Characterization of Bile Acid Absorption across the Unstirred Water Layer and Brush Border of the Rat Jejunum

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ABSTRACT We have examined the rate-limiting steps involved in bile acid absorption across the unstirred water layer and lipid cell membrane of the jejunal mucosa. Uptake of the polar bile acid taurocholate is limited solely by the cell membrane since this compound permeates the unstirred water layer more rapidly than the lipid cell membrane and stirring does not enhance uptake. With less polar bile acids which permeate the cell membrane relatively more rapidly, however, the unstirred water layer does exert resistance to mucosal uptake of these compounds. That the unstirred water layer is even more rate limiting to uptake from micellar solutions is indicated by the facts that the rate of bile acid absorption from such solutions is lower than from corresponding monomer solutions, stirring markedly enhances uptake from micellar solutions while increases in viscosity of the incubation media depress uptake and expansion of the micelle size further depresses absorption rates. We also have examined the important question of whether the micelle crosses the brush border intact once it reaches the aqueous-lipid interface. The observations that the calculated permeation rate of the micelle should be extremely low, the rate of mucosal cell uptake plateaus at a constant value when the critical micelle concentration is reached at the aqueous-lipid interface, and the different components of a mixed micelle are taken up at different rates indicate that uptake of the intact micelle does not occur; rather, bile acid absorption must be explained in terms of monomers in equilibrium with the micelle. Finally, after correction of the permeability coefficients of the various bile acids for the unstirred layer resistance the incremental partial molar free energy of solution of the hydroxyl group in the brush border membrane was calculated to equal −6126 $cal \cdot mole^{-1}$ indicating that passive diffusion of these compounds occurs through a very polar region of the cell membrane.

INTRODUCTION

It is now generally appreciated that absorption of fat from the diet involves at least five separate steps: (*a*) partial hydrolysis of triglyceride by pancreatic lipase, (*b*) micellar solubilization of the resultant fatty acids and β-monoglycerides with bile acids, (*c*) uptake of lipids from the mixed micelle into the mucosal cell, (*d*) reesterification of the fatty acid and β-monoglyceride followed by chylomicron formation and, finally, (*e*) release of the chylomicrons into the intestinal lymphatics (1–3). While considerable progress has been made in defining the physical-chemical characteristics of the intraluminal micellar phase (4, 5) and the biochemical steps operative within the cell during esterification and chylomicron formation (6, 7), there are virtually no precise data describing events in the region of the aqueous-lipid membrane interface during the actual process of absorption from micellar solutions.

This particular transport process, which is of fundamental importance to understanding absorption of a variety of lipids from the intestine, is unusually complex and difficult to study for at least three reasons. First, even in simple bile acid solutions there are at least three separate species—ionized monomers, protonated monomers, and micelles. Second, we have

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published a preliminary report indicating that diffusion through the unstirred water layer adjacent to the mucosal cell is rate limiting for absorption from micellar solutions (8). Thus, during this absorptive process one must characterize uptake of at least three chemical species through two separate absorption barriers. Third, commonly utilized techniques for determining transport rates of water-soluble compounds across the intestinal wall are of no value in studying this process since these methods cannot be utilized for determination of uptake rates of lipids that are trapped within the mucosal cell.

Recently we have developed methods that take into account unstirred water layers in the intestine and that allow quantification of essentially instantaneous uptake rates for both water-soluble and fat-soluble compounds into the intestinal absorptive cell (9). In this paper we will present data obtained using these techniques that provide information on three broad areas of fundamental importance to understanding absorption from micellar solutions. First, apparent passive permeability coefficients ($*P$) for ionized and unionized bile acid monomer movement into the jejunal mucosa are presented, and the relative resistance to absorption of these molecules offered by the unstirred water layer and the lipid membrane of the mucosal cell is defined. Second, several lines of experimental evidence are reported that indicate that under physiological conditions the unstirred water layer is rate limiting for absorption of molecules carried in the micelle. Third, data are presented which indicate that the intact micelle does not cross the cell membrane; rather, absorption must be explained in terms of monomer molecules in equilibrium with the micelle. Calculations based upon these permeability data also are presented that allow estimation of the incremental free energies ($\delta\Delta F_{w\rightarrow l}$) associated with the addition of various substituent groups to the permeating molecules.

METHODS

Chemicals. Radiolabeled[1] and unlabeled[2] bile acids were checked for purity and where necessary were purified as described previously (10). ^{3}H-labeled fatty acids[3] were found to be > 98% pure using thin-layer chromatography. Unlabeled fatty acids[4] and lecithin[5] were used as supplied from the manufacturer. Inulin-^{14}C (footnote 6) and ^{14}C- and ^{3}H-labeled dextran (footnote 6) were used to measure adherent mucosal fluid volume. The dextran was dialyzed against distilled water before use.

Incubation techniques. For these in vitro experiments essentially instantaneous unidirectional uptake rates for bile acids and fatty acids into the intestinal mucosa were determined using a previously described assay system (9). 180–220 g Sprague-Dawley rats fed ad libitum were killed by a blow on the head and bled. The entire small intestine was removed, flushed with iced saline, everted over a glass rod, and divided into 10 segments of equal length numbered from 1 to 10, proximal to distal. Segments 2 and 3 and segments 8 and 9 were used for the study of jejunal and ileal transport, respectively. After the segments were filled with cold Krebs bicarbonate buffer, sacs 2 cm in length were tied off and kept in iced buffer solution. Incubation solutions were prepared using Krebs bicarbonate buffer which was altered by omission of calcium. Preliminary experiments showed no differences in rates of tissue uptake of bile acids and fatty acids from calcium-containing and calcium-free solutions. Solutions were oxygenated with 5% CO_2 in oxygen. The ^{14}C- or ^{3}H-labeled compound whose uptake was being measured and a nonabsorbable marker containing the alternate label to measure the volume of the adherent mucosal fluid were added to the incubation solution. Incubation of sacs was carried out for 4 min under three different conditions of stirring of the incubation medium: completely unstirred, bubble stirred using a small stream of 5% CO_2 in oxygen, and vigorously stirred using a magnetic stirring bar to produce a distinct vortex in the incubation chamber (approximately 1800 rpm). After incubation sacs were removed and rinsed for 4 sec in cold saline. The two ends of each sac were cut off and the cylindrical center was placed in a tarred counting vial for determination of tissue dry weight. The tissue was then saponified and double-label counting was carried out as previously described (9). After correction for the mass of the test molecule present in the adherent mucosal solution, tissue uptake rates (J) were calculated and are expressed as the nmoles of the test molecule taken up into the mucosa per min/100 mg dry wt of tissue (nmoles$\cdot$min$^{-1}\cdot$100 mg^{-1}).

In previous validation experiments (9) we have shown (*a*) that the incubation time is sufficient for the unstirred water layers to become uniformly labeled with the nonabsorbable marker, (*b*) that the rate of uptake of the probe molecule is linear with respect to time between 2 and 5 min and extrapolates to zero at zero time, (*c*) that the rate of tissue uptake is linear with respect to the concentration of the test molecule in the incubation medium for passively absorbed substances, and (*d*) that the presence of bile acid micelles in the incubation medium does not alter the inherent permeability of the mucosa.

Histologic controls. Sections of the gut segments were taken routinely after incubation in buffer alone and in buffer containing the various additions of bile acids, phospholipids, etc., for histologic examination.

RESULTS

Transport characteristics of bile acid uptake into the jejunal and ileal mucosa. Previous publications using techniques based on the transmural movement of bile acids have established that jejunal absorption is passive while uptake across the ileum is active (11–13). Since the new technique used in the present study involves measurement of bile acid uptake directly into the absorptive cell, it is essential to show that this

[1] New England Nuclear, Boston, Mass.; Mallinckrodt Chemical Works, St. Louis, Mo.; Tracerlab Div., LFE Electronics, Waltham, Mass.

[2] Steraloids, Inc., Pawling, N. Y.; Calbiochem, Los Angeles, Calif.

[3] Amersham/Searle Corp., Arlington Heights, Ill.; Schwarz Bio Research Inc., Orangeburg, N. Y.

[4] Applied Science Laboratories, Inc., State College, Pa.

[5] Nutritional Biochemical Corp., Cleveland, Ohio.

[6] New England Nuclear Corp., Boston, Mass.

TABLE I

Evidence against Carrier-Mediated Transport of Bile Acid across the Jejunal Brush Border

Experimental measurement and conditions	Tissue uptake, J	
	Jejunum	Ileum
	nmoles·min⁻¹·100 mg⁻¹	
A. Flux of TC into the mucosal cell in the absence and presence of GC in the incubation solution		
TC (0.5 mM):GC (0)	11±1 (6)	100±12 (6)
TC (0.5 mM):GC (1.0 mM)	10±2 (6)	57±6 (6)
TC (1.0 mM):GC (0)	21±2 (6)	190±17 (6)
TC (1.0 mM):GC (1.0 mM)	21±3 (6)	108±8 (6)
TC (1.5 mM):GC (0)	35±5 (6)	241±27 (6)
TC (1.5 mM):GC (1.0 mM)	31±4 (6)	150±16 (6)
B. Flux of TC into the mucosal cell in the absence and presence of TC inside the cell		
Without TC inside mucosal cell	11±2 (5)	—
With TC inside mucosal cell	12±1 (5)	—
C. Flux of TC out of the mucosal cell in the absence and presence of TC outside the cell		
Without TC in incubation solution	72±8 (8)	—
With TC in incubation solution	68±4 (8)	—

In experiment A the flux of TC into the mucosal cell was measured in paired sacs incubated in solutions with and without unlabeled GC. The concentrations of TC and GC used in each study are shown above. In experiment B one member of a pair of sacs was preincubated for 10 min in a 1 mM solution of unlabeled TC after which flux into the mucosa was quantitated in both sacs from a solution containing 1 mM labeled TC. In experiment C the pairs of sacs were preincubated for 10 min in a 1 mM solution of labeled TC. After briefly rinsing the sacs, they were then incubated for 3 min in solutions with and without 1 mM unlabeled TC at the end of which the flux of labeled TC into the incubation solution was determined. The number of determinations is shown in parentheses. Mean ±1 SEM are given. In this and all subsequent tables and figures the following abbreviations are used: cholic (C), glycocholic (GC), taurocholic (TC), deoxycholic (DC), glycodeoxycholic (GDC), taurodeoxycholic (TDC), chenodeoxycholic (CDC), glycochenodeoxycholic (GCDC), and taurochenodeoxycholic (TCDC) acids.

uptake step has the appropriate transport characteristics. While most studies reported in this paper were undertaken in the jejunum, we also have included in this initial section studies performed in the ileum. This allows a critical comparison of the characteristics of uptake into the jejunum (presumably passive) and ileum (presumably active). These initial studies were undertaken using the probe molecule taurocholate since its critical micelle concentration (CMC)[7] is relatively high, about 4 mM (14), and, therefore, at concentrations less than this, one is dealing with a solution containing only ionized monomers.

The first series of experiments, shown in Fig. 1, show the kinetics of bile acid uptake into the jejunum and ileum. The rate of tissue uptake, J, in the jejunum is linear with respect to concentration while in the ileum J clearly describes a hyperbolic relationship to the

[7] *Abbreviation used in this paper:* CMC, critical micelle concentration.

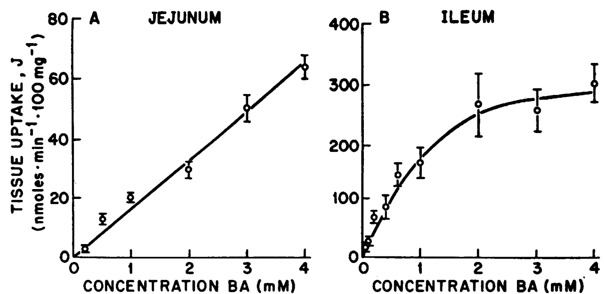

FIGURE 1 Characteristics of bile acid (BA) uptake into the mucosa of the jejunum and ileum. Sacs prepared from jejunum and ileum were incubated in Krebs bicarbonate buffer containing taurocholate-^{3}H at concentrations ranging from 0.05–4.0 mM. Mean values ±1 SEM for 6 to 30 determinations at each concentration are shown by the vertical bars.

concentration of the probe molecule in the bulk water phase. Additional data on possible carrier-mediated transport of bile acids in the intestine is given in Table I. In experiment A the uptake of radiolabeled taurocholate was measured in the presence and absence of a second unlabeled bile acid, glycocholate. Glycocholate depressed taurocholate uptake in the ileum but not in the jejunum. In experiments B and C, the unidirectional flux rates of radiolabeled taurocholate from outside-to-inside and from inside-to-outside the jejunal mucosal cell, respectively, were measured in the presence and absence of unlabeled taurocholate on the contralateral side. In neither instance was the unidirectional flux of taurocholate enhanced by the

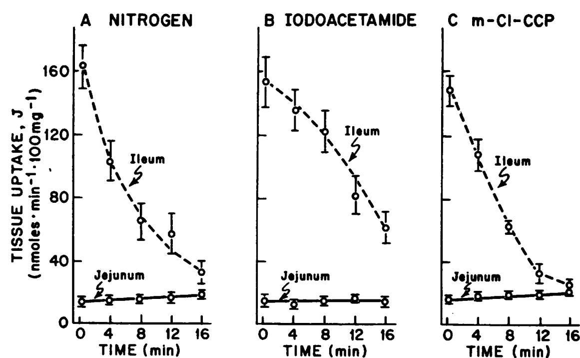

FIGURE 2 Effect of anoxia and metabolic inhibition on uptake of taurocholate across the jejunal and ileal brush border. Sacs prepared from jejunum and ileum were preincubated for 4–16 min either in buffer gassed with 5% CO_2 in nitrogen (A) or in oxygenated buffer containing iodoacetamide (5×10^{-3} M) (B) or m − Cl − CCP (1×10^{-5} M) (C). During the last 4 min of incubation taurocholate-^{3}H (1 mM) was added and J determined. Control sacs incubated for 4–16 min in oxygenated buffer showed no depression of uptake. Mean values ±1 SEM for six determinations at each time are shown by the vertical bars.

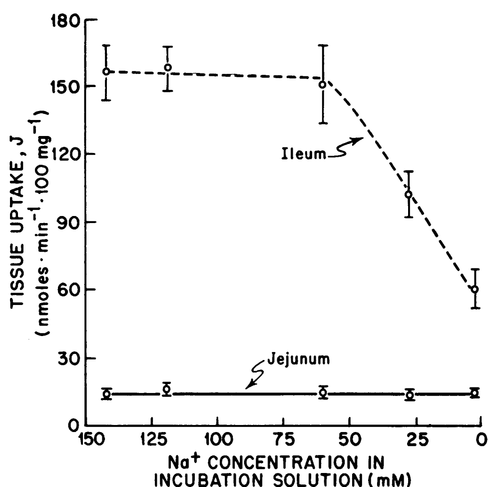

FIGURE 3 Effect of the Na^+ concentration in the incubation solution on taurocholate uptake across the brush border of the jejunum and ileum. Krebs bicarbonate buffer was used to determine J at 143 mM sodium concentration. $KHCO_3$ was substituted for $NaHCO_3$ to give the 118 mM sodium concentration. All solutions of lower sodium concentration were made by iso-osmotic substitution of mannitol for NaCl. The sacs were preincubated for 0.5 min in the solution of appropriate sodium concentration then transferred to an identical incubation solution containing taurocholate-^{3}H (1 mM) for determination of J over a 4 min period. Mean values ± 1 SEM for six determinations at each sodium concentration are shown by the vertical bars.

presence of bile acid on the opposite side of the brush border membrane.

Striking differences were observed in jejunum and ileum with respect to sensitivity to anoxia, metabolic inhibition, temperature, and sodium requirements. As illustrated in Fig. 2 transport of taurocholate into the ileum was markedly inhibited by the absence of oxygen and by exposure of the tissue to iodoacetamide and carbonyl cyanide *m*-chlorophenyl hydrazone (*m*-Cl-CCP).[8] Uptake in the jejunum, in contrast, was either not affected or else was slightly increased by these three manipulations.

In order to determine temperature coefficients jejunal and ileal uptake were measured at 0°, 27°, and 37°C. The Q_{10} value for taurocholate uptake in the ileum was 2.1 while those for uptake of various bile acids in the jejunum were much lower, e.g., taurocholate (1.2), cholate (1.3), glycocholate (1.3), taurodeoxycholate (1.4), and chenodeoxycholate (1.5) (means of four determinations at each temperature).

As shown in Fig. 3, ileal transport also was inhibited by lowering the concentration of Na^+ in the incubation media below 60 mEq/liter while jejunum absorption was unaffected. When ileal segments were exposed to sodium-free solutions for 4.5 min and bile acid uptake rates were then measured from sodium-containing buffer, transport rates were normal. Finally, the addition of α-aminoisobutyric acid, glycine, fructose, or dextrose to the incubation solution (all at 5 mM) failed to affect tissue uptake rates of taurocholate in either the jejunum or ileum.

Characterization of passive monomer absorption across the jejunal mucosa. Since these experiments indicated that bile acid transport into the jejunal mucosa was by passive diffusion and since the proximal small intestine is the primary site of fat absorption, this area of the bowel was utilized in all subsequent experiments designed to elucidate the interactions between the various species of bile acid in the bulk buffer solution and the unstirred water layer and lipid membrane of the mucosal cell. Initial studies were directed at characterization of bile acid monomer uptake. Since bile acid monomer solutions, i.e., solutions of bile acid below their CMC, contain both ionized and protonated species it was necessary to determine the passive permeability characteristics of the mucosa for each of these species as previously described from this laboratory (10). The rate of uptake, J, from dilute solutions is given by the formula:

$$J = (*P^-)([BA^-]) + (*P^0)([BA^0]), \qquad (1)$$

where $*P^-$ and $*P^0$ are the apparent permeability coefficients for the ionized and protonated bile acid monomers, respectively, and $[BA^-]$ and $[BA^0]$ are

TABLE II

Apparent Permeability Coefficients for Ionized Bile Acid Monomers ($*P^-$) and Unionized Monomers ($*P^0$)

Bile acid monomer	$*P^-$	$*P^0$
	$nmoles \cdot min^{-1} \cdot 100\ mg^{-1} \cdot mM^{-1}$	$nmoles \cdot min^{-1} \cdot 100\ mg^{-1} \cdot mM^{-1}$
TC	15±1.7 (25)	
GC	31±3.7 (20)	
C	48±5.1 (20)	264±76 (5)
TDC	26±2.3 (20)	
GDC	66±3.8 (20)	
DC	141±6.8 (20)	368±30 (5)
TCDC	35±2.5 (28)	
GCDC	49±3.3 (24)	
CDC	115±3.4 (20)	639±55 (5)

Apparent permeability coefficients ($*P$) in jejunum are shown for ionized and unionized monomers. Actual determinations were made at bile acid concentrations ranging from 0.25 to 1.0 mM, but all data are normalized to 1.0 mM. The number of determinations is shown in parentheses. Mean values ±1 SEM are given.

[8] E. I. du Pont de Nemours and Co., Wilmington, Del.

respective concentrations of these two species. Since J can be determined experimentally and $[BA^-]$ and $[BA^0]$ can be calculated from the Henderson-Hasalbach equation using the pK_a value of each bile acid (5), equation 1 contains only two unknowns, $*P^-$ and $*P^0$. By measuring J at two different pH values near the pK_a for a given bile acid and solving the pair of simultaneous equations, $*P^0$ and $*P^-$ can be derived. In practice $*P^0$ could be determined directly only for cholate, deoxycholate, and chenodeoxycholate because of the relatively high pK_a values of these bile acids (5.0–5.9 [5]). The conjugated bile acids have such low pK_a values that it would have been necessary to measure J at very acid pH values in order to estimate $*P^0$ directly for these compounds; since it has been shown that these low pH values alter the permeability of the mucosal membrane (10, 15, 16) this was not feasible.

As shown in Table II the apparent permeability coefficients of bile acid monomers vary in a regular manner with respect to molecular structure. First, for any given number of hydroxyl groups the unconjugated bile acid has the highest $*P^-$ value while the glycine conjugate has a lower value and the taurine conjugate is lower still. Second, for any particular unconjugated or conjugated bile acid, removal of a hydroxyl group enhances permeation significantly. Third, removal of the negative charge markedly increases permeation.

As shown in Fig. 4 the data in Table II may be transformed into a useful linear form by plotting the ln of the product ($*P$) (mol wt, $M)^{1/2}$ against N, the number of hydrogen bonds that a particular bile acid can form with water (10, 17). The linear regression curve for the $*P^-$ values has a negative slope so that the quantity $\ln {*PM^{1/2}}$ decreases by a factor of 0.386 for each additional hydrogen bond that a bile acid can form with water. While it is not possible to define accurately the regression curve for the protonated monomers from the limited data, these three points probably describe a second line that is essentially parallel to the curve for the ionized monomers.

The fact that the regression line in Fig. 4 has a significant slope strongly implies that the lipid membrane and not the unstirred water layer primarily is rate limiting for passive monomer absorption. However, to test this possibility further uptake, J, was measured from 1 mM solutions of taurocholate, taurodeoxycholate, and deoxycholate under three conditions of stirring: completely unstirred, bubble stirred, and vigorously stirred with a magnetic bar. Under these three respective conditions of mixing J equaled 14.2±0.6, 17.3±2.4, and 16.6±1.2 nmoles·min^{-1}·100 mg^{-1} for taurocholate; 18.3±1.4, 24.1±4.0, and 26.5±3.5 nmoles·min^{-1}·100 mg^{-1} for taurodeoxycholate, and 61.5±4.6, 127.1±5.6, and 156.7±8.3 nmoles·min^{-1}·100 mg^{-1} for deoxycho-

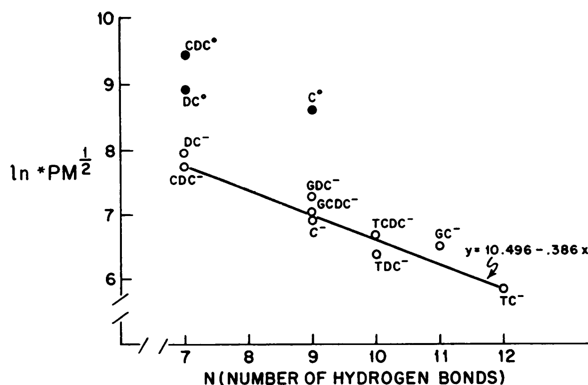

FIGURE 4 Relationship of apparent permeability coefficients, $*P$, for ionized (BA^-) and protonated (BA^0) bile acids to their hydrogen-binding capability. In this diagram $*P$ from Table III has been multiplied by the square root of the mol wt ($M^{1/2}$) for each bile acid and the ln of this product has been plotted against N, the number of hydrogen bonds that a particular bile acid monomer can form in water. Values of N were assigned as suggested by Stein (17).

late (n = 5 in all cases). Thus, it is clear that the unstirred water layer does exert significant resistance to absorption of even bile acid monomers if $*P$ is sufficiently high.

Determination of the rate-limiting step for bile acid absorption from micellar solutions. In view of these results it was anticipated that the major resistance to uptake from micellar solutions might be the unstirred water layer. Four different experimental tests of this possibility were undertaken.

Since these experiments were conducted using high concentrations of bile acid, however, it was first necessary to establish that such solutions did not alter the morphology or permeability characteristics of the epithelial cell membrane. Histological sections were made of intestinal sacs incubated with buffer alone and with solutions containing 20 mM concentrations of several conjugated bile acids. No differences in histologic appearance between the two preparations were observed with light microscopy. Next, it was shown that increasing concentrations of bile acid did not alter the adherent mucosal volume measured with inulin-^{14}C. Even with taurocholate and taurodeoxycholate concentrations as high as 20 mM the measured adherent mucosal volume remained constant (9); hence, the jejunal brush border did not manifest enhanced permeability to the marker in the presence of micellar solutions of bile acid. Finally, the permeability of a water soluble fatty acid, heptanoate was measured in the presence and absence of bile acid micelles and similar rates of tissue uptake were found (9). This finding provided additional evidence that bile acid micelles did not alter the permeability characteristics of the cell membrane under the conditions of these experiments.

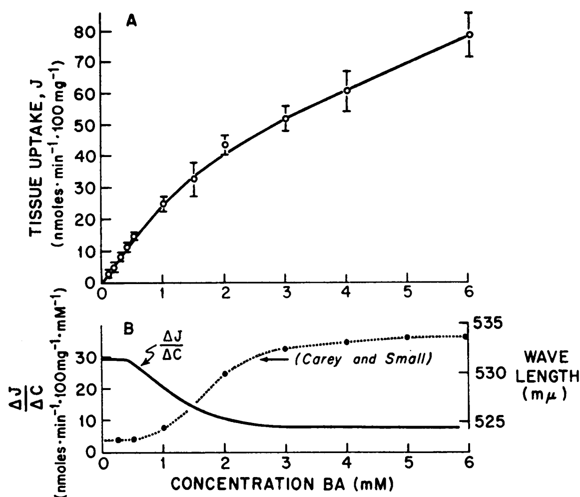

FIGURE 5 Effect of micelle formation on taurodeoxycholate uptake across the jejunal brush border. In panel A sacs were incubated in buffer containing taurodeoxycholate-^{3}H in concentrations ranging from 0.1 to 6 mM. Uptake rates, J, are means ±SEM for 5 to 32 determinations at each concentration. In panel B the slope of the curve from panel A, $\Delta J/\Delta C$, is superimposed upon the wavelength of maximum absorption of Rhodamine 6-G in the presence of taurodeoxycholate (from Carey and Small [14]).

The first type of experiment that was undertaken was to determine the manner in which uptake varied as the concentration of a bile acid in the perfusate was increased above its CMC. As shown in Fig. 5 A, in the case of taurodeoxycholate tissue uptake was linear with respect to concentration of bile acid in the perfusate up to a concentration of approximately 0.8 mM; above this level the slope of the curve declined. In Fig. 5 B the first derivative, $\Delta J/\Delta C$, of this curve is shown. The second superimposed curve represents the wave length of maximal absorption of the dye, Rhodamine G-6, as a function of the concentration of taurodeoxycholate present in solution with the dye. As interpreted by Carey and Small (14), the lowest concentration of bile acid at which the dye spectrum begins to shift represents the point at which micelle formation begins. It is apparent that this shift corresponds very closely to the concentration of bile acid at which $\Delta J/\Delta C$ breaks downward.

For comparison, the uptake rate for two other bile acids, taurocholate and taurodehydrocholate are shown in Fig. 6 along with the curve for taurodeoxycholate. Again J is linear for taurodeoxycholate and taurocholate up to the point where micelle formation begins; beyond this concentration J falls off. In the case of taurodehydrocholate, which does not form micelles, J is linear throughout the range of concentrations tested. These results probably reflect the lower diffusion rate for the micelle relative to the monomer and, therefore, are consistent with the thesis that the unstirred water layer becomes more rate limiting to bile acid uptake from micellar solutions than from monomer solutions.

A second test of this possibility is shown in Table III A where the effect of stirring on uptake from a monomer and two micellar solutions is given. Vigorous stirring has essentially no effect on uptake from a monomer solution of taurocholate but increased uptake from a 30 mM solution of taurocholate, from 151±18 nmoles·min^{-1}·100 mg^{-1} to 253±39 nmoles·min^{-1}·100 mg^{-1} and from a 30 mM solution of taurodeoxycholate from 84±13 nmoles·min^{-1}·100 mg^{-1} to 314±41 nmoles·min^{-1}·100 mg^{-1}.

If the unstirred water membrane is rate limiting for uptake from micellar solutions then such uptake should be inversely related to the viscosity of the unstirred water layer. That this third possibility is the case is shown by the data in Table III B; increasing the viscosity of the incubation media to 1.54 centipoise decreased uptake from micellar solutions of taurocholate and taurodeoxycholate.

Finally, the effect of expansion of the micelle size on uptake was next investigated. As shown by the solid line in Fig. 7 for unstirred conditions, when the bile acid concentration was kept constant at 20 mM and increasing amounts of phospholipid were added to expand the micelle there was a progressive fall in the rate of tissue uptake of bile acid. Vigorous stirring resulted in enhanced uptake at every level of phos-

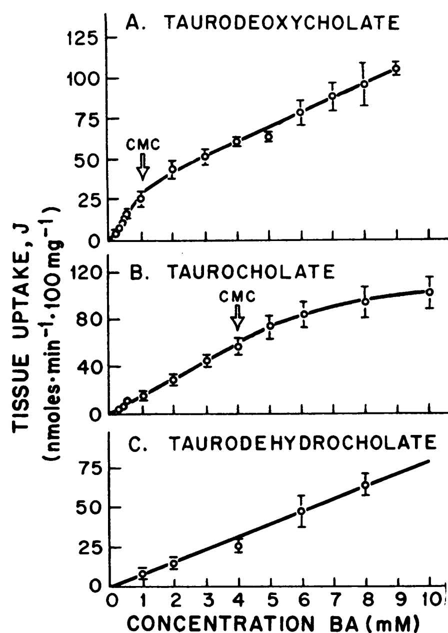

FIGURE 6 Effect of micelle formation on bile acid uptake across the jejunal brush border. The experimental conditions were the same as described in Fig. 5. The indicated CMC were obtained from reference 14.

pholipid, yet it is again apparent that under stirred conditions uptake was inversely related to the phospholipid : bile acid ratio and, hence, presumably to the size of the mixed micelle.

Characterization of events at the aqueous-lipid interface of the jejunal brush border. While the preceding experiments indicate that the unstirred water layer exerts a major resistance to absorption from micellar solutions, they do not answer the question of whether the micelle is absorbed intact through the brush border once it crosses the unstirred water layer and reaches the aqueous-lipid interface. This critical question was approached by two experimental means.

First, if the micelle crosses the cell membrane intact, then tissue uptake should continue to increase even when sufficiently high concentrations of bile acid were present in the bulk phase so that micelles crossed the whole thickness of the unstirred water layer and came into contact with the brush border membrane. This was found not to be the case. As shown in Fig. 6 J increased as the concentration of bile acid was elevated to levels as high as 10 mM. However at much higher concentrations, uptake leveled off at a constant value. Thus, in studies performed with bubble stirring, J equaled 125±11 and 127±14 nmoles·min^{-1}·100 mg^{-1} from taurocholate solutions of 14 and 20 mM, respectively. Similarly, values of 177±23 and 175±14 nmoles·min^{-1}·100 mg^{-1}, 342±39 and 358±26 nmoles·min^{-1}·100 mg^{-1}, and 192±12 and 199±11 nmoles·min^{-1}·100 mg^{-1} were obtained from 25 and 35 mM solutions of cholate, glycocholate, and taurodeoxycholate, respectively. Thus, tissue uptake ceases to increase with increases in bulk phase concentrations once the critical micelle concentration has been reached against the lipid membrane of the jejunal brush border.

TABLE III
Effect of Vigorous Stirring and Increased Viscosity on Bile Acid Uptake from Micellar Solutions

Bile acid and concentration	Control	Experimental
	nmoles·min^{-1}·100 mg^{-1}	
A. Stirring effect		
TC (1mM)	14±1 (6)	16±1 (6)
TC (30mM)	151±18 (6)	253±39 (6)
TDC (30 mM)	84±13 (6)	314±41 (6)
B. Viscosity effect		
TC (30 mM)	165±16 (6)	114±16 (6)
TDC (30mM)	70±6 (6)	56±4 (6)

In experiment A the control data were obtained with no stirring of the incubation solution while the data in the second column were obtained under vigorously stirred conditions using a magnetic bar. In experiment B bile acid uptake was measured from buffer alone (control) and from buffer to which PEG (7g/100ml) was added to increase the viscosity to 1.54 centipoise. The number of determinations is shown in parentheses. Mean values ±5 SEM are given.

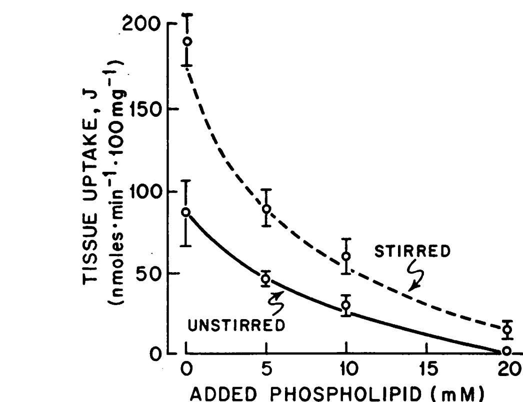

FIGURE 7 Effect of micelle expansion on taurodeoxycholate uptake across the jejunal brush border. Sacs were incubated in buffer containing 20 mM taurodeoxycholate-^{3}H and phospholipid (lecithin) at concentrations of 0, 5, 10, and 20 mM. Uptake rates, J, were determined under unstirred and vigorously stirred (magnetic bar) conditions. Mean values ±1 SEM for six determinations at each phospholipid concentrations are shown by the vertical bars.

The second approach to this problem was to measure tissue uptake of two components of a mixed micelle, the bile acid and a fatty acid. As shown in experiment A, Table IV, when the ratio of palmitate to taurocholate was progressively increased in the bulk buffer solution (column A) bile acid uptake remained essentially constant while fatty acid uptake increased in proportion to the concentration of fatty acid dissolved in the solution. As shown in the last column, the ratio of fatty acid : bile acid uptake divided by the ratio of fatty bile : bile acid in the incubation solution is much greater than the value of 1 that would be expected if the micelle were taken up intact. Similarly, in experiments B and C rates of uptake of the two probe molecules could be altered independently either by varying the rate of stirring or by varying the type of fatty acid present in the micelle. Again, the ratio of fatty acid : bile acid taken up into the tissue was always disproportionately higher than the ratio of these two test molecules in the bulk micellar solution.

DISCUSSION

Previous studies based on transmural movement have established that bile acid absorption across the jejunum is passive (11–13). Initial experiments reported here were undertaken to characterize the process of bile acid uptake into the jejunal mucosa and, for purposes of comparison, the ileal mucosa using our newer methodology. As demonstrated in Figs. 1–3 and Table I,

TABLE I
Rates of Bile Acid and Fatty Acid Uptake from Mixed Micelles

Composition of mixed micelles	Conditions of stirring	Tissue uptake, J: Bile acid	Tissue uptake, J: Fatty acid	Fatty acid:bile acid ratio: A. Incubation solution	Fatty acid:bile acid ratio: B. Tissue uptake	B/A
mM		*nmoles·min⁻¹·100 mg⁻¹*				
A. Varying fatty acid concentration						
TDC (20mM):palmitate (0.1 mM)	Stirred	226±46 (5)	2.9±0.4 (5)	0.005	0.012	2.4
TDC (20mM):palmitate (0.2 mM)	Stirred	215±35 (5)	4.5±0.7 (5)	0.010	0.020	2.0
TDC (20mM):palmitate (0.3 mM)	Stirred	216±22 (5)	8.0±0.4 (5)	0.015	0.037	2.5
TDC (20mM):palmitate (0.4 mM)	Stirred	165±23 (5)	11.2±0.9 (5)	0.020	0.067	3.4
B. Varying stirring with Oleate						
TDC (20mM):Oleate (0.5 mM)	Unstirred	86±17 (5)	7.3±1.1 (5)	0.025	0.084	3.4
TDC (20mM):Oleate (0.5 mM)	Stirred	233±17 (5)	23.0±2.6 (5)	0.025	0.098	3.9
C. Varying stirring with palmitate						
TDC (20mM):palmitate (0.5 mM)	Unstirred	80±10 (5)	10.6±2.4 (5)	0.025	0.132	5.3
TDC (20mM):palmitate (0.5 mM)	Stirred	232±32 (5)	22.5±4.0 (5)	0.025	0.096	3.8

Uptake rates, J, for both the bile acid and fatty acid in mixed micelles were determined under vigorously stirred (magnetic bar) and unstirred conditions. The last three columns show the molar ratios of fatty acid to bile acid in the incubation solution (A), the molar ratios of fatty acid to bile acid taken up into the tissue (B), and ratio B divided by ratio A. The number of determinations is shown in parentheses. Mean values ±1 SEM are given.

taurocholate absorption into the ileal mucosal cell manifests saturation kinetics and exhibits competitive inhibition by a structurally related bile acid. In addition, this transport system is sensitive to metabolic inhibition and anoxia, is depressed by a low sodium concentration in the mucosal perfusate, and has a high Q_{10} value. These features are all compatible with a carrier-mediated, energy-linked active transport system which presumably is present in the brush border of the ileal mucosal cell.

In contrast, movement of bile acid into the jejunal mucosa is not a saturable process and is not stimulated by the presence of bile acid on the contralateral side of the cell membrane. Jejunal uptake also is unaffected by anoxia, metabolic inhibition, or a low sodium environment and has a low Q_{10} value. These findings are compatible with a simple passive diffusion process for bile acid absorption into the mucosal cells of the proximal small intestine.

The system is at least one step more complex, however, since extending outward from the external surface of brush border is a series of unstirred water lamellae each progressively more mixed until they blend imperceptibly with the bulk solution of the incubating medium (18). Therefore, during absorption of bile acid into the jejunal mucosal cell the molecule must, in effect, permeate two membranes in series—the unstirred water "membrane" and the lipid cell membrane. The total resistance encountered as a bile acid molecule moves from the bulk water phase into the jejunal cell is the sum of the resistances engendered by each of these membranes; in particular situations either of these may become the major resistance and, therefore, the rate-limiting step to movement of a molecule from the bulk phase to the cell interior.

If C_1, C_2, and C_3, respectively, are taken as the concentration of the probe molecule in the bulk buffer phase, at the aqueous-lipid interface and just inside the cell membrane then the rate of movement, J, across the unstirred water layer is equal to

$$(C_1 - C_2)(D/d), \quad (2)$$

where D is the free diffusion coefficient for the probe molecule and d is the functional thickness of the unstirred water membrane. The rate of movement across the lipid cell membrane, in turn, is given by the expression

$$(C_2 - C_3)(P), \quad (3)$$

where P is the permeability coefficient of the probe molecule through the lipid membrane. Since C_3 is not measurable and is likely to be very low relative to C_1 under the conditions of these studies this term is considered equal to zero in all subsequent calculations so that equation 3 reduces to $J = (C_2)(P)$. Since under steady-state conditions the rates of movement across the two membranes must be equal it follows that

$$J = (C_1 - C_2)(D/d) = (C_2)(P). \quad (4)$$

It should be pointed out that P represents the permeability coefficient of the lipid membrane while the

term D/d, in effect, represents the permeability coefficient for the unstirred water membrane. Both terms have the units $cm \cdot sec^{-1}$.

Two extreme situations are encountered in biologic systems where either the unstirred water membrane or the lipid cell membrane is the major resistance to uptake and, therefore, is the rate-limiting step to absorption. In the situation where diffusion across the unstirred water layer is very rapid relative to the rate of membrane permeation, i.e., where $D/d \gg P$, C_2 approximately equals C_1 so that the rate of jejunal uptake essentially equals the product $(C_1)(P)$. In the opposite extreme, permeation of the cell membrane is very rapid relative to the rate of diffusion across the unstirred water layer, i.e., $P \gg D/d$ so that C_2 approaches zero and jejunal uptake essentially equals $(C_1)(D/d)$.

In the present study it is likely that the first extreme is represented by absorption of the very polar bile acid taurocholate from monomer solution since this monomer diffuses relatively rapidly through the unstirred water membrane but penetrates the lipid cell membrane very slowly. Two findings support this conclusion. First, the permeability coefficient D/d of the unstirred water membrane for the taurocholate monomer equals 2.7×10^{-4} $cm \cdot sec^{-1}$. Since the highest calculated value for the lipid membrane permeability coefficient (P) is only 5.8×10^{-5} $cm \cdot sec^{-1}$ it is evident that $D/d > P$ so that C_2 should approximately equal C_1.[9] Second, that this is the case is supported by the experimental data in Table III which shows no significant effect of stirring on taurocholate uptake and suggests that C_2 does indeed equal C_1 for this bile acid.

That uptake of the other bile acids with higher $^*P$ values also primarily is rate limited by the lipid cell membrane is suggested by the definitive negative slope evident in Fig. 4 where the function $\ln {^*PM^{\frac{1}{2}}}$ is plotted against a measure of the polarity of these different bile acids, in this case the number of hydrogen bonds (N) that each bile acid potentially can form with water. If the unstirred water membrane were rate limiting than a plot of the function $^*PM^{\frac{1}{2}}$ would be independent of N.[10] The log linear nature of this relationship derives from the fact that under conditions where the lipid cell membrane is rate-limiting $^*P$ is proportional to K, the lipid-aqueous partition coefficient for the bile acid: K, in turn, is proportional to $e^{-\Delta F_{w \to l}}$ where $\Delta F_{w \to l}$ is the free energy change involved in transferring one mole of bile acid from the unstirred water layer to the lipid phase of the cell membrane (20). Since differences in $\Delta F_{w \to l}$ are proportional to N, $\ln {^*PM^{\frac{1}{2}}}$ should vary in a linear manner with respect to N.

Despite this relationship, however, it is also apparent that stirring has a significant effect upon bile acid monomer uptake that is directly related to the $^*P$ value for the different bile acids; thus, vigorous stirring increases J by a factor of 1.1, 1.5, and 2.6 for taurocholate ($^*P$–15 $nmoles \cdot min^{-1} \cdot 100\ mg^{-1} \cdot mM^{-1}$), taurodeoxycholate ($^*P$–26 $nmoles \cdot min^{-1} \cdot 100\ mg^{-1} \cdot mM^{-1}$), and deoxycholate ($^*P$–141 $nmoles \cdot min^{-1} \cdot 100\ mg^{-1} \cdot mM^{-1}$), respectively. The unstirred water membrane, therefore, must exert a significant resistance to the absorption of even bile acid monomers if their permeation rates through the membrane are sufficiently high. It follows that because of this unstirred layer effect the slope of the line in Fig. 4 is artifactually low as are the $^*P$ values reported in Table II.

In view of these results it would be expected that the unstirred water layer would exert an even greater rate-limiting effect upon absorption of bile acid from micellar solutions since these large complexes would diffuse more slowly across the diffusion barrier. Four separate experimental results provide strong support for this possibility. First, $\Delta J/\Delta C$ suddenly decreases when the CMC is achieved in the bulk buffer solution; a finding that presumably reflects the slower free diffusion constant for bile acid in micellar form. Second, stirring has a much more profound effect upon uptake from micellar solutions than from monomer solutions. For example, stirring enhances taurocholate and taurodeoxycholate uptake from a monomer solution by a factor of only 1.1 and 1.5, respectively, but increases uptake from micellar solutions of these same bile acids by 1.7 and 3.7, respectively. Third, increases in the viscosity of the unstirred water layer depress bile acid uptake from micellar solutions. Fourth, progressive expansion of micellar size with another nonbile acid amphipath progressively depresses bile acid uptake yet stirring still markedly enhances absorption.

The final question of critical importance is whether, once the CMC is achieved at the aqueous-lipid interface (at C_2), the bile acid micelle can cross the lipid cell membrane intact. Three lines of evidence strongly suggest that this does *not* take place. First, from the

[9] D/d was calculated from the values of D (5.9×10^{-6} $cm^2 \cdot sec^{-1}$ [19]) and d (1.0×10^{-2} cm). For comparative purposes the value of $^*P$– for taurocholate must be converted from the units $nmoles \cdot min^{-1} \cdot 100\ mg^{-1} \cdot mM^{-1}$ to $cm \cdot sec^{-1}$. We have shown in unpublished data from this laboratory that under comparable experimental conditions the effective surface area of the unstirred water layer equals 4.31 cm^2/100 mg dry wt of tissue. Using this value $^*P$ for taurocholate equals 5.8×10^{-5} $cm \cdot sec^{-1}$.

[10] If the unstirred water membrane is rate limiting to uptake, $^*P$ would be proportional to the free diffusion constant, D, for each bile acid. It has been observed empirically that for relatively small molecules D is inversely proportional to $(M)^{\frac{1}{2}}$; therefore, the product $^*PM^{\frac{1}{2}}$ usually equals a constant (17).

relationship shown in Fig. 4 the theoretical permeability coefficient for a micelle can be derived. From such a calculation it can be determined that $*P$ for even the smallest micelle such as that formed by taurocholate (aggregation Nos. 4, 5 [14]) would be at least 1×10^{-9} the $*P$ for the corresponding monomer.[11] Second, when the concentration of bile acid in the bulk phase is raised to very high levels so that the CMC apparently is achieved at C_2, bile acid uptake into the tissue plateaus at a constant level. This again implies that the monomer but not the micelle is capable of penetrating the membrane. Third, when bile acid and fatty acid uptake from micellar solutions are both measured under conditions where the ratio of fatty acid to bile acid, the type of fatty acid and the degree of stirring are varied, uptake of these two components occurs at independent rates.

On the basis of all of these data events occurring during absorption of bile acid across the jejunum may be summarized as follows. Bile acid monomers are absorbed across the proximal small intestine by passive monomer diffusion. The major resistance to such absorption is the lipid cell membrane although for bile acids with high membrane permeability the unstirred water layer begins to influence the rate of uptake. This water layer exerts even more resistance to movement of micelles from the bulk solution so that as the concentration of bile acid in the bulk phase is raised above the CMC the micelles diffuse into a region of lower total bile acid concentration and presumably undergoes spontaneous disintegration. When the concentration of bile acid in the bulk phase is raised sufficiently high, the CMC can be achieved at C_2: since the monomers in equilibrium with the micelle, but not the micelle itself, cross the membrane tissue uptake ceases to increase at this point.

In previous studies performed in vivo we have shown that bile acid monomer uptake exhibits a similar relationship as shown in Fig. 4. In addition, we found that bile acid uptake continued to increase when the concentration in the bulk water phase was raised above the CMC and that expansion of the micelle by the addition of fatty acid or phospholipid consistently depressed bile acid absorption. The break in the curve describing $\Delta J/\Delta C$ (Fig. 6) was not found in the in vivo experiments; this very likely was the result of large scatter in the in vivo data since in these experiments each point on the curve represented a point derived from an individual animal (10).

Two other points warrant emphasis. Under physiological conditions during fat absorption the bile acid micelle is greatly expanded and so will have a very low diffusion coefficient. In addition, it is to be anticipated that the unstirred water layer will be even thicker than found in these in vitro studies; peristalsis should have essentially no effect upon the unstirred water layer since even extremely vigorous stirring is capable of only minor disruption of this membrane. These considerations along with the results found in both our in vitro and in vivo studies clearly imply that the unstirred water layer is of major importance in determining the rates of uptake of substances carried in the micelle under physiological conditions.

In addition to the implications of these findings with respect to bile acid and fat absorption, these data also can be used to calculate various thermodynamic parameters of the uptake step that yield information of fundamental importance to characterization of the diffusion pathway for these compounds through the intestinal mucosa. In order to make such calculations, it is first necessary to correct the apparent permeability coefficients given in Table II for unstirred layer resistance. By rearranging terms, equation 2 can be rewritten as:

$$C_2 = C_1 - J(d/D), \qquad (5)$$

where the term $J(d/D)$ represents the correction term for the unstirred layer effect. By calculating C_2 for each of the bile acids when the bulk phase concentration is 1 mM, true permeability coefficients (P) can be determined.[12] These equal taurocholate (17), glycocholate (38), cholate (67), taurodeoxycholate (31), glycodeoxycholate (111), deoxycholate (709), taurochenodeoxycholate (45), glycochenodeoxycholate (70), and chenodeoxycholate (331 nmoles·min^{-1}·100 mg^{-1}·mM^{-1}).

From these values it is apparent that the addition of the hydroxyl, glycine, and taurine groups to the bile acid molecule reduces permeation, on the average, by

[11] For this calculation the taurocholate micelle was assumed to be composed of five monomers with their various polar groups all interacting with the water phase so that the micelle contained five negative charges and forms essentially 60 hydrogen bonds. As can be calculated from the data in Table II for C, DC, and CDC the introduction of a negative charge into the molecule reduces the value of the quantity $\ln {*PM^{\frac{1}{2}}}$ by an average of 1.46; thus, for the taurocholate micelle $\ln {*PM^{\frac{1}{2}}} = 4.66 - (0.386)(48)$; from this value the apparent permeability coefficient of the micelle should equal approximately 1.9×10^{-8} nmoles·min^{-1}·100 mg^{-1}·mM^{-1}.

[12] In order to make these calculations the following values were used. Diffusion coefficients for the various bile acids were normalized to the published value for taurocholate, 5.9×10^{-6} cm^2·sec^{-1} (19) and were calculated from the relationshp $DM^{\frac{1}{2}} = 133.9 \times 10^{-6}$ where $M^{\frac{1}{2}}$ equals the square root of the mol wt of each bile acid. Unpublished work from this laboratory has shown that d equals approximately 1×10^{-2} cm over the sites of absorption in the villus tips under the conditions of stirring used in these experiments and that the effective surface area of the unstirred water layer equals 4.31 cm^2·100 mg^{-1}. This latter value was used to convert the units of the apparent permeability data in Table II to nmoles·sec^{-1}·cm^{-2}.

a factor of 4.1, 4.3, and 11.4, respectively. The addition of such polar substituent groups reduces permeation by increasing the free energy change, $\Delta F_{w \rightarrow 1}$, involved in transferring bile acid from the water phase to the lipid phase of the brush border membrane. The changes in $\Delta F_{w \rightarrow 1}$, i.e., the incremental free energies $\delta \Delta F_{w \rightarrow 1}$, can be calculated from these ratios and yield values of +874, +897, and +1498 cal·mole^{-1}, respectively, for the addition of the hydroxyl, glycine, and taurine groups to the bile acid nucleus (20).

The incremental free energy can be further resolved into terms that give a measure of the solute:water interaction ($\delta \Delta F_w$, incremental partial molar free energy of solution in water) and solute:membrane interactions ($\delta \Delta F_1$, incremental partial molar free energy of solution in the lipid membrane) so that (20)

$$\delta \Delta F_{w \rightarrow 1} = - \delta \Delta F_w + \delta \Delta F_1. \quad (6)$$

In the case of the hydroxyl group $\delta \Delta F_w$ equals approximately −7000 cal·mole^{-1} and, therefore, $\delta \Delta F_1$ for the jejunum must equal approximately −6126 cal·mole^{-1}. This value is lower than those calculated by Diamond and Wright (20) for isobutanol (−6000 cal·mole^{-1}), ether (−4900 cal·mole^{-1}), olive oil (−4200 cal·mole^{-1}), and cell membrane of *Nitella* (−3400 cal·mole^{-1}). Thus, $\delta \Delta F_1$ for the jejunal mucosa is strikingly more negative than would be anticipated if this membrane had the characteristics of a very nonpolar lipid layer as is commonly stated; indeed, these calculations indicate that the region of the lipid cell membrane through which passive permeation occurs must be quite polar since the addition of a hydroxyl group to the bile acid molecule increases solute:membrane interaction ($\delta \Delta F_1$ of −6126 cal·mole^{-1}) nearly as much as it increases solute:water interaction ($\delta \Delta F_w$ of −7000 cal·mole^{-1}). A similar finding has been reported in the rabbit gallbladder by Smulders and Wright (21). The important consequence of this fact with respect to passive absorption across the intestinal epithelial surface is emphasized by the fact that the addition of a hydroxyl group to a molecule reduces permeation across a nonpolar membrane such as found in *Nitella* by a factor of 1/420 (20) but diminishes uptake across the jejunum by a factor of only 1/4.1.

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REFERENCES

1. Senior, J. R. 1964. Intestinal absorption of fats. *J. Lipid Res.* **5**: 496.
2. Isselbacher, K. J. 1967. Biochemical aspects of lipid malabsorption. *Fed. Proc.* **26**: 1420.
3. Wilson, F. A., and J. M. Dietschy. 1971. Differential diagnostic approach to clinical problems of malabsorption. *Gastroenterology.* **61**: 911.
4. Hofmann, A. F., and D. M. Small. 1967. Detergent properties of bile salts: correlation with physiological function. *Annu. Rev. Med.* **19**: 333.
5. Carey, M. C., and D. M. Small. 1972. Micelle formation by bile salts: physical chemical and thermodynamic considerations. *Arch. Intern. Med.* In press.
6. Johnston, J. M. 1968. Mechanism of fat absorption. *Handb. Physiol.* **3** (Sect. 6): 1353.
7. Ockner, R. K., F. B. Hughes, and K. J. Isselbacher. 1969. Very low density lipoproteins in intestinal lymph: role in triglyceride and cholesterol transport during fat absorption. *J. Clin. Invest.* **48**: 2367.
8. Wilson, F. A., V. L. Sallee, and J. M. Dietschy. 1971. Unstirred water layers in intestine: rate determinant of fatty acid absorption from micellar solutions. *Science (Wash. D. C.).* **174**: 1031.
9. Sallee, V. L., F. A. Wilson, and J. M. Dietschy. 1972. Determination of unidirectional uptake rates for lipids across the intestinal brush border. *J. Lipid Res.* **13**: 184.
10. Schiff, E. R., N. C. Small, and J. M. Dietschy. 1972. Characterization of the kinetics of the passive and active transport mechanisms for bile acid absorption in the small intestine and colon of the rat. *J. Clin. Invest.* **51**: 1351.
11. Lack, L., and I. M. Weiner. 1961. In vitro absorption of bile salts by small intestine of rats and guinea pigs. *Am. J. Physiol.* **200**: 313.
12. Dietschy, J. M., H. S. Salomon, and M. D. Siperstein. 1966. Bile acid metabolism. I. Studies on the mechanisms of intestinal transport. *J. Clin. Invest.* **45**: 832.
13. Dietschy, J. M. 1968. Mechanisms for the intestinal absorption of bile acids. *J. Lipid Res.* **9**: 297.
14. Carey, M. C., and D. M. Small. 1969. Micellar properties of dihydroxy and trihydroxy bile salts: effects of counterion and temperature. *J. Colloid Interface Sci.* **31**: 382.
15. Rosen, H., A. Leaf, and W. B. Schwartz. 1964. Diffusion of weak acids across the toad bladder. Influence of weak acids across the toad bladder. *J. Gen. Physiol.* **48**: 379.
16. Wright, E. M., and J. M. Diamond. 1969. Patterns of non-electrolyte permeability. *Proc. R. Soc. Lond. B. Biol. Sci.* **172**: 227.
17. Stein, W. D. 1967. The Movement of Molecules Across Cell Membranes. Academic Press Inc., New York.
18. Dainty, J. 1963. Water relations of plant cells. *Adv. Bot. Res.* **1**: 279.
19. Woodford, F. P. 1969. Enlargement of taurocholate micelles by added cholesterol and monoolein: self-diffusion measurements. *J. Lipid Res.* **10**: 539.
20. Diamond, J. M., and E. M. Wright. 1969. Biological membranes: the physical basis of ion and nonelectrolyte selectivity. *Annu. Rev. Physiol.* **31**: 581.
21. Smulders, A. P., and E. M. Wright. 1971. The magnitude of nonelectrolyte selectivity in the gallbladder epithelium. *J. Membrane Biol.* **5**: 297.