Biochimica et Biophysica Acta, 406 (1975) 280–293

BBA 77072

CHARACTERIZATION OF THE PASSIVE AND ACTIVE TRANSPORT MECHANISMS FOR BILE ACID UPTAKE INTO RAT ISOLATED INTESTINAL EPITHELIAL CELLS

FREDERICK A. WILSON and L. LOCKIE TREANOR

Department of Medicine, Vanderbilt University School of Medicine, Veterans Administration Hospital, Nashville, Tenn. 37203 (U.S.A.)

(Received March 26th, 1975)

SUMMARY

The unstirred water layer has been shown to lead to an underestimation of apparent passive permeability coefficients (P(app)) and cause a significant overestimation of apparent K_m (K_m(app)) values for active transport processes in intestinal whole tissue preparations. Isolated cells offer several potential advantages in the study of transport processes including a decreased diffusion layer of water adjacent to their absorptive membranes. Initial studies in cells isolated from rat intestine involving measurements of CO_2 and lactate production and O_2 consumption showed that overall metabolic pathways were functioning. Next, unidirectional uptake rates of bile acids across the isolated cell membrane were determined following correction for extracellular fluid contamination with a non-absorbable marker. Using epithelial cells isolated from jejunum P(app) for eight bile acid monomers varied from 24.9 (taurocholate) to 1563 (deoxycholate) nmol/min/100 mg protein/mM. From these data the incremental free energy changes for the addition of a hydroxyl, glycine and taurine group to the bile acid molecule were calculated to be 982, 1040 and 1464 cal/mol, respectively, values similar to those obtained after correction for unstirred water layer resistance in whole tissue preparations. Following subtraction of the passive component in isolated ileal cells complete kinetic curves for taurocholate and taurodeoxycholate yielded V(app) values of 109 and 70 nmol/min per 100 mg, respectively. K_m(app) values of 0.24 mM (taurocholate) and 0.10 mM (taurodeoxycholate) are lower than usually recorded in whole tissue. Bile acid uptake into cells from ileum, but not jejunum, was affected by temperature, metabolic and competitive inhibition. These studies indicate that isolated epithelial cells are a metabolically viable, relatively purified intestinal preparation which discriminates between active and passive transport processes for bile acids under conditions where unstirred water layer artifacts are minimized.

INTRODUCTION

The maintenance of adequate concentrations of bile acids within the intestinal contents for effective micellar solubilization of long chain fatty acids and β-mono-

glycerides during fat digestion is dependent upon bile acid reabsorption in the intestine. Since the original observations that everted intestinal segments from the ileum but not from the jejunum were able to transport bile acids against a concentration gradient [1], it has been recognized that bile acids are actively absorbed across the ileum [2–6] and passively absorbed across the jejunum [2, 7]. It also has been demonstrated that apparent passive permeability coefficients (P(app)) for bile acid monomers crossing the proximal small bowel vary with the chemical structure of the bile acid molecule being tested. Likewise, the active transport of a given bile acid across the ileum, described in terms of apparent values for maximal transport velocity (V(app)) and Michaelis constant (K_m(app)), depends upon the number of hydroxyl groups on the steroid nucleus and whether or not the bile acid is conjugated [8]. More recent investigations described in detail the absorption of bile acids across the unstirred water layer adjacent to the brush border of the intestinal epithelium and the brush border membrane itself. These studies demonstrated that diffusion through the unstirred water layer constitutes a significant resistance to overall intestinal transport which results in gross underestimates of the true passive permeability coefficients, P [9], and overestimates of the K_m values for active transport processes in the intestine by several-fold [10].

The characterization of bile acid transport has until now been determined in vivo in man and experimental animals [11, 12] and by a variety of in vitro assay systems involving whole tissue preparations [2, 13]. It seemed reasonable to extend these observations to isolated intestinal epithelial cells for the following reasons: (1) cells isolated from the complex macrovillous structure of the intestinal wall potentially have a decreased layer of water for diffusion adjacent to their absorptive membranes; (2) isolated intact epithelial cells represent a sequential step in the breakdown from in vivo preparations to progressively smaller components of the mucosal cell that are more intimately involved in transport processes across the brush border membrane; (3) such preparations essentially represent a homogeneous cell population in which transport rates and metabolic processes are uninfluenced by other components of intestinal wall, and (4) the unique anatomical difference in bile acid transport affords the opportunity to validate the discriminatory capacity between active and passive processes for isolated cells. For these studies, metabolically viable cells were isolated from jejunum and ileum. The cells were incubated with bile acids and a non-absorbable marker to correct for extracellular bile acid contamination. First, these studies demonstrate that criteria for passive diffusion of bile acids were satisfied in mucosal epithelial cells isolated from jejunum. The apparent permeability coefficients, P(app), for the bile acids vary in a regular manner dependent upon their molecular structure and from these data an incremental free energy change is calculable. Second, for cells isolated from ileum, criteria for active transport of bile acids are met and when P(app) for the passive component is subtracted from total uptake, a kinetic curve results from which the kinetic parameters K_m(app) and V(app) are derived. Finally, the implication of these findings with respect to the intestinal unstirred water layer is discussed.

MATERIALS AND METHODS

Epithelial cell preparations

Intestinal epithelial cells were prepared by a method similar to that used by

Kimmich [14]. Female 180–220 g Sprague-Dawley rats fed regular chow 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 ten segments of equal length numbered from 1 to 10, proximal to distal. Segments 2–4 and 7–9 were used for the study of jejunum and ileum, respectively. The segments were incubated for 30 min at 37 °C in Tris buffer containing 1 mg/ml of hyaluronidase (Sigma Chemical Co., St. Louis, Mo.). The composition of the Tris buffer (pH 7.4) was 120 mM NaCl, 20 mM Tris·HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mg/ml bovine serum albumin. The resulting cellular suspension was strained through nylon stocking material and centrifuged at $200 \times g$ for 10 min. The pellet was washed twice with Tris buffer and finally was suspended in appropriate volume of buffer for metabolic and transport measurements.

Metabolic determinations

Measurement of $^{14}CO_2$ production from [1-^{14}C]glucose (New England Nuclear Corp., Boston, Mass.) was performed by a method similar to that of Kimmich [14]. The cell suspensions (1 ml) were added to 1 ml of Tris buffer containing 10 mg/ml of bovine serum albumin, 4 mM glucose, and 0.1 μCi [1-^{14}C]glucose. Incubations were carried out in siliconized 25-ml Erlenmeyer flasks equipped with center wells. The wells contained 0.2 ml of hydroxide of hyamine and a filter paper wick. The flasks were stoppered and incubated at 37 °C in a water bath while shaken at 100 oscillations/min. The reactions were terminated at appropriate time intervals with 0.5 ml of 1 M H_2SO_4 injected through the stopper. Blanks were prepared by adding H_2SO_4 to the buffer before the cell suspension, and incubations were carried out in the usual manner described for viable cells. After 1 h equilibration period, the hydroxide of hyamine and wick were removed, added to toluene-Triton scintillation fluid and counted for radioactivity. Values were expressed as nmol of radiolabeled CO_2 produced from the glucose/mg cell protein per time interval, e.g. nmol/mg per 15 min. Glycolytic activity was determined by measuring lactate formation from glucose. 1 ml of cell suspension was added to 3 ml of Tris buffer containing 5 mM glucose in 25-ml siliconized Erlenmeyer flasks. Incubations were carried out at 37 °C in a shaking bath at 100 oscillations/min. After the incubation interval, 0.5-ml aliquots were removed and added to 1 ml of 0.6 M $HClO_4$ to stop metabolic activity. The protein was sedimented and lactate was determined on an aliquot of the supernatant according to the method of Hohorst [15]. All measurements of lactate production were corrected for the amount of lactate present in cells at the start of the incubation. Lactate production was expressed in nmol/mg per 15 min. Oxygen consumption was recorded polarographically by a Gilson Oxygraph (Model K, Gilson Medical Electronics, Middleton, Wisc.) fitted with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) at 27 °C in an air-saturated Krebs buffer in a final volume of 1.6 ml. The measurements were started after 1–4 mg of cellular protein was added to Tris buffer. The current output from the electrode was calibrated by using solutions of known oxygen content, and results were expressed as μl of oxygen taken up per mg cell protein per 15 min.

Flux rate determinations

In these experiments, essentially instantaneous, unidirectional uptake rates for

bile acids were determined using isolated epithelial cells from jejunum and ileum. ^{14}C-labeled bile acid (Mallinckrodt Chemical Works, St. Louis, Mo.), ^{3}H-labeled bile acid (New England Nuclear Corp., Boston, Mass.), and unlabeled bile acid (Steraloids, Inc., Pawling, N.Y.; Calbiochem, Los Angeles, Calif.) included taurocholate, taurodeoxycholate, glycocholate, glycochenodeoxycholate, glycodeoxycholate, cholate, chenodeoxycholate, and deoxycholate. These compounds were found to have greater than 95 % purity using thin-layer chromatography [8]. ^{14}C-labeled dextran or ^{3}H-labeled dextran (New England Nuclear Corp., Boston, Mass.) were used as non-absorbable markers to measure the volume of adherent extracellular fluid. The dextran was dialyzed against distilled water before use. Incubations were carried out in Tris buffer gassed with 100 % O_2 at 37 °C in 17 × 100 mm polypropylene test tubes shaken at 100 oscillations/min. Generally, 0.3 ml of cell suspension was added to an equivalent volume of buffer containing both appropriate concentrations of ^{14}C- or ^{3}H-labeled bile acid and dextran containing the alternate label. Preliminary studies showed no differences in rates of cell uptake for ^{3}H-labeled and ^{14}C-labeled taurocholate. After a 1 or 2 min incubation, duplicate 200-μl samples were removed and released into 4 ml of iced buffer. The suspension was immediately centrifuged at $200 \times g$ for 5 min. The cellular pellet was resuspended in 2 ml of iced buffer and centrifuged again. Following the wash, the tissue pellet was saponified with 0.75 M NaOH at 80 °C for 30 min. After cooling to room temperature, a toluene-Triton scintillation fluid was added and radioactivity counted using an external standardization technique to correct for quenching of the two isotopes [13]. Following correction for mass of bile acid molecules carried over in the adherent fluid by means of the marker compound flux rates, J, were calculated and have the units nmol taken up into cells/min per 100 mg cellular protein.

Other methods

Protein content was assayed in aliquots of cell suspensions by the method of Lowry et al. [16] employing crystalline bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) as a standard. Cell viability for each experiment, estimated from the percentage of cells per microscopic field which excluded 0.2 % tryphan blue, usually ranged from 80 to 90 %.

RESULTS

Lactate and CO_2 production and O_2 consumption by isolated cells

Initial experiments were performed to assess metabolic function of isolated jejunal cells with respect to time and the effects of agents known to modify metabolic and transport activity. As shown in Fig. 1A, lactate production was linear for 40 min. When incubated with ouabain, 100 μM, the rate of lactate production was slower than control. The rate of $^{14}CO_2$ production from [1-^{14}C]glucose (shown in Fig. 1B) was linear for 45 min. Incubation of cells with 2,4-dinitrophenol, 200 μM, resulted in enhancement of CO_2 production, whereas similar studies with oligomycin, 5 μg/ml of buffer, resulted in inhibition of CO_2 production. Polarographic determinations of O_2 consumption were routinely performed to assess cell viability prior to use in other studies. Representative values are illustrated in Fig. 1C indicating essentially linear O_2 consumption for 45 min. Similar rates of metabolic function were obtained from cells isolated from ileum (values not shown).

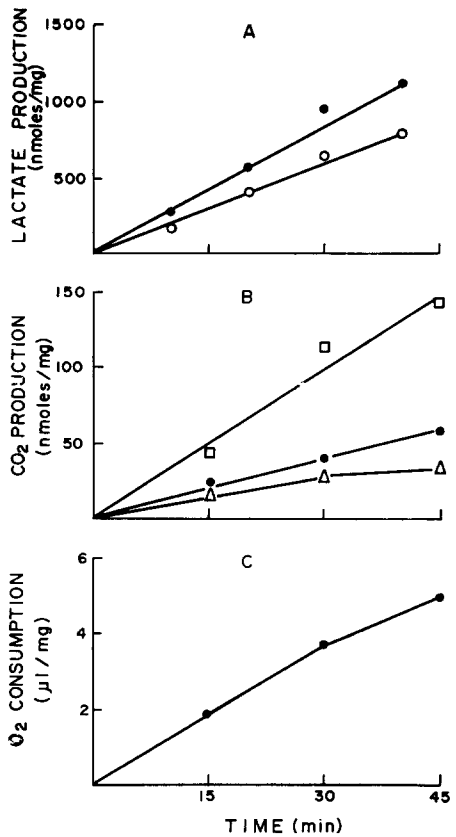

Fig. 1. Characteristics of lactate and CO_2 production from glucose and O_2 consumption with respect to time and the effects of various agents. Isolated cells were incubated in Tris buffer. Additions to the standard buffer were: panel A, 5 mM glucose (for controls, ● —●) and 100 μM ouabain (○—○); panel B, 5 mM glucose, 10 mg/ml bovine serum albumin (for controls, ● —●), 200 μM 2,4-dinitrophenol (□—□), and 5 μg/ml oligomycin (△—△); panel C, no additions.

Adherent extracellular fluid

During the measurement of cell uptake during very short incubations, bathing solution adherent to cells and carried over into the counting vial may result in significant errors in determination of uptake rates [13]. To provide a correction for the adherent extracellular fluid, it is important that equilibrium within the adherent cell fluid be obtained. The adherent cell fluid volume measured by ^{14}C-labeled dextran as a function of time of incubation (Fig. 2) shows that the dextran achieves equilibrium within the adherent fluid by 0.5 min and that equilibrium remains up to 6 min. Thus, during this period of incubation, the marker correctly estimates the adherent fluid volume.

Characteristics of bile acid uptake into isolated jejunal cells

The first series of experiments were performed to validate the assay system. Another potential source of error in the determination of uptake rates is the loss of

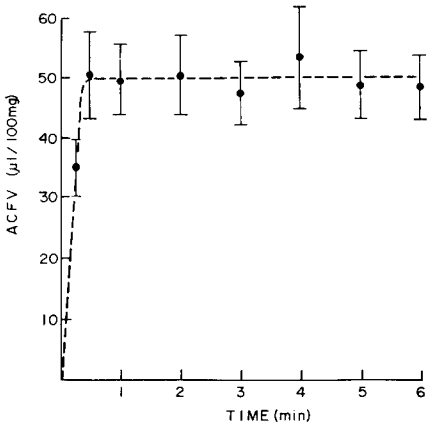

Fig. 2. Characteristics of the adherent cell fluid volume (ACFV) with respect to time. Isolated cells were incubated with Tris buffer containing ^{14}C-labeled inulin as the marker. Values are means ± S.E. for 40 determinations.

radioactivity following incubation of cells in radiolabeled solute. Experiments were performed as outlined in Table I, to assess whether loss of bile acid from isolated jejunal cells occurred during the centrifugation process. In experiment A, no difference was observed for the uptake of taurodeoxycholate when cell samples were left in iced buffer up to 3 min prior to centrifugation. Likewise, no difference was observed in cell uptake when centrifugation was varied from 5 to 10 min in experiment B. However, when the effect of repeated suspension and centrifugation was examined in experiment C, significant radioactivity was lost from the isolated cells. For this reason cells were subjected to a single wash prior to saponification and counting for radioactivity.

TABLE I

COMPARISON OF CELL UPTAKE DURING THE CENTRIFUGATION PROCESS

The uptake of 1 mM taurodeoxycholate was measured in paired samples of jejunal cells. Experiments A 1, B 1 and C 1 were performed in the usual manner. The other members of paired cell samples were: in experiment A 2, left in iced buffer for 3 min prior to centrifugation; in experiment B 2, centrifuged for 10 min; and in experiment C 2, resuspended and centrifuged on two occasions prior to saponification and counting for radioactivity. The number of determinations is shown in parentheses. Mean ± S.E. are given.

Experimental conditions		Cell uptake J, nmol/min per 100 mg	
(A)	Time of cells in iced buffer prior to centrifugation		
	(1) < 30 s	179.9±28.7	(12)
	(2) 3 min	174.8±17.9	(12)
(B)	Length of centrifugation		
	(1) 5 min	163.8±21.2	(12)
	(2) 10 min	181.4±19.8	(12)
(C)	Number of resuspensions and centrifugations		
	(1) One	180.0±39.2	(12)
	(2) Two	146.6±41.3	(12)

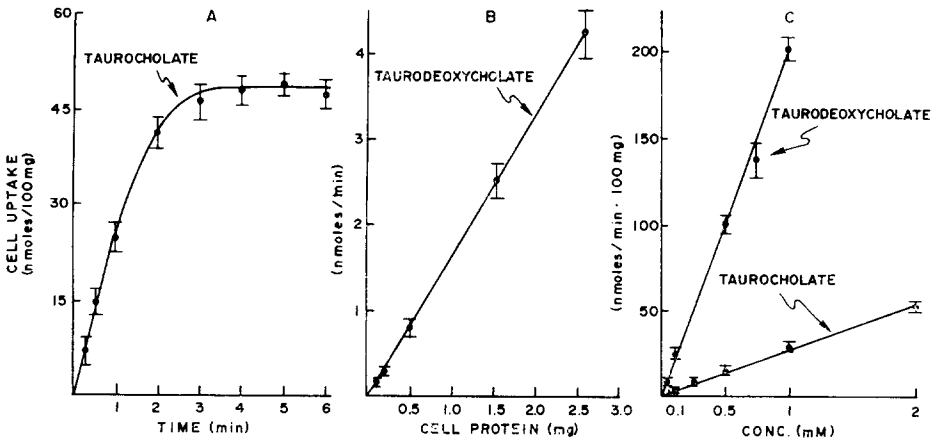

Fig. 3. Characteristics of bile acid uptake with respect to time, cell protein, and concentration. Isolated cells were incubated in Tris buffer containing ^{14}C-labeled taurocholate or ^{14}C-labeled taurodeoxycholate. Conditions for incubation were: panel A, 1 mM taurocholate; panel B, 1 mM taurodeoxycholate for 2 min; panel C, taurocholate and taurodeoxycholate for 2 min. Values are means ±S.E. for 8–24 determinations.

As shown in Fig. 3, the characteristics of uptake into isolated jejunal cells with respect to time, cell protein, and substrate concentration were next investigated. The time course (Fig. 3A) for taurocholate is essentially linear up to 2 min. Beyond this time period, cell uptake rates fall off presumably as intracellular levels of bile acid increase. Uptake rates for bile acid in the jejunum are perfectly linear with respect to cell protein. Taurodeoxycholate uptake is plotted in Fig. 3B. This determination also may be described by linear regression which has essentially zero intercepts. Thus, dividing cell uptake by cell protein is an appropriate method for normalizing determinations. Cell uptake rates for bile acids are linear with respect to their monomer concentrations, i.e. concentrations below their critical micellar concentration. Fig. 3C shows this relationship for taurocholate and taurodeoxycholate up to 2 and 1 mM, respectively.

Previous studies that showed bile acid flux across the brush border membrane of the jejunum to be passive employed whole tissue preparations. Since the technique used in the present study involves the measurement of bile acid uptake into the isolated absorptive cell, it is essential to show that this preparation has the appropriate transport characteristics. The third series of experiments, therefore, were directed at characterization of bile acid monomer uptake into isolated jejunal cells. As shown in Table II, the apparent permeability coefficients (P(app)) of bile acid monomers vary in a regular manner with respect to molecular structure. First, for any particular conjugated or unconjugated bile acid removal of hydroxyl group enhances permeation significantly. Second, for any given number of hydroxyl groups, the unconjugated bile acid has the highest P(app) value, the glycine conjugate has the second highest value, and the taurine the third highest.

As shown in Fig. 4, and as will be outlined in Discussion, the data in Table II may be transformed into a linear form by plotting the ln of product $(P(\text{app})) \cdot (M_r^{\frac{1}{2}})$ against n, the number of hydrogen bonds that a bile acid can form with water [8, 9].

The linear regression curve for P(app) has a negative slope so that the quantity ln P(app) · $M_r^{\frac{1}{2}}$ decreases by a factor of 0.752 for each additional hydrogen bond that a bile acid can form with water.

TABLE II

APPARENT PERMEABILITY COEFFICIENTS P (app) OF BILE ACID MONOMERS

Passive permeability coefficients vary with the chemical structure of the bile acid molecule. Bile acids represent a series of sterols which differ from one another primarily with respect to the presence or absence of conjugation with glycine or taurine and their degree of hydroxylation at the number 3, 7 or 12 carbon position on the cyclopentanophenanthrene nucleus. For example, the systematic names for the unconjugated bile acids, cholate, chenodeoxycholate and deoxycholate, are 3α-, 7α-, 12α-trihydroxycholanoic, 3α-, 7α-dihydroxy cholanoic, and 3α-, 12α-dihydroxy cholanoic acids, respectively. Mean values ±S.E. for apparent permeability coefficients P (app) are shown for bile acid monomers. Jejunal cells were incubated for 2 min at 37 °C in Tris buffer (pH 7.4) containing bile acid concentrations from 0.5 to 1 mM, but all data are normalized to 1 mM. The actual number of experimental determinations for each bile acid is given in parentheses.

Bile acid	P (app) (nmol/min/100 mg/mM)
Taurocholate	24.9 ± 1.8 (56)
Glycocholate	78.8 ± 3.5 (46)
Cholate	328.8 ± 51.9 (68)
Taurodeoxycholate	177.9 ± 16.5 (146)
Glycodeoxycholate	241.2 ± 25.8 (30)
Deoxycholate	1563.2 ± 124.7 (30)
Glycochenodeoxycholate	243.6 ± 25.8 (30)
Chenodeoxycholate	1428.0 ± 192.6 (20)

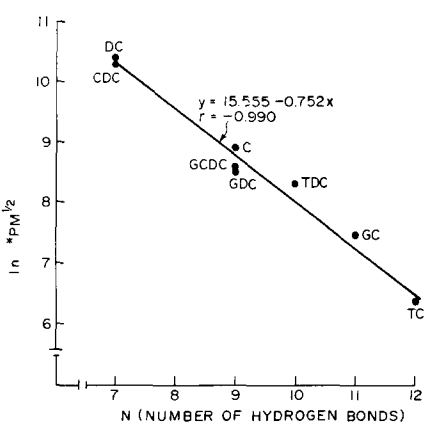

Fig. 4. Relationship of apparent permeability coefficients, P (app), at pH 7.4, to their hydrogen-binding capability. In the diagram P (app) (Table II) has been multiplied by the square root of the molecular weight ($M_r^{\frac{1}{2}}$) for each bile acid and the ln of this product has been plotted against n, the number of hydrogen bonds that a particular bile acid molecule can form in water. Values of n were assigned as suggested by Stein [33]. In this figure DC, CDC, C, GCDC, GDC, TDC, GC and TC represent deoxycholate, chenodeoxycholate, cholate, glycochenodeoxycholate, glycodeoxycholate, taurodeoxycholate, glycocholate and taurocholate, respectively.

Characteristics of bile acid uptake into isolated ileal cells

Cells isolated from the ileum were used in these studies to characterize the kinetics of the active system for bile acids. For kinetic analysis, ileal uptake, J, is given by the expression [8]

$$J = V(\text{app}) \frac{C_m}{K_m(\text{app}) + C_m} + (P(\text{app})) \cdot (C_m) \quad (1)$$

in which J, cell uptake in nmol/min per 100 mg protein; V(app), apparent maximal cell uptake in nmol/min per 100 mg protein; C_m, bile acid concentration in mmol/l in the incubation solution; K_m(app), apparent Michaelis constant in mmol/l; P(app), apparent permeability coefficient of the bile acid in nmol/min per 100 mg protein/mmol at pH 7.4. The first term gives the magnitude of the active flux and describes a rectangular hyperbola; the second term gives the magnitude of the passive flux and describes a straight line. It is apparent that in order to determine the rate of active uptake, the passive flux must be subtracted from the experimental uptake rate, J. The required permeability coefficients (P(app)) of various bile acids for ileal cells were obtained in two ways. First, with increasing C_m (3–4 times K_m(app)) the first term approximates a constant and J continues to increase in an essentially linear manner with a slope equal to P(app). Second, the passive permeability characteristics of the ileal cells for bile acids were assumed to be identical to those from the jejunum as described previously [8]. The passive permeability coefficients obtained by these two methods were averaged, and the average value was subtracted from the measured total uptake to yield the active component of bile acid uptake in ileal cells [8, 11].

The experiments, shown in Figs 5A and 5B, describe the kinetics of bile acid uptake into jejunal and ileal cells for taurocholate and taurodeoxycholate. The rate of cell uptake, J, in the jejunal cells is linear with respect to concentration while in the ileal cells J describes a hyperbolic component with respect to the concentration of the probe molecule in the incubation solution. However, a passive component of uptake by ileal cells was quantitatively important especially for taurodeoxycholate. The slope of the linear portion of the curve for ileal uptake approached that for jejunal uptake. The regression curve for taurocholate (J against C_m) that describes the passive permeability for the jejunum, $y = 28.4x + 1.1$, approached the one for the asymptotic linear component in the ileum, $y = 32.5x + 80.2$. Similarly, the linear regression curves for the P(app) values of taurodeoxycholate have a slope such that J increased by a factor of 185 and 194 for 1 mM change in concentration for the jejunum and ileum, respectively. When the average of the slopes was subtracted from ileal values, the kinetic curve for active transport (dashed line) resulted. The values of V(app) and K_m(app) were derived from the curves for taurocholate and taurodeoxycholate based on the Lineweaver-Burk plot.

The kinetics of bile acid uptake into jejunal and ileal cells support the previous studies that transport is active in the ileum and passive in the jejunum. To document further the presence of such transport mechanisms, a comparison of the characteristics of uptake into the jejunal and ileal cells was undertaken. Data on possible energy-dependent carrier-mediated uptake of bile acids are given in Table III. 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

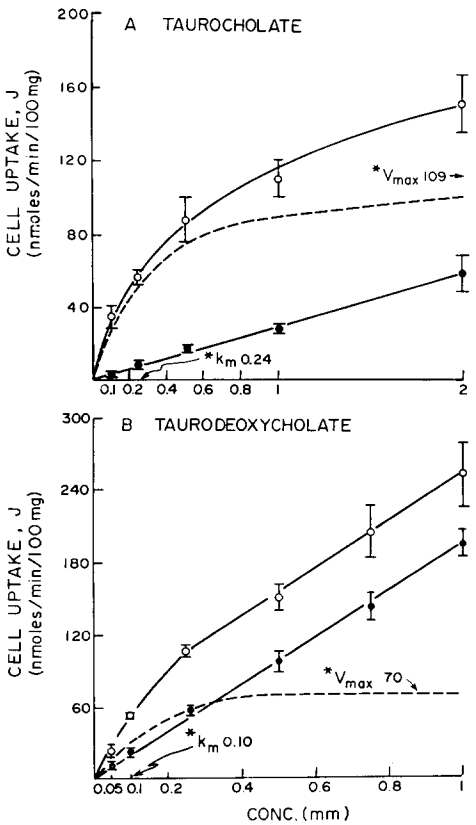

Fig. 5. Relationship between incubation concentration and uptake of taurocholate and taurodeoxycholate in jejunal (● — ●) and ileal (○ — ○) cells. The dashed lines represent the active ileal component calculated by subtracting an apparent passive permeability coefficient from experimentally determined rates of ileal cell uptake as described in the text. Values are means ±S.E. for 24 and 14 determinations for taurocholate and taurodeoxycholate, respectively.

uptake in ileal cells but not in jejunal cells. In experiment B, differences were observed in jejunal and ileal cells with respect to metabolic inhibition. Transport of taurocholate into the ileal cells but not jejunal cells was inhibited by the presence of oligomycin and ouabain. The effect of temperature on jejunal and ileal uptake was measured at 0 and 37 °C in experiment C. The Q_{10} value for taurocholate uptake was 1.1 and 1.6 for jejunal and ileal cells, respectively.

DISCUSSION

Studies with epithelial cells isolated from the rat intestine have been utilized widely to characterize metabolic and transport processes [14, 17–23], but have as yet to include passive and active absorption of bile acids. Since intact viable cells are required to study active transport processes, initial studies were undertaken to assess their metabolic function. As demonstrated in Fig. 1, the linear production of $^{14}CO_2$

TABLE III

CHARACTERISTICS OF BILE ACID UPTAKE INTO ISOLATED JEJUNAL AND ILEAL CELLS

In these experiments, cells isolated from jejunum and ileum were incubated for 2 min in Tris buffer containing radiolabeled taurocholate under the following conditions: with or without unlabeled glycocholate (A), with or without metabolic agents (B), and at 0 or 37 °C (C). The concentrations of bile acids and metabolic agents used in each study are shown above. Mean values ±S.E. are given. The number of determinations is shown in parentheses.

Experimental conditions		Cell uptake (J, nmol/min per 100 mg)			
		Jejunal		Ileal	
(A)	Uptake of taurocholate into cells in absence and presence of glycocholate				
	Taurocholate (0.5 mM) : Glycocholate (0)	14.7±1.7	(22)	54.0±6.9	(22)
	Taurocholate (0.5 mM) : Glycocholate (1.0 mM)	14.2±2.7	(22)	37.4±4.1	(22)
	Taurocholate (1.0 mM) : Glycocholate (0)	24.7±3.2	(22)	89.7±11.6	(22)
	Taurocholate (1.0 mM) : Glycocholate (1.0 mM)	23.3±2.7	(22)	63.6±5.8	(22)
	Taurocholate (1.5 mM) : Glycocholate (0)	34.8±1.4	(22)	109.6±12.2	(22)
	Taurocholate (1.5 mM) : Glycocholate (1.0 mM)	33.3±1.9	(22)	86.9±7.7	(22)
(B)	Uptake of taurocholate into cells in absence and presence of metabolic agents				
	Taurocholate (1.0 mM)	25.4±4.3	(16)	78.6±4.7	(16)
	Taurocholate (1.0 mM) : ouabain (100 μM)	23.7±4.5	(16)	59.1±5.7	(16)
	Taurocholate (1.0 mM) : oligomycin (5 μg/ml)	26.4±2.9	(16)	67.9±6.8	(16)
(C)	Uptake of taurocholate into cells at different temperatures				
	Taurocholate (1.0 mM) at 0 °C	27.5±2.8	(12)	45.3±5.7	(12)
	Taurocholate (1.0 mM) at 37 °C	31.7±3.9	(12)	104.7±11.3	(12)

and lactate and the linear consumption of O_2 with respect to time indicate that pathways of overall metabolism were functioning. The period of linear metabolic activity is comparable to that of cells prepared from rats in other investigations [17, 18]. The initial mean values for CO_2 production (42.5 nmol/mg per 15 min), lactate production (225 nmol/mg per 15 min), and O_2 consumption (1.9 μl/mg per 15 min) quantitatively compare with previous values for isolated intestinal cells [17–19]. The normal cell response toward metabolic inhibitors is emphasized in Fig. 1 which shows inhibition of CO_2 and lactate production by oligomycin and ouabain, respectively. Finally, enhancement of CO_2 production with low doses of an uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, suggest the cells' ability to couple metabolic activity to energy production [14].

Methods to measure flux rates have not included routinely non-absorbable markers to correct for contamination by extracellular test molecules. During short incubation periods, recent experiments have shown only a portion of the mass of labeled test material analyzed with tissue penetrating the cell membrane [13]. When membrane uptake is very rapid, the amount of compound in the extracellular fluid represents only a small percentage of the total amount analyzed in the sample (e.g. 8 % for deoxycholate), but when uptake is low relatively more of the total compound in the sample activity is due to contamination of the cell with incubation fluid (e.g. 60 % for taurocholate). Accurate correction for extracellular contamination of test molecule requires uniform labeling of the adherent fluid with the non-absorbable

marker. The fact that equilibrium within this space is not achieved until 0.5 min (as shown in Fig. 2) would suggest that at shorter incubation times, the marker would underestimate the adherent fluid volume. Another requirement for such determinations is that the marker of adherent fluid volume should be totally excluded from cell. The fact that an increase in activity of the test label (Fig. 3A) continues beyond 0.5 min up to 3–4 min while equilibrium is achieved for the marker label (Fig. 2) suggests that bile acid uptake across the epithelial cell membrane is occurring while the cell membrane penetration for the marker dextran is essentially zero. For these reasons, either a 1- or 2-min period of incubation was selected for transport studies.

The rate of absorption into the intestinal mucosal cell is determined by at least two major diffusion barriers: an unstirred water layer adjacent to the cell membrane and the cell membrane itself. In vitro studies have shown that the aqueous diffusion barrier in the small intestine may lead to significant underestimations of passive permeability coefficients [9, 24, 25] and cause marked overestimations of K_m(app) values for active transport [10]. Indeed, the unstirred water layer, and not the cell membrane, may be rate limiting for absorption of such physiologically important compounds as bile acids [9] and fatty acids [25]. The physical dimensions of the unstirred water layer have been resolved into two compartments, a superficial and deep layer. The physiologically important superficial layer overlying the villous tips varies in thickness from 115 to 334 μm, dependent upon the rate of mixing of the bulk mucosal solution [26].

Previous studies with red blood cells surrounded by an unstirred water layer of only 5.5 μm in thickness suggested that permeability coefficients in isolated cell systems do not require correction on account of the aqueous diffusion layer [27]. Our studies with isolated intestinal epithelial cells also suggest that unstirred water layer effects are minimized and that uptake is primarily rate limited by the cell membrane. The fact that the regression line in Fig. 4 has a significant slope implies that the lipid membrane is rate limiting for passive monomer absorption. If a diffusion layer of water adjacent to the cell membrane were rate limiting, the quantity $\ln P(\text{app}) \cdot M_r^{\frac{1}{2}}$ would be independent of functional groups on the parent molecule because, in diffusion limited situations, P(app) of lower molecular weight compounds is proportional to D, the free diffusion coefficient in water; D, in turn, is inversely proportionate to $M_r^{\frac{1}{2}}$. Thus, the quantity $PM_r^{\frac{1}{2}}$ would equal a constant. Clearly, this is not the case since $\ln PM_r^{\frac{1}{2}}$ decreases with increasing hydrogen bonding. The slope of this regression obtained with isolated cells is even greater than similar plots obtained from data using whole tissue preparations [8, 9]. From these observations it is concluded that because of the theoretically thin unstirred layer that exists around isolated individual cells the apparent permeability coefficients (P(app) given in Table I can be considered, in fact, to be true permeability coefficients (P).

On the basis of the above considerations it is possible to use the permeability coefficients in Table II to calculate actual incremental free energy changes. Under conditions where passive transport across the cell membrane is rate limiting P is proportional to K, the lipid membrane: 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 1 mol of bile acid from the aqueous phase adjacent to the cell membrane to the lipid phase of the cell membrane. The major determinant of changes in permeability for the homologous series of bile acids becomes the magnitude of the

incremental free energy change, $\delta \Delta F_{w \to l}$, that results from the addition or deletion of a functional group from the parent molecule. The value of $\delta \Delta F_{w \to l}$ for the functional groups on the bile acids, in turn, primarily is proportional to the capability of these groups to hydrogen bond with water [28]. This relationship in graphic form is presented in Fig. 4, where the slope of the line corresponds to a free energy change of about 500 cal/mol for each additional hydrogen bond. More specifically, the addition of a hydroxyl, glycine and taurine group to the bile acid molecule yields incremental free energy changes of 892, 1040 and 1464 cal/mol, respectively. These values are in close agreement to those incremental free energy changes obtained using P ratios following correction of apparent permeability coefficients for unstirred water layer resistance in whole tissue preparations [9].

One final point warrants emphasis. A potential deficiency in the use of isolated intestinal epithelial cells is the inability to evaluate separately the contribution of the brush border membrane and the other cell membranes to the process under study [29]. Recent analyses of the microvillous membranes and basolateral membranes have shown different lipid compositions for the two different regions of the plasma membranes of mouse intestinal epithelial cells [30]. Inasmuch as the rate of passive penetration of a cell membrane is determined by the lipid membrane : aqueous partition coefficient, consideration must be given the relative polarity of the basolateral membrane newly exposed to solute molecules in isolated cell experiments. The resemblance of $\delta \Delta F_{w \to l}$ values for the addition of functional groups to the bile acid molecule in isolated cells to everted intestinal segments suggests, however, that the microvillous membrane and basolateral membranes are similar at least with respect to their partitioning characteristics. Indeed these values suggest that the epithelial cell membranes of the rat jejunum. like several other epithelial surfaces, are relatively polar structures, more polar even than isobutanol, lecithin or ether in solution [26, 28, 31, 32].

The discriminating capacity of the isolated cell membrane is more rigorously tested by the unique feature of bile acid transport that the active system is localized anatomically to the ileum. The active absorption of bile acids in the ileal cells is supported by the kinetic curves shown in Fig. 5. When the passive component is subtracted from experimental data, J describes an apparent hyperbolic relationship to the concentration of the probe molecule in the bulk water phase. The V(app) value for taurocholate is greater than taurodeoxycholate, a finding noted previously [8, 11]. The K_m(app) values of 0.24 and 0.10 mM for taurocholate and taurodeoxycholate, respectively, are lower than usually described for in vivo [11] and whole tissue preparations [8, 10] and are consistent with the fact that the presence of the unstirred water layer in these preparations results in artifactually elevated values [10]. Finally, transport with ileal cells, as shown in Table III, satisfied many of the criteria for active transport not seen with jejunal cells. Thus, these data indicate that isolated epithelial cells are a metabolically viable, relatively purified intestinal preparation which discriminates between active and passive transport processes for bile acids under conditions where unstirred water layer artifacts are minimized.

ACKNOWLEDGEMENTS

The authors express their appreciation to Drs John M. Dietschy and Verney L. Sallee, The University of Texas Health Science Center, Dallas, Texas, for their

helpful comments during the preparation of this manuscript. F. A. W. is a Clinical Investigator for the Veterans Administration.

REFERENCES

1 Lack, L. and Weiner, I. M. (1961) Am. J. Physiol. 200, 313–317
2 Dietschy, J. M., Salomon, H. S. and Siperstein, M. D. (1966) J. Clin. Invest. 45, 832–846
3 Playoust, M. R. and Isselbacher, K. J. (1964) J. Clin. Invest. 43, 467–476
4 Holt, P. R. (1964) Am. J. Physiol. 207, 1–7
5 Lack, L. and Weiner, I. M. (1966) Am. J. Physiol. 210, 1142–1152
6 Heaton, K. W. and Lack, L. (1968) Am. J. Physiol. 214, 585–590
7 Dietschy, J. M. (1968) J. Lipid Res. 9, 297–309
8 Schiff, E. R., Small, N. C. and Dietschy, J. M. (1972) J. Clin. Invest. 51, 1351–1362
9 Wilson, F. A. and Dietschy, J. M. (1972) J. Clin. Invest. 51, 3015–3025
10 Wilson, F. A. and Dietschy, J. M. (1974) Biochim. Biophys. Acta 363, 112–126
11 Krag, E. and Phillips, S. F. (1974) J. Clin. Invest. 53, 1686–1694
12 Weiner, I. M. and Lack, L. (1962) Am. J. Physiol. 202, 155–157
13 Sallee, V. L., Wilson, F. A. and Dietschy, J. M. (1972) J. Lipid Res. 13, 184–192
14 Kimmich, G. A. (1970) Biochemistry 9, 3659–3668
15 Hohorst, H. J. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 266–282, Academic Press, New York
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
17 Leslie, G. I. and Rowe, P. B. (1972) Biochemistry 11, 1696–1703
18 Perris, A. D. (1966) Can. J. Biochem. 44, 687–693
19 Iemhoff, W. G. J., Van Den Berg, J. W. O., De Pijper, A. M. and Hulsmann, W. C. (1970) Biochim. Biophys. Acta 215, 229–241
20 Stern, B. K. and Jensen, W. E. (1966) Nature 209, 789–790
21 Reiser, S. and Christiansen, P. A. (1971) Biochim. Biophys. Acta 225, 123–139
22 Yousef, I. M. and Kuksis, A. (1972) Lipids 7, 380–386
23 Momtazi, S. and Herbert, V. (1973) Am. J. Clin. Nutr. 26, 23–29
24 Dietschy, J. M., Sallee, V. L. and Wilson, F. A. (1971) Gastroenterology 61, 932–934
25 Sallee, V. L. and Dietschy, J. M. (1973) J. Lipid Res. 14, 475–484
26 Westergaard, H. and Dietschy, J. M. (1974) J. Clin. Invest. 54, 718–732
27 Sha'afi, R. I., Rich, G. T., Sidel, V. W., Bossert, W. and Solomon, A. K. (1967) J. Gen. Physiol. 50, 1377–1399
28 Diamond, J. M. and Wright, E. M. (1969) Annu. Rev. Physiol. 31, 581–646
29 Reiser, S. and Christiansen, P. A. (1973) Life Sci. 12, 25–33
30 Kawai, K., Fujita, M. and Nakao, M. (1974) Biochim. Biophys. Acta 369, 222–233
31 Smulders, A. P. and Wright, E. M. (1971) J. Membrane Biol. 5, 297–318
32 Diamond, J. M. and Katz, Y. (1974) J. Membrane Biol. 17, 121–154
33 Stein, W. D. (1967) The Movement of Molecules Across Cell Membranes, pp. 65–125, Academic Press, New York