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Characterization of Bile Acid Binding to Rat Intestinal Brush Border Membranes*

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Summary. Studies were performed to characterize the binding[1] of bile acids to intestinal brush border membranes. Total ^{14}C-taurodeoxycholate binding was: 1) similar for brush borders prepared from jejunum and ileum, 2) linear with respect to monomer concentration, 3) uninhibited by a structural analog, and 4) not depressed by boiling or trypsin. A linear relationship existed between binding and the number of hydrogen bonds formed by a bile acid and the slope of the line corresponded to $\delta\Delta F$ of 300 cal/mol. The binding of bile acids to the $105{,}000 \times g$ supernatant fraction of sonicated brush borders was similar to the binding of phospholipid liposomes using gel chromatography. These data suggest that: 1) the kinetics and characteristics of binding of bile acid to ileal brush borders do not reflect the kinetics and characteristics of active ileal transport previously obtained in whole tissue preparations, but instead reflect the kinetics and characteristics of passive jejunal transport; 2) a determinant of binding is hydrogen bonding with water; 3) isolated intact brush borders are relatively polar membranes; and 4) binding to solubilized brush borders may represent partitioning between the aqueous phase and membrane lipid.

The characterization of bile acid intestinal transport has been determined *in vivo* in man and experimental animals (Schiff, Small & Dietschy, 1972; Krag & Phillips, 1974), by a variety of *in vitro* assay systems involving whole tissue preparations (Holt, 1964; Lack & Weiner, 1966; Dietschy, Salomon & Siperstein, 1966; Wilson & Dietschy, 1972) and more recently in isolated epithelial cells (Wilson & Treanor, 1975). These studies have shown that the ileal system satisfies many of the criteria for active transport, i.e. 1) to move bile acid against an electrochemical gradient, 2) to manifest saturation kinetics when uptake rates are measured as a function of bile acid concentration in the mucosal perfusate, 3) to exhibit competitive inhibition of uptake of one bile acid by another structurally related bile acid, and 4) to be inhibited by anaerobiosis

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1 Binding in this manuscript denotes the sum of specific membrane receptor-ligand interaction plus partitioning of ligand into membrane lipid.

or metabolic inhibitors. In contrast, bile acid absorption across the jejunum manifests linear kinetics, no competition between bile acids or metabolic inhibition, and therefore satisfies criteria for passive transport It also has been demonstrated that apparent passive permeability coefficients for bile acid monomers crossing the proximal small bowel vary with the state of ionization and the chemical structure of the bile acid molecule being tested. Likewise, the active transport of a given bile acid across the ileum described in terms of apparent values for maximal transport velocity and Michaelis constant depends upon the number of hydroxyl groups on the steroid nucleus and whether or not the bile acid is conjugated (Schiff *et al.*, 1972).

The extension of the study of bile acids to the characterization of their binding to brush border membranes represents a sequential step in the breakdown of the intestine from *in vivo* preparations to progressively smaller components of the mucosal cell that are more intimately involved in bile acid transport. In addition, the initial step in intestinal membrane transport must describe events occurring at the level of the brush border. The purpose of the study, therefore, was to study the binding of bile acids by isolated brush borders from jejunum and ileum and determine whether the kinetics and characteristics of binding reflected the kinetics and characteristics for bile acid transport previously described in whole tissue and cell preparations.

Materials and Methods

Materials

^{14}C-labeled bile acid (California Bionuclear Corp., Sun Valley, Calif.) and unlabeled bile acid (Steraloids, Inc., Pawling, N.Y.; Calbiochem, Los Angeles, Calif.) included taurocholate, taurodeoxycholate, taurochenodeoxycholate, glycocholate, glycodeoxycholate, cholate, deoxycholate, chenodeoxycholate and lithocholate. These compounds were found to have greater than 95% purity using thin-layer chromatography (Schiff *et al.*, 1972). A phospholipid preparation (Fleischer & Fleischer, 1967) comprising cardiolipin (20%), lecithin (40%) and phosphatidyl ethanolamine (40%) was a gift from Dr. Sidney Fleischer (Vanderbilt University, Nashville, Tenn.). Sephadex G-75 was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Plastic micro test tubes (0.4-ml capacity) were purchased from Beckman (catalog no. 314326).

Methods

Preparation of brush borders and basolateral membranes. Female 180 to 220 g Sprague-Dawley rats fed regular chow ad libitum were killed by a blow on the head and bled. The entire small bowel was removed, flushed with iced 0.9% NaCl solution, everted and divided into 10 segments of equal length, numbered 1 to 10, proximal to distal. Segments 2–4 and 7–9 were used for the study of jejunum and ileum, respectively. The mucosa

was scraped from these segments with a glass slide and placed in a Sorvall Omni-mixer with 75 vol of ice-cold 5 mM EDTA, pH 7.5. The scrapings were homogenized at speed setting no. 9 for 25 sec and centrifuged at $450 \times g$ for 10 min. The supernatant was set aside for later purification of basolateral membranes. Brush borders were prepared from the sediment by modification of the method of Forstner, Sabesin and Isselbacher (1968). The sediment was washed three times with EDTA buffer, sedimenting at $450 \times g$ between each wash. The final pellet was resuspended in 2 vol of 90 mM NaCl-0.8 mM EDTA buffer, allowed to stand for 30 min and then filtered in succession through No. 9 bolting silk (97 meshes per linear inch), No. 25 bolting silk (200 meshes per linear inch) and a pad of glass wool. After the filters had been washed with additional 5 mM EDTA buffer, brush borders in the total washing were sedimented by centrifugation at $450 \times g$ for 10 min and washed once in 2.5 vol of 2.5 mM EDTA buffer. After centrifugation the pellet was washed and suspended in appropriate buffers for membrane solubilization, enzyme determinations, microscopy and binding experiments.

The supernatant, previously set aside, was used for preparation of basolateral membranes according to the method of Quigley and Gotterer (1969) as adopted by Parkinson, Ebel, DiBona and Sharp (1972). The supernatant was centrifuged at $10{,}000 \times g$ for 10 min and the pellet was suspended in 2.5 mM EDTA, pH 7.4. This fraction was "aged" by a 3-hr incubation at 37 °C, layered over a gradient composed of 20, 30 and 40% sucrose and centrifuged in an SW 50L rotor at $50{,}000 \times g$ for 90 min at 4 °C. The membrane fraction between 20 and 30% sucrose (density interface, $d = 1.08/1.13$), designated as the M_1 band by Quigley and Gotterer (1969) and Parkinson *et al.* (1972), was removed by gentle aspiration with a bent needle and syringe and sedimented in 1 mM Tris buffer, pH 7.5, by centrifuging at $14{,}000 \times g$ for 30 min. The pellet was suspended in appropriate buffer for studies of relative membrane purity.

Preparation of solubilized brush border membranes. Brush border membranes were suspended in iced 20 mM Tris-1 mM EDTA buffer, pH 8, at a concentration of 1 to 2 mg protein per ml and sonicated with a Ystrom probe sonicator (Technic International, Bergenfield, N.J.) for 4 min at the highest setting. The sonicated brush borders were centrifuged in an SW 50L rotor at $105{,}000 \times g$ for 120 min at 4 °C. The supernatant was recovered and used for binding studies.

Microscopy. Brush border isolation was monitored at several stages by phase microscopy. Brush border and basolateral membrane pellets were fixed in 2% glutaraldehyde in phosphate buffer, pH 7.2. The pellets were washed twice and stored in phosphate-buffered 7.5% sucrose, pH 7.2. Post-fixation was performed in Millonig's phosphate-buffered 1% OsO_4 for 2 hr. En bloc staining was accomplished with 1% uranyl acetate. After dehydration in a graded series of ethanol and propylene oxide washings, embedment was made in Araldite. Thin sections were cut with a diamond knife on the LKB Ultratome and collected on 300-mesh unsupported copper grids. Sections were examined with a Phillips EM 200 electron-microscope operated at 60 KV and photographed at print magnifications of 9800 to 77,000.

"Marker" enzymes. Adenosine triphosphatase (ATPase) activity was determined by the amount of inorganic phosphate produced during the incubation of subcellular fractions with ATP. (Mg+Na+K)-ATPase determined in a medium consisting of (in mM): 4 Na_2ATP, 3.9 $MgCl_2$, 30.2 imidazole-glycylglycine, 160 NaCl, 25 KCl, 0.2 Na_2EDTA, pH 7.2. Ouabain-insensitive ATPase was measured by adding ouabain, 45 mg/250 ml. After incubation for 60 min at 37 °C, the reaction was stopped by the addition of ice-cold 6% trichloracetic acid. Inorganic phosphate was determined in the supernatant by the method of Taussky and Shorr (1953). Protein was determined according to the method of Lowry, Rosebrough, Farr and Randall (1951) employing crystalline bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) as a standard. The specific activity of ATPase was expressed as μmoles phosphate·mg protein^{-1}·hr^{-1}. (Na+K)-ATPase was defined as the

difference in the specific activities of (Mg+Na+K)-ATPase and ouabain-insensitive ATPase. Disaccharidases were determined as described by Dahlqvist (1964). Sucrose, lactose and maltose were employed as substrates.

Measurement of binding of ^{14}C-labeled bile acid to intact brush borders. Incubations were carried out at 0 °C in plastic micro test tubes (capacity 0.4 ml). All incubations were prepared using phosphate buffer (Robinson, 1949), pH 7.4, which was altered by the omission of Ca^{2+} and Mg^{2+}. Incubation medium contained ^{14}C-labeled bile acid at the concentrations stated in legends to Figures and Tables. Brush border membranes (0.1 to 0.8 mg of protein) suspended in phosphate buffer were added to complete a reaction volume of 200 µl. After 3 min of incubation, the tubes were centrifuged immediately in a Beckman Microfuge (Catalog No. 314300) for 2 min. Varying the length of centrifugation (0.5 to 6 min) did not vary the radioactivity sedimented. An aliquot (100 µl) of the supernatant fluid was added to a vial containing 10 cc of Ready-Solv Solution VI (Beckman Catalog No. 566436). The ^{14}C label was counted in a Packard Tri-Carb scintillation counter (model 2003). The quantity of bound bile acid is expressed as nanomoles of bile acid bound per mg of membrane protein. The amount of bound bile acid was calculated according to the method of Smigel, Frölich and Fleischer (1974) as follows:

$$\text{Bound bile acid in nmoles} = \frac{\text{total cpm-free cpm}}{\text{total cpm}} \cdot \text{bile acid added in nmoles} \qquad (1)$$

where total cpm = mean cpm of ^{14}C-labeled bile acid in 4 to 6 100-µl aliquots taken from tubes in which buffer was substituted for the membrane suspension; free cpm = cpm in a 100-µl aliquot from the assay tube that contained the membrane suspension. Replicate determinations were made in all binding experiments. Each experiment was carried out at least twice with different batches of membranes and labeled bile acid.

Measurement of binding of ^{14}C-labeled bile acid to solubilized brush borders and phospholipid liposomes. Binding of bile acid was measured by gel chromatography at 4 °C, using a 0.5 × 15 cm column of Sephadex G-75 according to the method of Hummel and Dreyer (1962). The column was equilibrated with 0.1 mM ^{14}C-labeled bile acid in 20 mM Tris-1 mM EDTA buffer, pH 8, before 100-µl samples of solubilized brush borders (containing 0.16 to 0.86 mg protein, 0.05 to 0.08 µg phosphorous) or phospholipid liposomes (containing 30 to 33.2 µg phosphorous) were applied. Protein concentration in the eluate was determined by absorbance measurements at 280 nm and by the method of Lowry *et al.* (1951). Phosphorus concentration was determined by digestion of 0.45 ml of eluate samples in 72% perchloric acid at 200 °C for 30 min in incineration tubes fitted in a heated aluminum block. After digestion the tubes were prepared for optical density determinations according to the method of Rouser and Fleischer (1967). The free bile acid concentration in equilibrium with the samples was determined from the radioactivity in the eluent ahead of and behind the protein peak, and the amount bound was determined by dividing the excess radioactivity in the protein and/or phosphorus peak by the specific activity of the bile acid (nmoles/cpm) and the protein and/or phosphorus concentration.

Results

Purification of Brush Borders

Initial experiments were performed to assess the relative purity of the brush border preparations. Brush borders are characterized by a high amount of disaccharidase activity (Schiller, David & Johnston, 1970;

ıble 1. Enzyme activities of mucosal homogenate, brush borders and basolateral membranes from rat jejunum and ileum[a]

	Lactase		Sucrase		Maltase		(Na+K)-ATPase	
	S.A. (IU[b]·g protein^{-1})	Recovery (%)[c]	S.A. (IU·g protein^{-1})	Recovery (%)	S.A. (IU·g protein^{-1})	Recovery (%)	S.A. (μmoles·hr^{-1}·mg protein^{-1})	Recovery (%)
Jejunum:								
ucosal homogenate	18.6± 1.1	100	139.2±14.0	100	391.7± 41.7	100	3.0±0.9	100
·ush borders	132.5± 4.4	14.3	678.7±81.7	9.8	2383.5±569.8	12.2	6.8±0.8	4.5
ısolateral membranes	6.4± 1.3	0.2	12.2± 1.6	0.1	65.2± 10.4	0.1	70.8±2.0	11.8
Ileum:								
ucosal homogenate	7.5± 3.1	100	29.8± 9.0	100	253.2± 55.4	100	2.2±0.3	100
·ush borders	74.2±10.7	6.8	271.1±60.6	6.2	1021.4±517.2	2.8	4.4±0.9	1.4
ısolateral membranes	3.1± 0.8	0.3	8.8± 1.8	0.2	63.7± 5.2	0.2	27.6±4.0	10.5

Mean values for six determinations ±1 SEM are given.
IU equals μmoles·min^{-1}.
Percentage of initial enzyme protein of mucosal homogenate recovered in brush border or basolateral embrane preparations.

Houghton & McCarthy, 1973). In contrast, basolateral membranes contain higher activities of (Na+K)-ATPase than brush borders (Quigley & Gotterer, 1969; Fujita, Ohta, Kawai, Matsui & Nakao, 1972). As shown in Table 1 A, the specific activities of jejunal brush border lactase, sucrase, maltase and (Na+K)-ATPase were 7.1, 4.9, 6.1 and 2.3 times higher, respectively, than in original homogenates of jejunal mucosal scrapings. Similarly, these specific activities were 9.9-, 9.1-, 4.0- and 2.0-fold higher in ileal brush borders than in ileal homogenates. To further assess the relative purity of brush borders, marker enzyme activity was compared to that seen in the basolateral membrane fraction [(equivalent to the M_1 band of Quigley and Gotterer (1969) and Parkinson *et al.* (1972)]. As can be seen in Table 1, this cell membrane fraction exhibited relatively low levels of disaccharidase activity and high activity for (Na+K)-ATPase. The jejunal brush border/basolateral membrane enzyme activities for lactase, sucrase, maltase and (Na+K)-ATPase of 20.7, 55.6, 36.5 and 0.09, respectively, indicated clear separation of the two membrane populations. Similar ratios of 23.9, 30.8, 16.0 and 0.16

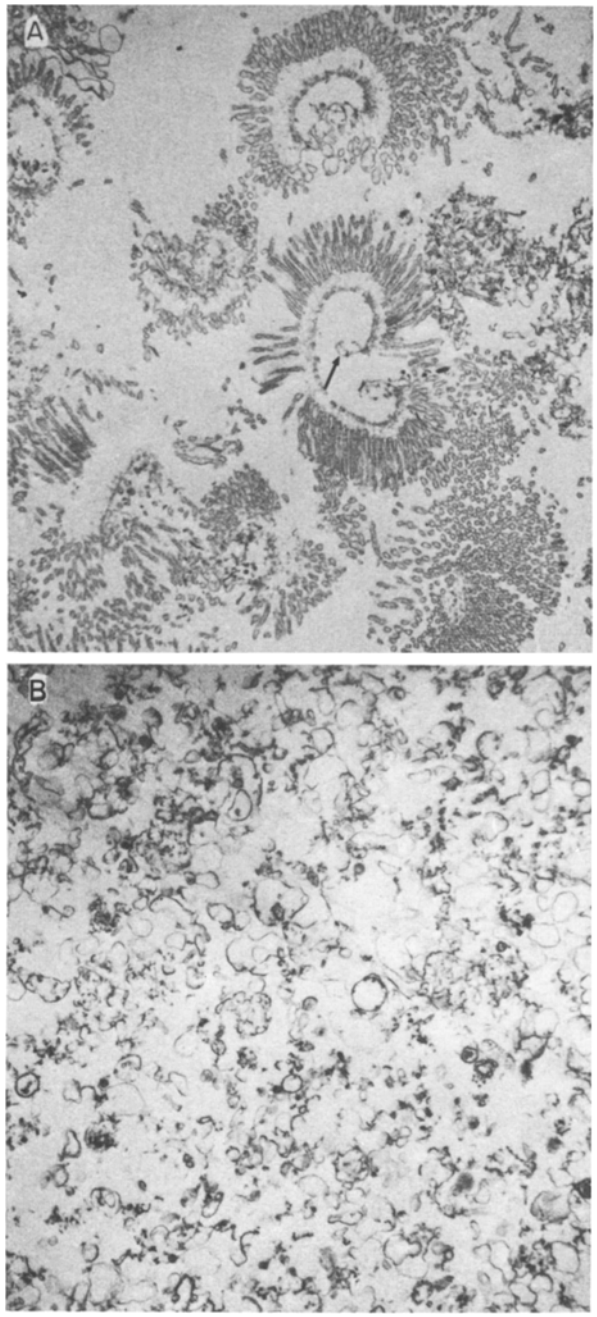

Fig. 1. Electron-micrographs of plasma membranes. In plate *A*, a membrane section consists of a homogeneous preparation of brush borders that contained only small amounts of fibrous material. Tags of vesicular membranous material occasionally were seen attached to the brush border (*see* arrow). Magnification 15,000×. In plate *B*, the basolateral membrane preparation consisted of empty vesicles. There was no adherent material such as glycocalyx or ribosomes and no structures recognizable as mitochondria. Magnification 26,600×

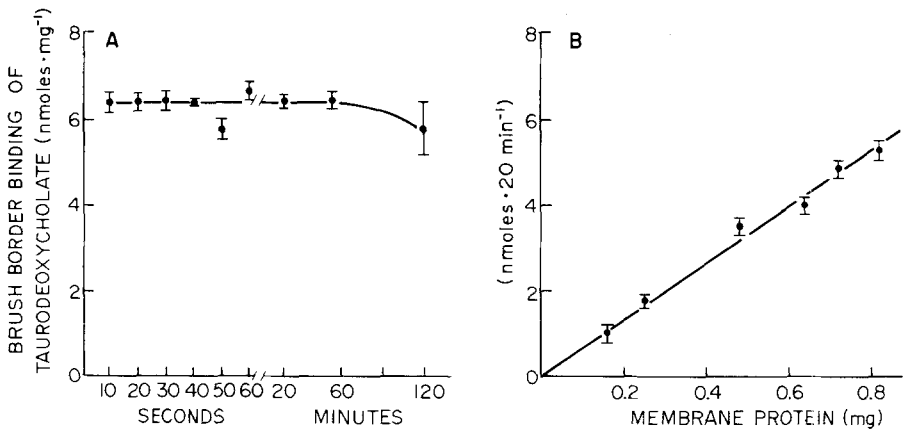

Fig. 2. Characteristics of bile acid binding with respect to time and membrane protein. Ileal brush borders were incubated in phosphate buffer containing ^{14}C-labeled taurodeoxycholate at 0 °C. Conditions for incubation were: panel *A*, 0.1 mM taurodeoxycholate from 10 sec to 120 min; panel *B*, 0.1 mM taurodeoxycholate for 3 min. Values are the means ±1 SE for 10 determinations

were seen for ileal brush border/basolateral membrane enzyme activities. Also shown in Table 1 are the percentages of initial enzyme content of the mucosal homogenates recovered in the brush borders and basolateral membranes. For example, 2.8–14.3% of initial mucosal disaccharidase content was found in the brush borders, whereas the recovery in the basolateral membranes was only 0.1–0.3%.

The successful isolation of purified brush borders and basolateral membranes is further indicated by their appearance under electron-microscopic examination. As shown in Fig. 1*A* brush border preparations were homogeneous and contained only small amounts of fibrous appearing material. Brush border membranes were not seen in the form of vesicles. Occasionally, however, vesicular membranous material remained attached to the brush borders (*see* arrow). The basolateral membrane preparation (the M_1 band) primarily consisted of empty vesicles (Fig. 1*B*). This fraction was not contaminated with clearly recognizable mitochondria or other cellular organelles.

Bile Acid Binding to Intact Brush Borders

The characteristics of bile acid binding to intact ileal brush borders with respect to time and membrane protein are demonstrated in Figs. 2 and 3. The binding of taurodeoxycholate is extremely rapid (Fig. 2*A*).

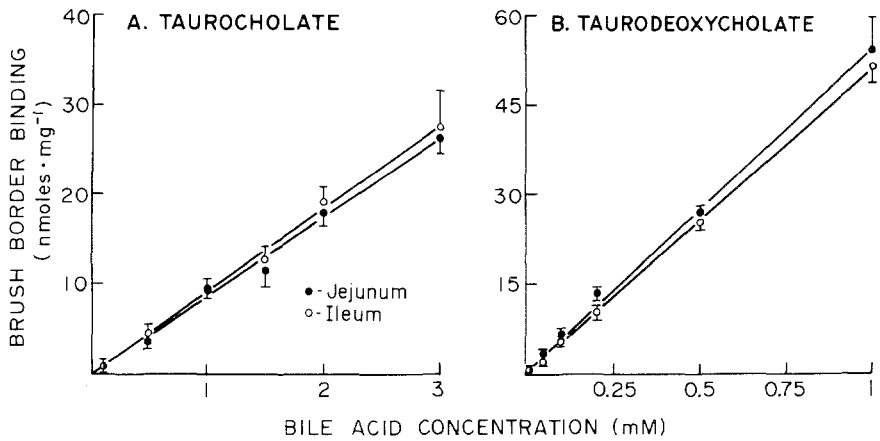

Fig. 3. Characteristics of bile acid binding with respect to monomer concentration. Jejunal (●—●) and ileal (○—○) brush borders were incubated in phosphate buffer containing ^{14}C-labeled bile acid at 0 °C for 3 min. Conditions for incubation were: panel *A*, taurocholate from 0.1 to 3 mM; panel *B*, taurodeoxycholate from 0.01 to 1 mM. Values are the means ± SE for 14 determinations. Slopes for the ileum and jejunum are not statistically different using a *t*-test ($p > 0.01$)

A steady-state level is achieved within seconds. Other members of the bile acid series also displayed essentially instantaneous maximal binding to brush borders that remained constant up to 48 hr of incubation at 0 °C. Bile acid binding is perfectly linear with respect to membrane protein. Taurodeoxycholate binding is plotted in Fig. 2*B*. This determination may be described by linear regression which has essentially zero intercepts. Thus, dividing binding by membrane protein is an appropriate method for normalizing determinations. The binding of bile acids to brush borders is linear with respect to their monomer concentrations, i.e. concentrations below the critical micellar concentration. Fig. 3 shows this relation for taurocholate and taurodeoxycholate up to 3 and 1 mM, respectively. Of note, is the essentially identical binding of bile acid to brush borders isolated from jejunum and ileum.

Previous studies that described kinetics and characteristics for passive bile acid flux across the jejunal brush border membrane and an active transport process in the ileum employed whole tissue and isolated intact epithelial cells. The lack of saturation kinetics when bile acid binding is measured with respect to monomer concentration and similar binding of bile acids to jejunal and ileal brush borders suggest that differences in the transport system for bile acids in ileum and jejunum are not explained by total binding to the isolated brush border membrane under

Table 2. The binding of 0.1 mM taurodeoxycholate (TDC) to ileal brush borders

Experimental conditions	Membrane binding of TDC (% of control)
A. Increasing concentrations of taurochenodeoxycholate (TCDC)	
TCDC (0)	100.0 (24)
TCDC (0.02 mM)	91.7 (24)
TCDC (0.1 mM)	92.3 (24)
TCDC (0.5 mM)	98.0 (24)
B. At different temperatures	
37 °C	100.0 (16)
24 °C	105.6 (16)
10 °C	96.6 (16)
0 °C	100.9 (16)
C. Presence of metabolic inhibitors	
No inhibitor	100.0 (12)
Ouabain (0.1 mM)	95.5 (12)
n-ethyl maleimide (1 mM)	104.5 (12)
Iodoacetamide (5 mM)	86.6 (12)
D. Following treatment of membranes	
No treatment	100.0 (12)
Boiling for 15 min	142.9 (12)
Trypsin (5.5 mM) for 15 min	96.1 (12)

In experiment A the binding of 0.1 mM TDC by brush borders was measured in solutions with and without unlabeled TCDC. The concentrations of TCDC in each study are shown. In experiment B brush borders were incubated with 0.1 mM labeled TDC at the temperatures indicated. In experiment C bile acid binding to brush borders was determined in the absence and presence of ouabain, 0.1 mM, *n*-ethyl maleimide, 1 mM, and iodoacetamide, 5 mM. In experiment D brush borders were treated with boiling or trypsin, 5.5 mM, prior to incubation with labeled TDC. Values are expressed as the percent of control. The numbers in parentheses represent the number of experimental determinations.

conditions of these experiments. To test this hypothesis further studies with ileal brush borders were performed to determine whether binding characteristics reflected those previously described for active bile acid transport (Schiff *et al.*, 1972; Wilson & Dietschy, 1972; Wilson & Treanor, 1975). Data on bile acid binding to ileal brush borders are given in Table 2. In experiment A, the binding of radiolabeled taurodeoxycholate was measured in the presence and absence of a second unlabeled bile acid, taurochenodeoxycholate. A fivefold excess of this structural analogue failed to depress the taurodeoxycholate binding to ileal brush borders. In experiment B, incubation of labeled bile acid with brush

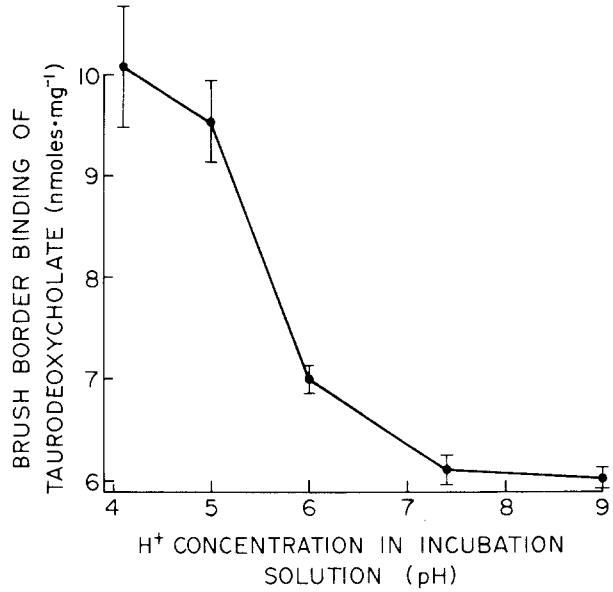

Fig. 4. Characteristics of bile acid binding with respect to pH of the incubation solution. Ileal brush borders were incubated in buffer containing ^{14}C-labeled taurodeoxycholate, 0.1 mM, for 3 min at 0 °C. The values are the means ±1 SE for 12 determinations

borders was unaffected by changes in temperature between 0 and 37 °C. In experiment C, bile acid binding was unaffected by the addition of the metabolic inhibitors, ouabain, N-ethylmaleimide and iodoacetamide. In experiment D, prior treatment of brush borders by boiling or with trypsin resulted in no decrease in bile acid binding from untreated membranes. In fact, boiling actually enhanced bile acid binding.

As shown in Fig. 4, the pH of the incubation medium is inversely related to bile acid binding. Data from Fig. 4 can be used to calculate binding of the monomer ionic and nonionic species using the formula:

$$\text{Total bile acid binding, } BA_{\text{total}} = (B^0[BA^0]) + (B^-[BA^-]) \qquad (2)$$

where total bile acid binding equals the binding constant for the pronated specie (B^0) times its concentration plus the binding constant for the ionized specie (B^-) times its concentration. Total binding can be determined experimentally and $[BA^0]$ and $[BA^-]$ can be calculated from the Henderson-Haselbach equation using the pk_a (Small, 1971) of taurodeoxycholate. By measuring binding at several pH's near the pk_a of taurodeoxycholate and solving for simultaneous equations, values of 6.1 and 4341 nmoles·mg^{-1}·0.1 mM^{-1} were determined for B^- and B^0, respec-

Table 3. The binding of bile acid monomers to ileal brush borders

Bile acid	Membrane binding (nmoles·mg^{-1}·0.1 mM^{-1})	
Taurocholate	0.84±0.19	(50)
Glycocholate	1.03±0.24	(11)
Cholate	2.50±0.40	(21)
Taurodeoxycholate	4.76±0.21	(36)
Glycodeoxycholate	5.86±0.41	(22)
Deoxycholate	7.54±0.35	(39)
Taurochenodeoxycholate	4.09±0.17	(11)
Chenodeoxycholate	8.02±0.35	(22)
Lithocholate	42.26±3.10	(11)

The binding of bile acids varies with their chemical structure. Bile acids represent a series of sterols which differ from one another with respect to their degree of hydroxylation at the number 3, 7, or 12 carbon position on the cyclopentanophenanthrine nucleus. For example, the systematic names for the unconjugated bile acids cholate, chenodeoxycholate, deoxycholate and lithocholate are 3α-, 7α-, 12α-trihydroxycholanoic, 3α-, 7α-dihydroxycholanoic, 3α-, 12α-dihydroxy cholanoic, and 3α-monohydroxy cholanoic acids, respectively. Mean values ±1 SE are given. The number in parentheses gives the number of single binding determinations for each bile acid.

tively. Thus, brush borders had a 714-fold higher affinity for the pronated than the ionized species of taurodeoxycholate.

Previous *in vitro* studies have shown that the passive flux across the jejunal mucosa is dependent upon the structure of the bile acid molecule. As shown in Table 3, the binding of bile acid monomers to ileal brush borders also varies in a similar manner with respect to molecular structure. First, for any given conjugated or unconjugated bile acid removal of the hydroxyl group enhances binding. Second, for a given number of hydroxyl groups the unconjugated bile acid has the highest binding, the glycine conjugate has the second highest binding and the taurine conjugate the third.

The data in Table 3 may be transformed into a linear form, as shown in Fig. 5, by plotting the log of the product (binding constant, B) (molecular weight $^{1/2}$, $m^{1/2}$) against N, the number of hydrogen bonds that a bile acid can form with water (Schiff *et al.*, 1972; Wilson & Dietschy, 1972; Wilson & Treanor, 1975). The linear regression for binding has a negative slope so that the quantity in $Bm^{1/2}$ decreases by a factor of 0.47 for each hydrogen bond that a bile acid can form with water. The slope of the line corresponds to an incremental free energy change,

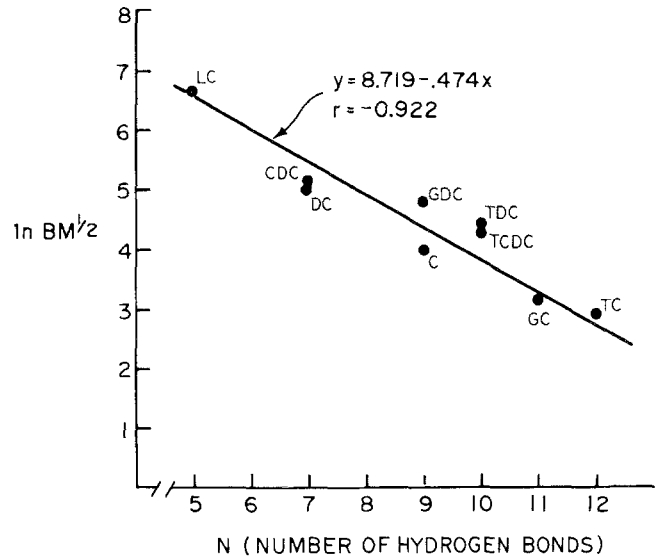

Fig. 5. The relationship of bile acid binding, B, to brush borders and their capacity to form hydrogen bonds with water. In this diagram, B (Table 3) has been multiplied by the square root of the molecular weight ($M_{\frac{1}{2}}$) for each bile acid and the ln of this product has been plotted against n, the number of hydrogen bonds. Values of n were assigned as suggested by Stein (1967). In this Figure LC, CDC, DC, GDC, C, TDC, TCDC, GC and TC represent lithocholate, chenodeoxycholate, deoxycholate, glycodeoxycholate, cholate, taurodeoxycholate, taurochenodeoxycholate, glycocholate and taurocholate, respectively

$\delta \Delta F_{w \to l}$, of about 300 cal/mol for each additional hydrogen bond. Thus, a determinant of bile acid binding to brush borders is the degree of hydrogen bonding in the bulk water phase.

Bile Acid Binding to Solubilized Brush Borders

The 105,000 × g supernatant fraction of sonicated ileal brush borders was passed over a Sephadex G-75 column equilibrated with ^{14}C-bile acid. A typical elution profile for the supernatant fraction is shown in Fig. 6. The peak protein and phosphorus concentrations appear in the same elution position within the void volume.

The binding of bile acid to sonicated brush border supernatant fraction and phospholipid liposomes is compared in Table 4. Not only is the binding similar for sonicated brush border membrane supernatant and phospholipid liposomes but the differences in binding is again dependent upon molecular structure of the bile acid molecule being tested as seen for binding to intact brush borders.

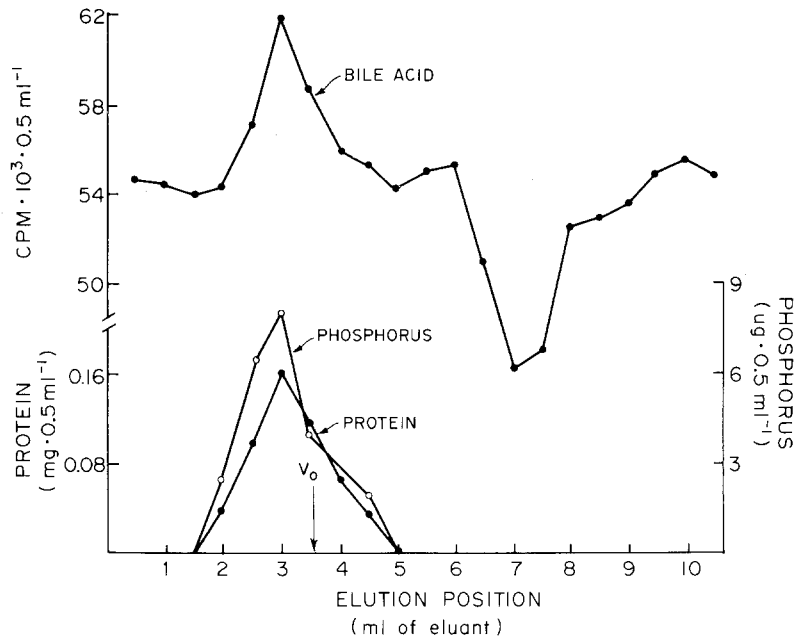

Fig. 6. Determination of binding of bile acid to 105,000 × *g* supernatant fraction of sonicated brush borders. The free ^{14}C-labeled bile acid concentration in equilibrium with the membrane fraction is 0.1 mM. The protein and phosphorus peak appear in the same elution position within the void volume

Table 4. Comparison of bile acid binding to solubilized brush borders and phospholipid liposomes

Bile acid	Binding	
	Solubilized brush borders	Phospholipid liposomes
	(nmoles·µg phosphorus^{-1}·0.1 mM^{-1})	
Taurocholate	0.17[a]	0.26
Cholate	0.31	0.34
Taurodeoxycholate	0.72	0.72

[a] Values represent the mean of two or three determinations.

Discussion

A detailed description of molecular events in intestinal transport of bile acids has been difficult because previous *in vivo* and *in vitro* assay systems have been complex. The brush border preparation reduces the

intestine to the membrane component directly involved in the transfer of bile acid from the intestinal lumen into the absorptive cell interior. The method of isolated brush borders described in this paper appears to provide membrane fractions that are purified with respect to starting homogenate and distinct from basolateral membranes in a manner previously reported (Forstner *et al.*, 1968; Quigley & Gotterer, 1969; Parkinson *et al.*, 1972).

The binding assay employed in this study involved the incubation of ^{14}C-labeled bile acid and intact brush borders, centrifuging down the membranes, counting the supernatant and calculating the total amount of bile acid bound to the membrane by multiplying the total amount added times the percent bile acid bound. Basically, this method is similar to that described for measuring kinetics of prostaglandin binding to liver plasma membranes (Smigel *et al.*, 1974) and partition coefficients in erythrocyte membranes (Metcalfe, Seeman & Burgen, 1968; Seeman, 1969). As recently pointed out by Katz and Diamond (1974), this method is valid if the fraction of the total amount of solute that is not in the supernatant is appreciable. The assay system also has the advantage over methods that directly measure the solute in the membranes by not having to correct for solute trapped in water adherent to the membranes or differences in counting due to tissue quenching.

Binding assays have provided a means to demonstrate specific ligand/membrane interaction for a variety of molecules that are transported across cell membranes presumably via a carrier system (Pardee, 1968; Eichholz & Howell, 1972). In models of carrier-mediated transport, a receptor is believed to represent the recognition site in the carrier complex, and it is generally assumed that binding of the ligand to the receptor is the first step in membrane translocation (Pardee, 1968). Previous *in vivo* and *in vitro* studies have suggested that bile acid transport across the ileum occurs via an active carrier-mediated transport process, whereas, transport across the jejunum occurs by simple passive diffusion (Dietschy *et al.*, 1966; Schiff *et al.*, 1972; Krag & Phillips, 1974). A logical extension of these earlier studies, therefore, is the search for a receptor involved in the active transport of bile acids across the mucosal cell membrane. In addition, the geographical uniqueness of the bile acid transport systems might allow for comparison of binding to jejunal and ileal brush borders and thus factor out nonspecific binding phenomena.

The data presented in this paper failed to demonstrate differences in total bile acid binding to brush borders isolated from jejunum and ileum or show that binding was saturable with respect to monomer

concentration. In addition, binding to ileal brush borders was affected by the state of ionization of the bile acid being tested and was unaffected by temperature, competitive or metabolic inhibition and prior treatment of membranes by boiling or trypsin in an attempt to inactivate a specific membrane receptor. Thus, the kinetics and characteristics of binding of bile acid to ileal brush borders did not reflect the kinetics and characteristics of active ileal transport previously described in whole tissue and isolated cell preparations, but instead reflected the kinetics and characteristics of passive jejunal transport (Schiff *et al.*, 1972; Wilson & Dietschy, 1972; Wilson & Treanor, 1975). This situation might arise from the presence of interfering nonspecific low affinity binding that occurs from partitioning between the aqueous phase and membrane lipid. Evidence of such a phenomenon occurring in bile acid binding to brush borders is derived from the application of the following formulations.

In situations where transport across biological membranes occurs by simple diffusion,

$$P = kD/d \tag{3}$$

where P is the permeability coefficient, k is the partition coefficient, D the diffusion coefficient and d the thickness of the membrane. K, in turn, can be related to thermodynamic properties (Diamond & Wright, 1969):

$$k = e^{-\Delta F_{w\to 1}/RT} \tag{4}$$

where $\Delta F_{w\to 1}$ is the change in energy in transferring 1 mole of solute from water to membrane lipid. Solving for Eqs. (3) and (4):

$$\ln \frac{Pd}{D} = \frac{-\Delta F_{w\to 1}}{RT}. \tag{5}$$

A number of unknowns exist in Eq. (5); however, experiments are done to measure permeation of molecules, one with and one without a particular functional group (P^+/P^0). By solving for simultaneous equations (Schiff *et al.*, 1972):

$$\delta \Delta F_{w\to 1} = RT \ln (P^+/P^0) \tag{6}$$

where $\delta \Delta F_{w\to 1}$ is the incremental free energy change associated with the transfer of 1 mole of a single functional group from the aqueous phase to the cell membrane. Assuming membrane binding is determined

in large part by k, then:

$$\delta \Delta F_{w \to l} = RT \ln (B^+/B^0) \tag{7}$$

where B^+/B^0 represent the ratios of binding coefficients for two solutes which differ only by the presence of a single functional group, i.e. OH, on one of the probe molecules. $\delta \Delta F_{w \to l}$ can be calculated from a single pair of probe molecules but the present studies allow for the determination of such data from a homologous series of compounds. As shown in Fig. 5, when the binding coefficients for the bile acid series are plotted logarithmically there is a linear relationship to the number (n) of hydrogen bonds a bile acid molecule may form with water. The values of n were assigned as suggested by Stein (1967). When the slope of this relationship is multiplied by the appropriate gas constant R and absolute temperature T, a $\delta \Delta F_{w \to l}$ of about 300 cal/mole is determined for each hydrogen bond. Thus, a determinant of bile acid binding to brush borders is the ability of the bile acid molecule to form hydrogen bonds with water. More specifically, the addition of a hydroxyl group to the bile acid molecule yields a $\delta \Delta F_{w \to l}$ of 420 cal/mol. This value is less than the incremental free energy change obtained using P ratios in whole intestinal tissue preparations (Wilson & Dietschy, 1972; Westergaard & Dietschy, 1974) and suggests that if bile acid penetration of the membrane is complete, the isolated brush borders represent a more polar phase than the whole intestinal tissue preparation.

To explore further the question of bile acid partitioning between the aqueous phase and membrane lipid, studies were performed to compare the binding of bile acid to solubilized brush borders and phospholipid, one of the major membrane lipid components (Kawai, Fujita & Nakao, 1974). The method employed, that of gel chromatography, has been used previously to measure binding of bile acid to native bovine serum albumin (Makino, Reynolds & Tanford, 1973). Not only was the binding similar for sonicated brush border membranes and phospholipid, but the differences in binding were again dependent upon molecular structure of the bile acid molecule being tested.

Taken together these data suggest that bile acid binding is determined at least in part by partitioning between the aqueous phase and membrane lipid. This physical phenomenon may have obscured specific ligand/receptor interaction responsible for active, carrier-mediated bile acid transport. It awaits perhaps for newer methods, such as recently described for bile acid binding to liver plasma membranes, to resolve this important question (Accatino & Simon, 1976).

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