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INTERACTION OF THE SUGAR CARRIER OF INTESTINAL BRUSH-BORDER MEMBRANES WITH $HgCl_2$

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Summary

$HgCl_2$ was used as an inhibitor and potential label for the glucose carrier of intestinal brush-border membranes. Half-maximal inhibition of Na^+-dependent D-glucose uptake was reached with micromolar concentrations of $HgCl_2$ when the protein concentration was 1.2 mg/ml. Similar concentrations were found to inhibit the binding of [^{3}H]phlorizin, a reversible competitive inhibitor of sugar transport. Inhibition was reversed by dithioerythritol but only marginally by EDTA. The data support the involvement of a sulfhydryl group in the inhibitory process.

Deoxycholate-extracted membranes, which are enriched in specific phlorizin binding activity, were used for labeling studies using $^{203}HgCl_2$. The polypeptides were separated by gel electrophoresis and analyzed by protein staining and autoradiography. Non-specific $^{203}HgCl_2$ labeling was minimized by pre-treatment with sulfhydryl reagents which do not inhibit phlorizin binding. Several bands, which are lost from the autoradiographic pattern during a negative purification of the phlorizin binding sites, could be ruled out as essential components of the sugar carrier. The polypeptide profile was also analyzed following proteolysis, which abolished phlorizin binding. Those radioactive bands of which apparent M_r values were altered by the treatment were considered as possible candidates. Finally, samples in which inhibition was reversed by thiols were also studied. The possible identity of the polypeptide(s) involved in glucose translocation is discussed in the light of these observations.

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Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoate); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

Introduction

D-glucose is transported across the brush-border membrane of epithelial cells from the small intestine by an Na^+-dependent system [1] which can be inhibited by the glucoside, phlorizin, in a competitive manner [2,3]. Research on the molecular mechanism of the transport process has been greatly stimulated in recent years by the availability of vesicular preparations of brush-border membranes [4,5]. In particular, substantial knowledge has been accumulated regarding the kinetic properties of the sugar transport and its inhibition by phlorizin [5—7]. However, little is known about the identity of the proteins responsible for the transport and the nature of the chemical groups involved. At least part of the difficulty lies in the fact that the sugar carriers are extremely scarce in this membrane. From [^{3}H]phlorizin binding studies it has been calculated that only 10—40 pmol binding sites are present per mg protein [6]. Thus, if an M_r of 100 000 is arbitrarily assumed, these phlorizin binding sites would constitute a maximum of 0.4% (w/w) of the protein.

The involvement of sulfhydryl groups in this transport process is reasonably well substantiated. Stirling [8], Schaeffer et al. [9] and Lerner et al. [10] found mercurials to inhibit sugar uptake into intestinal segments, and Klip et al. [11] found *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulfonate to be powerful inhibitors for D-glucose uptake and phlorizin binding in brush-border membrane vesicles. These observations prompted several investigators to attempt the identification of the transport protein(s) by labeling with radioactive *p*-chloromercuribenzoate followed by electrophoretic analysis [12,13]. However, *p*-chloro[^{203}Hg]mercuriphenylsulfonate is commercially available only at rather low specific activities (10—100 mCi/g Hg, Amersham). Considering the density of the carriers in the membrane (see above) and assuming a 1 : 1 stoichiometry for the reaction of *p*-chloromercuriphenylsulfonate with the carrier, one can calculate how much radioactivity is associated with the transport protein. Thus, when a 100 μg protein sample is used for gel electrophoresis, a maximum of 6—60 dpm will be associated with the inhibitory site. This amount of radioactivity is difficult to determine accurately with the detection methods currently available.

We report here an attempt to label the intestinal sugar carrier based on a combination of selective enrichment and labeling techniques. The present approach has the following advantages: (i) The inorganic mercuric ion (referred to hereafter as $HgCl_2$, even though a variety of stable complexes with Cl^-, OH^- and other anions co-exist in aqueous solution) was used instead of organomercurials. $HgCl_2$ was found to be a more powerful inhibitor of glucose uptake and phlorizin binding than either *p*-chloromercuribenzoate or *p*-chloromercuriphenylsulfonate, and its radioactive isotope is available at much higher specific activities. (ii) A membrane preparation enriched in phlorizin binding sites was used. Enrichment was achieved by controlled extraction with deoxycholate. (iii) The labeling procedure was correlated with phlorizin binding and D-glucose transport measurements. Therefore, optimal conditions could be used for the gel electrophoretic analysis.

Materials

$^{203}HgCl_2$ (1.4 Ci/g Hg) and [G-^{3}H]phlorizin (2.06 Ci/mmol) were purchased from New England Nuclear and D-[1-^{3}H]glucose (8.3 Ci/mmol) from Amersham. A 10 mM stock solution of $HgCl_2$ (Merck) was kept at room temperature. Solutions of *N*-ethylmaleimide (Fluka), 5,5′-dithiobis-(2-nitrobenzoic acid) (Fluka) and diazinedicarboxylic acid bis-dimethylamide (Calbiochem) were prepared fresh every day. A 5% (w/v) potassium deoxycholate solution (pH 9.0) was prepared from deoxycholic acid (Fluka) and kept at 4°C. The molecular weight standard proteins were from Boehringer, Mannheim. Trypsin and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone and dithioerythritol were from Sigma.

Methods

Brush-border membranes from rabbit small intestine were prepared according to the method of Schmitz et al. [4] as modified by Kessler et al. [14]. The final vesicles were frozen in solid CO_2/C_2H_5OH at a protein concentration of 20—25 mg/ml in 300 mM mannitol, 10 mM Hepes-Tris, pH 7.0 (buffer A) and 1 mM dithioerythritol and stored at —20°C. The membranes were thawed at 25°C within one week of preparation and washed in buffer A to remove all traces of dithioerythritol. When indicated, the vesicles were treated with deoxycholate at a ratio of 0.65 mg deoxycholate/mg protein as previously described [15]. When required, the deoxycholate-extracted membranes were subjected to further purification by alkaline treatment. The pelleted membranes were resuspended to a final concentration of approx. 1 mg/ml in an ice-cold solution of 300 mM mannitol containing enough NaOH to bring the pH up to 12. After 5 min of incubation at 0°C, the membranes were sedimented and resuspended in buffer A, washed once and finally resuspended in buffer A to approx. 10 mg protein/ml. For proteolysis, deoxycholate-treated membranes (1.2 mg protein/ml in buffer A) were exposed to trypsin (1 : 50, w/w, enzyme to membrane protein) at room temperature for 10 min. The reaction was stopped with 5 vols. buffer A containing 1 mM N-α-*p*-tosyl-L-lysine chloromethyl ketone followed by sedimentation of the membranes. Protein was determined according to the method of Bradford [16] using the Bio-Rad reagent.

Treatment with sulfhydryl group reagents. In all the incubations described below, the final concentration of membrane protein was 1.2 mg/ml. Intact vesicles or deoxycholate-treated membranes were incubated with the indicated concentrations of $HgCl_2$ or $^{203}HgCl_2$ in buffer A on ice for 5 min. The suspensions were then diluted with 5 vols. ice-cold buffer A, followed by centrifugation at 60 000 × *g* for 30 min. When indicated, the diluting solution contained, in addition, either 1 mM EDTA or 5 mM dithioerythritol.

Reaction of deoxycholate-extracted membranes with the appropriate concentrations of *N*-ethylmaleimide, DTNB or diazinedicarboxylic acid bis-dimethylamide was performed in buffer A at room temperature for 10 min. This was followed by a 5-fold dilution with ice-cold buffer A and sedimentation of the membranes. The pelleted membranes were finally resuspended in

buffer A to a final concentration of 10—20 mg protein/ml and used for binding or transport measurements and gel electrophoresis.

Binding and transport measurements. Phlorizin binding was determined as described [6,7,17] using membranes equilibrated at room temperature with buffer A containing 200 mM of either NaCl or KCl. The incubation was started by the addition of 5 μM [^{3}H]phlorizin (final concentration) and terminated after 15 s by dilution and filtration. The specific Na^+-dependent phlorizin binding was calculated by subtracting the amount of phlorizin bound in the presence of K^+ ('non-specific' binding, see Ref. 17) from that bound in the presence of Na^+ (total binding). D-Glucose transport was measured under two different conditions: (a) concentrative uptake was performed in buffer A at room temperature as described in Ref. 14, using 0.1 mM D-[1-^{3}H]glucose and 100 mM NaSCN; (b) tracer-exchange measurements were performed after equilibrating the vesicles in buffer A with 0.1 mM D-glucose and 100 mM of either NaCl or KCl at room temperature for 30 min. Transport was initiated by mixing 10-μl aliquots of the membrane suspension with 10 μl of the same buffer containing 1 μCi D-[^{3}H]glucose. The incubation was stopped at the desired times by dilution with 3 ml ice-cold stop solution (250 mM KCl/ 1 mM Tris-HCl, pH 7.0) followed by filtration.

Determination of sulfhydryl groups. The total amount of sulfhydryl groups was determined essentially by the method of Ellman [18]. The membranes (0.5 to 1.0 mg protein/ml) were dissolved in a solution containing 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM DTNB and 1% SDS. After 5 min the reaction was completed and the absorbance at 412 nm determined. After subtracting the contributing light-scattering, the amount of sulfhydryl groups was calculated using an extinction coefficient of 13 600 $M^{-1} \cdot cm^{-1}$.

SDS-polyacrylamide gel electrophoresis. This was performed on 1 mm thick, 8.5% slabs according to Laemmli [19]. The solubilizing mixture contained 2% SDS and 5 mM *N*-ethylmaleimide but was devoid of β-mercaptoethanol (see Results). After boiling for 90 s, 50 μg protein were applied to each slot. The gels were calibrated using rabbit phosphorylase *a* (M_r 92 500), bovine serum albumin (M_r 67 500), ovalbumin (M_r 45 000) and bovine chymotrypsinogen (M_r 25 000) as standards. For autoradiography, the Coomassie blue-stained gels were dried under vacuum and exposed to Kodirex X-ray film at room temperature for at least 10 h.

Results

1. Effect of $HgCl_2$ on D-glucose uptake

When an inwardly-directed electrochemical gradient of Na^+ is applied across the membrane of brush-border vesicles, D-glucose is transiently concentrated in the intravesicular space. This phenomenon, known as 'overshoot', is brought about by the coupling of glucose flux to the Na^+ gradient, which provides the energy for the uphill accumulation of the sugar. Eventually, the Na^+ gradient is dissipated and the glucose concentration inside the vesicles approaches that of the surrounding medium [5,14]. Fig. 1 shows the time course of glucose uptake when a NaSCN gradient was applied across the membrane. The effect of $HgCl_2$ on glucose accumulation was tested by pre-treating the membranes

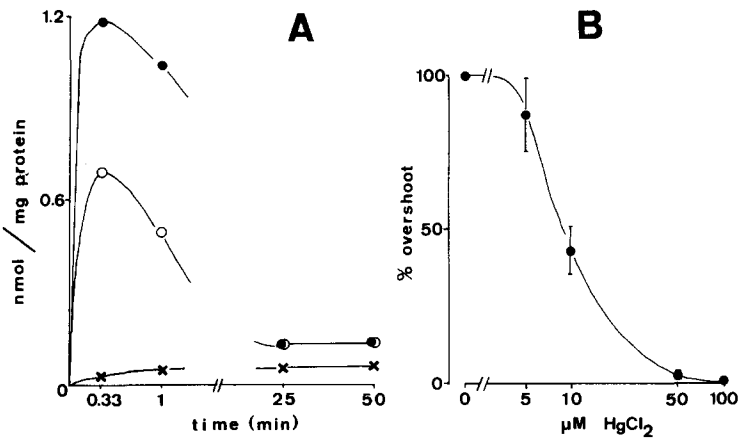

Fig. 1. (A) Effect of increasing $HgCl_2$ concentrations on the concentrative uptake of D-glucose by brush-border vesicles. Vesicles (1.2 mg protein/ml) were incubated without (•) or with 10 μM (○) or 50 μM $HgCl_2$ (×) for 5 min at 0°C and centrifuged after dilution. The resuspended pellets were used for D-glucose uptake determinations in the presence of an inwardly-directed NaSCN gradient. The points are the mean of duplicate determinations from a representative experiment. (B) Concentration dependence of the inhibition of concentrative D-glucose uptake by $HgCl_2$. The magnitude of the overshoot in vesicles, pre-treated at 1.2 mg protein/ml with various concentrations of $HgCl_2$, was determined at 20 s in experiments like that shown in A. The points are the mean ± S.E. of three experiments. When not indicated, the error bars were smaller than the symbols.

with various concentrations of the agent for 5 min at 0°C. This figure shows that, even at concentrations of $HgCl_2$ as low as 10 μM, the concentrative phase of the uptake was drastically reduced: the equilibrium value, however, remained unaltered. Higher concentrations of $HgCl_2$ inhibited further and eventually abolished the overshoot completely, while gradually decreasing the amount of glucose trapped at equilibrium. In the experiment illustrated in Fig. 1A, over 50% reduction in the equilibrium value was obtained by pre-treatment with 50 μM $HgCl_2$. The results indicate that the milder treatment with $HgCl_2$ affected glucose transport but did not break the vesicles, whereas higher concentrations, in addition, made the vesicles leaky to the sugar. Experiments similar to that described above were performed at several $HgCl_2$ concentrations and the amount of glucose taken up after 20 s was compared to that of untreated vesicles. The results of three such experiments are shown in Fig. 1B. An average half-maximal inhibition of the overshoot was reached with approx. 8 μM $HgCl_2$, which correspond to 6.7 nmol/mg protein.

Inhibition by $HgCl_2$ could be due either to a direct effect on the sugar carrier or to a reduction of the driving force provided by the electrochemical gradient of Na^+. The latter mechanism, which can be brought about by an increase in ionic conductance, has been found to mediate the inhibition of the overshoot produced by low concentrations of organomercuricals [11,20] and other sulfhydryl reagents [21]. To define the mechanism underlying the reduction in the overshoot by $HgCl_2$, the uptake of D-glucose was measured in the absence of ionic gradients under tracer-exchange conditions. Smaller amounts of glucose are taken up under these conditions than during the overshoot, due to the lack of driving force provided by the gradient. Hence, the contribution of

uptake through parallel pathways becomes significant and must be considered. Whereas tracer exchange through the carrier requires Na^+, the entry of glucose through alternative pathways occurs even in the absence of this cation. Thus, the fraction of the total flux that is carrier-mediated was calculated by subtracting the amount of glucose transported in the presence of K^+ from that measured in Na^+ media. Fig. 2A illustrates a typical experiment showing the time-course of D-[1-^{3}H]glucose tracer-exchange. Sugar taken up through the Na^+-dependent system amounted to 80% of the total flux at 4 s, but only to approx. 40% at 8 s. Pre-treatment of the vesicles with 25 μM $HgCl_2$ markedly reduced the rate of D-glucose uptake. This inhibitory effect was selective in that only the Na^+-dependent component, but not the Na^+-independent one, was affected. In fact, the Na^+-independent fraction of the uptake was observed to increase slightly after treatment with $HgCl_2$ (see value at 60 s). This increased leakiness was more pronounced when concentrations of $HgCl_2$ above 25 μM were used. Similar observations have been reported recently [20]. An increase in the uptake of L-glucose, which is not effectively transported by the carrier, has also been observed after pre-treatment with $HgCl_2$ (Biber, J., unpublished data).

The sensitivity of the tracer exchange to $HgCl_2$ was determined by exposing the vesicles to increasing concentrations of the inhibitor. Fig. 2B shows the pooled data of three such experiments performed in duplicate. Only the Na^+-dependent flux is illustrated, and the values are given as percent of the flux in untreated vesicles. The uptake was determined at 4 and 8 s, and essentially identical results were obtained. Half-maximal inhibition was achieved with

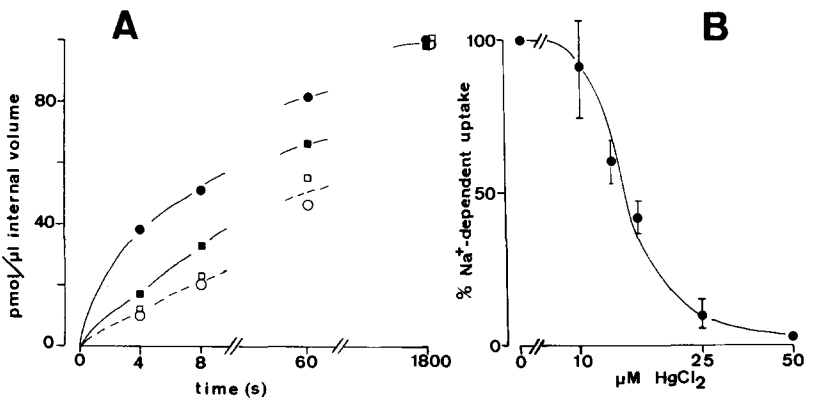

Fig. 2. A. Inhibition by $HgCl_2$ of D-glucose uptake under equilibrium conditions. Vesicles (1.2 mg protein/ml) were treated with (■,□) or without (●,○) 25 μM $HgCl_2$ for 5 min at 0°C followed by dilution and centrifugation. D-[1-^{3}H]glucose tracer-exchange was measured after equilibrating the vesicles with 0.1 mM D-glucose and 100 mM of either NaCl (●,■) or KCl (○,□) in buffer A. The internal volume of the vesicles was calculated using the equilibrium values at 30 min and the specific activity of the incubation solution. The points are the mean of duplicate determinations from a typical experiment. B. Concentration dependence of the inhibition by $HgCl_2$ of Na^+-requiring D-glucose uptake under equilibrium conditions. Vesicles (1.2 mg protein/ml) were treated with the concentrations of $HgCl_2$ shown on the abscissa as described and then used for tracer-exchange experiments like that illustrated in A. The Na^+-dependent part of the influx at 4 s was calculated by subtracting the Na^+-independent fraction from the total flux. The points are the mean ± S.E. of three experiments performed in duplicate. Error bars smaller than the symbols are not illustrated.

approx. 14 μM $HgCl_2$. A comparison of Figs. 1B and 2B indicates that the overshoot was more sensitive to inhibition by $HgCl_2$ than the tracer exchange: the former was more than 50% inhibited by 10 μM of the inhibitor, whereas the latter was barely affected at this concentration.

2. Effect of $HgCl_2$ on phlorizin binding

A deoxycholate-extracted preparation of brush-border vesicles recently developed in our laboratory [15] appears suitable for labeling studies of the glucose carrier. Unfortunately, extraction with deoxycholate renders the membranes leaky so that no transport measurement is feasible. Therefore, if the inhibitory effect of $HgCl_2$ is to be studied in this preparation, other parameters such as the binding of [^{3}H]phlorizin must be determined. The results of this analysis are depicted in Fig. 3. Only the Na^+-dependent component of the binding is illustrated (see Methods). $HgCl_2$ was found to be a potent inhibitor of phlorizin binding to deoxycholate-treated membranes. Half-maximal inhibition was brought about by 20 μM $HgCl_2$. In order to test whether the inhibition was the result of the reaction of $HgCl_2$ with SH groups and whether gross, irreversible changes of the binding site occurred, $HgCl_2$-treated membranes were washed in buffer A containing 5 mM dithioerythritol, and then used for phlorizin binding determinations. These results are also shown in Fig. 3. Substantial recovery of the binding was observed, particularly at the lower inhibitor concentrations. In contrast, no significant recovery of the binding was obtained after washing with 1 mM EDTA membranes pre-treated with 50 μM $HgCl_2$, and only 24% recovered after 25 μM $HgCl_2$.

For comparison, a parallel series of experiments was performed using intact vesicles. Pre-treatment with increasing concentrations of $HgCl_2$ also effectively reduced phlorizin binding to these membranes. In three experiments performed in duplicate, binding was found to be 79.5 ± 13.4% of the control value after

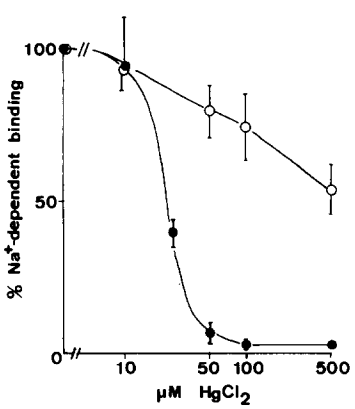

Fig. 3. Inhibition of phlorizin binding by $HgCl_2$ and reversal by dithioerythritol. Deoxycholate-extracted membranes (1.2 mg protein/ml) were treated for 5 min at 0°C with the concentrations of $HgCl_2$ shown on the abscissa, followed by dilution with either buffer A (•) or buffer A containing 5 mM dithioerythritol (○) and finally centrifuged. The phlorizin binding was measured as described in Methods. The Na^+-dependent fraction was calculated by subtracting the Na^+-independent fraction from total binding and is expressed as percent of the control. The points are the mean ± S.E. of three experiments performed in duplicate. Error bars smaller than the symbols are not illustrated.

10 μM $HgCl_2$, and complete inhibition was caused by 50 μM $HgCl_2$. Also, considerable reversal of the inhibition was achieved with 5 mM dithioerythritol.

3. Can inhibition by $HgCl_2$ be protected by substrates?

$HgCl_2$ is expected to react indiscriminately with sulfhydryl groups of a variety of membrane proteins. In order to identify the glucose carrier, a means must be devised to differentiate between the label bound to this and to other proteins. This could, in principle, be done by protecting the critical groups from being labeled, by addition of substrates or competitive inhibitors during the incubation with $HgCl_2$, provided the latter inhibits at the substrate binding site. Unfortunately, our efforts to protect phlorizin binding from inhibition were unsuccessful. Deoxycholate-treated membranes exposed to 50 μM $HgCl_2$ in the presence of 100 mM NaCl, 100 mM NaCl plus 5 mM D-glucose or 100 mM NaCl plus 100 μM phlorizin, retained less than 10% of the binding of untreated membranes, in spite of the fact that the concentrations of glucose and phlorizin used were more than 5-fold above those needed for half-saturation of the transport sites [5,6].

4. Reaction with sulfhydryl residues

An alternative approach to enhance the specificity of the labeling consists of minimizing the number of sulfhydryl residues available during the labeling steps. This can be achieved by prior treatment of the membranes with sulfhydryl agents that do not react with the essential residues of the glucose carrier. We tested a variety of reagents for their ability to reduce the number of sulfhydryl residues, and simultaneously determined their potency as inhibitors of phlorizin binding. The results of this survey are presented in Table I. The first column shows that up to 40% of the total membrane sulfhydryl groups could react with some of the agents, e.g., 5 mM diazinedicarboxylic acid bis-dimethylamide or 2.5 mM DTNB. However, phlorizin binding to these membranes remained virtually intact (second column). To determine if the essential sulfhydryl groups were still susceptible to inhibition by $HgCl_2$, the membranes pre-treated with the different agents were exposed to a subsequent treatment with 50 μM $HgCl_2$. The last column of the table shows that binding of phlorizin was completely abolished by the second treatment.

5. Effect of $HgCl_2$ on polypeptide electrophoretic pattern

A necessary condition for the identification of the glucose carrier is that the label should remain attached to the proteins during analytical procedures. For this reason, reducing agents were omitted from the solubilizing solution used for gel electrophoresis, the method chosen for polypeptide fractionation (see below). Since this could seriously alter the migratory behavior of the bands, preliminary experiments were performed to test the effect of $HgCl_2$ and of the lack of β-mercaptoethanol on the gel pattern. Deoxycholate-extracted membranes were treated with varying $HgCl_2$ concentrations under the same conditions used for inhibition studies, boiled in 2% SDS, and subjected to SDS-polyacrylamide gel electrophoresis (this treatment both solubilized and dissociated oligomeric proteins, such as the sucrase-isomaltase complex). The results of a typical experiment are shown in Fig. 4. Even though the individual

TABLE I

EFFECT OF VARIOUS REAGENTS ON THE SULFHYDRYL CONTENT AND PHLORIZIN BINDING PROPERTIES OF DEOXYCHOLATE-EXTRACTED BRUSH-BORDER MEMBRANES

Deoxycholate-extracted membranes (1.2 mg protein/ml) were treated with the concentrations of the agents listed, for 10 min at room temperature. After centrifugation, some membranes were used for the determination of sulfhydryl group content, while the rest were incubated a further 5 min at 0°C in buffer A with or without 50 μM $HgCl_2$. After dilution and centrifugation, the pellets were resuspended and used for phlorizin binding determinations. Only the Na^+-dependent fraction of the binding is given. The data are given as percent of the control (unreacted) values. The values are the mean ± S.E. of three experiments performed in duplicate (n.d., not determined). The number of SH groups which reacted with Ellman's reagent in control membranes was taken as 100%. Diamide, diazinedicarboxylic acid bis-dimethylamide; DTNB, 5,5′-dithiobis-(2-nitrobenzoate).

Agent	Concentration mM	SH groups reacted %	Phlorizin binding %	Phlorizin binding after $HgCl_2$ %
Diamide	0.5	14.9 ± 1.3	107.0 ± 6.5	1.6 ± 1.6
	2.5	31.9 ± 3.9	88.2 ± 11.5	2.8 ± 2.8
	5.0	39.4 ± 4.0	93.6 ± 12.9	n.d.
Iodoacetamide	0.5	11.0 ± 0.4	102.9 ± 7.3	4.6 ± 2.4
	2.5	19.9 ± 1.1	86.7 ± 5.8	3.2 ± 3.2
DTNB	0.5	19.8 ± 2.3	99.7 ± 2.6	8.7 ± 7.4
	2.5	41.1 ± 2.9	83.0 ± 7.2	6.3 ± 6.0
N-Ethylmaleimide	0.5	31.9 ± 1.3	106.2 ± 8.2	3.9 ± 2.9
	2.5	38.8 ± 4.4	82.2 ± 9.2	7.1 ± 2.7

bands are not as sharp, the overall pattern of control membranes is essentially the same as that observed in the presence of β-mercaptoethanol (see Refs. 15 and 17). The migration of certain bands was altered by treatment with $HgCl_2$, most changes occurring at concentrations higher than 25 μM. Among the major changes are: (a) the bands labeled I and S gradually fade whilst new bands with lower mobility increase in intensity; (b) the actin-like band, A, disappears as a series of new bands form in the M_r 35 000 to 40 000 region. At high $HgCl_2$ concentrations the fastest of these bands attains a staining intensity comparable to that of the original actin band. These new bands formed by $HgCl_2$ are not the result of peptide bond cleavages, since the normal pattern was recovered when the membranes were washed with dithioerythritol prior to electrophoresis. It is pertinent to point out that other organomercurial reagents such as *p*-chloromercuriphenylsulfonate can also enhance actin mobility, and that this behavior is reversed by dithioerythritol [13]. Faster migration of a band could result from an increase in the amount of SDS bound, whereas slower mobility could reflect aggregate formation. Interestingly, the alterations in electrophoretic mobility produced by $HgCl_2$ (50 μM) were only marginally reversed by washing with 1 mM EDTA.

These observations prompted us to select 25 μM $^{203}HgCl_2$ to label deoxycholate-treated membranes. At this concentration only minor alterations in the electrophoretic profile were observed so that most bands were still easily identifiable. Moreover, this concentration was sufficient to produce nearly complete inhibition of phlorizin binding.

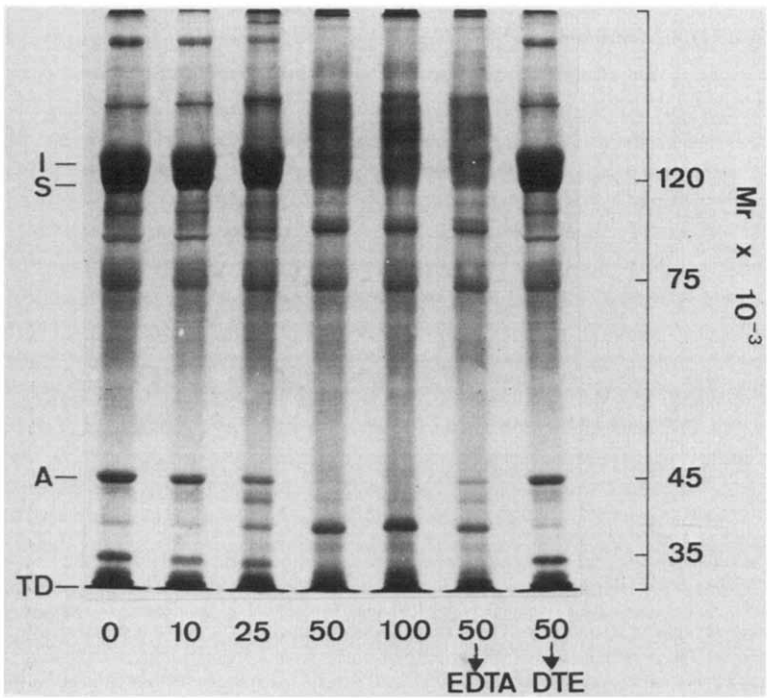

Fig. 4. Effect of $HgCl_2$ on the electrophoretic behavior of brush-border membrane polypeptides. Deoxycholate-extracted vesicles (1.2 mg protein/ml) were incubated for 5 min at 0°C with the concentration (μM) of $HgCl_2$ indicated at the bottom of the gel. After dilution with buffer A, the membranes were centrifuged and the pellets used for electrophoresis in 8.5% acrylamide gels. When indicated, the buffer used for dilution contained, in addition, 1 mM EDTA or 5 mM dithioerythritol (DTE). The gels were stained with Coomassie blue. I, isomaltase-containing band; S, sucrase-containing band; A, actin-like protein; TD, tracking dye (bromophenol blue). The molecular weight scale is shown to the right.

6. *Labeling with* $^{203}HgCl_2$

Deoxycholate-treated membranes were labeled with 25 μM $^{203}HgCl_2$ and analyzed by SDS-polyacrylamide gel electrophoresis as described under Methods. Redistribution of the label from the original binding site to sulfhydryl groups which become exposed upon protein denaturation was prevented by blocking the latter with *N*-ethylmaleimide. The label, however, was not displaced by the alkylating agent, since no radioactivity could be detected in the running buffer, the staining or de-staining solutions. Fig. 5 shows the labeling pattern of control deoxycholate-extracted membranes. Most of the bands contain radioactivity but the labeling is selective inasmuch as the amount of $^{203}HgCl_2$ bound is not proportional to the abundance of the polypeptides. Most of the label is found in the S-I bands, in the region corresponding to M_r 60 000 to 70 000, and near the running front of the gel. A small amount of aggregated material was regularly observed on top of the gel.

Pre-treatment of membrane sulfhydryl groups with 5 mM diazinedicarboxylic acid bis-dimethylamide did not change the polypeptide composition of the membranes but reduced the amount of label bound to some bands, such as actin and the bands of M_r approx. 100 000 migrating ahead of sucrase. In

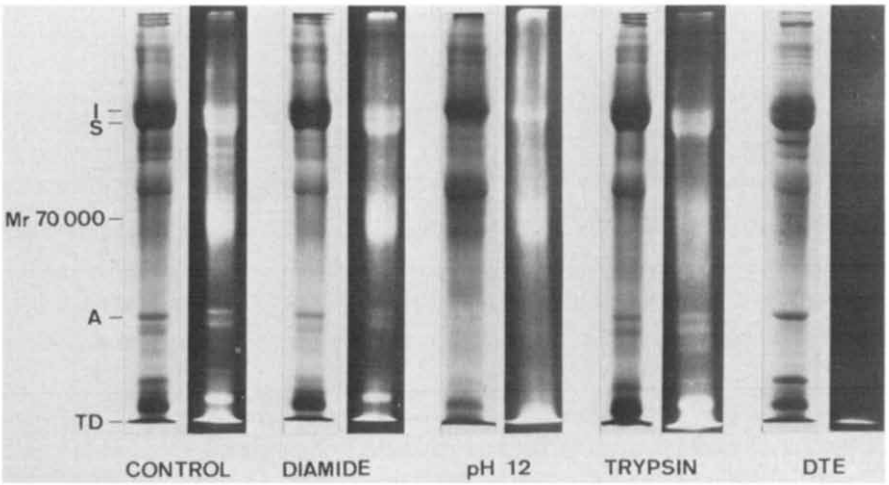

Fig. 5. Labeling of deoxycholate-extracted membranes with $^{203}HgCl_2$. Coomassie blue staining (left) and autoradiographic (right) patterns are shown for membranes treated with $^{203}HgCl_2$ under various conditions: control, membranes (1.2 mg protein/ml) were incubated with 25 μM $^{203}HgCl_2$ for 5 min at 0°C and washed in buffer A; diazinedicarboxylic acid bis-dimethylamide (diamide), prior to $^{203}HgCl_2$-treatment the membranes were incubated with 5 mM diamide for 10 min at room temperature and then washed in buffer A; pH 12, prior to $^{203}HgCl_2$-treatment the membranes were extracted at pH 12 in 300 mM mannitol for 5 min at 0°C, then centrifuged and washed once in buffer A; trypsin, prior to $^{203}HgCl_2$-treatment the membranes (1.2 mg protein/ml) were incubated for 10 min at room temperature with trypsin (1 : 50, w/w) and the reaction stopped with 1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; dithioerythritol (DTE), membranes labeled as described for the control were washed once with buffer A containing 5 mM dithioerythritol. The region of M_r 70 000 is indicated. All other details as in Fig. 4.

contrast, ^{203}Hg binding to other bands was essentially unaffected. Similar results were obtained by pre-treatment with DNTB or *N*-ethylmaleimide.

Brief exposure of deoxycholate-extracted membranes to solutions of pH 12 releases several polypeptides whilst the phlorizin binding sites are preserved [15]. Binding of the glycoside to these alkali-extracted membranes was also found to be inhibited by $HgCl_2$ (data not shown). The Coomassie blue and labeling patterns of alkali-extracted membranes are shown in Fig. 5. Several of the labeled bands have been removed so that most of the ^{203}Hg is found in the isomaltase-containing band (I), the M_r 60 000 to 70 000 region and co-migrating with the tracking dye. Minor labeled components are observed elsewhere in the gel.

Trypsin abolishes phlorizin binding to deoxycholate-treated vesicles [17]. This effect, which is also produced by other proteolytic enzymes, is presumably associated with the fragmentation of the binding protein. The autoradiographic pattern of ^{203}Hg-labeled trypsin-treated membranes is shown in Fig. 5. Treatment with the enzyme brought about two main alterations in the radiographic profile: the labeling intensity in the M_r 60 000—70 000 area decreases with the concomitant appearance of ^{203}Hg binding material in the M_r 50 000—55 000 region. The latter may have derived from the former. Increased labeling near the gel front and minor changes in other bands were also noted.

Fig. 5 also shows the results of washing $^{203}HgCl_2$-treated membranes with

dithioerythritol. As noted before, dithioerythritol reversed the effect of the inhibitor on the electrophoretic profile of the membranes (Fig. 4). It also removed over 95% of the bound isotope, and simultaneously restored most of the phlorizin binding activity (Fig. 3). Accordingly, an amount of protein similar to that applied to the control gel produced only a barely-detectable radiographic image. In contrast, EDTA did not reverse the changes in electrophoretic behavior induced by $HgCl_2$, nor was it able to restore glycoside binding. Only 20—25% of the bound $^{203}HgCl_2$ was removed by EDTA and the labeling pattern was only slightly affected (not shown).

Discussion

1. The nature of the sites involved in the inhibition of sugar uptake and phlorizin binding

$HgCl_2$ proved to be a very effective irreversible inhibitor of glucose transport in brush-border vesicles. It reacts rapidly under mild conditions, and is remarkably more potent than other mercurial [11] or non-mercurial (Table I) sulfhydryl reagents. The potency of mercury as an inhibitor was found to differ when sugar uptake was measured whilst imposing an inward Na^+ gradient and under equilibrium conditions (Figs. 1 and 2). In the former case, the D-glucose flux is known to be strongly dependent on the magnitude and polarity of the electrochemical Na^+ gradient. Thus, shunting of the membrane conductance could explain the reduction in the overshoot observed at very low $HgCl_2$ concentrations. In fact, several sulfhydryl reagents have been shown to increase Na^+-permeability in brush-border vesicles [20,21]. This difficulty is eliminated by measuring the uptake under equilibrium (tracer-exchange) conditions, and the data thus obtained should better reflect the interaction of the sugar carrier with mercury. In keeping with this prediction, mercury displayed a similar potency to inhibit sugar tracer-exchange and phlorizin binding to the carrier when both were measured in the absence of either electrical or ionic gradients (Figs. 2 and 3).

When equal amounts of protein were used, similar concentrations of $HgCl_2$ were required to inhibit phlorizin binding to intact and deoxycholate-extracted brush-border membranes. In both cases, the inhibition was largely reversed by dithioerythritol but only slightly by EDTA. Thus, the same site(s) appears to have reacted in both preparations. The chemical nature of the group which reacted in the inhibitory process cannot be unequivocally established but it is reasonable to assume that a sulfhydryl side chain is involved. In fact, although $HgCl_2$ can, in principle, react with a variety of chemical groups, including phosphoryl and carboxyl moieties, its affinity for sulfhydryl side chains is much higher [22]. The stability constant for the dissociation of $HgCl_2$ and a simple thiol has been reported to be as high as $1 \cdot 10^{-40}$ to $1 \cdot 10^{-44}$ [22], so that when the thiol is present in an excess over $HgCl_2$ (as was the case in most of our experiments) no other group is expected to react. The observed reversal by a competing thiol (Fig. 3) is also consistent with this notion. A similar conclusion was reached by Stirling [8] and by Lerner et al. [10] using intestinal segments.

The affected sulfhydryl group does not appear to be located at either the

sugar or Na^+ binding sites, or at the hydrophobic region which is believed to interact with the aglycone moiety of phlorizin [3]. This conclusion is based on the finding that neither glucose nor phlorizin was capable of protecting the inhibition of glycoside binding by $HgCl_2$. Previous work from this and other laboratories [10,11] had suggested that the reactive sulfhydryl group is not easily accessible from the outer milieu. Since the transport and inhibitor binding sites must be externally exposed, formation of the critical mercaptide probably occurs at a site more deeply located in the membrane.

2. Labeling of the sugar carrier with $^{203}HgCl_2$

The enormous stability of the mercaptide bond allowed us to attempt the identification of polypeptides involved in sugar translocation. Thus, rather lengthy separation techniques such as gel electrophoresis could be employed while the mercury remained attached to the proteins. In the light of the criticisms raised in the Introduction concerning previous reports of labeling with organomercurials, the feasibility of our approach must be stressed. Firstly, deoxycholate-treated vesicles provide an experimental system in which the glucose carrier is enriched several-fold compared to the systems used in the past. Secondly, a detailed preliminary analysis of the inhibitory properties of $HgCl_2$ permitted us to select the optimal conditions for the labeling process, therefore reducing non-specific binding of $HgCl_2$. Thirdly, prior to the labeling step, we were able to block with various reagents sizeable amounts of sulfhydryl groups not involved in D-glucose transport or phlorizin binding. Finally, the availability of radioactive $^{203}HgCl_2$ of specific activities far greater than those of organomercurials allowed the application of substantial amounts of radioactivity to the gels. Assuming that only one cysteinyl residue per protein need react to bring about inhibition, and considering the density of transport sites in deoxycholate-treated membranes [15], it is possible to calculate the maximum amount of radioactivity in the gel which is related to transport inhibition. Using $^{203}HgCl_2$ with a specific activity of 1.4 Ci/g Hg, and applying 50 μg of protein to one slot of the gel (see Methods), up to 1.4 nCi will be associated with the inhibitory site. A band containing this amount of radioactivity can be clearly discerned by autoradiography after a relatively short exposure time [23].

In order to identify the glucose carrier protein, several known features of the system must be taken into account. We had earlier shown that phlorizin binding to deoxycholate-treated membranes is enriched after removal of extrinsic proteins with alkali and abolished by proteolysis with trypsin [15,17]. In the present work we found that treatment with several reagents can reduce the number of sulfhydryl groups by up to 40% without appreciably inhibiting phlorizin binding (Table I) and that subsequent addition of $HgCl_2$ abolished the binding. Moreover, dithioerythritol, but not EDTA, was found to reverse the inhibition. Considering these properties, the band(s) corresponding to the glucose transport proteins must fulfill the following criteria: (a) it must be labeled; (b) in membranes treated with either DTNB, diazinedicarboxylic acid bis-dimethylamide or *N*-ethylmaleimide prior to the addition of $^{203}HgCl_2$, the band should not only remain labeled, but also represent a larger fraction of the total counts; (c) the band must be substantially enriched after treatment at

pH 12; (d) most of the label must remain bound after washing with EDTA but should be removed by dithioerythritol; (e) the electrophoretic mobility of the band must increase after proteolysis with trypsin (an exception to this prediction could occur if the proteolytic cleavage took place at a site contained in a loop bounded by a disulfide bond: because the samples were applied to the gel without prior reduction, the proteolytic fragment could be held together by the disulfide bridge).

Analysis of Fig. 5 shows that most of the bands stained by Coomassie blue are also labeled by $^{203}HgCl_2$, but that the relative specific activities of the various bands vary greatly. In addition, some of the bands meet some of the criteria listed above but only a diffuse band of M_r 60 000 to 70 000 fulfills them all. Knowing that approx. 90% of the added radioactivity was incorporated by the membranes it is possible to estimate the minimum fraction of the counts which is related to transport and glycoside binding inhibition. Under optimal conditions, i.e., when the carrier has been enriched 6- to 7-fold by combined deoxycholate and alkaline treatment, and assuming that only one sulfhydryl group is labeled, approx. 2% of the isotope is bound to the transport protein. This figure must be multiplied by the number of sulfhydryl residues in the protein, if more than one are present and labeled, which is not unexpected for a polypeptide of high molecular weight. Even though the limitations inherent in the autoradiography of ^{203}Hg make a precise quantitation difficult, the amount of radioactivity found in the M_r 60 000 to 70 000 region is consistent with the above predictions.

Naturally, the data discussed above do not provide final evidence to implicate a particular polypeptide in the process of sugar translocation. They are, nevertheless, useful to rule out some components as possible candidates (e.g., S-band, actin or the bands of M_r 20 000 to 30 000). Klip et al. [15] recently described efforts to partially purify and identify the sugar transport component with a band in the electrophoretic pattern of brush-border membranes. Interestingly, after ruling out a number of bands present in the original preparation, they suggested that a band of M_r approx. 70 000 could be involved in sugar transport. This polypeptide, named 7f in Ref. 15, displayed the same diffuse broad profile and is presumably analogous to the M_r 60 000 to 70 000 band observed in this work *.

These results are in obvious disagreement with previous reports [12,13] in which the glucose transport activity was linked to proteins of lower M_r which, at least in one case, were identified as actin (actin is almost completely removed from brush-border membrane vesicles by treatment with deoxycholate without sizeable loss of phlorizin binding capacity [15]). Although more than one type of polypeptide chain could be involved in sugar translocation, so that the data are not necessarily contradictory, more evidence is needed for the final elucidation of the number and the nature of the constituents of the transport system.

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* Unfortunately, the electrophoretic system used in the present paper did by necessity differ in some details from that used earlier (i.e., no β-mercaptoethanol was added to SDS), so that the patterns are not quite identical. Nevertheless, their striking similarity (see, for example, Fig. 4 of this paper and Fig. 3 of Ref. 15) allows their comparison.

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