Effects of Ion Substitution on Bile Acid-dependent and -independent Bile Formation by Rat Liver

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ABSTRACT To characterize the transport mechanisms responsible for formation of canalicular bile, we have examined the effects of ion substitution on bile acid-dependent and bile acid-independent bile formation by the isolated perfused rat liver. Complete replacement of perfusate sodium with choline and lithium abolished taurocholate-induced choleresis and reduced biliary taurocholate output by >70%. Partial replacement of perfusate sodium (25 of 128 mM) by choline reduced bile acid-independent bile formation by 30% and replacement of the remaining sodium (103 mM) by choline reduced bile acid-independent bile formation by an additional 64%. In contrast, replacement of the remaining sodium (103 mM) by lithium reduced bile acid-independent bile formation by only an additional 20%, while complete replacement of sodium (128 mM) by lithium reduced bile formation by only 17%, and lithium replaced sodium as the predominant biliary cation. Replacement of perfusate bicarbonate by Tricine, a zwitterionic amino acid buffer, decreased bile acid-independent bile formation by ≥50% and decreased biliary bicarbonate output by ~60%, regardless of the accompanying cation. In separate experiments, replacement of sodium by lithium essentially abolished Na,K-ATPase activity measured either as ouabain-suppressible ATP hydrolysis in rat liver or kidney homogenates, or as ouabain-suppressible ^{86}Rb uptake by cultured rat hepatocytes.

These studies indicate that bile acid(taurocholate)-dependent bile formation by rat liver exhibits a specific requirement for sodium, a finding probably attributable to the role(s) of sodium in hepatic sodium-coupled taurocholate uptake and/or in maintenance of Na,K-ATPase activity. The surprising finding that bile acid-independent bile formation was substantially unaltered by complete replacement of sodium with the permeant cation lithium does not appear to be explained by Na,K-ATPase-mediated lithium transport. Although alternative interpretations exist, this observation is consistent with the hypothesis that much of basal bile acid-independent bile formation is attributable to an ion pump other than Na,K-ATPase, which directly or indirectly mediates bicarbonate transport.

INTRODUCTION

Canalicular bile formation is an energy requiring process thought to result from active solute transport followed by osmotic water flow. Na,K-ATPase, one of the best characterized cellular ion pumps, is located in liver on the sinusoidal and lateral plasma membrane (1, 2), a position analogous to its location in most other epithelia. Secondary active transport processes energized by the transmembrane electrochemical sodium gradient attributable to Na,K-ATPase are currently believed to play a major role in absorption or secretion by a variety of epithelial tissues (3). Although Na,K-ATPase is also postulated to be important in bile formation (4–6), the precise role of this enzyme in bile acid-dependent (BADF)[1] and bile acid-independent (BAIF) bile formation remains poorly understood.

Recent studies on whole liver (7, 8), isolated hepatocytes (9, 10), and cultured hepatocytes (11–13) indicate that conjugated bile acids are concentratively transported into hepatocytes via a sodium-dependent, ouabain-suppressible mechanism, presumably powered by the transmembrane electrochemical sodium

Portions of this work were presented at the 82nd Annual Meeting of the American Gastroenterological Association, New York, 18–20 May 1981, and were published in abstract form in 1981. *Gastroenterology.* **80:** 1353a.

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Received for publication 10 September 1981 and in revised form 23 April 1982.

[1] *Abbreviations used in this paper*: BADF, bile acid-dependent bile formation; BAIF, bile acid-independent bile formation; B/P ratios, bile-to-plasma ratios.

gradient established by Na,K-ATPase. Therefore, it is likely that sodium and Na,K-ATPase are essential for bile acid-dependent bile formation.

Sodium transport and Na,K-ATPase also have generally been assumed to play a central role in bile acid-independent bile formation (4–6). Circumstantial evidence in support of this hypothesis includes the observations that (*a*) output of sodium, which accounts for a greater proportion of bile osmolality than any other solute (14, 15), is linearly related to BAIF (16), (*b*) BAIF and hepatic Na,K-ATPase activity change in parallel following administration of certain agents (17–23), and (*c*) inhibitors of Na,K-ATPase diminish bile flow (24, 25). However, several studies have failed to demonstrate a consistent relationship between BAIF and Na,K-ATPase activity after treatment with drugs and hormones (26, 27), and some authors have reported an increase in BAIF following administration of Na,K-ATPase inhibitors (28, 29). Therefore, the role of Na,K-ATPase-mediated ion transport in BAIF remains controversial. Indeed, the identity of those solutes other than bile acids that are actively transported into bile is currently unknown. Sodium-coupled transport of chloride has been proposed as a major mechanism for BAIF; however, studies in our laboratory have provided no evidence in support of this hypothesis (30). Hardison and Wood (31) recently reported evidence of a role for bicarbonate transport in BAIF, although the mechanism involved and its relationship to Na,K-ATPase are unknown. To better characterize the transport processes responsible for canalicular bile formation and to assess the role of Na,K-ATPase, we have examined the effects of ion substitution on BADF and BAIF by the isolated perfused rat liver.

METHODS

Chemicals and radioisotopes. Ouabain, taurocholate, lithium chloride, choline chloride, choline bicarbonate, and Tricine (*N*-tris [hydroxymethyl]methylglycine) were obtained from Sigma Chemical Co., St. Louis, MO. ^{86}RbCl, [^{14}C]sucrose, [^{3}H]PEG-900, and [^{3}H]taurocholate were obtained from New England Nuclear, Boston, MA. Fluosol-43 was obtained from Alpha Therapeutics Corp. (Los Angeles, CA). Lithium bicarbonate was prepared by bubbling CO_2 through lithium hydroxide until the pH fell below 8.0. Bicarbonate concentration was confirmed using a Natelson microgasometer.

Analytical techniques. Sodium, potassium, and lithium were measured by flame photometry and chloride by electrometric titration. Bicarbonate was measured as total CO_2 using a Natelson microgasometer. Perfusate arterial and venous oxygen partial pressures were measured on a Corning blood gas analyzer (Corning Medical, Medfield, MA). Oxygen consumption was calculated from the arterial-venous P_{O_2} difference, perfusate flow rate, liver weight, and the oxygen carrying capacity of the fluorocarbon emulsion used (as determined by the manufacturer). Protein concentration was measured by the method of Lowry et al. (32). Bile acid concentrations in bile before taurocholate infusion were measured by the 3α-hydroxysteroid dehydrogenase assay (33).

Lactate dehydrogenase (LDH) activity in cultured hepatocytes was measured as previously described (11). Na,K-ATPase and Mg-ATPase activities of rat liver and kidney homogenates [prepared as previously described (34) with a Sorvall Omnimixer, Sorvall Biomedical Div., DuPont Co., Newtown, CT] were measured by continuously recording

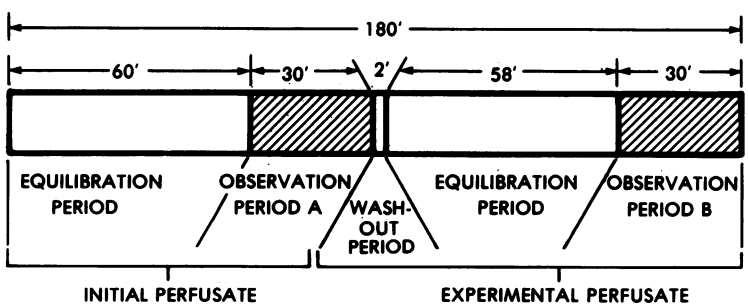

FIGURE 1 Protocol for studying the effect of ion substitution on BAIF by the isolated perfused rat liver. All livers were perfused for a total of 3 h. During the first 90 min, perfusion was conducted in a recirculating mode using a 20% (vol/vol) fluorocarbon emulsion-containing perfusate with a balanced electrolyte composition (initial perfusate). Base-line output was recorded during the final 30 min of this period (period A). At 90 min, the liver was flushed free of the control perfusate using an oxygenated, nonrecirculating wash solution, with or without fluorocarbon. From 92 to 180 min the liver was again perfused with a fluorocarbon emulsion-containing perfusate (experimental perfusate) in a recirculating mode. Bile flow during the final 30 min of this second period (period B) was compared with that in observation period A. The solute composition of the perfusates for the various studies are given in Tables I, II, and IV. The wash solution always contained the same solute concentrations as the experimental perfusate. The wash solution and experimental perfusate were identical to the initial perfusate for control livers and differed for experimental livers. Liver weight was measured at the end of the 3-h perfusion.

TABLE I
*Effect of Sodium Elimination on BADF by the Isolated Perfused Rat Liver**

	Sodium chloride perfusion‡	Lithium chloride perfusion§
Base line‖		
Bile flow, *μl/min/g*	0.55±0.14	0.38±0.06
Bile acid output, *nmol/min/g*	0.67±0.21	1.49±1.13
Infusion 1¶		
Infusion rate, *nmol/min/g*	18.3±2.1	18.0±1.7
Perfusate concentration, *μM*	27±30	35±31
Increase in bile flow, *μl/min/g*	0.21±0.18	0.01±0.08**
Bile acid output, *nmol/min/g*	22±12	5±4‡‡
Infusion 2¶		
Infusion rate, *nmol/min/g*	63±17	63±12
Perfusate concentration, *μM*	52±43	167±102
Increase in bile flow, *μl/min/g*	0.38±0.16	0.01±0.07§§
Bile acid output, *nmol/min/g*	60±10	14±1§§
Infusion 3¶		
Infusion rate, *nmol/min/g*	199±172	193±152
Perfusate concentration, *μM*	278±391	981±1,015
Increase in bile flow, *μl/min/g*	0.31±0.22	−0.14±0.08§§
Bile acid output, *nmol/min/g*	72±21	8±3§§

* All data is given as mean±SD of three perfusions normalized for liver weight.

‡ Perfusate contained 103 mM NaCl/25 mM choline bicarbonate/2.28 mM K_2SO_4/2.5 mM $CaCl_2$/2.12 mM $MgSO_4$/10 mM glucose/20% fluorocarbon emulsion.

§ Perfusate contained 103 mM LiCl/25 mM choline bicarbonate/2.28 mM K_2SO_4/2.5 mM $CaCl_2$/2.12 mM $MgSO_4$/10 mM glucose/20% fluorocarbon emulsion.

‖ Base-line bile flow and bile acid output were measured during the last 30 min of the initial 90 min perfusion before taurocholate infusion.

¶ The data shown are for the last 20 min of each 30 min taurocholate infusion.

** $P < 0.05$ compared with sodium perfused livers.

‡‡ $P < 0.01$ compared with sodium perfused livers.

§§ $P < 0.001$ compared with sodium perfused livers.

spectrophotometry (35) using Tris-ATP and K^+-phosphoenolpyruvate instead of the respective sodium salts. NaN_3 was omitted from the assay buffer. Na,K-ATPase-mediated ATP hydrolysis was taken to be the difference between total ATP hydrolysis and ATP hydrolysis observed in the presence of 1 mM ouabain, which has been shown to completely suppress rat liver plasma membrane Na,K-ATPase activity (36). In some assays, sodium chloride in the assay buffer (present at a concentration of 120 mM) was completely replaced by lithium chloride or choline chloride.

Radioactivity was measured by liquid scintillation counting in Aquasol (New England Nuclear) using external or internal standardization for quench correction.

Isolated perfused rat liver studies. The surgical technique and operation and design of the perfused rat liver apparatus used in these studies have been described in detail (37, 38). Briefly, nonfasting male Sprague-Dawley rats (180–220 g) fed standard lab chow (Purina rodent chow, Ralston Purina Co., St. Louis, MO) were anesthetized with ether and prepared for perfusion by cannulation of the common bile duct, portal vein, and intrathoracic portion of the inferior vena cava. Immediately after cannulation of the portal vein, *in situ* perfusion was begun with Krebs-Henseleit solution (39), warmed to 37°C and bubbled with 95% O_2/5% CO_2. The liver was then excised and transferred to a thermostatically controlled cabinet maintained at 37°C. Perfusate (Tables I, II, and IV) was circulated by means of a peristaltic pump (LKB Multiperpex 2115, Bromma, Sweden) and passed in sequence through a membrane oxygenator (40) consisting of 4.6 m of Silastic tubing (i.d. 0.147 cm, o.d. 0.196 cm, Dow Corning Corp., Midland, MI) coiled in an oxygenated glass jar, a temperature-sensitive probe, a stainless steel filter screen (Millipore Corp., Bedford, MA), a flow-through pH electrode (Markson Science Inc., Del Mar, CA), a bubble trap/pressure gauge, and finally the liver via the portal vein cannula. A constant perfusion rate of 25 ml/min was maintained in all studies and net portal perfusion pressure (measured as the difference between pressure during liver perfusion and pressure in the absence of a liver) ranged from 3 to 4 cm H_2O. The membrane oxygenator was gassed with 95% O_2/5% CO_2 (when perfusate contained bicarbonate) or 100% O_2 (when Tricine replaced bicarbonate). pH was monitored throughout perfusions and maintained between 7.35 and 7.45 by slight changes in pCO_2 or addition of small amounts of bicarbonate or hydroxide as necessary. Bile was collected in pretared 1.5-ml polypropylene containers (Bio-Rad Laboratories, Richmond, CA), and measured gravimetrically assuming a density of 1 g/ml.

This system using perfusates containing 20% (vol/vol) fluorocarbon emulsion (Fluosol-43) as an oxygen carrier, has been extensively validated (37, 38). Viability of the perfused liver after 3 h of perfusion has been demonstrated by normal appearance on electron microscopy, stable perfusate lactate dehydrogenase and transaminase activity, and normal hepatic oxygen consumption. For the purpose of these studies, it was further demonstrated that hepatic oxygen consumption averaged 2.76±0.22 μmol/g liver per min at 1 h and was essentially unchanged (2.54±0.20 μmol/g liver per min) at 3 h in control perfusions (41).

In studies designed to assess the effects of sodium elimination on BADF, livers were perfused for 3 h in a recirculating mode using sodium chloride- or lithium chloride-containing fluorocarbon perfusates (Table I) with 25 mM choline bicarbonate as buffer. After a 90-min equilibration period, 360 nmol of taurocholate dissolved in sodium chloride or lithium chloride was added to the perfusate as a bolus to achieve an immediate perfusate concentration of ~7 μM. This was immediately followed by a series of three graded 30-min infusions of taurocholate (18, 63, and 196 nmol/min per g liver). All taurocholate solutions contained trace amounts of [^{3}H]taurocholate of known specific activity. Perfusate and bile samples for scintillation counting were taken immediately following initiation of the taurocholate infusion and every 10 min thereafter for determination of perfusate and bile taurocholate concentration and biliary taurocholate output. The increment in bile flow over base line during the last 20 min of each infusion period was compared between sodium chloride- and lithium chloride-perfused livers.

The effects of sodium or bicarbonate replacement on BAIF by the perfused rat liver were examined using the protocol shown in Fig. 1. Perfusate composition for each experiment is given in Tables II and IV. In selected studies, 1–2 μCi of

TABLE II

Electrolyte Composition of Perfusate and Bile in Sodium Substitution Studies

Number of perfusions	Sodium substitution protocol	Period A									
		Perfusate (mM)*									
		Na^+	$Chol^+$	Li^+	K^+	Ca^{+2}	Mg^{+2}	Cl^-	HCO_3^-	SO_4^{-2}	PO_4^{-3}	Glucose
8	Control	128	0	0	4.6 (5.6)‖	2.5	2.12	108	25	4.4	0 (1.0)‖	10
4	Partial choline	128	0	0	5.6	2.5	2.12	108	25	4.4	1.0	10
4	Complete choline	103	25	0	4.6 (5.6)	2.5	2.12	108	25	4.4	0 (1.0)	10
9	Lithium + choline	103	25	0	4.6 (5.6)	2.5	2.12	108	25	4.4	0 (1.0)	10
6	Complete lithium	128	0	0	5.6	2.5	2.12	112.6	25	2.12	1.0	10

* Perfusate electrolyte composition at the beginning of periods A and B is expressed as millimoles/liter total perfusate volume (20% of perfusate volume consisted of fluorocarbon emulsion).

‡ Data presented as mean ± SD.

§ Concentrations of K^+, Ca^{+2}, Mg^{+2}, Cl^-, HCO_3^-, SO_4^{-2}, PO_4^{-3} and glucose were identical to those in period A.

‖ Figure in parentheses indicate that 1 mM KH_2PO_4 was added to the perfusate for some livers. Results did not differ for these livers compared to those perfused without KH_2PO_4.

¶ $P < 0.05$ compared to control livers.

** $P < 0.001$ compared to control livers.

[^{14}C]sucrose and [^{3}H]PEG-900 were added to the perfusate and radioactivity in bile and perfusate was measured at 15-min intervals throughout the entire perfusion allowing calculation of bile/perfusate ratios (B/P). Bile secretory pressure was measured in certain studies at the end of the 3-h perfusion by elevating the bile duct cannula above the liver hilum to the point where bile stopped flowing. As a replacement for bicarbonate we chose to use Tricine, a zwitterionic amino acid buffer (42) that has been used successfully in mammalian cell culture systems (43, 44) and in the isolated perfused rat liver (31).

Cultured hepatocyte studies. Transport studies in cultured hepatocytes were performed as described (11). Briefly, rat hepatocytes were prepared by collagenase perfusion and plated on collagen-coated plastic cover slips (0.6–0.8 mg cell protein/cover slip) (45, 46). All experiments were performed

TABLE III

*Effect of Sodium Substitution on BAIF by the Isolated Perfused Rat Liver**

Number of perfusions	Sodium substitution protocol	Liver weight/ body weight	Period A Bile flow	Period B Bile flow	Bile flow in period B/A × 100
		%	μl/min/g	μl/min/g	%
8	Control‡	3.9±0.5	0.674±0.200	0.677±0.203§	101±12
4	Partial choline‡	3.8±0.3	0.916±0.057	0.638±0.076‖	70±5¶
4	Complete choline‡	4.2±0.2	0.489±0.074	0.171±0.085‡‡	36±22¶
9	Lithium + choline‡	4.5±0.5**	0.486±0.083	0.387±0.070‡‡	80±12¶
6	Complete lithium‡	3.9±0.5	0.937±0.222	0.771±0.208§	83±18**

* All data is presented as mean±SD and is normalized to liver weight.

‡ See Table II for perfusate composition.

§ Not significant compared with bile flow in period A by paired *t* test.

‖ $P < 0.02$ compared with bile flow in period A by paired *t* test.

¶ $P < 0.005$ compared with control livers.

** $P < 0.05$ compared with control livers.

‡‡ $P < 0.002$ compared with bile flow in period A by paired *t* test.

TABLE II (*Continued*)

Period B										
Perfusate (mM)*§				Bile (mM)‡						
Na^+	Li^+	K^+	Cl^-	Na^+	$Chol^+$	Li^+	Na^+	Li^+	K^+	Cl^-
153±19	—	9.2±2.2	111±26	128	0	0	154±16	—	9.9±5.0	110±9
159±9	—	9.1±0.3	96±6	103	25	0	132±9¶	—	11.4±0.9	110±8
134±12	—	12.2±1.1¶	106±6	0	128	0	4.0±3.4**	—	12.3±0.4	—
132±9	—	11±0.7¶	106±10	0	25	103	2.6±0.8**	107±23**	14.7±1.6¶	94±15¶
158±26	—	6.9±1.6	105±20	0	0	128	0.5±1.3**	141±7**	14.9±1.8¶	103±8

44–50 h after plating and viability was tested by trypan blue exclusion and release of lactate dehydrogenase into the incubation medium. Transport experiments were initiated by preincubating the cells in 26 × 33-mm wells (Flow Laboratories, Inc., Rockville, MD) containing a balanced electrolyte solution (130 mM NaCl/3.4 mM KCl/0.8 mM $MgSO_4$/1.2 mM $CaSO_4$/0.8 mM K_2HPO_4/10 mM Hepes/5 mM $NaHCO_3$/5 mM glucose, adjusted to pH 7.4 with 1 M NaOH) or a sodium-free solution in which sodium chloride had been replaced with lithium chloride and sodium bicarbonate with choline bicarbonate (pH adjusted with 1 M LiOH). Ouabain (5 mM) was added to sodium-containing medium for some incubations. After a 1-h preincubation, cover slips were transferred to wells containing 3 ml of medium identical to that used in the preincubation step except that 1–2 μCi of ^{86}Rb, a potassium analog, had been added. At time points ranging from 1 min to 30 min, uptake was stopped and extracellular isotope removed by dipping the cover slips for 30 s in each of eight beakers containing 25 ml of ice-cold incubation medium without isotope. Previous studies have demonstrated that this wash procedure removes extracellular isotope while causing minimal loss of intracellular isotope (11). The cells were then scraped from the cover slip and radioactivity and protein were quantitated. Cellular uptake was calculated as nanomoles Rb per milligram cell protein, assuming that potassium in the incubation medium was equivalent to rubidium. Na,K-ATPase-dependent ^{86}Rb uptake was taken to be the difference between ^{86}Rb uptake by cultured hepatocytes in the presence and absence of 5 mM ouabain.

Calculations and statistics. In ion substitution studies in the perfused liver, bile flow in observation period B (B) was expressed as a percentage of bile flow in observation period A (A), i.e., B/A × 100, and this percentage was compared between control and experimental livers using Student's *t* test. In addition, paired *t* tests were used to calculate the significance of differences between bile flow in period A and period B for each group of livers. ^{86}Rb uptake rates in cultured hepatocytes were taken to be the slope of the linear portion of the uptake curve calculated by linear regression analysis. Na,K-ATPase-mediated ^{86}Rb uptake was the difference between slopes in the absence and presence of 5 mM ouabain. Student's *t* test was used to calculate the significance of differences between control and experimental studies.

RESULTS

General observations. Bile formation by control livers not receiving a taurocholate infusion fell from an initial value of 1.21±0.33 μl/min per g to 0.80±0.22 μl/min per g at 1 h, concomitant with a decrease in biliary bile acid concentration to 1.9±1.2 mM. Because considerable variation was observed in basal bile formation by different livers (Table III), the effect of ion substitution on BAIF was studied using a protocol (Fig. 1) that permitted each liver to serve as its own control. In selected studies, the perfusion technique was modified by addition of 1 mM KH_2PO_4 to the perfusate (Table II) and by cannulation of the bile duct with PE-50 instead of PE-10 tubing. Bile flow tended to be increased by these modifications (0.92±0.26 μl/min

TABLE IV
Perfusate and Bile Composition in Bicarbonate Substitution Studies

	Period A	Period B				
	All livers	Control livers	Tricine control livers	Sodium experimental livers	Lithium experimental livers	Choline experimental livers
		(4)*	(4)	(4)	(4)	(3)
Perfusate composition (*mM*)‡						
Sodium	128	128	115	118	0	0
Lithium	0	0	0	0	118	0
Choline	0	0	0	0	0	118
Potassium	4.6	4.6	4.6	4.6	4.6	4.6
Calcium	2.5	2.5	2.5	2.5	2.5	2.5
Magnesium	2.12	2.12	2.12	2.12	2.12	2.12
Chloride	108	108	95	123	123	123
Bicarbonate	25	25	25	0§	0	0
Tricine	0	0	10	10	10	10
Sulfate	4.4	4.4	4.4	4.4	4.4	4.4
Glucose	10	10	10	10	10	10
Bile composition (*mM*)‖						
Bicarbonate	—	20.6±4.5	18.4±2.8	12.2±2.8¶	11.9±1.9¶	—**

* Number of livers.
‡ Electrolyte concentration at initiation of perfusion is given as millimoles/liter of total perfusate (20% of perfusate volume consisted of fluorocarbon emulsion).
§ Bicarbonate concentration when perfusate was made up. Actual perfusate concentration at 3 h was 11 mM, a finding probably attributable to hepatic CO_2 production.
‖ Data given as mean±SD.
¶ $P < 0.01$ compared with control livers.
** Quantity of bile too small to allow measurement.

per g vs. 0.69±0.12 μl/min per g at 1 h); however, the effect of ion substitution on BAIF was unaltered and the results from all perfusions have therefore been combined (Table III).

Sucrose bile/plasma (B/P) ratios in control livers averaged 0.21±0.07 at 1 h and gradually rose with time to average 0.25±0.13 at 2 h and 0.49±0.19 at the end of 3 h. PEG-900 B/P ratios in control livers averaged 5.68±1.59 at 1 h and 5.94±1.86 at 3 h. Bile secretory pressure at the end of 3 h of perfusion averaged 11.5±0.9 cm and was 7.4±1.6 cm above perfusion pressure in control livers.

TABLE V
*Effect of Bicarbonate Substitution on BAIF by the Isolated Perfused Rat Liver**

No. of perfusions	Liver weight/ body weight	Period A	Period B			
		Bile flow	Cation	Buffer	Bile flow	Bile flow in period B/A × 100
	g	*μl/min/g*			*μl/min/g*	%
4	3.9±0.5	0.574±0.116	Sodium	Bicarbonate	0.576±0.069	102±11
4	3.6±0.2	0.684±0.175	Sodium	Bicarbonate and tricine	0.708±0.144	105±10
4	4.4±0.3	0.799±0.196	Sodium	Tricine	0.402±0.048	52±12‡
4	5.3±1.2§	0.568±0.179	Lithium	Tricine	0.245±0.237	38±12‡
3	4.6±0.2§	0.652±0.063	Choline	Tricine	0.071±0.060	11±10‡

* All data is given as mean±SD and is normalized for liver weight. See Table IV for perfusate composition.
‡ $P < 0.002$ compared with control (sodium chloride/sodium bicarbonate) perfused livers.
§ $P < 0.02$ compared with control (sodium chloride/sodium bicarbonate) perfused livers.

TABLE VI

*Effect of Sodium Elimination on ATPase Activity in Rat Liver and Kidney Homogenates**

	Liver		Kidney	
	Na,K-ATPase	Mg-ATPase	Na,K-ATPase	Mg-ATPase
	μmol P_i/mg/h	μmol P_i/mg/h	μmol P_i/mg/h	μmol P_i/mg/h
Sodium	1.9±1.3	8.4±1.0	6.8±3.2	19.9±1.5
Lithium‡	0.14±0.12§••	8.4±1.6	0.29±0.24‖••	18.3±1.7
Choline‡	0.29±0.26¶••	8.7±1.5	0.77±0.64‖••	18.6±1.3

* Data given as mean±SD of six homogenates.

‡ Lithium and choline reaction mixtures were sodium free and contained 120 mM LiCl or 120 mM choline chloride, respectively, in place of NaCl.

§ $P < 0.01$ compared to assay with sodium.

‖ $P < 0.001$ compared to assay with sodium.

¶ $P < 0.02$ compared to assay with sodium.

•• Not significantly different from zero.

Transient increases in perfusion pressure were noted after switching from control perfusate to sodium-free perfusate containing choline and/or lithium, presumably related to changes in hepatic vascular resistance due to inhibition of Na,K-ATPase (47). Perfusion pressure subsequently returned toward base line despite

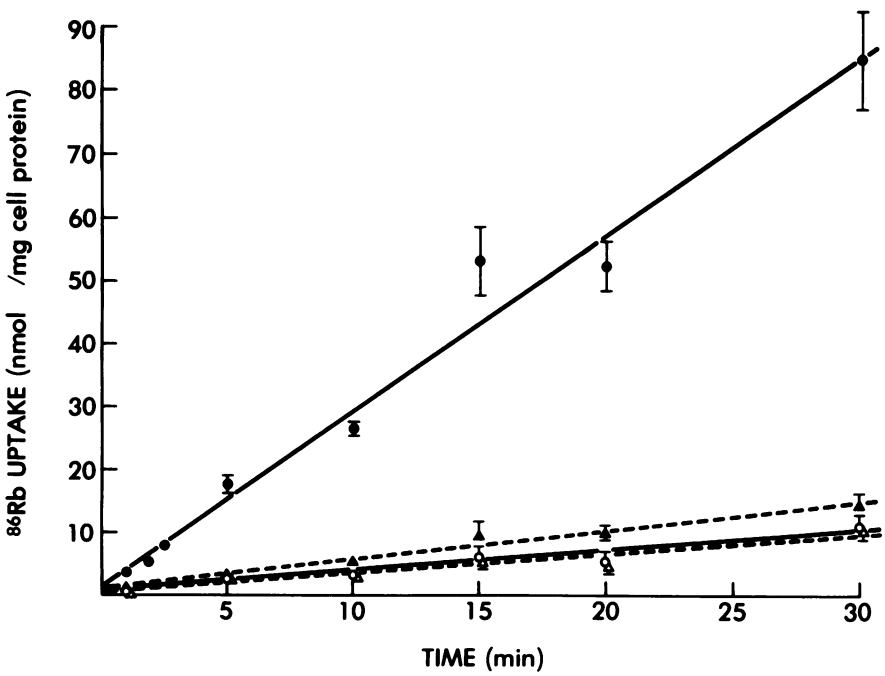

FIGURE 2 ^{86}Rb uptake by cultured rat hepatocytes. Uptake of ^{86}Rb (considered equivalent to uptake of potassium) was measured by incubating hepatocytes in a balanced electrolyte solution containing 5 mM K and tracer doses of ^{86}Rb after a 1-h preincubation in identical media without radioactive Rb. 5 mM ouabain was added to some solutions. Sodium chloride solutions contained 130 mM NaCl and 5 mM $NaHCO_3$. Lithium chloride solutions contained 130 mM LiCl and 5 mM choline bicarbonate. After incubation periods of 1–30 min, cells were removed from the radioactive medium, washed in ice-cold medium, and radioactivity was counted. Uptake is given as mean ±SE. Sodium chloride medium, (●); sodium chloride plus 5 mM ouabain, (▲); lithium chloride medium (○); lithium chloride plus 5 mM ouabain (△).

continued sodium-free perfusion. A transient choleresis was observed in both control and experimental livers beginning with the wash-out procedure at 90 min and lasting 30–45 min and was most marked in the control livers. This choleresis was observed in perfusions using both fluorocarbon-containing and fluorocarbon-free wash solution (Fig. 1, legend), and thus was not attributable to transient hypoxia or changes in perfusate osmolality. However, perfusate bicarbonate concentrations fell up to 20% during the first 90 min of perfusion and were restored to base line by the perfusate change at 90 min. Since bile flow is sensitive to changes in perfusate bicarbonate (see below), this may have accounted for at least a portion of the choleresis observed in these studies. Bile flow and B/P ratios of sucrose and PEG-900 did not differ between studies using fluorocarbon-containing and fluorocarbon-free wash solutions, and thus all data has been combined for presentation.

Liver weight expressed as a percentage of body weight tended to be greater following a 3-h perfusion with choline and/or lithium-containing perfusate than with control perfusate (Table III). This increase achieved statistical significance only for perfusate in which sodium (128 mM) was replaced by choline (25 mM) plus lithium (103 mM) or in which both sodium and bicarbonate were replaced by choline or lithium and Tricine, respectively.

Effects of sodium elimination on BADF. As shown in Table I, the choleretic response to infused taurocholate during sodium chloride perfusion was virtually abolished during lithium chloride perfusion. Biliary bile acid output was reduced by 77–89% during lithium chloride perfusion despite higher resultant taurocholate concentrations in the perfusate. During the third infusion period in which taurocholate was infused at rates equaling or exceeding T_m, toxicity was observed in all livers as indicated by falling bile flow and biliary taurocholate output.

Effects of sodium substitution on BAIF. Partial replacement (25 of 128 mM) of perfusate sodium by choline reduced bile flow compared with base line (B/A) by 30% (Table III). Replacement of the remaining 103 mM of sodium by choline further reduced bile flow by an additional 64% (Table III) and sodium essentially disappeared from bile (Table II). Thus, bile flow in period B in the presence of 128 mM choline was ~25% of that in the presence of 128 mM sodium. In contrast, replacement of the remaining 103 mM of perfusate sodium by lithium further reduced bile flow by only an additional 20% (Table III) and lithium replaced sodium as the predominant biliary cation (Table II). Total replacement of 128 mM sodium by 128 mM lithium reduced bile flow by only 17% (Table III). Bile flow in period B in the presence of 103 mM lithium and 25 mM choline was therefore ~65% of that in the presence of 128 mM sodium, while bile flow in the presence of 128 mM lithium was ~83% of that in the presence of 128 mM sodium.

Sodium largely disappeared from bile during sodium-free perfusion and was replaced by lithium during lithium perfusion (Table II). Biliary electrolyte composition was otherwise unaltered with the exception of the potassium concentration, which rose during perfusion with lithium- or choline-containing media to ~16±5 mM (Table II), concomitant with a rise in the perfusate potassium concentration to 17.5±5.7 mM, observations consistent with the known effects of Na,K-ATPase inhibition on extracellular potassium concentrations.

Sucrose and PEG-900 B/P ratios were unaltered by choline or lithium perfusion. Neither partial nor complete choline perfusion altered sucrose or PEG-900 B/P ratios (data not shown). In livers perfused with 103 mM lithium and 25 mM choline, sucrose B/P ratios averaged 0.23±0.05 at 1 h, 0.26±0.06 at 2 h, and 0.43±0.07 at 3 h, while PEG-900 B/P ratios averaged 5.56±0.92 at 1 h and 5.17±2.30 at 3 h. In livers perfused with 128 mM lithium, sucrose B/P ratios averaged 0.14±0.05 at 1 h, 0.21±0.07 at 2 h, and 0.29±0.06 at 3 h, while PEG-900 B/P ratios averaged 4.64±1.27 at 1 h and 4.00±1.10 at 3 h. None of these values differed significantly from control values except for the sucrose B/P ratios during the last 45 min of perfusion with 128 mM lithium which were lower ($P < 0.05$) than control values. Bile secretory pressure exceeded net portal perfusion pressure by 4 cm or more in most studies, averaging 5.9±3.2 cm above perfusion pressure in all studies.

Effects of bicarbonate elimination on BAIF. As shown in Tables IV and V, replacement of perfusate bicarbonate with Tricine reduced bile flow significantly, regardless of the accompanying cation. In sodium-perfused livers, bile flow fell 49%, biliary bicarbonate concentration fell 41%, and bicarbonate output fell 63%. These changes were not attributable to Tricine toxicity, as the addition of 10 mM Tricine to bicarbonate-containing perfusates did not reduce bile flow. In contrast, in preliminary studies addition of 25 mM Tricine to bicarbonate-containing perfusates did reduce bile flow by a mean of 31% (data not shown) suggesting that higher concentrations of Tricine can exert a modest cholestatic effect. Sucrose B/P ratios did not differ between control and bicarbonate-substituted livers.

Effects of sodium elimination on ATP hydrolysis. Na,K-ATPase-mediated ATP hydrolysis in rat liver and kidney homogenates was reduced 92 and 94% by substitution of lithium for sodium and 85 and 89% by substitution of choline for sodium (Table VI). Because

of the inherent imprecision in measuring Na,K-ATPase, particularly when it constitutes a small fraction of total ATPase activity (35), there is considerable uncertainty associated with measurement of Na,K-ATPase activity in the absence of sodium. In fact, Na,K-ATPase activity in the presence of lithium did not differ significantly from zero. In contrast, Mg-ATPase-mediated ATP hydrolysis was unaffected by sodium elimination.

Effects of sodium elimination on ^{86}Rb uptake by cultured hepatocytes. We used ouabain-suppressible ^{86}Rb uptake as a measure of Na,K-ATPase activity in these studies as rubidium has been shown to substitute effectively for potassium (48) and has been used successfully to measure Na,K-ATPase-mediated potassium influx in diverse tissues such as cardiac muscle (49), lymphocytes (50), fibroblasts (51), liver slices (52), and cultured hepatocytes (53, 54). Na,K-ATPase-mediated uptake of ^{86}Rb was taken to be the ouabain-suppressible fraction of total uptake. Ouabain was used in a concentration of 5 mM as preliminary studies indicated that 1 mM ouabain did not produce maximal suppression. LDH release by the cultured cells averaged 4.5±1.8%/h and was not altered by addition of ouabain or replacement of sodium.

As illustrated in Fig. 2, ^{86}Rb uptake was linear to 30 min. ^{86}Rb uptake averaged 2.78±0.17 nmol/min per mg cell protein in control medium and 0.45±0.04 nmol/min per mg in the presence of 5 mM ouabain. When sodium was replaced by lithium, ^{86}Rb uptake in the absence (0.314±0.042 nmol/min per mg) and presence (0.299±0.048 nmol/min per mg) of 5 mM ouabain did not differ significantly. Ouabain-suppressible ^{86}Rb uptake in lithium-containing medium (0.015±0.064 nmol/min per mg) did not differ from zero and was <0.7% of ouabain-suppressible ^{86}Rb uptake in sodium-containing medium (2.33±0.18 nmol/min per mg).

DISCUSSION

These experiments were designed to characterize the transport mechanisms responsible for formation of canalicular bile by the isolated perfused rat liver. The roles of sodium and Na,K-ATPase were assessed by replacement of perfusate sodium with choline, a large and relatively impermeant monovalent organic cation, or lithium, a relatively permeant monovalent inorganic cation that resembles sodium in both hydrated ionic radius and chemical properties. We found that substitution of lithium or choline for sodium essentially abolished Na,K-ATPase activity measured either as ouabain-suppressible ATP hydrolysis in liver homogenate or as ^{86}Rb transport by cultured hepatocytes, and also effectively eliminated taurocholate-induced choleresis by the perfused rat liver. In contrast, lithium perfusion only modestly reduced BAIF, suggesting that basal BAIF exhibits no specific requirement for sodium or Na,K-ATPase. We also confirmed the observation of Hardison and Wood (31) that replacement of perfusate bicarbonate by Tricine substantially reduces BAIF. Before attempting interpretation of these observations, it is appropriate to comment upon the experimental design and assumptions involved in these experiments.

The isolated perfused rat liver has been used extensively to study hepatic metabolism and transport. Fluorocarbon was used as an oxygen carrier in these studies because it permits ion substitution experiments that would be difficult or impossible with erythrocyte-containing perfusate. Comparative studies have indicated that bile flow[2] (55), oxygen consumption, gluconeogenesis, lactate and ketone body production, and ATP concentration (56) are similar for livers perfused with erythrocytes or fluorocarbon. Moreover, bile flow in these studies (1.21±0.33 μl/min per g at 30 min, 0.80±0.22 μl/min per g at 60 min in control livers) falls within the reported range [0.39–1.5 μl/min per g at 20–30 min (55, 57–61) and 0.31–1.4 μl/min per g at 60–120 min (58–61)] for isolated rat livers perfused without bile acid infusions. Bile secretory pressure exceeded perfusion pressure in these studies by 7.4±1.6 cm of water, a figure also within the range (4–9 cm of water) reported by previous investigators for erythrocyte-perfused livers (62, 63). The absolute magnitude of the bile secretory pressure (11.5±0.9 cm), however, was less than has been observed by others (16–20 cm) (62, 63). Livers perfused concurrently[2] with erythrocyte-containing perfusate exhibited bile secretory pressures (10.5 cm) similar to those observed in fluorocarbon-perfused livers. Thus, the lower bile secretory pressures observed in these studies are probably attributable to methodologic differences (e.g., time of measurement, perfusion pressure, lack of bile acid administration) rather than the use of fluorocarbon per se.

The B/P ratio of radiolabeled sucrose was measured in these studies to monitor possible changes in tight junction permeability. The ratios observed in control livers (0.21±0.07 at 1 h, 0.49±0.19 at 3 h) were higher than those reported for the intact rat [0.106–0.21 (64–67)], in which total bile flow is higher, but were within the reported range for the isolated rat liver [0.16–0.56 (68)] and did not differ from concurrent experiments using erythrocyte-containing perfusate.[2] The gradual increase in B/P ratio of sucrose observed during the 3 h perfusion is attributable in part to the expected increase with decreasing rates of bile formation (64) and may also reflect a gradual increase in junctional

[2] Van Dyke, R. W., and B. F. Scharschmidt. Unpublished observations.

permeability of the isolated liver. The important observation for the interpretation of these experiments is that sucrose B/P ratios did not differ between control and experimental livers.

It has been suggested that PEG-900 may represent a marker of total canalicular secretion, unlike solutes, such as erythritol, that are postulated to measure only net (secretion minus absorption) water flow (68, 69). PEG-900, therefore, was also used in these studies, in an attempt to assess potential changes in the relative contribution of secretion and absorption to total bile flow.

The terms BADF and BAIF as used in the context of this study also deserve comment. Given a plot of bile flow (ordinate) vs. bile acid output (abscissa), BAIF is conventionally defined as the extrapolated "y" intercept and BADF as the product of the slope and bile acid output. This approach, which in itself may be subject to error (70), was not applicable to these studies since taurocholate infusion failed to induce a choleresis during sodium-free perfusion. BAIF was therefore taken to be basal bile output after 1 h, by which time biliary bile acid concentration had fallen to 1.9±1.2 mM. This appears to be a reasonable approach since bile flow attributable to basal bile acid output estimated from the measured bile flow (0.8 μl/min per g), bile acid concentration (1.9 mM) and the slope of the line relating bile flow and bile acid output [0.009–0.017 μl/nmol (58, 60, 63)] is 0.014–0.026 μl/min per g or <5% of total. This calculated percentage may represent an underestimate when applied to very low biliary bile acid concentrations (70) where bile acid osmotic activity and thus BADF may be increased severalfold. However, basal bile output (and presumably that portion of bile flow attributable to basal bile acid output) was unaffected by ion substitution, and therefore the observed changes in bile flow are primarily attributable to changes in BAIF.

An important assumption underlying the interpretation of these experiments is that substitution of lithium for sodium effectively abolishes Na,K-ATPase-dependent ion transport in the perfused liver. This assumption is based on several considerations. We, and others, have shown that Na,K-ATPase manifests a high degree of cation selectivity and functions poorly if at all in the absence of sodium. In this study, Na,K-ATPase-mediated ATP hydrolysis in liver homogenates (Table VI) and Na,K-ATPase-mediated ^{86}Rb uptake in cultured hepatocytes (Fig. 2) were reduced to nearly unmeasurable levels after substitution of lithium for sodium. Although lithium has been shown to compete for either the potassium (71) or the sodium (72–74) site on Na,K-ATPase in other tissues, it does so poorly. In lithium-loaded human erythrocytes, the K_m for lithium is an order of magnitude higher than for sodium and maximal cation pumping is <25% of that seen with sodium (72, 73), while ATP hydrolysis by pig kidney Na,K-ATPase in lithium medium is only 5% of that in sodium medium (74). Nagel (75) has presented direct evidence that in frog skin lithium equilibrates passively across the cell membrane. It therefore appears reasonable to assume that lithium perfusion effectively eliminates Na,K-ATPase activity as well as sodium or lithium gradients capable of powering secondary active transport mechanisms.

In light of these considerations, it is not surprising that complete substitution of lithium and choline for perfusate sodium virtually abolished taurocholate-induced choleresis (BADF) and markedly decreased taurocholate transport. Hepatic uptake of taurocholate is known to be dependent upon both sodium and Na,K-ATPase (7–13). Residual taurocholate transport observed in the absence of sodium (14.0 nmol/min per g liver at a perfusate concentration of 167 μM taurocholate) is in rough agreement with that predicted (3.6 nmol/min per g liver) based on the observed sodium-independent uptake of taurocholate in cultured rat hepatocytes (0.125 nmol/min per mg cell protein per mM taurocholate) (11) assuming that 17% of liver weight is protein (13).

In contrast to the effect of sodium-free perfusion on taurocholate-induced choleresis and taurocholate transport, the effect of sodium-free perfusion on BAIF was quite unexpected. Specifically, substitution of lithium for sodium reduced BAIF by ≤20% and lithium replaced sodium as the predominant biliary cation (Tables II and III). This suggests that much of basal BAIF in the isolated perfused rat liver exhibits no specific sodium requirement and can be largely dissociated from Na,K-ATPase activity, or any other ion pump with a specific sodium requirement. This finding does not indicate that sodium is not important for BAIF. Indeed, in the intact organism, sodium accounts for a larger proportion of bile osmolality than any other solute (14, 15), and replacement of sodium with choline, a relatively impermeant cation, markedly reduced bile flow (Table III). These findings are consistent, however, with the view that much sodium movement associated with BAIF is passive (76). These observations do not exclude a role for Na,K-ATPase-mediated sodium transport in BAIF. Indeed, slightly lower bile flow was consistently noted during lithium perfusion (Tables I–V). This might reflect either the elimination of a Na,K-ATPase-dependent solute transport mechanism or, equally as likely, nonspecific effects of sodium removal and/or lithium replacement. The data presented here also do not exclude the additional possibility of a versatile ion pump other than Na,K-ATPase that might mediate both sodium and lithium active transport and thus BAIF. However, we

are not aware of a precedent for such a pump in other mammalian systems.

Other explanations for this unexpected finding also deserve consideration. It is conceivable, for example, that residual intracellular sodium might support some Na,K-ATPase activity even during sodium-free perfusions. However, bile flow was measured after 1 h of sodium-free perfusion, at which time sodium concentration in both perfusate (1.3±1.7 mM) and bile (0.5±1.3 mM) was very low and electrochemical gradients adequate to support ion transport were probably dissipated. This explanation therefore does not seem likely. Similarly, the observation that lithium perfusion did not increase the B/P ratio of radiolabeled sucrose nor alter bile secretory pressure argues against altered junctional permeability as an explanation for maintenance of bile flow during lithium perfusion. Finally, the B/P ratio of PEG-900 was similar to that previously reported for intact rats and the perfused rat liver (68, 69). Thus, it does not seem likely that lithium perfusion reduced both secretion and reabsorption so as to leave net flow unchanged.

If Na,K-ATPase and sodium-dependent transport mechanisms are not solely responsible for BAIF, what other transport mechanisms might be important? Hardison and Wood (31) observed that bicarbonate and/or proton transport may participate in BAIF. Similarly, we found that removal of bicarbonate from perfusate decreased BAIF by ~50% and biliary bicarbonate output by 63% (Table IV). Presumably, the remaining bicarbonate in bile originated from hepatic metabolic CO_2 production. Two mechanisms have been postulated for bicarbonate transport in mammalian cells, and either or both might mediate hepatic bicarbonate transport. Sodium/hydrogen exchange driven by the transmembrane electrochemical sodium gradient attributed to Na,K-ATPase (77) has been identified as an important mechanism for bicarbonate transport in the renal tubule (78, 79). Although the operation of such a mechanism during lithium perfusion cannot be rigorously excluded (77), it appears unlikely in light of the considerations outlined above. A primary hydrogen ion (or bicarbonate) pump is another possible mechanism for bicarbonate transport. Such a pump has been identified in mitochondria (80), bacteria (81), turtle urinary bladder (82), and the rat renal tubule (83, 84). Our findings suggest that a similar pump may play a role in BAIF.

ACKNOWLEDGMENTS

The authors would like to thank Dr. D. M. Bissell, Ms. Okhi Choun, and Mr. Mark Malamud for preparing the cultured hepatocytes.

This work was supported by National Institutes of Health research grant AM-26270, training grant AM-07007, Liver Core Center grant AM-26743, Research Career Development Award AM-00323 (Dr. Scharschmidt), research fellowship grant AM-06743 (Dr. Van Dyke), a grant from the American Liver Foundation (Dr. Van Dyke), and the Walter C. Pew Fund for Gastrointestinal Research.

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