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DISTRIBUTION OF SULFHYDRYL GROUPS IN INTESTINAL BRUSH BORDER MEMBRANES

LOCALIZATION OF SIDE-CHAINS ESSENTIAL FOR GLUCOSE TRANSPORT AND PHLORIZIN BINDING

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Summary

1. Brush border membrane vesicles from rabbit small intestine were found to contain 46 nmol SH groups/mg protein, 52% of which could react with 4,4′-dithiodipyridine, a membrane permeating probe. Only 18% of the total SH-groups reacted with the impermeant probe 5,5′-dithiobis(2-nitrobenzoic acid), indicating that only this fraction is externally located.

2. Brush border membrane vesicles could be disrupted by a gentle treatment with deoxycholate, releasing most of their electron-dense core material. In deoxycholate-treated vesicles most of the SH groups that reacted with 4,4′-dithiodipyridine react with 5,5′-dibiobis(2-nitrobenzoic acid), suggesting that both membrane surfaces became exposed to the extravesicular medium.

3. In intact vesicles (1.2 mg protein/ml), the binding of phlorizin (a competitive inhibitor of the monosaccharide transport system) was 50% inhibited by 67 μM of the penetrating organomercurial *p*-chloromercuribenzoate, but was about ten times less sensitive to the poorly permeating *p*-chloromercuriphenylsulfonate. In contrast, binding of phlorizin to leaky (deoxycholate-treated) membranes was equally susceptible to either reagent.

4. Mercurial inhibition of phlorizin binding could be reversed by dithioerythritol in both sealed and leaky membranes, whereas the less permeant thiol L-glutathione (reduced form) could only revert the inhibition in leaky membranes.

Abbreviations: *p*-Cl-HgBzO$^-$, *p*-chloromercuribenzoate: *p*-Cl-HgPhSO$_3^-$, *p*-chloromercuriphenylsulfonate; DTNB. 5,5′-dithiobis(2-nitrobenzoic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

5. In sealed vesicles, concentrative D-glucose uptake (measured in the presence of a NaSCN gradient) was more sensitive than phlorizin binding to mercurial inhibition. Moreover, *p*-chloromercuribenzoate was only slightly more potent than *p*-chloromercuriphenylsulfonate. This effect of the mercurials is thought to be indirect, due to the collapse of the Na^+ electrochemical gradient that drives concentrative sugar uptake, since

6. D-Glucose transport under equilibrium conditions could be inhibited over 60% by 100 μM *p*-chloromercuribenzoate but was only marginally affected by the same concentration of *p*-chloromercuriphenylsulfonic acid. These effects are comparable to those produced by the mercurials on phlorizin binding.

7. The results are interpreted as indicating that an SH group essential for D-glucose uptake and phlorizin binding is found close to or at the cytoplasmic side of the membrane. In addition, more superficially located SH groups appear to be involved in maintaining the impermeability of the membrane to small ions.

Introduction

D-Glucose is taken up through the brush borders of intestinal epithelial cells by means of a specialized transport system which involves membrane protein(s). This uptake is tightly coupled to the co-transport of Na^+ [1], so that the movement of the cation down its electrochemical gradient provides the driving force for the accumulation of the sugar inside the cell. The concentration of the internalized sugar can thus exceed that of the surrounding medium several times. This monosaccharide uptake system is effectively blocked by micromolar concentrations of the glycoside phlorizin which acts in a competitive fashion [2,3].

Although the particular proteins responsible for the sugar translocation have not yet been identified, attempts have been made to determine the involvement of certain amino acid side-chains in the transport process. This has been studied by measuring the effect on monosaccharide accumulation of a variety of reagents that combine with specific groups of proteins. Schaeffer et al. [4] and Lerner et al. [5] have described that addition of *p*-Cl-$HgPhSO_3^-$ to the mucosal side of intestinal segments reduces sugar uptake; similar results were obtained by Stirling [6] using $HgCl_2$. The effect of both reagents was reversed by dithiothreitol suggesting that the mercurials acted on membrane SH groups [5,6]. These reagents could be affecting the sugar uptake by a direct interaction with the translocating protein, but also by an increase of the membrane permeability and hence a decrease of the Na^+ gradient. Also, the complexity of the preparation used makes the assignment of a precise site of action of the SH reagents very difficult.

An alternative approach which circumvents some of these difficulties is the estimation of the binding of phlorizin to isolated brush borders or to membrane vesicles derived from them. Although no such studies have been performed with intestinal membranes, this type of information is available for renal brush borders, which possess a glucose carrier system analogous to that of the intestine [7]. Binding of the glycoside to isolated renal brush borders was

found to be decreased by *N*-ethylmaleimide [8]; similarly, a partial reduction of phlorizin binding to purified brush border membranes was observed after treatment with *N*-ethylmaleimide [9—11] and more markedly with the organomercurials *p*-Cl-HgBzO$^-$ [10], *p*-Cl-HgPhSO$_3^-$ [9], or mersalyl [11].

It was the aim of this study to establish whether SH groups are also involved in the binding of phlorizin and the transport of D-glucose in intestinal brush border membranes. Furthermore, we wished to investigate the location of the essential SH side-chains of the protein with respect to the plane of the membrane. This latter question was approached in two ways: (a) by using SH reagents of similar reactivity but different ability to penetrate through the membrane, and (b) by comparing the effect of the reagents on sealed vesicles and leaky membranes. The organomercurials *p*-Cl-HgBzO$^-$ and *p*-Cl-HgPhSO$_3^-$ were selected for the first approach, since they share a common reaction mechanism and strong avidity for SH groups, yet they show marked differences in their solubility properties [12]. Since at pH 7 the sulfonated analog is considerably more hydrophilic than *p*-Cl-HgBzO$^-$, its action is restricted to the more superficial SH groups of the membrane [13]. Conversely, *p*-Cl-HgBzO$^-$ — being more lipid soluble — can in addition reach those SH groups that are more deeply embedded in the bilayer and, after crossing the membrane, react with SH groups at the opposite side [13]. For the second approach we have developed a preparation of leaky brush border membranes by treating the vesicles with small amounts of deoxycholate [14]. In these membranes both surfaces become readily available for chemical modification by water-soluble reagents. The susceptibility of their phlorizin binding to the action of the mercurials was compared to that of sealed brush border vesicles.

Materials and Methods

Potassium deoxycholate was prepared from deoxycholic acid (Fluka AG) by neutralization with KOH. [G-^{3}H]Phlorizin (2.06 Ci/mmol) was purchased from New England Nuclear, and D-[1-^{3}H]glucose (8.3 Ci/mmol) from Amersham. *p*-Cl-HgBzO$^-$ (free acid) and *p*-Cl-HgPhSO$_3^-$ (monosodium salt) were obtained from Sigma. Stock solutions of these reagents (5 mM *p*-Cl-HgBzO$^-$ titrated to pH 10 with NaOH, and 10 mM *p*-Cl-HgPhSO$_3^-$) were made in H_2O and stored at 4°C in the dark for not longer than two weeks. A 10 mM stock solution of $HgCl_2$ (Merck) in H_2O, was kept at room temperature. DTNB, 4,4′-dithiodipyridine, and reduced glutathione were purchased from Fluka AG; solutions of these reagents were prepared fresh every time. Dithioerythritol was bought from Sigma.

Brush border membrane vesicles from small intestines of rabbits were prepared according to the method of Schmitz et al. [15] as modified by Kessler et al. [16]. The vesicles were resuspended at a concentration of 20—25 mg protein/ml in a medium containing 300 mM D-mannitol, 10 mM Hepes-Tris pH 7.0 (buffer A) and 1 mM dithioerythritol. 2-ml aliquots were then frozen in solid CO_2/ethanol and kept at —20°C. Inclusion of the thiol was essential for the preservation of the vesicles. The membranes were thawed within a week of preparation by immersion in a 25°C water bath for a few minutes. They were then washed in buffer A to remove all traces of dithioerythritol.

Reaction with mercurials. The reaction of organomercurials with protein SH groups is essentially irreversible ($K_d \leqslant 10^{-20}$ M) [17]. Therefore, the comparison of dose-response curves of two different preparations is valid only if the ratio of membrane protein to reagent is identical in both samples [12]. This was the case in all our experiments, in which the protein concentration was kept constant at 1.2 mg/ml. Intact vesicles or deoxycholate-treated membranes were incubated in buffer A containing the desired concentration of either *p*-Cl-$HgBzO^-$, *p*-Cl-$HgPhSO_3^-$ or $HgCl_2$ on ice for 5 min. Identical results were obtained if the incubation with the mercurial was prolonged for 30 min, indicating that the reaction was already complete at 5 min. The suspensions were then diluted 5—10-fold with buffer A. When indicated, this buffer contained in addition 1 or 5 mM dithioerythritol, or 1 mM glutathione. After 10 min incubation at 0°C the membranes were sedimented at 60 000 × *g* for 30 min and the supernatants were carefully aspirated. The pellets were resuspended in buffer A and used for phlorizin binding or glucose uptake measurements.

Binding and transport measurements. Phlorizin binding was determined essentially as described [14] with membranes equilibrated at room temperature for 30 min with buffer A containing 200 mM of either NaCl or KCl. The binding was started by the addition of 5 μM [G-^{3}H]phlorizin and terminated after 15 s by dilution and filtration. Na^+-dependent phlorizin binding was calculated by subtracting the amount of ligand bound in the presence of K^+ (unspecific binding, see Ref. 14) from that bound in the presence of Na^+ (total binding). Only the Na^+-dependent component of the binding, which is thought to represent interaction of the glycoside with the glucose carrier, is reported. In some preparations the reagents produced changes in the unspecific phlorizin binding; however, these are not reported since the origin of this binding and its variations are unknown.

Two types of glucose transport measurements were performed: Concentrative glucose uptake, measured in the presence of an inwardly directed NaSCN gradient, and uptake under equilibrium conditions (tracer exchange) in which case the medium composition was the same inside and outside the vesicles. Concentrative uptake determinations were performed at room temperature as described before [16] using 0.1 mM D-[1-^{3}H]glucose in buffer A at pH 7.0. Tracer exchange measurements were performed after equilibrating the vesicles (20 mg protein/ml) in buffer A with 0.1 mM D-glucose and either NaCl or KCl (0.1 M) for 30 min at room temperature. D-[1-^{3}H]glucose uptake was initiated by mixing 10-μl aliquots of the suspension with 10 μl of the same buffer containing 1 μCi of the isotope. Transport was stopped at the indicated times by dilution with 3 ml of ice-cold 250 mM KCl, 1 mM Tris-HCl (pH 7.0) followed by filtration in Sartorius filters (0.6 μm pore size). These were washed with 5 ml of stopping solution and counted. Protein was measured as in [18] using the Bio Rad reagent.

Determination of total and exposed membrane SH groups. Two reagents (DTNB and 4,4′-dithiodipyridine) were used for this purpose. DTNB (2 mM) was dissolved in buffer A and the pH was adjusted back to 7.0 with Tris base. A nominally 2-mM solution of 4,4′-dithiodipyridine was made by vigorously stirring the reagent in buffer A for 30 min at room temperature, followed by sedimentation of the undissolved reagent in a clinical centrifuge. Exposed SH

groups were determined incubating the membranes (1—3 mg protein/ml) in the reagent solutions for 10 min at room temperature. The membranes were cooled in an ice bath, sedimented at 60 000 × *g* for 30 min and the absorbance of the supernatants was measured against reagent blanks. The total number of SH groups was determined in membranes suspended in reagent solutions to which SDS (1% final concentration) was added, and omitting the sedimentation step. After incubating at least 20 min at room temperature the absorbance of the solubilized preparations was measured against reagent blanks containing SDS. Any residual contribution of light scattering was subtracted. The concentration of SH groups was calculated using extinction coefficients of 13 600 $M^{-1} \cdot cm^{-1}$ at 412 nm for DTNB [17] and 21 300 $M^{-1} \cdot cm^{-1}$ at 324 nm for 4,4′-dithiodipyridine.

Results

Effect of deoxycholate on brush border vesicle morphology

Brush border membrane vesicles prepared by the method of Kessler et al. [16] are known to preserve their native right side-out configuration and to be more than 90% sealed, based on the resistance of internally trapped actin to proteolytic attack [14]. Fig. 1A shows the morphology of these vesicles, which has been described in detail before [16]. Particularly important for the present argument is the presence of a large amount of electron dense material in the intravesicular space, which is thought to originate from the actin filaments that constitute the matrix of the microvilli [20]. Fig. 1B shows that the dense core is lost from the vesicles upon deoxycholate extraction. In addition, small membrane fragments and discontinuities in the profile of some vesicles are also evident. We interpret these observations as indicating that membrane continuity is lost upon detergent treatment, allowing the release of the actin-rich core. This interpretation is in agreement with previous biochemical studies which showed that deoxycholate treatment releases most of the M_r = 45 000 'actin-like' protein from the vesicles and renders the remaining fraction susceptible to proteolysis and to extraction by mild perturbants [21]. Also, the ability of the vesicles to retain glucose is lost [14]. Thus, both types of evidence indicate that, as opposed to intact vesicles which are sealed, deoxycholate-treated vesicles are leaky and expose both sides of the bilayer to the external medium.

Total and exposed SH groups of intact and deoxycholate-treated membranes

Two different reagents, DTNB and 4,4′-dithiodipyridine, were used to determine the degree of exposure and the total content of SH groups in normal and deoxycholate-extracted membranes. Both react with membrane SH groups by a similar mechanism (i.e., disulfide-sulfhydryl exchange reactions) but they differ in their ability to traverse biological membranes. Thus, Ellman's reagent (DTNB) which is negatively charged at physiological pH, is thought to permeate membranes only very slow, if at all [22,23], whereas 4,4′-dithiodipyridine, which is neutral, quickly traverses lipid bilayers [23]. Table I shows the results of these determinations. A similar fraction of the SH groups could react with 4,4′-dithiodipyridine in both types of membranes prior to solubiliza-

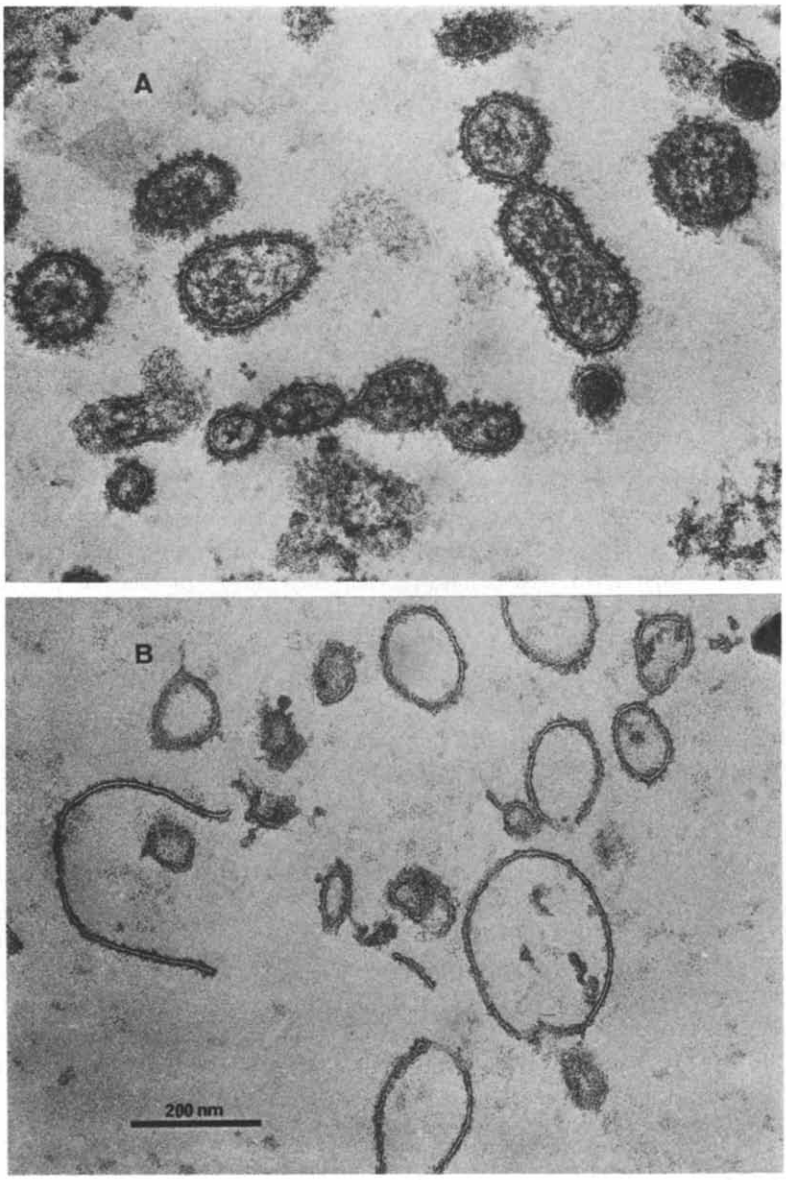

Fig. 1. Electron micrographs of thin sections of brush border membranes. Bursh border membranes or deoxycholate-treated membranes were rapidly frozen using a propane-jet freezing method; freeze-substitution was performed in methanol containing osmium tetroxide and uranyl acetate (Müller, M. et al., submitted for publication). (A) Intact brush border vesicles. Note the presence of considerable amounts of electron-dense material in the intravesicular space. (B) Deoxycholate-treated brush border membranes. The dense core material has been largely removed and discontinuities are observed in the vesicular profile. ×119 700.

tion in SDS (last column, Table I). This fraction (about one half of the total) can be defined as the complete set of SH groups which are 'reactive' in the native proteins (see Discussion). In contrast, only one-third of these 'reactive' SH groups were detected by DTNB in the vesicles whereas a full 80% was

TABLE I

ACCESSIBILITY OF MEMBRANE SULFHYDRYL GROUPS TO PERMEANT AND IMPERMEANT REAGENTS

Total SH groups were determined after dissolving the membranes (1—3 mg protein/ml) in 1% SDS. Exposed SH groups were determined in the absence of detergents as described under Methods. The values are the mean ± one S.E. of the number of determinations shown in parenthesis.

Preparation	Reagent	Total SH groups (nmol/mg protein)	Exposed SH groups (nmol/mg protein)	%
Sealed brush border vesicles	DTNB	38.6 ± 0.9 (8)	7.2 ± 0.6 (6)	18.6
	4,4'-Dithiodipyridine	46.0 ± 0.8 (8)	24.1 ± 1.0 (5)	52.2
Deoxycholate-treated membranes	DTNB	39.0 ± 1.8 (8)	16.2 ± 0.9 (8)	41.5
	4,4'-Dithiodipyridine	53.7 ± 1.3 (8)	28.4 ± 1.0 (7)	52.9

accessible to this reagent in deoxycholate-treated vesicles, as would be expected for leaky membranes.

The effect of mercurials on phlorizin binding

Intact vesicles. Treatment of brush border vesicles with *p*-Cl-HgBzO$^-$ resulted in a concentration-dependent inactivation of the Na^+-dependent binding of phlorizin (Fig. 2A). Half maximal inhibition was achieved at 67 μM when the protein concentration was 1.2 mg/ml. Most of the inhibition could be reversed by subsequent addition of a molar excess of dithioerythritol, particularly at the lower inhibitor concentrations. Only two thirds of the binding could be recovered by addition of the thiol after 500 μM *p*-Cl-HgBzO$^-$ was used, suggesting that irreversible denaturation of some phlorizin binding proteins took place at this concentration. The less permeant *p*-Cl-HgPhSO$_3^-$ was markedly less effective as an inhibitor of phlorizin binding to intact vesicles than *p*-Cl-HgBzO$^-$ (Fig. 2B). Concentrations in excess of 500 μM were required for half maximal inhibition. The inhibition was also partly reversed by dithioerythritol.

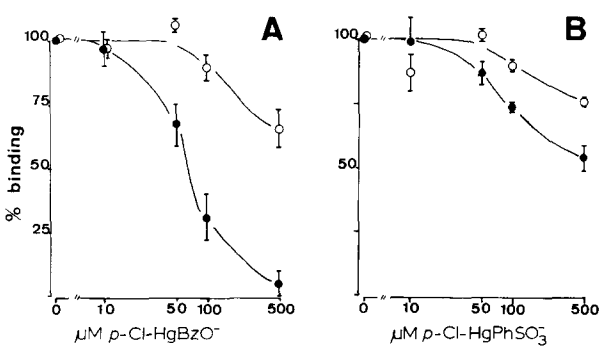

Fig. 2. (A) Concentration dependence of the inhibition of Na^+-dependent phlorizin binding to intact brush border vesicles by *p*-Cl-HgBzO$^-$. Membranes (1.2 mg protein/ml) were treated for 5 min at 0°C with the concentration of the mercurial shown in the abscissa (log scale), and then diluted with 5—10 vols. ice-cold buffer A without (•) or with (○) 5 mM dithioerythritol, and centrifuged after a further 10 min incubation at 0°C. The points are the mean ± one S.E. of three experiments performed in duplicate. (B) Concentration dependence of the inhibition of Na^+-dependent phlorizin-binding to intact brush border vesicles by *p*-Cl-HgPhSO$_3^-$. All other details as in (A).

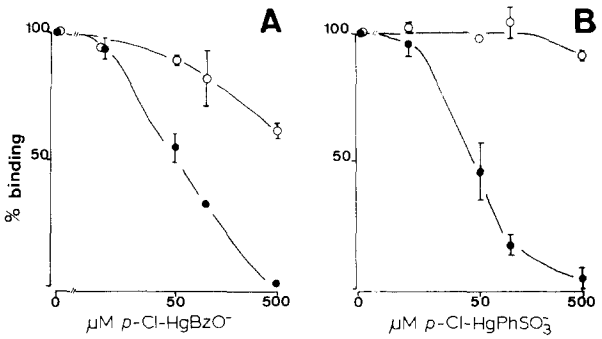

Fig. 3. (A) Concentration dependence of the inhibition of Na^+-dependent phlorizin binding to deoxycholate-treated membranes by *p*-Cl-HgBzO$^-$. Deoxycholate-treated membranes (1.2 mg protein/ml) were treated for 5 min at 0° C with the concentration of the mercurial shown in the abscissa (log scale), and then diluted with 5—10 vols. ice-cold buffer without (•) or with (○) 5 mM dithioerythritol, and centrifuged after a further 10 min incubation at 0° C. The points are the mean ± S.E. of three experiments performed in duplicate. When not illustrated, the S.E. was smaller than the symbol. (B) Concentration dependence of the inhibition of Na^+-dependent phlorizin binding to deoxycholate-treated membranes by *p*-Cl-HgPhSO$_3^-$. Other details as in (A).

Deoxycholate-treated membranes. In leaky membranes, the susceptibility of phlorizin binding to inhibition by *p*-Cl-HgBzO$^-$ was comparable to that found in sealed vesicles (compare Figs. 3A and 2A). Binding was 50% inhibited by 57 μM *p*-Cl-HgBzO$^-$ and substantial reversal by dithioerythritol was observed at the lower mercurial concentrations, whereas only 60% of the original binding could be recovered by addition of the thiol after 500 μM *p*-Cl-HgBzO$^-$ was employed. The curve relating *p*-Cl-HgPhSO$_3^-$ concentration to binding inhibition in deoxycholate-treated membranes was not significantly different from that obtained using *p*-Cl-HgBzO$^-$ (compare Figs. 3A and B). This is in contrast with the marked differential sensitivity of intact vesicles towards these mercurials. Again, dithioerythritol managed to reverse most of the inhibition.

These observations can be explained by postulating the existence in the phlorizin binding protein of a critical SH group which is not readily accessible from the outer milieu. In agreement with this suggestion, we found that $HgCl_2$, which is thought to permeate membranes freely [12], was also a powerful inhibitor of phlorizin binding. Moreover, no differences were observed in the susceptibility of sealed and leaky membranes to this agent.

Reversal of mercurial inhibition of phlorizin binding by different thiols

Assuming that the critical SH group which is being blocked is not immediately accessible from outside the vesicles, it is expected that the inhibition provoked by a penetrating mercurial should be reversed by permeating but not by impermeant thiols. To test this prediction we inhibited the binding of phlorizin to intact vesicles with $HgCl_2$ and measured the ability of dithioerythritol and glutathione to displace the inhibitor (Table II). Intestinal membranes are resistant to penetration by extracellular glutathione [24] while they are generally believed to be permeable to small molecular weight thiols such as dithioerythritol [5]. To minimize splitting of glutathione to the more penetrating products cysteinylglycine or cysteine, the vesicles were exposed to the thiol at low temperature (0—4° C) and for a short time (10 min). As expected,

TABLE II

REVERSIBILITY OF MERCURIAL-INHIBITED PHLORIZIN BINDING BY TWO DIFFERENT THIOLS

Membranes (1.2 mg/ml) were incubated with 0.05 mM $HgCl_2$ for 5 min at ice temperature in mannitol-Tris-Hepes buffer, pH 7.0; then diluted with 5—10 vols. of buffer with or without the indicated thiol (1 mM); spun down and tested for phlorizin binding. n = number of experiments.

	% Na^+-dependent binding ($\bar{x} \pm$ S.E.)
Sealed vesicles	
Control	100 (5)
$HgCl_2$	0 ± 0 (5)
$HgCl_2$, then dithioerythritol	79.2 ± 5.0 (5)
$HgCl_2$, then glutathione	10.7 ± 6.4 (4)
Deoxycholate-treated membranes	
Control	100 (4)
$HgCl_2$	3.2 ± 2.2 (4)
$HgCl_2$, then dithioerythritol	88.2 ± 9.5 (4)
$HgCl_2$, then glutathione	80.7 ± 13.8 (4)

dithioerythritol was considerably more effective than the same concentration of glutathione, which produced only a marginal reversal.

The inhibition of leaky membranes by mercurial treatment was also found to be reversed by dithioerythritol (lower half of Table II). Moreover, glutathione was practically equipotent with dithioerythritol in these membranes, supporting the notion that the relevant site faces the intravesicular medium, which in the case of the deoxycholate-treated membranes is connected to the exterior through discontinuities in the bilayer.

Effect of mercurials on D-glucose transport

Phlorizin is believed to interact with the D-glucose transport protein at two distinct sites: a sugar-binding site, which is involved in the translocation of monosaccharides, and an aglycone-binding site, which is probably hydrophobic [3]. In addition, the protein displays a Na^+-binding site which is also involved in phlorizin binding. It is expected that modifications of either the sugar or Na^+ sites which bring about inactivation of glycoside binding will also inactivate transport of monosaccharides. Conversely, changes in the aglycone-binding moiety should not necessarily result in transport inhibition. To gain insight about the site of action of the mercurials, we measured their effect on D-glucose uptake by intact vesicles. In this system, the uptake is generally measured during the imposition of a transmembrane electrochemical gradient of Na^+ [25,16]. The coupled influx of Na^+ and glucose leads to a transient accumulation of the latter inside the vesicles, a phenomenon which is known as overshoot. Fig. 4 shows the effect of increasing concentrations of p-Cl-$HgBzO^-$ on the uptake of D-glucose when a NaSCN gradient was imposed. Low concentrations of the mercurial reduced the magnitude of the overshoot (which is an indication of the flux of glucose) but did not alter the final equilibrium value (which is a measure of the internal volume) indicating that the vesicles were not disrupted. Higher mercurial concentrations increased the leakiness of the membranes (judged again by their ability to retain monosaccharides). In three

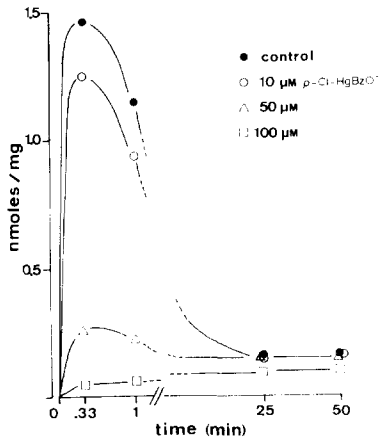

Fig. 4. Effect of increasing concentrations of *p*-Cl-HgBzO$^-$ on the uptake of D-glucose by intact brush border vesicles in the presence of a NaSCN gradient. Vesicles (1.2 mg protein/ml) were treated with the concentration of the mercurial indicated and centrifuged after dilution with buffer A as described under Methods. The pellets were resuspended and used for net D-glucose uptake determinations in the presence of an inwardly directed electrochemical gradient of Na^+. Abscissa: time in min. Ordinate: nmol D-glucose uptake/mg protein.

similar experiments, 100 μM *p*-Cl-HgBzO$^-$ reduced the trapped volume by less than 25% and 500 μM by about 50%.

Contrary to what was observed for the inhibition of phlorizin binding, *p*-Cl-HgPhSO$_3^-$ was almost as effective as *p*-Cl-HgBzO$^-$ to inhibit the uptake of D-glucose. The overshoot was 50% inhibited by 23 μM *p*-Cl-HgBzO$^-$ or by 42 μM *p*-Cl-HgPhSO$_3^-$. Moreover, both values are significantly ($P < 0.05$) lower than those found for the inhibition of phlorizin binding.

Since a gradual increase in the unspecific permeability to D-glucose was noted at high concentrations of both mercurials, it is conceivable that lower concentrations could increase the permeability to smaller particles, such as inorganic ions *. This would produce a quick collapse of the electrochemical Na^+ gradient, which is the force that drives the concentration of glucose above equilibrium levels during the overshoot. We tested this possibility by measuring the uptake of glucose under conditions of equilibrium (i.e., no glucose, Na^+ or potential gradients were present), and the results are shown in Fig. 5. Tracer uptake was determined in membranes equilibrated with NaCl or with KCl, to enable us to estimate the fraction of the flux that took place via the Na^+-dependent cotransport system. As expected, the concentration of D-glucose in the vesicles never exceeded the final equilibrium concentration, which was the same in Na^+ or K^+ media. The initial rate of tracer uptake was several times higher in Na^+ than in K^+ media, and only the Na^+-dependent part of the uptake was altered by 100 μM *p*-Cl-HgBzO$^-$ (Fig. 5). In six experiments

* In fact, SH reagents such as *N*-ethylmaleimide or cupric phenanthroline have been found to enhance the Na^+ permeability of these membranes (Biber, J., Ph.D. Thesis, Eidgenössische Technische Hochschule, Zürich).

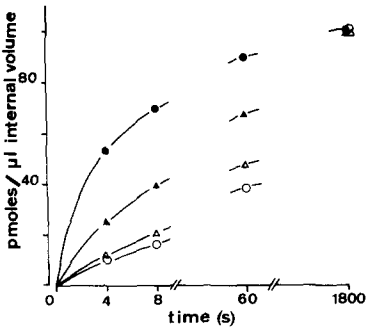

Fig. 5. Effect of 100 µM *p*-Cl-HgBzO$^-$ on the uptake of D-glucose by vesicles under equilibrium conditions. Vesicles (1.2 mg protein/ml) were incubated with (triangles) or without (circles) 100 µM PCMB as described, diluted with buffer A and centrifuged. The pellets were resuspended in buffer A and then equilibrated with 0.1 mM D-glucose and 100 mM of either NaCl (filled symbols) or KCl (open symbols) for 30 min at room temperature. Finally, D-[1-^{3}H]glucose uptake was measured under equilibrium conditions as described in Methods. Abscissa: time course in s. Ordinate: pmol D-glucose uptake/µl intravesicular volume. The latter was calculated from the amount of isotope trapped at 1800 s. The points are the mean of duplicate determinations from a typical experiment.

like the one illustrated in Fig. 5, we compared the efficacy of 100 µM *p*-Cl-HgBzO$^-$ or *p*-Cl-HgPhSO$_3^-$ on the uptake of glucose measured at 4 s under equilibrium conditions. At this concentration, the mercurials produced remarkably different effects on the binding of phlorizin (Fig. 2) while their effects on the overshoot were found to be similar, i.e., essentially complete inhibition. The Na^+-dependent part of the tracer uptake was reduced to 38.2 ± 7.1% of the control flux by *p*-Cl-HgBzO$^-$, a value consistent with the effect of this mercurial on phlorizin binding. Strikingly, *p*-Cl-HgPhSO$_3^-$ had only an insignificant effect on the uptake, which was still 93.4 ± 6.7% of the control. This is in sharp contrast with the effect of the same concentration of this reagent on the overshoot. Neither reagent significantly affected the unspecific (Na^+-independent) uptake of glucose.

Discussion

Morphological and biochemical evidence has been presented demonstrating that brush border vesicles become leaky after treatment with deoxycholate. Whether the detergent also caused major changes in the structure and arrangement of membrane components is at present unknown, but measurements of the interaction of phlorizin with DOC-treated membranes showed that various parameters of the binding were unaffected [21], supporting the view that no drastic changes occurred. Therefore, a short treatment of the vesicles with deoxycholate provides a useful preparation in which both sides of the membrane are exposed.

The data of Table I can be rationalized by defining the existence of four different populations of SH groups in the brush border membrane: (a) extravesicular groups, which are accessible to non-penetrating reagents in sealed membranes; (b) intravesicular groups, which can be reached by non-penetrating reagents only in leaky membranes; (c) groups located inside the bilayer, which

will only react with lipid-soluble reagents. These three groups can be collectively termed 'reactive' SH groups. (d) 'Buried' or 'cryptic' sites which cannot be reached unless the membrane is disrupted and the proteins denatured by strong surfactants. This latter group constitutes about 50% of the total SH group content.

The total SH group content of the vesicles in SDS solution was slightly but significantly higher when determined with 4,4'-dithiodipyridine than with DTNB. This can be explained if the negatively charged DTNB were unable to reach some of the SH groups which remain in hydrophobic parts of the protein-detergent complexes. The difference between the reagents was even more noticeable in the case of the deoxycholate-extracted membranes, which are enriched in integral, and therefore presumably more hydrophobic, proteins.

All the 'reactive' SH groups are expected to interact with 4,4'-dithiodipyridine in both types of membranes. Under the conditions used, they constitute roughly 50% in both membranes. Abouth 80% of the 'reactive' groups were also detected by DTNB in deoxycholate-extracted membranes, but only about one third reacted in intact vesicles. This implies that most of the 'reactive' sites are located at the inner surface of the vesicles, with only a small fraction available from the outside, and about 20% within the bilayer.

The inhibition of phlorizin binding and sugar uptake brought about by mercurials is most probably due to their interaction with SH side-chains of proteins. Although mercurials can form stable complexes with a variety of groups present in proteins and lipids, they react almost exclusively with SH groups when their molar concentrations are similar [12,17]. In the present work, substantial inhibitions were observed at mercurial concentrations of between 10 and 100 μM, when the membranes were present at a concentration of 1.2 mg protein/ml, which corresponds to about 60 μM SH groups (see Table I). In addition, the inhibition was largely reversed by added thiols, which are expected to displace the mercurials from membrane mercaptides.

The SH groups involved in the inhibition of the sugar carrier are not readily accessible from the outer milieu; penetrating reagents or disrupted membranes need be used to achieve inhibition. A similar conclusion was reached by Lerner et al. [5] as well as by Schaeffer et al. [4] when comparing the sensitivity of the amino acid and sugar transport-systems of intestinal segments towards SH reagents. Whether the relevant groups are located within the bilayer or facing the cytoplasmic medium has not been defined. However, the latter possibility seems more likely, in view of the increased inhibitory potency of p-Cl-$HgPhSO_3^-$ when applied to leaky membranes, and of the ability of the rather large thiol glutathione to reverse the inhibition in the same membranes. This interpretation is consistent with the earlier proposal [14] that the glucose carrier is an asymmetric transmembrane protein which exposes to the cytoplasmic medium a portion that is relevant to its transport function. Fragmentation of this cytoplasmic portion by trypsin eliminates the Na^+-dependent binding of phlorizin [14].

The small inhibitory effect of p-Cl-$HgPhSO_3^-$ on phlorizin binding to sealed vesicles can be attributed to either slow penetration of the reagent or, more likely, to the prior breakdown of some vesicles (see above), as a result of mercurial reaction with external groups. It is this reaction of p-Cl-$HgPhSO_3^-$

with extravesicular SH groups which may increase the ionic conductance and consequently collapse the electrochemical gradient that normally drives the overshoot.

Phlorizin is generally believed to bind at the outer surface of the brush border membrane [26], although the evidence supporting this contention is not yet conclusive. Because the SH group that is affected by the mercurials is not located at this surface, it appears that the reaction takes place at a site other than the glycoside-binding site. Preliminary experiments in which the inhibition of the binding by mercurials could not be protected by the presence of glycoside agree with this interpretation. Hence, a long range conformational change originating near the inner surface of the membrane seems to abolish the binding at the opposite surface.

Finally, given the relatively low concentrations of mercurial required for inhibiting glucose translocation and phlorizin binding, it is pertinent to recommend their use for the labelling and eventual identification of the molecular component responsible for monosaccharide transport.

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