Studies of Relationships among Bile Flow, Liver Plasma Membrane NaK-ATPase, and Membrane Microviscosity in the Rat

EMMET B. KEEFFE, BRUCE F. SCHARSCHMIDT, NANCY M. BLANKENSHIP, and ROBERT K. OCKNER, *Department of Medicine and Liver Center, University of California, School of Medicine, San Francisco, California 94143*

ABSTRACT Liver plasma membrane (LPM) NaK-ATPase activity, LPM fluidity, and bile acid-independent flow (BAIF) were studied in rats pretreated with one of five experimental agents. Compared with controls, BAIF was increased 24.6% by thyroid hormone and 34.4% by phenobarbital, decreased by ethinyl estradiol, but unchanged by propylene glycol and cortisone acetate. Parallel to the observed changes in BAIF, NaK-ATPase activity also was increased by thyroid hormone (40.8%) and decreased by ethinyl estradiol (26.2%). In contrast, NaK-ATPase activity failed to increase after phenobarbital but did increase 36% after propylene glycol and 34.8% after cortisone acetate. Thus BAIF and NaK-ATPase activity did not always change in parallel. The NaK-ATPase K_m for ATP was not affected by any of these agents.

LPM fluidity, measured by fluorescence polarization using the probe 1,6-diphenyl-1,3,5-hexatriene, was found to be increased by propylene glycol, thyroid hormone, and cortisone acetate, decreased by ethinyl estradiol, and unaffected by phenobarbital. Thus in these cases, induced changes in LPM fluidity paralleled those in NaK-ATPase activity. In no case did Mg-ATPase or 5'-nucleotidase activities change in the same direction as NaK-ATPase, and the activity of neither of these enzymes correlated with LPM fluidity, thus indicating the selective nature of the changes in LPM enzyme activity caused by the agents.

These findings indicate that LPM fluidity correlates with NaK-ATPase activity and may influence the activity of this enzyme. However, the nature of the role of LPM NaK-ATPase in bile secretion is uncertain and needs further study.

INTRODUCTION

Canalicular bile currently is thought to be formed by the active transport of solutes from the liver cell into the bile canaliculus, resulting in the generation of an osmotic gradient and, secondarily, water flow (1–3). In all species studied, 30–60% of this water movement depends on the transport of bile acids (1). The remainder of canalicular bile flow has been termed bile acid-independent flow (BAIF),[1] and has been attributed to the transport of sodium from the hepatocyte into the canaliculus by canalicular membrane NaK-ATPase (4).

Since the direct measurement of sodium transport across the canalicular membrane has not been possible, indirect methods have been required to examine this hypothesis. Frequently employed in such studies has been an assessment of the correlation in rats between experimentally induced changes in BAIF and NaK-ATPase activity of canaliculi-enriched liver plasma membranes (LPM). Agents administered over several days have been reported to increase (phenobarbital, thyroid hormone, taurocholate) or decrease (ethinyl estradiol) both BAIF and LPM NaK-ATPase activity (5–9). However, results of these experiments in different laboratories have not been entirely consistent. For example, phenobarbital treatment also has been associated with either no change (10–12) or a decrease (13) in LPM NaK-ATPase activity, and dexa-

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[1] *Abbreviations used in this paper*: BAIF, bile acid-independent flow; LPM, isolated liver plasma membrane.

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methasone has been shown to decrease BAIF while increasing NaK-ATPase activity (14).

We have attempted to clarify these apparent inconsistencies by systematically comparing NaK-ATPase activity, measured by a more reproducible coupled enzyme assay (15), with BAIF after treatment with five agents. In addition, because several studies have suggested that the activity of NaK-ATPase may be at least partially dependent on the lipid composition and fluidity of the plasma membrane (6, 16, 17), we evaluated the possibility that these membrane properties might correlate with experimental changes in NaK-ATPase activity. The results show that NaK-ATPase activity is uniformly correlated with LPM fluidity, but that the relationship between NaK-ATPase activity and BAIF is quite inconsistent. This latter finding, along with recent cytochemical data localizing NaK-ATPase to the sinusoidal and lateral hepatocyte surfaces (18, 19), suggests that current concepts of bile formation need reevaluation.

METHODS

Animals and chemical agents. Nonfasted male Sprague-Dawley rats were used in all experiments and fed standard chow (Berkeley Standard Diet, Feedstuff Processing Co., San Francisco, Calif.). Animals were kept in wire-bottom cages and maintained in a constant temperature environment (25°C) with alternating 12-h light and dark cycles.

Drugs and hormones were administered to experimental animals as follows: (*a*) Na-3,3'5-triiodo-L-thyroxine (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 0.5 mM NaOH (150 μg/ml) and injected intraperitoneally in three 50 μg/100 g body wt doses at 48 h intervals. (*b*) 17α-ethinyl estradiol (Sigma Chemical Co.) was dissolved in propylene glycol (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) (5 mg/ml) and injected subcutaneously for 5 d in daily doses of 0.5 mg/100 g body wt. (*c*) Phenobarbital sodium (J. T. Baker Chemical Co., Philippsburg, N.H.) was dissolved in 0.9% NaCl (40 mg/ml) and injected intraperitoneally for 5 d in daily doses of 8 mg/100 g body wt. (*d*) Cortisone acetate (Merck Sharp & Dohme Canada Ltd, Montreal, Quebec) in a saline suspension (25 mg/ml) was injected intraperitoneally for 5 d in daily doses of 10 mg/100 g body wt. Control animals were injected with vehicle in the same manner as experimental animals. Propylene glycol (0.1 mg/100 g body wt) served as both control for ethinyl estradiol and a separate experiment agent. Bile flow studies and LPM preparations were begun at 9:00 a.m., 24 h after the last injection. All animals weighed 260–370 g at the time of sacrifice.

Bile flow studies. Under anesthesia, the common bile duct was cannulated with polyethylene tubing (PE 90, Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. Y.) tapered at the tip. The cannula was positioned and secured just below the confluence of the major hepatic ducts and exteriorized through the closed abdominal incision. After operation animals were placed in restraining cages (20) and body temperature maintained at 37°C by a heating lamp. Bile was then collected and weighed in tared tubes for 3 h in eight 15-min, and two 30-min intervals. At the completion of bile secretory studies, the liver was removed and weighed. Total bile acids were determined on aliquots from each collection period by the enzymatic method using purified 3-α-hydroxy-steriod dehydrogenase (Worthington Biochemical Corp., Freehold, N. J.) as described by Talalay (21) and modified by Amirand and Small (22). BAIF was determined by extrapolating the calculated linear regression of bile flow vs. bile acid excretion to zero bile acid excretion.

Preparation of LPM. LPM were prepared from control and experimental animals by discontinuous sucrose density gradient centrifugation by a modification of the methods of Song et al. (23) and Boyer and Reno (24) as described (15). These animals had not been used for the bile flow studies. The final protein concentration was 1.3–3.0 mg of protein/ml, as measured by the method of Lowry et al. (25) using human serum albumin (Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) as a standard.

Transmission electron microscopy was performed by Dr. Albert Jones of the Departments of Medicine and Anatomy, and the Liver Center, University of California, San Francisco. Two LPM preparations were fixed overnight at 4°C in 4% glutaraldehyde, concentrated by Millipore filtration (Millipore Corp., Bedford, Mass.), postfixed in 2% osmium tetroxide buffered with 0.1 M cacodylate, pH 7.4, dehydrated in ethanol, and embedded in Epon 812 (Shell Oil Co., Houston, Tex.). Thin sections were stained with uranyl acetate lead citrate and examined with a Philips EM-300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N. J.).

ATPase assays. The specific activity of Mg-ATPase and NaK-ATPase was determined on homogenates and LPM preparations in duplicate or triplicate assays after overnight storage at 4°C. A coupled enzyme assay, previously validated by us for use in LPM, was employed in which the production of ADP in the ATPase reaction is linked to NADH oxidation with pyruvate kinase and lactic dehydrogenase (15). The apparent K_m for ATP and NaK-ATPase activity at V_{max} were calculated by nonlinear regression analysis of reaction rates measured over a range of ATP concentration from 0.25 to 5.0 mM. In addition, the conventional ATPase assay was performed on each sample as outlined (15). Because the coupled and the conventional assay gave parallel results, only those ATPase activities determined by the coupled technique are presented.

Marker enzyme assays. All assays were performed on LPM stored overnight at 4°C. Alkaline phosphatase was measured by a modification of the method of Pekarthy et al. (26) in cuvettes containing 1.0 ml reaction mixture consisting of 46.7 μmol of glycine (pH 10.5), 2.3 μmol of $MgSO_4$ and $ZnSO_4$, 5.6 μmol of *p*-nitrophenylphosphate, and the enzyme preparation (30–60 μg of LPM protein). The formation of *p*-nitrophenol at 37°C was estimated on a recording spectrophotometer at 410 nm and was linear over 15 min. 5'-Nucleotidase, a membrane marker, was determined as described (9) except that the reaction was initiated by the addition of LPM. The method of Tisdale (27) for succinate-cytochrome *c* reductase, a marker for mitochondria, and the method of Masters et al. (28) for NADPH-cytochrome *c* reductase, a marker for microsomes, were modified as outlined by Wannagat et al. (9).

Studies of membrane fluidity. Membrane fluidity of the LPM preparations was determined by fluorescence polarization as described by Shinitzky and co-workers (29, 30) using an Elscint MVla microviscosimeter (Elscint Inc., Hackensack, N. J.). The fluorescent hydrocarbon, 1,6-diphenyl-1,3,5,-hexatriene (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was used as a probe for monitoring fluidity of the hydrophobic core of the LPM. The probe was kept as a 2 mM stock solution in tetrahydrofuran and was diluted 1:2,000 in phosphate-buffered saline with vigorous mixing im-

mediately before use. Approximately 200 μg of LPM protein was incubated in this probe-phosphate-buffered saline mixture for 30 min at 37°C on a metabolyte water bath shaker (New Brunswick Scientific Co., Inc., Edison, N. J.) at 100 oscillations/min. The fluorescence polarization of this mixture was then determined, while LPM preincubated in phosphate-buffered saline without probe was used as a blank. The Elscint microviscosimeter measures the intensity of emitted fluorescent light oriented parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the incident polarized light. Fluorescence polarization, which is calculated by the formula $(I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$ approaches zero in a fluid medium allowing rapid rotation of probe molecules and approaches a limiting value (0.46 for diphenylhexatriene) in a viscous environment. Fluorescence polarization is thus inversely related to membrane fluidity and directly related to microviscosity.

LPM lipid studies. Lipids were extracted from LPM according to the method of Folch et al. (31). LPM total cholesterol was quantified as outlined (9), and phospholipid was measured by the method of Bartlett (32), as modified by Marinetti (33).

Statistical methods. Student's *t* test was used for statistical analysis of differences between experimental and respective control groups (34); *P* values of <0.05 were considered to be statistically significant. The Michaelis-Menton constants, V_{max} and apparent K_m, were determined on a digital computer (Hewlett Packard Co., Palo Alto, Calif.) using a nonlinear least squares program provided by the University of California Cardiovascular Research Institute. In all statistical analyses, propylene glycol, thyroid, phenobarbital, and cortisone acetate treatment groups were compared to saline control, while propylene glycol also served as control for ethinyl estradiol-treated animals.

RESULTS

Effects of drugs and hormones on body and liver weight. Over the 5-d treatment periods, ethinyl estradiol and cortisone acetate significantly decreased body weight by 11.5 and 7.7%, respectively, while phenobarbital resulted in a 15.9% gain in body weight (Table I). Liver weight of animals treated with phenobarbital was 39.4% greater and cortisone acetate 18.5% greater than control. The greater liver weight in these two treatment groups could not be explained by differences in total body weight at time of sacrifice.

Bile flow studies. Thyroid hormone and phenobarbital significantly increased both total bile flow and BAIF, while ethinyl estradiol decreased both. The changes in total bile flow observed in these studies primarily reflect the changes in BAIF, because none of the experimental agents significantly altered bile acid excretion (Table II). Thyroid hormone did result in a 41% increase in bile acid excretion that was comparable to that noted by Layden and Boyer (8), but did not reach statistical significance in our studies. When bile flow and bile acid excretion were expressed per gram liver rather than per 100 g body wt, the effect of phenobarbital on bile flow was smaller and statistically insignificant, while the thyroid hormone and ethinyl estradiol-induced changes remained significant. Cortisone acetate and propylene glycol failed to influence any aspect of bile formation measured in these studies.

LPM characteristics. Two LPM preparations from control animals were studied by electron microscopy and revealed membrane vesicles and paired membrane sheets joined by tight junctions (Fig. 1). Intracellular organelles were only rarely identifiable. Specific activities of the LPM enzymes, NaK-ATPase, Mg-ATPase, and 5'-nucleotidase were enriched 50±9- (mean±SE), 27±3-, and 23±2-fold compared to homogenate in all groups. The degree of enrichment of these enzymes was not significantly different from control for any treatment group. Control LPM only were further characterized enzymatically by determination of alkaline phosphatase specific activity with the LPM: homogenate ratio (mean±SE) showing 27.8±4.8-fold enrichment.

In contrast to these LPM enzymes, LPM: homogenate ratios of organelle enzyme marker activity in all treatment groups were low, confirming that there was

TABLE I
Effects of Drugs and Hormones on Body and Liver Weight

Treatment	Change in body weight	*P*	Liver weight	*P*
	%		g	
Controls:				
Saline (6)	+0.3±0.4	—	11.9±0.7	—
Propylene glycol (9)	+2.7±1.4	NS	13.6±0.7	NS
Thyroid (6)	−1.1±0.9	NS	12.7±0.2	NS
Ethinyl estradiol (9)	−11.5±1.5	<0.001	12.0±0.8	NS
Phenobarbital (12)	+15.9±2.2	<0.001	16.6±0.5	<0.001
Cortisone acetate (15)	−7.7±1.0	<0.001	14.1±0.4	<0.02

Adult male Sprague-Dawley rats received control or experimental injections as described in Methods. Change in body weight reflects percent change from base line to completion of 5-d treatment period. Sample sizes are indicated in parentheses and all values are expressed as mean±SE. *P*, significance of difference from appropriate control.

TABLE II
Effects of Drugs and Hormones on BAIF and Bile Acid Excretion

Treatment	BAIF	Bile acid excretion
	μl/min/100 g body wt	*μmol/min/100 g body wt*
Controls:		
Saline (6)	4.92±0.29	0.114±0.010
Propylene glycol (6)	4.79±0.87	0.123±0.006
Thyroid (5)	6.13±0.29*	0.161±0.023
Ethinyl estradiol (6)	3.71±0.21*	0.116±0.010
Phenobarbital (7)	6.61±0.42*	0.116±0.005
Cortisone acetate (7)	4.43±0.29	0.117±0.006

Bile was collected from conscious restrained rats after 5 d of control or experimental injections, and BAIF and bile acid excretion determined as described in Methods. Sample sizes are indicated in parentheses and all values are expressed as mean±SE.

* Significantly different from control at $P < 0.01$ to <0.001.

no important mitochondrial or microsomal contamination (Table III). The percent recovery of these organelle enzyme markers, as shown in Table IV, was not significantly different from control for any treatment group. Finally, the yield of LPM protein, as shown in Table III, was significantly increased by thyroid hormone when expressed both per gram liver and per 100 g body wt.

Effects of drugs and hormones on LPM activity. The results of various treatment programs on LPM NaK-ATPase activity are shown in Table III. As indicated in Table II and Table III, thyroid hormone increased both BAIF and NaK-ATPase activity, while ethinyl estradiol decreased both. However, the increased BAIF in phenobarbital-treated rats was not associated with a change in ATPase activity. Conversely, propylene glycol and cortisone acetate, which increased ATPase, did not affect BAIF. Thus, LPM NaK-ATPase activity and BAIF changed in parallel in only two of the five treatment groups.

To investigate whether the observed changes were specific for NaK-ATPase activity or merely represented non-specific effects on all LPM enzymes, the activities of Mg-ATPase and 5'-nucleotidase were studied. In no case did Mg-ATPase or 5'-nucleotidase change in the same direction as NaK-ATPase (Table III), thus indicating the selective nature of altered LPM enzyme activity.

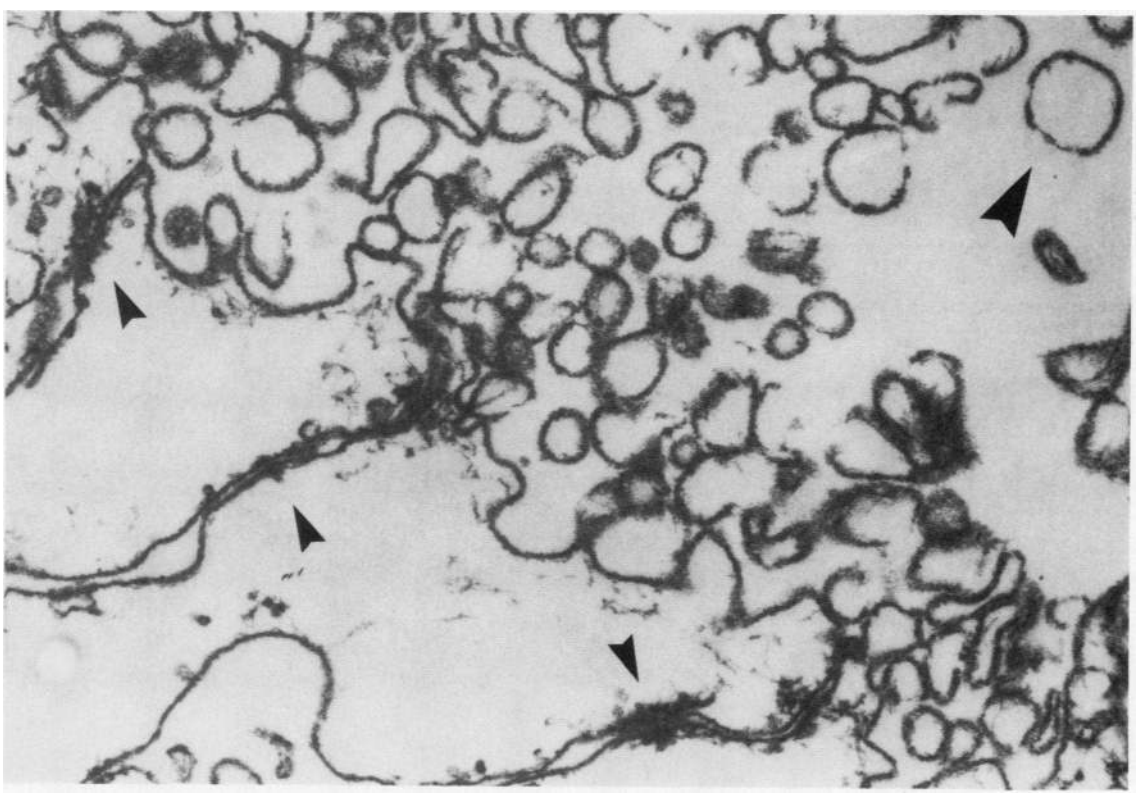

FIGURE 1 Electron micrograph of LPM prepared from the liver of a control animal. Paired membrane strips joined by tight junctions (small arrows) and membrane vesicles (large arrow) predominate, thus suggesting that lateral surfaces as well as sinusoidal and/or canalicular surfaces are present. Intracellular structures such as nuclei and mitochondria are not evident (×40,000).

TABLE III

Effect of Drugs and Hormones on LPM Protein Yield, LPM Enzyme Activity, and Intracellular Organelle Enzyme Markers

Treatment	LPM protein		NaK-ATPase	Mg-ATPase	5'-Nucleotidase	Succinate-cytochrome *c* reductase	NADPH-cytochrome *c* reductase
	mg protein/g liver	*mg protein/ 100 g body wt*	*μmol Pi/mg protein/h*			*LPM:homogenate ratio*	
Controls:							
Saline	0.39±0.03 (22)	1.66±0.14 (22)	20.40±1.37 (27)	72.00±5.39 (27)	66.93±5.28 (11)	0.13±0.06 (6)	0.23±0.02 (6)
Propylene glycol	0.50±0.04 (9)	2.06±0.19 (9)	27.75±2.04* (9)	56.62±4.67 (9)	42.01±5.71* (9)	0.23±0.04 (6)	0.15±0.02‡ (6)
Thyroid	0.70±0.10§ (6)	2.33±0.28‡ (6)	28.73±2.42‡ (6)	60.65±3.37 (6)	70.41±7.62 (6)	0.17±0.04 (6)	0.19±0.02 (6)
Ethinyl estradiol	0.38±0.04 (9)	1.63±0.15 (9)	20.47±2.59‡ (9)	149.67±11.73§ (9)	55.54±7.23 (6)	0.13±0.03‡ (6)	0.15±0.02 (6)
Phenobarbital	0.31±0.03 (12)	1.56±0.16 (12)	20.34±1.34 (12)	52.42±3.73‡ (12)	30.40±2.34§ (12)	0.37±0.09 (9)	0.24±0.04 (9)
Cortisone acetate	0.49±0.05 (15)	1.94±0.19 (15)	27.50±1.49* (12)	68.53±5.91 (12)	38.40±3.88§ (15)	0.16±0.03 (12)	0.14±0.02‡ (12)

Values are mean±SE. The number of individual LPM preparations studied is shown in parenthesis. Significance of difference vs. control:
* $P < 0.01$.
‡ $P < 0.05$.
§ $P < 0.001$.

TABLE IV

Effect of Drugs and Hormones on Percentage of Total Hepatic Enzyme Activity Recovered in LPM

Treatment	NaK-ATPase	Mg-ATPase	5'-Nucleotidase	Succinate-cytochrome *c* reductase	NADPH-cytochrome *c* reductase
			% recovery		
Controls:					
Saline	22.6±8.9 (6)	7.5±1.4 (6)	7.3±0.6 (6)	0.03±0.01 (6)	0.06±0.01 (6)
Propylene glycol	14.9±5.3 (6)	5.8±0.4 (6)	5.8±1.0 (6)	0.08±0.02 (6)	0.05±0.01 (6)
Thyroid	10.6±2.0 (6)	8.4±1.3 (6)	8.6±1.5 (6)	0.07±0.02 (6)	0.07±0.01 (6)
Ethinyl estradiol	19.8±7.4 (6)	11.7±1.8* (6)	6.1±1.0 (6)	0.04±0.01 (6)	0.05±0.01 (6)
Phenobarbital	10.8±1.9 (12)	5.3±0.6 (12)	5.3±0.6 (12)	0.10±0.03 (9)	0.05±0.01 (9)
Cortisone acetate	12.3±2.2 (12)	7.7±1.4 (12)	6.3±0.5 (15)	0.06±0.01 (12)	0.05±0.01 (12)

Values (mean±SE) were obtained by the following calculation: (total LPM enzyme activity/gram liver)/(total homogenate enzyme activity/gram liver) × 100. The number of individual LPM preparations studied is shown in parentheses. Significance of difference vs. control:
* $P < 0.002$.

TABLE V
Effect of Drugs and Hormones on ATPase Enzyme Kinetics

Treatment	NaK-ATPase		Mg-ATPase	
	K_m	V_{max}	K_m	V_{max}
	mM	*μmol Pi/mg LPM protein/h*	*mM*	*μmol Pi /mg LPM protein/h*
Controls:				
Saline (12)	0.43±0.07	26.45±1.38	0.35±0.01	76.02±0.64
Propylene glycol (6)	0.49±0.08	30.89±1.66	0.54±0.04*	59.01±1.30*
Thyroid (6)	0.45±0.02	31.30±0.36*	0.44±0.06	67.09±2.83*
Ethinyl estradiol (9)	0.25±0.09	22.66±1.96*	0.50±0.20	164.09±2.12*
Phenobarbital (12)	0.56±0.08	22.29±1.01	0.38±0.03	55.81±1.33*
Cortisone acetate (12)	0.48±0.07	30.45±1.41	0.47±0.01*	74.87±0.65

Values are mean±SE.
* Significance of difference from control (propylene glycol is control for ethinyl estradiol) at $P < 0.05$ to <0.001.

As shown in Table IV, percent recovery of each enzyme in LPM from liver homogenate after the various treatment programs was essentially unaffected by the various treatments. Therefore, changes noted in enzyme specific activity in Table III do not seem likely to simply represent drug-induced changes in enzyme recovery.

The effect of drug and hormone treatment on ATPase enzyme kinetics is shown in Table V. The NaK-ATPase K_m for ATP was not affected by any of the agents tested. Comparing each treatment group, mean NaK-ATPase activity at V_{max} ranged from 9 to 30% (mean 14%) greater than its respective specific activity on the standard assay (Table III). In each case, V_{max} paralleled measured specific activity with the changes in V_{max} after thyroid and ethinyl estradiol treatments reaching significance.

Changes in Mg-ATPase activity at V_{max} were generally similar to those in specific activity. Unlike NaK-ATPase, the Mg-ATPase K_m for ATP was significantly increased by propylene glycol and cortisone acetate, but there were no consistent associated changes in V_{max}.

Effects of drugs and hormones on LPM microviscosity. LPM microviscosity was studied to evaluate the possibility that experimentally induced changes in NaK-ATPase activity and/or BAIF might be associated with changes in this physical property of the liver cell membrane. Results are shown in Table VI expressed as fluorescence polarization, and changes in NaK-ATPase activity and BAIF are indicated for comparison. The increased NaK-ATPase activity and BAIF seen with thyroid hormone were associated with decreased LPM microviscosity, while reciprocal

TABLE VI
Effects of Drugs and Hormones on BAIF, NaK-ATPase Activity, and LPM Microviscosity

Agent	BAIF	NaK-ATPase activity	Fluorescence polarization
	μl/min/100 g body wt	*μmol Pi/h/100 g body wt*	
Controls:			
Saline	4.92±0.29	33.40±2.63	0.317±0.003
Propylene glycol	4.79±0.87 (−)	56.17±4.93* (↑)	0.299±0.004‡ (↓)
Thyroid	6.13±0.29* (↑)	64.92±6.93* (↑)	0.294±0.003* (↓)
Ethinyl estradiol	3.71±0.21* (↓)	34.39±6.71§ (↓)	0.326±0.002§ (↑)
Phenobarbital	6.61±0.42‡ (↑)	32.10±4.26 (−)	0.307±0.003 (−)
Cortisone acetate	4.43±0.29 (−)	52.94±5.40* (↑)	0.300±0.003‡ (↓)

Values are mean±SE. Fluorescence polarization was determined on a microviscosimeter as outlined in Methods. Significance of difference from control indicated by arrows (↑, ↓) and no difference by (−). NaK-ATPase activity is expressed per 100 g body wt; significance of difference from control is indicated:
* $P < 0.001$.
‡ $P < 0.01$.
§ $P < 0.02$.

changes were observed with ethinyl estradiol. However, in phenobarbital-treated animals, bile flow increased without significant change in either ATPase activity or microviscosity, while in propylene glycol and cortisone acetate-treated rats the increased ATPase activity and decreased membrane microviscosity were not accompanied by a change in bile flow. The activity of the other LPM enymes, Mg-ATPase and 5'-nucleotidase, showed no consistent relationship to LPM microviscosity.

Effect of drugs and hormones on LPM cholesterol and phospholipid content and ratio. Because the cholesterol:phospholipid ratio is an important determinant of membrane fluidity, the content of these two lipids were determined in LPM from each of the treatment groups and their molar ratio was calculated (Table VII). Ethinyl estradiol administration caused an increase in LPM cholesterol and cholesterol:phospholipid ratio, consistent with the previously noted increase in microviscosity (Table VI). Conversely, propylene glycol, thyroid, and cortisone acetate treatments decreased both LPM microviscosity and cholesterol, but did not alter the cholesterol:phospholipid ratio.

DISCUSSION

These studies lead to two important conclusions: (*a*) the postulated role of LPM NaK-ATPase activity in the generation of BAIF needs to be reevaluated, since enzyme activity and BAIF did not consistently change in parallel; and (*b*) fluidity of LPM may be an important determinant of NaK-ATPase activity, since in each treatment group LPM fluidity paralleled the activity of this enzyme.

TABLE VII
Effect of Drugs and Hormones on LPM Total Cholesterol and Phospholipids

Treatment	Total cholesterol	Phospholipid	Cholesterol: phospholipid ratio
	μmol/mg protein		
Controls:			
Saline (6)	0.233±0.018	1.074±0.209	0.25±0.04
Propylene glycol (5)	0.132±0.010*	0.804±0.026	0.17±0.01
Thyroid (6)	0.139±0.005*	0.636±0.026	0.22±0.01
Ethinyl estradiol (5)	0.297±0.044*	1.137±0.131	0.26±0.02*
Phenobarbital (10)	0.203±0.006	1.063±0.099	0.20±0.02
Cortisone acetate (12)	0.156±0.009*	0.787±0.029	0.20±0.01

Values are mean±SE. Sample sizes are indicated in parentheses.
* Significance of difference vs. control at $P < 0.01$ to <0.001.

The formation of BAIF has been attributed to active transport of sodium by canalicular LPM NaK-ATPase (1). Substantial evidence indirectly supports this hypothesis: (*a*) LPM enriched in canaliculi (23, 35) are also enriched in NaK-ATPase activity compared to homogenate (5, 7, 9, 24); (*b*) canalicular bile flow is linearly related to biliary sodium output both in the basal state and after experimental manipulation of bile flow (36–38); and (*c*) drugs or hormones given over several days have been reported to increase or decrease BAIF with parallel changes noted in LPM NaK-ATPase activity (5–9). In agreement with others, we found that thyroid hormone increased both BAIF and NaK-ATPase activity and that ethinyl estradiol decreased both. However, phenobarbital choleresis was not associated with a change in ATPase activity, while cortisone acetate and propylene glycol increased ATPase activity without altering BAIF. Thus, our studies of BAIF and NaK-ATPase activity along with those of some others (10–14) do not support a direct correlation between NaK-ATPase activity and BAIF in all experimental situations.

Three additional factors need to be considered in the interpretation of these indirect studies in which BAIF and LPM NaK-ATPase activity are compared: (*a*) the portion of the hepatocyte surface membrane harvested by LPM preparative techniques; (*b*) potential in vivo regulatory factors of NaK-ATPase activity; and (*c*) the specific location of NaK-ATPase in the liver cell membrane. First, it is likely that various membrane preparative techniques yield LPM comprised of different proportions of sinusoidal, lateral, and canalicular surfaces, and thus account for some of the inconsistencies among different laboratories. Morphologic studies suggest that lateral surfaces isolate as membrane sheets joined by tight junctions and that sinusoidal and canalicular surfaces both isolate as membrane vesicles (39). In the present study, both membrane sheets and vesicles were present on electron microscopy, suggesting that lateral surfaces as well as sinusoidal and/or canalicular surfaces were represented. The 27.8-fold enrichment of alkaline phosphatase, shown cytochemically to be present only on the canalicular surface (18, 19) supports the presence of canalicular membranes among the membrane vesicles in our LPM.

A second important factor in the interpretation of these studies employing an indirect approach of comparing BAIF, an in vivo event, to NaK-ATPase activity, an in vitro measurement, is failure to account for possible in vivo regulatory factors of NaK-ATPase activity, such as bile acids and calcium ions (24, 40).

Finally, in cytochemical studies of the rat hepatocyte employing a modification of the Ernst technique, NaK-ATPase was localized to the sinusoidal and lateral,

but not canalicular, portions of the hepatocyte surface membrane (18, 19). The absence of NaK-ATPase on the canalicular surface constitutes strong evidence against the hypothesis that BAIF is generated by ATPase-mediated transport of intracellular sodium across the canalicular membrane. NaK-ATPase in sinusoidal and lateral membranes might still play a role in bile formation, either by contributing to paracellular Na^+ and water flow (41), or, as in other secretory epithelia, by generation of an electrochemical sodium gradient which in turn supplies the driving force for bile flow via a sodium-coupled transport system (42).

Of particular interest in the present studies is the demonstration that alteration of a physical property of LPM, i.e., fluidity, correlated directly with a functional property, i.e., NaK-ATPase activity. LPM fluidity was determined by measurement of microviscosity by fluorescence polarization using the probe diphenylhexatriene, which localizes to the hydrophobic core of the membrane (43, 44). In a previous study, using electron spin resonance, LPM fluidity in ethinyl estradiol-treated rats was found to be decreased in association with decreased NaK-ATPase activity and bile flow (6). In addition to having confirmed the associated decrease in membrane fluidity and NaK-ATPase activity after ethinyl estradiol treatment, we have established the converse, i.e., that fluidity and enzyme activity are both increased in rats treated with propylene glycol, thyroid hormone, and cortisone acetate. Furthermore, ethinyl estradiol treatment resulted in increased LPM cholesterol and cholesterol:phospholipid ratio, which is one of several membrane compositional factors that determine fluidity (17). An increase in this ratio results in a more highly ordered membrane with increased microviscosity or lowered fluidity. As expected, propylene glycol, thyroid, and cortisone acetate treatments decreased both LPM microviscosity and cholesterol content, but did not alter the cholesterol:phospholipid ratio (Tables VI and VII).

The parallel relationship between membrane fluidity and NaK-ATPase activity has received further support from investigations showing decreased LPM NaK-ATPase activity and fluidity after in vitro exposure to chlorpromazine and some of its metabolites (45) and, in newborn rat LPM, after lithocholate exposure in utero (46). However, work in progress evaluating the effects of bile acids on LPM fluidity by electron spin resonance suggests that inhibition of NaK-ATPase activity by taurocholate and taurochenodeoxycholate may not be accompanied by parallel changes in fluidity (unpublished observations). Thus one must be cautious in attributing altered NaK-ATPase activity to changes in fluidity of the LPM core per se. Analysis of fluidity in other regions of the LPM as well as more information regarding the mechanism by which changes in fluidity are produced will be necessary to better understand the interrelationships among membrane physical properties, NaK-ATPase activity and bile flow.

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