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Substrate Specificity of the H^+-Sucrose Symporter on the Plasma Membrane of Sugar Beets (*Beta vulgaris* L.)[1]

Transport of Phenylglucopyranosides

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ABSTRACT

Previous results (TJ Buckhout, Planta [1989] 178: 393–399) indicated that the structural specificity of the H^+-sucrose symporter on the plasma membrane from sugar beet leaves (*Beta vulgaris* L.) was specific for the sucrose molecule. To better understand the structural features of the sucrose molecule involved in its recognition by the symport carrier, the inhibitory activity of a variety of phenylhexopyranosides on sucrose uptake was tested. Three competitive inhibitors of sucrose uptake were found, phenyl-α-D-glucopyranoside, phenyl-α-D-thioglucopyranoside, and phenyl-α-D-4-deoxythioglucopyranoside (PDTGP; K_i = 67, 180, and 327 micromolar, respectively). The K_m for sucrose uptake was approximately 500 micromolar. Like sucrose, phenyl-α-D-thioglucopyranoside and to a lesser extent, PDTGP induced alkalization of the external medium, which indicated that these derivatives bound to and were transported by the sucrose symporter. Phenyl-α-D-3-deoxy-3-fluorothioglucopyranoside, phenyl-α-D-4-deoxy-4-fluorothioglucopyranoside, and phenyl-α-D-thioallopyranoside only weakly but competively inhibited sucrose uptake with K_i values ranging from 600 to 800 micromolar, and phenyl-α-D-thiomannopyranoside, phenyl-β-D-glucopyranoside, and phenylethyl-β-D-thiogalactopyranoside did not inhibit sucrose uptake. Thus, the hydroxyl groups of the fructose portion of sucrose were not involved in a specific interaction with the carrier protein because phenyl and thiophenyl derivatives of glucose inhibited sucrose uptake and, in the case of phenyl-α-D-thioglucopyranoside and PDTGP, were transported.

The active transport of sucrose into the phloem tissues of higher plants drives the export of photosynthate carbon and energy to nonphotosynthetic tissues. Numerous studies of sucrose uptake into intact tissues suggest that uptake is carrier-mediated and catalyzed by a H^+-sucrose symport (9). Confirmation of this mechanism of sucrose uptake has been obtained in PM[3] preparations from sugar beet leaves (2, 3, 13), *Ricinus* cotyledons (19), and spinach leaves (18). In these examples, sucrose uptake into PM vesicles is dependent on a pH gradient, alkaline inside, is electrogenic, and is specific for the sucrose molecule. It also has been demonstrated that sucrose uptake induces a transient alkalization of the exterior medium and that the characteristics of sucrose-induced alkalization and pH-dependent sucrose uptake are identical (4, 17). The stoichiometry of sucrose to H^+ transport is 1:1 (4, 17). At this time, however, equating the mechanism of sucrose uptake in isolated vesicles with that of phloem loading *in vivo* is not possible.

The chemical features on the sucrose molecule that are involved in the specific interaction of sucrose with the protein carrier are critical to the understanding of the molecular mechanism of sucrose transport. Phloem loading in sugar beets (8) and maize (10) was shown to be specific for the sucrose molecule. Sucrose uptake by *Ricinus* cotyledons (12) and celery petioles (5) or efflux in broad bean leaf discs (6, 15) was not greatly affected by common mono- and disaccharides. In protoplasts isolated from soybean cotyledons, uptake of sucrose was inhibited by a 100-fold excess of maltose, inulobiose and methyl-β-D-fructofuranoside (16). Maltose also inhibited sucrose uptake into sugar beet leaf discs (14) but did not alter sucrose uptake into PM vesicles isolated from sugar beet leaves (2, 3), although in this later study the maltose concentration was only 10-fold greater than sucrose.

By far the most extensive study of the chemical specificity of the sucrose transport system was conducted with soybean protoplasts (11). The findings of that study indicate that a hydrophobic interaction between sucrose and the carrier and the hydroxyl groups at positions C3, C4, and C6 of the glucose residue are involved in substrate recognition by the symport carrier. In this study, we investigated the structural features of the sucrose molecule that confer transport specificity to the H^+-sucrose symport on the sugar beet PM.

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[3] Abbreviations: PM, plasma membrane(s); ΔpH, pH gradient; PDTGP, phenyl-α-D-4-deoxythioglucopyranoside; PTGP, phenyl-α-D-thioglucopyranoside; PTMP, phenyl-α-D-thiomannopyranoside; K_i, inhibitor constant.

MATERIALS AND METHODS

Plant Material and Isolation of PM

Sugar beets (*Beta vulgaris* L. cv Mono HYE 4[4] [Michigan Sugar Co., Saginaw, MI] and cv Tina EE [Kleinwanzlebener Saatzucht, Göttingen, FRG]) were grown in growth chambers as described by Buckhout (2) or in hydroponic culture as described by Bush (3). Fully expanded, basal leaves from 56- to 168-d-old plants were harvested 1 to 3 h after the beginning of the light period, and PM vesicles were isolated by aqueous two-phase partitioning (2) from 180 g fresh weight of sugar beet leaves with the midrib removed. Fifty-two-gram phase systems were used. For experiments involving sucrose- or analog-induced H^+ movement, the PM isolation procedure was modified as described by Slone and Buckhout (17). Briefly, K_2SO_4 (0.5 or 2 mM) was substituted for 1 mM KCl in the homogenization medium, and the PM vesicles were washed in an unbuffered medium containing 350 mM sorbitol and 0.5 or 2.0 mM K_2SO_4 (medium A) or medium A buffered with 2 mM Hepes-1,3-bis[tris(hydroxymethyl)methylamino]-propane, pH 7.8 (medium B). Medium A was used for preparation of vesicles in proton uptake experiments and medium B in acetic acid uptake assays (see below). The vesicles were resuspended in their respective wash media at a protein concentration of 15 to 20 $mg \cdot mL^{-1}$, and 105- to 130-μL aliquots were frozen at −80°C. Protein concentration was determined by the method of Bradford (1).

Assay for ΔpH-Dependent Sucrose Uptake

Sucrose uptake was determined at 15°C by the filtration method as described by Buckhout (2). ΔpH-dependent uptake was defined as the difference in sucrose uptake in vesicles diluted in pH 6.0 buffer in the absence and presence of carbonyl cyanide *m*-chlorophenylhydrazone at a final concentration of 5 μM. The sucrose incorporation was determined at 0.5, 1, 2, and 4 min, and net sucrose influx was determined from these data by linear regression analysis.

Analysis of K_i

The effects of hexose analogs on sucrose uptake were tested as follows. Analogs were dissolved in water or when necessary in 50% methanol to a final concentration of 0.1 M. Control analyses for each analog were conducted with the appropriate solvent. Typically, four inhibitor concentrations were used (0, 250, 500, and 750 μM), and the inhibition was tested against three sucrose concentrations (250, 500, and 750 μM). In some cases (PTGP and PTMP), the available quantity of inhibitor did not allow for a full analysis as described above. In these cases, the analysis at 750 μM inhibitor was omitted. Data were plotted by the method of Dixon and Webb (7), and inhibitor constants were determined graphically from the intersection of the lines. In all cases, lines were drawn using linear regression analysis and the intersections calculated mathematically.

[4] The mention of vendor or product does not imply that they are endorsed or recommended by U.S. Department of Agriculture over vendors of similar products not mentioned.

Assay of Analog- and Sucrose-Induced Proton Uptake

Working stock solutions (0.1–0.2 M) of the phenylhexopyranosides were prepared in 20 to 50% methanol and stored at −20°C. PTMP preparations contained trace amounts of an alkaline contaminant; therefore, before its use in proton uptake assays, the pH of the PTMP stock solution was adjusted by titration with Dowex-50 cation exchange beads to a pH value close to the stable value of the complete reaction medium (see below). PTGP and PDTGP solutions were slightly acidic; however, no correction of the pH of the solution was necessary.

Methods used for the analysis of analog- and sucrose-induced proton flux were those of Slone and Buckhout (17). PM vesicles (100–120 μL approximately 1 mg protein) prepared in medium A were diluted 11- to 12-fold in medium A supplemented with valinomycin and lacking K_2SO_4. Stock solutions (13 μL) of phenylhexopyranosides or sucrose dissolved in 20 to 50% methanol were added to the reaction after the pH stabilized, typically between 6.6 and 6.8. The pH

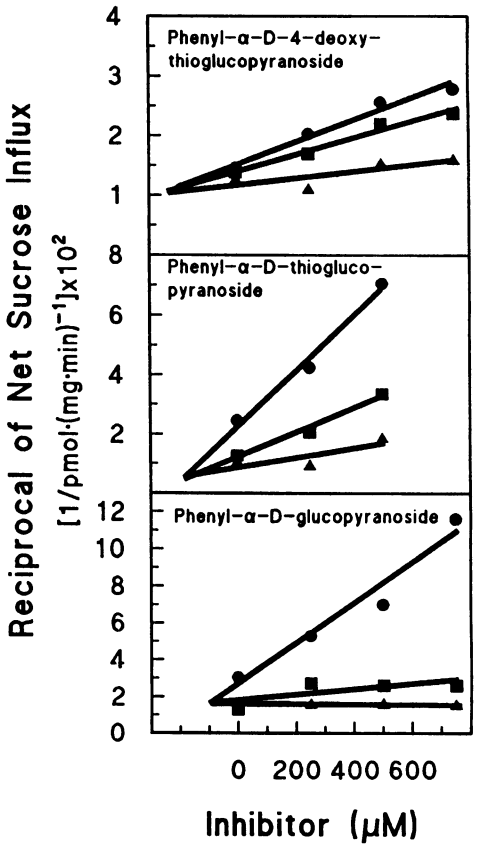

Figure 1. Dixon plot analysis of the inhibition of sucrose uptake by the phenylhexopyranosides, phenyl-α-D-glucopyranoside, PTGP, and PDTGP. Net sucrose influx was determined at 250 (●), 500 (■), and 750 (▲) μM in the presence of 0, 250, 500, and 750 μM inhibitor as described in "Materials and Methods." K_i values of 67, 180, and 327 μM were determined for phenyl-α-D-glucopyranoside, PTGP, and PDTGP, respectively. The K_m for net sucrose influx was approximately 500 μM. Values are the average of three determinations from three separate experiments.

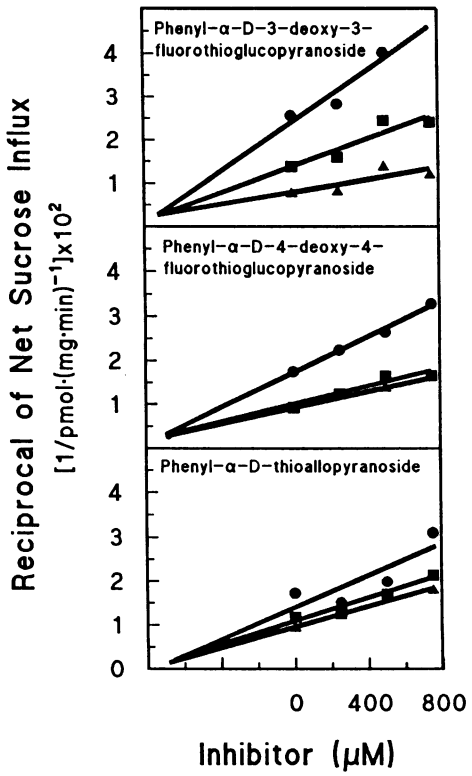

Figure 2. Dixon plot analysis of the inhibition of net sucrose influx by the phenyl-α-D-3-deoxy-3-fluorothioglucopyranoside, phenyl-α-D-4-deoxy-4-fluorothioglucopyranoside, and phenyl-α-D-thioallopyranoside. K_i values of 766, 626, and 857 μM were determined, respectively. Further details of the experimental protocol are given in the legend to Figure 1 and in "Materials and Methods." Values are the average of three determinations from three separate experiments.

of the reaction was measured with a combination, semimicro-pH electrode (Accu-pHast; Fisher Scientific, Silver Spring, MD) and an Orion 701A pH meter (Fisher Scientific) and was recorded continuously with an analog chart recorder. Calibration of pH changes was performed at the end of each experiment by addition of known quantities of HCl or NaOH to the reaction mixture. The final volume of the reaction mix after the first addition of phenylhexopyranosides or sucrose was 1.3 mL. Further details are described in the legend to Figure 6.

Assay for Acetic Acid Uptake

Uptake of [^{14}C]acetic acid was conducted essentially as described before (2, 3), except that nigericin was added to one of a pair of samples to determine bound acetate. Nitrocellulose filters (0.45 μm) were used to collect the vesicles. The assay contained 30 μL PM vesicles (approximately 0.45 mg protein) and 570 μL buffer (350 mM sorbitol, 2 mM Hepes-1,3-bis[tris(hydroxymethyl)methylamino]propane adjusted to pH 6.0 with Mes and 5.5 μM acetic acid, 0.8 μCi). Valinomycin (0.5 μM) was also included to prevent trapping of acetic acid (3) and to maintain conditions similar to the H^+ uptake assay.

Materials

Unless otherwise stated, the sources for chemicals was as previously stated (2, 17). Hexose derivatives were obtained commercially or synthesized. The identity of the synthesized compounds has previously been confirmed (ref. 11 and references therein).

RESULTS

Determination of K_i

After a number of compounds were analyzed for their ability to inhibit sucrose uptake, nine compounds were further analyzed for the determination of inhibitor constants and the mechanism of inhibition. The results of these analyses could be grouped into three general categories. The first group, which consisted of PTGP (Fig. 1; K_i = 180 μM), phenyl-α-glucopyranoside (Fig. 1; K_i = 67 μM), and PDTGP (Fig. 1; K_i = 327 μM) inhibited sucrose uptake competitively with a K_i less than the K_m for sucrose, which was approximately 500 μM. A second group consisting of phenyl-α-D-3-deoxy-3-fluorothioglucopyranoside (Fig. 2), phenyl-α-D-4-deoxy-4-fluorothioglucopyranoside (Fig. 2), and phenyl-α-D-thioallopyranoside (Fig. 2) competitively inhibited sucrose uptake with K_i values between 600 and 800 μM. A final group of compounds had no effect on sucrose accumulation (Fig. 3). A summary of the chemical structures and the corresponding inhibition constants, where appropriate, are presented in Figure 4.

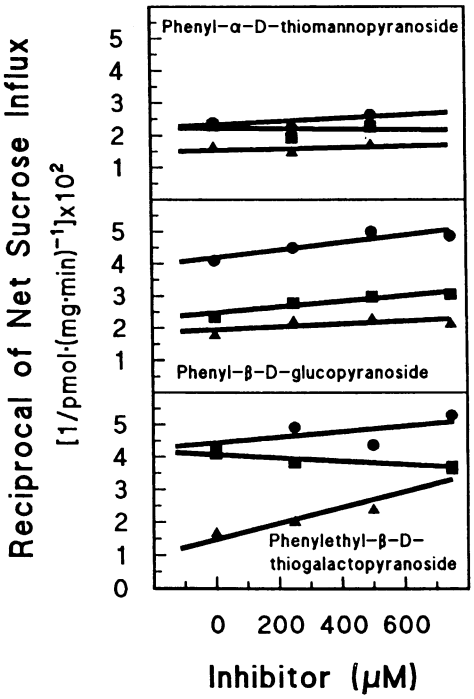

Figure 3. Dixon-plot analysis of the inhibition of net sucrose influx by the PTMP, phenyl-β-D-glucopyranoside, and phenylethyl-β-D-thiogalactopyranoside. K_i values for these compounds could not be determined. Further details of the experimental protocol are given in the legend to Figure 1 and "Materials and Methods." Values are the average of three determinations from three separate experiments.

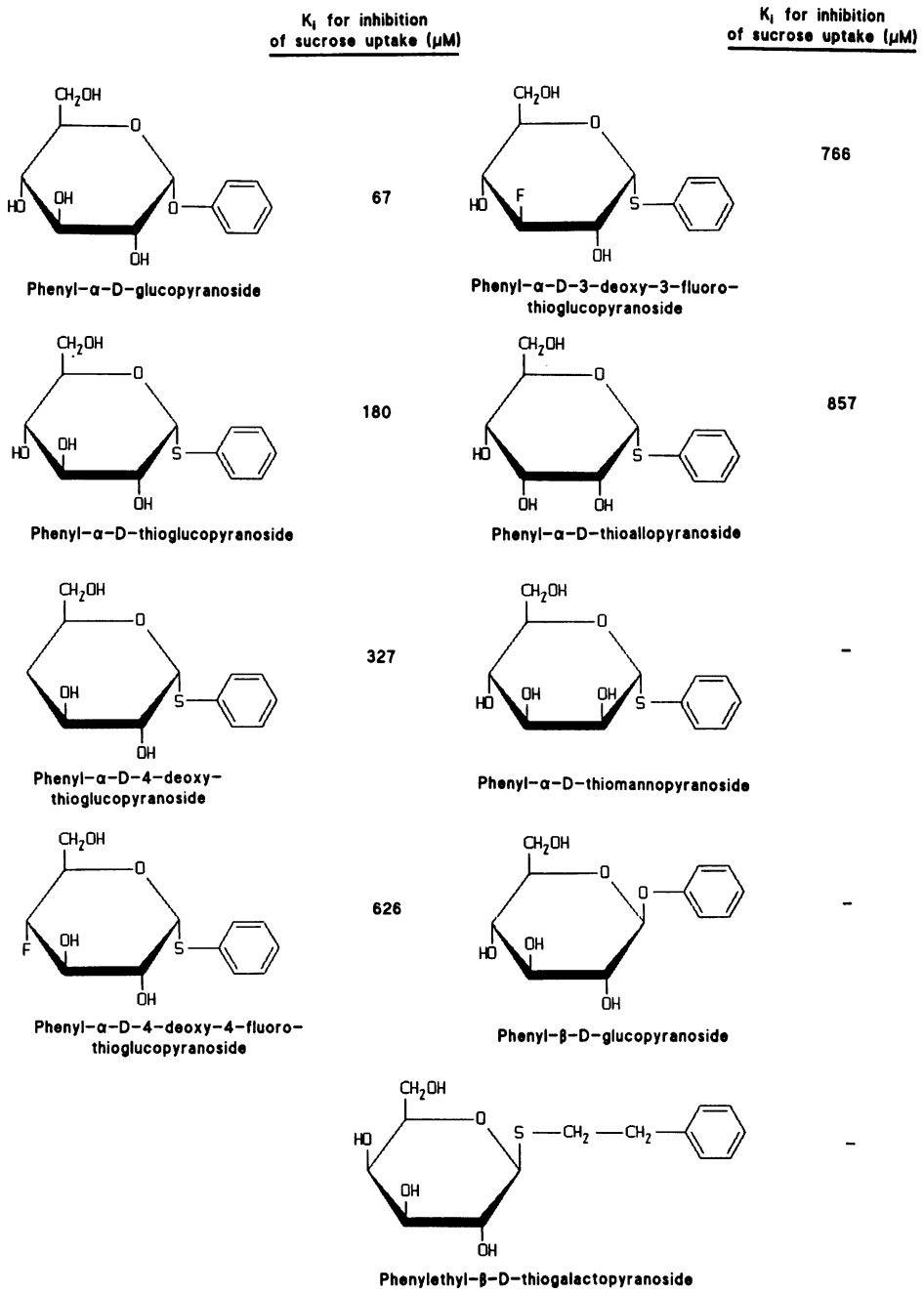

Figure 4. Summary of chemical structures and K_i values for the phenylhexopyranoside analogs tested. K_i values were calculated from Dixon plots presented in Figures 1 to 3. –, No inhibition was detected.

To exclude the possibility that inhibition by phenylhexopyranosides was due to a breakdown in the pH gradient, the effect of these compounds on acetate uptake was tested. Neither PTGP, PDTGP, nor PTMP (Fig. 5) or phenyl-α-glucopyranoside (data not shown) influenced the accumulation of acetate within the vesicles, which indicates that the inhibition of sucrose uptake was not due to dissipation of the ΔpH gradient. The fact that the inhibition was competitive also supported this conclusion. Thus, the inhibitory effect of these compounds on sucrose uptake was most likely due to their reversible binding to the sucrose-binding site on the symport carrier.

Analysis of Analog- and Sucrose-Induced H^+ Uptake

To confirm that the inhibition by the phenylglucopyranoside derivatives was due to binding at the sucrose-binding site, the ability of these compounds to induce an alkalization of the extravesicular medium was tested. Such an alkalization would be consistent with the operation of a H^+ symport. For technical reasons, only a small number of these compounds

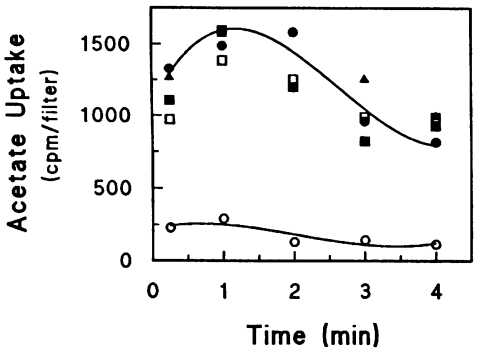

Figure 5. Analysis of acetate uptake by PM vesicles in the presence or absence of various phenylhexopyranosides. PM vesicles, washed in a medium buffered at pH 7.8, were diluted in pH 6.0 or 7.8 buffer containing radiolabeled acetic acid with or without test compounds (1 mM) and nigericin. At the indicated times, a portion of the vesicles was collected on nitrocellulose filters and acetate uptake determined. See "Materials and Methods" for further details. O, Control; ■, plus PTGP; □, PTMP; ▲, PDTGP, all without nigericin. For clarity, acetate uptake into vesicles diluted in buffer containing nigericin is shown only for control vesicles (O). Results obtained for test compound were statistically similar. Differences between samples with and without nigericin represent the amount of acetic acid taken up into the vesicles, which is a measure of pH gradient size.

could be tested in the H^+ uptake assay. PTGP at 1 mM was slightly more effective than sucrose in inducing alkalization of the extravesicular medium (Fig. 6, *cf.* A, B, and C). PDTGP was less effective than sucrose or PTGP (Fig. 6D), and PTMP did not induce alkalization (Fig. 6A). Because of the slight acidification caused by trace contaminants in the PTGP solution (see "Materials and Methods"), the rate of alkalization induced by PTGP was slightly underestimated, and the difference between sucrose- and PTGP-induced alkalization was greater than observed. Sucrose- and PTGP-induced alkalization was inhibited by carbonyl cyanide *m*-chlorophenylhydrazone (*cf.* E and F), which indicates that transport caused movement of H^+.

These results indicate that a H^+-phenylglucopyranoside symport exists on the PM. The inhibitory response following alternate and consecutive additions of PTGP and sucrose was consistent with transport of these substrates by the same transport protein, assuming that the H^+-sucrose symporter was reversible and that PTGP and sucrose did not mutually affect transport by negative allosteric effects. The fact that PTGP was also transported into soybean protoplasts (11) supports this conclusion.

DISCUSSION

A comparison of the chemical structures of the phenylhexopyranosides with their ability to inhibit sucrose uptake allows a number of conclusions with regard to chemical structures required for recognition by the sucrose symporter. Most strikingly, the data indicate that the fructosyl hydroxyl residues of the sucrose molecule participate in no specific interaction with the carrier protein. This is illustrated by the fact that PTGP and phenyl-α-glucopyranoside effectively inhibit su-

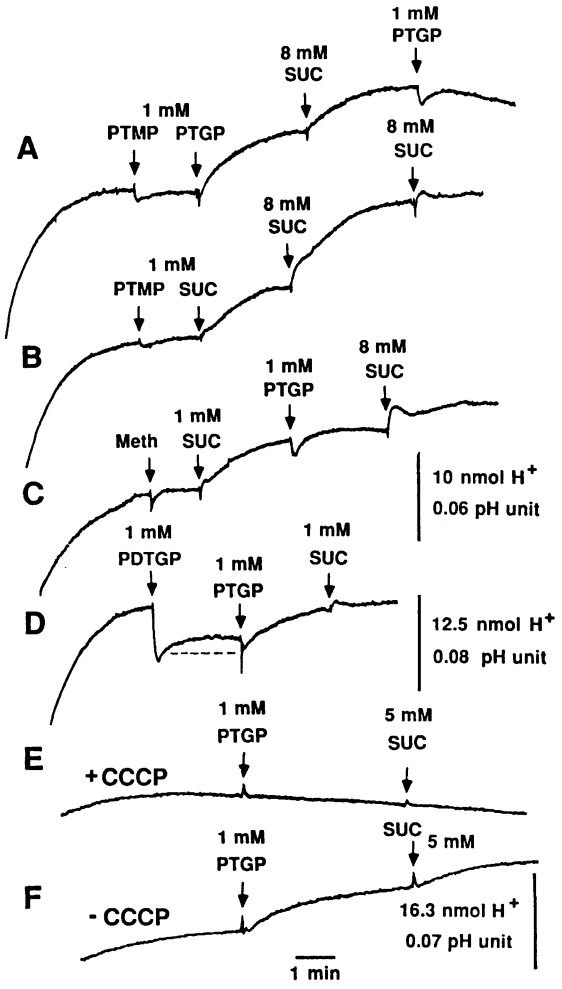

Figure 6. Phenylhexopyranoside- and sucrose-induced alkalization in PM isolated from sugar beet leaves. PM vesicles, washed in medium A (350 mM sorbitol, 4 mM K^+), were diluted into the same medium without K^+ but containing 0.5 μM valinomycin. The pH of the reaction mixture was recorded continuously. Sucrose (SUC), PTGP, PTMP, and PDTGP were added as indicated at the arrows. Addition of methanol (Meth), the solvent, is also shown (C). Final PM protein concentration: A to D, 1.3 mg·mL^{-1}; E and F, 1.6 mg·mL^{-1}. The three sets of traces, A, B, and C; E and F; and D are representative data from three separate experiments using three different batches of PM. The magnitude of the pH change and the buffering capacity of each experiment are indicated by vertical bars. The starting pH was approximately 6.8 for A to D and 6.6 for E and F. Note that under these conditions, valinomycin establishes a membrane potential (inside negative) for the first several minutes (our unpublished observations and ref. 17) and then participates with K^+ in providing charge compensation. A pH gradient is imposed across the vesicle membrane, estimated at pH 7.4 to 7.8 inside and pH 6.8 or 6.6 outside. See "Materials and Methods" for further details. CCCP, Carbonyl cyanide *m*-chlorophenylhydrazone.

crose uptake and that PTGP is transported by the sucrose symporter, although the phenyl ring contains no free hydroxyl groups. Hitz *et al.* (11), in their study of the structural requirement for sucrose uptake into soybean protoplasts, first noted that a large aspect of the interaction of the sucrose molecule with the protein carrier occurs through a hydrophobic interaction between hydrophobic region of the fructose portion of the molecule with no specific interactions of the fructosyl hydroxyl groups with the carrier. As in soybean (11), this hydrophobic surface can be provided by a phenyl ring. Interestingly, these phenyl-substituted compounds had in some cases a greater affinity for the carrier than the natural substrate, sucrose.

In contrast, all alterations of the pyranose portion of the phenylhexopyranosides resulted in a reduction or a complete loss in the inhibitory activity. Not surprisingly, changing the conformation from α to β at the C1 of glucose transformed the molecule from an active inhibitor of the sucrose symporter (phenyl-α-D-glucopyranoside) to an inactive compound (phenyl-β-D-glucopyranoside). Similarly, reversing the orientation or substitution of the hydroxyl group at position C2, C3, or C4 greatly decreased the inhibitory activity of the resulting compound. These results indicate that the hydroxyl groups of glucose are required for maintenance of a specific conformation of sucrose and/or for the interaction of sucrose with the sucrose symport carrier.

These conclusions agree for the most part with those of Hitz *et al.* (11), but some differences are noted. First, in soybean protoplasts, thioglucopyranoside derivatives were more active inhibitors of sucrose uptake than glucopyranoside derivatives, whereas the sugar beet sucrose carrier was inhibited approximately equally by phenylthio- and phenylglucopyranosides. Second, although removal of the hydroxyl at C4 of glucose (PDTGP) decreased the inhibitory activity compared with the parent compound (PTGP) in both soybean and sugar beet, substitution of the C4 hydroxyl with a fluoro group restored some of the original activity in soybean but resulted in a further decrease in inhibitory activity in sugar beet. Because both hydroxyl and fluoro substitutions can undergo hydrogen bonding, it appears that the fluoro substitution prevents, possibly based on the somewhat greater hydrophobicity of a fluoro compared with a hydroxyl group, optimal interaction with the sucrose carrier. Thus, the structural features of the sucrose molecule required for transport are similar in both soybean and sugar beet, but some specifics of the interaction of the sucrose molecule with the protein carrier appear to differ.

In summary, although phenyl-α-D-gluco- and thioglucopyranosides are chemically different from sucrose, their three-dimensional structures are such that they bind to and are transported by the PM H^+-sucrose symporter. Determination of specific substrate interactions will help define amino acids involved in sucrose binding on the PM sucrose transport proteins.

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