Direct Determination of the Driving Forces for Taurocholate Uptake into Rat Liver Plasma Membrane Vesicles

MICHAEL C. DUFFY, BENNETT L. BLITZER, and JAMES L. BOYER, *Liver Study Unit, Department of Medicine, Yale University School of Medicine, New Haven, Connecticut 06510*

ABSTRACT To determine directly the driving forces for bile acid entry into the hepatocyte, the uptake of [^{3}H]taurocholic acid into rat liver plasma membrane vesicles was studied. The membrane preparation contained predominantly right-side-out vesicles, and was highly enriched in plasma membrane marker enzymes.

The uptake of taurocholate at equilibrium was inversely related to medium osmolarity, indicating transport into an osmotically sensitive space. In the presence of an inwardly directed sodium gradient (NaCl or sodium gluconate), the initial rate of uptake was rapid and taurocholate was transiently accumulated at a concentration twice that at equilibrium (overshoot). Other inwardly directed cation gradients (K^+, Li^+, choline$^+$) or the presence of sodium in the absence of a gradient (Na^+ equilibrated) resulted in a slower initial uptake rate and did not sustain an overshoot. Bile acids inhibited sodium-dependent taurocholate uptake, whereas bromsulphthalein inhibited both sodium-dependent and sodium-independent uptake and D-glucose had no effect on uptake. Uptake was temperature dependent, with maximal overshoots occurring at 25°C. Imposition of a proton gradient across the vesicle ($pH_o < pH_i$) in the absence of a sodium gradient failed to enhance taurocholate uptake, indicating that double ion exchange (Na^+-H^+, OH^--anion) is unlikely. Creation of a negative intravesicular potential by altering accompanying anions or by valinomycin-induced K^+-diffusion potentials did not enhance taurocholate uptake, suggesting an electroneutral transport mechanism. The kinetics of taurocholate uptake demonstrated saturability with a Michaelis constant at 52 μM and maximum velocity of 4.5 nmol · mg^{-1} · protein · min^{-1}.

These studies provide definitive evidence for a sodium gradient-dependent, carrier-mediated, electrically neutral transport mechanism for hepatic taurocholate uptake. These findings are consistent with a model for bile secretion in which the basolateral enzyme Na^+,K^+-ATPase provides the driving force for "uphill" bile acid transport by establishing a transmembrane sodium gradient.

INTRODUCTION

Bile acids, the major organic solutes in bile, undergo an extensive enterohepatic circulation that involves a series of transport steps in both liver and intestine (1). Uptake of bile acid anions from sinusoidal blood into the hepatocyte involves movement against both a 10–50-fold chemical gradient (2–4) and an unfavorable electrical gradient (transmembrane potential −30 to −40 mV) (5). Because bile acids are major choleretic substances and determine the biliary excretion of phospholipid and cholesterol, the driving forces controlling these transport steps (hepatic uptake, intracellular transport, and canalicular excretion) are critical to the formation of bile, as well as to the maintenance of the integrity of the enterohepatic circulation.

Previous studies in the intact dog (6) suggest that a saturable, carrier-mediated transport system exists for bile acid uptake from portal blood into the hepatocyte. Hepatic uptake of the bile acid, taurocholate, is sodium dependent in the isolated perfused rat liver

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Portions of this work were presented at the Annual Meeting of the American Association for the Study of Liver Diseases, 7 November 1981, and published in abstract form: *Hepatology (Baltimore)*, 1981, 1:507.

Dr. Duffy was supported by U. S. Public Health Service training grant AM 07356. Dr. Blitzer was a recipient of U. S. Public Health Service clinical investigator award 5K08-AM00661. Dr. Duffy's current address is GI Unit, University of Connecticut Health Center, Farmington, CT 06032; Dr. Blitzer's current address is Liver Study Unit, University of Cincinnati College of Medicine, Cincinnati, OH 45267. Address correspondence to Dr. Duffy.

Received for publication 10 March 1982 and in revised form 27 April 1983.

(7) and in isolated rat hepatocytes (8, 9). However, these experimental approaches do not clearly separate sinusoidal membrane events from intracellular processes, e.g., metabolism and protein binding. In addition, they do not differentiate between sodium dependence due to a direct sodium-coupled carrier and rheogenic potential-driven uptake or double ion exchange (e.g., Na^+-H^+ and OH^--anion$^-$), as has been suggested for some sodium-dependent transport systems in epithelia (10).

For these reasons, we used plasma membrane vesicles prepared from rat liver to better define the driving forces for hepatic bile acid uptake. Plasma membrane vesicles are a valuable technique for elucidation of transport mechanisms in epithelia, since they eliminate the problems of intracellular metabolism and permit direct analysis of driving forces. Similar techniques have recently been used to study hepatic amino acid transport (11–13).

The aims of the present study were to: (*a*) prepare and characterize a vesicle preparation from liver plasma membranes; (*b*) determine the driving forces for taurocholate uptake into these vesicles by analysis of the effects of cation gradients, pH gradients, and induced electrical potentials on uptake; and (*c*) define the kinetics of sodium-dependent and independent taurocholate transport into these vesicles. A preliminary report of this work has appeared in abstract form (14).

METHODS

Animals. Male albino Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 175–235 g were used for all preparations. The animals had free access to water, were fed Purina rodent chow ad lib. (Ralston Purina Co., St. Louis, MO) and housed in a constant temperature-humidity-controlled room with 12-h light-dark cycles. Nonfasted animals were killed by decapitation between 8 and 9 a.m.

Membrane preparation. Liver plasma membranes were isolated by a modification of the discontinuous sucrose gradient methods of Song et al. (15) and Boyer and Reno (16). After decapitation, the livers from three to four animals were rapidly removed, chilled on ice, and weighed. 30 g of liver were divided into 5-g portions, minced, rinsed with 1 mM $NaHCO_3$ buffer (pH 7.4), and homogenized with 7 up-and-down strokes in 40 ml of 1 mM $NaHCO_3$ buffer in a loose-fitting Dounce homogenizer (Kontes Glass Co., Vineland, NJ). The homogenates were combined, further diluted with $NaHCO_3$ buffer to a final volume of 870 ml, filtered twice through 60-gauge cheesecloth, and centrifuged in a Sorvall RC-5B centrifuge (GSA rotor) at 1,500 *g* for 10 min (Du Pont Co., Instruments Product Div., Sorvell Biomedical Div., Wilmington, DE). The resulting nuclear pellet containing the plasma membranes was resuspended in 5.5 vol of density-1.26 sucrose to yield a final density of 1.22. 16 ml of this suspension was overlayered with 6 and 4.5 ml of density 1.18 and 1.16 sucrose, respectively, and centrifuged in a fixed angle rotor (Spinco 30, Beckman Instruments, Inc., Palo Alto, CA) at 66,000 *g* for 60 min at 4°C in a Beckman L5-65 ultracentrifuge. Plasma membranes recovered from the density 1.18–1.16 interface were washed with 4–5 vol of 1 mM $NaHCO_3$ at 2,900 *g* for 10 min. The pellet was washed again in 5 vol of 1 mM $NaHCO_3$ at 11,700 *g* for 10 min at 4°C. The final pellet was resuspended in 1 mM $NaHCO_3$ buffer for enzyme analysis or in membrane suspension media (300 mM sucrose, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, 0.2 mM $CaCl_2$) for vesicle transport studies (1.5–4 mg protein/ml) and vesiculated by homogenization with 30 up-and-down strokes in a 7.5-ml capacity tight-fitting Dounce homogenizer (Kontes Glass Co.), followed by passage through a 27-gauge needle three times.

Electron microscopy. Membranes were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1.5 h at 4°C and then pelleted at 27,000 *g* in a SW 50.1 rotor for 15 min. After postfixation in 1% osmium tetroxide, specimens were stained *en bloc* with 0.5% uranyl acetate in 0.15 M NaCl, dehydrated in graded ethanol solutions, and embedded in Spurr's medium. Ultrathin sections were cut with a diamond knife on an Ultracut microtome (American Optical Corp., Southbridge, MA), stained with uranyl acetate and lead citrate, and examined with a Zeiss 10 B electron microscope (Carl Zeiss, Inc., New York).

The specimens for freeze-fracture were fixed in 1% glutaraldehyde cryoprotected with glycerol to a final concentration of 25% (vol/vol) for 1–1.5 h at 4°C and frozen in Freon 22 cooled by liquid nitrogen at its melting point, and then stored in liquid nitrogen. The specimens were fractured in a Balzers freeze etch unit, BAF301 (Balzers High Vacuum, Balzers, Hudson, NH) at −130°C stage temperature in a vacuum of at least 10^{-6} torr. Immediately after fracturing, a 250-Å thickness of platinum was shadowed at an angle of 45° and the specimen was coated with a 50-Å thickness of carbon. Another 150-Å-thick layer of carbon was applied at −40°C to give a strong replica. After cleansing in hypochlorite solution overnight, the replicas were washed with distilled water and examined with a Carl Zeiss, Inc., 10B transmission electron microscope. The orientation of the vesicles was assessed by analysis of the particle density of the fracture faces as previously described (17, 18).

Marker enzyme assays. All assays were performed on membrane preparations stored overnight at 4°C. The mitochondrial and microsomal markers, NADH and NADPH cytochrome c reductases (EC 1.6.2.4) respectively, were measured by a modification of the method of Omura and Takesue (19). In cuvettes containing 1.0-ml reaction mixture consisting of 100 μmol KH_2PO_4 (pH 7.5), 0.5 μmol KCN, 0.05 μmol oxidized yeast cytochrome c, 0.1 μmol of either NADH or NADPH, and 30–100 μg membrane protein, the reduction of cytochrome c was continuously monitored at 550 nm at 30°C in a Gilford 250 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Corning Glass Works, Oberlin, OH). The plasma membrane markers Na^+,K^+-ATPase (EC 3.6.1.3) and Mg^{2+}-ATPase (EC 3.6.1.4) were measured by a recording spectrophotometric method (20). Alkaline phosphatase (EC 3.1.3.1) was determined as previously described (21). Protein was determined by the method of Lowry et al. (22), with bovine serum albumin, fraction V, as the standard.

Transport assay. Uptake of [^{3}H]taurocholic acid (New England Nuclear, Boston, MA) or [^{3}H]L-alanine (New England Nuclear) was measured by a rapid Millipore filtration technique (Millipore/Continental Water Systems, Bedford, MA). 20 μl membrane suspension containing 20–80 μg protein were preincubated at 25°C for at least 5 min. Uptake was initiated by the addition of 80 μl incubation medium

(125 mM NaCl, 50 mM sucrose, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, 0.2 mM $CaCl_2$) containing either [^{3}H]taurocholic acid or [^{3}H]L-alanine preincubated at 25°C. The final incubation solution therefore contained 100 mM NaCl, 100 mM sucrose, 10 mM Hepes/KOH, 10 mM $MgSO_4$, and 0.2 mM $CaCl_2$. After incubation for 5 s to 30 min, uptake was terminated by the addition of 3.5 ml ice-cold stop solution (100 mM NaCl, 100 mM sucrose, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, 0.2 mM $CaCl_2$) and vesicle-associated ligand was separated from free ligand by immediate rapid filtration (1 ml/s) through a 0.45-μM Millipore filter (HAWP) that had been presoaked in cold stop solution and prefiltered with 3 ml of 1 mM taurocholate to diminish nonspecific filter binding. The filter was washed three times with 3.5 ml stop solution, dissolved in 6 ml Redisolv HP (Beckman Instruments, Inc.), and counted in a Beckman LS 7000 liquid scintillation counter. Nonspecific binding was determined in each experiment by adding the cold stop solution before the addition of the incubation solution, and this blank was subtracted from all determinations. All incubations were performed in triplicate, with either freshly prepared membranes kept on ice or membranes that had been frozen in liquid nitrogen, thawed at 37°C, and placed on ice. Storage of the vesicles in liquid nitrogen for up to 1 wk resulted in no loss of transport activity compared with freshly prepared vesicles. Uptake of taurocholate was found to vary linearly with protein between 20 and 100 μg. Most observations were confirmed with at least three separate membrane preparations. Unless otherwise specified, the results shown are from a single representative experiment.

Analysis of intravesicular contents. Vesicles were incubated in a buffered medium containing 6 μM [^{3}H]taurocholate and 100 mM NaCl for 45 s. After termination of uptake, the filters were extracted by boiling in 3 ml H_2O for 5 min. The extract was pelleted and the supernatant was removed, lyophilized, resuspended in water, and subjected to thin-layer chromatography (23).

Chemicals. [^{3}H]Taurocholic acid (3.39 Ci/mmol) and [^{3}H]L-alanine (82.7 Ci/mmol) were obtained from New England Nuclear. The [^{3}H]taurocholic acid was >95% pure by thin-layer chromatography (23). Sodium taurocholate was purchased from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, CA), Valinomycin and Hepes (*N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid) from Sigma Chemical Co. (St. Louis, MO), and bromsulphthalein (BSP)[1] from Hynson, Westcott, and Dunning (Becton, Dickinson & Co., Baltimore, MD). All inorganic chemicals were of reagent grade or of the highest purity available.

Statistical analysis. The *t* test was used to test for significant differences amongst means (24). The kinetic data were analyzed on a Northstar Horizon Minicomputer (Northstar Computers, Inc., Berkeley, CA) using a program that performed a nonlinear weighted last-squares fit of the individual data points to a rectangular hyperbola (25).

RESULTS

Electron microscopy. Examination of the final membrane preparation by transmission electron microscopy demonstrated a predominantly vesicular morphology (Fig. 1), with vesicle diameters ranging from 0.1 to 0.4 μm. Sheets of unvesiculated membrane and occasional intact canaliculi were also seen. Analysis of membrane particle density by freeze-fracture electron microscopy revealed that the majority of the vesicles were oriented as in the intact cell (right-side-out) (Fig. 2 and Table I), confirming the findings of Sips et al. (26).

Marker enzymes. The specific activities for the plasma membrane and subcellular organelle marker enzymes (Table II) show that this preparation is significantly enriched in all of the plasma membrane markers tested, with relatively more enrichment of the basolateral (sinusoidal) marker Na^+,K^+-ATPase than the apical (canalicular) markers Mg^{2+}-ATPase, or alkaline phosphatase. There was no enrichment in mitochondrial or microsomal enzyme markers.

Demonstration of vesicle uptake. To distinguish between binding to the membrane and uptake into the vesicles, the equilibrium uptake of taurocholate was determined under conditions in which the intravesicular volume was altered osmotically. Total uptake at equilibrium (Fig. 3) was proportional to the reciprocal of the osmolarity of the incubation solution, indicating transport into an osmotically sensitive space. Extrapolation of the regression line back to the ordinate (theoretical zero intravesicular volume) demonstrates that binding accounts for at least 50% of the equilibrium uptake under isotonic conditions.

The degree of binding was also assessed by hypotonic lysis. Washing the filters with deionized water rather than with the usual isotonic stop solution decreased the mean uptake from 10.5±1.8 to 4.4±1.4 pmol/mg, suggesting that at least 42% of the measured uptake is due to binding, a finding consistent with estimates obtained from the osmotic data.

By osmotic methods (data not shown), binding was estimated to account for 20% of total [^{3}H]L-alanine uptake. From their respective equilibrium uptakes, the calculated apparent intravesicular volumes were 0.8 and 8–12 μl/mg protein, respectively, for L-alanine and taurocholate. These data indicate that the degree of binding is significantly greater for taurocholate than for alanine and suggest that the osmotic methods may underestimate the degree of binding of taurocholate.

When the intravesicular contents were assessed by thin-layer chromatography (see Methods), 96% of the radioactivity had the same R_f as the taurocholate standard, indicating that [^{3}H]taurocholate did not undergo degradation during transport.

Effect of cation gradients on uptake of taurocholate. The effect of cation gradients on vesicle uptake of 1 μM [^{3}H]taurocholate is illustrated in Fig. 4. The presence of an inwardly directed 100-mM NaCl gradient (extravesicular > intravesicular) produced a rapid initial rate of uptake, with a maximal accumulation occurring at 45 s, followed by a slow decline as tau-

[1] *Abbreviation used in this paper:* BSP, bromsulphthalein.

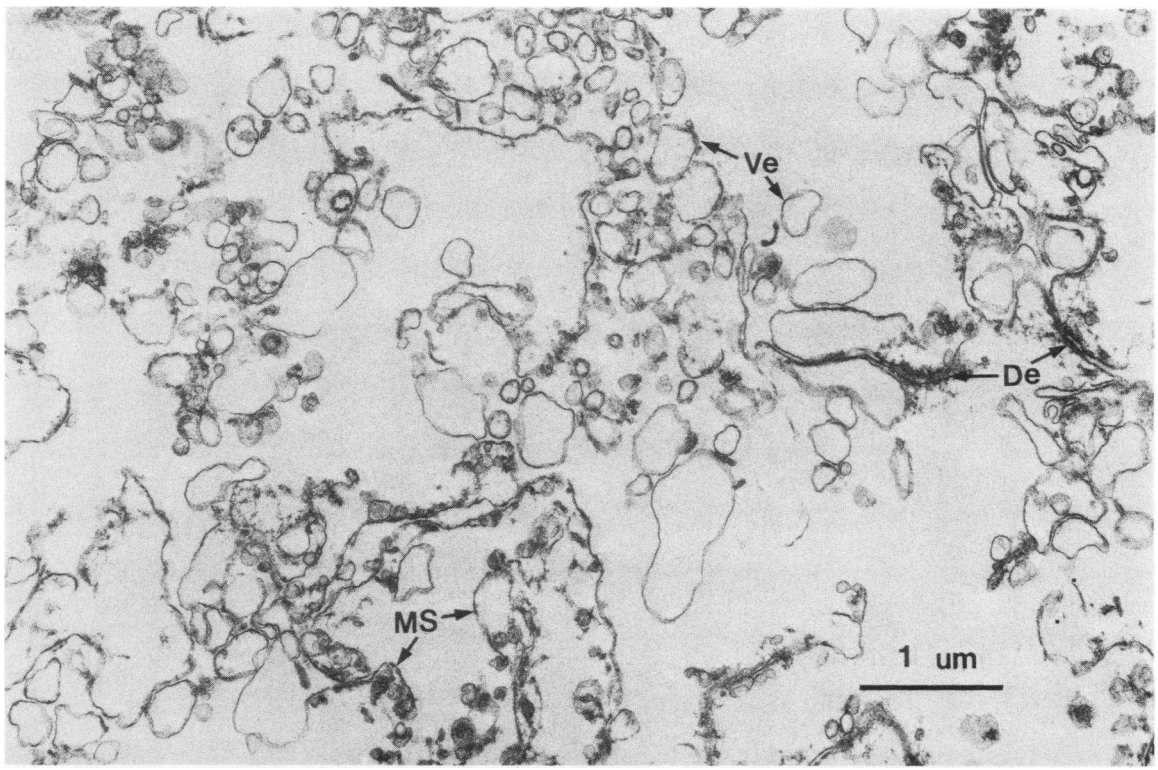

FIGURE 1 Transmission electron micrograph (× 18,750) of a vesiculated liver plasma membrane preparation showing many vesicles (*Ve*), as well as occasional desmosomes (*De*) and nonvesiculated membrane sheets (*MS*).

rocholate effluxed from the vesicles, reaching equilibrium by 30 min. The transient accumulation of taurocholate at a concentration twice that at equilibrium (overshoot) directly demonstrates that a NaCl gradient is capable of driving taurocholate into the vesicles against a concentration gradient. In contrast, the presence of an inwardly directed KCl gradient gave a fivefold slower initial rate of uptake (5 s) and no overshoot was observed. Uptake in the presence of inwardly directed LiCl or choline Cl gradients (Table III) was similar to that seen in the presence of a KCl gradient. The initial rate of taurocholate uptake under Na^+-equilibrated conditions ($[Na^+]$ inside the vesicles = $[Na^+]$ in the incubation solution) (Fig. 4) was much slower than under Na^+ gradient conditions, and an overshoot was not observed. These data indicate that a Na^+ gradient is specifically required for uphill transport of taurocholate. Furthermore, the specific requirement for sodium indicates that cation diffusion potentials are not significant driving forces for taurocholate uptake.

Uptake of taurocholate as a function of sodium concentration was studied (Fig. 5). The rate of uptake of taurocholate increased with increasing concentrations of Na^+ up to 100 mM, suggesting that the Na^+-dependent transport system was then saturated (Michaelis constant [K_m] for Na^+ = 16±5 mM).

Effect of temperature. The initial rates of taurocholate uptake were significantly faster at 25°, 30°, and 37°C than at either 15° or 4°C. Maximal overshoots were obtained at 25°C. At 4°C, the transport of taurocholate into the vesicles was much slower, and the uptake did not reach equilibrium by 30 min. These data demonstrate temperature dependence of the uptake process.

Specificity of uptake. The specificity of taurocholate uptake was examined by the addition of various unlabeled compounds to the incubation media (Table IV). Each of the three bile salts tested (chenodeoxycholate, cholate, glycocholate) inhibited the initial rate of [^{3}H]taurocholate uptake in the presence of a NaCl gradient but not in the presence of a KCl gradient, indicating an inhibition of only the sodium-dependent portion of uptake. BSP, an organic anion whose uptake is not sodium dependent (27), inhibited both the sodium-dependent and sodium-independent uptake of taurocholate. No significant inhibition was observed with the addition of D-glucose.

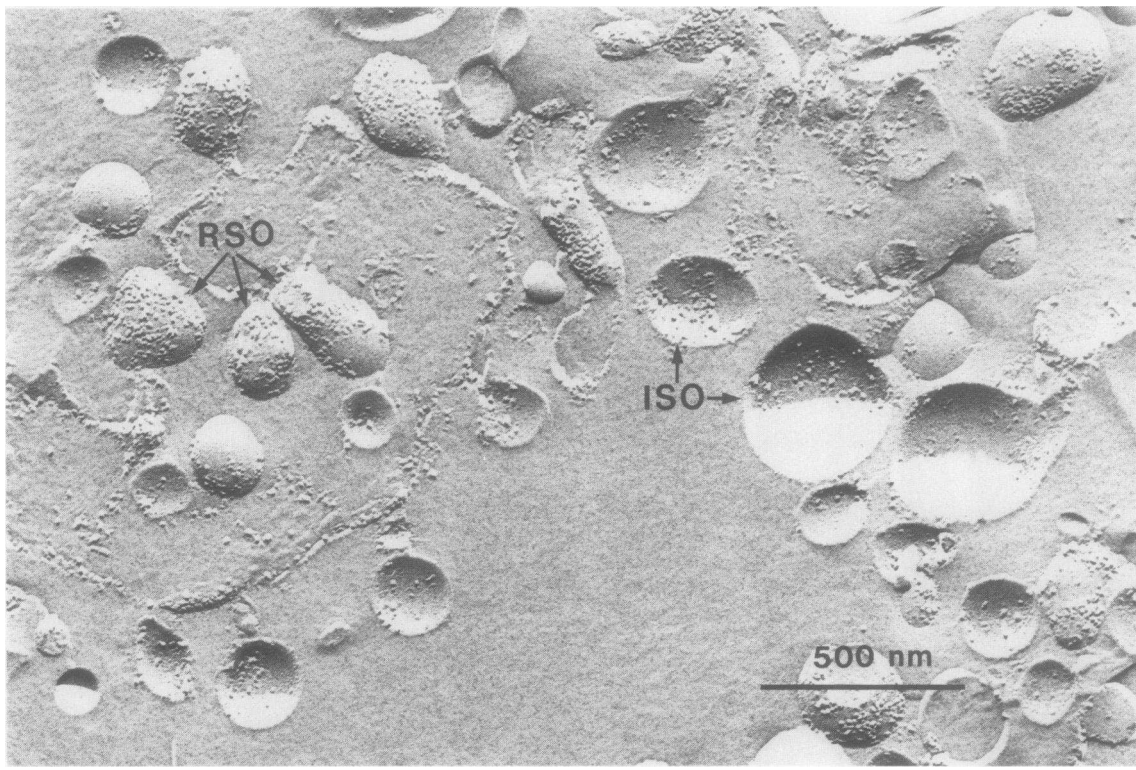

FIGURE 2 Freeze-fracture electron micrograph of liver plasma membrane preparation illustrated in Fig. 1. Right-side-out (*RSO*) and inside-out (*ISO*) vesicles are identified according to particle density and curvature (17, 18). The fracture faces belonging to the inner half of the membrane bilayer (P-face) and outer half of the membrane bilayer (E-face) were found to have high and low particle densities respectively for basolateral and canalicular membrane surfaces in the intact hepatocyte. Convex fracture faces with high particle density and concave fracture faces with low particle density were assumed to be RSO vesicles, and the converse to represent ISO vesicles.

Effect of pH gradients. To determine whether the observed Na^+ dependence of uphill taurocholate transport was due to a double ion exchange mechanism (e.g., Na^+-H^+ exchange and OH^--taurocholate exchange), the effect of pH gradients across the vesicle on taurocholate uptake under Na^+-equilibrated conditions was examined. Imposition of an inwardly directed proton gradient (Fig. 6) across the vesicle (pH 6.0_o [outside] < pH 7.5_i [inside]) did not enhance the initial rate of taurocholate uptake and failed to produce an overshoot. Increasing the gradient (pH 4_o vs. pH 7.5_i) also failed to increase taurocholate uptake (data not shown). These studies indicate that a pH gradient cannot drive taurocholate uphill.

TABLE I
Orientation of Plasma Membrane Vesicles

Orientation	No. of vesicles	Percentage
Right-side out	693	74
Inside out	176	19
Indeterminant	71	7
Totals	940	100

Vesicles from two membrane preparations were examined by freeze-fracture electron microscopy. Orientation was determined by analysis of the particle density of the fracture faces from six replicas (45 photomicrographs).

Effect of electrical potential. The influence of induced electrical potentials on taurocholate uptake was examined in two experimental approaches. First, Na^+-coupled taurocholate uptake was determined under conditions in which the intravesicular charge was altered by substitution of accompanying anions covering a range of lipid permeabilities. More permeable anions should produce more negative intravesicular potentials, which would be expected to affect rheogenic transport processes (28). The initial rate of taurocholate uptake (Fig. 7) was highest when chloride, which

TABLE II

Enzyme-specific Activities of Plasma Membrane and Subcellular Organelle Markers

	Plasma membrane markers		
	Mg^{2+}-activated ATPase	$(Na^+ + K^+)$activated ATPase	Alkaline phosphatase
	μmol Pi/mg·h	*μmol Pi/mg·h*	*μmol PNP/mg·h*
Homogenate	2.22±0.38 (5)	0.79±0.34 (5)	0.23±0.03 (4)
Plasma membrane	24.24±3.49 (5)	14.44±4.74 (5)	3.48±1.25 (4)
Enrichment ratio	10.92	18.28	15.24

	Subcellular organelle markers	
	NADPH cytochrome c reductase	NADH cytochrome c reductase
	μmol/mg·min	*μmol/mg·min*
Homogenate	0.031±0.004 (6)	0.197±0.028 (5)
Plasma membrane	0.014±0.002 (6)	0.196±0.040 (5)
Enrichment ratio	0.45	0.99

The data are expressed as mean±SD. The numbers in parentheses are the numbers of membrane preparations assayed. PNP, paranitrophenol.

has an intermediate lipid permeability, was used. Significantly slower initial rates of uptake (5 s) were observed in the presence of more permeant anions (thiocyanate and nitrate) as well as the less permeant anions (sulfate and gluconate). However, both chloride and the relatively impermeant anion gluconate sustained a sodium-dependent overshoot. Thiocyanate, the most permeant anion, resulted in the slowest uptake. Thus, there was no correlation between the uptake of tau-

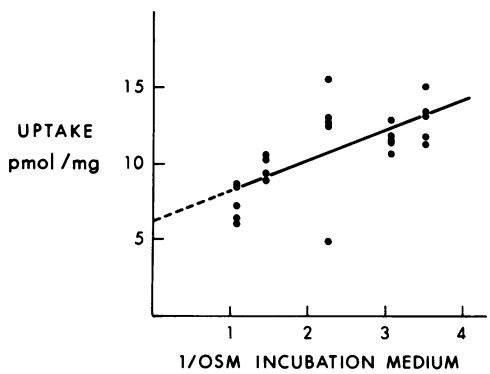

FIGURE 3 Effect of medium osmolarity (*OSM*) of taurocholate uptake at equilibrium. Vesicles were loaded with 300 mM sucrose, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, and 0.2 mM $CaCl_2$. The final extravesicular medium contained 100 mM NaCl, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, 0.2 mM $CaCl_2$, and 1 μM [^{3}H]taurocholic acid, with sucrose added to give the indicated osmolarities. The uptake of taurocholate was determined after incubation at 25°C for 30 min. The individual uptakes±SD from one experiment are shown, with the regression line calculated by least squares analysis. The dotted line represents extrapolation to theoretical zero intravesicular volume $r = 0.67$ and $P < 0.001$.

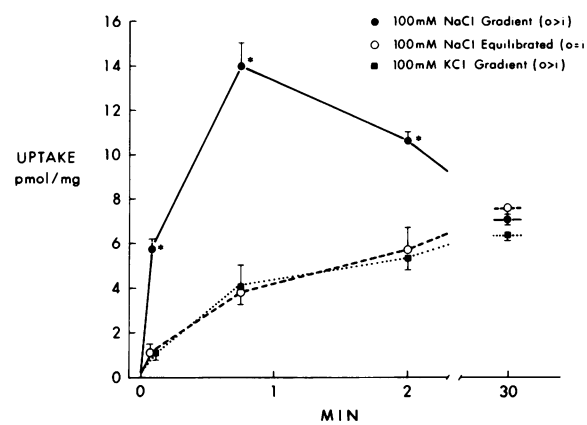

FIGURE 4 Effect of cation gradients on 1 μM taurocholate uptake. Vesicles were loaded with 300 mM sucrose, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, and 0.2 mM $CaCl_2$. The final extravesicular medium contained 1 μM [^{3}H]taurocholic acid, 100 mM sucrose, 10 mM $MgSO_4$, 0.2 mM $CaCl_2$, and either 100 mM NaCl or 100 mM KCl. In the Na^+-equilibrated experiment, the vesicles were loaded with 100 mM NaCl in the buffered sucrose solution and incubated in the same medium containing 1 μM [^{3}H]taurocholic acid. Each point represents the mean±SD for three determinations from a single membrane preparation. Prior experiments in which multiple initial time points were assessed established that the maximal uptake in the presence of a NaCl gradient occurred at 45 s. *, $P < 0.001$.

TABLE III
Effect of Cation Gradients on 1 μM Taurocholate Uptake

Gradient (100 mM) (outside > inside)	Incubation time		
	5 s	45 s	30 min
	pmol/mg membrane protein		
NaCl	5.4±1.5	13.0±2.9	7.7±0.4
KCl	2.1±0.6	6.1±1.7	8.7±0.7
LiCl	1.5±0.4	5.5±1.3	7.0±0.2
Choline Cl	2.5±0.5	5.0±1.7	7.4±0.3

Values given are the mean±SD for three determinations.

rocholate and the relative lipid permeabilities of the accompanying anions, findings most consistent with an electroneutral transport process.

To generate intravesicular potentials more directly, valinomycin-induced K^+ diffusion potentials were used. The vesicles were loaded with 50 mM K^+ gluconate and a negative interior charge was created by exposing the vesicles to valinomycin, an ionophore that increases membrane conductance for K^+ ions. Valinomycin (1 μg/ml) enhanced the uptake of L-alanine (Fig. 8), a neutral amino acid previously shown to be

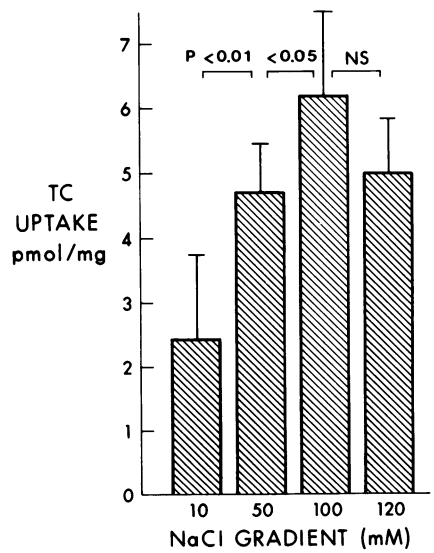

FIGURE 5 Effect of sodium gradient on initial rate of taurocholate uptake. Vesicles loaded with 300 mM sucrose, 10 mM Hepes/KOH (pH 7.5), 0.2 mM $CaCl_2$, and 10 mM $MgSO_4$ were incubated for 5 s in an extravesicular medium containing 1 μM [^{3}H]taurocholic acid, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, and 0.2 mM $CaCl_2$ with the concentrations of NaCl shown, with sucrose added to maintain the same osmolarity. Each point is the mean±SD for five determinations from a single membrane preparation.

TABLE IV
Inhibition of Taurocholate Uptake

Gradient	pmol/mg (±SD)	Percentage of control
100 mM NaCl		
Control	12.5±1.3	100
100 μM Glyocholate	7.3±1.0	58
100 μM Chenodeoxycholate	3.4±1.0	27
100 μM Cholate	8.1±1.1	65
100 μM BSP	1.1±0.5	9
100 μM D-glucose	11.2±0.8	90
100 mM KCl		
Control	1.6±0.6	100
100 μM Glyochoclate	1.6±0.4	100
100 μM Chenodeoxycholate	1.9±0.7	119
100 μM Cholate	1.9±0.5	119
100 μM BSP	0.7±0.3	44
100 μM D-glucose	2.0±0.5	125

The uptake of 1 μM taurocholate (at 10 s) was assessed in the presence of a 100-μM NaCl gradient or 100-mM KCl gradient with and without the addition of unlabeled inhibitors to the incubation media. The final concentration of each inhibitor was 100 μM. The buffers were the same as for Fig. 4; n = 5 determinations.

transported into liver plasma membrane vesicles by an electrogenic process (enhanced by negative intravesicular potentials) (12). In contrast, valinomycin had

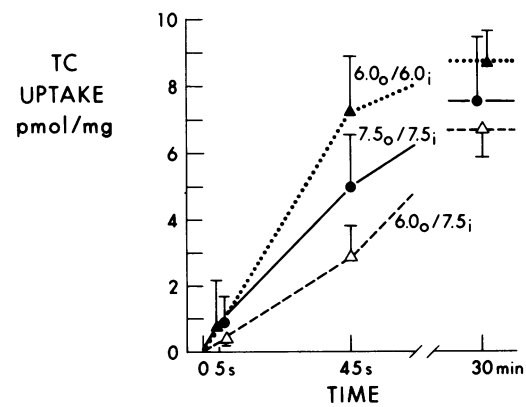

FIGURE 6 Effect of pH gradients on taurocholate uptake in the absence of a sodium gradient. Vesicles were loaded with 100 mM NaCl, 50 mM sucrose, 10 mM $MgSO_4$, 0.2 mM $CaCl_2$ and 50 mM Hepes/KOH (pH 7.5 or 6.0) and incubated in an extravesicular medium containing 1 μM [^{3}H]taurocholic acid in the same buffered salt solution. The pH of the membrane suspensions and incubation solutions are shown. The final extravesicular incubation medium pH for the ($6.0_o/7.5_i$) experiment was 6.6. Each point represents the mean±SD for three determinations from one experiment. No significant differences in uptake rates were observed for pH $6.0_o/7.5_i$ or pH $6.0_o/6.0_i$ compared with control (pH $7.5_o/7.5_i$).

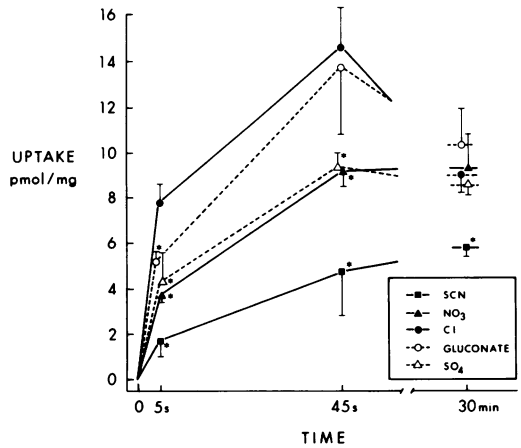

FIGURE 7 Effect of anion substitution on taurocholate uptake. Vesicles loaded with 300 mM sucrose, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, and 0.2 mM $CaCl_2$ were incubated in an extravesicular medium containing 1 μM [^{3}H]taurocholic acid, 100 mM sucrose, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, 0.2 mM $CaCl_2$, and either 100 mM NaSCN, 100 mM $NaNO_3$, 100 mM NaCl, 100 mM Na gluconate, or 50 mM Na_2SO_4. The means±SD for three determinations from a single experiment are shown. The asterisks indicate significant differences ($P < 0.05$–0.001) from the control (NaCl) value determined by individual t tests.

no effect on [^{3}H]taurocholate uptake (Fig. 8), providing further evidence that sodium-dependent taurocholate transport is an electroneutral process. In addition, creation of a positive intravesicular charge by reversing the 50 mM K^+ gluconate gradient (K^+ outside > K^+ inside) in the presence of valinomycin had no effect on the uptake of taurocholate. When higher concentrations of valinomycin were used (20 μg/ml), uptake of taurocholate was decreased compared with control, regardless of the direction of the K^+ gluconate gradient, consistent with a toxic effect of valinomycin at this concentration. Thus, both the anion substitution and valinomycin data provide evidence that the Na^+-coupled uptake of taurocholate into these vesicles takes place by an electroneutral process.

Kinetics. The kinetics of [^{3}H]taurocholate uptake (1–100 μM) into liver plasma membrane vesicles were studied (Fig. 9). In the presence of an inwardly directed KCl gradient, the initial rate of taurocholate uptake increased linearly with increasing taurocholate concentrations, consistent with passive diffusion of the bile acid into the vesicles. In contrast, uptake of taurocholate in the presence of a NaCl gradient was more rapid and curvilinear. Subtraction of the diffusional component of uptake (KCl gradient) from uptake in the presence of a NaCl gradient yields the Na^+-dependent component, a rectangular hyperbola conforming to Michaelis-Menten saturation kinetics. The derived kinetic parameters consisted of a K_m of 52±15 μM and a maximum velocity of 4.5±0.6 nmol · mg^{-1} · protein · min^{-1} (mean±SEM).

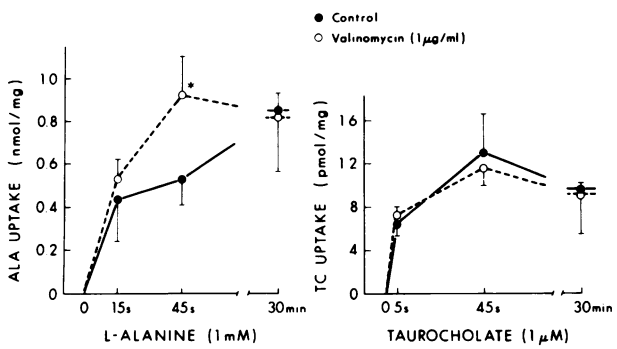

FIGURE 8 Effect of valinomycin-induced potassium diffusion potentials on taurocholate (*TC*) uptake and L-alanine (*ALA*) uptake. Vesicles were loaded with 50 mM K gluconate, 200 mM sucrose, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, and 0.2 mM $CaCl_2$, and incubated in a medium containing either 1 μM [^{3}H]taurocholic acid or 1 mM [^{3}H]L-alanine, 100 mM Na gluconate, 100 mM sucrose, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, and 0.2 mM $CaCl_2$. Valinomycin in ethanol was added to the membranes 30 min before initiation of the uptakes, in a concentration of 1 μg/ml of membrane suspension (0.2% ethanol), with ethanol (0.2%) added to controls. Each point represents the mean±SD for three determinations from a single representative experiment. A total of eight taurocholate and three alanine experiments were performed. The asterisks indicate that $P < 0.02$.

DISCUSSION

According to current concepts of the mechanisms of bile formation (29, 30), the primary driving force for taurocholate uptake into hepatocytes is the sodium gradient, which is generated by the activity of the basolateral enzyme, Na^+,K^+-ATPase (31). The present study provides direct evidence for this hypothesis and establishes that vesicles prepared from a liver membrane preparation highly enriched in the basolateral marker Na^+,K^+-ATPase contain a sodium-coupled transport mechanism for taurocholate. Furthermore, this coupled transport system is capable of uphill transport, as demonstrated by the overshoot phenomenon in the presence of an inwardly directed sodium gradient. No overshoot was observed in the absence of a sodium gradient (Na^+ equilibrated) or when potassium, lithium, or choline gradients were substituted for sodium. Uptake of taurocholate in the presence of a K^+ gradient was linear with increasing concentrations of taurocholate, suggesting a diffusion-dependent process. The finding of overshoots with both NaCl and Na-gluconate gradients is consistent with an active, sodium-coupled co-transport system. The mechanism appears to involve a directly coupled sodium-anion

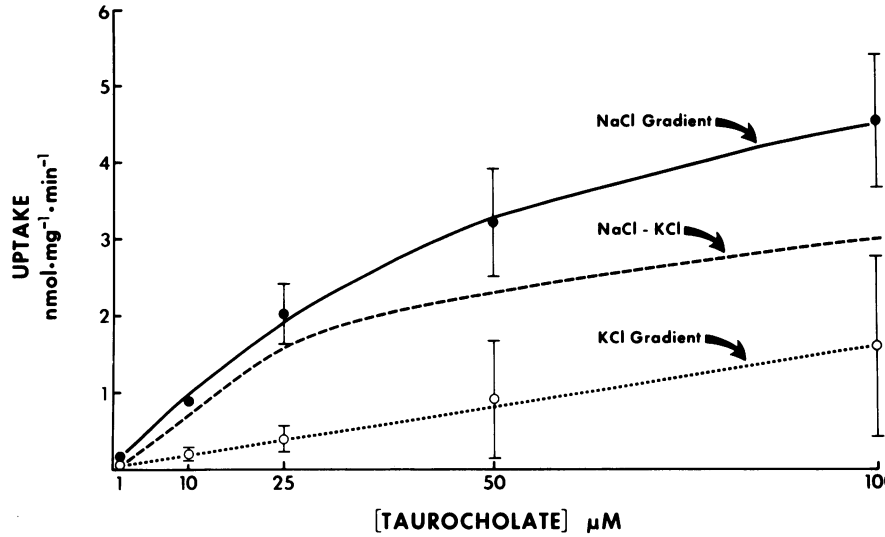

FIGURE 9 Kinetics of taurocholate uptake. Vesicles loaded with 300 mM sucrose, 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, 0.2 mM $CaCl_2$ were incubated for 5 s in an extravesicular medium containing 10 mM Hepes/KOH (pH 7.5), 10 mM $MgSO_4$, 0.2 mM $CaCl_2$, with [^{3}H]taurocholic acid in the concentrations indicated and either 100 mM NaCl or 100 mM KCl. Each point represents the mean±SD for five membrane preparations. The dashed line is the sodium-dependent uptake generated by subtraction of the diffusional component (KCl gradient) from the uptake in the presence of a NaCl gradient. The kinetic parameters were generated from a nonlinear weighted, least-squares fit of the individual sodium dependent uptakes from each experiment to a rectangular hyperbola.

carrier, since uptake is not influenced by induced electrical potentials or by pH gradients. The latter finding rules out a Na^+-H^+ exchange mechanism with secondary bile acid-OH^- exchange (10). Other bile acids inhibited Na^+-dependent taurocholate uptake, whereas BSP inhibited both Na^+-dependent and Na^+-independent uptake and D-glucose had no effect on uptake. These studies demonstrate carrier specificity. The kinetic data demonstrating saturability with respect to the anion (taurocholate) and the cation (Na^+) and the finding of temperature dependence both support a carrier-mediated transport mechanism. These observations are also consistent with recent studies in isolated hepatocytes demonstrating that bumetanide and furosemide, compounds that inhibit sodium-coupled chloride transport in rectal gland tissue (32) and Na^+-coupled K^+ transport in avian erythrocytes (33), inhibit the sodium-dependent uptake of taurocholate (9). Altogether, these data provide compelling evidence in favor of the current model of bile formation in which Na^+,K^+-ATPase maintains a sodium gradient across the basolateral membrane and thereby provides the driving force for Na^+-coupled uptake of bile acids by the hepatocyte.

Ruifrok and Meijer (34) and Inoue et al. (35) have also demonstrated a sodium-dependent transport system for taurocholate uptake into vesicles prepared from rat liver plasma membranes. The K_m for taurocholate observed in our study of 52 μM is similar to the 56 μM reported by Inoue et al. (35). Ruifrok and Meijer (34) reported a K_m of 250 μM, but they utilized supraphysiological concentrations of taurocholate in their kinetics experiment, which may account for their higher K_m. The K_m for taurocholate in all of these vesicle studies is somewhat higher than the 19 μM reported by Schwarz et al. (8), the 21 μM observed in our own laboratory (9) in isolated hepatocyte suspensions, and the 27 μM reported by Scharschmidt and Stephens (36), using hepatocyte monolayers.

Our findings of an electroneutral transport mechanism differ from those of Ruifrok and Meijer (34) and Inoue et al. (35), who found a relationship between anion permeability and taurocholate uptake in their preparations, suggesting electrogenic transport. However, Ruifrok and Meijer sampled later time points rather than initial rates in their anion substitution experiments. More importantly, in neither of these previous studies was direct assessment of the effect of alterations in transmembrane potential by ionophores, such as valinomycin on taurocholate uptake presented. The anion substitution and valinomycin data in the present study provide strong evidence for an electroneutral transport process, suggesting that the carrier co-transports sodium and taurocholate in a 1:1 ratio.

This observation is in agreement with the findings of Scharschmidt and Stephens (36) in cultured rat hepatocytes where ^{22}Na and taurocholate were transported into hepatocytes in an equimolar ratio. The uptake of taurocholate into renal vesicles also has been shown to be electroneutral (37). The effect of electrical potential on taurocholate transport in the ileum is also controversial. Rouse and Lack (38) reported electroneutral transport in guinea pig ileal membrane vesicles based on anion substitution data, whereas Lucke et al. (39) demonstrated electrogenic transport in rat ileal vesicles using anion substitution and valinomycin-induced diffusion potentials.

Although our anion substitution data clearly show no correlation between lipid permeability and uptake of taurocholate, the reason for the observed differences in uptake rates between the different anions remains to be explained. It is possible that the decrease in uptake seen with highly permeable anions such as thiocyanate is due either to co-transport of the anion with sodium, hereby producing a rapid dissipation of the sodium gradient, or to an interaction between the anions and the carrier or the membrane itself. No specific requirement exists for chloride however, as overshoots were also obtained with the relatively impermeant anion gluconate.

The carrier demonstrated specificity in that all of the bile acids tested inhibited taurocholate uptake, whereas D-glucose, a substance found not to be actively transported in the liver in vivo (40), did not. Bile acids inhibited only the sodium-dependent portion of taurocholate uptake, whereas BSP inhibited both the sodium-dependent and sodium-independent uptake, suggesting different mechanisms of inhibition for bile acids and BSP. Although many studies have suggested separate carrier mechanisms for uptake of bile salts and BSP (27, 41), some studies question the concept of totally separate carriers. Taurocholate has been demonstrated to decrease hepatic uptake and storage of BSP in the intact dog (42) and competitively to inhibit BSP uptake in isolated hepatocytes (43), suggesting the possibility of a shared carrier for BSP and taurocholate uptake. In addition, Ware et al. (44) have demonstrated a direct physicochemical interaction between BSP (particularly unconjugated BSP) and taurocholate. Our data demonstrating inhibition of taurocholate uptake of BSP do not allow any conclusions to be drawn regarding the mechanism or mechanisms of the inhibition.

It is uncertain why taurocholate uptake was not enhanced when sodium ions were equilibrated across the vesicles compared with potassium-equilibrated conditions. Although an overshoot would not be expected in these ion-equilibrated experiments, most other sodium-coupled anion carriers demonstrate an increase in the initial rate of uptake in the presence of sodium reflecting a more rapid entry of the anion via the coupled carrier mechanism. It is possible that the high degree of binding of taurocholate ($\geq$50%) may have obscured small differences in initial rates of uptake under such conditions. Ruifrok and Meijer (34) using rat liver plasma membrane vesicles and Wilson et al. (37) using renal brush border membrane vesicles also failed to demonstrate enhancement of taurocholate uptake under Na^+-equilibrated conditions.

Few transport studies have been performed using liver plasma membrane vesicles. The most definitive to date are the demonstrations by Van Amelsvoort et al. (11–13) that rat liver sinusoidal membranes transport L-alanine by a sodium-coupled electrogenic process, a finding we have confirmed in the present study. In their later studies (13), Na^+-dependent alanine transport appeared to correlate best with fractions enriched in Na^+,K^+-ATPase, and less well with liver plasma membranes enriched in canalicular membrane markers. Our own membrane preparation obtained from a low speed nuclear pellet by a modification of the Song et al. (15) procedure is probably more heterogeneous, as it is enriched in both sinusoidal and canalicular membrane markers. The method has the advantage of rapid preparation (4–5 h), so that loss of membrane transport processes is minimized. This membrane population contains fragments from mitochondrial and microsomal membranes, although it is only enriched in plasma membranes.

Electron microscopy of this preparation clearly demonstrates a heterogeneous population of vesicles, lateral cell membranes, and some intact canaliculi. Freeze-fracture photomicrographs demonstrated that most of the vesicles maintain the same orientation as that of the intact cell. Freeze fracture techniques have been used previously (18) to determine the orientation of brush border vesicles prepared from kidney cortex and small intestine. Recently Sips et al. (26) have reported similar studies for liver plasma membrane vesicles, indicating that the majority of vesicles are oriented right-side out. These findings are similar to our own and are in accord with a previous report by Higgins and Evans (45), who found that >90% of rat liver plasma membrane vesicles prepared by a different technique are oriented right-side out, using an antiserum to the ectoenzyme 5′-nucleotidase.

Finally, preliminary studies from this laboratory using more highly purified sinusoidal and canalicular membrane vesicles confirm the present findings and support the concept that electroneutral sodium-coupled taurocholate uptake is occurring across the sinusoidal membrane (Meijer, P. J., and J. L. Boyer, unpublished observations). In addition, the orientation of the sodium gradient across the sinusoidal membrane

corresponds to the direction of taurocholate transport into the cell, and, as already indicated, the kinetics of this process (K_m) are similar to findings in isolated cell suspensions and cultured hepatocytes. On the basis of morphometric studies (46), the total basolateral surface area in the starting homogenate would be expected to be approximately seven times that of the canalicular membrane. Since our preparation is more enriched in basolateral markers (20-fold over homogenate) than in canalicular markers (10–15-fold over homogenate), the bulk of the vesicles are probably derived from the basolateral membrane domain.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Ms. Oi Cheng Ng for preparing the electron micrographs, Dr. Peter Aronson for many helpful discussions regarding vesicle transport, Dr. Gary Rudnick for advice regarding analysis of the transport kinetics, Mrs. Rose Allen for her advice concerning the marker enzyme assays, and Dr. James Wade for help with freeze-fracture analysis.

This work was supported by U. S. Public Health Service research grant R01 AM 25036.

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