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SMALL-INTESTINAL Na^+/D-GLUCOSE COTRANSPORT

INACTIVATION OF SUGAR TRANSPORT AND PHLORIZIN BINDING BY THIOL-GROUP AND AMINO-GROUP REAGENTS

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It has previously been shown that mercurials acting from the cytoplasmic side or from within the hydrophobic part of the membrane inactivate the small intestinal Na^+/D-glucose cotransporter by blocking essential SH-groups (Klip, A., Grinstein, S. and Semenza, G. (1979) Biochim. Biophys. Acta 558, 233–245). Another (set of) sulfhydryl(s) which are critical for phlorizin binding and sugar transport function and which may lie on the luminal side of the brush border membrane, can be blocked by DTNB and 4,4′-dithiopyridine but not by *N*-ethylmaleimide. In addition, modification of amino groups by fluorescamine, reductive methylation and (under certain conditions) DIDS also lead to inactivation of the carrier's binding and transport functions. No evidence was obtained that any of the above groups is directly involved in the binding of either Na^+/D-glucose or phlorizin, since none of these compounds prevented inactivation of the cotransporter.

Introduction

Studying structure-function relationships in the small-intestinal Na^+/D-glucose cotransporter is turning out to be a very complicated task. Among the major reasons for the difficulties encountered are the minute concentrations in which it occurs in the brush border membrane (approximately 0.1–0.4% of the intrinsic membrane proteins, Refs. 1, 2) and its instability towards solubilization by detergents (e.g., Weber, J.; Takesue, S. and Takesue, Y., unpublished data). Thus, indirect approaches are being tried, for example its inactivation by more or less selective chemical reagents in situ, i.e., in the original membrane (either sealed, right side-out [3], brush border vesicles [4]) or membrane fragments, derived therefrom by deoxycholate extraction [5].

With the proviso that the reagent used is specific and the conditions are mild, this approach can indicate whether a given type of chemical groups in the cotransporter or in neighbouring membrane components is essential to its function - either the Na^+-dependent D-glucose transport or Na^+-dependent phlorizin binding. In addition, this approach may, is some cases, yield some information on the location of the essential group(s). For example, protection by substrate(s) indicates that the group(s) is (are) probably located within the substrate binding sites(s). It is also possible to obtain information on the sidedness of the essential

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Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

group(s) with respect to the plane of the membrane, either by comparing its reactivity in sealed vesicles towards chemically similar reagents with widely different permeabilities or by comparing its reactivity towards a reagent that is relatively impermeant to vesicles, but that has easy access to both sides of membrane fragments. Using this approach, we have previously produced evidence for the occurrence of essential thiols on the cytosolic and/or hydrophobic surface of the cotransporter (based on reactivity with organomercurials and $HgCl_2$, with no protection by substrates [6,7]).

In this paper we present evidence for the occurrence in the cotransporter or in neighbouring membrane components of a different (group of) essential thiols to those described previously [6,7].

Materials

[G-^{3}H]Phlorizin (6.3 Ci/mmol) was purchased from New England Nuclear, D-[1-^{3}H]glucose (8.3 Ci/mmol) from Amersham. Solutions of fluorescamine (Fluka) in dioxane, dansylaziridine (Fluka) in ethanol and 5,5′-dithiobis-(2-nitrobenzoic acid) (Fluka), 4,4′-dithiopyridine (Fluka), *N*-ethylmaleimide (Fluka) and sodium cyanoborohydride (EGA) in water were prepared fresh every day. Dithioerythritol was bought from Sigma, unlabelled phlorizin from Roth and formaldehyde from Fluka. Bradford's protein assay reagent was from Bio-Rad. DIDS was kindly provided by Dr. A. Rothstein (Toronto). Potassium deoxycholate was prepared as a 5% solution (pH 9) from deoxycholic acid (Fluka).

Methods

Rabbit intestinal brush border membranes vesicles were prepared according to the method of Schmitz et al. [8], as modified by Kessler et al. [4], using frozen small intestines. The vesicles were stored at −20°C as described in Ref. 6. Extraction of the membrane vesicles with deoxycholate followed a slightly modified version of that described in Ref. 5: Usually 3 mg protein/ml were treated with potassium deoxycholate (0.6 mg/mg protein) in 100 mM D-mannitol, 25 mM potassium phosphate (pH 8.0) at 4°C for 10 min in the presence of 1 mM dithioerythritol. After centrifugation at 60000 × *g* for 30 min (Kontron TGA 75, 30000 rpm with rotor Ti 50) the pellet was resuspended in the mannitol/phosphate buffer and spun down again. The final pellet was either resuspended in the mannitol/phosphate buffer or in a buffer used for transport and binding experiments (see below). This procedure yielded about the same degree of solubilization of protein (approx. 70%) as reported in Ref. 5.

Protein was determined according to the method of Bradford [9] using the Bio-Rad reagent.

Treatment with sulfhydryl-reactive reagents. All the incubations described below were performed in 25 mM phosphate, pH 8.0, 100 mM D-mannitol at a protein concentration of 1 mg/ml. All reagents were added as concentrated solutions. Membrane suspensions (intact vesicles or deoxycholate treated membranes) were incubated with the SH-reagent for 10 min at 20°C. To terminate the reaction, membrane suspensions were diluted with 5 vol. of ice-cold 10 mM Tris-HCl, pH 7.0 or 7.5, 100 mM D-mannitol and centrifuged at 60000 × *g* for 30 min. After one or two washes, the pelleted membranes were finally resuspended at a protein concentration of 10 to 20 mg/ml. When indicated, incubations were performed in the presence of deoxycholate and then washed twice. When necessary, the diluting solutions contained in addition 5 mM dithioerythritol.

Treatment with amino group-modifying reagents. The procedures were in principle the same as for the thiols with the following modifications.

Fluorescamine was added as a solution in dioxane, the final concentration of dioxane did not exceed 1%. This concentration was without effect on the functional parameters measured. The incubation was for 5 min at 20°C in the described phosphate-mannitol buffer adjusted to pH 7.6. With DIDS the membranes were incubated for 15 min at two different temperatures (see Table III).

Reductive methylation with formaldehyde and $NaCNBH_3$ was performed at pH 8.2 for 20 min at 20°C. For this reaction we carried out two types of control: either the vesicles were first incubated with the hydride and then after sedimentation and resuspension were exposed to the aldehyde in a second incubation, or vice versa.

Binding and transport measurements. Phlorizin binding and D-glucose uptake were determined

under non-gradient conditions as described in Ref. 6. Phlorizin binding was measured by incubating membrane suspensions with 5.5 μM [^{3}H]phlorizin for 10 s, after they had been preequilibrated (60 min room temperature) in either 0.2 M NaCl or 0.2 M KCl with 100 mM D-mannitol, 10 mM Tris-HCl, pH 7.0. D-Glucose uptake (2 or 5 s) was measured by incubating vesicles with 0.1 mM D-[^{3}H]glucose after they had been pre-equilibrated as above. The sodium-specific components of binding or uptake was calculated by substracting the non-specific component (measured in the presence of K^+) from the total binding or uptake (measured in the presence of Na^+).

Results

1. Effect of disulfides on D-glucose transport and phlorizin binding

Klip et al. [6,7] have shown that SH-reagents like organic mercurials and $HgCl_2$ are good inhibitors of the Na^+-dependent D-glucose transport and of the Na^+-dependent phlorizin binding of intestinal brush border membrane vesicles, indicating an essential role of SH-group(s) in these processes. This (these) SH-group(s) is (are) located at the intracellular side and/or in the hydrophobic layer of the brush border membrane. These authors failed to detect any inactivation of phlorizin binding when either brush border membrane vesicles or deoxycholate-extracted membranes were reacted with the highly specific SH-reagent DTNB (Ellman's reagent) at pH 7.0.

We have now investigated whether this and another disulfide (4,4'-dithiopyridine) under slightly different experimental conditions can inactivate Na^+-dependent D-glucose transport and phlorizin binding, and whether it would be possible to obtain some indication as to which side of the membrane these disulfide-reactive SH-groups are located.

As shown in Table I, both DTNB and 4,4'-dithiopyridine when reacted with intact brush border vesicles or with deoxycholate-extracted membranes at pH 8.0 do inhibit Na^+-dependent phlorizin binding. In contrast, at pH 7.0 under the reaction conditions of Ref. 7 they have little or no effect (Table I and Ref. 7).

These disulfides inhibit also Na^+-dependent D-glucose transport in intact vesicles. Fig. 1 shows that 1 mM and 2 mM 4,4'-dithiopyridine inhibits D-glucose transport by 22% and 65%, respectively, in brush border membrane vesicles. The decreased Na^+-dependent D-glucose transport by the disulfides investigated was not due to a non-specific membrane damage. In fact, neither the equilibrium values of glucose uptake nor the uptake velocity in the presence of K^+ (i.e., in the absence

TABLE I

EFFECT OF DISULFIDES ON THE Na^+-DEPENDENT PHLORIZIN BINDING TO THE SMALL-INTESTINAL Na^+/D-GLUCOSE COTRANSPORTER

The treatment of brush border vesicles (1 mg protein per ml) with the disulfides DTNB and 4,4'-dithiopyridine (4-PDS) was either performed in 100 mM mannitol, 10 mM Tris-HCl (pH 7.0) or 100 mM mannitol, 25 mM potassium phosphate (pH 8.0) for 10 min at room temperature in the presence (column C) or absence (columns A and B) of 0.6 mg deoxycholate per mg protein. Deoxycholate extraction prior to the modification reactions (column B) was done as described in Methods. All reactions were stopped by adding ice-cold 100 mM mannitol, 10 mM Tris-HCl (pH 7.0) and washing the membranes twice in this buffer. Phlorizin binding was determined as described in Methods. The means $\pm$S.D. of (n) experiments are given. A, intact vesicles; B, membranes extracted with deoxycholate, washed, reacted with the disulfide and washed; C, membranes reacted with the disulfide in the presence of deoxycholate and washed.

Reaction with	Percent inactivation of the Na^+-dependent phlorizin binding onto		
	A	B	C
DTNB			
Control, pH 7	0	0	0
1.0 mM, pH 7	0	n.d.	n.d.
Control, pH 8	0	0	0
0.5 mM, pH 8	n.d.	n.d.	18 (2)
1.0 mM, pH 8	31 $\pm$ 4 (3)	22 $\pm$ 5 (4)	60 $\pm$ 3 (4)
4-PDS			
Control, pH 8	0	0	0
0.25 mM, pH 8	n.d.	n.d.	29 $\pm$ 10 (3)
0.5 mM, pH 8	n.d.	n.d.	68 $\pm$ 5 (4)
1.0 mM, pH 8	42 $\pm$ 10 (7)	55 $\pm$ 2 (4)	94 $\pm$ 6 (10)
2.0 mM, pH 8	74 $\pm$ 5 (3)	86 $\pm$ 1 (3)	n.d.

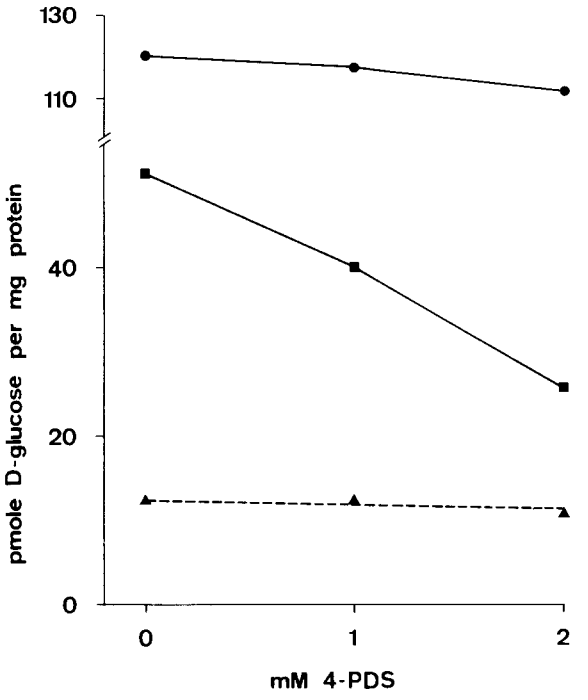

Fig. 1. Inactivation of the Na^+-dependent D-glucose uptake by 4,4'-dithiopyridine (4-PDS). After brush border vesicles had reacted with 0 to 2 mM 4,4'-dithiopyridine at pH 8.1 they were washed once as described under Methods and examined for residual D-glucose short time (5 s) uptake and for equilibrium values (60 min points). ■———■, uptake of D-glucose measured after 5 s (vesicles loaded with Na^+); ▲- - - - -▲, uptake of D-glucose measured after 5 s (vesicles loaded with K^+); ●———●, D-glucose equilibrium values (60 min) of Na^+-loaded vesicles.

of Na^+) were affected by the treatment (which rules out a gross damage of the membrane). A (possible) increase of the Na^+ permeability (and thus a faster collapse of $\Delta\tilde{\mu}_{Na^+}$) can likewise be ruled out as the reason for the decreased Na^+-dependent D-glucose uptake velocity, because the uptakes were measured in Na^+-pre-equilibrated vesicles (i.e., in the absence of $\Delta\tilde{\mu}_{Na^+}$). (Mercurials are known to lead to a fast collapse of $\Delta\tilde{\mu}_{Na^+}$ in these membranes in addition to inactivation of the carrier itself [6,7,10]). We conclude that one or more SH-group(s) in these membrane vesicles is (are) necessary for the functioning of the Na^+/D-glucose cotransporter; it (they) react(s) with disulfides at pH 8.0, but hardly so or not at all at pH 7.0.

The data in Table I allow no firm conclusion to be drawn as to the location of the SH-group(s) with respect to the plane of the membrane. In fact, the difference in reactivity observed between the two reagents in intact vesicles was also observed in deoxycholate-extracted membranes (B,C). However, the extent of inactivation of the cotransporter brought about by 4,4'-dithiopyridine or by DTNB in intact vesicles (A) was not statistically different from that brought about in deoxycholate-extracted membranes (B). This observation may indicate an easy accessibility of these SH-groups from the luminal side, since the negatively charged DTNB is (at pH 8) probably less permeant than 4,4'-dithiopyridine. As to the other SH-groups which react with mercurials they are located at the cytosolic and/or at the hydrophobic surface of the cotransporter [6,7].

Ligands of the transport system (D-glucose at 100 mM, or phlorizin at 1 mM, each in the presence of 100 mM NaCl) do not afford any detectable protection against the inactivation by these disulfides (data not shown). It therefore seems probable that the SH-group(s) involved probably do not occur at the substrate-binding site(s) (see Discussion).

Up to 70% of the inactivation brought about by DTNB or 4,4'-dithiopyridine is reversed by 5 mM dithioerythritol (see Methods).

Finally, if the membranes were reacted with the disulfides in the presence of deoxycholate (0.6 $mg \cdot mg^{-1}$ protein, Table I, column C), then the inactivation of phlorizin binding increased by approximately a factor of 2 with respect to either intact vesicles or deoxycholate-extracted membranes from which most of the detergent had been removed prior to the reaction (compare column C with B and A). This indicates that deoxycholate reversibly exposed additional SH-group(s).

2. Reaction of the SH-groups with other SH-reactive reagents

Other SH-reagents like diamide, iodoacetamide and *N*-ethylmaleimide are reported not to inactivate phlorizin binding in intestinal brush border membranes [7]. *N*-Ethylmaleimide in the presence of 0.6 mg deoxycholate/mg protein at pH 8.0 also does not inhibit phlorizin binding (not shown). In contrast, dansylaziridine, a lipophilic

SH-reagent [11] inactivated phlorizin binding by $50 \pm 8\%$ ($n = 3$) when intact vesicles are incubated with 1 mM reagent.

If intact vesicles had been pretreated with 2 mM *N*-ethylmaleimide, the Na^+-dependent D-glucose transport could still be inhibited by 2 mM 4,4'-dithiopyridine to a similar extent as without *N*-ethylmaleimide pretreatment (Table II). All these data suggested a differential reactivity of the SH-groups involved (either directly or indirectly) in D-glucose transport.

3. Effect of amino-reactive reagents on the D-glucose transporter

With the following amino-reactive reagents we investigated a possible role of amino groups in the transport process.

(i) Fluorescamine. The inactivation of the D-glucose transport system by fluorescamine was determined by exposing intact vesicles to increasing concentrations of the reagent. Fig. 2 shows the inactivation of the Na^+-dependent component of both D-glucose transport and phlorizin binding (expressed as percent of values observed with untreated vesicles). The concentrations necessary for

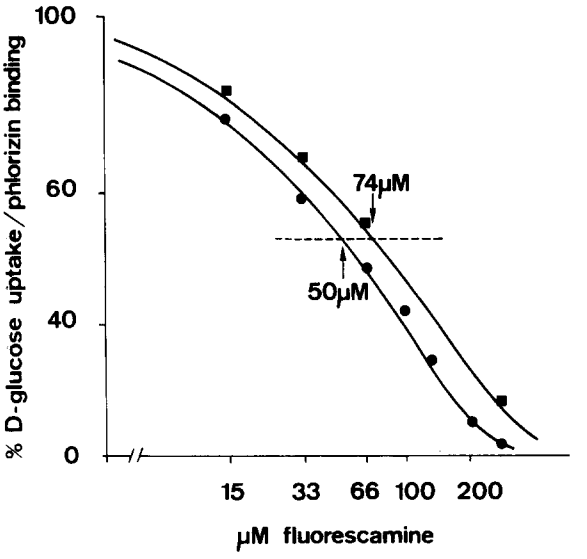

Fig. 2. Inactivation of the Na^+-dependent sugar carrier by fluorescamine. Brush border vesicles were incubated with increasing concentrations of fluorescamine (0–275 μM), washed twice and assayed for Na^+-dependent D-glucose influx (2 s) or Na^+-dependent phlorizin binding (10 s) under equilibrium conditions as described in Methods. The half-maximal inactivation concentrations of 50 μM (phlorizin binding) and 74 μM (D-glucose influx) fluorescamine were derived from the graph. ●——●, Na^+-dependent phlorizin binding; ■——■, Na^+-dependent D-glucose influx.

TABLE II

SUGAR TRANSPORT RATES AFTER MODIFICATION OF BRUSH BORDER VESICLES WITH *N*-ETHYLMALEIMIDE AND 4,4'-DITHIOPYRIDINE

Brush border vesicles (1 mg protein per ml) were first treated with 2 mM *N*-ethylmaleimide (NEM) in 100 mM mannitol, 10 mM Tris-HCl (pH 7.0) at 20°C for 10 min. The reaction was stopped by diluting the incubations with ice-cold 100 mM mannitol, 10 mM Tris-HCl (pH 8.0) and sedimenting the membranes by centrifugation. After resuspension in the same buffer, the vesicles were incubated for another 10 min at 20°C either in the presence or absence of 2 mM 4,4'-dithiopyridine (4-PDS). After a further washing step, D-glucose transport was determined as described in Fig. 1. The means ±S.D. of three experiments are given.

Treatment	Percent inhibition of the Na^+-dependent D-glucose transport
Controls	0
2 mM NEM	11 ± 8
2 mM 4-PDS	46 ± 7
(1) 2 mM NEM (2) 2 mM 4-PDS	51 ± 18

half-maximal inhibition of the D-glucose transport (approx. 74 μM) and of the phlorizin binding (approx. 50 μM) were not significantly different. Complete inactivation was achieved at about 300 nmol fluorescamine per mg protein. No inhibition was observed when fluorescamine had been inactivated (hydrolysis for 5 min) prior to being added to the membrane suspension.

Fluorescamine forms stable and fluorescent adducts with primary amino groups [12]. Although side reactions of fluorescamine with other nucleophiles (like SH-groups) were reported [13], additional data suggested that fluorescamine inactivates the D-glucose transport by reacting with amino groups (see below).

(ii) 4,4'-Diisothiocyano stilbene-2,2'-disulfonic acid (DIDS). Disulfonic stilbenes are potent and specific inhibitors of anion transport in red cells [14,15]. DIDS carries two isothiocyanate groups which are capable of covalent reaction with amino

groups; it was found to react with a critical group of the anion carrier [14,15]. The inhibition of Na^+-dependent phlorizin binding after brush border membranes have been treated with DIDS under various conditions is shown in Table III. As can be seen, DIDS inhibited this binding only if deoxycholate was present during the incubation and the temperature was kept above 4°C. If the reaction with DIDS was performed in the presence of 0.6 mg deoxycholate per mg protein at 26°C, half maximal inactivation of the Na^+-dependent phlorizin binding was reached at ca. 90 nmol DIDS per mg protein. The presence of less deoxycholate (0.1 to 0.3 mg/mg protein) during the modification resulted in a reduction of the inactivation (data not shown). If the reaction was carried out in the absence of detergent, then no decrease in phlorizin binding was observed, independently of the temperature (4°C or 26°C) and of the membrane preparation (intact vesicles or deoxycholate-extracted and washed membranes). This effect of DIDS in the presence of deoxycholate was virtually irreversible. Extensive washing with buffer (in the presence or absence of serum albumin) failed to reverse the inactivation, indicating a covalent reaction of DIDS with the membrane.

TABLE III

EFFECT OF DIDS ON THE Na^+-DEPENDENT PHLORIZIN BINDING

The incubation of intact brush border vesicles, deoxycholate-extracted vesicles and of vesicles plus 0.6 mg deoxycholate (DOC) per mg protein for 15 min at 4°C or 26°C with DIDS and the two washing steps were done as outlined for Table I. Na^+-dependent phlorizin binding (mean ± S.D. of (n) experiments: each experiment was carried out in triplicate) was determined as described in Methods. n.d., not determined.

Modification of	Percent inhibition of the Na^+-dependent phlorizin binding after treatment with DIDS at			
	4°C		26°C	
Intact vesicles with				
50 nmol DIDS/mg protein	2	(2)	5	(2)
100 nmol DIDS/mg protein	8 ± 8	(3)	5	(2)
200 nmol DIDS/mg protein	n.d.		0	
DOC-treated, washed vesicles with				
100 nmol DIDS/mg protein 200 nmol DIDS/mg protein 300 nmol DIDS/mg protein	3 ± 3	(3)	2 ± 3	(3)
Vesicles in the presence of DOC with				
50 nmol DIDS/mg protein	1		n.d.	
75 nmol DIDS/mg protein	n.d.		35	
100 nmol DIDS/mg protein	0		73	
200 nmol DIDS/mg protein	5		76	
250 nmol DIDS/mg protein	n.d.		95	

(iii) Reductive methylation. By reductive methylation with cyanoborohydride, amino groups can be modified specifically and under mild conditions [16]. Both D-glucose transport and phlorizin binding were clearly inactivated after reductive methylation of intact brush border membranes and therefore this result strongly suggested that NH_2-groups were involved in the transport of D-glucose (Table IV). Compared to control vesicles D-glucose uptake was inhibited by 28% and phlorizin binding by 31% after the vesicles have been treated with 30 mM $NaCNBH_3$ and 5.4 mM formaldehyde (Table IV).

(iv) Lack of protection by Na^+/D-glucose or phlorizin. It seems clear, therefore, that in addition to thiols, amino groups also play a functional role in the transport process of D-glucose in the intestine. However, as in the case of the SH-groups there was no substrate protection of these amino groups. In the reactions studied here fluorescamine, DIDS or formaldehyde could not be prevented from reacting with the critical amino groups by adding D-glucose or phlorizin (both in the presence of sodium) to the incubations (data not shown).

The overall kinetic parameters (K_m, V_{max}) of this transport system differ depending on whether they are measured in the presence of an inwardly directed electrochemical gradient of Na^+ ($[Na^+]_{out} \gg [Na^+]_{in}$), or whether they are measured under equilibrium conditions (Refs. 17, 18 and references quoted therein). It could therefore be possible that substrate(s) may protect the carrier from inactivation under one set of conditions, but not under another. Most of the above reagents act relatively slowly and thus equilibrium conditions had to be used. However, an exception is fluorescamine which reacts very rapidely [19] and which therefore can be tested also during the dissipation of an initial $[Na^+]_{out} \rightarrow [Na^+]_{in}$ gradient. When 100 mM NaSCN was added together

TABLE IV

REDUCTIVE METHYLATION OF BRUSH BORDER VESICLES WITH SODIUM CYANOBOROHYDRIDE

Brush border vesicles (1 mg/ml) in 300 mM mannitol, 25 mM phosphate (pH 8.2) were allowed to react for 20 min at room temperature in the indicated sequence with $NaCNBH_3$ and formaldehyde with a washing step between the two incubations. They were subsequently washed twice and examined for Na^+-dependent D-glucose transport activity (2 s initial influx or 2 s influx divided by the 60 min equilibrium value) and for Na^+-dependent phlorizin binding as described in Methods.

Treatment of the vesicles	Initial Na^+-dependent D-glucose flux (2 s) (%)	Initial flux/ Equilibrium (%)	Na^+-dependent phlorizin binding (%)
Controls	100 ± 6	100 ± 6	100 ± 11
First incubation with 30 mM $NaCNBH_3$, second incubation with 5.4 mM formaldehyde	76 ± 6	79 ± 6	87 ± 9
First incubation with 5.4 mM formaldehyde, second incubation with 30 mM $NaCNBH_3$	76 ± 3	84 ± 4	101 ± 11
Single incubation with 30 mM $NaCNBH_3$, and 5.4 mM formaldehyde simultaneously	49 ± 5	59 ± 6	65 ± 12

with the fluorescamine to the intact vesicles, fluorescamine still inactivated the D-glucose transport but again neither D-glucose nor phlorizin afforded any protection.

4. Differentiation between SH- and NH_2-groups

Both SH-reactive and NH_2-reactive reagents inhibited the Na^+-dependent phlorizin binding. However, because of the possibility that these reagents were not reacting specifically, it was necessary to establish whether sulfhydryl groups were the target of both types of reagents. Using $HgCl_2$ as a general and reversible SH-reactive reagent [7] and fluorescamine as the amino-reactive reagent [12,13] we designed the experiments shown in Table V to distinguish between the nucleophiles (SH and NH_2) involved in the transport of D-glucose. If the target of fluorescamine were SH-group(s), it should be possible to protect them from reacting with fluorescamine by using a prior reaction with Hg^{2+}.

Inactivation of the cotransporter by $HgCl_2$ alone is almost totally reversible by dithiothreitol [7]. However, the inactivation of the phlorizin binding was not reverted by dithiothreitol after intact vesicles had been treated either first with fluorescamine and then with $HgCl_2$ or vice versa. Because $HgCl_2$ has a very high affinity for SH-groups [20] and because it was not used in excess over the total number of free SH-groups [6], it could be assumed that under these conditions virtually all free SH-groups were complexed by the Hg^{2+} but that the amino-groups remained unaffected. Further, it seems unlikely that SH-groups

TABLE V

DIFFERENTIATION BETWEEN SH- AND NH_2-GROUPS

As given under Methods, brush border vesicles were incubated in 100 mM mannitol, 25 mM potassium phosphate (pH 7.6) with 66 μM fluorescamine (5 min at room temperature), 25 μM $HgCl_2$ (5 min on ice), 10 mM dithioerythritol (DTE) (10 min at room temperature) in the order indicated. Finally, they were sedimented, washed twice and assayed for Na^+-dependent phlorizin binding. Each determination was carried out in triplicate.

Vesicles treated with	Na^+-dependent phlorizin binding (5 s values) (%)
None	100
66 μM fluorescamine	40
66 μM fluorescamine, then 10 mM DTE	40
25 μM $HgCl_2$	10
25 μM $HgCl_2$, then 10 mM DTE	90
First 66 μM fluorescamine, second 25 μM $HgCl_2$, then 10 mM DTE	27
First 25 μM $HgCl_2$, second 66 μM fluorescamine, then 10 mM DTE	30

blocked by Hg^{2+} would still react with fluorescamine. In fact, protection of SH-groups was reported in Ref. 21, where *p*-chloromercuribenzene sulfonate was able to protect SH-groups from reacting with dinitrofluorobenzene. These results showed that it is possible to differentiate between the SH- and NH_2-groups and therefore led to the conclusion that both nucleophiles are indeed involved (either directly or indirectly) in the Na^+-dependent transport of D-glucose and binding of phlorizin.

Discussion

The small-intestinal Na^+/D-glucose cotransporter is easily inactivated by proteases [5] acting from the cytosolic side of the membrane and by mercurials acting from the cytosolic side and/or the hydrophobic core. This inactivation is much smaller when reagents act from the extracellular, luminal side of the membrane. Thus, the portions of the small-intestinal cotransporter which are exposed on the two sides of the membrane are not identical — or, to put it differently, the insertion of this cotransporter with respect to the plane of the membrane is permanently asymmetric. This important concept (which was not unexpected in view of the current views of the biogenesis and insertion of intrinsic membrane proteins [22–29] and of what is known of other 'simple' carrier transport systems (e.g., Refs. 15, 30–35) has obvious functional implications: a stable structural asymmetry clearly renders most unlikely a diffuse or 'rotative' mode of operation and favours instead a 'channel' or 'pore'.

Furthermore, it would be odd if an asymmetric structure would be functionally totally symmetrical. Indeed, the Na^+/D-glucose cotransporter has been shown recently to display different kinetic properties (K_m values, *trans* inhibition by substrates, etc.) on both sides of the membrane [17,18].

In the present paper we have proceeded with the functional mapping of the reactivity of the Na^+/D-glucose cotransporter and shown that SH-groups other than those reacting with mercurials are also essential for the Na^+-dependent D-glucose translocation or the Na^+-dependent phlorizin binding. We provide also some indication (Table I) that these additional thiols are likely to be located at the extracellular, luminal side (rather than at the cytosolic side) of the membrane.

Thiols are not the only nucleophiles in the Na^+/D-glucose cotransporter whose substitutions lead to irreversible inactivation. One or more amino group is also essential (Fig. 2, and Tables III and IV). As the reagents used in the present work were highly permeant, no information can be provided as to the sidedness of this (these) amino group(s) with respect to the membrane.

No protection was afforded by Na^+/D-glucose or phlorizin against inactivation by the SH-reactive or NH_2-reactive reagents tested. This lack of protection was observed for all reagents in the absence of a membrane potential difference, and for fluorescamine in its presence also. This indicates that the thiols reacting with disulfides (present paper), those reacting with mercurials (Refs. 6,7) and the essential amino groups investigated in the present paper are probably not involved in the binding of either substrate, although they may play a role in the 'translocation step', perhaps via a dithiol-disulfide exchange, as recently suggested for H^+-symport cotransporters [36].

Alternatively, their substitution may induce a non-functional conformational state in the cotransporter which could take place by reaction of the agents within the cotransporter itself or with groups belonging to neighboring components which then in turn affect in the cotransporter.

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