

Ursodeoxycholate Stimulates $\text{Na}^+\text{-H}^+$ Exchange in Rat Liver Basolateral Plasma Membrane Vesicles

Richard H. Moseley, Nazzareno Ballatori, Daniel J. Smith, and James L. Boyer

Department of Medicine, University of Michigan School of Medicine, Ann Arbor, Michigan, 48105; and Liver Center, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract

$\text{Na}^+\text{:H}^+$ and $\text{Cl}^-\text{:HCO}_3^-$ exchange are localized, respectively, to basolateral (bLPM) and canalicular (cLPM) rat liver plasma membranes. To determine whether these exchangers play a role in bile formation, we examined the effect of a choleretic agent, ursodeoxycholate (UDCA), on these exchange mechanisms. ^{22}Na (1 mM) and ^{36}Cl (5 mM) uptake was determined using outwardly directed H^+ and HCO_3^- gradients, respectively. Preincubation of bLPM vesicles with UDCA (0–500 μM) resulted in a concentration-dependent increase in initial rates of amiloride-sensitive pH-driven Na^+ uptake, with a maximal effect at 200 μM . UDCA (200 μM) increased V_{max} from 23 ± 2 (control) to 37 ± 7 nmol/min per mg protein; apparent K_m for Na^+ was unchanged. Preincubation with tauroursodeoxycholate (200 μM), taurocholate (10–200 μM) or cholate, chenodeoxycholate, or deoxycholate (200 μM) had no effect on pH-driven Na^+ uptake. UDCA (200 μM) had no effect on either membrane lipid fluidity, assessed by steady-state fluorescence polarization using the probes 1,6-diphenyl-1,3,5-hexatriene, 12-(9-anthroyloxy) stearic acid, and 2-(9-anthroyloxy) stearic acid (2-AS), or $\text{Na}^+\text{,K}^+\text{-ATPase}$ activity in bLPM vesicles. In cLPM vesicles, UDCA (0–500 μM) had no stimulatory effect on initial rates of HCO_3^- -driven Cl^- uptake. Enhanced basolateral $\text{Na}^+\text{:H}^+$ exchange activity, leading to intracellular HCO_3^- concentrations above equilibrium, may account for the bicarbonate-rich choleresis after UDCA infusion.

Introduction

Ursodeoxycholate (UDCA),¹ a $3\alpha,7\beta$ dihydroxy bile acid that is increasingly used to dissolve cholesterol gallstones in man, induces a marked choleretic response in rats that is associated with a selective increase in canalicular bicarbonate secretion

A preliminary report of this work was presented at the 36th meeting of the American Association for the Study of Liver Diseases, Chicago, IL, 1985, and appeared in abstract form (1985. *Hepatology* [Baltimore]. 5:1017).

Address correspondence to Richard H. Moseley, M.D., Gastroenterology Division, Department of Medicine, Veterans Administration Medical Center, 2215 Fuller Road, Ann Arbor, MI 48105.

Received for publication 13 March 1986 and in revised form 2 April 1987.

1. *Abbreviations used in this paper:* 2-AS, 2-(9-anthroyloxy) stearic acid; 12-AS, 12-(9-anthroyloxy) stearic acid; bLPM, basolateral liver plasma membrane vesicle; cLPM, canalicular liver plasma membrane vesicle; DPH, 1,6-diphenyl-1,3,5-hexatriene; MES, 2-(N-morpholino)-ethanesulfonic acid; TC, taurocholate; TUDCA, tauroursodeoxycholate; UDCA, ursodeoxycholate.

(1, 2). This property, as well as the observation that bicarbonate is required for the maintenance of bile acid-independent bile formation in the isolated perfused rat liver model (3–5), suggests that bicarbonate transport may be important in the generation of hepatic bile flow. Preliminary observations in the fluorocarbon-perfused rat liver also indicate that UDCA-stimulated choleresis is dependent on sodium and can be inhibited by amiloride, a competitive inhibitor of $\text{Na}^+\text{:H}^+$ exchange, and analogues of amiloride (6, 7). In addition, we have recently identified and characterized basolateral $\text{Na}^+\text{:H}^+$ and canalicular $\text{Cl}^-\text{:HCO}_3^-$ exchange on these respective domains of the hepatocyte plasma membrane (8, 9). Altogether, this evidence, although circumstantial, is consistent with a role for $\text{Na}^+\text{:H}^+$ and $\text{Cl}^-\text{:HCO}_3^-$ exchange in bile formation, as discussed in recent reviews (10, 11). It is therefore possible that bile acids such as UDCA might exert their actions via direct effects on these ion exchange mechanisms. To further explore the role of membrane transport processes in this critical hepatocellular function, we examined the effects of UDCA on $\text{Na}^+\text{:H}^+$ and $\text{Cl}^-\text{:HCO}_3^-$ exchange activities in purified rat liver basolateral (bLPM) and canalicular (cLPM) plasma membrane vesicles. The effects on $\text{Na}^+\text{:H}^+$ exchange activity of the structurally related taurine conjugate, tauroursodeoxycholate (TUDCA), as well as the bile acids, taurocholate (TC), cholate, deoxycholate, and chenodeoxycholate were also examined.

Methods

Materials. ^{22}Na was obtained from Amersham Corp., Arlington Heights, IL. ^{36}Cl was obtained as a 0.2–3.0 M HCl solution from New England Nuclear, Boston, MA, and was neutralized with tetramethylammonium hydroxide to a pH of 7.5 before use. Amiloride was a gift from Merck, Sharp and Dohme Research Laboratories (West Point, PA). UDCA and TUDCA were generously provided by Dr. Kenichi Kitani and were obtained from Tokyo Tanabe Co., Tokyo, Japan. Concentrated stock solutions of UDCA and TUDCA were prepared in propylene glycol, and equivalent volumes of propylene glycol were added to all control incubations. All other chemicals and reagents were obtained from Sigma Chemical Co., St. Louis, MO. All water used in preparing media was deionized and filtered through 0.45- μm Millipore filters. Valinomycin was stored in 95% ethanol.

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Kingston, NY) weighing 200–250 g were used for all studies. The animals had free access to water and Purina rodent chow and were housed in a constant temperature-humidity environment with alternating 12-h light and dark cycles. Fed animals were routinely sacrificed by decapitation between 7:30 and 8:30 AM.

Isolation of cLPM and bLPM. The method for isolation of the cLPM and bLPM as well as their biochemical and morphologic characterization have been described in detail (8, 12). A virtually complete separation of bLPM and cLPM by this method is reflected by the absence in cLPM of $\text{Na}^+\text{,K}^+\text{-ATPase}$, glucagon-stimulatable adenylate cyclase, and intact secretory component (12). By freeze fracture analysis, ~80% of cLPM and 73% of bLPM vesicles exhibit right-side-out configuration (8, 12).

Vesicles were preloaded with desired media by resuspending freshly isolated membrane vesicles by tight homogenization (10 up-and-down strokes, type B Dounce homogenizer [Kontes Glass Co., Vineland, NJ]) in membrane resuspension media as defined in the figure legends. Vesicles were then frozen and stored in liquid nitrogen (protein concentration > 5 mg/ml) before use.

Before transport studies, the frozen vesicle suspensions were quickly thawed in a 37°C water bath, diluted to the desired protein concentration, and passed repeatedly (15×) through a 25-gauge needle.

²²Na and ³⁶Cl uptake determination. For measurements of ²²Na and ³⁶Cl uptake, 20 μl (100–130 μg protein) of the membrane vesicle suspension was added at 25°C to 80 μl reaction mixture containing the radiolabeled material plus the appropriate medium. Uptake was terminated at specific time intervals by the addition of 3 ml of ice-cold stop solution consisting of 204 mM sucrose, 150 mM K gluconate, 0.2 mM Ca gluconate, 5 mM Mg gluconate, and 10 mM Hepes/Tris, pH 7.5. Membrane vesicle-associated ligand was separated from free ligand by immediate and rapid filtration through a 0.45-μm Millipore filter (HAWP). The filter was washed twice with 3 ml stop solution, dissolved in Redisolv HP (Beckman Instruments, Inc., Palo Alto, CA) and counted in a Beckman LS 2000 liquid scintillation counter. Non-specific binding of isotope to filter and membrane vesicles was determined in each experiment by addition, at 0–4°C, of reaction mixture and stop solution to 20 μl membrane suspension. This membrane/filter blank was subtracted from all determinations. Because of the presence of an electrogenic component to Na⁺ uptake in bLLPM vesicles (8, 13), all studies were performed under voltage-clamp conditions, in which the contribution of the membrane potential to the observed pH gradient-dependent Na⁺ uptake is minimized by the addition of the K⁺ ionophore, valinomycin (5 μg/mg protein) in the presence of 100 mM K⁺ on both sides of the vesicle membrane. Under these conditions, amiloride-sensitive pH-driven Na⁺ uptake (attributable to Na⁺:H⁺ exchange) is preferentially studied. To assess the effect of various bile acids on initial rates of pH-stimulated Na⁺ uptake, uptake was determined at 15 s. For experiments requiring preincubation of bile acids with membrane vesicles, concentrated solutions of bile acids in propylene glycol were used and equivalent amounts of propylene glycol served as control. Preliminary studies utilizing the pH-sensitive dye, acridine orange, demonstrated no effect of bile acids or propylene glycol on the rate of dissipation of the imposed pH gradient in bLLPM vesicles.

Unless otherwise indicated, all experimental data were obtained from triplicate analysis of three or more separate membrane preparations. All values are expressed as mean±SE. The data were compared by Student's *t* test; differences were considered to be statistically significant when *P* < 0.05.

Na⁺, K⁺-ATPase assays. LPM preparations were assayed for Na⁺, K⁺-ATPase activity using a previously described coupled enzyme assay in which the production of ADP by the ATPase reaction is linked to NADH oxidation using pyruvate kinase and lactic dehydrogenase as coupling enzymes (14).

Fluorescence polarization measurements. Fluorescence polarization was studied with a SLM 8000 photon-counting spectrofluorometer (SLM Instruments, Inc., Urbana, IL) using 1,6-diphenyl-1,3,5-hexatriene (DPH; Eastman Kodak Co., Rochester, NY) and 12-(9-anthroyloxy) stearic acid (12-AS; Molecular Probes, Inc., Junction City, OR) as probes for the hydrophobic core of the membrane (15), and 2-(9-anthroyloxy) stearic acid (2-AS; Molecular Probes, Inc.) as a probe for the surface of the membrane. For insertion into the membrane, an aliquot of probe was first dried on the inner surface of a test tube, an aliquot of membrane suspension added, and the tube vortexed. Measurements were performed as described by Lakowicz (16), and anisotropy (*r*) calculated using Perrin's equation: $r = (I_{||} - I_{\perp}) / (I_{||} + 2I_{\perp})$. All membrane suspension samples were prepared in triplicate and three readings taken on each sample at 1-min intervals. LPM suspensions containing no probe (blanks) were similarly prepared to check light scattering. Raman light scattering accounted for < 5% of

the intensity of the fluorescence signal in the presence of probe and therefore was discounted in these experiments.

Results

The effect of UDCA on pH-driven Na⁺ transport was first studied under conditions in which the bile acid was present only in the extravesicular medium. As shown in Table I, no effect on Na⁺ uptake was observed under these conditions. However, when membrane suspensions were preincubated with UDCA at 25°C for 10 min, a concentration-dependent increase in pH-driven Na⁺ uptake was observed with a maximal effect at 200 μM UDCA (Fig. 1). In subsequent experiments (data not shown), this requirement for preincubation was confirmed with no stimulation observed after 1 min preincubation, but enhancement of pH-driven Na⁺ uptake at 5 min was similar to that shown at 10 min. Preincubation of membrane vesicles with UDCA had no effect on the degree of uptake at equilibrium.

To determine if UDCA was selective in increasing Na⁺:H⁺ exchange activity, we next examined the effects of the structurally related taurine conjugate of UDCA, TUDCA, and TC on pH-driven Na⁺ uptake in bLLPM. As shown in Fig. 2, TUDCA and TC did not significantly increase pH-driven Na⁺ uptake above control levels, as compared with UDCA. Additionally, the similar inhibition by amiloride, a competitive inhibitor of Na⁺:H⁺ exchange (17), of pH-driven Na⁺ uptake, regardless of the bile acid studied, is consistent with a selective enhancement of Na⁺:H⁺ exchange by UDCA. The selective nature of the observed enhancement of Na⁺:H⁺ exchange activity by UDCA is suggested even further by studies with other bile acids. As shown in Table II, neither cholate, deoxycholate, nor chenodeoxycholate significantly increased pH-driven Na⁺ uptake above control levels, as compared with UDCA.

The component of Na⁺ flux in bLLPM vesicles that is observed under voltage clamp conditions has been previously attributed to amiloride-sensitive Na⁺:H⁺ exchange (8). Never-

Table I. Lack of Effect of UDCA on pH-driven Na⁺ Uptake in bLLPM Vesicles without Preincubation

15-s uptake values	
pmol/mg protein	
Control	232±48
25 μM UDCA	243±50
50 μM UDCA	204±59
100 μM UDCA	217±31
200 μM UDCA	233±49

Vesicles were preloaded with a pH 5.9 buffer (sucrose, 82 mM; 2-(*N*-morpholino)-ethanesulfonic acid (MES), 91 mM; Tris, 29 mM; Hepes, 14 mM; K gluconate, 100 mM; and Ca gluconate, 0.2 mM) and treated with valinomycin (5 μg/mg protein). Initial rates of sodium uptake (1 mM) were determined at 25°C by diluting vesicles into reaction medium of pH 7.9 (sucrose, 70 mM; Hepes, 76 mM; Tris, 70 mM; K gluconate, 100 mM; Ca gluconate, 0.2 mM) containing ²²Na with and without varying concentrations of UDCA. Data represent the mean±SE of triplicate analysis of three separate membrane preparations. UDCA had no effect on Na⁺:H⁺ exchange under these conditions (*P* = NS).

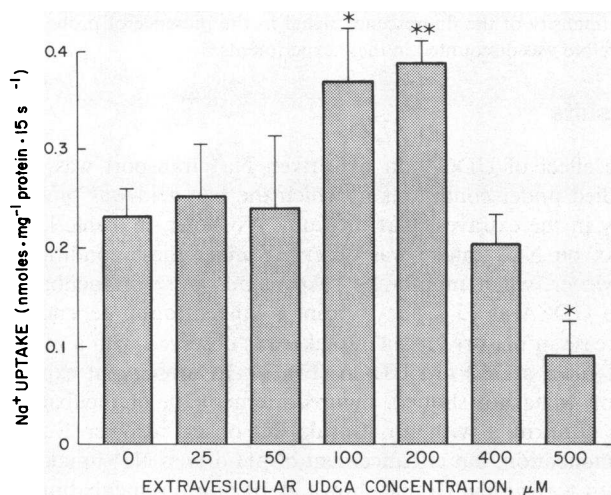


Figure 1. Concentration-dependent effect of UDCA on pH-driven Na^+ uptake in bLPM vesicles with preincubation. Vesicles, preloaded with pH 5.9 buffer (sucrose, 82 mM; MES, 91 mM; Tris, 29 mM; Hepes, 14 mM; K gluconate, 100 mM; Ca gluconate, 0.2 mM) and treated with valinomycin (5 $\mu\text{g}/\text{mg}$ protein) were preincubated with and without varying concentrations of UDCA for 10 min at 25°C. 15-s uptakes of Na^+ (1 mM) were then determined at 25°C by diluting vesicles fivefold into reaction medium of pH 7.9 containing ^{22}Na (sucrose, 70 mM; Hepes, 76 mM; Tris, 70 mM; K gluconate, 100 mM; Ca gluconate, 0.2 mM) and appropriate concentrations of UDCA. Data represents the mean \pm SE of triplicate analysis from at least three separate membrane preparations (except data at 500 μM UDCA, for which two separate membrane preparations were studied). UDCA (100 and 200 μM) significantly increased (* $P < 0.05$ and ** $P < 0.001$, respectively), whereas 500 μM significantly decreased (* $P < 0.05$) pH-driven Na^+ uptake.

theless, to study whether UDCA alters Na^+ permeability in bLPM vesicles, Na^+ flux was measured under pH-equilibrated conditions in the presence and absence of UDCA. As illustrated in Fig. 3, at a concentration and under preincubation conditions that result in maximal enhancement of pH-driven Na^+ uptake, UDCA had no effect on Na^+ flux.

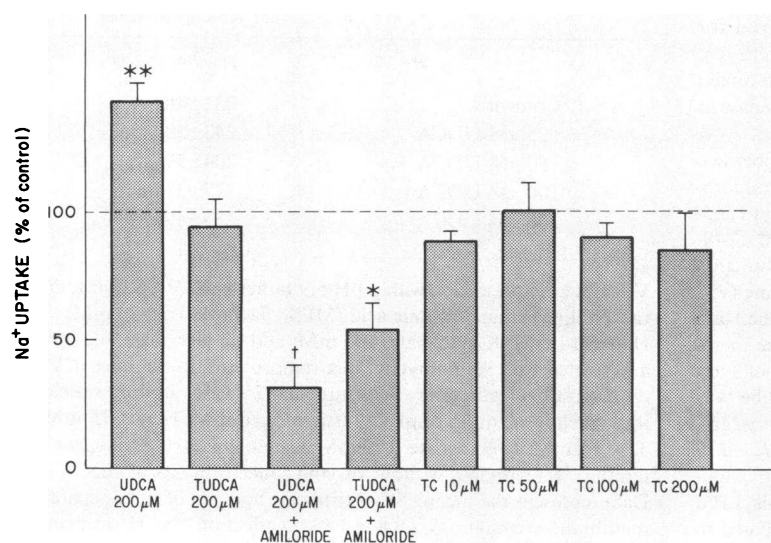


Table II. Effect of Bile Acids on pH-driven Na^+ Uptake in bLPM Vesicles

	15-s uptake value pmol/mg protein
Control	266 \pm 36
200 μM UDCA	353 \pm 26*
200 μM deoxycholate	316 \pm 22
200 μM chenodeoxycholate	295 \pm 19
200 μM cholate	276 \pm 40

Vesicles, preloaded with a pH 5.9 buffer (sucrose, 82 mM; MES, 91 mM; Tris, 29 mM; Hepes, 14 mM; K gluconate, 100 mM; Ca gluconate, 0.2 mM) and treated with valinomycin (5 $\mu\text{g}/\text{mg}$ protein) were preincubated with designated concentrations of bile acids for 10 min at 25°C. 15-s uptakes of Na^+ (1 mM) were then determined, at 25°C, by diluting vesicles fivefold into reaction medium of pH 7.9 containing ^{22}Na (sucrose, 70 mM; Hepes, 76 mM; Tris, 70 mM; K gluconate, 100 mM; Ca gluconate, 0.2 mM) and equivalent concentrations of bile acids. Data represents the mean \pm SE of triplicate analysis from at least three separate membrane preparations (range, 3–5 membrane preparations). Only UDCA resulted in a significant increase in pH-driven Na^+ uptake above control.

* $P < 0.05$.

In contrast to the observed enhancement of $\text{Na}^+:\text{H}^+$ exchange in bLPM vesicles, under the same optimal preincubation conditions, UDCA did not have any stimulatory effect on $\text{Cl}^-:\text{HCO}_3^-$ exchange in cLPM vesicles. As shown in Fig. 4, preincubation of membrane vesicles with either 200 μM UDCA or 200 μM TUDCA did not increase HCO_3^- gradient-driven Cl^- uptake above control values. In fact, a small degree of *cis*-inhibition of HCO_3^- gradient-driven Cl^- uptake resulted from preincubation with either bile acid. Similar results were obtained at both lower (50, 100 μM) and higher (400, 500 μM) concentrations of UDCA, at various preincubation times as well as in the absence of any preincubation (Table III).

Recent findings in rat colonic brush border membrane vesicles (18) suggest that membrane lipid fluidity may exert a

Figure 2. Effect of UDCA, TUDCA, and TC on pH-driven Na^+ uptake in bLPM vesicles. Vesicles, preloaded with pH 5.9 buffer, were treated with valinomycin (5 $\mu\text{g}/\text{mg}$) and then incubated in varying concentrations of bile acids for 10 min at 25°C. 15-s uptakes of Na^+ (1 mM) were determined at 25°C by diluting vesicles into reaction medium of pH 7.9. Amiloride (5 mM) was present in the designated experiments. Results are expressed as percentage of control uptake, and each bar represents the mean \pm SE of triplicate analysis from at least three separate membrane preparations (range 3–11 membrane preparations). Whereas UDCA significantly enhanced pH-driven Na^+ uptake (** $P < 0.001$), neither TUDCA ($P = \text{NS}$) nor TC (10–200 μM ; $P = \text{NS}$) stimulated $\text{Na}^+:\text{H}^+$ exchange. Amiloride inhibits $\text{Na}^+:\text{H}^+$ exchange despite the presence of UDCA († $P < 0.005$) or TUDCA (* $P < 0.05$).

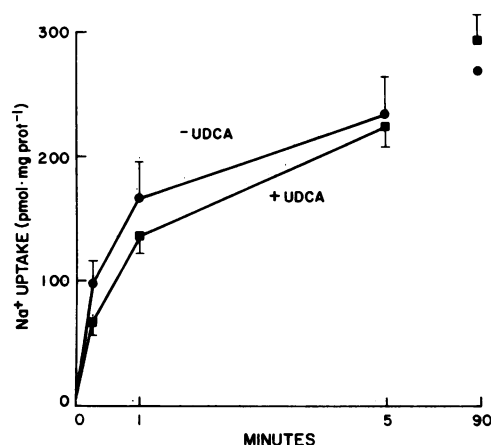


Figure 3. Effect of UDCA on Na^+ flux in bLPM vesicles. Vesicles, preloaded with 250 mM sucrose, 50 mM Hepes/Tris, pH 7.5, were incubated in equivalent amounts of propylene glycol (control) and UDCA in propylene glycol for 10 min at 25°C. Sodium (1 mM) uptake was determined at 25°C by diluting vesicles into a reaction medium of identical composition containing ^{22}Na . Data represent the mean \pm SE of triplicate analysis of at least three separate membrane vesicle preparations. UDCA had no effect on pH-equilibrated Na^+ flux.

modulatory effect on $\text{Na}^+:\text{H}^+$ exchange activity. To determine whether the enhancement of $\text{Na}^+:\text{H}^+$ exchange activity observed with UDCA was the result of an alteration in lipid fluidity, we assessed the effects of UDCA on membrane lipid

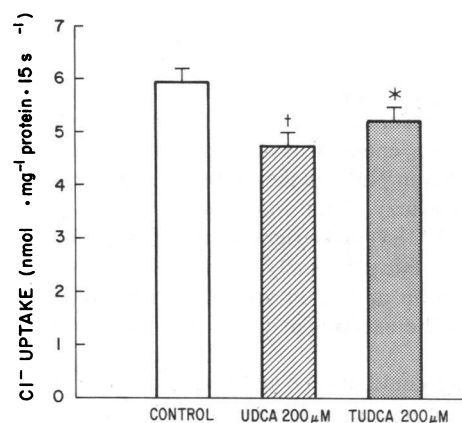


Figure 4. Lack of stimulatory effect of UDCA on $\text{Cl}^-:\text{HCO}_3^-$ exchange in cLPM vesicles. Vesicles preloaded in a pH 7.7 buffer containing 50 mM HCO_3^- (150 mM, sucrose; 100 mM TMA gluconate; 50 mM, choline bicarbonate; 35 mM, Tris; 35 mM, Hepes; 0.2 mM, Ca gluconate; and 5 mM, Mg gluconate) were incubated in equivalent amounts of propylene glycol (control), UDCA in propylene glycol, or TUDCA in propylene glycol for 10 min at 25°C. Initial rates (15 s) of chloride (5 mM) uptake were determined at 25°C by diluting vesicles into reaction medium of pH 6.0 containing ^{36}Cl (184 mM, sucrose; 100 mM, TMA gluconate; 30 mM, Tris; 14 mM, Hepes; 90 mM, MES; 0.2 mM, Ca gluconate; 5 mM, Mg gluconate). Data represent the mean \pm SE of 11 determinations in three separate membrane vesicle preparations. UDCA (200 μM) and TUDCA (200 μM) significantly decreased ($\dagger P < 0.005$; $*P < 0.05$, respectively) HCO_3^- driven Cl^- uptake.

Table III. Effect of UDCA on $\text{Cl}^-:\text{HCO}_3^-$ Exchange in cLPM Vesicles (15-s Uptake Values)

	Preincubation duration		
	0 min	5 min	10 min
	nmol/mg protein		
Control	4.7 \pm 0.2	4.5 \pm 0.1	—
50 μM UDCA	3.9 \pm 0.2*	3.6 \pm 0.2*	3.7 \pm 0.1
100 μM UDCA	4.1 \pm 0.1*	3.7 \pm 0.1*	3.3 \pm 0.2
200 μM UDCA	3.4 \pm 0.2*	3.5 \pm 0.2*	3.4 \pm 0.3
400 μM UDCA	4.0 \pm 0.3	3.5 \pm 0.3*	3.2 \pm 0.4
500 μM UDCA	3.8 \pm 0.3*	3.1 \pm 0.3*	3.0 \pm 0.1

Vesicles were loaded in a pH 7.7 buffer containing 50 mM HCO_3^- (150 mM, sucrose; 100 mM, TMA gluconate; 50 mM, choline bicarbonate; 35 mM, Tris; 35 mM, Hepes; 0.2 mM, Ca gluconate; and 5 mM, Mg gluconate) and, where noted, incubated in equivalent amounts of propylene glycol (control) and UDCA in propylene glycol, at 25°C. Initial rates (15 s) of chloride (5 mM) uptake were determined as in Fig. 4. Data represents the mean \pm SE of triplicate analysis of three separate membrane vesicle preparations. * $P < 0.05$.

fluidity after appropriate preincubations by steady-state fluorescence polarization using the probes DPH, 12-AS, and 2-AS. At a concentration of UDCA that resulted in a 32% increase over control in pH-driven Na^+ uptake (mean of four separate membrane preparations), no effect on anisotropy of the fluorescent probes was observed (Table IV).

TC, as well as taurochenodeoxycholate and deoxycholate, cause an immediate and reversible concentration-dependent inhibition of $\text{Na}^+,\text{K}^+:\text{ATPase}$ activity in isolated rat liver plasma membranes (19). To determine the degree of specificity of UDCA-induced stimulation of $\text{Na}^+:\text{H}^+$ exchange activity in bLPM, the effect of UDCA on $\text{Na}^+,\text{K}^+:\text{ATPase}$ activity in isolated membrane vesicles was examined. As illustrated in Table V, incubation of plasma membranes with UDCA had no significant effect on $\text{Na}^+,\text{K}^+:\text{ATPase}$ activity.

Finally, the effect of increasing extravesicular concentrations of Na^+ on the initial rates of pH-driven Na^+ uptake under control and UDCA-stimulated conditions was studied. pH-stimulated Na^+ uptake was enhanced by UDCA (200 μM) at all external Na^+ concentrations studied (Table VI). Regres-

Table IV. Effect of UDCA on Anisotropy (r) of Fluorescent Probes in Basolateral Membrane Vesicles

	Control	Propylene glycol	UDCA	TUDCA
			200 μM	200 μM
DPH	0.2256 \pm 0.003	0.2256 \pm 0.003	0.2254 \pm 0.003	0.2252 \pm 0.003
12-AS	0.0626 \pm 0.003	0.0627 \pm 0.003	0.0629 \pm 0.003	0.0628 \pm 0.003
2-AS	0.1024 \pm 0.003	0.1024 \pm 0.003	0.1024 \pm 0.003	0.1024 \pm 0.003

Data represent the mean \pm SD of nine readings from each of three separate membrane preparations. UDCA had no effect on anisotropy of the fluorescent probes.

Table V. Effect of UDCA on Na,K-ATPase Activity of Liver Plasma Membrane Preparation

	Control	Propylene glycol	UDCA
			200 μ M
Na ⁺ ,K ⁺ -ATPase activity (μ mol Pi/mg \cdot h)	16.1 \pm 2.3	16.9 \pm 4.4	15.9 \pm 4.4

Data represent the mean \pm SD from five separate membrane preparations. UDCA had no effect on Na⁺,K⁺-ATPase activity.

sion lines from a Lineweaver-Burke plot, calculated by least squares analysis, revealed a Michaelis constant (K_m) of 18 \pm 5 mM and a V_{max} of 36 \pm 7 nmol/min per mg protein under UDCA-stimulated conditions, compared with a K_m of 18 \pm 2 mM and a V_{max} of 23 \pm 2 nmol/min per mg protein under control conditions.

Discussion

The bicarbonate-rich choleresis observed after UDCA infusion provides a unique model for investigating the potential cellular mechanisms responsible for bile formation. Using liver plasma membrane vesicles, the present studies provide direct evidence for a stimulatory effect of UDCA on basolateral membrane Na⁺:H⁺ exchange. This evidence includes (a) a concentration-dependent enhancement of amiloride-sensitive pH gradient-driven Na⁺ uptake in basolateral membrane vesicles preincubated with ursodeoxycholate, (b) no enhancement of pH gradient-driven Na⁺ uptake with the less choleric (20), structurally related taurine conjugate of UDCA, or with TC, cholate, chenodeoxycholate and deoxycholate, and (c) no effect of UDCA on Na⁺ flux under pH-equilibrated conditions, Na⁺,K⁺-ATPase activity, or membrane lipid fluidity, as assessed by steady-state fluorescence polarization.

If stimulation of Na⁺:H⁺ exchange activity is to be ob-

Table VI. Effect of UDCA on Kinetics of pH-driven Na⁺ Uptake in bLLPM Vesicles

Na ⁺	Na ⁺ uptake (nmol/mg protein/15 s)	
	UDCA	Control
	200 μ M	
500 μ M	0.24 \pm 0.01	0.16 \pm 0.01
1 mM	0.47 \pm 0.03	0.26 \pm 0.02
2 mM	0.81 \pm 0.12	—
4 mM	1.5 \pm 0.4	—
5 mM	2.2 \pm 0.2	2.0 \pm 0.4
10 mM	3.5 \pm 0.3	2.0 \pm 0.3
25 mM	6.7 \pm 3.4	3.2 \pm 1.3

pH-stimulated (pH 5.9_{inside}/pH 7.9_{outside}) Na⁺ uptake was determined, under conditions similar to that in Fig. 1, at increasing extravesicular concentrations of Na⁺ gluconate. 15-s uptakes were determined. The data represent the mean \pm SE of triplicate analysis from three separate membrane preparations.

served, UDCA must be preincubated with membrane vesicles for up to 10 min, suggesting that UDCA does not interact with the exchanger on the outer membrane surface. Because the majority of membrane vesicles exhibit right-side-out configuration, the site of interaction may be localized to the inner aspect of the membrane. Uptake of UDCA into the vesicle interior might then be necessary for its effect to be observed. A similar mechanism, in which a bile salt might modify ion movement by accumulating within hepatocytes, was recently proposed to explain the hypercholeric effect of UDCA when administered to the intact animal (21). The exact mechanism by which UDCA is transported into the hepatocyte is not known, although conjugation may be the rate-limiting step in its overall transport from plasma to bile (22). Uptake of the dihydroxy unconjugated UDCA might be expected to proceed primarily via a nonsaturable sodium-independent process, given the finding that sodium-dependent, ouabain-suppressible uptake of bile acids in cultured rat hepatocytes is associated with increased ring hydroxylation and amino acid conjugation (23). Preliminary observations in isolated rat hepatocytes support this contention (24).

The kinetic studies, demonstrating an increase in V_{max} without an alteration in K_m for Na⁺, combined with the absence of changes in membrane lipid fluidity, suggest that UDCA may result in a minor allosteric modification of the exchanger that increases its efficiency or its turnover. Such an effect would be similar to the modulating effects of intracellular protons on Na⁺:H⁺ exchange (25). Indeed, UDCA uptake into hepatocytes has been associated with intracellular acidification (24). Because kinetic features were studied in the presence of a steep pH gradient, the observed effect of UDCA is more likely to be the result of increased turnover of Na⁺:H⁺ exchange rather than a change in the modifier site. In fact, it is unlikely that the effect of UDCA on Na⁺:H⁺ exchange in membrane vesicles in the present study is directly related to intravesicular acidification and changes in the imposed pH gradient, because preincubation with bile acids with similar pK_a's to UDCA (cholate, chenodeoxycholate) did not increase initial rates of pH-driven Na⁺ uptake (Table II). In addition, the high buffering capacity of the intravesicular media (140 mM Tris, Hepes, and MES) make significant changes in intravesicular pH unlikely. Parenthetically, in view of the high extracellular concentration of Na⁺ in vivo, it is not surprising that modulation of the exchanger does not occur through changes in K_m .

Na⁺:H⁺ exchange could also be affected by changes in its membrane lipid environment, and recent work in the intestine has noted this relationship (17, 26). The absence of appreciable changes in membrane lipid fluidity rules against this effect in the present study. In addition, lack of an effect on the Na⁺,K⁺-ATPase activity at UDCA concentrations which result in maximal stimulation of Na⁺:H⁺ exchange differs somewhat from previous studies that demonstrated both inhibitory and stimulatory effects of bile salts on plasma membrane Na⁺,K⁺-ATPase activity or fluidity (18, 27). However, higher concentrations of bile salts were used in these reports. The findings in the present study suggest that the effects of UDCA cannot be ascribed to nonspecific effects on plasma membrane fluidity and enzyme activity.

In cLPM vesicles, neither TUDCA nor UDCA had a direct stimulatory effect on Cl⁻:HCO₃⁻ exchange. In fact, UDCA

(0–500 μM) and TUDCA (200 μM) exerted a small but significant inhibition of HCO_3^- gradient-driven Cl^- uptake into cLPM vesicles (Fig. 4 and Table III). This *cis*-inhibition by UDCA suggests the existence of an organic anion exchanger on the canalicular membrane, the presence of which was previously undetectable (9). Clearly, additional studies are required to address this question and determine if the canalicular membrane of the rat liver contains an anion exchange transport system similar to that recently described for bile acids in rat intestinal basolateral membrane vesicles (28).

The specificity of the stimulation of $\text{Na}^+:\text{H}^+$ exchange by UDCA suggests that this membrane antiport could play a role in the choleresis observed with this bile acid and lends further support to a proposed model of bile formation (8, 9, 11) in which basolateral $\text{Na}^+:\text{H}^+$ exchange, in conjunction with cytosolic carbonic anhydrase, acts as a driving force for the canalicular secretion of HCO_3^- via canalicular $\text{Cl}^-:\text{HCO}_3^-$ exchange. This model predicts that the activity of the $\text{Na}^+:\text{H}^+$ exchanger will generate intracellular OH^- (and in turn, HCO_3^-), and therefore raise intracellular HCO_3^- above its equilibrium concentration. The net secretion of HCO_3^- , with Na^+ entering the canalculus passively via the paracellular pathway, would increase the osmotic activity of bile and thereby obligate the secretion of water and possibly of other electrolytes (8). Therefore, agents that enhance $\text{Na}^+:\text{H}^+$ exchange should increase canalicular HCO_3^- secretion and bile flow, whereas agents that inhibit $\text{Na}^+:\text{H}^+$ exchange should result in intracellular HCO_3^- concentrations closer to equilibrium concentration, and decrease canalicular HCO_3^- secretion and diminish bile flow.

To date, however, no direct evidence has been obtained to confirm a role for $\text{Na}^+:\text{H}^+$ exchange in bile formation in contrast to other diverse cellular functions, including intracellular pH control, cell volume regulation, and cell growth and proliferation (11, 29, 30). Nevertheless, several additional observations with known cholestatic and choleretic agents support this model. Amiloride and amiloride analogues significantly diminish UDCA-stimulated bile flow and bicarbonate output in perfused rat livers (6, 7) although a primary effect on basal bile flow is less clear (31). The carbonic anhydrase inhibitor, acetazolamide, has also been shown to decrease both UDCA-stimulated bile flow and biliary bicarbonate concentration in similar preparations (32). Ethinyl estradiol, a potent cholestatic agent, produced almost complete inhibition of $\text{Na}^+:\text{H}^+$ exchange activity in rat liver sinusoidal membrane vesicles (33). Finally, both glucocorticoids and thyroid hormone, known choleretic agents *in vivo* (34, 35), stimulate $\text{Na}^+:\text{H}^+$ exchange in other epithelia (36, 37). Whereas caution is warranted in ascribing physiologic relevance to these observations, the findings in the present study demonstrate that UDCA, a known choleretic agent, can stimulate $\text{Na}^+:\text{H}^+$ exchange *in vitro* at bile salt concentrations within the range reported for both liver tissue and cultured hepatocytes after UDCA administration (38, 39), and well below the critical micellar concentration of UDCA.

In conclusion, preincubation of isolated basolateral membrane vesicles with ursodeoxycholate results in a concentration-dependent increase in amiloride-sensitive $\text{Na}^+:\text{H}^+$ exchange. These findings have been discussed primarily with respect to an evolving model of bile formation, in which secondary active $\text{Na}^+:\text{H}^+$ exchange acts as a driving force for the

net canalicular secretion of HCO_3^- . However, the physiologic significance of these *in vitro* findings must eventually depend on *in vivo* studies that can more directly establish a role for or against electrolyte transport in bile formation, such as isolated hepatocyte couplets (40). A recent report that ursodeoxycholate-induced choleresis in the isolated perfused rat liver is not dependent on bicarbonate *per se* but is the result of H^+ (or OH^-) transport (41) supports this interpretation.

Acknowledgments

We thank Carolyn Barrett and Steven Murphy for excellent technical assistance, John W. Dobbins for helpful discussions, and E. Brooks Comstock for efficient typing of the manuscript.

This work was supported in part by grants AM-07356, AM-31493, AM-34989, and AM-07616 from the National Institutes of Health. Dr. Moseley was a recipient of an American Liver Foundation Postdoctoral Research Fellowship Award.

References

1. Dumont, M., S. Erlinger, and S. Uchman. 1980. Hypercholeresis induced by ursodeoxycholic acid and 7-ketolithocholic acid in the rat: possible role of bicarbonate transport. *Gastroenterology*. 79:82–89.
2. Kitani, K., and S. Kanai. 1982. Effect of ursodeoxycholate on the bile flow in the rat. *Life Sci*. 31:1973–1985.
3. Van Dyke, R. W., J. E. Stephens, and B. F. Scharschmidt. 1982. Effect of ion substitution on bile acid-dependent and bile acid-independent bile formation by the isolated perfused rat liver. *J. Clin. Invest.* 70:505–517.
4. Anwer, M. S., and D. Hegner. 1983. Role of inorganic electrolytes in bile acid independent canalicular bile formation. *Am. J. Physiol.* 244:G116–G124.
5. Hardison, W. G. M., and C. A. Wood. 1978. Importance of bicarbonate in bile salt-independent fraction of bile flow. *Am. J. Physiol.* 235:E158–E164.
6. Lake, J. R., R. W. Van Dyke, and B. F. Scharschmidt. 1987. Effects of Na^+ replacement and amiloride on ursodeoxycholic acid-stimulated choleresis and biliary bicarbonate secretion. *Am. J. Physiol.* 252:G163–G169.
7. Renner, E., J. R. Lake, E. Cragoe, R. W. Van Dyke, and B. F. Scharschmidt. 1985. Ursodeoxycholic acid (UDCA) choleresis; relationship to biliary HCO_3^- secretion and further evidence of a role for Na^+/H^+ exchange. *Hepatology (Baltimore)*. 5:1011. (Abstr.)
8. Moseley, R. H., P. J. Meier, P. S. Aronson, and J. L. Boyer. 1986. Na-H exchange in rat liver basolateral but not canalicular plasma membrane vesicles. *Am. J. Physiol.* 250:G35–G43.
9. Meier, P. J., R. Knickelbein, R. H. Moseley, J. W. Dobbins, and J. L. Boyer. 1985. Evidence for carrier-mediated chloride/bicarbonate exchange in canalicular rat liver plasma membrane vesicles. *J. Clin. Invest.* 75:1256–1263.
10. Scharschmidt, B. F., and R. W. Van Dyke. 1983. Mechanisms of hepatic electrolyte transport. *Gastroenterology*. 85:1199–1214.
11. Moseley, R. H., and J. L. Boyer. 1985. Mechanisms of electrolyte transport in the liver and their functional significance. *Semin. Liver. Dis.* 5:122–135.
12. Meier, P. J., E. S. Sztul, A. Reuben, and J. L. Boyer. 1984. Structural and functional polarity of canalicular and basolateral plasma membrane vesicles isolated in high yield from rat liver. *J. Cell Biol.* 98:991–1000.
13. Fuchs, R., T. Thalhammer, M. Peterlik, and J. Graf. 1986. Electrical and molecular coupling between sodium and proton fluxes in basolateral membrane vesicles of rat liver. *Pfluegers Arch. Eur. J. Physiol.* 406:430–432.
14. Scharschmidt, B. F., E. B. Keeffe, N. M. Blankenship, and R. K. Ockner. 1979. Validation of a recording spectrophotometric

method for measurement of membrane-associated Mg- and NaK-ATPase activity. *J. Lab. Clin. Med.* 93:790-799.

15. Shinitzky, M., and Y. Barenholz. 1978. Fluidity parameters of lipid regions determined by fluorescence polarization. *Biochim. Biophys. Acta* 515:367-394.

16. Lakowicz, J. R. 1983. Principles of Fluorescence Spectroscopy. Plenum Press, New York.

17. Kinsella, J. L., and P. S. Aronson. 1981. Amiloride inhibition of the Na⁺-H⁺ exchanger in renal microvillus membrane vesicles. *Am. J. Physiol.* 241:F374-F379.

18. Brasitus, T. A., P. K. Dudeja, P. K. Worman, and E. S. Foster. 1986. The lipid fluidity of rat colonic brush-border membrane vesicles modulates Na⁺/H⁺ exchange and osmotic water permeability. *Biochim. Biophys. Acta* 855:16-24.

19. Scharschmidt, B. F., E. B. Keeffe, D. A. Vessey, N. M. Blankenship, and R. K. Ockner. 1981. In vitro effect of bile salts on rat liver plasma membrane, lipid fluidity, and ATPase activity. *Hepatology (Baltimore)* 1:137-145.

20. Kitani, K., and S. Kanai. 1981. Biliary transport maximum of tauroursodeoxycholate is twice as high as that of taurocholate in the rat. *Life Sci.* 29:269-275.

21. Kitani, K., and S. Kanai. 1982. Effect of ursodeoxycholate on the bile flow in the rat. *Life Sci.* 31:1973-1985.

22. Zouboulis-Vafiadis, I., M. Dumont, and S. Erlinger. 1982. Conjugation is rate limiting in hepatic transport of ursodeoxycholate in the rat. *Am. J. Physiol.* 243:G208-G213.

23. Van Dyke, R. W., J. E. Stephens, and B. F. Scharschmidt. 1982. Bile acid transport in cultured rat hepatocytes. *Am. J. Physiol.* 243:G484-G492.

24. Corbic, M., M. Dumont, M. Peignoux, and S. Erlinger. 1985. Uptake of ursodeoxycholate and tauroursodeoxycholate in isolated rat hepatocytes. *Hepatology (Baltimore)* 5:983. (Abstr.)

25. Aronson, P. S., J. Nee, and M. A. Suhm. 1982. Modifier role of internal H⁺ in activating the Na-H exchanger in renal microvillus membrane vesicles. *Nature (Lond.)* 299:161-163.

26. Dudeja, P. K., E. S. Foster, and T. A. Brasitus. 1986. Regulation of Na⁺-H⁺ exchange by transmethylation reactions in rat colonic brush-border membranes. *Biochim. Biophys. Acta* 859:61-68.

27. Wannagat, F. J., R. D. Adler, and R. K. Ockner. 1978. Bile acid-induced increase in bile acid-independent flow and plasma membrane NaK-ATPase activity in rat liver. *J. Clin. Invest.* 61:297-307.

28. Weinberg, S. L., G. Burckhardt, and F. A. Wilson. 1986. Taurocholate transport by rat intestinal basolateral membrane vesicles:

evidence for the presence of an anion exchange transport system. *J. Clin. Invest.* 78:44-50.

29. Mahnensmith, R. L., and P. S. Aronson. 1985. The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. *Circ. Res.* 56:773-788.

30. Henderson, R. M., J. Graf, and J. L. Boyer. 1987. Na:H exchange regulates intracellular pH in isolated rat hepatocyte couplets. *Am. J. Physiol.* 252:G109-G113.

31. Erlinger, S., D. Dhumeaux, P. Berthelot, and M. Dumont. 1970. Effect of inhibitors of sodium transport on bile formation in the rat. *Am. J. Physiol.* 219:416-422.

32. Garcia-Marin, J. J., M. Dumont, M. Corbic, G. de Couet, and S. Erlinger. 1985. Effect of acid-base balance and acetazolamide on ursodeoxycholate-induced biliary bicarbonate secretion. *Am. J. Physiol.* 248:G20-G27.

33. Arias, I. M., Y. Adachi, and T. Tran. 1983. Ethinyl estradiol cholestasis: a disease of the sinusoidal domain of the hepatocyte plasma membrane. *Hepatology (Baltimore)* 3:872. (Abstr.)

34. Miner, P. B., E. Sutherland, and F. R. Simon. 1980. Regulation of hepatic sodium plus potassium-activated adenosine triphosphatase activity by glucocorticoids in the rat. *Gastroenterology* 79:212-221.

35. Layden, T. J., and J. L. Boyer. 1976. The effect of thyroid hormone on bile salt-independent bile flow and Na⁺,K⁺-ATPase activity in liver plasma membranes enriched in bile canaliculi. *J. Clin. Invest.* 57:1009-1018.

36. Kinsella, J., T. Cujdik, and B. Sacktor. 1984. Na⁺-H⁺ exchange activity in renal brush border membrane vesicles in response to metabolic acidosis: the role of glucocorticoids. *Proc. Natl. Acad. Sci. USA* 81:630-634.

37. Kinsella, J., and B. Sacktor. 1985. Thyroid hormones increase Na⁺-H⁺ exchange activity in renal brush border membranes. *Proc. Natl. Acad. Sci. USA* 82:3606-3610.

38. Reichen, J., and G. Paumgartner. 1976. Uptake of bile acids by perfused rat liver. *Am. J. Physiol.* 23:734-742.

39. Scharschmidt, B. F., and J. E. Stephens. 1981. Transport of sodium, chloride and taurocholate by cultured rat hepatocytes. *Proc. Natl. Acad. Sci. USA* 78:986-990.

40. Graf, J., A. Gautam, and J. L. Boyer. 1984. Isolated rat hepatocyte cell couplets—a primary secretory unit for electrophysiologic studies of bile secretory function. *Proc. Natl. Acad. Sci. USA* 81:6516-6520.

41. Garcia-Marin, J. J., M. Corbic, M. Dumont, G. de Couet, and S. Erlinger. 1985. Role of H⁺ transport in ursodeoxycholate-induced biliary HCO₃⁻ secretion in the rat. *Am. J. Physiol.* 249:G335-G341.