JCI The Journal of Clinical Investigation

Characterization of the kinetics of the passive and active transport mechanisms for bile acid absorption in the small intestine and colon of the rat

Eugene R. Schiff, Neal C. Small, John M. Dietschy

J Clin Invest. 1972;51(6):1351-1362. https://doi.org/10.1172/JCI106931.

Research Article

Bile acid uptake occurs via passive diffusion in all regions of the intestine and via active absorption in the ileum. Determination of the passive permeability coefficient for ionized monomers ($^{*}P^{-}$) demonstrated that permeability decreased by a factor of 3.4, 6.8, and 8.1 for the addition of a hydroxyl, glycine, or taurine group, respectively, to the steroid nucleus. Removal of the negative charge increased permeation by a factor of 4.4; however, permeability coefficients for the protonated monomers showed the same relative decrease with addition of a hydroxyl group. The calculated incremental free energies of solution ($\delta\Delta F_{W\rightarrow 1}$) associated with these additions equaled + 757 (hydroxyl), + 1178 (glycine), and + 1291 (taurine) cal/mole. Passive permeability coefficients for the transverse colon showed the same relative relationships among the various bile acids. After making appropriate corrections for passive permeability across the ileum, apparent values for the maximal transport velocity ($^{*}V_{max}$) and Michaelis constant ($^{*}K_{m}$) of the active transport system were measured. $^{*}V_{max}$ depended upon the number of hydroxyl groups on the steroid nucleus; values for the trihydroxy bile acids were high (1543-1906 pmoles/min per cm) while those for the dihydroxy (114-512 pmoles/min per cm) and monohydroxy (45-57 pmoles/min per cm) acids were lower. In contrast, $^{*}K_{m}$ values were related to whether the bile acid was conjugated; unconjugated bile acids had values ranging from 0.37 […]

Characterization of the Kinetics of the Passive and Active Transport Mechanisms for Bile Acid Absorption in the Small Intestine and Colon of the Rat

EUGENE R. SCHIFF, NEAL C. SMALL, and JOHN M. DIETSCHY

From the Department of Internal Medicine, The University of Texas Southwestern Medical School at Dallas, Dallas, Texas 75235

ABSTRACT Bile acid uptake occurs via passive diffusion in all regions of the intestine and via active absorption in the ileum. Determination of the passive permeability coefficient for ionized monomers ($*P^-$) demonstrated that permeability decreased by a factor of 3.4, 6.8, and 8.1 for the addition of a hydroxyl, glycine, or taurine group, respectively, to the steroid nucleus. Removal of the negative charge increased permeation by a factor of 4.4; however, permeability coefficients for the protonated monomers showed the same relative decrease with addition of a hydroxyl group. The calculated incremental free energies of solution ($\delta\Delta F_{w\to 1}$) associated with these additions equaled +757 (hydroxyl), +1178 (glycine), and +1291 (taurine) cal/mole. Passive permeability coefficients for the transverse colon showed the same relative relationships among the various bile acids. After making appropriate corrections for passive permeability across the ileum, apparent values for the maximal transport velocity ($*V_{max}$) and Michaelis constant ($*K_m$) of the active transport system were measured. $*V_{max}$ depended upon the number of hydroxyl groups on the steroid nucleus; values for the trihydroxy bile acids were high (1543–1906 pmoles/min per cm) while those for the dihydroxy (114–512 pmoles/min per cm) and monohydroxy (45–57 pmoles/min per cm) acids were lower. In contrast, $*K_m$ values were related to whether the bile acid was conjugated; unconjugated bile acids had values ranging from 0.37 to 0.49 mM, while values for the conjugated bile acids were approximately half as high (0.12–0.23 mM).

Dr. Schiff was a trainee in Gastroenterology during these studies and was supported by U. S. Public Health Service Training Grant No. T01-AM-5490. His current address is the Department of Internal Medicine, Veterans Administration Hospital, 1201 NW 16th Street, Miami, Fla. 33125.

Dr. Dietschy is a Markle Scholar in Academic Medicine.

Received for publication 9 August 1971 and in revised form 4 January 1972.

INTRODUCTION

It is now well established that bile acids are absorbed at all levels of the gastrointestinal tract, yet even early studies suggested regional differences in relative rates of uptake (1, 2). In addition, the transport mechanisms operative in this absorptive process have been shown to vary in different areas of the bowel. Lack and Weiner, for example, demonstrated that everted intestinal segments from the ileum, but not from jejunum, were able to transport taurocholic acid and glycocholic acid against a concentration gradient (3). This finding suggested the presence of an active transport system localized to the distal half of the small intestine; absorption observed in vivo across the jejunum presumably represented passive movement of bile acid down the chemical gradient that exists between the intestinal contents and portal blood (4, 5).

Still more recent studies have characterized these two transport systems in considerably more detail. The ileal system has been shown (*a*) to move bile acid against an electrochemical gradient; (*b*) to manifest saturation kinetics when uptake rats are measured as a function of bile acid concentration in the mucosal perfusate; (*c*) to exhibit competitive inhibition of uptake of one bile acid by another structurally related bile acid; and (*d*) to be inhibited by anaerobiosis or metabolic inhibitors (6–10). Hence, on the basis of these characteristics it appears that the terminal small bowel contains transport sites capable of active bile acid absorption. In contrast, absorption across the jejunum appears to be passive in that such movement has been shown (*a*) to occur only down existing activity gradients; (*b*) to have a linear

relationship to the concentration of bile acid in the mucosal perfusate; (*c*) not to manifest competitive phenomenon; and (*d*) to be uninfluenced by anaerobiosis or metabolic inhibition (6, 11).

Since under physiological conditions bile acids of various chemical structures are absorbed across the intestine, these systems are ideal for studying the relationship between chemical structure and kinetic characteristics of the active and passive transport processes. Such information is important not only as it relates to general problems of transport and cell permeability, but, in addition, it is of significance in better understanding the enterohepatic circulation under normal conditions and in disease states. Yet, essentially no data currently are available that allow precise comparisons of the kinetics of these two transport processes.

For this reason the present studies were undertaken. In this paper data are presented that allow a comparison of the passive apparent permeability coefficients (*P)[1] for both ionized and protonated bile acid monomers across the small and large intestine of the rat. In addition, experimental results are provided that illustrate the effects of altering the micellar characteristics of the bulk water phase on such passive uptake rates. Apparent values for the maximal transport velocity ($*V_{max}$) and Michaelis constant ($*K_m$) for the active transport of various bile acids have been determined are related to the specific molecular structure of the major classes of bile acids. Finally, the implication of these findings with respect to the normal enterohepatic circulation of bile acid is discussed.

METHODS

Chemicals and reagents. Radiolabeled bile acids, both tritiated and ^{14}C-labeled, were obtained from a variety of commercial sources.[2] In addition, in earlier experiments some bile acids were labeled with tritium by catalytic exchange.[3] In all cases the bile acids were purified by recrystallization and/or by repeated preparative thin-layer chromatography on 20 × 40 cm plates using three different solvent systems (12, 13) until > 98% of the radioactivity ran in a single area that co-chromatographed with appropriate, authenticated bile acid standards. In a number of instances it was possible to measure absorption rates using both a ^3H-labeled and ^{14}C-labeled bile acid; in such experiments identical rates of uptake were obtained. In addition, samples of the serosal fluid in a number of studies were concentrated and subjected to thin-layer chromatography. The radioactivity was found to run with the appropriate bile acid indicating that flux rates reflected movement of bile acid and not movement of labeled trace contaminants. Unlabeled bile acids obtained from two sources[4] were checked for purity using thin-layer chromatography after spotting approximately 100 μg. Where significant contamination was present purification was accomplished by recrystallization (unconjugated bile acids) or by preparative thin-layer chromatography. Mixed egg lecithin (14), monooleate,[5] and oleic acid[5] were used as supplied without further purification.

In the in vivo experiments bile acids were dissolved in either Krebs' bicarbonate buffer equilibrated with 95% oxygen: 5% CO_2 or in a phosphate-Tris buffer (dibasic sodium phosphate, 5 mM; Tris-[2-amino-2-hydroxymethyl-1, 3-propanediol], 5 mM; sodium chloride, 140 mM). In in vitro studies only Krebs' bicarbonate buffer was used. The phosphate-Tris buffer usually was titrated with HCl or NaOH to pH values varying from 6.0 to 8.0 as noted in specific experiments. Preliminary experiments indicated no difference in rates of absorption from Krebs' bicarbonate buffer and the phosphate-Tris buffer.

Incubation techniques. For in vitro experiments unidirectional flux rates for bile acids were determined using a previously described apparatus that allows perfusion of the mucosal and serosal surfaces of an intestinal segment with oxygenated buffer under conditions where the transmural potential difference essentially is zero (6). The small intestine of female Sprague-Dawley rats weighing 200–240 g was divided into 10 segments of equal length, numbered from 1 to 10, proximal to distal. The second and ninth segments usually were used for the study of jejunal and ileal bile acid transport, respectively. The volumes of the perfusate in this system were such that the concentration of bile acid in the mucosal solution decreased less than 2% (and in most cases, less than 1%) during the experimental period when measurements of mucosal-to-serosal flux rates were performed. In addition, after an equilibration period of 20–35 min, the appearance of bile acid in the serosal fluid became linear with respect to time and remained so for 2–3 hr; all measurement of flux rates, however, were made during the linear period from 40 to 70 min. Thus, using this system bile acid flux rates could be measured under circumstances where the appearance of the probe molecule in the serosal compartment was linear with respect to time, where the concentration of the molecule in the mucosal solution remained essentially constant during the experimental period and where the transmural potential difference essentially equaled zero. These flux rates, J, are expressed as the picomoles of bile acid moving across the bowel wall per minute per centimeter length of intestine (pmoles/min per cm).

In order to quantitate the flux rates of bile acids in vivo, rats were anesthetized with phenobarbital and the common bile duct was cannulated. Small bowel segment number 2 or number 9 or the transverse colon was isolated between ligatures, and proximal and distal catheters were implanted (6). After flushing clear, the test segment was returned to the abdominal cavity, and the abdomen was closed with several clips. With the animal maintained under anesthesia the segment was perfused with phosphate-Tris buffer solutions containing radiolabeled bile acids at a constant rate of 0.50 ml/min. At this flow rate there was essentially no change in either the pH or the concentration of bile acid during the passage of the buffer solution through the test

[1] *Abbreviations used in this paper:* CMC, critical micelle concentration; J, flux rates; $*K_m$, apparent Michaelis constant; *P, passive apparent permeability coefficients; $*V_{max}$, apparent maximal transport velocity.

[2] Mallinckrodt Chemical Works, St. Louis, Mo.; New England Nuclear Corp., Boston, Mass.; Nuclear-Chicago Corporation, Des Plaines, Ill.; Tracerlab, Richmond, Calif.

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

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

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

segment. Body temperatures of the anesthetized animals were maintained at 37°C during the perfusion by means of a heat lamp. Bile samples were collected directly into counting vials at 3-min intervals. The rate of appearance of the labeled bile acids in the bile usually became linear with respect to time after 10–14 min; the rate constant was calculated from the linear portion of the curve. As demonstrated previously (6, 10), we also have shown by direct perfusion of radiolabeled bile acids into the portal blood system that liver transport of bile acids into the bile is not rate-limiting in these experiments; indeed, the liver has been shown to clear bile acids in a linear fashion at over 10 times the maximal rates seen for intestinal transport. Thus, under the conditions of these experiments the rate of appearance of radiolabeled bile acid in the biliary drainage is an accurate measure of the rate of intestinal absorption. At the end of the perfusion, the intestinal segment was removed and its length determined. Flux rates, J, from these studies also are expressed as the picomoles of bile acid absorbed per minute per centimeter length of intestine (pmoles/min per cm).

Counting techniques. Samples of the perfusate from the in vitro experiments as well as the bile specimens were counted in Bray's solution using appropriate internal standards for quench correction (15).

Statistical methods. Most data are presented as mean values ±SEM for n determinations. The data that relate rates of active absorption of bile acids to their concentrations in the perfusate generally fit a hyperbolic function. Rather than utilize one of the standard procedures for linear transformation, a computer program was devised[6] that allows direct fitting of these kinetic data to a hyperbolic function assuming that the rate of absorption, J, equals zero when the concentration of bile acid in the perfusate also equals zero. An analysis of these curves, as described in the Appendix, gives mean values and variance for the apparent maximal transport velocity, $*V_{max}$, and the apparent Michaelis constant, $*K_m$, as well as a coefficient of dependency that has values from 0 to 1.0 and describes the relative fit of the data to a hyperbolic curve (1.0 represents a perfect fit).

RESULTS

These experiments were undertaken to characterize the transport processes involved in active and passive bile acid absorption, and both in vivo and in vitro techniques were utilized. However, since it was important to characterize passive absorption from bile acid solutions of high concentration and since in vitro intestine tolerates such concentrations poorly (16–18), data on passive absorption were obtained primarily using the in vivo perfusion technique. On the other hand, kinetic data on the active transport system could be measured at low concentrations of bile acid, and further, it was desirable to obtain such information under conditions where precise control of the electrochemical gradient across the bowel wall was possible; for these reasons data on the active system was obtained primarily in the in vitro perfusion system. In both cases, however, measurements also were obtained in the alternate system for comparative purposes.

Characterization of passive monomer absorption across the jejunum. It is well established that bile absorption across the jejunum is passive (11). Using the in vivo perfusion technique, measurements of the relative rates of absorption of various bile acids across the second intestinal segment were first undertaken. Since bile acid solutions contain molecules present in three species—ionized monomers, protonated monomers, and micelles—it was necessary to characterize passive permeability characteristics of the jejunal mucosa for each of these species separately. In a very dilute solution of bile acid below its critical micelle concentration (CMC) the relative amounts of ionized and protonated monomers is determined by the pH of the medium and the pK_a of the bile acid. The rate of passive absorption from such solutions necessarily must equal the sum of the passive rates of absorption of the two species across the rate-limiting membrane—presumably the luminal membrane of the columnar absorptive cell—of the intestine. Hence, the rate of uptake, J, from dilute solutions is given by the expression:

$$J = (*P^-)(C_m^-) + (*P^\circ)(C_m^\circ), \quad (1)$$

where $*P^-$ and $*P^\circ$ are the apparent permeability coefficients for the ionized and protonated bile acid monomers, respectively, and C_m^- and C_m° are the respective concentrations of these two species. Permeability coefficients in this study are expressed as the picomoles of bile acid absorbed per minute per 1-cm length of intestine when the concentration of bile acid in the mucosal perfusate is normalized to 1 mM, i.e., pmoles/min per cm per mM. Since J can be determined experimentally and $[C_m^-]$ and $[C_m^\circ]$ can be calculated from the Henderson-Hasselbach equation using appropriate published values for the pKa of each bile acid (19), the equation contains two unknowns, $*P^-$ and $*P^\circ$. However, by determining J at two different values of the pH of the buffered perfusate, a pair of simultaneous equations can be solved for the two unknowns. In practice, such experiments usually were undertaken using one solution at a pH that varied from 7.0 to 8.0 and another at a pH value that approached the pK_a for a particular bile acid; thus, permeability coefficients for each bile acid were calculated using pairs of perfusates at varying pH values. By this means it was determined that the values of $*P^-$ and $*P^\circ$ for the ionized and protonated forms for a given bile acid were constant at all concentrations of the molecules in the perfusate that were less than the CMC. That is, uptake, J, was linear with respect to concentration of bile acid in the mucosal perfusate (C_m) so that $\Delta J/\Delta C_m$ (which equals $*P$) was constant; hence, the kinetics of this process are consistent with passive absorption.

[6] Dr. Wanzer Drane, Biostatistics Department, The University of Texas Southwestern Medical School, Dallas, Tex. (see Appendix).

As shown in column A of Table I, the apparent permeability coefficients for ionized monomers varied in a regular but complex manner with respect to the molecular structure of the bile acid. For example, taurocholate, glycocholate, and cholate had $*P^-$ of 39±2, 51±4, and 254± 10 pmoles/min per cm per mM, respectively. A similar pattern of permeability was seen with the dihydroxy bile acids and with lithocholate; the unconjugated ion was always much more permeable than the glycine conjugate which, in turn, was more permeable than the taurine conjugate. In addition, however, as is also apparent in this table, removal of a hydroxyl group from the steroid nucleus markedly enhanced permeability. $*P^-$ for taurocholate, taurodeoxycholate, and taurolithocholate, for example, equaled 39±2, 114±7, and 387±39 pmoles/min per cm per mM, respectively.

TABLE I

Rates of Passive Absorption of Bile Acids Across the Jejunum of the Rat

Bile acid	Apparent permeability coefficients, *P ($\Delta J/\Delta C_m$), for A. Ionized monomers <CMC ($*P^-$)	B. Protonated monomers <CMC ($*P^\circ$)	C. Ionized bile acids in micelles
	pmoles/min per cm per mM		
C	254± 10 (7)	1,543±157 (7)	190± 42 (6)
GC	51± 4 (19)	590 (*)	69± 16 (19)
TC	39± 2 (12)	360 (*)	80± 15 (10)
DC	1,237±192 (7)	3,795±718 (5)	1,305±180 (5)
GDC	133± 9 (9)	1,470 (*)	125± 48 (9)
TDC	114± 7 (14)	890 (*)	115± 16 (20)
CDC	974± 25 (8)	3,900±512 (5)	1,050±175 (5)
GCDC	142± 18 (6)	1,470 (*)	166± 38 (8)
TCDC	138± 9 (7)	890 (*)	139± 41 (6)
LC	2,550 (*)	9,600 (*)	—
GLC	429± 61 (6)	3,660 (*)	—
TLC	387± 39 (4)	2,220 (*)	—
GHC	208± 18 (5)	—	—
THC	180± 20 (5)	—	—

Mean values ±1 SEM are given for the apparent passive permeability coefficients, *P, of ionized bile acid monomers, protonated bile acid monomers, and ionized bile acids in micelles. The number in parentheses gives the number of single animal determinations in each group. In this table and in 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), taurochenodeoxycholic (TCDC), lithocholic (LC), glycolithocholic (GLC), taurolithocholic (TLC), glycodehydrocholic (GHC), and taurodehydrocholic (THC) acids. The values indicated by the asterisk (*) were not determined experimentally but were calculated using the theoretical plot ln $*PM^{\frac{1}{2}}$ versus N, the number of potential hydrogen bonds that particular bile acids can form with water.

It is apparent that these marked differences in permeability cannot be the consequence merely of changes in molecular weight (M); removal of the taurine group from taurodeoxycholate, for example, increased $*P^-$ over 10-fold while the molecular weight is reduced by a factor of only 0.79. Similarly, removal of a hydroxyl group consistently increased permeability by two- to fivefold yet this deletion does not significantly alter the molecular weight. As reviewed recently by Diamond and Wright (20), and as will be outlined in the Discussion, the major determinant of changes in permeability in such a homologous series is the magnitude of the incremental free energy, $\delta\Delta F_{w\to 1}$, that results from the addition or removal of a functional group from the parent molecule. The value of $\delta\Delta F_{w\to 1}$ for the functional groups on the common bile acids, in turn, primarily is proportional to the capability of these groups to hydrogen bond with water. Hence, the function ln $*PM^{\frac{1}{2}}$ for each bile acid is inversely related to the number of hydrogen bonds, N, that bile acid can form. N for each bile acid is calculated as suggested by Stein (21). When the values of $*P^-$ in Table I are plotted in this form, as shown in Fig. 1, there is a linear relationship evident such that the value of the quantity ln $*PM^{\frac{1}{2}}$ decreases by a factor of 0.605 for each group added to the bile acid molecule that has the potential for forming an additional hydrogen bond. While the theoretical considerations of this graphic presentation will be discussed later, this plot is introduced at this point since it allows transformation of permeability data into a linear form that is useful for comparing permeability characteristics of one portion of the intestine to different species of bile acids or of different portions of the gastrointestinal tract to the same species.

As shown in column B of Table I the permeability coefficients for the protonated monomers of cholic acid, deoxycholic acid, and chenodeoxycholic acid were considerably higher than the corresponding ions and equaled 1543±157, 3795±718, and 3900±512 pmoles/min per cm per mM, respectively. These three values also are plotted in Fig. 1 and describe a second curve that is essentially parallel to, but displaced upward from the curve for the ionized monomers.

It was technically feasible to determine values of $*P^-$ and $*P^\circ$ for these three unconjugated bile acids because of their high pK_a values that vary from approximately 5.0 to 5.9 (19). Relatively small shifts downward in the pH of the buffered perfusion solution, e.g. from a pH of 8.0 to 6.6 in the case of cholic acid, resulted in significant increases in the rates of absorption, J; hence, it was possible to calculate values for both $*P^-$ and $*P^\circ$ from equation 1.

The pK$_a$ values for the glycine conjugates (3.9 to 4.2 [19]) and taurine conjugates (1.8 to 1.9 [19]) are so low, however, that shifts in the pH of the buffered perfusing solution in the range of 8.0 to 6.0 did not alter J for these bile acids. The rate of absorption from a 1 mM solution of taurocholic acid, for example, averaged 41±3, 38±7, and 40±8 pmoles/min per cm for perfusates with pH values of 8.0, 7.0, and 6.0, respectively. When the pH of the incubation media was lowered further in an attempt to determine directly the values of *P° for these substances, there clearly was an alteration in the permeability characteristics of the mucosal membrane. Uptake rates, J, from a 1 mM glycocholic acid solution, for example, equaled 50±6, 40±3, and 23±6 pmoles/min per cm at perfusate pH values of 6.0, 5.5, and 4.5, respectively. Because of these difficulties the values of *P° given for the conjugated bile acids in column B of Table I are derived from the upper curve in Fig. 1 and not from direct experimental determinations.

Effects of micelles in the perfusing media on passive bile acid absorption across the jejunum. The effect of micelles in the perfusing buffer on passive bile acid absorption was next undertaken. Since these studies involved the infusion of relatively high concentrations of bile acid, it was necessary to establish that under the conditions of these in vivo experiments such solutions

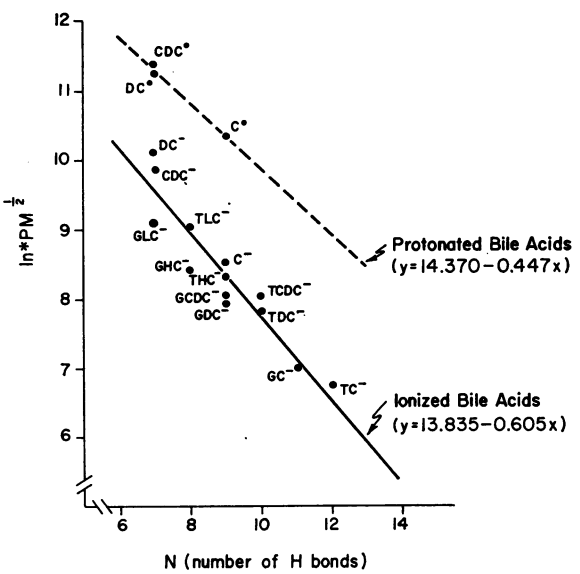

FIGURE 1 Relationship of apparent permeability coefficients, *P, for ionized and protonated bile acids to their hydrogen binding capability. In this diagram *P (columns A and B of Table I) has been multiplied by the square root of the molecular weight ($M^{\frac{1}{2}}$) for each bile acid and the ln of this product has been plotted against N, the number of hydrogen bonds that a particular bile acid monomer can form in water. Values of N were assigned as suggested by Stein (21).

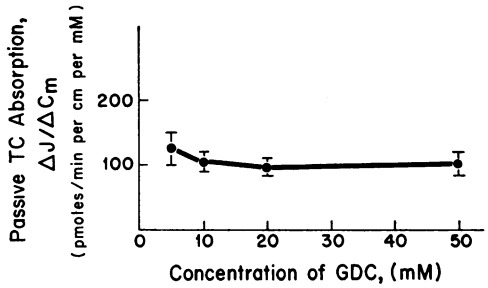

FIGURE 2 Effect of increasing concentrations of GDC on the passive absorption of TC. In this study the concentration of the probe molecule, TC, was constant at 1 mM while the concentration of GDC was varied from 5 to 50 mM.

did not alter the morphology or inherent permeability characteristics of the jejunal mucosa. Two types of studies were performed. First, histological sections were prepared from the test segments at times corresponding to the midpoint of the experimental infusion; these were coded and read independently by a pathologist.[7] No significant differences in histology were found in those segments perfused with buffer alone or with micellar solutions (including those with other lipid constituents) of all bile acids except deoxycholate. At concentrations in the range of 4–10 mM this latter bile acid caused loss of surface epithelium from the tips and for variable distances down the lateral surfaces of the villi. Second, the permeability of the jejunal membrane was tested with a labeled probe molecule in the presence of micellar solutions of varying concentration. As shown in Fig. 2, using taurocholate as the probe molecule, $\Delta J/\Delta C_m$ did not change significantly in the presence of micellar solutions of glycodeoxycholate up to 50 mM. Similar results were found with other bile acids and indicated that the presence of micelles in the perfusate under these in vivo conditions did not alter the permeability of the rate-limiting membrane.

The rates of absorption of three representative bile acids as a function of the concentration of bile acid in the perfusing media are shown in Fig. 3. For simplicity, these studies were all undertaken at a pH of 8.0 where essentially only the ionized species was present, and nonionic diffusion could be disregarded. As is apparent, the rate of absorption did not change markedly as the concentration of bile acid in the buffered perfusion media was increased above the CMC. A linear curve was fitted (by the method of least squares) to the points above the CMC for each bile acid, and the slope of this line ($\Delta J/\Delta C_m$) is given in column C of Table I. Comparison of these values with those in column A reveals few sig-

[7] Dr. Edward Eigenbrodt, Department of Pathology, The University of Texas Southwestern Medical School at Dallas.

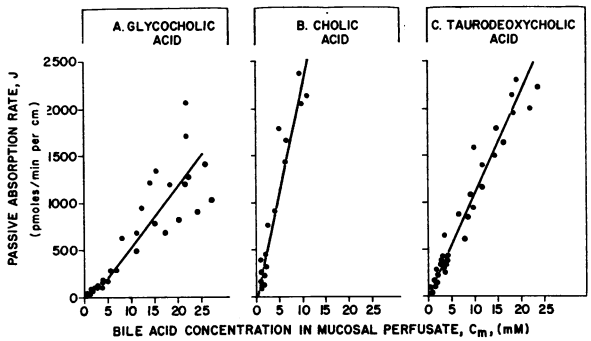

FIGURE 3 Passive absorption rates, J, for ionized bile acids across the rat jejunum in vivo as a function of bile acid concentration in the mucosal perfusate. In these studies the perfusate was phosphate-Tris buffer at a pH of 8.0.

nificant differences. $\Delta J/\Delta C_m$ for absorption of taurocholate and glycocholate from micellar solutions is higher than the corresponding values for absorption of these ions from monomer solutions, but in all other instances the two values are essentially identical. Thus, there were no dramatic differences between the slopes $\Delta J/\Delta C_m$ describing bile acid uptake from solutions containing only ionized monomers and those containing micelles.

In contrast to these results, when the micelle was greatly expanded with various lipids there was a consistent decrease in the rate of passive uptake of bile acid across the jejunum. As shown in Table II, the rate of uptake from micellar solutions of taurocholate and taurodeoxycholate was decreased 73 and 39%, respectively, by the addition of an equimolar amount of phospholipid. Similar results are apparent with expansion of the micelle with monooleate and oleic acid.

TABLE II

Effect of Phospholipid, Monoglyceride, and Fatty Acid on Passive Intestinal Absorption of Bile Acids from Micellar Solutions

Test bile acid and concentration	Amphipaths and concentration	$\Delta J/\Delta C_m$
mM	*mM*	*pmoles/min per cm per mM*
TC (10)	0	99±10
TC (10)	PL (10)	27± 3
TDC (10)	0	100±14
TDC (10)	PL (10)	61±11
TC (10)	0	91± 4
TC (10)	MG (5) + OA(1)	35± 5
TDC (10)	0	121± 7
TDC (10)	MG (5) + OA(1)	102± 8

All studies were carried out with bile acids dissolved in phosphate-Tris buffer titrated to a pH of 8.0. Solutions were equilibrated with the added amphipaths for 12-14 hr at 35-37°C before use. Each value represents the mean±1 SEM for six to eight determinations. The abbreviations represent monooleate (MG), oleic acid (OA), mixed egg lecithins (PL).

Table III shows the results of similar studies where uptake from mixed micelles, whose composition resembled that of those found in the intestine under physiological conditions, is compared to uptake from micellar solutions of a single bile acid. Again, in each instance, the addition of three other bile acids, monooleate and oleic acid decreased the rate of bile acid uptake.

Passive monomer absorption across the jejunum in vitro. For purposes of comparison, values of $*P^-$ for the 14 bile acids listed in Table I were determined across the second intestinal segment using the in vitro perfusion apparatus with the transmural potential difference set at zero. Representative $*P^-$ values equaled 31±3, 93±8, 176±25, and 520±17 pmoles/min per cm per mM for taurocholate, taurodeoxycholate, cholate, and deoxycholate, respectively. These values generally were lower than the corresponding values determined in vivo so that the regression curve fitting the plot ln $*PM^{\frac{1}{2}}$ against N was $y = 12.999 - 0.536x$. This curve has a slightly lower intercept but a similar slope as the curve for ionized monomers shown in Fig. 1. Hence, the same relationship between passive permeability of different bile acids across the jejunum can be demonstrated both in vivo and in vitro. Histologic controls revealed no significant changes in these studies where the absolute mucosal solution concentration was kept very low, usually < 0.2 mM.

Characteristics of the active transport system across the ileum. Prior studies from this and other laboratories have shown that active transport of bile acid takes place at all levels of the ileum (intestinal segments 6 to 10) (3, 6). However, since maximal rates usually are found in the ninth segment, this area of the terminal small intestine was utilized in these studies in which the kinetics of the active system were characterized for the various bile acids under in vitro conditions where the electrochemical gradient across the bowel was maintained at zero.

Under these circumstances absorption, J, across the ileal wall is given by the expression:

$$J = *V_{max}\frac{C_m}{*K_m + C_m} + (*P^-)(C_m), \qquad (2)$$

where the first component gives the magnitude of the active flux and the second component the magnitude of the passive flux. In order to obtain accurate curves that describe the rate of active absorption as a function of bile acid concentration in the mucosal perfusate, it was necessary to correct for the passive component, $(*P^-)$

TABLE III

Rates of Passive Absorption of Bile Acids in Mixed Micelles Containing Several Bile Acids, Monoglyceride, and Fatty Acid

Test bile acid	Presence of mixed micelle	$\Delta J/\Delta C_m$
		pmoles/min per cm per mM
TC	0	95±11
TC	+	75± 5
GC	0	98± 6
GC	+	59± 4
TDC	0	115± 6
TDC	+	98± 7
GDC	0	125±18
GDC	+	69±12

All studies were carried out with bile acids and other amphipaths dissolved in phosphate-Tris buffer titrated to a pH of 8.0. The first value in each pair represents the control value of $\Delta J/\Delta C_m$ for the particular bile acid alone (5 mM). The second value (+ mixed micelle) represents $\Delta J/\Delta C_m$ for that same bile acid from a solution also containing the other three bile acids (5 mM each), monooleate (5 mM), and oleic acid (1 mM). Each value represents the mean ±1 SEM for determinations in five animals.

(C_m). This, in turn, required values for permeability coefficients, $*P^-$, of the various bile acids in the ileum.

These values were determined by three different methods. First, it was assumed that the ileum was symmetrical with respect to passive permeability, and $*P^-$ for the major bile acids were determined by measuring unidirectional flux rates from the serosal to the mucosal surface in the ninth intestinal segment. Second, the passive permeability characteristics of the ileum for bile acids were assumed to be identical to those in the jejunum. If this assumption is correct, then $*P^-$ values for the ileum could be obtained by correcting the $*P^-$ values obtained in the jejunum, in vitro, for the difference in the relative mucosal surface area per centimeter length in these two areas of the bowel (22); hence,

$$*P^-_{\text{ileum}} = (*P^-_{\text{jejunum}})(0.80). \quad (3)$$

Third, at values of C_m that are 4–5 times $*K_m$, the first term in equation 2 approaches a constant value equal to $*V_{max}$. At still higher values of C_m, however, J continues to increase in an essentially linear manner with a slope equal to $*P^-$. Thus, by this means it was possible to determine directly $*P^-$ values in the ileum for taurocholate, glycocholate, and cholate. The data for the passive permeability coefficients in the ileum determined by these three methods were all similar and when combined and plotted as ln $*PM^{\frac{1}{2}}$ against N gave a linear regression curve described by the formula $y = 12.941 - 0.550x$. This curve was used to correct all values of J determined at various values of C_m for the passive component.

With this correction, data on the active transport of various bile acids across the ileum were obtained as shown in the representative examples in Fig. 4. The solid lines are the best fit, hyperbolic functions of these data. The values of $*V_{max}$ and $*K_m$ as well as coefficients of dependency derived from these curves for 11 bile acids are given in Table IV.

Since each point in Fig. 4 represents an experimental value determined in the intestine of a separate animal, there is scatter in the data and corresponding variance in the values of $*V_{max}$ and $*K_m$. Nevertheless, these data allow three major conclusions of importance. First, the coefficient of dependency is > 0.837 for all trihydroxy and dihydroxy bile acids indicating that the data fit well the expected hyperbolic function of a carrier-mediated transport system. The conjugates of lithocholate had lower values since, because of limited solubility, it was impossible to measure J at higher values of C_m. Second, there is striking dependence of the values of $*V_{max}$ on the number of hydroxyl groups on the steroid nucleus. The three trihydroxy bile acids have the highest maximal transport rates which vary from 1543 to 1906 pmoles/min per cm. The six dihydroxy compounds have much lower values that all fall in the range of 114–512 pmoles/min per cm, while $*V_{max}$ for the two monohydroxy bile acids are lower still. Third, in contrast to this relation-

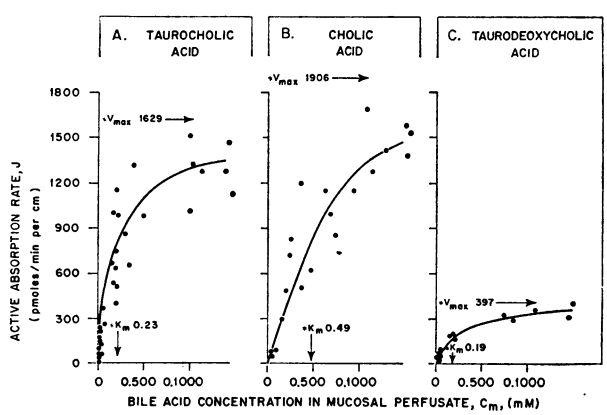

FIGURE 4 Active absorption rates, J, of ionized bile acids across the ileum in vitro as a function of bile acid concentration in the mucosal perfusate. The values plotted represent the experimentally determined rates of transmural movement minus the correction for mucosal-to-serosal passive flux as described in the text. The solid lines represent the best fit hyperbolic function computer drawn as described in the Appendix.

TABLE IV

Apparent Kinetic Characteristics of the Active Transport System for Bile Acids Across the Ileum of the Rat

Bile acid	Coefficient of dependency	$*V_{max}$	$*K_m$
		pmoles/min per cm	*mM*
C	0.909	1906±462	0.49±0.26
GC	0.853	1543±326	0.18±0.10
TC	0.897	1629±236	0.23±0.07
DC	0.861	224±169	0.37±0.16
GDC	0.837	114± 32	0.16±0.20
TDC	0.985	397± 27	0.19±0.04
CDC	0.850	512±150	0.38±0.08
GCDC	0.890	173± 40	0.21±0.06
TCDC	0.994	337± 13	0.12±0.01
GLC	0.500	45± 8	0.09±0.04
TLC	0.550	57± 21	0.09±0.07

Mean values ±1 SEM for apparent values for $*V_{max}$ and $*K_m$ for the active transport of various bile acids. The coefficient of dependency describes the fit of the experimental data to a hyperbolic curve assuming that the rate of transport equals zero where the bile acid concentration is zero.

ship between nuclear hydroxyl groups and $*V_{max}$, the values of $*K_m$ are independent of the number of hydroxyl groups but are apparently related to the presence or absence of a conjugated group at the C_{24} position. $*K_m$ for the three unconjugated bile acids cholate, deoxycholate, and chenodeoxycholate, for example, were high and in the range of 0.37–0.49 mM. All conjugated bile acids, on the other hand, had values < 0.23 mM.

Histologic controls also were performed in these studies and showed no differences between segments incubated with bile acids and those incubated in buffer alone except for deoxycholate at concentrations > 0.75 mM. With this bile acid there again was spotty loss of some surface epithelium.

Since data in everted gut sacs have shown a gradient of increasing apparent active transport down the length of the ileum (3, 6), it was of interest to determine whether this was due to a difference in $*V_{max}$ or $*K_m$ for bile acid transport at different levels of the terminal small intestine. Using the seventh intestinal segment $*V_{max}$ and $*K_m$ for taurocholate was determined to equal 405±72 pmoles/min per cm and 0.21±0.05 mM, respectively. Hence, the higher rate of transport in the ninth intestinal segment clearly was a manifestation of a higher maximal transport rate and not a difference in apparent affinity of the carrier for the molecule.

A limited number of observations were carried out in vivo to quantitate values of $*V_{max}$; for taurocholate and taurodeoxycholate maximal transport rates averaged 1450 (n = 3) and 425 (n = 3) pmoles/min per cm, respectively. Thus, as in the case of passive absorption, there is reasonably good agreement between the values obtained in vitro and in vivo.

Characterization of passive monomer absorption across the transverse colon. Experiments performed in this laboratory both in the everted gut sac and in the in vitro perfusion apparatus have shown the colon is unable to transport bile acids against an electrochemical gradient;[8] these findings confirm earlier reports that colon does not contain an active transport mechanism (8). In vivo experiments, therefore, were undertaken to quantify the passive permeability coefficients of bile acids in the colon. In the representative bile acids taurocholate, taurodeoxycholate, glycodeoxycholate, and cholate, for example, mean values of 12±2, 40±8, 75±14, and 180±9 pmoles/min per cm per mM, respectively, were found. When values for the nine common tri- and dihydroxy bile acids were plotted as ln $*PM^{\frac{1}{2}}$ against N a linear regression curve was obtained that equaled $y = 12.912 - 0.612x$.

DISCUSSION

In previous publications from this laboratory the specific transport mechanisms operative in the absorption of bile acids from the small and large bowel have been reviewed (6, 11); these mechanisms include passive ionic and nonionic absorption, which takes place at all levels of the gastrointestinal tract, and active absorption, which occurs only in the ileum. The study of bile acid transport is more complex than the study of many other water soluble compounds that exist in solution as a single species where molecular activity can be approximately equated to simple concentration. Since bile acids may exist in solution as ionized and protonated monomers or in complex micells, it is important to quantitate the transport characteristics of each of these species and to differentiate events in the bulk water phase from those in the lipid cell membrane that may cause alterations in rates of transport.

Using paired experiments in which absorption of bile acid was measured from perfusates of two different pH values, apparent passive permeability coefficients, *P, were determined for the ionized and protonated monomers of the major conjugated and unconjugated bile acids. Use of such a technique obviates the possibility that the observed uptake actually represented exclusive absorption of the protonated species present in very small amounts even in relatively alkaline perfusates. The values of $*P^-$ and $*P^\circ$ in Table I vary in a regular manner with respect to three alterations in molecular structure: (*a*) the presence of a negative charge reduces

[8] Unpublished observations from this laboratory.

permeability by a factor of approximately 4.4; (*b*) conjugation of bile acids with glycine or taurine lowers permeability on the average by a factor of 6.8 and 8.1, respectively; and (*c*) addition of a nuclear hydroxyl group decreases permeability by a mean factor of 3.4.

Theoretically, passive absorption of bile acids might take place by diffusion through polar regions of the cell surface, by diffusion across the lipid phase of the cell membrane or by some type of passive, facilitated diffusion. However, we previously have shown that there is no evidence of carrier-mediated diffusion and that osmotically induced water flow does not significantly alter the rate of passive bile acid absorption across the jejunum (6); hence, absorption of these molecules must take place by diffusion through the lipid cell membrane. If this is the case, then the variations in permeability rates among the different bile acids noted above must be explicable in terms of the intramolecular forces that determine solute: water and solute: lipid interactions.

Under conditions where membrane penetration is limited by the rate of diffusion through the membrane interior (20),

$$P = \left(\frac{D}{d}\right)e^{-\Delta F_{w\to 1}/RT}, \qquad (4)$$

where P is the permeability coefficient, D the diffusion constant, d the thickness of the cell membrane, $\Delta F_{w\to 1}$ the free energy change in transferring 1 mole of bile acid from the perfusate to the lipid cell membrane, R the gas constant, and T the absolute temperature. Assuming that in these studies *d* remains constant and D is inversely proportional to $M^{\frac{1}{2}}$ (21) then the quantity ln $*PM^{\frac{1}{2}}$ will be proportional to $\Delta F_{w\to 1}$. In the bile acid series alterations in $\Delta F_{w\to 1}$ caused by the addition of various functional groups to the molecule primarily are related to the number of hydrogen bonds that these respective groups can form with water. Hence, as shown in Fig. 1, in the case of ionized monomers there is a linear inverse relationship between the quantity ln $*PM^{\frac{1}{2}}$ and N. For the addition of each potential hydrogen bond ln $*PM^{\frac{1}{2}}$ is reduced by a factor of 0.605; thus, for example, the addition of each hydroxyl group (N = 2) to a bile acid, decreases $*PM^{\frac{1}{2}}$ by 3.35.

It is of interest that the regression curves (ln $*PM^{\frac{1}{2}}$ against N) that describe passive permeability of the ileum and colon, $y = 12.941 - 0.550x$ and $y = 12.912 - 0.612x$, respectively, are similar to the one for the jejunum ($y = 13.835 - 0.605x$). The decrease in *y* intercepts between the jejunum and the ileum and colon very likely represents only a decrease in relative mucosal surface area per unit length of intestine in these areas of the bowel. The similar slopes, however, indicate that the permeability characteristics of these regions with respect to passive bile acid absorption are essentially identical.

As also shown in Fig. 1, removal of the negative charge results in a second curve that is displaced upward but is parallel to the curve for ionized bile acid. Thus, a protonated bile acid is more permeable than the corresponding ion but the pattern of relative permeability among the various protonated bile acids is the same as that for the ionized species. It can be predicted from these two curves that a series of bile acids with two negative charges would have very low permeability coefficients that would describe a third curve with the approximate formula, $y = 13.3 - 0.60x$.

As outlined by Diamond and Wright in their comprehensive analysis of the determinants of cell membrane permeation, the change in $\Delta F_{w\to 1}$, i.e. the incremental free energy of solution, $\delta\Delta F_{w\to 1}$, caused by the addition of a functional group to a molecule can be calculated from the permeability coefficients of the two species using the equation (20):

$$\delta\Delta F_{w\to 1} = -(RT)\left(\ln\frac{P^{+}}{P^{\circ}}\right) \qquad (5)$$

where P^{+} and P° are the permeability coefficients for the molecule with and without the substituent group, respectively. Using the *P from this study, mean values of $\delta\Delta F_{w\to 1}$ for the hydroxyl, glycine, and taurine groups equal + 757, + 1178, and + 1291 cal/mole, respectively. Hence, these results are in general agreement with those reported by Diamond and Wright in that the addition of any substituent group (except for $-CH_2-$ in their series) is associated with a positive value of $\delta\Delta F_{w\to 1}$ and a corresponding decrease in permeability through the cell membrane.

The effect of micelles in the mucosal perfusate on the rate of bile acid absorption is particularly relevant to the physiological situation that exists in vivo during digestion. From the data presented in Tables I (column C), II, and III, two generalizations are possible: first, $\Delta J/\Delta C$ does not abruptly fall as the concentration of bile acid in the bulk perfusate is raised above the CMC, and second, expansion of micelle size by the addition of nonbile acid constituents decreases the rate of bile acid absorption. If one assumes that only the bile acid monomer penetrates the cell membrane then the first result is surprising since J should equal the product of *P times the concentration of monomer in the perfusate. Since the concentration of monomer approaches a constant value in solutions above the CMC, J also should plateau at a constant value. This finding could be explained by assuming either that the mucosal cell is capable of absorbing the intact micelle or that there is a diffusion barrier present between the bulk water phase of the perfusate

and the cell membrane. While the current studies were not designed to answer this important question, we have published preliminary data indicating that the latter explanation is the correct one (23–25).

One other feature of passive penetration of the intestine also deserves emphasis. When the pH of the perfusate was decreased from 6.0 to 4.5, apparent permeability coefficients decreased significantly. This observation is similar to that described previously by Rosen, Leaf, and Schwartz (26) in the toad bladder and by Wright and Diamond (27) in the gall bladder where the cell membrane was shown to become less permeable to various probe molecules as the pH of the perfusate was lowered to near 4.0. It is apparent, therefore, that shifts in pH may alter rates of passive absorption both by producing changes in the bulk phase solution, i.e. altering the relative concentration of the ionized and protonated forms of weak acids and bases, and by effecting the permeability characteristics of the membrane itself.

The kinetics of the ileal active transport system, like those of passive absorption, are strikingly dependent on molecular structure. This is best shown in Fig. 5 where the value of $*V_{max}$ for each bile acid has been plotted against its respective $*K_m$. Statistical analysis, as described in the Appendix, reveals a significant clustering of values as indicated by the dashed lines. These data show two major relationships. First, $*V_{max}$ primarily is determined by the number of hydroxyl groups on the steroid nucleus but is independent of whether the bile acid is conjugated or not; hence, $*V_{max}$ for the trihydroxy acids $>>$ dihydroxy $>$ monohydroxy. Second, in contrast, $*K_m$ values primarily depend on whether the C_{24} position is conjugated, but are independent of the number of nuclear hydroxyl groups, thus the $*K_m$ for all of

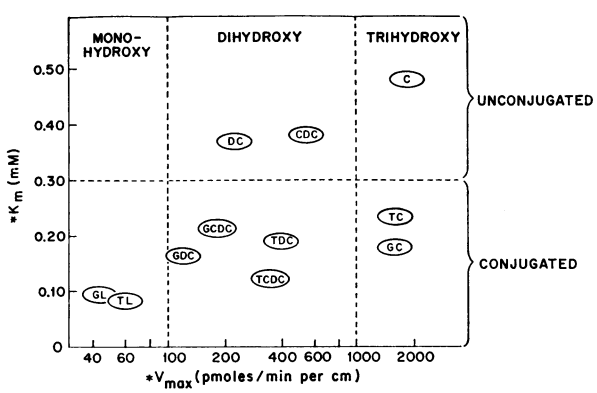

FIGURE 5 Relationship between $*K_m$ and $*V_{max}$ values for the active transport of bile acid across the ileum. The dashed lines delineate clusters of data that are significantly different from one another according to the statistical methods outlined in the Appendix. $*V_{max}$ was plotted on a log scale to compress the x axis.

the conjugated bile acids are about half the value of the unconjugated bile acids. Hence, it is of interest that this active ileal transport system apparently absorbs most avidly those bile acids that are least absorbed by passive mechanisms so that the active and passive systems complement one another and bring about nearly complete absorption of bile acid from the small intestinal contents.

Having defined in detail the characteristics of the several absorptive mechanisms for bile acid it is possible to define, in general terms, the interaction of these mechanisms in the intact animal and, presumably, in man. First, bile acid absorption across the jejunum is passive and due to a combination of ionic and nonionic diffusion. The quantitative importance of these two processes for any bile acid depends upon the pH of the luminal contents and upon the relative values of $*P^-$, $*P^\circ$, and the pK_a for that bile acid. To simplify these comparisons, the latter three values may be used to calculate a single term, the pH^*, that gives the pH for a particular bile acid at which the passive ionic and nonionic absorption rates will be exactly equal. The approximate values of pH^* for the common bile acids in dilute solutions are taurocholic acid, 2.8; taurodeoxycholic acid, 2.8; taurochenodeoxycholic acid, 2.7; glycocholic acid, 5.0; glycodeoxycholic acid, 5.7; glycochenodeoxycholic acid, 5.2; cholic acid, 5.8; deoxycholic acid, 5.8; and chenodeoxycholic acid, 6.5. At pH values $<$ pH^* nonionic absorption becomes the dominant process. The pH in the jejunum would have to drop below 2.7–2.8 before significant nonionic absorption of the taurine conjugates could take place; hence, absorption of these conjugated bile acids must be exclusively via passive ionic diffusion. On the other hand, the pH^* values for the glycine conjugates are within the pH range encountered physiologically, at least in the proximal portion of the jejunum, so that nonionic absorption of these bile acids is possible; however, more distally in the jejunum the pH is above the pH^* values for even the glycine conjugates so that ionic diffusion again becomes the dominant process in this area of the bowel.

Second, the presence of mixed micelles containing various lipids markedly reduces the rate of absorption of bile acids (Tables II and III). Thus, permeability coefficients determined in simple, dilute solutions cannot be extrapolated to estimate the quantity of bile acids absorbed across the jejunum under physiological conditions as we previously have attempted to do (6). The facts that the jejunum normally contains only conjugated bile acids, that conditions are such that only passive ionic diffusion (and a limited amount of nonionic diffusion in the case of the glycine conjugates) takes place, and that large mixed micelles containing lipid are present readily explains the comparatively low rate of bile acid absorption in the proximal small intestine. Yet, it should be

emphasized that currently there are no quantitative data on the extent of bile acid absorption across the jejunum under physiological conditions.

Third, in the ileum passive ionic, passive nonionic, and active absorption all take place. Passive ionic absorption presumably would increase with the removal of lipid from the mixed micelle. Unconjugated bile acids are formed at this level of bowel and because of their higher pH° values their presence provides a means for some nonionic bile acid absorption to occur despite the relatively high pH in this region. Active absorption also takes place with the highest maximal transport velocities (V_{max}) being for the conjugated trihydroxy bile acids. It should be emphasized that the $*K_m$ of this system is so low (approximately 0.2 mM for the conjugated bile acids) that essentially $*V_{max}$ velocities will continue even when the intraluminal concentration of bile acid has dropped to levels as low as 0.5–1.0 mM.

Fourth, within the colon presumably only passive ionic absorption takes place. Nevertheless, since the predominant bile acids in the colonic contents (deoxycholate and lithocholate) have relatively high $*P^-$ values it is theoretically possible that the rate of such passive absorption might be considerable. However, if the bile acids are precipitated (e.g. lithocholate) or bound to particulate matter in the stool then only small amounts of bile acid would be present in stool water and the rate of absorption would be correspondingly low.

One final point regarding these studies requires emphasis. The values of the apparent permeability coefficients and those of $*V_{max}$ and $*K_m$ reported here accurately describe the passive and active permeability characteristics of the intestinal membrane of the rat as it exists in vitro and in vivo. Accordingly, these values are useful in understanding the various transport phenomena operative in maintenance of the enterohepatic circulation of bile acid. Recently, however, we have shown that the resistance encountered by a molecule as it is absorbed into the mucosal cell is composed of two components: a resistance that is engendered by the lipid cell membrane and another resistance that is the result of an unstirred layer adjacent to the membrane (23–25). Hence, the data reported here represent the kinetic characteristics of the "resisting membrane," i.e. lipid membrane plus unstirred water layer, and therefore, they cannot be construed as the kinetic parameters of the lipid cell membrane itself. Because of this situation it is to be anticipated that the *P values are lower than the permeability coefficients of the lipid membrane, that the calculated values of $\delta\Delta F_{w\to 1}$ are underestimates of the actual values, and that the values of $*K_m$ are higher than the Michaelis constants of the carrier in the membrane.

APPENDIX

The following method, formulated by Dr. Wanzer Drane (the Biostatistics Department, University of Texas Southwestern Medical School, Dallas, Tex.), was used for a statistical analysis of the curves describing the active absorption of bile acid in the ileum.

The formula for an equilateral hyperbola (the inverse law) which when plotted includes the origins (0,0) is

$$J = *V_{max}\frac{[S]}{*K_m + [S]},$$

where J is the rate of absorption (picomoles/minute per centimeter), $*V_{max}$ the apparent maximal transport rate (picomoles/minute per centimeter), $*K_m$ the apparent Michaelis constant (millimolars), and [S] the concentration of bile acid in the perfusate (millimolars). This is the well known Michaelis-Menten equation. It can be seen that when $[S] = *K_m$ then

$$J = *V_{max}\frac{*K_m}{*K_m + *K_m},$$

$$J = *V_{max}\cdot\tfrac{1}{2},$$

and when [S] increases without limit $[S]/(*K + [S])$ approaches unity and J approaches $*V_{max}$.

Of several methods fitting this equation to data the one chosen is the method of least squares. That is, if J_i is the ith response to a corresponding substrate concentration $[S]_i$, then over a set of n such response-substrate pairs, the function

$$SSE(V,K) = \sum_{i=1}^{i=n}\left(J_i - V\frac{[S]_i}{K + [S]_i}\right)^2$$

is evaluated for different (V,K) pairs until the smallest value SSE(V,K) is found. The (V,K) pair corresponding to the minimum of SSE(V,K) are $*V_{max}$ and $*K_m$, respectively. Since $J_i - *V_{max}[S]_i/(*K_m + [S]_i)$ is a deviation of the actual response J_i from the theoretical response $*V_{max}[S]_i/(*K_m + [S]_i)$, $SSE(*K_m, *V_{max})$ is the sum of squared deviations and to make SSE(V,K) a minimum is to find the "least squares" value of the pair $(*V_{max}, *K_m)$. For a more detailed exposition see Mounter and Turner (28) and Draper and Smith (29).

Plotting $(*V_{max}, *K_m)$ as indicated in Fig. 5, indicates a bilateral separation of the points into five groups as denoted by the dashed lines. Making use of the large sample statistical properties of the least square estimates $(*V_{max}, *K_m)$, Duncan's new multiple range test was twice employed firstly on the several $*V_{max}$ and secondly on the corresponding $*K_m$ (30–32). The dashed lines separate those points which are significantly separated. We have taken this to be evidence of clustering or grouping as indicated.

ACKNOWLEDGMENTS

The authors wish to acknowledge with appreciation that Dr. Donald M. Small, Boston, Mass., made available manuscripts containing recently determined values of bile acid pK_a's, CMC's, etc. before these were published.

This work was supported by research grant HE-09610 and training grant T01-AM-5490. Dr. Neal C. Small, who was a medical student at The University of Texas Southwestern Medical School during these studies, was supported by funds from a grant from the John and Mary Markle Foundation.

REFERENCES

1. Searle, G. W., and R. D. Baker. 1956. Bile salt absorption in the small intestine. *Fed. Proc.* **15**: 166.
2. Webling, D. D'A. 1966. The site of absorption of taurocholate in chicks, using polyethylene glycol as a reference substance. *Aust. J. Exp. Biol. Med. Sci.* **44**: 101.
3. Lack, L., and I. M. Weiner. 1961. *In vitro* absorption of bile salts by small intestine of rats and guinea pigs. *Amer. J. Physiol.* **200**: 313.
4. Tidball, C. S. 1964. Intestinal and hepatic transport of cholate and organic dyes. *Amer. J. Physiol.* **206**: 239.
5. Sullivan, M. F. 1965. Bile salt absorption in the irradiated rat. *Amer. J. Physiol.* **209**: 158.
6. 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.
7. Playoust, M. R., and K. J. Isselbacher. 1964. Studies on the transport and metabolism of conjugated bile salts by intestinal mucosa. *J. Clin. Invest.* **43**: 467.
8. Holt, P. R. 1964. Intestinal absorption of bile salts in the rat. *Amer. J. Physiol.* **207**: 1.
9. Lack, L., and I. M. Weiner. 1966. Intestinal bile salt transport: structure-activity relationships and other properties. *Amer. J. Physiol.* **210**: 1142.
10. Heaton, K. W., and L. Lack. 1968. Ileal bile salt transport: mutual inhibition in an in vivo system. *Amer. J. Physiol.* **214**: 585.
11. Dietschy, J. M. 1968. Mechanisms for the intestinal absorption of bile acids. *J. Lipid Res.* **9**: 297.
12. Hofmann, A. F. 1962. Thin-layer adsorption chromatography of free and conjugated bile acids and silicic acid. *J. Lipid Res.* **3**: 127.
13. Gregg, J. A. 1966. New solvent systems for thin-layer chromatography of bile acids. *J. Lipid Res.* **7**: 579.
14. Vogel, A. I. 1956. Practical Organic Chemistry. Longmans, Green and Company, New York. 3rd edition.
15. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**: 279.
16. Parkinson, T. M., and J. A. Olson. 1963. Inhibitory effects of bile acids on the uptake, metabolism, and transport of water-soluble substances in the small intestine. *Life Sci.* **2**: 393.
17. Dietschy, J. M. 1967. Effects of bile salts on intermediate metabolism of the intestinal mucosa. *Fed. Proc.* **26**: 1589.
18. Low-Beer, T. S., R. E. Schenider, and W. O. Dobbins. 1970. Morphological changes of the small-intestinal mucosa of guinea pig and hamster following incubation *in vitro* and perfusion *in vivo* with unconjugated bile salts. *Gut.* **11**: 486.
19. Small, D. M. 1971. The physical chemistry of cholanic acids. *In* The Bile Acids. D. P. Nair, and D. Kritchevsky, editors. Plenum Publishing Corporation, New York. **1**: 247.
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. Stein, W. D. 1967. The Movement of Molecules Across Cell Membranes. Academic Press Inc., New York.
22. Boyne, R., B. F. Fell, and I. Robb. 1966. The surface area of the intestinal mucosa in the lactating rat. *J. Physiol.* **183**: 570.
23. Wilson, F. A., and J. M. Dietschy. 1971. The role of micelle uptake during bile acid and fat absorption by the intestinal mucosa. *Clin. Res.* **19**: 406.
24. Sallee, V. L., and J. M. Dietschy. 1971. The role of bile acid micelles in absorption of fatty acids across the intestinal brush border. *J. Clin. Invest.* **50**: 80a. (Abstr.)
25. 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 (Washington).* **174**: 1031.
26. Rosen, H., A. Leaf, and W. B. Schwartz. 1964. Diffusion of weak acids across the toad bladder. *J. Gen. Physiol.* **48**: 379.
27. Wright, E. M., and J. M. Diamond. 1969. Patterns of non-electrolyte permeability. *Proc. Roy. Soc. Ser. B. Biol. Sci.* **172**: 227.
28. Mounter, L. A., and M. E. Turner. 1963. The evaluation of Michaelis constants and maximal velocity kinetic studies of enzymic reactions. *Enzymol. Acta Biocatal.* **25**: 225.
29. Draper, N. R., and H. Smith. 1967. Applied Regression Analysis. John Wiley & Sons, Inc., New York.
30. Duncan, D. B. 1951. A significance test for differences between ranked treatments in an analysis of variance. *Va. J. Sci.* **2**: 171.
31. Duncan, D. B. 1955. Multiple range and multiple F tests. *Biometrics.* **11**: 1.
32. Harter, H. L. 1960. Critical values for Duncan's new multiple range test. *Biometrics.* **16**: 671.