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CHEMICAL MODIFICATION OF THE SMALL INTESTINAL Na^+/D-GLUCOSE COTRANSPORTER BY AMINO GROUP REAGENTS

EVIDENCE FOR A ROLE OF AMINO GROUP(S) IN THE BINDING OF THE SUGAR

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Some amino group reagents inactivate the small-intestinal Na^+/D-glucose cotransporter, as measured either as a catalyst of Na^+-dependent D-glucose transport or as a Na^+-dependent phlorizin ligand. The amino group(s) studied in this paper are not identical with those investigated previously (Biber, J., Weber, J. and Semenza, G. (1983) Biochim. Biochim. Acta 728, 429–437): these are protected from inactivation by the simultaneous presence of Na^+ plus sugar substrates. They are thus likely to be located within the substrate-binding site.

Introduction

In recent years much work has been carried out with the goal of solubilizing, partially purifying and/or identifying (components of) the Na^+/D-glucose cotransporter of brush border membranes (Refs. 1–4 and Takesue, S., Takesue, Y. and Semenza, G., unpublished observations).

Another approach — selective inactivation of the cotransporter in situ, i.e., not removed from the original membrane, with the use of 'specific reagents' — has also provided new information on some properties of the cotransporter: e.g., that it is inserted asymmetrically with respect to the plane of the membrane [5–7], that SH-groups [6–8] and tyrosine residues [9] are essential to its function. Some of these SH-group(s) are neither located within the substrate-binding site nor accessible from the luminal side of the membrane [6,7], others seem to be localized differently [8] (see Note added in proof). Proteases inactivate the cotransporter when acting from the cytosolic, but not from the luminal side of the membrane [3,10].

In the present paper we present evidence that the cotransporter is inactivated by various amino-group reagents (cf. Ref. 8). The inactivation by some of these reagents is delayed or prevented in the presence of transportable sugars. We conclude that the small-intestinal Na^+/D-glucose cotransporter, in all probability, has (an) amino group(s) in or very close to the sugar-binding site.

Materials and Methods

[^{3}H]Phlorizin (6.3 Ci/mmol) and D-[1(n)-^{3}H]-glucose (15.0 Ci/mmol) were purchased from New England Nuclear. Fluorescamine (Fluram) was from Fluka, deoxycholic acid from Merck, dithioerythritol from Sigma, *p*-sulfophenylisothio-

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid

cyanate from EGA and sodium cyanoborohydride from Aldrich. 1-Fluoro-2,4-dinitrobenzene, phenylisothiocyanate, methylacetimidate, methyl-4-mercaptobutyrimidate, isethionylacetimidate and dimethylsuberimidate were all bought from Pierce. All reagents were of higest purity available. SDS-polyacrylamide gel electrophoresis was done as described elsewhere [11,12].

Vesicle preparation. Brush border membrane vesicles from rabbit small intestine were prepared according to the method of Kessler et al. [13], a modification of the procedure of Schmitz et al. [14]. The vesicles were resuspended at a protein concentration of 20–40 mg/ml in a medium containing 300 mM mannitol and 10 mM potassium phosphate, pH 7.6. Aliquots containing 10 to 50 mg protein were quickly frozen in solid CO_2 and stored not longer than two weeks at −20°C. Protein was determined using the Bio-Rad Protein Assay following the Coomassie blue binding method of Bradford [15].

Modification reactions. Frozen vesicles were thawed at room temperature and washed in the buffers described below (30 min, $60000 \times g$). Modifications were routinely performed at a protein concentration of 2 mg/ml at room temperature in: (a) 300 mM mannitol, 10 mM potassium phosphate, pH 7.6 (5 min) in the case of fluorescamine; (b) 300 mM mannitol, 25 mM potassium phosphate, pH 8.2 (20 min) for the reductive methylation; (c) 100 mM potassium chloride, 100 mM triethanolamine-HCl, pH 8.5 (30 min) in all other cases (imidate, fluorodinitrobenzene, isothiocyanates).

When indicated, additional sugar or salt was present. The stock solutions of some reagents were prepared immediately before use in water and their pH controlled (imidates, formaldehyde, sodium cyanoborohydride and *p*-sulfophenylisothiocyanate). Others were prepared in dioxane (fluorescamine, fluorodinitrobenzene, phenylisothiocyanate) so that the final concentration of dioxane during the modification was 1%; the organic solvent had no detectable influence on transport and binding measurements. Incubations were finished by adding two parts of ice-cold 300 mM mannitol solution to one part reaction mixture and sedimenting the vesicles 30 min at $60000 \times g$. Additionally they were washed twice in 300 mM mannitol, 10 mM Hepes-Tris, pH 7.0 0°C) and finally resuspended at a protein concentration of about 15 mg/ml. The amount of free, unmodified amino groups was estimated with 2,4,6-trinitrobenzenesulfonate [16] with additional 0.1% Triton X-100 in the buffer.

Deoxycholate treatment. The preparation of deoxycholate extracted membrane fragments from the vesicles followed the procedure of Klip et al. [3]: to 2 mg/ml vesicles in 100 mM potassium phosphate, 250 mM potassium chloride, pH 8.5 on ice, 0.1% potassium deoxycholate was added. After a centrifugation (30 min, $60000 \times g$) the pellet was washed in the appropriate buffer under the same conditions and used for modification. Deoxycholate treatment after modification reactions led to different extraction patterns (detected by SDS-polyacrylamide gel electrophoresis) and could therefore not be used.

Uptake measurements. Brush-border membrane or deoxycholate fragments in 300 mM mannitol, 10 mM Hepes-Tris, pH 7.0 were preincubated with either 200 mM NaCl or 200 mM KCl for at least one hour on ice. Influx of D-glucose (16.7 μM) after 2 s and 60 min or binding of phlorizin (3.61 μM) after 5 or 10 s was determined with the short-time uptake apparatus of Kessler et al. [17] (which is now commercially available from Innovativ Labor AG., 8134 Adliswil, Switzerland). Filtration of the vesicles with Sartorius filters and counting of the radioactivity is also described in detail elsewhere [17]. The sodium specific uptake was calculated by substracting the potassium values from the sodium values. Usually, the potassium values of D-glucose uptake which were around 5% compared to the sodium values did not rise after modification.

Results

Small-intestinal brush-border membrane vesicles have some 350 nmol amino groups per mg protein (by determination with trinitrobenezesulfonate) of which approximately one-third belong to the lipids [18]. In order to investigate the effect, if any, of amino reagents on the Na^+/D-glucose cotransporter, brush-border membrane vesicles or deoxycholate-extracted membranes were reacted with the reagents chosen (see under

Materials and Methods and in the legends) and then washed free of the remaining reagent. Zero-trans D-glucose uptake into these vesicles (Table I) was measured after preeqilibration in Na^+-rich buffer, at low D-glucose outer concentration (both conditions minimizing a possible indirect effect of the reagent used via non-specific increase of ion permeability and decrease of $\Delta\psi$) and at very short incubation times (2 s): the data were thus not distorted by deviation from linearity or by non-specific increase of ion permeabilities.

Phlorizin is a well-known fully competitive inhibitor and ligand of the Na^+/D-glucose cotransporter [10,19]. Phlorizin binding to vesicles was likewise measured after salt equilibration. In spite of the similar conditions, phlorizin binding was usually found to have been inactivated slightly more than D-glucose initial uptake (see Table I). This might have been due to a larger loss of D-glucose than of phlorizin in washing of the control vesicles, or to some minor systematic error.

Methylacetimidate substitution and reductive methylation [8,20,21] belong to the most specific reactions for amino groups. Fluorescamine is known to react with thiols [22] in addition to amino groups. However, as it has been shown elsewhere [8] fluorescamine inhibition of the Na^+/D-glucose cotransporter is due to a reaction with amino group(s). Fluorodinitrobenzene also can react with thiols in addition to amino groups.

TABLE I

INHIBITION OF THE Na^+-DEPENDENT D-GLUCOSE TRANSPORT AND OF THE Na^+-DEPENDENT PHLORIZIN BINDING AFTER TREATMENT OF BRUSH-BORDER MEMBRANES WITH VARIOUS REAGENTS THAT MODIFY AMINO GROUPS

Intestinal brush-border vesicles (1 to 2 mg protein/ml) in the indicated buffer (compare methods) were reacted with the reagents listed below at room temperature. After injecting 2 vol. ice-cold 300 mM mannitol to stop the modification, vesicles were sedimented for 30 min at 60000 × *g* and then washed twice in uptake buffer (300 mM mannitol, 10 mM Hepes-Tris, pH 7.0). Finally, resuspended at a protein concentration between 12 and 20 mg/ml, they were assayed for D-glucose influx (16.86 μM) or phlorizin binding (3.61 μM) under nongradient conditions i.e. inside and outside of the vesicle membrane 200 mM NaCl or KCl in the uptake buffers. Appropriate controls were done exactly the same way but without the modifying reagent or in the case of the reductive methylation with the two reagents one after the other instead of incubating them at the same time with the vesicles. Mean values and their standard deviations of *n* different experiments (*n*) [a] or mean values and standard deviations of *n* determinations in one experiment (*n*) [b] are presented as percent of the respective controls.

Treatment	Initial Na^+- dependent D-glucose flux (%)	Phlorizin binding (%)
Appropriate controls ± dithioerythritol (DTE)	100	100
Methylacetimidate 30 mM 30 min 20°C, pH 8.5	50 ± 3 (8) [a]	51 ± 7 (6) [b]
Reductive methylation with 30 mM $NaCNBH_3$, 5.4 mM formaldehyde 20 min 20°C, pH 8.2	64 ± 7 (3) [b]	60 ± 18 (3) [b]
Fluorescamine 66 μM 5 min 20°C, pH 7.6	55 ± 6 (3) [a]	47 ± 9 (5) [a]
Fluorodinitrobenzene 2 mM 30 min 20°C, pH 8.5	56 ± 10 (4) [a]	48 ± 11 (3) [b]
Same with addition of 5 mM DTE	57	
Phenylisothiocyanate 1 mM 30 min 20°C, pH 8.5	53 ± 7 (4) [a]	
Phenylisothiocyanate 0.6 mM 30 min 20°C, pH 8.5		64 ± 9 (3) [a]

The reaction with thiols, however, is reversed by dithioerythritol [23]. Finally, also phenylisothiocyanate is a well-known amino reagent. Inspection of Table I clearly shows therefore, that the reaction of amino groups (and not of thiols) had led to irreversible inactivation of the Na^+/D-glucose cotransporter, whether it is measured via D-glucose transport, or via phlorizin binding.

Higher concentrations of the reagents lead to complete inactivation (data not shown).

The pH-dependence of the inactivation by fluorodinitrobenzene and by the isothiocyanate derivatives used also agrees with amino groups being the target of this reaction (Fig. 1). (The increased inactivation rate by isothiocyanates at pH values below 7 remains unexplained and may indicate additional reaction with other group(s) or internal catalysis of the coupling step by a deprotonated side chain).

The inactivation by other imidates is shown in Table II. It is clear that the reactivity of the critical amino group(s) in the cotransporter can be differ-

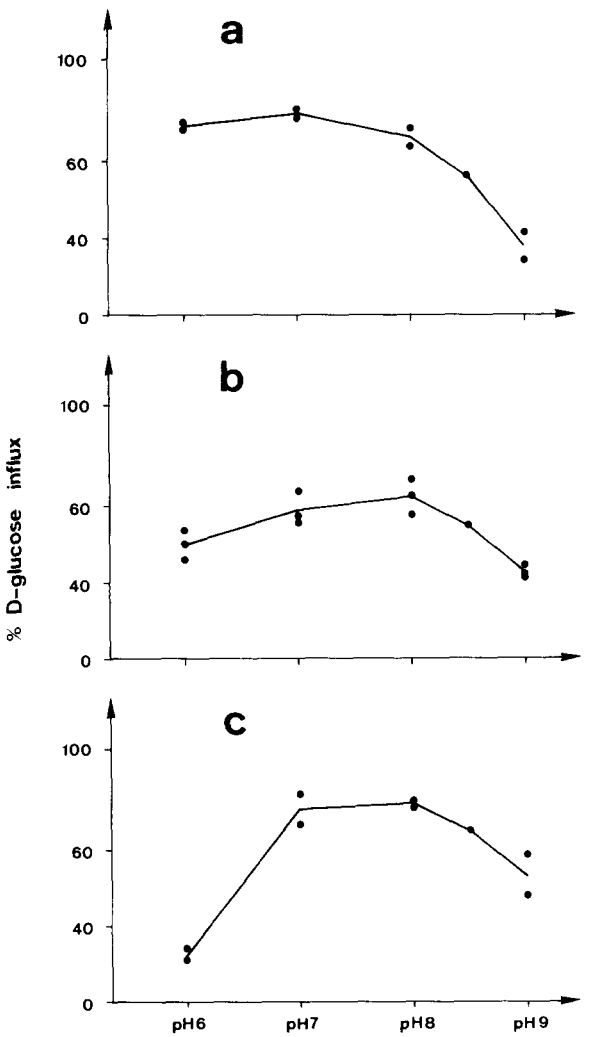

Fig. 1. Inactivation of Na^+-dependent D-glucose transport in brush-border membrane vesicles by fluorodinitrobenzene, phenylisothiocyanate or *p*-sulfophenylisothiocyanate at different pH values. Brush-border membrane vesicles (2 mg protein per ml) in 100 mM triethanolamine, 20 mM Mes, 100 mM NaCl, 100 mM L-glucose, pH 6.0, 7.0, 8.0, 8.5 or 9.0 were treated for 30 min at room temperature with (a) 2 mM fluorodinitrobenzene, or (b) 1 mM phenylisothiocyanate, or (c) 10 mM *p*-sulfophenylisothiocyanate. After reaction, the washing procedure was carried out and the D-glucose uptake measured as described in the legend to Table II. The D-glucose concentration was 16.86 μM. The concentration of NaCl and other salts were identical at both sides of the membrane. The initial flux (●——●) is expressed as percent of the untreated controls.

TABLE II

THE DIFFERENT INHIBITION POWER OF VARIOUS IMIDOESTERS ON THE Na^+-DEPENDENT D-GLUCOSE TRANSPORT

2 mg protein/ml of intestinal brush-border membrane vesicles were incubated for 30 min at room temperature in 100 mM triethanolamine-HCl, 100 mM NaCl (pH 8.5) with several imidates. Care was taken to adjust the imidate stock solution's pH back to 8.5 (10-fold concentrated in buffer; prepared immediately before use). Modification reactions were terminated by adding 2 vol. ice-cold 300 mM mannitol to 1 vol. incubation solution. Vesicles were afterwards sedimented 30 min at 60000 × *g* and washed twice in 300 mM mannitol, 10 mM Hepes-Tris, pH 7.0 to get rid of remaining reagents and NaCl. 2-s influx values for 16.86 μM D-glucose plus or minus 200 mM NaCl (equilibrated) were used to estimate the initial Na^+-dependent D-glucose transport activity. Mean values of three determinations are given. Free membrane amino groups were assayed by trinitrobenzenesulfonate [16].

Reagent	Initial Na^+-dependent D-glucose flux (%)	Unmodified amino groups left (%)
Controls	100	100
Methylacetimidate 30 mM	51	53
Isethionylacetimidate 30 mM	52	57
Methyl-4-mercaptobutyrumidate 30 mM plus dithioerythritol 5 mM	2	48
Dimethylsuberimidate 15 mM	13	65

ent from that of the bulk of the membrane amino groups. Particularly noteworthy is their higher reactivity with methyl-4-mercaptobutyrimidate (+dithioerythritol).

Protection by substrates

Table III shows the degree of inactivation of Na^+-dependent D-glucose transport in the presence of Na^+ or K^+ and of L- or D-glucose. The affinity of L-glucose for the cotransporter is very low [13] or nil; that of D-glucose depends on the presence of its co-substrate, Na^+. It would therefore be expected that, if the reacting amino group(s) are located within the glucose-binding site or interact with it, then the simultaneous presence of D-glucose and Na^+ will produce protection against inactivation; also, D-glucose without Na^+ may afford some degree of protection. This is the case

TABLE III

RETARDATION OF THE Na^+-DEPENDENT D-GLUCOSE TRANSPORT INACTIVATION BY ADDING D-GLUCOSE DURING THE MODIFICATION REACTION

Small-intestinal brush-border membrane vesicles (2 mg protein per ml) were reacted for 30 min at room temperature in 100 mM NaCl or KCl, 100 mM D- or L-glucose, 100 mM triethanolamine-HCl, pH 8.5 with: (a) 2 mM fluorodinitrobenzene (plus 1% dioxane, end concn.); or (b) 30 mM methylacetimidate; or (c) 2 mM phenylisothiocyanate (plus 1% dioxane). After reaction the vesicles were washed free of reagents, salts and sugars, by spinning them down and washing them twice with 300 mM mannitol, 10 mM Hepes-Tris, pH 7.0. Na^+-dependent D-glucose initial influx was measured after preequilibration in 10 mM Hepes-Tris, pH 7.0, 300 mM mannitol plus either 200 mM NaCl or KCl; the initial outer concentration of D-glucose was 16.86 μM. The data given are the means of three determinations ± S.D. (Fluorodinitrobenzene and phenylisothiocyanate were added as a 100-fold concentrated dioxane solution; methylacetimidate was dissolved at a 10-fold concentration in the buffer immediately before use, the final pH value was readjusted).

(a) Fluorodinitrobenzene

Modification buffer containing	Initial Na^+-dependent D-glucose flux (pmol/mg per 2 s)		Remaining transport activity (%)
	Control	2 mM fluorodinitrobenzene	
KCl + L-glucose	6.10 ± 0.40	3.67 ± 0.08	60 ± 4
NaCl + L-glucose	6.28 ± 0.04	3.79 ± 0.09	61 ± 2
KCl + D-glucose	5.38 ± 0.03	3.55 ± 0.06	66 ± 2
NaCl + D-glucose	5.55 ± 0.16	4.40 ± 0.14	79 ± 3

(b) Methylacetimidate

Modification buffer containing	Initial Na^+-dependent D-glucose flux (pmol/mg per 2 s)		Remaining transport activity (%)
	Control	30 mM methylacetimidate	
KCl + L-glucose	5.66 ± 0.40	2.84 ± 0.07	50 ± 4
NaCl + L-glucose	5.57 ± 0.25	2.91 ± 0.12	52 ± 3
KCl + D-glucose	5.62 ± 0.18	3.88 ± 0.03	69 ± 2
NaCl + D-glucose	5.65 ± 0.28	4.24 ± 0.26	75 ± 6

(c) Phenylisothiocyanate

Modification buffer containing	Initial Na^+-dependent D-glucose flux (pmol/mg per 2 s)		Remaining transport activity (%)
	Control	2 mM phenyl isothiocyanate	
KCl + L-glucose	6.10 ± 0.40	1.58 ± 0.05	26 ± 2
NaCl + L-glucose	6.18 ± 0.04	1.88 ± 0.05	30 ± 1
KCl + D-glucose	5.38 ± 0.03	1.80 ± 0.02	33 ± 1
NaCl + D-glucose	5.55 ± 0.16	2.56 ± 0.05	46 ± 2

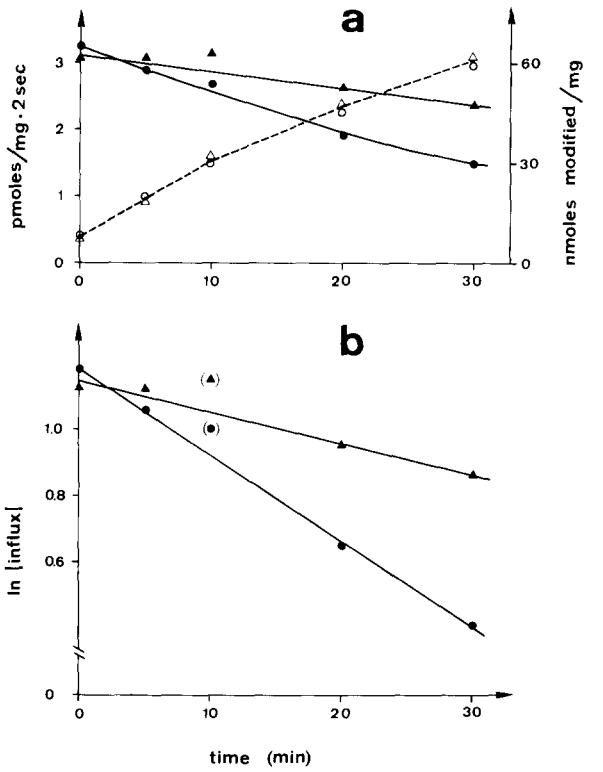

Fig. 2. Inhibition of the Na^+-dependent D-glucose transport after treating brush-border membranes with 2 mM fluorodinitrobenzene in the presence or absence of D-glucose. The experiment was performed exactly the same way as described in Table II and III; the modifications were stopped after 5, 10, 20 or 30 min. ●———●, D-glucose influx values after modification in the presence of 100 mM L-glucose; ▲———▲, the influx values after modification in the presence of 100 mM D-glucose. ○— — —○ and △— — —△, the corresponding numbers of dinitrophenylamino groups in the vesicles, estimated by a molar extinction coefficient of 14000 at 366 nm. (a) Linear plots. (b) Logarithmic plots. Slopes of linear regressions are −0.0092/30 min and −0.0259/30 min.

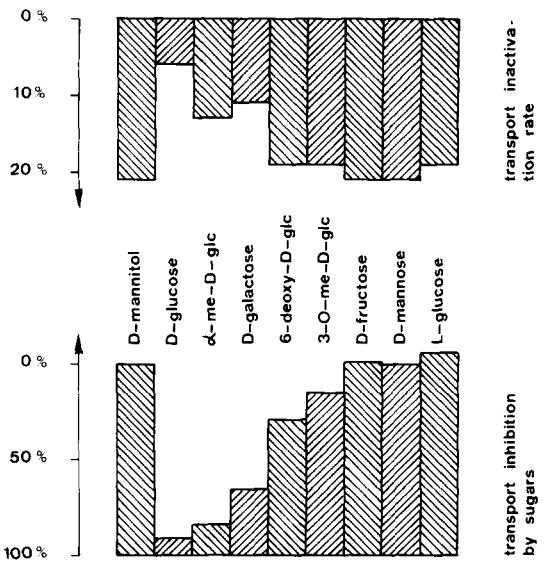

Fig. 3. Effect of different sugars on the Na^+-dependent D-glucose transport inactivation rate by methylacetimidate. 2 mg protein/ml of brush-border membranes were incubated for 30 min at room temperature in 100 mM triethanolamine-HCl, 100 mM NaCl, 100 mM sugar, pH 8.5 with 15 mM methylacetimidate as described in Table II and assayed for D-glucose influx (upper part of the picture). The direct interaction of the sugars with the glucose transporter was evaluated by measuring the D-glucose influx inhibition in the modification buffer plus these various sugars under the same conditions as for the modification with methylacetimidate (lower part of the picture).

(Table III, a, b, c). The inactivation by fluorodinitrobenzene and its protection by D-glucose was investigated more closely (Fig. 2). The pseudo-first-order constant of cotransporter inactivation was, under the conditions chosen, 0.22 $l \cdot mol^{-1} \cdot s^{-1}$. In the presence of 100 mM D-glucose plus 100 mM Na^+ it was reduced to 0.08 $l \cdot mol^{-1} \cdot s^{-1}$.

If the critical amino group(s) is (are) located within the substrate-binding site of the cotransporter then for the various monosaccharides there should be a correlation between their ability to protect this amino group and their competition with D-glucose for the substrate-bindig site of untrated vesicles. The data in Fig. 3 show that the more potent a sugar is as an inhibitor of D-glucose transport, the better it is in protecting the cotransporter from inactivation by methylacetimidate.

Inactivation of phlorizin binding and sugar transport by phenylisothiocyanate and sulfophenylisothiocyanate in intact vesicles and in deoxycholate extracted membranes (Fig. 4)

Contrary to our expectation, the two reagents have quite different reactivities with the critical amino group(s) in the cotransporter; a difference that is not related to their expected different permeabilities across the membrane. In fact, the same difference in reactivity is found in deoxycholate disrupted membranes as well as in intact, sealed vesicles. The fact that each of the two reagents (but in particular, the water soluble sulfophenylisothiocyanate) reacts to the same extent in vesicles

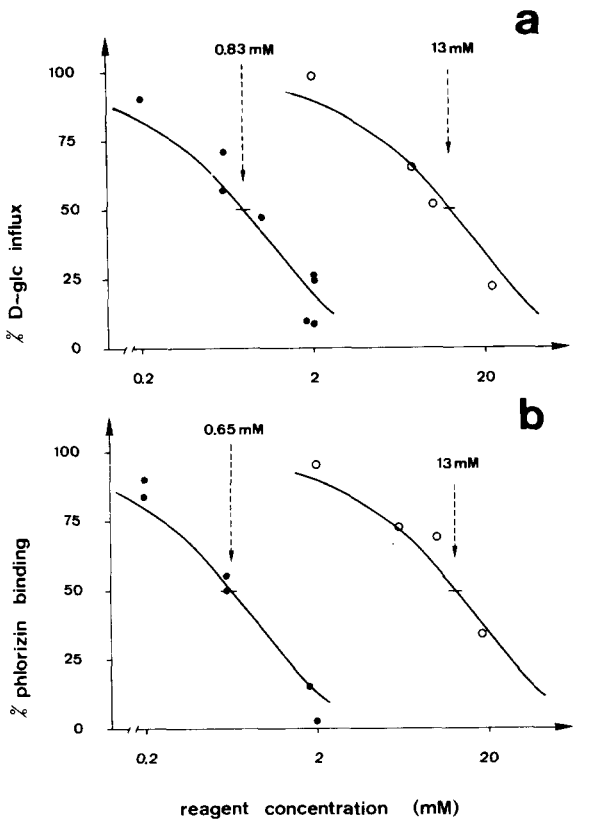

Fig. 4. Modification of the sugar transport system by isothiocyanates with different hydrophobicities. 2 mg protein/ml of (a) intact brush border membrane vesicles or (b) deoxycholate-extracted membranes were incubated with 0.2 to 2 mM phenyl-isothiocyanate (●), or 2 to 22 mM *p*-sulfophenylisothiocyanate (○) for 30 min at room temperature in 100 mM triethanolamine-HCl, 100 mM KCl, 100 mM mannitol, pH 8.5. After the usual stop and wash steps (see Table II), the intact vesicles (a) were examined for Na^+-dependent D-glucose influx (16.86 μM D-glucose under equilibrium conditions) and the deoxycholate-treated membranes (b) were investigated for Na^+-dependent phlorizin binding (3.61 μM phlorizin). The experimentally found points were fitted by the theoretically calculated curve for second-order kinetics.

as in deoxycholate-extracted membranes shows that the reactive amino group is freely accessible from the outer, luminal side of the membrane. The difference in intrinsic reactivity between phenyl-isothiocyanate and sulfophenyl-isothiocyanate will be discussed below.

Having established that the critical amino group(s) of the cotransporter must be located within or near the sugar-binding site (since protection by sugars fulfilled the diagnostic criteria, as mentioned above) we expected that phlorizin and its congeners that are high-affinity ligands of the substrate-binding site would also protect the transport activity from inactivation by methylacetimidate. This, however, was not found to be the case (Table IV). A possible explanation is offered in the Discussion.

Attempts at identifying a labeled band in SDS-polyacrylamide gel electrophoresis patterns

After modification with fluorodinitrobenzene, some 60 nmol of dinitrophenyl groups are bound per mg of brush border membrane protein (Fig. 2), in the case of methylacetimidate even more rea-

TABLE IV

EFFECT OF PHLORIZIN AND DERIVATIVES ON THE METHYLACETIMIDATE INHIBITION REACTION

The experiments were performed the same way as in Table II with 100 mM triethanolamine-HCl, pH 8.5, 100 mM NaCl and 0.3 mM phloretin, phlorizin or 4'-deoxyphlorizin or 100 mM D-glucose and 1% ethanol in the incubation buffer (30 min room temperature).

Modification buffer containing NaCl plus	Initial Na^+-dependent D-glucose flux (pmol/mg per 2 s)		Remaining transport activity (%)	Blocked amino groups (%)
	Control	30 mM methylacetimidate		
–	5.06 ± 0.02 = 100%	2.67 ± 0.10	53 ± 2	44
Phloretin 0.3 mM	4.61 ± 0.06 = 91%	2.22 ± 0.07	48 ± 2	46
Phlorizin 0.3 mM	4.44 ± 0.01 = 88%	2.50 ± 0.11	56 ± 3	45
Deoxyphlorizin 0.3 mM	4.94 ± 0.03 = 98%	2.64 ± 0.06	53 ± 1	46
D-Glucose 100 mM	5.10 ± 0.13 = 101%	3.73 ± 0.16	73 ± 4	44

gent molecules block amino groups (Table II). On the other hand only 14 pmol D-glucose transporter per mg protein are found (see Discussion): hence less than 0.1% of the membrane associated modification reagent is responsible for the D-glucose protectable carrier modification, an amount that almost certainly cannot be seen on SDS-polyacrylamide gel electrophoresis. Nevertheless, gels were run with fluorodinitro[^{3}H]benzene and [^{14}C]ethylacetimidate reacted vesicles (radioactive labelled methylacetimidate was not commercially available, therefore the very similar ethylacetimidate was used). The highly water-soluble imido-ester labels the brush border vesicles rather unselectively; in contrast, probably as a result of its hydrophobic nature, fluorodinitrobenzene reacts, in addition to actin and other cytoskeletal components, with a class of (intrinsic membrane) proteins that are tightly associated with the membrane and that run on an SDS-polyacrylamide gel electrophoresis in a region of the gel corresponding to molecular weights of 60000 to 90000. As to a possible effect of D-glucose on the labeling pattern, none was detected (as expected) [24].

Discussion

The Na^+/D-glucose cotransporter accounts for approximately 0.1% of the intrinsic proteins of the small-intestinal brush-border membrane, as determined by the binding of phlorizin (approx. 14 pmol per mg protein) under optimal conditions of Na^+ concentration and $\Delta\psi$ across the membrane (negative inside the vesicles) [10,19]. The identification, isolation and characterization of such a minute component is a formidable task. Some progress has been achieved by solubilization and reconstitution, which have led to the suggestions of bands with molecular weights of 60000–70000 [2] and 160000 [1] as components of the carrier. In another approach a protein of 110000 was suggested as the target of radiation inactivation [25]. Recently, a band (apparent M_r 72000) in SDS-polyacrylamide gel electrophoresis has been indicated by photoaffinity labeling and by other criteria as a possible candidate of (a component of) the Na^+/D-glucose cotransporter [3,26].

An alternative approach towards the goal of obtaining information on the positioning and on some aspects in structure-function relationship in this cotransporter is the use of selective or semi-selective reagents for protein modification. The use of SH-reagents has allowed us to establish an essential role of a SH group in this protein in phlorizin binding. These thiols are most probably not a part of the substrate binding site, but are located either at the cytosolic or at the hydrophobic surface and on the luminal side of the cotransporter [6–8] (see Note added in proof). Thus, it was also established that this cotransporter is oriented in an asymmetric fashion across the membrane; this is in keeping with the current ideas on biosynthesis and vectorial membrane insertion of intrinsic proteins and with the similar asymmetric insertion of other membrane carriers (e.g., Refs. 27–29). The detection of a permanent structural asymmetry in this cotransporter is also relevant to its mode of functioning as it rules out 'rotating' or 'diffusive' models and is matched by its functional, i.e., kinetic asymmetry [30,31].

In the present paper we have investigated whether substitution of amino groups in the translocator would affect its function (either Na^+-dependent D-glucose transport or Na^+-dependent phlorizin binding) and if so, where, as far as its function is concerned, this amino group(s) is located with respect of the membrane. The latter question was approached in two ways: (i) by using amino group reagents of similar reactivity but of different permeabilities across the brush border membrane; (ii) by comparing the effect of such reagents on sealed, right-side-out [13,32] membrane vesicles or on deoxycholate-disrupted membranes [3].

This 2-fold approach was necessary because partial extraction of the membranes with deoxycholate may have altered in part the original positioning of the cotransporter in the membrane. Likewise, a double functional test (D-glucose transport and phlorizin binding) was needed when working with sealed vesicles; a loss of D-glucose transport alone may have been simulated by non-specific damage of the membrane structure.

The results presented above can be summarized as follows: all amino reagents used inhibit irreversibly the Na^+/D-glucose cotransporter, whereas its activity is monitored by D-glucose transport or by phlorizin binding. Whenever a reagent was

known to react with other types of groups, appropriate controls showed that the inactivation of the transporter was related to reaction with amino group(s) (Tables I, II, Fig. 1).

Within the Na^+/D-glucose cotransporter at least one of this critical amino group(s) is located at the substrate binding site, or is indirectly essential for substrate binding. This is shown by the partial protection afforded, particularly in the presence of Na^+, by D-glucose and by other monosaccharides known to interact with the cotransporter. Notable in this respect are the need for the presence of Na^+ (the interaction of D-glucose with the cotransporter is known to require Na^+ [30,31]) (Table III, a, b, c) and also the good parellelism between the ability of monosaccharides to interact with the cotransporter (as shown by its competition with D-glucose for transport) and their ability to afford protection against inactivation by methylacetimidate (Fig. 3). It is also noteworthy that the protection by D-glucose plus Na^+ is present with all reagents tested in this respect (methylacetimidate, fluorodinitrobenzene, isothiocyanates).

Barnett et al. [33] and Hosang et al. [34] have suggested that position 6 of the monosaccharide (or position 6 of the glucosyl moiety of phlorizin) interacts with the corresponding subsite in the substrate binding site of the cotransporter by accepting a H-bond from it. It is possible, but of course we have no direct evidence for it, that the critical amino group in the transporter substrate binding site may be this H-donor.

Whether this is actually the case, must, however, remain an open question at the moment. In fact, contrary to the monosaccharides, phlorizin and phlorizin derivatives at high relative concentrations afford little or no protection against inactivation by methylacetimidate (Table IV). This is noteworthy particularly in the case of 4'-deoxyphlorizin, which, unlike phlorizin, interacts with the Na^+/D-glucose cotransporter equally well at pH 8.5 and at neutral pH [35]. The inefficiency of phlorizin and phlorizin derivatives can be due, in principle, to a number of reasons. One reason could be that the cotransporter when loaded with Na^+ plus transportable substrate may occur predominantly in a form reacting little or not at all with amino group reagents, whereas when loaded with Na^+ and phlorizin (a non-transportable ligand) may occur in a form as reactive as the free cotransporter.

The observation that the water soluble sulfophenylisothiocyanate (partition coefficient water/cyclohexane, 99 : 1) has equal access to the critical amino group in sealed, right-side-out membrane vesicles as in deoxycholate disrupted membranes (Fig. 4) strongly indicates that this amino group is located at or near the outer, luminal surface of the membrane. This agrees, also, with the pattern of protection specificity by monosaccharides being identical with that of their interaction with the cotransporter from the outside. In fact, although this has been shown for the erythrocyte sugar carrier [36] and not for the cotransporter studied here, it is conceivable that the substrate specificity of translocators may be different at both sides of the membrane. The small-intestinal Na^+/D-glucose transporter does have a stable structural [5–7] and functional [30,31] asymmetry.

The difference in reactivity between phenylisothiocyanate and sulfophenylisothiocyanate (Fig. 4) and also among the imidates (Table II) requires some comment. Considerations on the basis of Hammett's substituent rules indicated that the reactivities of the two isothiocyanates should be nearly the same — indeed the sulfonate derivative should react approx. 20% better. Fig. 4 shows that it is phenylisothiocyanate instead, that reacts better (by more than one order of magnitude). As this large difference is not due to the different permeabilities of the membrane for the reagents (it is present in the deoxycholate-extracted membrane fragments also), the higher reactivity of phenylisothiocyanate must be sought in its higher lipophilicity (partition coefficient in water/cyclohexane, approx. 3 : 97), which indicates a hydrophobic microenvironment close to or around this amino group. (Phenylisothiocyanate is indeed used for the substitution of amino groups occurring in an hydrophobic environment [37]).

The reactivities of imidoesters (Table II) also agree with a hydrophobic environment occurring in the immediate neighborhood of this (these) amino group(s) of the cotransporter: whereas methylacetimidate and isethionylacetimidate react to about the same extent, methyl-4-mercaptobutyrimidate (clearly the most hydrophobic of the three) reacts much faster.

Likewise, the different reactivities of chloromercuribenzoate and chloromercuriphenylsulfonate (with thiols, Ref. 6) may be related with the occurrence of hydrophobic domains. These thiols (contrary to the amino group(s) studied here) are not related, however, with the substrate binding sites.

The occurrence of (an) amino group(s) within or very near to the sugar-binding site can explain satisfactorily the present observations and does not contradict others. However, additional, possible explanations for the inactivation of the cotransporter by amino group reagents must also be considered. One obvious possibility is that the critical amino group(s) may not itself (themsevles) bind the sugar, but influence the binding, via, e.g., a conformational change. In addition, as the apparent K_m values for D-glucose strongly depend on the binding of Na^+ to its own site [31,38,39] a still more indirect mechanism must also be envisaged: substitution of this (these) amino group(s) may affect Na^+ binding first (we do not mean with this that amino groups act as ligands for Na^+ – nitrogen is an unlikely candidate for this); the inactivation of Na^+ binding would in turn decrease the binding of the sugar.

Note added in proof (Received May 10th, 1983)

It has been reported recently [40] that membrane vesicles from the outer renal cortex lose phlorizin binding sites upon reduction with dithiothreitol. In similar experiments, using vesicles from intestinal brush borders, we failed to detect this effect, which very probably reflects the already noted dissimilarities (see, for example, Ref. 30 and references quoted therein) between the two kindred systems.

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