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Transport of sugars across the plasma membrane of beetroot protoplasts

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Abstract. Protoplasts isolated from beetroot tissue took up glucose preferentially whereas sucrose was transported more slowly. The ^{14}C-label from [^{14}C]glucose and [^{14}C]sucrose taken up by the cells could be detected rapidly in phosphate esters and, after feeding of [^{14}C]glucose was found also in sucrose. The temperature-dependent uptake process (activation energy E_A about 50 kJ·mol^{-1}) seems to be carrier mediated as indicated by its substrate saturation and, for glucose, by competition experiments which revealed positions C_1, C_5 and C_6 of the D-glucose molecule as important for effective uptake. The apparent K_m(20° C) for glucose (3-O-methylglucose) was about 1 mM whereas for sucrose a significantly lower apparent affinity was determined ($K_m$about 10 mM). When higher concentrations of glucose (5 mM) or sucrose (20 mM) were administered, the uptake process followed first-order kinetics. Carrier-mediated transport was inhibited by N,N'-dicyclohexylcarbodiimide, Na-orthovanadate, *p*-chloromercuribenzenesulfonic acid, and by uncouplers and ionophores. The uptake system exhibited a distinct pH optimum at pH 5.0. The results indicate that generation of a proton gradient is a prerequisite for sugar uptake across the plasma membrane. Protoplasts from the bundle regions in the hypocotyl take up glucose at higher rates than those derived from bundle-free regions. The results favour the idea that apoplastic transport of assimilates en route of unloading might be restricted to distinct areas within the storage organ (i.e. the bundle region) whereas distribution in the storage parenchyma is symplastic.

Key words: Assimilate allocation – *Beta* (sugar uptake) – Protoplast (sugar uptake) – Sugar uptake.

Introduction

In many higher plants, sucrose is not only the main translocate but can also accumulate in storage organs like sugarcane or sugar beets. For the process of loading in "source" leaves, a transfer of sucrose from the photosynthesizing cells towards the sieve tubes across the apoplast is most likely (Giaquinta 1983). However, the existence of a symplastic pathway cannot be excluded (Richter 1985) especially since Schmalstig and Geiger (1985) and Kaiser and Martinoia (1985) have indicated that unloading of the phloem in developing leaves ("sink" leaves) could be restricted to the symplast. Conclusive evidence for both hypotheses is still lacking even taking into consideration the fact that at present the regulation of solute transfer can be understood best by an interaction with membrane transport.

Several approaches have been used to study the regulation of sugar uptake in the storage tap-root of *Beta vulgaris*. Uptake experiments with tissue slices from the tap-root were the main basis for the "apoplast-pathway" hypothesis. Prior to final storage in the vacuole, sucrose has to cross at least two membranes (Kursanov 1974). Under this presupposition, lack of tracer randomization in experiments with position-labelled sucrose was taken as evidence that invertase does not exist in the apoplast (Giaquinta 1977; Wyse 1979). This conclusion is not unequivocal because the data

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Abbreviations: CCCP = Carbonylcyanide *m*-chlorophenylhydrazone; DCCD = N,N'-dicyclohexylcarbodiimide; DOG = deoxyglucose; Mes = 2-(N-morpholino)ethanesulfonic acid; 3-OMG = 3-O-methylglucose; PCMBS = *p*-chloromercuribenzenesulfonic acid; SDS = Sodium dodecyl sulfate; Tris = 2-amino-2-(hydroxymethyl)-1,3-propanediol

could also account for phloem unloading by a symplastic pathway, which interpretation in turn is supported by the (preliminary) results of Mierzwa and Evert (1984) about plasmodesmatal frequency in sugar beets. Wyse (1985), on the other hand, failed to detect any plasmodesmatal connections between the sieve element-companion cell complex of the phloem and that of the storage-parenchyma cells. According to this author, since only 70% of the asymmetrically labelled sucrose arrived chemically unaltered in the storage cells, up to 30% must have been hydrolysed prior to storage. Saftner et al. (1983) described a carrier system for sucrose uptake (K_m3.6 mM) whereas Wyse had reported a nonsaturable sucrose concentration-uptake relationship only, probably as a consequence of secondary effects like plasmoptysis in tissue discs which had been "equilibrated" in distilled water (Zamski and Wyse 1984).

Considering this confusing situation a re-examination of the sugar-uptake mechanism into beet storage tissue was highly desirable. Using isolated vacuoles of beetroot and sugar beet it has already been shown in this laboratory that sucrose uptake at the tonoplast obeys partially a saturation isotherm with optimal activity at pH 7.0 and in the presence of MgATP (Doll et al. 1979; Willenbrink et al. 1984). Therefore, we have tried to characterize sugar transport at the plasmalemma of beetroot cells using isolated protoplasts derived from different regions of the beetroot tissue.

The authors are well aware of the fact that isolated protoplasts are in many respects an artificial test system (Galun 1981), thus posing certain limitations upon the data obtained by this technique. On the other hand, cell-wall resistances and the complex nature of this tissue must also be considered as an obstacle for the interpretation of uptake data. In order to avoid misinterpretations, data from protoplast studies were compared with those from similar experiments on tissue discs.

Materials and methods

Plant material and chemicals. Red beets (*Beta vulgaris* L., uniform seed type "Rote Kugel, new globe or little ball"; van Waveren Pflanzenzucht, Göttingen, FRG) were cultivated in the experimental fields of the Max Planck Institut für Züchtungsforschung, Köln-Vogelsang, or in the greenhouse of the Botanical institute (under supplementary artificial light, HQL lamps, 400 W). Sorbitol, sodium dodecyl sulfate (SDS), sucrose and xylose were purchased from Merck (Darmstadt, FRG), spermidine and trypsin inhibitor from Serva (Heidelberg, FRG), and phenylmethyl silicone oil Wacker AP 100 from Kahmann and Ellerbrock (Bielefeld, FRG). Carbonylcyanide *m*-chlorophenylhydrazone (CCCP), N,N'-dicyclohexylcarbodiimide (DCCD), nigericin, valinomycin, phlorizin and *p*-chloromercuribenzenesulfonic acid (PCMBS) were obtained from Sigma (München, FRG). Radiochemicals were provided by Amersham Buchler (Braunschweig, FRG; [U-^{14}C]glucose, 9.99 GBq·mmol^{-1}; [U-^{14}C]3-O-methylglucose, 10.9 GBq·mmol^{-1}; [U-^{14}C]sucrose, 20.42 GBq·mmol^{-1}) and by NEN (Dreieichenhain, FRG; dextrancarboxyl [carboxyl-^{14}C], 3.1 MBq·mmol^{-1}). Specific activity was changed for experiments as indicated by appropriate dilution with nonlabelled substances. All other chemicals used were standard commercial products of analytical grade.

Isolation of protoplasts. Protoplasts were isolated from the hypocotyl tissue as previously described (Willenbrink et al. 1984) using the following enzymes: Pectolyase (Seishin Pharmaceutical, Tokyo, Japan), Rohament CT (Röhm and Haas, Darmstadt, FRG), Macerozyme (Welding, Hamburg, FRG). In the two-step release procedure the originally used slight infiltration of the enzyme medium I was omitted. Rohament CT and Macerozyme have been used in enzyme medium II (second step). Protoplasts were collected after 16 h of incubation at about 22° C by gently shaking the suspension, filtering through a nylon (80 µm mesh), and sedimenting in a centrifuge (Minifuge 2, Heraeus Christ, Hanau, FRG) at 50–70·*g* (15 min). The sediment was cautiously resuspended twice in 0.8 M sorbitol + 20 mM 2-(N-morpholino)ethanesulfonic acid (Mes)-2amino-2-(hydroxymethyl)-1,3-propanediol (Tris) (or Mes-NaOH, depending on the subsequent experiments) + 5 mM spermidine + 1 mM dithioerythritol (DTE) + 0.1% trypsin inhibitor (corresponds to 55 trypsin inhibitor units per litre) + 0.1% bovine serum albumin (BSA) + 5% White's medium (v/v; Nickell and Maretzki 1969), and settled under gravity. Finally, the sedimented protoplasts were resuspended twice in 0.8 M sorbitol ± buffer (type, concentration, and pH according to the experiment). A 1-ml aliquot of the final suspension contained 10^6 protoplasts throughout, and the average yield was 50% as calculated from the betanin ratio.

Microscopy. The number of protoplasts was counted in a haemocytometer (Neubauer, Walter Graf, Wertheim, FRG). Viability was tested using Evans blue (Gaff and Okong'O-ogala 1971), fluorescein diacetate (Widholm 1972), and by monitoring cyclosis (Nomarski interference contrast optics, Kamiya 1959). Efficiency of enzyme digestion was controlled with calcofluor white ST (Nagata and Takebe 1970).

Uptake experiments. Uptake of various ^{14}C-labelled sugars was measured as described by Getz (1985). Briefly, the uptake period was initiated by the addition of the [^{14}C]sugar to the protoplasts suspended in 0.8 M sorbitol ± buffer as indicated. Samples (usually three 0.1-ml portions) were withdrawn at intervals. Protoplasts were separated from the medium by 2–3 min centrifugation through silicone oil (AP 100) in a table-top centrifuge (Microfuge 11; Beckman Instruments, München, FRG). Radioactivity was determined by liquid scintillation spectrometry (Supersolve from Zinsser, Frankfurt, FRG; LS 7500 from Beckman) in an aliquot of the pellet which was redissolved in 1.3% (w/v) aqueous SDS solution. Uptake rates were linear for at least 40 min and therefore they were determined by linear regression analysis of their slopes. Membrane integrity was monitored in parallel experiments using [^{14}C]dextran (usually 37 kBq·ml^{-1} suspension medium); when radioactivity in the pellet increased with time of [^{14}C]dextran incubation, the plasma membrane was considered leaky, and the protoplast isolate was discarded. Competition experiments were carried out according to Heldt and Rapley (1970) as described by Kaiser and Heber (1984). For inhibitor studies, control sets were ad-

ministered with the same amount of solvent as the corresponding inhibitor set (normally 0.1% ethanol, v/v). Unless stated otherwise, protoplasts were incubated with either the inhibitor or the solvent for 10 min prior to the addition of the ^{14}C-sugar.

Further assays. Aliquots of the samples dissolved in 1.3% SDS were used to assay the betanin content by the method of Leigh and Branton (1976), and protein by the micromethod of Neuhoff et al. (1979). Total glucose, fructose and sucrose content was estimated enzymatically (Combitest; Boehringer, Mannheim, FRG); osmotic pressure was measured with a semimicro-osmometer (type M; Knauer, Berlin, West Germany). Oxygen consumption of protoplast suspensions was assayed with an oxygen probe (type E 012; WTW, Weilheim, FRG). Products of sugar uptake in the protoplasts were separated by paper chromatography (Pharr et al. 1977). Chromatograms were scanned with a windowless methane-flow counter (type LB 2722; Berthold/Frieseke, Wildbad, FRG) and distribution of radioactivity was estimated planimetrically (MOP AM 02; Kontron, München, FRG). For control experiments with tissue discs, the method of Grant and Beevers (1964) was adapted to our experimental conditions. Preparation of tissue discs and experiments were carried out under sterile conditions.

Results

General observations. Isolated protoplasts (Fig. 1) responded to changes in osmotic pressure in the medium. They excluded widely Evan's blue (90%), exhibited esterase activity nearly independently of medium-pH (in the range of pH 4 to 9; data not shown) and showed cytoplasmic streaming. The respiratory activity of the isolated protoplasts was about 6 $\mu mol \cdot g^{-1}FW \cdot h^{-1}$ (Table 1). Generally, less than 5% of freshly isolated protoplasts were "contaminated" with cellulose remainders, as indicated by calcofluor-white-ST staining. Some general properties of mature beetroot tissue are listed in Table 1. The osmotic pressure of the tissue sap compares well with data from the literature (Tomos et al. 1984). The average density of beetroot protoplasts was determined in order to facilitate the selection of the appropriate silicone oil. Betanin content of isolated protoplasts and tissue discs was measured routinely for estimation of protoplast yield. Knowledge of protein content of isolated protoplasts and of the cell number per unit betanin allows for a tentative comparison of our uptake data for beetroot with those for other plant tissues. The large standard deviations in Table 1 indicate that such comparisons are restricted, since these parameters vary depending on tissue region, on age, and on cultivation conditions (field or greenhouse, respectively; data not shown).

Uptake of sugars into protoplasts and tissue discs. Uptake from a 1 mM [^{14}C]glucose solution into isolated protoplasts was linear with time for about

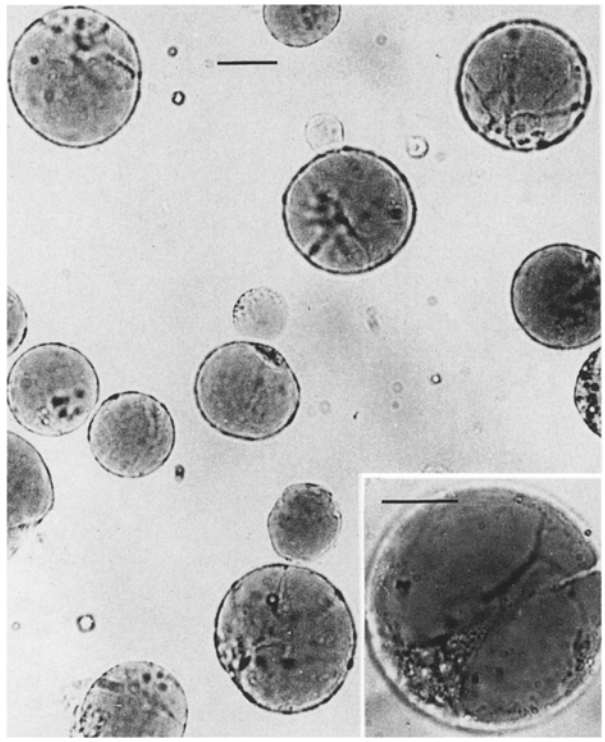

Fig. 1. Micrograph (bright light) of bundle protoplasts from red beet. Note transcellular strands and heterogenity of protoplast size. Bar = 10 μm. *Insert:* storage protoplast (bar = 20 μm)

40 min (Fig. 2). The presence of chloramphenicol did not affect uptake rates and linearity (data not shown). Maximal uptake rates were 0.4 μmol glucose $\cdot g^{-1}FW \cdot h^{-1}$ and 0.06 μmol sucrose $\cdot g^{-1}FW \cdot h^{-1}$ from 1 mM ^{14}C-sugar solutions. For tissue discs, corresponding uptake rates were 0.66 μmol glucose $\cdot g^{-1}FW \cdot h^{-1}$ and 0.26 μmol sucrose $\cdot g^{-1}FW \cdot h^{-1}$. In-situ uptake, as estimated from increasing sucrose contents of beetroots with age, was 0.1 to 0.23 $\mu mol \cdot g^{-1}FW \cdot h^{-1}$ (i.e. 0.2 to 0.46 $\mu mol \cdot g^{-1}FW \cdot h^{-1}$ hexose units). Aeration of the discs for 24 h prior to incubation did not alter glucose uptake but increased ^{14}C-uptake from sucrose, from 0.26 to 0.44 $\mu mol \cdot g^{-1}FW \cdot h^{-1}$. Sugar uptake into isolated protoplasts was not affected by aeration. Chromatographic analysis of products resulting from the incubation of tissue discs with [^{14}C]glucose showed about 50% of the radioactivity to be in the sugar fraction, and 25% each in the amino-acid and organic-acid fractions. These proportions were not significantly altered, with the incubation time of 1–5 h. The sugar (neutral) fraction was composed of sucrose (79%), of glucose (20%) and of fructose (1%). For protoplasts, Table 2 shows the distribution pattern of labelled sugars and phosphate esters. Labelled and unlabelled reference substances were cochromatographed for

Table 1. General properties of mature[a] red-beet storage tissue

Examined feature	Mean value	(±SE)	Unit
Osmotic pressure of tissue sap[c]	550	(± 30)	mosmol·kg^{-1}
Density of beetroot protoplasts[b, d, g]	1.096	(± 0.009)	g·cm^{-3}
Betanin content of tissue[d]	1441.0	(± 590.0)	units·g^{-1}FW
Betanin/protein-ratio in protoplasts			
of conductive tissue[d, e]	225.0	(± 129.0)	units betanin·mg^{-1} (protein)
of conductive tissue[d, f]	410.0	(± 145.0)	units betanin·mg^{-1} (protein)
of bundle-free tissue[d, e]	365.0	(± 99.0)	units betanin·mg^{-1} (protein)
of bundle-free tissue[d, f]	306.0	(± 74.0)	units betanin·mg^{-1} (protein)
Protein content of protoplasts[d]	4.41	(± 1.8)	mg·g^{-1}FW
Cell number[d]	4925.0	(±1965.0)	unit^{-1}betanin
Sugar content of protoplasts[c]	140	− 180	mM sucrose
	0.3	− 5.3	mM glucose
	0.03	− 0.5	mM fructose
O_2 consumption of protoplast suspensions[c]	6.0	(± 2.3)	µmol·g^{-1}FW

[a] Plant age 85–145 d; [b] determined by weighing empty and filled (with densely packed protoplast pellet) glass micropipettes; [c] n≧10; [d] n≧30; [e] preparations were made from 85- to 115-d-old plants; [f] 115- to 145-d-old plants were used for protoplast isolation; [g] in 0.8 M sorbitol

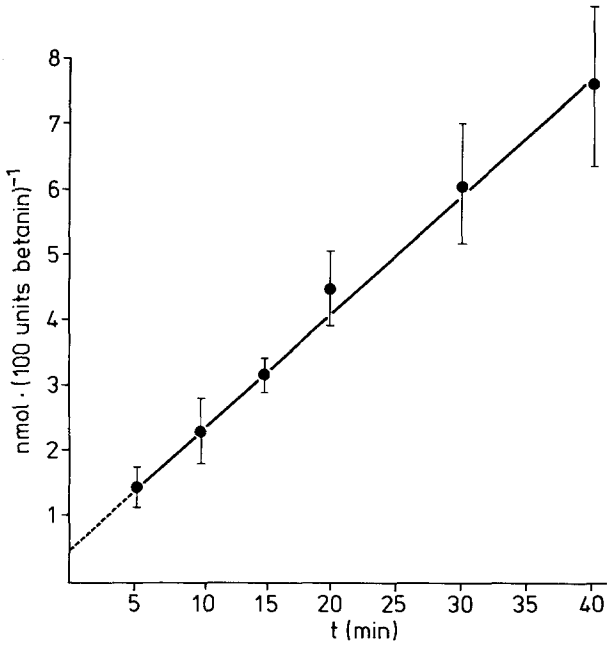

Fig. 2. Time course of glucose uptake into beetroot protoplasts. Uptake conditions: 0.8 M sorbitol + 10 mM Mes-Tris (pH 5) ± 0.02% (w/v) chloramphenicol + 1 mM D-glucose (9.25 kBq·mmol^{-1}); 20 ± 2° C. Mean values (±SD; n = 5) for protoplasts, derived from an average sample of whole beetroot tissue

Table 2. Products of glucose and sucrose uptake into beetroot protoplasts. Mean values from at least six experiments. About $2 \cdot 10^6$ protoplasts per ml incubation medium (as in **Fig. 2**) were incubated for different times in sugar as indicated (74 kBq [^{14}C]glucose and 185 8kBq [^{14}C]sucrose, respectively, per mole). Uptake was stopped by silicone-layer-filtering centrifugation into 5 µl of 2.5 M sorbitol, containing 1% (w/v) SDS, or by washing the protoplasts three times with 0.8 M sorbitol (icecold) prior to sonication (2 min) and addition of an equivalent amount of methanol

Reference-substance	% radioactivity (±SD) after incubation with:			
	2.5 mM [^{14}C]glucose			10 mM [^{14}C]sucrose
	5 min	35 min	60 min	60 min
Maltose	29(±8)	21(±12)	31(±14)	14(± 9)
Sucrose	25(±8)	16(± 8)	21(±12)	47(±17)
Glucose	20(±5)	20(±10)	10(± 5)	9(± 4)
Phosphate-esters	5(±1)	23(±11)	24(± 6)	15(± 8)
Others	21(±2)	20(± 6)	14(± 4)	15(± 4)

tentative identification. Obviously, both [^{14}C]glucose and [^{14}C]sucrose are rapidly metabolized in the protoplasts. It is noteworthy that about 25% of the label from [^{14}C]glucose is recovered in sucrose within 5 min, whereas within this period about 50% of the [^{14}C]sucrose is metabolized (data not shown) within 60 min.

Concentration dependence of sugar uptake. Glucose uptake does not increase linearily with solute concentration (Fig. 3). On the other hand, uptake into beetroot protoplasts followed saturation kinetics only at lower concentrations whereas beyond 5 mM glucose a linear, diffusion-like component became apparent. Transformation of the data according to Lineweaver-Burk yielded apparent K_m

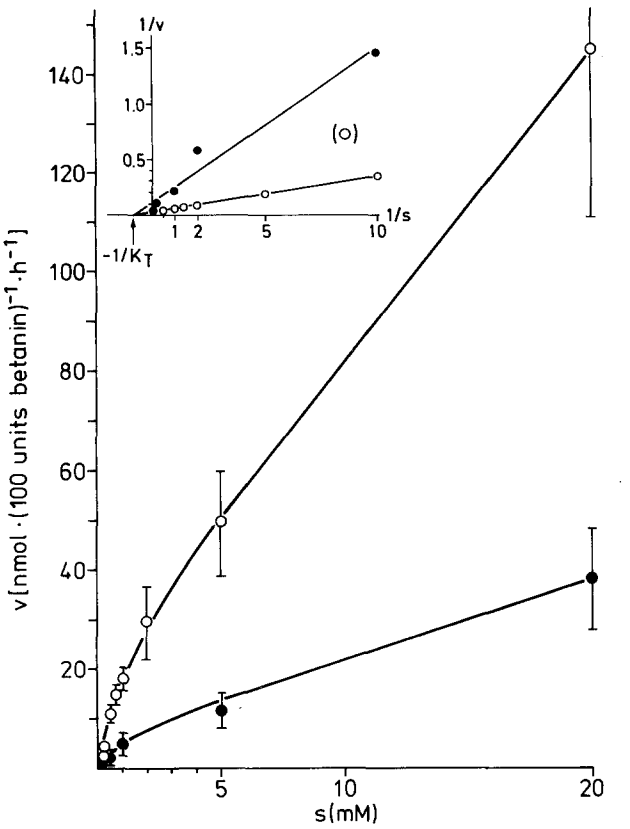

Fig. 3. Concentration dependence of glucose uptake into beetroot protoplasts from different tissue regions. Mean values (±SD, *bars*) of at least four independent experiments each with three parallel samples. Uptake conditions were as described in **Fig. 2**, glucose concentrations as indicated (at 0.1–2 mM: 37 kBq·mmol^{-1}; at higher concentrations: 92.5 kBq·mmol^{-1}). *Insert:* Lineweaver-Burk plot of uptake data. o—o, Bundle protoplasts; •—•, storage protoplasts. Apparent K_m= 1.0 mM (±0.4); V_{max}=46 and 9.3 nmol·(100 units betanin)$^{-1}$·h^{-1} for bundle and storage protoplasts, respectively. Corresponding data for glucose uptake into tissue discs are: K_m(app.)=1.2 mM, V_{max}=54 nmol·(100 units betanin)$^{-1}$·h^{-1} (1.2 µmol·g^{-1}FW·h^{-1})

values of about 1 mM (±0.4 mM; Fig. 3, insert). Uptake experiments with the non-metabolizable glucose analogue 3-O-methylglucose (3-OMG; cf. Reinhold and Kaplan 1984) confirmed these results: the kinetic profile of 3-OMG uptake from low concentrations (up to 5 mM) was not equal to that of glucose but was at least similar (Fig. 4). It should be noted that extracts from protoplasts incubated with [^{14}C]3-OMG exhibited only one detectable ^{14}C-spot on the chromatograms.

Sucrose transport into the protoplasts principally followed the same kinetics as those for glucose, but with a lower apparent affinity (K_m 10 mM), whereas in the case of storage protoplasts (see below) only the diffusion-like component seemed to be present (Fig. 5).

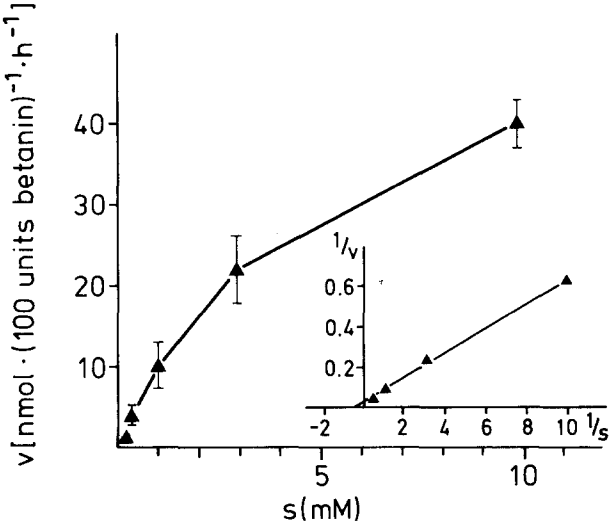

Fig. 4. Concentration dependence of 3-OMG uptake into bundle protoplasts. Conditions as described in **Fig. 3.** Mean values (±SD) of four experiments with three parallel samples each. *Insert:* Lineweaver-Burk transformation of the uptake data. At 3 and 10 mM OMG: 92.5 kBq·mmol^{-1}. Apparent K_m= approx. 2 (±0.8) mM; V_{max}=approx. 31 nmol·(100 units betanin)$^{-1}$·h^{-1}

Glucose and sucrose uptake were temperature dependent. A Q_{10}-value of approx. 2 was estimated for the range of 12 to 22° C. According to Kamamoto et al. (1971) these levels for the activation energy (E_A) indicate involvement of an enzyme-catalyzed reaction. Incubation experiments with tissue discs reconfirmed the kinetic features shown for beetroot protoplasts (glucose uptake by tissue discs: apparent K_m=approx. 1.2 mM, V_{max}=approx. 54 nmol·(100 units betanin)$^{-1}$·h^{-1},=1.2 µmol·g^{-1}FW·h^{-1}; see below).

Sugar uptake into protoplasts isolated from different hypocotyl regions. For sugar-beet roots Richter and Ehwald (1983) have reported that no detectable barrier exists for solute exchange between the xylem vessels and the free space (cell-wall compartment) of storage parenchyma. Consequently, the sugar concentration of xylem exudates should resemble that of the free space in the bundle region as well. Since in the case of sugar-beet roots the concentration of sugar in xylem exudates hardly exceeds 3 mM (Fife et al. 1962), we compared uptake rates for glucose and sucrose in both fractions, bundle protoplasts and storage protoplasts, in the range of K_m for glucose uptake (1 mM).

In both preparations, glucose uptake exceeded sucrose uptake. There was obviously a strong pref-

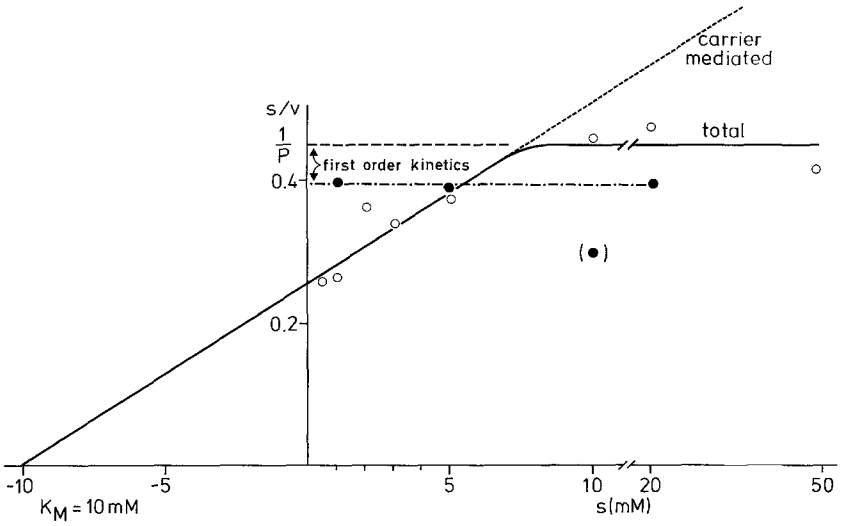

Fig. 5. Hanes-plot of the concentration dependence of sucrose uptake. Mean values of five (storage protoplasts, ●—●) or 10 (bundle protoplasts, o—o) experiments with three parallel samples each. Uptake conditions as described for glucose uptake (**Fig. 3**). Apparent K_m for bundle protoplasts = approx. 10 mM; V_{max} approx. 40 nmol·(100 units betanin)$^{-1}$·h^{-1}

Table 3. Comparison of sugar uptake into beetroot protoplasts of different tissue origin. Uptake conditions: 0.8 M sorbitol + 10 mM Mes-Tris (pH 5.5) + 0.02% (w/v) chloramphenicol; 9.25 kBq·mmol^{-1} sugar; T = 20 ± 2° C; n = 4 (bundle-free region, glucose), n = 10 (others). a = nmol sugar·mg^{-1} protein·h^{-1}; b = nmol sugar·(100 units betanin)$^{-1}$·h^{-1}

Protoplast origin	Uptake from:			
	1 mM glucose		1 mM sucrose	
	a	b	a	b
Average of hypocotyl	21.3	7.1	11.0	3.6
Bundle-free region	15.3	5.1	9.8	3.2
Conductive tissue	80.1	25.9	11.8	4.3

Table 4. Competitive inhibition of D-glucose uptake

Competing substance	Uptake[a] (±SD)	% inhibition	
		Protoplasts[a]	Discs[b]
None	4.3 ± 1.6	0	0
D-Glucose	2.1 ± 0.9	100	100
L-Glucose	3.7 ± 1.0	27	0
2-DOG	2.8 ± 1.1	68	100
3-OMG	2.6 ± 0.6	78	100
D-Galactose	3.1 ± 0.8	55	89
1-OMG	3.7 ± 1.2	27	n.d.
6-DOG	3.4 ± 1.1	41	n.d.
Glucuronic acid	4.0 ± 2.2	13	n.d.
D-Xylose	4.2 ± 1.0	5	n.d.
Sucrose	4.4 ± 0.1	0	25
D-Fructose	4.3 ± 0.4	0	11

[a] Means of at least four experiments (three replicates each using protoplasts isolated from whole beetroot tissue. Reaction mixture: 0.8 M sorbitol + 10 mM Mes-Tris (pH 5) + 0.2 mM [^{14}C]-D-glucose ± 1.8 mM competing substance, 22 ± 2° C. Uptake expressed as nmol (glucose)·(100 units betanin)$^{-1}$·h^{-1}.

[b] Means of three experiments. Uptake conditions: 0.5 M sorbitol + 10 mM $CaCl_2$ pH 5.5 (without buffer) + 1 mM [^{14}C]-D-glucose (37 kBq·mmol^{-1}) ± 9 mM competing sugar, 18 ± 2° C. Uptake in controls was 0.65–0.83 µmol (glucose)·g^{-1} FW·h^{-1} (equals 45–58 nmol·(100 units betanin)$^{-1}$·h^{-1}. n.d. = not determined

erence for glucose transport into protoplasts derived from the bundle region (Table 3). This can be deduced also from the higher V_{max} evaluated for the bundle protoplasts as compared with storage protoplasts (Fig. 3). Taking into account that sucrose is composed of two hexose units, glucose uptake into bundle protoplasts is still 3–4 times greater than sucrose uptake.

Specifity of the uptake system. Table 4 shows the degree of competition (in %) of the added substrates with D-glucose for the carrier binding site. Uptake of D-glucose into isolated protoplasts and, more distinctly, into tissue discs is fully inhibited by 3-OMG and by 2-deoxyglucose (DOG). The influence of the other substrates on glucose transport is less pronounced. Among these substrates the following order of effectiveness in competing with glucose can be seen: D-galactose > 6-DOG > 1-O-methylglucopyranoside (1-OMG) > glucuronic acid xylose = D-fructose = sucrose. While uptake of D-glucose into isolated protoplasts sometimes appeared to be affected by the presence of L-glucose, uptake into tissue discs was not.

Sucrose uptake (0.5–3.0 mM) could not be inhibited by D-glucose. On the contrary, a slight stimulation was observed in the presence of 4.5 to 27 mM D-glucose (data not shown).

Influence of pH and of mono- and divalent cations on sugar transport. Glucose uptake from 1 mM so-

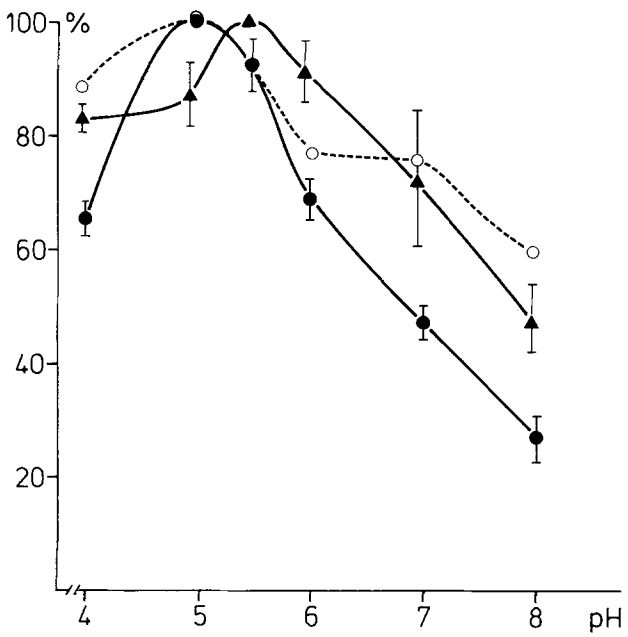

Fig. 6. pH-dependence of sugar uptake into isolated protoplasts (●——●, o-----o) and tissue discs (▲——▲) from red-beet hypocotyls. Means of at least four experiments (±SD, *bars*). Uptake conditions: 1 mM D-glucose (●——●) or 3 mM sucrose (o-----o; 9.25 kBq·mmol^{-1}) in 0.8 M sorbitol and 10 mM Mes-Tris (pH 4–6) or 10 mM Tris-Mes/HCl (pH 6–9) for protoplasts; 2 mM D-glucose (37 kBq·mmol^{-1}) in 0.5 M sorbitol + 10 mM $CaCl_2$ + buffers as follows: disodiumcitrate (pH 4, 5), Mes-Tris (pH 6) and Hepes-HCl (pH 7, 8) for tissue discs. 100% = 8.2 nmol·(100 units betanin)$^{-1}$·h^{-1} for protoplasts (glucose and sucrose), and 45 nmol·(100 units betanin)$^{-1}$·h^{-1} for discs

lution has been measured in the presence of 10, 20, 40 and 100 mM K^+ or Na^+ and of 2 and 5 mM Mg^{2+} or Ca^{2+}. Uptake appeared slightly stimulated by 2 or 5 mM Mg^{2+} (about 20%) and by 20 mM K^+ (about 50%), but not by Na^+ or Ca^{2+} (data not shown). At 100 mM K^+ or Na^+, the uptake rate was lowered (data not shown).

Uptake of glucose and sucrose was optimal at pH 5.0. This is illustrated in Fig. 6 for protoplasts and for tissue discs. Sugar uptake proves to be clearly dependent on the pH of the surrounding medium. This is not caused by a decreased stability of the protoplasts in the different pH media (cf. "General observations").

Influence of inhibitors on glucose uptake. Uptake experiments in the presence of ionophores, uncouplers and inhibitors of the plasma-membrane ATPase should help to clarify the mechanism and the energy dependence of glucose transport. Glucose uptake into protoplasts was inhibited by phlorizin (Table 5) – a specific inhibitor of hexose carriers in animal (Rosenberg and Wilbrandt 1957)

Table 5. Inhibition of glucose uptake into isolated protoplasts. Means of at least four experiments with protoplasts isolated from an average sample of whole beetroot tissue (three replicates each). Uptake conditions: 0.5 mM D-glucose in 0.8 M sorbitol + 10 mM Mes-Tris (pH 5) + 0.1% (v/v) ethanol as inhibitor-solvent. Uptake in controls = 7 nmol·(100 units betanin)$^{-1}$·h^{-1}

Inhibitor	Concentration (M)	% (control)
Phlorizin[a]	(10^{-5})	90
	(10^{-4})	83
	($5 \cdot 10^{-4}$)	75
	(10^{-3})	61
CCCP	(10^{-8})	72
	(10^{-7})	37
	(10^{-6})	15
Nigericin	(10^{-8})	92
	(10^{-7})	84
	(10^{-6})	50
	(10^{-5})	47
	(10^{-4})	39
Valinomycin (±5 mM K^+)	(10^{-8})	106
	(10^{-6})	72
	(10^{-5})	46
	(10^{-4})	13
Na_3VO_4[c]	(10^{-5})	85
	(10^{-4})	76
	($5 \cdot 10^{-4}$)	58
DCCD[b]	(10^{-7})	79
	(10^{-5})	51
	(10^{-4})	29
	(10^{-3})	20
PCMBS[c]	(10^{-5})	81
	(10^{-4})	67
	(10^{-3})	52

[a, b] Controls for bundle protoplasts: [a]20 and [b]60 nmol·(100 units betanin)$^{-1}$·h^{-1}
[c] Without ethanol

and plant cells (Felle et al. 1983). The inhibition pattern widely resembles the pattern published by Felle et al. (1983) for *Riccia fluitans*.

Uncouplers like CCCP change the proton electrochemical potential on the membrane directly. Glucose uptake into storage tissue of red beet was severely inhibited by the presence of this uncoupler, as demonstrated in Table 5. While tissue discs needed nearly 10^{-5} M CCCP for half-maximal inhibition (Table 6), glucose transport into isolated protoplasts was reduced by 50% in the presence of about 10^{-7} M CCCP. 2,4-Dinitrophenol (DNP) proved to be much less effective (Table 6).

Sodium-orthovanadate (Na_3VO_4) is reported to be a relatively specific inhibitor of plasmalemma ATPases (O'Neill and Spanswick 1984a). Comparatively high concentrations of this chemical were needed to show a distinct effect on glucose uptake

Table 6. Inhibition of glucose uptake into discs of red-beet tissue. Means of three experiments. Uptake conditions: 0.5 M sorbitol + 10 mM $CaCl_2$ pH 5.5 (without buffer) + 1 mM [^{14}C]-D-glucose (37 kBq·mmol^{-1}), 18 ± 2° C. Uptake value for control sets: approx. 55 nmol·(100 units betanin)$^{-1}$·h^{-1}

Inhibitor	Concentration (M)	% (control)
CCCP	(10^{-6})	67
	(10^{-5})	42
	(10^{-4})	29
DNP	(10^{-5})	81
Na_3VO_4	(10^{-3})	87
PCMBS	(10^{-4})	56
	(10^{-3})	35
Phlorizin	(10^{-4})	92
	($5 \cdot 10^{-4}$)	90
	(10^{-3})	76

Table 7. Influence of inhibitors of glucose uptake on respiratory activity of isolated beetroot protoplasts. Means of three experiments. Incubation conditions: 0.8 M sorbitol, pH 5 + about 10^6 protoplasts per ml, 22 ± 2° C

Inhibitor	Concentration (M)	O_2 consumption[a]	Inhibition (%)
Control	(−)	3.5	0
CCCP	(10^{-6})	3.4	3
CCCP[b]	(10^{-5})	3.1	11
CCCP[c]	(10^{-5})	0.9	75
Valinomycin	(10^{-5})	3.3	8

[a] nmol O_2·(100 units betanin)$^{-1}$·h^{-1}
[b] Measured after 25 min of incubation
[c] Preincubation of protoplasts in 10^{-5} M CCCP for 40 min

into beetroot protoplasts (Table 5). This finding has to be discussed with respect to the less-concentrated inhibitor solutions which are able to inhibit the plasma-membrane ATPase completely in native and reconstituted-vesicle preparations of red beet (O'Neill and Spanswick 1984b).

N,N'-Dicyclohexylcarbodiimide (DCCD), also a putative inhibitor of plasma-membrane ATPases (Pedemonte and Kaplan 1986), decreased glucose uptake sharply (50% inhibition at about 8.10^{-6}; Table 5). Another inhibitor of ATPases, diethylstilbestrol (DES), mainly lysed protoplasts when used in concentrations higher than $5 \cdot 10^{-4}$ M. Lower concentrations yielded no reproducible results (data not shown).

Glucose uptake into isolated protoplasts and into tissue discs was reduced to half-maximal by $1.4 \cdot 10^{-3}$ M and $3 \cdot 10^{-3}$ M PCMBS, respectively, concentrations reported elsewhere for the *specific inhibition* of *sucrose carriers* (Delrot 1981; M'Batchi and Delrot 1984).

Influence of inhibitors of glucose uptake on O_2 consumption by isolated protoplasts. Uncouplers may increase O_2 consumption of protoplasts by uncoupling electron transport from phosphorylation in mitochondria. Inhibitors could therefore perturb the activity of mitochondrial ATP synthesis and thus block the plasma-membrane ATPases, too, by cutting off the energy supply.

In fact, the respiratory activity of isolated protoplasts was affected by CCCP (Table 7), but it was reduced instead of being enhanced by concentrations which eliminate glucose transport in uptake experiments.

Concentrations of valinomycin half-maximal for inhibition of glucose transport decreased the respiratory activity of isolated protoplasts only slightly (8%), as shown in Table 7.

Discussion

Isolated protoplasts are artificially altered plant cells, especially when they are derived from tissues rich in plasmodesmata like the storage tissue of red beet. More important than the viability tests used in this study is the fact that the protoplasts exhibited normal metabolic activities with respect to sugar uptake: the amount of sugar taken up by protoplasts corresponded well with uptake rates into tissue discs and also with in-situ uptake rates. Furthermore, the concentration dependence for glucose uptake into protoplasts and into tissue discs also corresponded well. Hence, isolated protoplasts, which offer the advantage of direct accessibility of solutes and other substances to the plasma membrane, are considered as a suitable instrument for membrane transport studies.

According to Ehwald et al. (1980) and to Richter and Ehwald (1983), the central vacuole occupies about 90% of the beet storage tissue, the free space 2–6%, and the cytoplasm 4–8%. Assuming that the cytosol comprises approx. 50% of the total cytoplasm, it equals about 2–4% of the tissue. Based on these estimations an uptake rate into bundle protoplasts from 1 mM [^{14}C]glucose solution of only 20 nmol·(100 units betanin)$^{-1}$·h^{-1} would increase the cytosolic glucose concentration by 5–10 mM, provided that only about 10% of the [^{14}C]glucose is retained in the cytosol as a consequence of the rapid metabolism and transport of resynthesized [^{14}C]sucrose into the vacuole (cf.

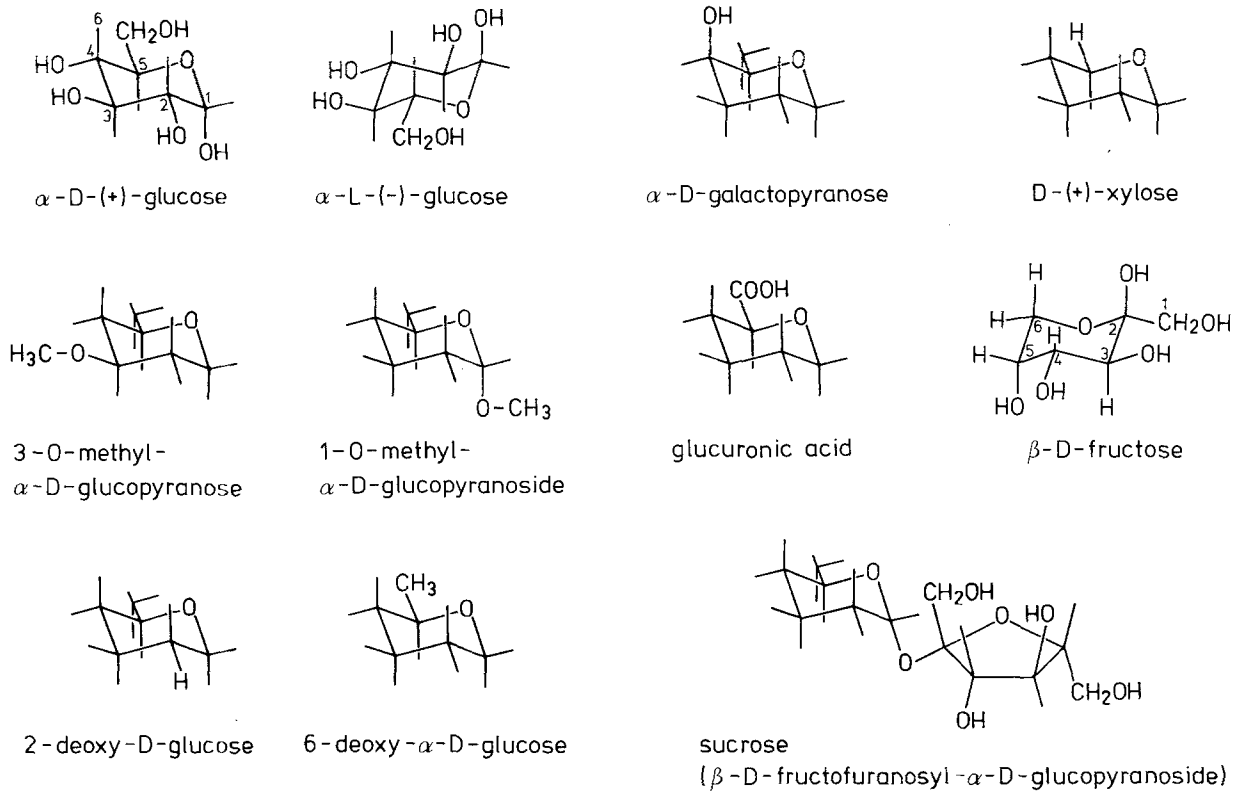

Fig. 7. Molecular structure of molecules used for competition studies. Positions of C-atoms in the pyranose-chair form are numbered only in α-D(+)-glucose. In the other molecules only substituent alterations compared with α-D-(+)-glucose are indicated

Table 2). Since the glucose concentration in protoplasts amounts to 0.3–5.3 mM (Table 1) whereas the vacuolar concentration was estimated to be 1 mM (isolated vacuoles, data not shown), the basic cytosolic level of free glucose must be assumed to exceed the overall protoplast concentration. Hence, from an external solution of 1 mM, glucose would be taken up against a concentration gradient. This indicates an active transport system at the plasma membrane, because even high turnover rates for glucose (Table 2) do not suffice to decrease internal levels of glucose below those administered externally (up to about 10 mM), a situation which would allow downhill passive permeation. This view is supported by the only slightly altered K_m value for the uptake of non-metabolizable 3-OMG compared with the value for glucose (Figs. 3, 4).

For a closer characterization of glucose uptake, a possible competitive inhibition by the glucose analogue 3-OMG and by 2-DOG was studied (Table 4). Further alterations of the D-glucose molecule, however, as shown in Fig. 7, were not recognized by the carrier, and could not compete with D-glucose for the binding site. This means that uptake of D-glucose into red-beet protoplasts is very specific. The D-glucose-carrier is able to discriminate between the enantiomers: L-glucose had almost no effect on transport of D-glucose. This compares quite well with data characterizing the glucose carrier of suspension-cultured cells of *Saccharum* (Maretzki and Thom 1972). The positions C_2, C_3 and C_4 of the D-glucose molecule seem to be of minor importance for binding to the glucose carrier of the beetroot plasmalemma (Table 4, Fig. 7). Substitutions in the positions C_1, C_5 and C_6, however, are crucial in this regard, because 1-OMG, glucuronic acid, D-fructose and 6-DOG did not affect glucose uptake. We did not test the importance of the pyranose-chair form for binding capacity to the glucose carrier, but we do not agree with Zamski and Wyse (1985) that D-fructose could serve as representative for furanose forms. Angyall et al. (1976) have shown that solutions of D-fructose are mainly composed of the pyranose-chair form (about 75% at 27° C). In this context, it should be mentioned that in contrast to the data reported here for beetroot protoplasts, the glucose-uptake system in other green plants accepts both glucose and fructose, as has been shown by Gogarten and Bentrup (1984) and by Komor et al. (1985).

In the presence of 1 mM phlorizin, glucose transport into red-beet protoplasts was inhibited by 40% (Table 5). Considering that 70% inhibition of 3-OMG transport by phlorizin was determined as maximal for *Riccia fluitans* (Felle et al. 1983), one might assume 1 mM of this agent to be the concentration for half-maximal inhibition in the case of red-beet protoplasts.

Comparing the present results with the criteria for carrier-mediated transport (Reinhold and Kaplan 1984), we conclude that glucose transport into red-beet protoplasts is mediated by a specific plasma-membrane carrier.

Sucrose uptake from a 10 mM [^{14}C]sucrose solution yielded a rate of only 20–30 nmol·(100 units betanin)$^{-1}$·h^{-1} (Fig. 5). Based (i) on the most unfavorable assumption of 20% exchange transport of sucrose at the tonoplast (Doll 1979) and (ii) on the fact that 50% of label is retained in sucrose, this would mean an increase in the cytosolic sucrose concentration of about 5 to 10 mM. Sucrose transport across the plasma membrane of red-beet storage tissue would therefore occur only until the concentration between the cytoplasm and the medium becomes equilibrated. The accumulation step might be attributed to the tonoplast in this case, as pointed out by Wyse (1979) for sugar-beet taproot discs, and as indicated by Schmalstig and Geiger (1985) for sugar-beet sink leaves. Apart from the lower apparent affinity of the presumed plasma-membrane carrier for sucrose (K_m 10 mM) compared with the glucose carrier (K_m 1 mM), uptake values for sucrose transport into isolated protoplasts are not sufficient to explain in-situ uptake rates. Uptake of [^{14}C]sucrose is not inhibited by glucose, and sucrose does not alter significantly the uptake of [^{14}C]glucose into isolated bundle protoplasts. This may indicate the presence of two different carriers at the plasma membrane of red-beet storage tissue. Whether or not the slight stimulation of sucrose transport in the presence of glucose reflects a cooperative effect, remains to be determined.

The results for protoplasts were confirmed by those obtained for tissue discs, where a preference for glucose uptake could also be found. However, after an aeration period of 24 h prior to the uptake experiment, ^{14}C uptake from [^{14}C]sucrose into tissue discs was remarkably enhanced and was nearly equal to the rate measured for [^{14}C]glucose uptake. We suggest that aeration of tissue discs induced (cell-wall bound) acid-invertase activity and that, according to Eschrich (1980), sucrose hydrolysis in the apoplast might precede sugar uptake into the parenchyma cells adjacent to the phloem. The report that only 70% of [^{14}C]glucosyl-labelled sucrose administered to sugar-beet taproot discs remains chemically unaltered (Wyse 1979) strengthens this suggestion.

Sugar uptake across the plasma membrane of red beet is clearly pH-dependent (Fig. 6); this may indicate the importance of protons as a driving force for glucose uptake into red beet. Our results of experiments with uncouplers like DNP and CCCP and the K^+/H^+-exchange-mediating ionophore nigericin support this view. A proton-pumping plasmalemma ATPase, responsible for maintainance of a pH gradient between apoplast and cytoplasm, has been established meanwhile for red-beet storage tissue (O'Neill and Spanswick 1984a, b). This ATPase may be involved in secondary active glucose uptake across the red-beet plasma membrane, since in our experiments uptake is reduced by putative inhibitors of such ATPases. Rather high concentrations of Na-orthovanadate, however, are needed for inhibition of glucose transport in beetroot protoplasts, whereas only micromolar amounts are sufficient to block proton-pumping of plasmalemma ATPases in beetroot vesicle preparations (O'Neill and Spanswick 1984a). We therefore think that the vesicles used by O'Neill and Spanswick were turned inside out and hence, Na_3VO_4 had direct access to the binding site at the ATPase (Cantley et al. 1978). This may explain in part the lower concentrations for total inhibition of ATPase activity. Additionally, we tested Na_3VO_4 under pH conditions optimal for glucose uptake (pH 5.0). Acidic pH, however, promotes formation of decavanadate, a yellow polymerization product of orthovanadate (Gallagher and Leonard 1982). Thus the actual concentration of the inhibitory +V − oxidation state was presumably lower than indicated in Tables 5 and 6.

The possible involvement of the plasmalemma ATPase in glucose transport in beet roots is substantiated by the inhibitory action of DCCD and PCMBS; the latter has frequently been reported to inhibit sucrose transport specifically (Giaquinta 1976; Maynard and Lucas 1982; M'Batchi and Delrot 1984). From data presented by Vara and Serrano (1982) and Serrano (1983) it can be concluded that plant plasma-membrane ATPases possess essential sulfhydryl groups. Therefore, the glucose-transport-inhibiting effect of PCMBS in our experiments is not surprising.

While the slight stimulation of glucose uptake in the presence of 20 mM K^+ may reflect an improved membrane stability caused by K^+ (Jolivet et al. 1983), its inhibition by higher cation concentrations (100 mM K^+ or Na^+) may be simply ex-

plained by depolarization of the membrane potential (interior negative) as a consequence of cation uptake. Whether or not the strongly inhibiting effect of valinomycin (Tables 5, 6) on glucose uptake is caused by hyperpolarization of the membrane potential induced by this ionophore is unknown at present.

A perfect separation of functionally different cells of the beet hypocotyl is not yet attainable. Nevertheless, the data presented here allow the conclusion that phloem unloading of sucrose into the apoplast of beet roots might be restricted to the bundle region. The parenchyma cells adjacent to the bundles may preferentially absorb sugars from the apoplast, mainly glucose, since its uptake significantly exceeds that of sucrose into protoplasts isolated from the bundle region. We therefore suggest that sucrose hydrolysis occurs in the apoplast, but is limited to the phloem-unloading site.

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