J. Membrane Biol. 51, 47–73 (1979)

Partial Purification of the Sugar Carrier of Intestinal Brush Border Membranes. Enrichment of the Phlorizin-Binding Component by Selective Extractions

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Received 14 May 1979; revised 30 July 1979

Summary. The [^{3}H] phlorizin-binding component of brush border vesicles was enriched *in situ* by negative purification. Several procedures, known to effect selective solubilization of membrane components, were used separately or in combination to remove proteins unrelated to the binding. Deoxycholate ruptured the vesicles and released 67% of their protein, thereby increasing the specific [^{3}H] phlorizin-binding activity of the pellet three- to fourfold. Extracting the deoxycholate-pellets with either NaI or alkaline solutions released up to 38% of the deoxycholate-insoluble protein without significantly affecting phlorizin binding. The polypeptide composition of the membranes at the different stages was analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis. A number of polypeptides present in the original vesicles could be ruled out as essential components of the [^{3}H] phlorizin binding entity.

Intact and deoxycholate-treated vesicles were subjected to proteolytic attack. Papain liberated sucrase and isomaltase from intact vesicles, but affected neither other Coomassie-stained bands nor phlorizin binding. Neither the protein composition nor the binding properties of sealed vesicles were influenced by trypsin or chymotrypsin. However, all the proteolytic enzymes tested on deoxycholate-treated membranes substantially reduced [^{3}H] phlorizin binding and produced concomitantly the disappearance of several bands from the electrophoretic profile.

Pretreatment of vesicles with papain, followed by deoxycholate extraction and incubation in alkaline media, increased the specific binding activity of the membranes up to ninefold by removing close to 90% of the protein. A limited number of polypeptides are suggested as possible candidates for the glycoside-binding site of intestinal brush borders.

Phlorizin and other structurally related glycosides are among the most potent inhibitors of monosaccharide transport in small intestinal epithelia [1, 13]. Inhibition by the glycoside is of competitive nature [1, 13] whereas the aglycone, phloretin, inhibits noncompetitively [13]. This has led to the suggestion [13] that the glucoside moiety of phlorizin is directed to the sugar-binding site of the transport entity. Phlorizin binds with high affinity to membrane vesicles derived from rabbit intestinal brush-border membranes. The dissociation constant of the binding reaction agrees with the K_i value for inhibition of D-glucose uptake, and both constants are similarly sensitive to changes in the transmembrane electro-

0022-2631/79/0051-0047 $05.40

chemical gradient of Na^+ [38, 41]. The binding capacity of a purified brush-border preparation has been reported to range between 10 and about 40 pmol/mg protein [38, 41], similar to the values found for renal brush-border vesicles [3, 4, 7, 16].

The identification and isolation of the membrane components responsible for glucose uptake and glycoside binding in the intestine has become the goal of several investigators [10, 26, 35]. Some of these efforts, based mostly on the labeling of glucose or phlorizin protectable thiol groups of membrane proteins, have, however, yielded contradictory results [26, 35]. Other studies [10, 11], utilizing membrane solubilization followed by reconstitution of transport competent vesicles, are only in a preliminary stage and do not allow the final identification of the specific sugar-transport component in small intestinal brush borders [11].

A major inherent difficulty in these studies has been the scarcity of transporters in the membrane. If the maximum number of phlorizin-binding sites referred to above is considered and a 1:1 stoichiometry is assumed, the fraction of the total protein involved in sugar translocation can be estimated if a hypothetical molecular weight is assigned. Considering that the molecular weights of most membrane proteins fall in the range of 25,000 to 250,000, an intermediate value of 100,000 can be used for the calculation. In this case, the transporter would constitute only about 0.4% of the total protein in the membrane. It seems, therefore, that a partial purification of the transport component would be not only an essential stage for its isolation but also a first step towards its identification. Towards this aim, we undertook the fractionation of brush border membranes and assessed the fate of the sugar-transport system by determining phlorizin binding to the resulting membrane fractions. Selective solubilization of membrane components was achieved by means of detergent extraction, protein perturbants, and limited proteolysis. The produced membranes were analyzed for protein and lipid composition as well as for glycoside binding.

Materials and Methods

Materials

A 5% potassium DOC[1] stock solution (pH 9.0) was prepared from deoxycholic acid as described by MacLennan [27]. G-[^{3}H] Phlorizin was purchased from New England Nu-

[1] *Abbreviations:* DOC, deoxycholate; DTE, dithioerythritol; DMMA, dimethyl maleic anhydride; pCMBS, *p*-chloromercuriphenyl sulfonic acid; PMSF, phelylmethylsulfonylfluoride; TLCK, N-α-*p*-tosyl *L*-lysine chloromethylketone; LIS, lithium 3,5 diiodosalicylate; S, Coomassie blue-stained band containing sucrase; *I*, band containing Isomaltase; *A*, band containing actin-like protein.

clear (2.06 Ci/mmol), and D-[1-^{3}H] glucose from Amersham (8.3 Ci/mmol); DMMA, deoxycholic acid and formic acid were obtained from Fluka AG.; pCMBS, DTE, Tris, bovine trypsin and chymotrypsin, PMSF; and TLCK were from Sigma; Triton X-100 and $NaDodSO_4$ were from Serva, Heidelberg; papain and the molecular weight standards were from Boehringer, Mannheim; LIS from Eastman; iodoacetamide from BDH; nonradioactive phlorizin from Roth. The Bio Rad reagent was used for protein determination by the method of Bradford [5].

Methods

Brush border membrane vesicles from frozen small intestines of rabbits were prepared daily by the calcium precipitation method of Schmitz *et al.* [29] as modified by Kessler *et al.* [20]. All the treatments described below were performed at 0 to +4 °C unless otherwise stated.

DOC extraction. Prior to DOC extraction, the membranes were washed once with a solution containing 250 mM KCl and 10 mM Tris-HCl, pH 7.5, and then resuspended to about 3 mg protein/ml in a similar solution buffered to pH 8.5. The solution was then made 0.75 mM in DTE, and finally the required amount of DOC was added. When not indicated otherwise, the concentration of DOC used was 0.5 mg/mg protein. The suspension was gently shaken, incubated 10 min on ice, and finally centrifuged at $60{,}000 \times g$ for 30 min. The supernatant was aspirated and the membranes were washed once in 300 mM D-mannitol and 10 mM Tris-HCl (buffer *A*) pH 7.0 with 0.75 mM DTE. Whenever present, DTE was used at this concentration.

Extraction of DOC-treated membranes with perturbing agents. Extraction of proteins from the DOC-treated membranes was performed by resuspending the washed DOC pellets to a final concentration of about 0.75 mg protein per ml in one of the following solutions: (a) 1 M NaI in buffer *A*, pH 7.5, with DTE; (b) 5 mM pCMBS in buffer *A*, pH 7.5; (c) 300 mM D-mannitol, 0.75 mM DTE and enough NaOH to bring the pH to 12. The resuspended materials were then homogenized by repeated passage through a 25-gauge needle, incubated for 10 min at 0 °C and spun down at $60{,}000 \times g$ for 30 min. The supernatants were aspirated and the pellets were washed once in buffer *A*, pH 7.0, with DTE. Samples treated with NaI were diluted with 3 volumes of water before centrifugation, and in some experiments the samples incubated at pH 12 were neutralized by addition of a 1 M Tris-HCl, pH 7.5, solution before centrifugation. For DMMA extraction, the DOC pellet was resuspended in 3 ml of distilled water at room temperature (final concentration 0.75 mg protein/ml) and 4 mg solid DMMA were added while maintaining the pH at 8 by addition of NaOH. The sample was then spun down and washed as above.

Proteolysis. Intact vesicles (3 to 5 mg protein/ml) or DOC-treated vesicles (0.75 to 2 mg protein/ml) were incubated with trypsin (1:50 proteinase to membrane protein wt/wt) in buffer *A*, pH 7.5, with DTE for 10 min at room temperature. The reaction was stopped by a 5-min incubation with a 20-fold molar excess of TLCK followed by a threefold dilution with ice-cold buffer *A*, pH 7.0, with DTE and centrifugation at $60{,}000 \times g$ for 30 min. The pellets were washed once more with the same buffer containing 2 mM TLCK. Similar conditions were used for digestion with chymotrypsin, but only dilution and washing were used to stop proteolysis. Papain was activated in buffer *A*, pH 6.8, containing 1 mM sodium EDTA and 5 mM cysteine for 30 min at room temperature while bubbling nitrogen through the mixture. Proteolysis was carried out for either 10 min at room temperature or for 30 min at 37 °C (*see* text) in the same medium, using either intact vesicles (3 to

5 mg protein/ml) or DOC-extracted vesicles (0.75 to 2 mg protein/ml) and a 1:50 (wt/wt) ratio of papain:membrane protein. The reaction was stopped as above with TLCK, which is known to be a potent irreversible inhibitor of papain and trypsin.

Transport and binding measurements. D-[1-^{3}H] Glucose uptake in the presence of a NaSCN gradient was determined essentially as described [38], using 300 mM mannitol. [G-^{3}H] Phlorizin binding measurements were performed at room temperature according to Toggenburger *et al.* [41] with the following modifications: intact brush-border vesicles (final concentration 10 to 15 mg protein/ml) or membranes treated with DOC and other agents (2 to 5 mg protein/ml) were preincubated for at least 30 min at room temperature with 100 mM of either NaCl or KCl in buffer *A*, pH 7.0, with DTE. The binding reaction was initiated by mixing 10-μl aliquots of these suspensions with 10 μl of the same medium containing twice the final [^{3}H] phlorizin concentration desired. After 5 sec of vigorous mixing in a vortex and a further 10 sec of incubation, binding was stopped by diluting with 2.5 ml of an ice-cold solution containing 250 mM KCl, 0.1 mM nonradioactive phlorizin, 1 mM Tris-HCl, pH 7.5, filtering the diluted sample through a prerinsed Sartorius filter (0.6 μm pore size) and washing once with 5 ml of the same solution. The filters were dissolved and counted as described [38]. *Total* binding refers to that measured in solutions containing 100 mM NaCl; *unspecific* binding was determined in Na-free solutions containing 100 mM KCl; *Na^+-specific* binding was calculated as the difference between the total and the unspecific binding. *Specific binding activity* represents the amount of Na^+-dependent bound [^{3}H] phlorizin divided by the protein content of the sample.

Protein was determined by the method of Bradford [5]. To ensure complete solubilization of the proteins, the membranes were dissolved in 50 μl of formic acid prior to the addition of the reagent. In some cases the method of Kalb and Bernlohr [19] was used for protein determination. In this case, light scattering was avoided by dissolving the membranes in 1 ml 0.1% $NaDodSO_4$. Bovine γ-globulin was used as a standard.

Polyacrylamide (8.5% wt/wt) gel electrophoresis (PAGE) was performed in 1-mm thick slabs as described [24]. Samples (generally 50 μg protein/slot) were boiled for 45 sec unless otherwise stated. Samples that had been exposed to trypsin or papain were mixed with 2 mM TLCK before addition of the solubilizing solution. Supernatants were concentrated for electrophoresis by precipitation with 12% trichloroacetic acid, followed by washing of the precipitate with 100 mM Tris-HCl, pH 7.5, and resuspension in 5% $NaDodSO_4$. The gels were stained with Coomassie blue *R* and scanned in an Integraph CH 174 densitometer. The gels were calibrated with rabbit phosphorylase *a* (mol wt 92,500), bovine serum albumin (mol wt 67,500), ovalbumin (mol wt 45,000) and bovine chymotrypsinogen (mol wt 25,000) as standards.

Results

Effects of DOC on Membrane Integrity and Composition

Selective protein solubilization from different membranes has been achieved by controlled extraction with detergents [31, 42]. We have followed this approach to enrich the [^{3}H] phlorizin-binding activity of brush border membranes by selectively removing proteins unrelated to the binding components with DOC. Figure 1*A* shows the percent of protein found in the supernatant after exposing the vesicles to increasing

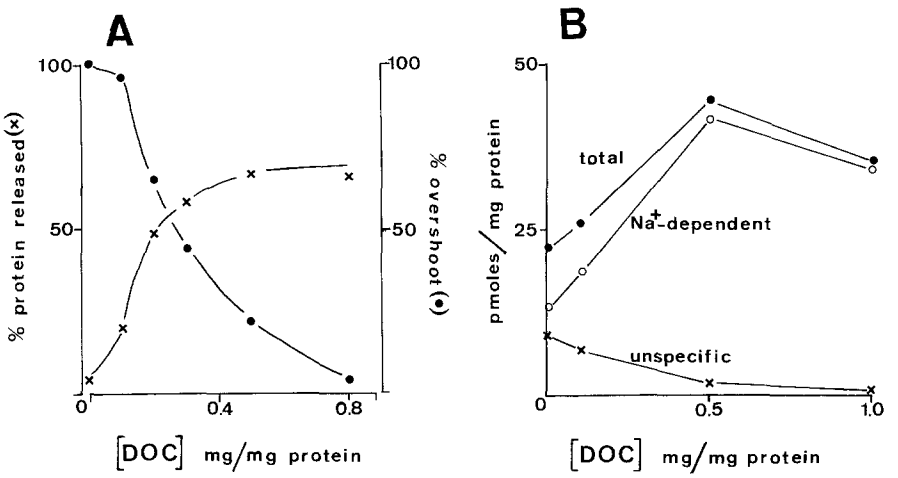

Fig. 1. (*A*): Effect of increasing concentrations of DOC on brush-border membrane integrity. Brush-border vesicles were incubated with the concentrations of DOC indicated on the abscissa and centrifuged as described in Methods. The fraction of the protein remaining in the supernatant is illustrated (×). The points are the mean of 4 determinations. The pellets were washed and used for the determination of D-glucose uptake activity. The overshoot was determined at 1 min and the results, determined as glucose taken up per mg protein in the pellet are shown as the fraction of the overshoot measured in control vesicles (●). Each point is the mean of 4 determinations. (*B*): Effect of increasing concentrations of DOC on [^{3}H] phlorizin binding. After extraction with the amounts of DOC indicated on the abscissa, the membranes were washed and used for [^{3}H] phlorizin-binding determinations. Binding was measured at room temperature using 5 μM [^{3}H] phlorizin in the presence (total binding) and in the absence (unspecific binding) of Na^+. The Na^+-dependent component of the binding (○) was determined by subtracting unspecific (×) from total (●) binding. Ordinate: pmol [^{3}H] phlorizin bound/mg protein in the extracted pellet. The points are the mean of duplicate determinations of a typical experiment

amounts of DOC followed by centrifugation as indicated under Methods. Protein release increased gradually as the ratio of DOC to protein (wt/wt) was raised from 0 to 0.8. Intact brush-border vesicles were found to contain 14.7 ± 0.62 μg phospholipid phosphorus per mg protein. Considering that phospholipids constitute 68% by weight of the total lipid of these membranes[2], and assigning the phospholipids an average mol wt of 750, the vesicles contain about 1.9 mg protein/mg lipid. Phosphorus-containing material was found to be released by DOC concomitantly with the protein. Upon treatment with 0.5 mg DOC/mg protein, the membranes lost 67.8% of the protein but only 37% of their phospholipids, so that the ratio of protein/lipid decreased from the original value of 1.9 to around 1.0 after detergent treatment. These results can be

[2] H. Hauser, *personal communication.*

rationalized if the detergent produced an opening of the vesicles allowing proteins trapped in the interior to leak into the supernatant (*see* Discussion).

In order to assess the degree of leakiness induced by DOC, the ability of the vesicles to take up D-glucose was measured. In the presence of an inwardly directed NaSCN gradient, D-glucose is known to be transiently accumulated inside brush-border vesicles, attaining a concentration which is several-fold higher than that in the medium. This phenomenon is commonly referred to as *overshoot*. The concentrative phase eventually subsides and the intravesicular sugar concentration becomes that of the surrounding medium (equilibrium). Figure 1*A* shows that the overshoot of glucose transport is gradually lost upon pretreatment of the membranes with increasing amounts of DOC. The uptake determined at equilibrium displayed a similar behavior. These findings are consistent with the above proposal that the vesicles are disrupted by DOC, resulting in a loss of their secluded volume and entrapped contents. Alternative explanations, such as damage or solubilization of the transport entity, are unlikely insofar as specific phlorizin binding is still displayed by the DOC-treated membranes (*see* below).

Effect of DOC on [^{3}H] Phlorizin Binding

Binding of [^{3}H] phlorizin to intact brush-border membranes has been measured in the presence of a NaSCN gradient [38, 41]. Under these conditions, at least 2 components can be discerned: a Na^+-dependent, glucose-inhibitable, high-affinity binding ($K_d = 4.7 \pm 2.4$ μM, mean ± SD) and a lower affinity component which does not require Na^+ ions and is insensitive to monosaccharides. The former will be referred to as Na^+-dependent and the latter as unspecific binding. We have chosen a concentration of 5 μM [^{3}H] phlorizin which is close to the K_d, to study the effect of DOC-pretreatment on glycoside binding. Figure 1*B* shows the effect that exposure to different concentrations of the detergent has on the binding measured after 15 sec of incubation with the ligand. The figure shows that the total binding of [^{3}H]phlorizin increased as the concentration of DOC used to extract the membranes was raised. In contrast, the unspecific component of the binding diminished with increasing amounts of detergent, accounting for only 4 to 11% (range of 8 experiments) of the total binding when 0.5 mg DOC/mg protein was used, as compared to over 40% in untreated vesicles. The calculated

Na^+-dependent binding increased progressively as the concentration of detergent was elevated, reaching maximum values at 0.5 mg DOC/mg protein. This enhanced specific binding activity can be accounted for by the amount of protein released from the membranes by the detergent, if it is assumed that all the phlorizin binding sites remain in the membrane. For instance, when 0.5 mg DOC/mg protein was used, $67.8 \pm 2.4\%$ of the protein (mean $\pm$ SE of 8 experiments) was lost to the supernatant. The membranes, retaining 32.2% of the protein displayed a specific glucoside-binding activity which was 3.37 ± 0.4 ($n=8$) fold higher than untreated membranes. Hence the original binding capacity (calculated as the product of the specific binding times the recovered protein) was conserved. Enriched phlorizin binding was also observed in DOC-extracted renal brush-border vesicles [39]. At higher concentrations of DOC (1.0 mg/mg protein) both the total and the Na^+-dependent binding begin to fall, probably as a result of either solubilization of, or damage to, the receptor. For this reason, 0.5 mg DOC/mg protein was used for the further characterization of the binding components.

To evaluate whether treatment with the detergent altered the properties of the binding sites, we compared the affinity of control and of DOC-extracted membranes for [^{3}H] phlorizin as well as the Na^+-dependence and D-glucose sensitivity of the binding. To allow a valid comparison, the binding properties of control vesicles were first characterized in the absence of an electrochemical gradient. Figure 2*A* shows the amount of [^{3}H] phlorizin bound to intact vesicles that were pre-equilibrated in either 100 mM NaCl (total binding) or 100 mM KCl (unspecific binding). The total binding increased but did not saturate when the concentration of ligand in the medium was increased from 2.5 to 40 μM. However, when the linear unspecific component was subtracted from the total binding, a saturation curve was obtained. By plotting this curve according to Scatchard, we calculated a K_d of 8.6 μM for the Na-dependent binding sites in the absence of either chemical or potential gradient. This value is very close to the one arrived at under gradient conditions (*see* above) which provides evidence that the same binding sites are being analyzed in both instances. Figure 2*B* shows that the [^{3}H] phlorizin concentration dependence of glycoside binding to detergent-treated membranes is essentially identical to that of intact vesicles: total binding increased without reaching saturation due to the contribution of two components, a Na^+-dependent and an unspecific one. The latter, albeit smaller than in control vesicles, also increased linearly with increasing [^{3}H] phlorizin concentrations. As before, an isotherm could be calculated

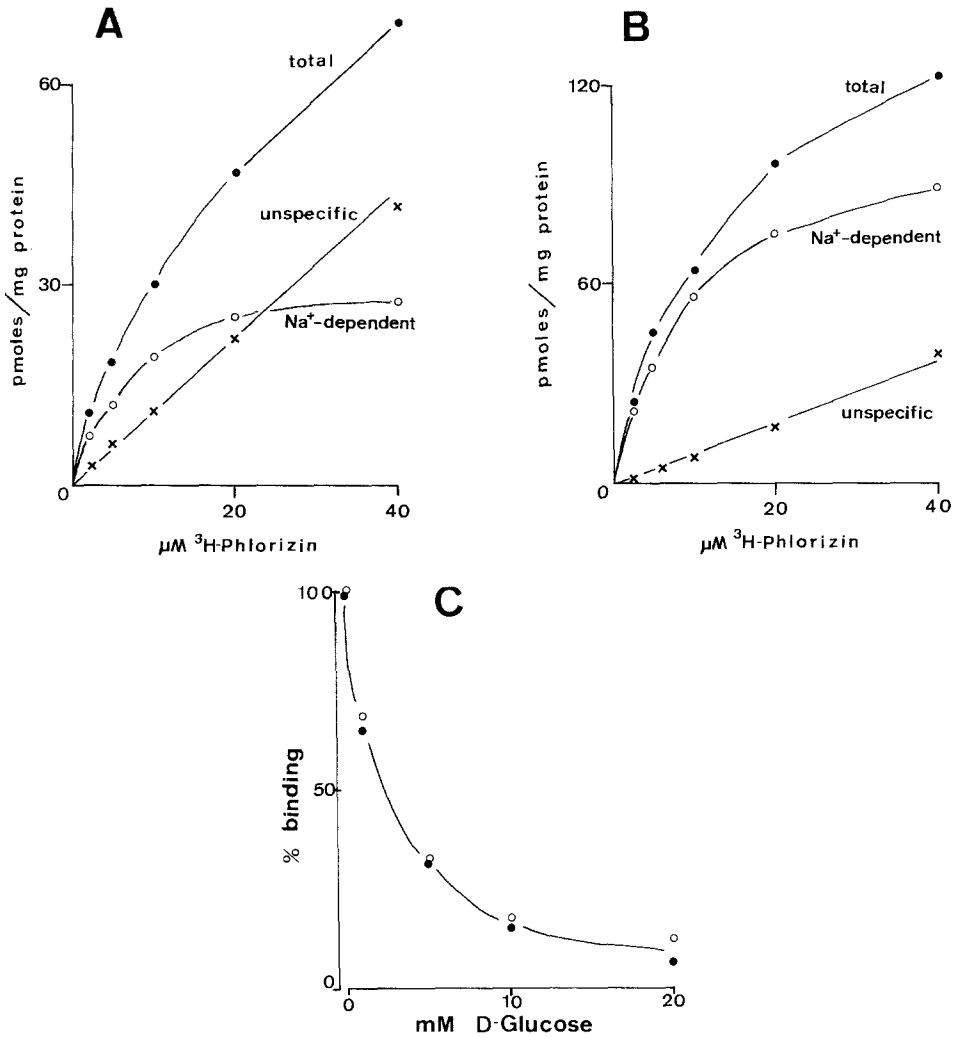

Fig. 2. Properties of [^{3}H] phlorizin binding to intact and DOC-extracted vesicles. (*A*): Concentration dependence of [^{3}H] phlorizin binding to intact brush-border vesicles. Binding was measured for 15 sec at room temperature using the concentrations of [^{3}H] phlorizin shown in the abscissa. Total (●) and unspecific (×) binding were measured as described under Methods; Na^+-dependent binding (○) was calculated from their difference. Ordinate: pmol [^{3}H] phlorizin bound/mg protein. The points are the means of two determinations. (*B*): Concentration dependence of [^{3}H] phlorizin binding to DOC-treated membranes. Brush-border vesicles were treated with 0.5 mg DOC/mg protein, centrifuged, and washed. The pellets were used for [^{3}H] phlorizin binding determination as in *A*. (*C*): Inhibition of [^{3}H] phlorizin binding by D-glucose. Intact brush-border vesicles (●) were incubated with 5 μm [^{3}H] phlorizin and D-glucose at the concentrations indicated in the abscissa for 15 sec at room temperature. Na^+-dependent [^{3}H] phlorizin binding was calculated by subtracting unspecific from total binding as in *A* and *B*. To facilitate comparison between the two types of membranes, the data are expressed as the fraction of the binding found in the absence of D-glucose. Each point is the mean of two determinations from a typical experiment. *A* and *B* were obtained with different batches of vesicles

for the Na^+-dependent part of the binding from which a K_d-value of 9.5 μM was derived. The close agreement of the K_d-values found for the native and detergent-treated membranes suggests that the original population of binding sites was retained.

The inhibition of [^{3}H] phlorizin binding to normal and extracted membranes by D-glucose is illustrated in Fig. 2*C*. The binding values were normalized to facilitate the comparison. It is clear that both preparations are affected to the same extent by the monosaccharide, supporting the notion that their binding sites are identical. By linearization of these data the K_i for D-glucose as a competitive inhibitor of [^{3}H] phlorizin binding was found to be 0.45 mM under the conditions used. This value is similar to those found for renal membranes [7, 14, 16]. As a further test of the identity of the glycoside-binding sites in the DOC pellet and in control vesicles, the Na^+-dependence of their [^{3}H] phlorizin binding was compared. Even though unspecific binding in the DOC-treated membranes was reduced (*see* Fig. 1*B*) this study was possible since the residual binding in the absence of Na^+ was not inhibited by glucose in either preparation. Parallel increases in glucose inhibitable binding to both sets of membranes were found as the amount of Na^+ in the medium was varied from 0 to 100 mM. In four determinations, half maximal stimulation was reached by control vesicles at 43.5 mM NaCl, and at 49 mM by the DOC-treated membranes.

Polypeptide Composition of Normal and DOC Extracted Membranes

Assuming that the [^{3}H] phlorizin-binding site is of proteic nature (*see* below), the observed enhancement in glycoside-binding activity should be reflected in an enrichment of certain polypeptides in the DOC-extracted membranes. To explore this possibility, we have compared the polypeptide composition of intact and DOC-treated membranes by $NaDodSO_4$-PAGE. The gels were stained with Coomassie blue, and the staining intensity was taken as a measure of the protein content of each band. Figure 3*A* shows the results of these studies. The molecular-weight scale is depicted to the right of the gels. Since the gel system employed (8.5% polyacrylamide) only resolved proteins of mol wt above 20,000, polypeptides smaller than this limit were not analyzed in this study. A nomenclature of the bands, to be used throughout this report, is given to the left of the first gel. For simplicity, the assumption is made that each band is composed of a single type of polypeptide. Those

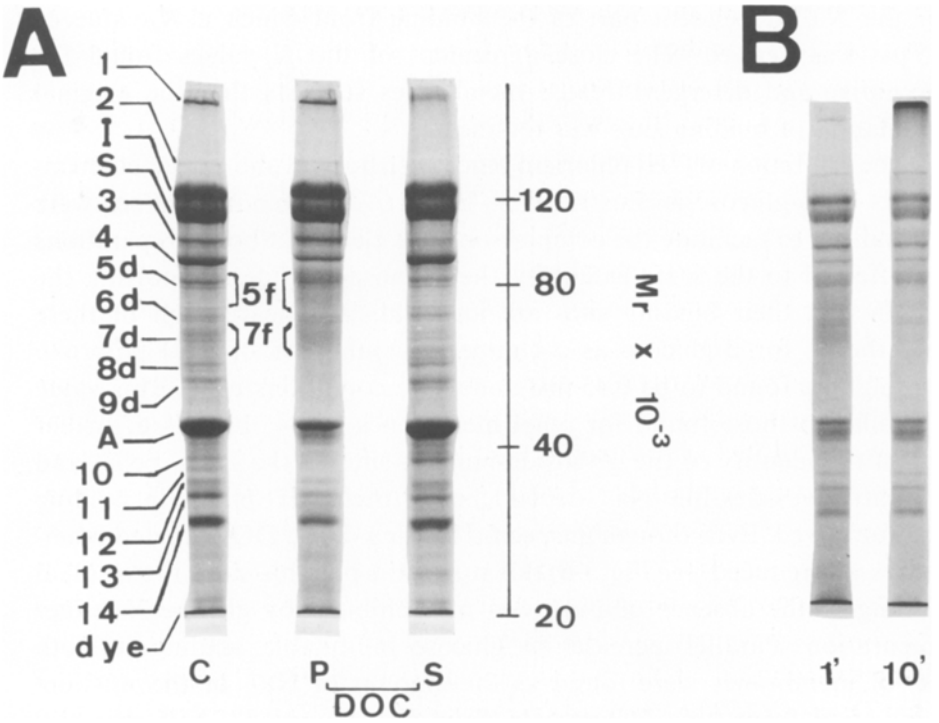

Fig. 3. (*A*): Effect of DOC on the NaDodSO$_4$-PAGE protein pattern of brush-border membranes. The samples were boiled for 45 sec in dissociating solution containing bromophenol blue as tracking dye, and analyzed on 8.5% polyacrylamide gels according to Laemmli [24]. The gels were stained with Coomassie blue. Gel *C*: control, untreated brush-border vesicles, 50 μg protein. The nomenclature to be used throughout this communication is shown on the left: *I* = isomaltase, *S* = sucrase, *A* = actin-like protein; doublets are identified by the letter *d* following the band number and diffuse, or *fuzzy* bands are labeled *f*. Brush border membranes were treated with DOC (0.5 mg/mg protein), and centrifuged. A sample of the pellet (50 μg protein) is shown in gel *P*. The supernatant was concentrated by precipitation with trichloroacetic acid and analyzed on gel *S* (50 μg protein). The molecular weight scale is shown to the right. (*B*): Heat-dependent protein aggregation of DOC-treated membranes. Two identical samples (40 μg protein) of DOC-extracted and washed membranes were mixed with solubilizing solution and boiled for 1 or 10 min (gels 1′ and 10′). Note the aggregate on top of gel 10′

bands which have been previously identified with defined proteins are labeled in capital letters. For the sake of clarity, all other major bands have been numbered. Ill-defined or *fuzzy* areas are followed by the letter *f*, and sharp contiguous bands (doublets) are labeled *d*. Bands that are very faint on the original vesicle sample, but become more intense during the purification procedure, are included in the numbering system (e.g., *5f* anf *7f*), whereas those that become visible only after the extractions,

are not numbered and will be discussed later. Notice that doublet *5d* is superimposed on the fuzzy region *5f*, and *7d* on *7f*. The electrophoretic pattern of control brush-border vesicles is shown in gel *C*. Two main bands labeled *I* and *S* (mol wt 125,000 and 112,000) have been previously shown to contain isomaltase and sucrase, respectively [30]. The isomaltase polypeptide is known to be anchored to the bilayer through a hydrophobic stalk. Sucrase, which forms a 1:1 complex with isomaltase, is bound to the latter at an extramembranal site, but is probably not otherwise attached to the membrane [6]. The most prominent band resolved by the gel (about 20% of the total protein) is labeled *A* (mol wt 45,000) because of its similarity to actin [26, 40]. Gel *P* displays the protein profile of washed DOC-treated membranes, and gel *S* shows the proteins that were liberated to the supernatant by the detergent. Similar amounts of protein were applied to each of the three gels (*C, P* and *S*) so that it is not to be expected that gels *P* and *S* complement each other to match gel *C* qualitatively. However, a similar loading of the gels is essential to compare the samples, thus allowing conclusions to be drawn regarding enrichment or depletion of the bands. A detailed account of the distribution of the bands in the pellet is given in Table 3 and in the Discussion. The most conspicuous difference is seen in the distribution of band *A*, most of which is found in the supernatant. In addition, a band (mol wt 42,000) that migrates immediately ahead of the residual band *A* became apparent after extraction of the membranes with DOC. Isomaltase, an intrinsic protein, is present both in the pellet and in the supernatant. This suggests that some areas of the membranes are being solubilized by the detergent. In both pellet and supernatant the original proportion of *S* to *I* is preserved, suggesting that the enzymes are solubilized as the native 1:1 complex (*see also* [30]). A marked enrichment was noted in the areas of components *5f* and *7f* of the pellet, together with a substantial depletion of regions *7d, 8d, 9d, 10, 11, 12, 13* and *14*. The depleted areas in the pellet correlate with enhanced staining in the corresponding areas of the supernatant, indicating that extraction rather than aggregation or degradation had taken place. To further test whether degradation occurred upon addition of the detergent – due for instance to activation of intrinsic proteases – the extraction was repeated in the presence of 0.3 mM PMSF and 10 mM iodoacetamide. The results obtained in the presence of these protease inhibitors were indistinguishable from those of Fig. 3*A*. Furthermore, when samples of the total suspension in DOC (before centrifugation) were analyzed, the gel patterns were identical to those of untreated vesicles. In addition,

no new bands could be detected in the low molecular weight region after DOC treatment, even when the samples were analyzed in 15% polyacrylamide gels[3].

A peculiar feature of the electrophoretic pattern of DOC-treated membranes became apparent when the degree of heating after addition of the electrophoresis solubilizer was varied (Fig. 3*B*). Boiling the samples for periods of between 15 sec and 1 min produced gel patterns in which band *1* was the component of highest molecular weight; no significant amount of aggregated material was detectable on the top of the gel. However, as the boiling period was prolonged, an aggregate developed which did not enter the gel. Interestingly, not all the polypeptides contributed to the same extent to the aggregated material, since all bands did not fade to a similar degree. Band *1*, and most of the bands encompassed in the *5* to *7* region (mol wt 82,000 to 63,000) seem to be the principal components of the aggregate, whereas the rest of the bands were only minimally changed. Aggregation occurred even in the presence of reducing agents, so that formation of disulfide bonds is an unlikely explanation. Heat-dependent changes in the aggregation state of other membrane proteins in $NaDodSO_4$ have been reported [15, 28]. To prevent the formation of these aggregates, all the samples in the experiments described below were boiled for only 45 sec. Shorter heating periods were avoided because incomplete dissociation of the sucrase/isomaltase complex is observed sometimes.

Use of Membrane Perturbants

In order to remove externally and internally adsorbed proteins, DOC-treated membranes – in which both surfaces are exposed to the medium – were subjected to various protein extraction procedures which have proved selective in red cell [18, 37] and adipocyte membranes [32]. Following extraction with 0.5 mg DOC/mg protein, the membranes were washed and resuspended in media containing the perturbing agents and finally centrifuged. The first column in Table 1 shows the fraction of the protein that was released by the different agents. A substantial amount of protein was liberated even under relatively mild conditions, such as exposure to media adjusted to pH 11. By analogy with the red cell membrane, it is conceivable that the perturbants caused fragmentation of the vesicles into smaller structures which might not sediment under the centrifugation

[3] J. Biber, *personal communication.*

Table 1. Effect of perturbing agents on DOC-extracted membranes[a]

Agent or condition	Protein released to the supernatant (%)	Specific binding activity in the pellets (%)	Binding recovery in the pellets (%)
DOC-control	3.2±1.8	100.0	96.8
NaI	28.6±4.7	127.5±16.7	91.0
pH 11	38.4±5.1	146.4± 6.9	90.2
pH 12	55.8±3.4	164.8±12.2	72.8
pCMBS	29.0±5.6	47.9± 1.4	34.0
DMMA	56.9±6.8	8.8± 2.0	3.8

[a] DOC-treated membranes were exposed to several agents and centrifuged as described under Methods. The fraction of the protein remaining in the supernatant is given in the first column. The washed pellets were used for [^{3}H] phlorizin-binding determination. Specific binding activity and binding recovery (calculated by multiplying the specific binding activity by the amount of protein remaining in the pellet) are expressed as percent of the values found for DOC-treated membranes. Values are the mean of 3 experiments performed in duplicate, ±1 SE.

conditions used. However, the contribution of these fragments to the total protein solubilized is probably small since no significant phospholipid phosphorus could be detected in the supernatants.

[^{3}H] Phlorizin binding was tested in the extracted membranes. The treated pellets were washed, resuspended, and used for the determination of glycoside binding as described. The specific binding activities remaining in the pellets after the different extractions are listed in the second column of Table 1. The total recoveries, calculated as the product of the remaining protein times the specific binding activity, are included in the third column of this Table. Although NaI and pH 11 removed a considerable fraction of the protein, neither treatment significantly decreased the recovery of [^{3}H] phlorizin binding to the membrane pellet. This resulted in specific binding activities higher than that of the control. Substantial recovery of the binding was also obtained after incubation at pH 12, provided the solution was neutralized after 1 to 3 min. Longer incubations in this alkaline medium resulted in a progressive loss of binding capacity (more than 50% after 45 min) even though no further protein was released. Reaction of membrane thiol groups with pCMBS released 29% of the protein but simultaneously lowered the specific binding activity by 52%, which implies that 66% of the binding sites were solubilized or inactivated. DMMA was the most effective solubilizing agent, removing more than half of the protein.

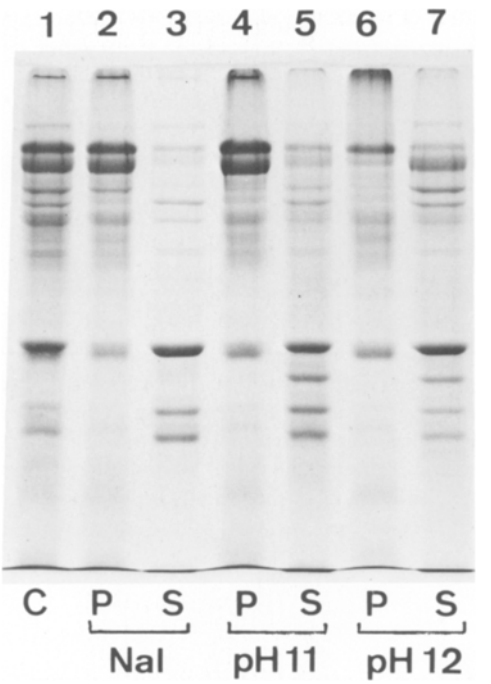

Fig. 4. Effect of perturbing agents on the protein composition of DOC-treated membranes. DOC-treated membranes (*C*) were resuspended in solutions containing 1 M NaI or enough NaOH to raise the pH to 11 or 12, and centrifuged. Samples of the pellets (*P*) and supernatants (*S*) were used for electrophoresis as described in Fig. 3*A*. Gel *1*: DOC-treated membranes. Gel *2*: Pellet after NaI extraction. Gel *3*: Supernatant of NaI extraction. Gel *4*: Pellet after extraction at pH 11. Gel *5*: pH 11 supernatant. Gel *6*: Pellet after extraction at pH 12. Gel *7*: pH 12 supernatant

Unfortunately, no significant binding activity could be recovered in the DMMA-extracted pellet. As was the case for pCMBS, it is at present not known whether reaction with the anhydride inactivated or solubilized the [^{3}H]phlorizin-binding site.

The protein composition of the pellets and supernatants separated after the extraction with the different agents was analyzed by gel electrophoresis, and the results are shown in Fig. 4. A detailed account of the fate of the different bands is given in Table 3.

Other observations that are noteworthy are: (i) The polypeptide of mol wt 42,000, which was barely visible before the extraction due to the presence of *A* (mol wt 45,000), remains in the pellet and becomes very conspicuous. (ii) In agreement with previous findings [8], we observed that sucrase/isomaltase complex was dissociated by alkaline pH. Because isomaltase is anchored to the bilayer [6], dissociation releases

only sucrose into the supernatant (gels *6* and *7*, Fig. 4), accounting for a substantial fraction of the protein solubilized (Table 1). (iii) An aggregate on top of the gels was usually observed in samples of membranes treated with alkali (gels *4* and *6*). These aggregates could not be eliminated by addition of DTE (5 mM), EDTA (10 mM) or N-ethylmaleimide (5 mM) to the membranes before solubilization, nor by increasing the concentration of $NaDodSO_4$ or mercaptoethanol in the solubilizing solution. (iv) The supernatants of samples treated at high pH (gels *5* and *7*) displayed a band of mol wt 40,000 which was not present in the original material (gel *1*) and is probably a product of the degradation of a larger polypeptide. (v) Small amounts of *S* and *I* were found in the supernatants from NaI and pH treatments. This is probably due to the formation of small membrane fragments which do not sediment after centrifugation at $60{,}000 \times g$ for 30 min.

Use of Proteases

To further investigate the identity and localization of the [^{3}H] phlorizin binding component, we subjected the brush border membranes to the action of diverse proteases. Table 2 indicates the fraction of the protein liberated from either intact or DOC-extracted vesicles by trypsin, chymotrypsin or papain. Only an insignificant fraction of protein was released from control vesicles by either trypsin or chymotrypsin. After 10 min

Table 2. Effect of proteases in intact and DOC-treated membranes[a]

		Protein released to the supernatant (%)	Specific binding activity in the pellets (%)	Binding recovery in the pellet (%)
Vesicles	Control	2.3±1.6	100.0	97.7
	Trypsin	3.8±1.9	118.7±8.4	114.2
	Chymotrypsin	8.2±3.6	113.7±4.5	104.4
	Papain	27.8±3.9	165.8±7.6	121.4
DOC-treated membranes	Control	2.8±2.7	100.0	97.2
	Trypsin	10.9±2.0	19.6±7.3	17.5
	Chymotrypsin	11.0±2.3	21.6±4.8	19.2
	Papain	46.0±5.9	26.4±1.4	14.3

[a] Brush-border vesicles or DOC-treated membranes were exposed to the proteases as described under Methods. After centrifugation the supernatants were analyzed for protein and the pellets were washed and used for determinations of [^{3}H] phlorizin binding. Other details are as in Table 1. Values are the mean of 4 determinations ±1 SE

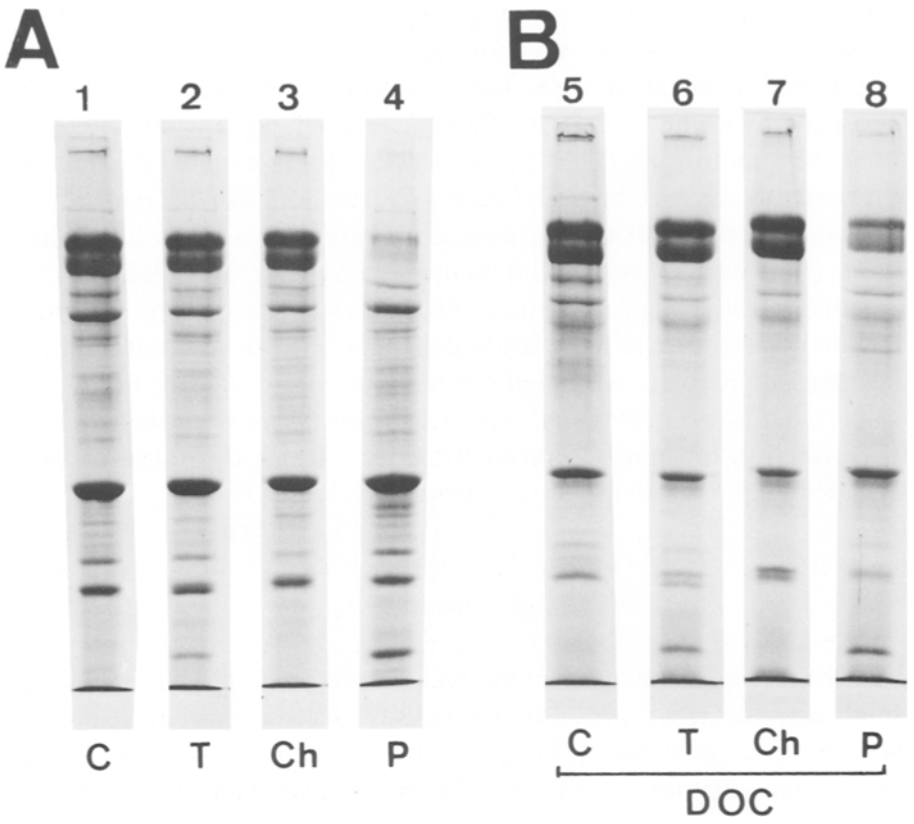

Fig. 5. Effect of proteases on intact and DOC-extracted membranes. Intact vesicles or DOC-treated membranes were incubated in the absence (*C*) or presence of 1:50 wt/wt of trypsin (*T*), chymotrypsin (*Ch*), or papain (*P*) for 10 min at room temperature. After centrifugation and washing, samples containing 50 μg protein were analyzed as in Fig. 3*A*. (*A*): Proteolysis of intact vesicles. Gel *1*, untreated vesicles; Gel *2*; trypsin-treated vesicles; Gel *3*; chymotrypsin-treated vesicles; Gel *4*; papain-treated vesicles. (*B*): Proteolysis of DOC-extracted membranes. Gel *5*; control DOC-extracted membranes; Gel *6*; trypsin-treated membranes; Gel *7*; chymotrypsin-treated membranes; Gel *8*; papain-treated membranes

at room temperature, papain released 27.8% of the protein from these vesicles. In contrast, following DOC treatment, both trypsin and chymotrypsin were able to release about 11% of the protein, and the amount released by papain also increased.

The electrophoretic pattern of the membranes subjected to proteolysis is shown in Fig. 5. Consistent with the negligible release of protein, no major changes in the pattern of intact vesicles were observed after trypsin or chymotrypsin action, with the possible exception of band *2* which is fainter after proteolysis. A band with an apparent molecular

weight similar to that of trypsin was found in vesicles treated with this protease even after repeated washing. Whether this is a fraction of tenaciously bound enzyme, or a product of the hydrolysis of a membrane component has not been analyzed. Papain removed a substantial fraction of bands *1*, *S* and *I* from these membranes, in agreement with previous findings [38]. It should be noted that all the gels in Fig. 5*A* contain the same amount of protein. Because *S* and *I* constitute an important fraction of the original protein, the remaining bands appear "enriched" after papain treatment. Apart from the appearance of several low mol wt bands (notably one of 38,000 to 42,000 and another of 22,000, which is probably residual papain), no other major changes were noticed. Importantly, *A* was not degraded after incubating intact vesicles with any of the proteases, in spite of the fact that it proved to be susceptible to proteolysis after solubilizing the membranes with DOC [23].

More noticeable changes were observed when DOC-treated membranes were proteolyzed (Fig. 5*B*). Even though the same amount of protein was again applied to all the gels, the staining intensity of the control gel in this and several other similar experiments seemed higher, probably due to the presence in the proteolyzed samples of a sizeable amount of small polypeptides which are abnormally fixed or stained. Trypsin drastically affected bands *2*, *3* and *7f* and partially removed bands *4*, *A* and *14*, and generated a series of low molecular weight polypeptides. Essentially the same results were observed using chymotrypsin, which in addition cleaved bands *6d* and *13*, and with papain, that also released the sucrase/isomaltase complex. A summary of the effect of the proteases on the DOC-treated material is presented in Table 3.

Binding of [^{3}H] phlorizin to protease-treated membranes was tested and the results are pooled in Table 2. Unspecific binding was not affected by the enzymes in either native or DOC-treated membranes, and is therefore not included. The recovery of Na^+-dependent binding in sealed vesicles treated with the proteases was as good or even better than in untreated membranes. In the case of papain, which liberated 27.8% of the protein, the specific binding activity was proportionally increased. Similar experiments have been performed in rat renal brush-border membranes with conflicting results [16, 39]. In agreement with our findings, Thomas and Kinne [39] reported no effect of proteases on phlorizin binding, while Glossman and Neville [16] observed inactivation. The latter group, however, prepared their vesicles under hypotonic conditions in which lysis could occur, so it is conceivable that the intravesicular surface became accessible to the proteases. In fact, it has been shown

Table 3. Effect of perturbants and proteases on the protein pattern of DOC-treated membrane[a]

Treatment	Bands																		
	1	2	I	S	3	4	5d	5f	6d	7d	7f	8d	9d	A	10	11	12	13	14
DOC[b]	+	+	+	+	+	−	−	+	−	− −	+	− −	− −	−	− −	− −	− −	−	−
NaI[c]	+	− −	+	+	+	−	−	+	− −	− −	+	− −	− −	− −	− −	− −	− −	− −	− −
pH 11[c]	+	− −	+	+	−	−	−	+	− −	− −	+	− −	− −	− −	− −	− −	− −	− −	− −
pH 12[c]	+	− −	+	−	−	−	−	+	− −	− −	+	− −	− −	− −	− −	− −	− −	− −	− −
pCMBS[c]	+	− −	+	+	+	−	−	+	− −	− −	+	− −	− −	− −	− −	− −	− −	− −	− −
Trypsin[d]	+	− −	+	+	− −	−	−	+	−	− −	−	− −	− −	−	− −	− −	− −	+	−
Chymotrypsin[d]	+	− −	+	+	− −	−	−	+	− −	− −	−	− −	− −	−	− −	− −	− −	− −	?
Papain[c]	−	− −	−	−	− −	−	+	+	−	− −	−	− −	− −	?	?	?	?	?	?

[a] Membranes treated with diverse agents were analyzed by $NaDodSO_4$-PAGE and stained with Coomassie blue as in Figs. 3–5. The nomenclature of the bands is defined in Fig. 3*A*. The intensity of the bands was visually compared in gels loaded with equivalent amounts of protein. The bands were marked "+" if enriched or not affected; "−" if partially depleted; "− −" if completely removed and "?" when their intensity was difficult to determine due to the presence of unidentified proteolysis products.

[b] Changes in the DOC-extracted membranes relative to intact brush-border vesicles.

[c] DOC-pretreated membranes were extracted with different perturbing agents. Their pattern is compared with that of DOC-treated membranes.

[d] DOC-pretreated membranes were exposed to the proteases. Their protein pattern is compared with that of DOC-treatment membranes.

that only the cytoplasmic region of the hexose carrier of rabbit intestinal brush borders is susceptible to proteases [23 and *see* below].

Na^+-dependent [^{3}H] phlorizin binding was substantially reduced in DOC-treated membranes following proteolysis. Only a small fraction of the binding was resistant to inactivation under the conditions used, perhaps due to incomplete vesicle disruption at this detergent concentration (Fig. 1*A*). The same explanation could apply to the residual band *A* found in DOC-treated membranes after proteolysis (Fig. 5*B*).

Combined Use of Proteases, DOC and Alkaline Extraction

The bands containing *S* and *I* constitute a large fraction of the protein remaining in the membranes after extraction with DOC followed by either NaI or pH 11 treatments (Fig. 4). Since these bands were found to be selectively removed from intact vesicles by papain without appreciable effects on [^{3}H] phlorizin binding, it should be possible to combine a preliminary proteolysis step with the above treatments in order to achieve a further purification of the glycoside-binding molecules. One out of four experiments with similar results is illustrated in Fig. 6. To completely remove *S* and *I*, papain treatment was extended to 30 min and incubation was carried out at 37 °C instead of room temperature. Under these conditions 32% of the protein was solubilized from the membrane, and only traces of *I* were found in the gels (gel *2*, Fig. 6). Distinct darkening was observed in the regions of the gel corresponding to mol wt 49,000 to 57,000 and 38,000 to 42,000, and also in the low mol wt region (29,000 and lower). To maintain a ratio of DOC to lipid equivalent to that used for unproteolyzed membranes, the amount of detergent added was calculated on the basis of protein determinations performed prior to proteolysis and assuming that no lipid was lost in this step. Papain pretreatment induced several changes in the extraction pattern with DOC (compare gel *3*, Fig. 6 with gel *P*, Fig. 3*A*). Among these, the absence of band *7f* and the presence of the intensely stained area between mol wt 49,000 and 57,000 indicated by the asterisk (which was generated by the protease), are the most remarkable. That the latter is a proteolytic product of the former is reasonable in view of their staining intensities and the fact that both are broad bands. Band *14* was more easily eluted by DOC after papain treatment so that only traces remained in the pellet. Alkali-treatment solubilized band *2* and most of bands *A*, *13* and *14* (gel *4*).

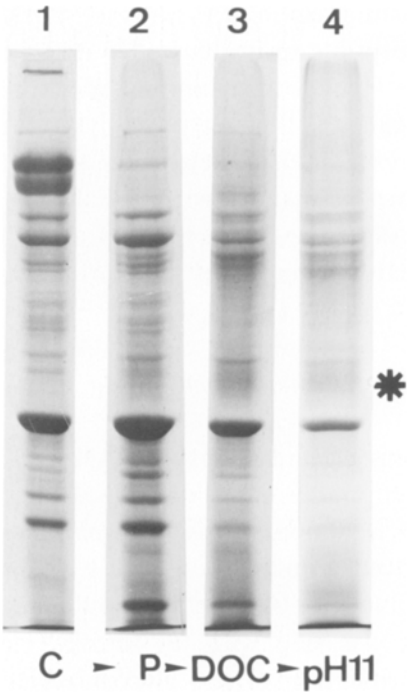

Fig. 6. Sequential exposure of brush-border vesicles (*C*) to papain (*P*), DOC and alkaline solution. Gel *1*, untreated brush-border vesicles; Gel *2*; brush border-vesicles exposed to papain (1:50 wt/wt) for 30 min at 37 °C; Gel *3*; papain-treated vesicles extracted with DOC; Gel *4*; papain-treated vesicles were extracted with DOC, centrifuged, and the pellet was extracted at pH 11. The asterisk indicates the position of a polypeptide band (mol wt 49,000 to 57,000) that was generated by the treatment with papain and preserved throughout the extraction sequence

After the combined treatment with these agents, 89.7 ± 2.3% (mean ± SE of 5 experiments) of the protein was removed from the membranes yet most of the [^{3}H] phlorizin binding was recovered in the pellet. Thus, the specific binding activity was enriched up to 1.3-fold after papain, 5.7-fold if proteolysis was followed by DOC extraction, and 8.9-fold with subsequent treatment at pH 11. Adequate recoveries were only obtained if care was taken to completely inactivate papain before the addition of DOC. This was achieved by repeated washing in TLCK media. When this highly enriched membrane preparation was subjected to treatment with trypsin, more than 85% of the binding was lost. Concomitantly with the loss of binding, the intensity of bands *3*, *5d* and that of the broad band of mol wt 49,000 to 57,000 were sharply decreased, pointing to their possible involvement in glycoside binding (*see* Discussion).

Use of Other Detergents

We tested the possibility of enriching the [^{3}H] phlorizin binding site in brush-border membranes by extraction with detergents other than DOC. Triton X-100 has been used for the solubilization and reconstitution of glucose transport activity using both intestinal [10] and renal [9, 22] brush borders, and is therefore expected to preserve also [^{3}H] phlorizin bindings activity. We found that membranes treated with 0.05, 0.1 and 0.4 mg Triton X-100 mg protein lost 4, 22 and 65% of their protein, respectively. Unspecific binding increased dramatically with the lower Triton X-100 concentration, becoming up to 4.5-fold larger than in control vesicles, and decreased with 0.4 mg Triton X-100/mg protein to about 0.5 times the control value. In contrast, Na^+-dependent binding was markedly reduced even by the lowest detergent concentration used (65% inhibition at 0.05 mg Triton X-100/mg protein). Similar findings were reported by Thomas and Kinne [39] in renal brush-border membranes. These results can be explained by either selective solubilization or gradual inactivation of the binding component. Preliminary attempts to measure glycoside binding to the solubilized material were unsuccessful.

LIS, a potent dissociating agent, has been used at low concentrations to differentially release polypeptides from red cell membranes [36]. We measured the effect of 50 mM LIS on [^{3}H] phlorizin binding to brush-border membranes and found Na^+-dependent binding to be completely abolished. Because only a small fraction of the protein was released, it is likely that LIS inactivated glycoside binding. Similarly, no Na^+-dependent binding was found after incubating the vesicles with 0.1 mg $NaDodSO_4$/mg protein for 10 min at room temperature.

Discussion

Opening of the Vesicles by DOC

Brush-border membrane vesicles were found to contain 1.92 mg protein/mg lipid, as estimated from the phospholipid phosphorus content. This value is unusually high compared with those found for other animal plasma membrane preparations [17], which range between 0.7 and 1.5 mg protein/mg lipid. The higher protein content of brush-border membranes could indicate that additional nonmembrane proteins are present in this

preparation, either entrapped or loosely adsorbed inside the vesicles. This seems likely since the vesicles are believed to pinch off during the homogenization of brush-border microvilli, which are composed of a protein-rich core surrounded by the plasma membrane. In fact, fragments of electron-dense core material are found inside the vesicles in micrographs of thin sections [20]. In our vesicular preparation, an actin-like protein, which is a major component of the core filamentous material [40], is also the most abundant protein species (band *A* in Fig. 3). DOC treatment released proportionally more protein than phospholipid, and this was accompanied by a simultaneous loss of ability to retain D-glucose, indicating that membrane integrity was lost. Preliminary microscopic observations[4] show that after extraction with 0.5 mg DOC/mg protein, the size and shape of the vesicles are not grossly altered but discontinuities appear in most of the membranes. On the basis that most of the actin-like polypeptide is solubilized from the membrane during extraction (Fig. 3), a selective release of the core material can be envisaged to follow vesicle breakdown.

Disposition of Polypeptides in the Membrane

The availability of a vesicle preparation which is sealed to proteases before but not after detergent treatment allows analysis of the localization of particular polypeptides in the membrane. As has been deduced by independent methods [6, 12], most of the protein mass of the sucrase/isomaltase complex is located at the outer face of the brush border membrane and is removed from sealed vesicles by papain without changes in membrane permeability [38]. Band *1* also must face the external medium, since it is not found in the gels after papain treatment. The remaining polypeptides do not expose any bonds to the exterior which are susceptible to cleavage by the proteases used. Bands *2, 3, 4, 6d, 7f, 13, 14* and *A* are affected by proteolysis only after DOC pretreatment (Table 3). Even though this could have resulted from conformational changes induced by the detergent, it is easier to correlate the acquisition of susceptibility to cleavage with the loss of membrane tightness. The latter would imply that at least a portion of these proteins is exposed to the intravesicular (normally cytoplasmic) medium.

The mode of interaction of the polypeptides with the membrane can be deduced from the effects of membrane perturbants on their solubil-

[4] M. Müller, S. Grinstein, A. Klip, and G. Semenza, *unpublished observations.*

ity properties. A given group of polypeptides (Table 3) was released from DOC-treated vesicles by a variety of agents which do not perturb hydrophobic interactions. Therefore this group, composed of bands *2, 3, 6d, A, 13* and *14*, is probably only attached loosely to the membrane and can be classified as extrinsic on peripheral [34]. Bands *1, I, 5f, 7f*, and the band of mol wt 42,000 exposed upon removal of *A*, stay in the membrane after all these treatments and are presumably intrinsic. It is interesting to note that the latter group of proteins display ill-defined contours in the electrophoresis, whereas the peripheral polypeptides yield sharp bands. This reflects a property of the polypeptides rather than a consequence of the extraction, because the same features are observable in the total DOC pellet. The occurrence of *fuzzy* bands in $NaDodSO_4$-PAGE patterns of other proteins has been attributed to heterogeneity in the carbohydrate content of glycoproteins [36] and to the continuous formation of reversible disulfide bonds during the run [25].

Partial Purification of the Phlorizin-Binding Component

The specific phlorizin-binding activity of the brush-border membranes was progressively increased as a selective elution of proteins was accomplished. Even though activation of the binding could have occurred, we regard this as an unlikely possibility since the apparent affinity of the sites did not change significantly after detergent treatment (Fig. 2). Thus it is simpler to assume that the increased specific binding activity reflects the removal of unrelated proteins.

Based on the quantitative considerations mentioned in the introduction and assuming a single polypeptide chain of mol wt 100,000, the phlorizin binding component was calculated to represent approximately 0.4% of the total protein in intact vesicles. This value should increase to about 1.6% after enrichment by DOC treatment, to about 2.3% after NaI or pH 11 treatment, and to a maximum of 3.2% following extraction of the DOC pellet with pH 11, if no solubilization of the binding sites occurred. The progressive intensification found in the staining of several bands is quantitatively compatible with these calculations. Assuming that the staining intensity of the bands is a true measure of their protein content and that only one polypeptide type is present in each band, it is possible to make certain predictions regarding the identity of the phlorizin-binding polypeptides in the electropherograms: (i) The band(s) representing the phlorizin-binding entity, which should be barely visible in the original preparation (0.4% of the total protein),

are expected to be enriched three- to fourfold in the DOC pellet with respect to the original vesicles. (ii) They are also expected to remain and be enriched in the pellet after the extraction with NaI or alkaline media. (iii) The part of the molecule responsible for the binding must stay in the membrane after proteolysis of intact vesicles with proteases which do not affect the binding. (iv) Finally, it is possible that the mobility of the relevant bands changes after proteolysis from inside the vesicles (after DOC) which greatly reduces Na^+-dependent glycoside binding. However, cleavage at a site near the end of the polypeptide chain could inactivate the binding without appreciably altering its electrophoretic mobility.

According to these criteria, those bands showing at least one "– –" or "–" sign in the upper part of Table 3 are probably not essential components of the binding site, whereas those showing only "+" signs are viable candidates. The former include bands *2, S, 3, 4, 5d, 6d, 7d, 8d, 9d, A, 10, 11, 12, 13* and *14*. The possible candidates are bands *1, I, 5f, 7f* and the mol wt 42,000 band. Judging from their molecular weight and relative staining intensity, the number of copies of any of them suffices to account for the known number of phlorizin-binding sites.

Because phlorizin binding to intact vesicles is unaffected by papain – which removes all but a piece of isomaltase – it is possible to exclude the outermost part of this glycosidase as a possible binding component. However, the involvement of the remaining membrane-binding fragment, which is too small to be resolved by the gel system used, cannot be ruled out. Band *1* also is cleaved by externally added papain, which means that if this polypeptide is involved in glycoside binding, it is still functional after papain treatment. From the possible candidates listed above, only *7f* is degraded by trypsin and chymotrypsin after DOC treatment to an extent consistent with the reduction in phlorizin binding. Although it does not rule out bands; *1*, *5f* and the mol wt 42,000 polypeptide as likely possibilities, this finding is compatible with a purported role of *7f* in glycoside binding.

A maximum ninefold enrichment of phlorizin-binding sites was achieved by pretreating vesicles with papain followed by DOC extraction and finally incubating in alkaline solutions. In this preparation the binding component could constitute over 3.5% of the total protein and should be very conspicuous in the gel pattern. Only a reduced number of bands are seen in the gel (Fig. 6) and some of them, such as bands *3* and *4*, can be practically ruled out based on the arguments listed above. Interestingly, band *7f*, which was suggested above as a possible

element of the binding component, is either very faint or not present in the final pellet, and a new band of mol wt 49,000 to 57,000 appears instead. Whether the latter is a product of the proteolysis of the former was already considered (*see* p. 65). Therefore, if *7f* is involved in the binding, its fragmentation by external proteolysis with papain would not inactivate it.

The notion that the phlorizin binding component is a part of the monosaccharide-transport system in both intestinal and renal brush borders has been well substantiated [3, 13, 21, 33, 38, 41]. Whether additional components exist and whether they co-purify with the glycoside binding element throughout the procedure described, is not known. To answer these questions, we attempted to reconstitute D-glucose transport activity from the purified material. Unfortunately, no resealing of the DOC-treated membranes could be achieved by either incubation in media with high ionic strength, freezing and thawing, or sonication with or without exogenous lipids. Thus it remains to be defined whether additional elements of the transporter are being inactivated or solubilized by the extractions, leaving only an active glycoside binding site attached to the membrane.

Even though no final identification of the phlorizin-binding site was accomplished, several polypeptides have been eliminated as possible candidates and a considerable (ninefold) enrichment was obtained. This is, to our knowledge, the highest degree of purification achieved for a Na^+-coupled carrier system. The difficulty encountered in the purification of this type of carriers – due to their scarcity in the membrane – is best illustrated by the fact that only a tenfold enrichment is required for the purification to near homogeneity of the more abundant Na^+-independent sugar carrier of the red cell [2]. The purified brush-border membrane preparation obtained should prove an excellent starting material for either further purification or for labeling studies. The preparation has the additional advantage that unspecific phlorizin binding, which is high in the original vesicles, has been drastically reduced. These features, combined with the use of DOC – which proved to be more suitable for these studies than other ionic and non-ionic detergents – should contribute to the final identification and purification of the monosaccharide transport system.

We wish to thank Drs. D. Diedrich, R. Cherry, and M. Kessler for valuable suggestions and critical reading of the manuscript. We also wish to thank Ms. U. Zillian for help in typing the manuscript.

This work was supported in part by the Swiss National Science Foundation, Berne.

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