortened to this period.

For inhibition studies, [U-^{14}C]sucrose, [*methyl*-^{14}C]4-MPαG and [*methyl*-^{14}C]4-MPα2dG were supplied at a specific radioactivity of 1 MBq mmol^{-1}. Investigations of up take of labelled substrates over time employed a specific radioactivity of 0.5 MBq mmol^{-1} for each substrate. PCMBS (Sigma) was used at 2 mM, and FCCP (Fluka) at 5 μM. [U-^{14}C] Sucrose (22.3 GBq mmol^{-1}) and [6,6′(n)-^{3}H]sucrose (202 GBq mmol^{-1}) were purchased from Amersham International (Amersham, UK).

In our application of the double isotope technique of Inui and Christensen [21], [6,6′-^{3}H]sucrose and [*methyl*-^{14}C]4-MPαG were used at a concentration ratio of 1:1 over a range of substrate concentrations. After a 1 h incubation, the ratios of the activities of the isotopes in each sample were measured by scintillation counting.

During these experiments, [6,6′(n)-^{3}H]sucrose, [*methyl*-^{14}C]4-MPαG and [*methyl*-^{14}C]4-MPα2dG, were supplied at a specific radioactivities of 2.5 MBq mmol^{-1}, 1 MBq mmol^{-1} and 2 MBq mmol^{-1}, respectively.

All TLC was carried out on silica-gel 60 F_{254} (Merck 5554). AnalaR solvents were purchased from Merck and, unless otherwise stated, reagents were purchased from Aldrich. Solids were dried in vacuo over phosphorus pentoxide and solvents were dried before use according to Perrin and Armarego [22].

Synthesis of sucrose analogues

4-HPβF (I)

(I) was synthesised by exploiting the invertase-catalysed fructosyl transfer to hydroquinone first reported by Nakamura [23]. Yeast invertase (EC 3.2.1.26) immobilised on Fractogel® TSK DEAE-650 (S) (Merck), and equilibrated in sodium phosphate buffer (10 mM, pH 7.0), was prepared by the method of Woodward and Wiseman [24].

Enzymatic synthesis

Nitrogen saturated sodium acetate/acetic acid buffer (1 litre, 20 mM, pH 5.0) was added to sucrose (68.5 g, 0.2 mol) and hydroquinone (66 g, 0.6 mol, 3 equiv.) under an atmosphere of nitrogen, and the solution agitated with warming to dissolve the solids. Matrix-bound enzyme (20 ml) was added while sparging, and the reaction mixture stirred gently at room temperature. The progress of the reaction was monitored by TLC (solvent system CH_3CN/H_2O, 9:1 (v/v), sprayed with 5% (v/v) sulphuric acid in ethanol and developed by charring). Product R_F 0.53 and hydroquinone R_F 0.9.

The enzyme was removed by filtration under nitrogen and the solution concentrated in vacuo to approx. 250 ml. The precipitated hydroquinone was removed by filtration and the filtrate was washed five times with diethyl ether (250 ml) and the aqueous phase evaporated in vacuo to a mobile syrup. The syrup was divided into aliquots (100 ml) which were extracted ten times with one volume of CH_3CN/H_2O, 9:1 (v/v), using a counter-current distribution method. The turbid upper phase was dried in vacuo to a syrup, dissolved in a minimum of methanol and purified by flash chromatography on silica eluting with diethyl ether (2–3 vol.) to remove hydroquinone, followed by CH_3CN/H_2O, 9:1 (v/v). Fractions (10 ml) were analysed by TLC, pooled and evaporated in vacuo. Recrystallisation from CH_3CN/H_2O, 9:1 (v/v), yielded (I) as colourless crystals in low yield (24 preps. gave 1.5 g, 5.5 mmol product), m.p. 118–121°C (found: C, 51.7% and H, 6.06%; $C_{12}H_{16}O_7$ calculated: C, 52.9% and H, 5.93%). $[\alpha]_D^{20} = -121.00$ (*c.* 1.0, MeOH).

NMR ^1H (500 MHz, D_2O) δ 3.52–3.58 (m, 1H, H-5), 3.62 (dd, 2H, *J* 10, 30 Hz, H-1), 3.82 (dd, 1H, *J* 5, *J* 12 Hz, H-6), 3.95 (dd, 1H, *J* 5, *J* 10 Hz, H-6′), 4.17 (t, 1H, *J* 8 Hz, H-4), 4.29 (d, 1H, *J* 8 Hz, H-3), 6.82, 7.09 (2d, 4H, *J* 10 Hz, H-2′, 3′, 5′, 6′).^{13}C (50.2 MHz, D_2O) δ 60.84, 63.38 (C-1, 6), 75.32, 76.71, 82.44 (C-3, 4, 5), 106.81 (C-2), 116.59, 124.56 (C-2′, 3′, 5′, 6′ aryl), 147.05, 152.98 (C-1′, 4′). *m/z* (C.I.) 272(M^+, < 10%), 180(25), 145(12), 110(base peak), 55(10). ν_{max} (KBr disk) 3395, 3325, 835, 1222, 1511, 929, 875, 857 and 820 cm^{-1}.

4-HPαG (II)

An anomerically enriched sample of (II) was synthesised, essentially following the method of Helferich and Reischel [25], from hydroquinone monobenzoate [26] and β-D-glucosepentaacetate. Traces of the β-anomer contaminant were removed by treatment with β-glucosidase (EC 3.2.1.21) (7 U ml^{-1} in sodium acetate/acetic acid buffer 50 mM (pH 5.0)). The resulting (II) was purified by flash chromatography on silica, eluting with diethyl ether and then with CH_3CN/H_2O, 9:1 v/v). Fractions were analysed by TLC (silica gel 60; CH_3CN/H_2O, 9:1). Recrystallisation from hot ethyl acetate yielded colourless crystals, m.p. 204–206°C (Found: C, 52.7% and H, 5.83%; $C_{12}H_{16}O_7$ calculated: C, 52.9% and H, 5.93%). $[\alpha]_D^{20} = +179°$ (*c.* 1.0, H_2O).

NMR ^1H (500 MHz, D_2O) δ 3.48 (t, 1H, *J* 10 Hz, H-6), 3.64–3.81 (m, 4H, H-2, 3, 4, 5), 3.88 (t, 1H, *J* 10 Hz, H-6′), 5.44 (d, 1H, *J* 5 Hz, H-1), 6.84, 7.04 (2d, 4H, *J* 10 Hz, H-2′, 3′, 5′, 6′). ^{13}C (50.2 MHz, D_2O) δ 60.99 (C-6), 70.09, 71.89, 73.11, 73.75 (C-2, 3, 4, 5), 99.12 (C-1), 117.06, 120.01 (C-2′, 3′, 5′, 6′ aryl), 152.07, 150.79 (C-1′, 4′). *m/z* (C.I.) 272 (M^+ < 10%), 180 (23), 162 (10), 145 (11), 110 (base peak), 55 (12). ν_{max} (KBr disk) 3419, 3370, 1515, 1223, 917, 830 and 781 cm^{-1}.

The syntheses of 4′-methoxyphenyl α-D-glucopyranoside (4-MPα2dG), 2-deoxy(4′-methoxyphenyl) α-D-glucopyranoside (4-MPα2dG), and their *methyl*-^{14}C analogues has been previously described [27].

Results and Discussion

In agreement with Daie [4], the uptake of sucrose into celery phloem was shown to be linear over at least 80 min (Fig. 2), but an extrapolation of this curve does not intersect the origin. In her detailed description of the isolated celery phloem system, Daie has already produced evidence that such linear uptake of sucrose into celery phloem over 1 h can neither result from metabolism of the compound, nor be the product of an equilibrium established between uptake and leakage. We therefore felt justified in using the slopes obtained as initial rates for our kinetic analysis, and believe that intersection of the vertical axis could be attributable to a rapidly saturated binding component.

[*methyl*-^{14}C]4-MPαG was also taken up in a linear fashion over 60 min (Fig. 2B), but the rate declined beyond this and, as with sucrose, the extrapolation of the linear portion of this plot does not pass through the origin. Transport of [*methyl*-^{14}C]4-MPαG was also found to be sensitive to FCCP (5 μM) and PCMBS (2 mM) (Fig. 2B), both of which are potent inhibitors of active transport of sucrose in plants [14,28].

With respect to sucrose, isolated phloem of *A. graveolens* L. cv. 'Sutton's solid white' was found in this study to display a K_m and V_{max} of 6.9 ± 0.4 mM and 8.5 ± 1.0 μmol g^{-1} h^{-1}, respectively. Using the same

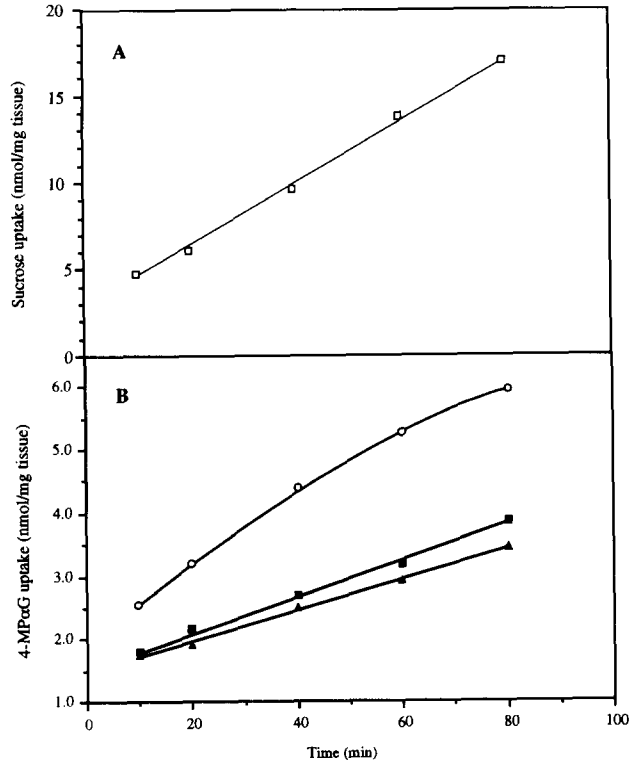

Fig. 2. Transport of sucrose (50 mM) (□), and of 4-MPαG (40 mM) (○), into isolated celery phloem. The effects of 5 μM FCCP (▲), and of 2 mM PCMBS (I), upon the uptake of 4-MPαG are also shown.

technique with tissue isolated from *A. graveolens* L. cv. 'Utah 5270', Daie [4] found K_m and V_{max} values of 5 mM and 15 μmol g^{-1} h^{-1}. We believe that the disparities between these and our values are sufficiently small to be attributable to varietal or environmental differences. Our values are similar to those for several intact tissues [29–31].

TABLE I

Inui and Christensen-type double labelling experiment to study the simultaneous uptake of [methyl-^{14}C]4-MPαG and [U-^{3}H]sucrose

The ratio of sucrose: 4-MPαG, in the incubating medium, was maintained at 1.0 for all concentrations. The ratios recovered from the phloem tissue were analysed using the *F*-test for sample homogeneity.

[Sucrose], [4-MPαG]/(mM):	40	10	5	2.5
^{3}H dpm/^{14}C dpm	8.86	8.18	8.31	8.59
	8.93	8.73	8.18	8.69
	8.34	7.08	8.51	7.98
	8.23	7.86	7.91	7.93
	7.85	7.61	8.63	7.79
$\bar{x}$ ($n = 5$)	8.44	7.89	8.31	8.20
s^2	0.164	0.305	0.064	0.136

$\bar{X}$ ($n = 20$) = 8.21
$S_w^2 = 0.209$
$F_{3,16} = \frac{S_b^2}{S_w^2} = 1.321$

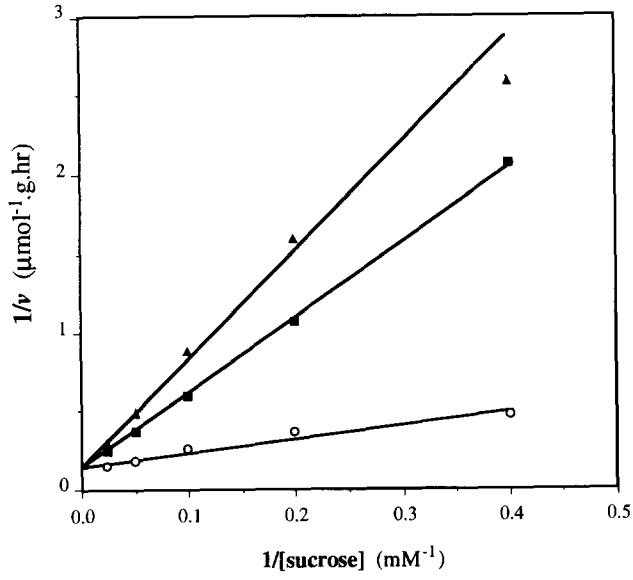

Fig. 3. Lineweaver-Burk plot of sucrose uptake in the presence of 4-HPαG (30 mM) (■) and of 4-MPαG (30 mM) (▲), and in the absence of inhibitor (○).

Uptake of sucrose (10 mM) in the presence of 4-HPβF (30 mM) was not significantly different from that of controls ($t_{(n=23)} = 1.309$, $P < 0.2$). However, 4-HPαG (30 mM) exhibited classical competitive inhibition of sucrose uptake with a K_i of 6.7 ± 0.1 mM (Fig. 3). This result contrasts with the reports of significant inhibition of sucrose uptake by another sucrose analogue that lacks the glucosyl moiety, methyl β-D-fructofuranoside [15], although the kinetic nature of this effect was not elucidated.

4-MPαG was also shown to display competitive inhibition of sucrose uptake, with a K_i of 6.0 ± 0.2 mM (Fig. 3). Interestingly, the increase in hydrophobicity of the aglycone by methylation of the *p*-hydroxyl is accompanied by a decrease in K_i. This is consistent with involvement of the aryl group in a hydrophobic interaction with the transport protein in a manner similar to that proposed for the binding of the fructosyl moiety of sucrose [17].

The uptake of [*methyl*-^{14}C]4-MPαG was shown to be competitively inhibited by sucrose (Fig. 4) with a K_i of 7.2 ± 0.8 mM. The K_m calculated for 4-MPαG was found to be 5.7 ± 0.2 mM, whilst V_{max} was estimated at 4.5 ± 0.3 μmol g^{-1} h^{-1}. The V_{max}/K_m for sucrose ($1.23 \cdot 10^{-3}$ dm^3 g^{-1} h^{-1}) is therefore over 1.5-times the value for 4-MPαG ($0.789 \cdot 10^{-3}$ dm^3 g^{-1} h^{-1}) reflecting that, while 4-MPαG binds more strongly to the carrier, it is transported less rapidly.

The results of a double isotope experiment that followed the simultaneous uptake of [*methyl*-^{14}C]-4-MPαG and [6,6′(n)-^{3}H]sucrose are detailed in Table I. Given that the transport of both species has been shown to meet the Michaelis-Menten equation (Fig. 3),

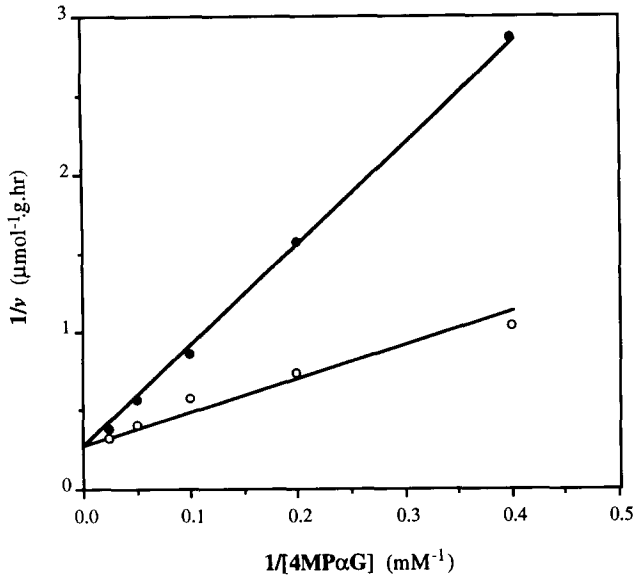

Fig. 4. Lineweaver-Burk plot of 4-MPα G uptake in the presence (•) and absence (○), of sucrose (30 mM).

if the transport site for these substances is identical and is the sole transport mechanism, then for all concentrations [A] and [B], where [A]/[B] is constant and where v_A and v_B represent the initial rates of uptake of A and B, respectively, v_A/v_B must be constant [21].

The ratios of sucrose to 4-MPα G recovered from the phloem tissue (Table I), following incubation, were analysed using the F-test for sample homogeneity. This investigates, by an analysis of the variances (s^2), the null hypothesis that the obtained ratios originate from the same population. Variances taken within each of the four samples, and between them, may be compared using the F ratio for the required degrees of freedom (V). In this case, with $n = 5$ within each concentration group and $k = 4$ for the number of concentrations, V_w (degrees of freedom for variances taken within the groups $k(n-1)) = 16$ and V_b (degrees of freedom for variances taken between the groups $(k-1)) = 3$. Hence the ratio $F_{3,16}$ is given as follows:

$$S_b^2 = \frac{n\sum(X-\bar{X})^2}{k-1} \qquad S_w^2 = \frac{\sum_k\sum_n(x-\bar{x})^2}{k(n-1)}$$

$$F_{3,16} = \frac{S_b^2}{S_w^2} = 1.321$$

For the null hypothesis to be accepted at the 5% confidence level, the statistic must be less than 3.24 (from tables). Our result of 1.321 is well within this range and therefore there is no significant difference between the ratios A/B. This is conclusive proof that both substrates are entering the phloem on the same carrier, and that there is only one type of transporter for sucrose in this tissue.

Furthermore, these results show that the stringent requirements of this experiment for initial rates, and Michaelis-Menten kinetics, must have been met.

4-MPα 2dG (20 mM) was shown to exhibit classical non-competitive inhibition of sucrose uptake (Fig. 5), altering the apparent V_{max} without a change in K_m ($K_i = 2.5 \pm 0.1$ mM). The low rate of uptake of [*methyl*-^{14}C]4-MPα 2dG, 1.4 μmol mg^{-1} h^{-1} at 10 mM, was not sensitive to either FCCP (5 μM) or PCMBS (2 mM) (Fig. 6), nor was uptake of even lower concentrations inhibited by sucrose (30 mM). This value is similar to the rates of uptake of 4-MPα G (40 mM) noted in the presence of PCMBS and FCCP: 1.7 and 1.5 μmol mg^{-1} h^{-1} respectively (Fig. 2B). Furthermore, the double isotope experiment, which followed the simultaneous uptake of [*methyl*-^{14}C]4-MPα 2dG and [6,6′(n)-^{3}H]sucrose, failed to show constant ratios over all concentrations, and consequently the $F_{3,16}$ statistic bears out this very significant difference between samples (Table II). Together, these observations suggest that this analogue does not bind to the carrier at the same site as sucrose and neither is it actively transported into the phloem.

It has been reported that a similar analogue, phenyl α-D-2-deoxythioglucopyranoside, displayed competitive inhibition of sucrose uptake into soybean cotyledon protoplasts, with a K_i similar to that of phenyl α-D-thioglucopyranoside [17]. Our results, in contrast, clearly demonstrate that the same substitution at C-2 of *p*-methoxyphenyl α-D-glucopyranoside profoundly

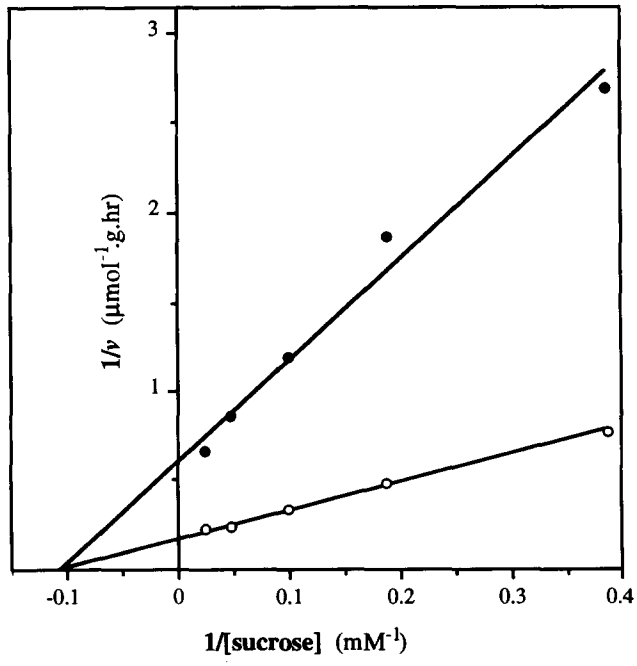

Fig. 5. Lineweaver-Burk plot of sucrose uptake in the presence of 4-MPα 2dG (20 mM) (•), and in the absence of inhibitor (○).

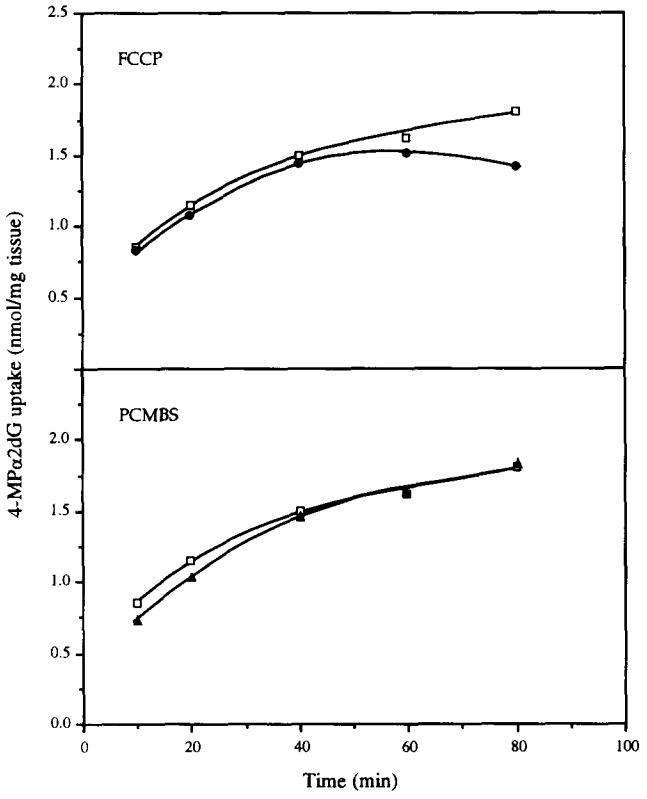

Fig. 6. Transport of 4-MPα2dG (10 mM) into isolated celery phloem in the presence of 5 μM FCCP (●), of 2 mM PCMBS (▲), and in the absence of inhibitor (□).

changes the interaction of the compound with the sucrose carrier of isolated phloem tissue.

It is noteworthy, however, that Hitz et al. [17] determined K_i values from the slope of a plot of $(V_o/V_1 - 1)$ versus concentration of the inhibitor, a method which does not allow discrimination between competitive and non-competitive inhibition. The term K_i appears in the following rearrangements of the Michaelis-Menten equation, where Eqn. 1 describes competitive inhibition and Eqn. 2 non-competitive inhibition:

$$\frac{1}{V_i} = \frac{1}{V_{max}} + \frac{K_m(1+[I]/K_i)}{V_{max}[S]} \quad (1)$$

$$\frac{1}{V_i} = \frac{(1+[I]/K_i)}{V_{max}} + \frac{K_m(1+[I]/K_i)}{V_{max}[S]} \quad (2)$$

Where V_o represents the rate of uptake of a substrate (S) in the absence of inhibitor, the slope of the plot $(V_o/V_i - 1)$ versus concentration of the inhibitor gives K_i provided that $[S] \ll K_m$, which ensures that the contribution of the $1/V_{max}$ term to the value of $1/V_i$ becomes negligible. Under these conditions it can be seen from Eqns. 1 and 2 that, irrespective of whether the inhibition is competitive or non-competitive,

$$\frac{1}{V_i} \text{ tends to } \frac{K_m(1+[I]/K_i)}{V_{max}[S]}$$

Thus, values of K_i can be determined with reasonable accuracy employing this method, but a caveat must be entered that it cannot distinguish between competitive and non-competitive inhibition. The effects of phenyl α-D-2-deoxythioglucopyranoside and a number of other compounds, on the uptake of sucrose into soybean cotyledon protoplasts seem to have been investigated solely by this approach, under which circumstances inhibition was assumed, rather than proven, to be competitive. We therefore believe it is possible that this assumption was incorrect and has led to an underestimation of the importance of the hydroxyl at position C-2 on the glucosyl moiety in the binding of sucrose to its carrier in plant plasma membranes.

Of the aryl glucosides investigated by Hitz et al. [17], only phenyl α-D-glucopyranoside and phenyl α-D-thioglucopyranoside were conclusively demonstrated to be competitive inhibitors of sucrose uptake into soybean cotyledon protoplasts. More recently, Dixon plots of the inhibition of sucrose uptake into isolated plasma membrane vesicles of *Beta vulgaris* by these and three other compounds (phenyl α-D-3-deoxy-3-fluoro-thioglucopyranoside, phenyl α-D-4-deoxythioglucopyranoside and phenyl α-D-4-deoxy-4-fluoro-thioglucopyranoside) revealed a pattern that may be consistent with competitive inhibition [8]. However, it should not be overlooked that, while the Dixon plot ($1/v$ vs. [I]) can discriminate between non-competitive and competitive inhibition, it cannot distinguish the latter from cases of mixed inhibition in which the K_i for the non-competitive component is larger than that of the

TABLE II

Inui and Christensen-type double labelling experiment to study the simultaneous uptake of [methyl-^{14}C]4-MPα2dG and [U-^{3}H]sucrose

The ratio of sucrose: 4-MPαG, in the incubating medium, was maintained at 1.0 for all concentrations. The ratios recovered from the phloem tissue were analysed using the *F*-test for sample homogeneity.

[Sucrose], [4-MPα2dG]/(mM):	20	10	5	2.5
^{3}H dpm/^{14}C dpm	23.53	51.88	81.59	110.5
	19.52	62.00	78.04	111.5
	11.89	60.63	91.92	116.3
	21.66	77.69	93.90	118.7
	11.74	81.35	95.85	93.63
$\bar{x}$ ($n = 5$)	17.67	66.71	88.26	112.3
s^2	24.45	122.8	50.35	24.77
$\bar{X}$ ($n = 20$) = 70.69				
$S_w^2 = 69.49$	$F_{3,16} = \frac{S_b^2}{S_w^2} = 112.5$			

competitive component [32]. Thus, the data described by Hecht et al. [8] provide evidence that the analogues of phenyl α-D-thioglucopyranosides, substituted at hydroxyl groups at C-3 and C-4, exhibit patterns of inhibition of sucrose uptake that retain at least a competitive component, but do not allow one to conclude that alterations in K_i, as a result of these substitutions, reflect changes in the affinity for the sucrose binding site of the carrier. The study of Hecht et al. did not report the inhibitory effects of such analogues substituted at C-6 and C-2, and our findings suggest that substitution at the latter position of the methoxyphenyl α-D-glucopyranoside prevents recognition by the sucrose carrier.

We believe that inhibitory effects of analogues bearing long hydrophobic aglycone groups are particularly vulnerable to misinterpretation. Their striking similarity to other amphipathic compounds that have been demonstrated to be non-ionic detergents suggests that they too may be capable of disrupting biological membranes [33,34]. The observed decrease in apparent K_i with increasing chain length of these analogues [17] may reflect increased affinity for the lipid bilayer rather than for the sucrose carrier. Until the effects of these compounds on sucrose transport have also been demonstrated to result from competitive inhibition it may be unwise to draw any firm conclusions regarding binding to the protein.

Our results are perhaps surprising in light of findings that provision of 2-deoxyglucose to mesophyll cells of *Zea mays* [35,36] and *Beta vulgaris* [36] resulted in the formation of 2-deoxysucrose, which was transported in the vascular system to regions remote from the point of synthesis. Loading of sucrose into the phloem is thought to occur by active transport from the apoplasm [1]. However, an absolute requirement for the glucosyl C-2 hydroxyl for recognition by the sucrose carrier would seem to preclude this route for the uptake of 2-deoxysucrose into phloem tissue. Furthermore, the ability of 2-deoxysucrose to increase efflux of [^{14}C]sucrose from leaf discs of *Vicia faba* was found to be only 17% that of sucrose, which could suggest that this analogue may well not be recognised by the carrier [37]. The possibility that the movement of 2-deoxysucrose formed in mesophyll cells may have followed a symplasmic route, independent of the sucrose transporter [38], cannot be ruled out. Indeed, Sammler et al. [35] stated their belief that this was the case. This anomaly could best be resolved by a thorough kinetic investigation of the interaction of 2-deoxysucrose with the sucrose carrier.

Conclusions

Sucrose up take into isolated celery phloem is mediated by a single carrier, the binding site of which also recognises aryl α-D-glucopyranosides. Although the pattern of interactions with the sucrose carrier displayed by the analogues described in this study are broadly concordant with the model for specificity proposed by Hitz et al. [17], a prominent departure from this is apparent. Substitution at the C-2 position of methoxyphenyl α-D-glucopyranoside to form 4-MPα2dG results in a loss of ability to bind to the recognition site of the carrier. We believe that the true effects of substitution at this position have not been previously recognised because the nature of the inhibition by the analogues could not be determined by the method employed.

Acknowledgements

SDJG was grateful for a research studentship from Dow Elanco Ltd., Letcombe. This work has also been supported by the University of Oxford. We are indebted to Sylvia Priest, Chris Dudley and the glasshouse staff of DowElanco for the provision of high quality celery plants. We are grateful to Dr. Keith Dalziel, FRS, for assistance with kinetic analysis, and to Dr. John Liebeschuetz (DowElanco) for his support and encouragement.

References

1 Giaquinta, R.T. (1983) Annu. Rev. Plant Physiol. 34, 347–387.
2 Komor, E. (1977) Planta 137, 119–131.
3 Petzold, U., Neumann, S. and Dahse, I. (1989) Biochem. Physiol. Pflanz. 185, 27–40.
4 Daie, J. (1987) Planta 171, 474–482.
5 Buckhout, T.J. (1989) Planta 178, 393–399.
6 Bush, D.R. (1989) Plant Physiol. 89, 1318–1323.
7 Lemoine, R. and Delrot, S. (1989) FEBS Lett. 249, 129–133.
8 Hecht, R., Slone, J.H., Buckhout, T.J., Hitz, W.D. and Van Der Woude, W.J. (1992) Plant Physiol. 99, 439–44
9 Warmbrodt, R.D., Van Der Woude, W.J., Buckhout, T.J. and Hitz, W.D. (1989) Planta 180, 105–115.
10 Williams, L.E., Nelson, S.J. and Hall, J.L. (1990) Planta 182, 540–545.
11 Williams, L.E., Nelson, S.J. and Hall, J.L. (1992) Planta 186, 541–550.
12 Delrot, S. and Bonnemain, J.L. (1981) Plant Physiol. 67, 560–564.
13 M'Batchi, B. and Delrot, S. (1988) Planta 174, 340–348.
14 Lin, W., Schmitt, M.R., Hitz, W.D. and Giaquinta, R.T. (1984) Plant Physiol. 75, 936–940 (I), 941–946 (II).
15 Schmitt, M.R., Hitz, W.D., Lin, W. and Giaquinta, R.T. (1984) Plant Physiol. 75, 941–946.
16 Hitz, W.D., Schmitt, M.R., Card, P.J. and Giaquinta, R.T. (1985) Plant Physiol. 77, 291–295.
17 Hitz, W.D., Card, P.J. and Ripp, K.G. (1986) J. Biol. Chem. 261, 11986–11991.
18 Kaiser, G. and Heber, U. (1984) Planta 161, 562–568.
19 Card, P.J. and Hitz, W.D. (1984) J. Am. Chem. Soc. 108, 5348–5350.
20 Card, P.J., Hitz, W.D. and Ripp, K.G. (1986) J. Am. Chem. Soc. 108, 158–161.
21 Inui, Y. and Christensen, H.N. (1966) J. Gen. Physiol. 50, 203–224.
22 Perrin, D.D. and Armarego, W.L.F. (1988) Purification of Laboratory Chemicals, 3rd. Edn., Pergamon, Oxford.

23 Nakamura, S. (1965) Sci. Rep. Tokyo Kyoiku Daigaku 12, 1–18.
24 Woodward, J. and Wiseman, A. (1978) Biochim. Biophys. Acta 527, 8–16.
25 Helferich, B. and Reischel, W. (1938) Ann. Chem. 533, 278–290.
26 Robertson, A. and Waters, R.B. (1930) J. Chem. Soc. 2, 2729.
27 Griffin, S.D.J. and Donaldson, I.A. (1993) J. Lab. Comp. Radiopharm. 33, 557–562.
28 Lichtner, F.T. and Spanswick, R.M. (1981) Plant Physiol. 67, 869–874.
29 Estruch, J.J., Pereto, J.G., Vercher, Y. and Beltran, J.P. (1989) Plant Physiol. 91, 259–265.
30 Grimm, E., Bernhardt, G., Rothe, K. and Jacob, F. (1990) Planta 182, 480–485.
31 Hampson, S.E., Loomis, R.S. and Rains, D.W.(1978) Plant Physiol. 62, 846–850.
32 Dixon, M. and Webb, E.C. (1979) Enzymes, 3rd. Edn., Longman, London.
33 Baron, C. and Thompson, T.E. (1975) Biochim. Biophys. Acta 382, 276–285.
34 Saito, S. and Tsuchiya, T. (1984) Biochem. J. 222, 829–832.
35 Sammler, P., Ehwald, R. and Göring, H. (1974) Biochem. Physiol. Pflanzen 165, 291–302.
36 Pavlinova, O., Göring, H., Turkina, M.V. and Ehwald, R. (1978) Fiziol. Rast. 25, 213–221.
37 Delrot, S., Roques, N., Descotes, G. and Mentech, J. (1991) Plant Physiol. Biochem. 29, 25–29.
38 Robards, A.W. and Lucas, W.J. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 369–419.