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Research Article

The relationship between bile flow and Na+,K+-ATPase activity in liver plasma membranes enriched in bile canaliculi was studied in rats treated with ethinyl estradiol, phenobarbital, or 20-methyl cholanthrene. In comparison with controls (1.49+/-0.12 microliter/min per g liver), bile flow was significantly diminished by ethinyl estradiol, increased by phenobarbital, and unchanged by 20-methyl cholanthrene or the solvent, propanediol (0.92+/-0.31, 2.50+/-0.21, 1.62+/-0.18, and 1.64+/-0.30 microliter/min per g liver, respectively). The corresponding values for canalicular Na+,K+-ATPase activity were 80.7+/-19.2, 50.0+/-18.4, 231.7+/-42.6, 82.7+/-30.7, and 143.6+/-55.3 micronmol Pi/h per g liver. Canalicular Na+,K+-ATPase activity was significantly correlated (r=0.785, n=31) with bile flow. These findings support the hypothesis that a fraction of bile flow is related to Na+,K+-ATPase activity and canalicular Na+ transport.

Relationship between Bile Flow and Na^+,K^+-Adenosinetriphosphatase in Liver Plasma Membranes Enriched in Bile Canaliculi

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ABSTRACT The relationship between bile flow and Na^+,K^+-ATPase activity in liver plasma membranes enriched in bile canaliculi was studied in rats treated with ethinyl estradiol, phenobarbital, or 20-methyl cholanthrene. In comparison with controls (1.49±0.12 μl/min per g liver), bile flow was significantly diminished by ethinyl estradiol, increased by phenobarbital, and unchanged by 20-methyl cholanthrene or the solvent, propanediol (0.92±0.31, 2.50±0.21, 1.62±0.18, and 1.64±0.30 μl/min per g liver, respectively). The corresponding values for canalicular Na^+,K^+-ATPase activity were 80.7±19.2, 50.0±18.4, 231.7±42.6, 82.7±30.7, and 143.6±55.3 μmol P_i/h per g liver. Canalicular Na^+,K^+-ATPase activity was significantly correlated ($r = 0.785$, $n = 31$) with bile flow. These findings support the hypothesis that a fraction of bile flow is related to Na^+,K^+-ATPase activity and canalicular Na^+ transport.

INTRODUCTION

The formation of canalicular bile is thought to result from transport of solutes across the canalicular membrane of the hepatocyte and osmotic water flow (1). Although a fraction of bile flow is related to the secretion of bile acids, a bile acid-independent fraction is believed to result from active transport of sodium into the bile canaliculi (1, 2). It has been suggested that the formation of the bile acid-independent fraction is regulated by the enzyme Na^+,K^+-stimulated adenosinetriphosphatase (Na^+,K^+-ATPase) (2, 3). This view is supported by the finding that Na^+,K^+-ATPase is present in fractions of liver plasma membranes enriched in bile canaliculi (3). Furthermore, a parallel effect of thyroid hormone on Na^+,K^+-ATPase activity and on bile acid-independent bile flow has recently been demonstrated (4). In the present study, bile acid-independent bile formation was decreased by ethinyl estradiol or increased by phenobarbital administration to rats, as previously shown by other investigators (5, 6). Subsequently, Na^+,K^+-ATPase activity in canalicular membranes and bile flow were investigated in the same animals.

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METHODS

Animals and treatments. Male specific-pathogen free Sprague-Dawley rats (Tierfarm Hartmut and Vos, Tuttlingen, W. Germany) were maintained on a standard rat diet (Altromin 300R, Altromin GmbH, Lage, W. Germany) and tap water ad lib. The body and liver weights are given in Table I. Seven rats were treated for 5 days with daily subcutaneous injections of 5 mg/kg body wt 17-ethinyl estradiol (Sigma Chemical Co., St. Louis, Mo.) dissolved in 5 mg/ml propanediol. This dosage schedule is known to induce cholestasis (7). Five animals were treated for 4 days with daily intraperitoneal injections of 80 mg/kg body wt phenobarbital (Siegfried AG, Zofingen, Switzerland) dissolved in 80 mg/ml propanediol. This dosage has been used previously to induce a maximal increase in bile flow (8). Five rats received daily intraperitoneal injections of 20 mg/kg body wt of 20-methyl cholanthrene (Sigma Chemical Co.) suspended in 20 mg/ml propanediol. This dosage has been shown to induce microsomal enzymes without affecting bile flow (8). Seven rats received equal volumes (1 ml/kg body wt) of the solvent, propanediol, only, whereas seven additional animals received no treatment at all. All injections were given between 8:00 and 8:30 a.m. 24 h after the final treatment, each animal was anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg body wt). Body temperature was monitored with a rectal thermometer and maintained at 37.5±0.5°C. The common bile duct was cannulated with polyethylene 10 tubing and the bile was collected in tared tubes and then weighed. Bile collection was begun 5–10 min after cannulation of the common bile duct. Immediately after completion of bile collection, heparin (2,500 IU/kg body wt) was injected into the vena cava and the animal was sacrificed by severing the thoracic aorta and vena cava. The liver was perfused with ice-cold physiologic saline to eliminate erythrocytes; it was then removed and weighed.

TABLE I

Body and Liver Weight and Bile Flow in the Different Treatment Groups

	Control	Propanediol	Ethinyl estradiol	Phenobarbital	20-Methyl cholanthrene
n	7	7	7	5	5
Body weight before treatment, *g*	231±9	243±14	239±18	273±29*	272±26*
Body weight after treatment, *g*	236±8	247±16	239±15	275±34*	280±27*
Liver weight, *g*	9.8±0.4	11.8±1.5*	14.3±2.6*	12.7±1.4‡	13.8±0.8§
Bile flow, *μl/min/g liver*	1.49±0.12	1.64±0.30	0.92±0.31‡	2.50±0.21§	1.62±0.18

Data are expressed as mean±SD.
Significance of difference from control group:
* $P < 0.05$.
‡ $P < 0.01$.
§ $P < 0.001$.

Preparation of liver plasma membranes (LPM)[1] *rich in bile canaliculi.* The method of Song et al. (9) as modified by Boyer and Reno (3) was used with the following modifications. The liver was homogenized in 1 mM $NaHCO_3$ buffer (pH 7.4) containing 0.5 mM $CaCl_2$ as proposed by Ray (10). The LPM fraction, obtained according to the method of Boyer and Reno (3), was resuspended in $NaHCO_3$ buffer and centrifuged once at 1,500 *g* and twice at 10,000 *g* for 10 min in a Sorvall RC-5 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The final pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.4) by aspirating the pellet three times through a 23-gauge hypodermic needle. To confirm the presence of canalicular membranes, aliquots of this suspension were processed for electron microscopy according to the method of Baudhuin et al. (11) (Fig. 1).

Enzyme assays. All enzyme assays were performed immediately after LPM preparation. Na^+,K^+-ATPase activity was determined according to a modification of Wheeler and Whittam's method (12). The incubation medium contained 120 mM Na^+, 12.5 mM K^+, 5 mM Mg^{2+}, 1 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetate, and 5 mM ATP in 125 mM Tris-HCl buffer (pH 7.4). Total ATPase activity was assayed in this incubation medium at 37°C for 5 min. The non-Na^+,K^+-stimulated ATPase was determined under identical conditions in the presence of 1 mM ouabain octahydrate (Sigma Chemical Co.). The latter represented Mg^{2+}-stimulated ATPase activity (3). The reaction was terminated with 35% wt/vol TCA and the samples were immediately transferred to an ice bath. After centrifugation at 15,000 *g* for 5 min, an aliquot of the supernate was analyzed for inorganic phosphorus by the method of Berenblum and Chain as modified by Lindberg and Ernster (13). Blanks for the spontaneous degradation of ATP and for the phosphorus content of the membrane fractions were substracted. Na^+,K^+-ATPase activity was obtained from the difference between total and Mg^{2+}-stimulated ATPase activity.

The activity of the LPM marker enzyme 5'-nucleotidase (EC 3.1.3.5) (4, 9) was measured in homogenates and LPM fractions according to the method of Bodansky and Schwartz (14). Mitochondrial contamination was assessed by determining the mitochondrial marker enzyme succinate dehydrogenase (EC 1.3.99.1) using the method of Earl and Korn (15). Glucose-6-phosphatase (EC 3.1.3.9), a microsomal marker enzyme, was assayed by the method of De Duve et al. (16). The ATPase assays were performed in triplicate and the marker enzyme assays in duplicate. All enzyme assays exhibited zero-order kinetics at the time intervals and protein concentrations used.

Protein was estimated by a modification of the Lowry method (17) using bovine serum albumin as standard.

Statistical analysis. All results are expressed as mean ±SD. Means of two samples were compared by Student's *t* test after testing the equality of variances by a *F*-test (18). If the variances were unequal, the modification of the *t* test described by Welch (19) was used. Regression analysis was performed by the method of least squares (18). $P < 0.05$ was considered statistically significant.

RESULTS

In accordance with previous reports, ethinyl estradiol (5, 7) and phenobarbital (6, 8) had marked effects on bile formation. In comparison with control animals, bile flow was decreased 35% by ethinyl estradiol and increased 68% by phenobarbital treatment. Neither 20-methyl cholanthrene nor propanediol significantly altered bile flow (Table I).

The protein content of the liver homogenate was slightly increased by the two enzyme-inducing agents, phenobarbital and 20-methyl cholanthrene (Table II). Protein recovery in the canalicular LPM fraction was significantly increased by phenobarbital administration as well as by treatment with the solvent only (Table II). The purification ratios, calculated from 5'-nucleotidase activity in the LPM fractions and the homogenate, were 19±4, 24±3, 40±7, 25±10, and 19±5 in ethinyl estradiol-, phenobarbital-, 20-methyl cholanthrene-, and solvent-treated and control rats, respectively (Table III). Microsomal contamination was not altered by any of the treatments as judged by the activity of the corresponding marker enzyme, glucose-6-phosphatase (Table III). Mitochondrial contamination was assessed by the determination of succinate dehydrogenase activity in homogenate and LPM fractions in both control groups and after ethinyl estradiol treatment. No significant increase

[1] *Abbreviation used in this paper*: LPM, liver plasma membranes.

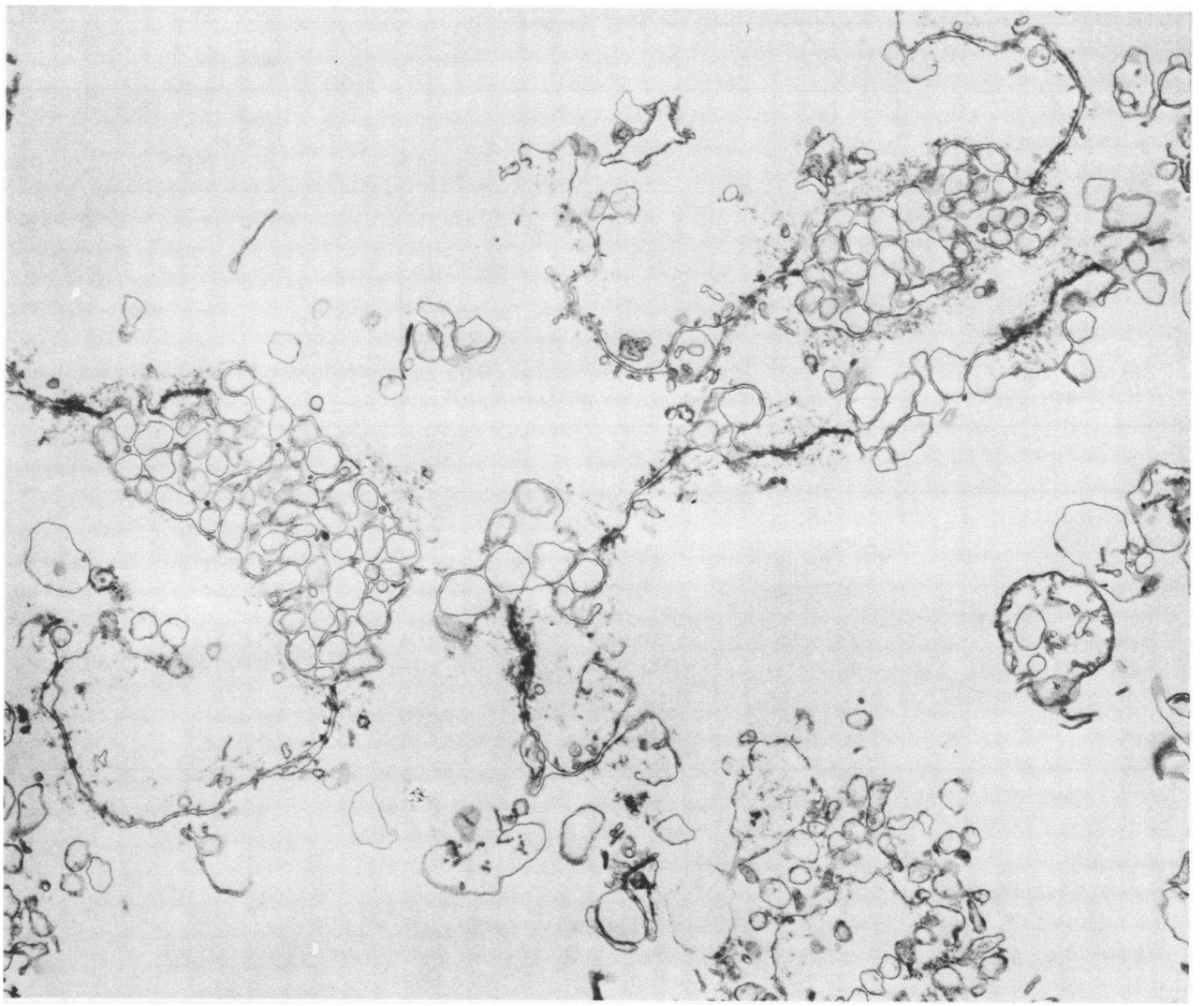

FIGURE 1 Electron micrograph of LPM fraction enriched in bile canaliculi. Magnification ×15,000.

in mitochondrial contamination was observed (Table III).

Whereas Mg^{2+}-ATPase was not significantly altered by the different treatments, both ethinyl estradiol and phenobarbital exhibited marked effects on LPM Na^+,K^+-ATPase. Thus, Na^+,K^+-ATPase activity was decreased 43% by ethinyl estradiol and increased 81% by phenobarbital treatment as compared to the control group. Whereas 20-methyl cholanthrene had no effect on Na^+,K^+-ATPase activity, propanediol increased enzyme activity by 24%. This increase, however, was not statistically significant (Table IV). When the effects of the different treatments on LPM Na^+,K^+-ATPase activity were related to gram of liver, the following figures for enzyme activity were obtained: 50.0±18.4, 231.7±42.6, 82.7±30.7, 143.6±55.3, and 80.7±19.7 μmol P_i/h per g liver in ethinyl estradiol-, phenobarbital-, 20-methyl cholanthrene-, and propanediol-treated and control rats, respectively. Na^+,K^+-ATPase activity in LPM fractions was significantly correlated with bile flow ($r = 0.785$; $P < 0.001$). The equation describing the dependence of bile flow (y) on canalicular Na^+,K^+-ATPase activity calculated per gram liver (x) was $y = 0.901 + 0.006 \cdot x$ (Fig. 2).

DISCUSSION

Canalicular bile consists of at least two fractions (1, 2), one of which depends on the secretion of bile acids (2). The other fraction is thought to result from active transport of sodium across the canalicular membrane of the hepatocyte (1, 2). Na^+,K^+-ATPase, an enzyme

TABLE II

Protein Content in Homogenate and LPM Fraction after the Different Treatments

	Control	Propanediol	Ethinyl estradiol	Phenobarbital	20-Methyl cholanthrene
n	7	7	7	5	5
Homogenate, *mg/g liver*	155±13	154±20	156±14	179±24	232±49‡
LPM, *mg/g liver*	2.20±0.20	3.22±1.12*	2.50±0.54	3.54±0.75‡	2.14±0.54

Data are expressed as mean±SD.
Significance of difference from control group:
* $P < 0.05$.
‡ $P < 0.01$.

TABLE III

Activity of Marker Enzymes (Micromoles of Substrate Liberated per Hour and per Milligram Protein) in Homogenate and LPM Fraction after the Different Treatments

	Control	Propanediol	Ethinyl estradiol	Phenobarbital	20-Methyl cholanthrene
n	7	7	7	5	5
5′ nucleotidase					
Homogenate	4.9±1.4	5.4±3.4	3.7±1.6	2.4±0.4‡	2.7±0.6‡
LPM	91.7±26.3	113.6±22.1	66.4±11.2*	55.9±2.9*	106.5±21.0
Glucose-6-phosphatase					
Homogenate	14.2±2.5	15.0±3.3	17.5±1.4	18.6±2.8*	16.3±1.5
LPM	0.21±0.11	0.27±0.15	0.27±0.14	0.32±0.19	0.28±0.16
Succinate dehydrogenase					
Homogenate	7.6±1.7	7.9±1.4	7.9±1.9	Not done	Not done
LPM	0.24±0.13	0.30±0.39	0.45±0.53	Not done	Not done

Data are expressed as mean±SD.
Significance of difference from control group:
* $P < 0.05$.
‡ $P < 0.01$.

TABLE IV

Activity of Mg^{2+}-and Na^+,K^+-Stimulated ATPase (Micromoles P_i Liberated per Hour and per Milligram Protein) in Homogenate and LPM Fraction after the Different Treatments

	Control	Propanediol	Ethinyl estradiol	Phenobarbital	20-Methyl cholanthrene
n	7	7	7	5	5
Mg^{2+}-ATPase					
Homogenate	2.9±0.3	3.8±0.5	3.9±0.2	4.2±0.8	4.0±0.9
LPM	69.2±9.7	69.1±11.8	74.9±8.8	61.9±9.4	79.4±16.9
Na^+,K^+-ATPase					
Homogenate	1.3±1.1	1.5±0.6	0.9±0.7	1.7±0.4	1.3±0.3
LPM	36.5±6.2	45.3±9.4	20.8±10.1*	66.2±13.6*	38.2±6.1

Data are expressed as mean±SD.
Significance of difference from control group:
* $P < 0.05$.

which regulates the transport of sodium across a variety of biological membranes (20), has previously been demonstrated in LPM fractions rich in bile canaliculi (3, 4). The finding of a close relationship between Na^+,K^+-ATPase activity in canalicular membranes and bile formation measured in the same rats (Fig. 2) supports the hypothesis that a fraction of bile is dependent on this enzyme and is formed by active transport of sodium across the canalicular membrane (1, 2, 4). It is evident, however, that alternative explanations are possible for the striking association between canalicular Na^+,K^+-ATPase activity and bile

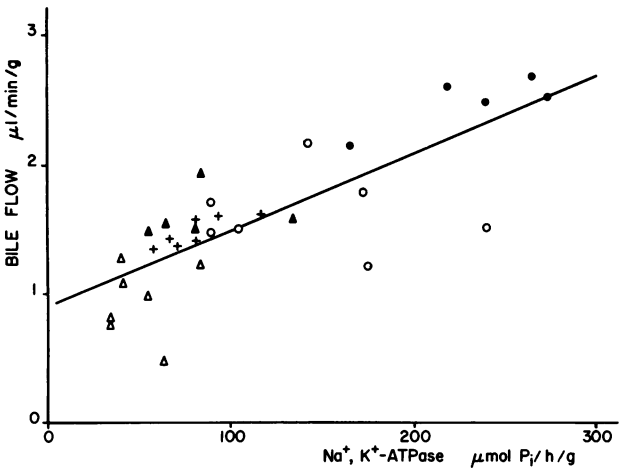

FIGURE 2 Relationship between bile flow (y) and canalicular Na^+,K^+-ATPase activity calculated per gram liver (x) in control (+) animals, propanediol- (0), phenobarbital- (●), 20-methyl cholanthrene- (▲), and ethinyl estradiol- (△) treated rats. The equation describing the regression line was $y = 0.901 + 0.006x$ ($r = 0.785$, $n = 31$).

formation. Thus, the changes in enzyme activity might have occurred in response to changes in bile flow. Ethinyl estradiol and phenobarbital could also have independent effects on bile flow and canalicular Na^+,K^+-ATPase activity. These explanations, however, appear unlikely, since cardiac glycosides inhibit the enzyme activity in vitro (3) in concentrations which also inhibit bile acid-independent bile flow in the isolated perfused rat liver (1) and in the rabbit (2). It must be noted, though, that, depending on the dose, ouabain can also be choleretic (21) and that this effect has been attributed to an inhibition of Na^+,K^+-ATPase of sinusoidal membranes (22).

In comparing the effects of different treatments on canalicular Na^+,K^+-ATPase activity, possible alterations of plasma membranes by the different pharmacological agents affecting their preparation and purification must be considered. The similar purification ratios of the LPM marker enzyme 5'nucleotidase, obtained after phenobarbital and ethinyl estradiol treatment, as well as an unaltered electron microscopic appearance of the LPM fractions, argue in favor of a satisfactory and reliable purification. This is further confirmed by the relatively small mitochondrial and microsomal contamination as judged from the specific activity of the marker enzymes, succinate dehydrogenase and glucose-6-phosphatase, respectively (Table III). The activities of these marker enzymes are comparable to those obtained by the original procedure of Song et al. (3, 4, 9), although we performed the washing procedures at 10,000 *g* as compared to the originally described 1,500 *g* (9). Previous experiments in our laboratory have shown that mitochondrial contamination was not further diminished when all washes were performed at 1,500 *g*, but that the protein yield was significantly reduced (unpublished observations). Thus, the use of a centrifugal field of 10,000 *g* for the second and third washing procedure permitted an increase in the yield of protein in the canalicular-enriched membrane fraction by a factor of about five as compared to the original procedure (3, 4, 9). A further factor explaining the increase in protein yield is the inclusion of Ca^{2+} into the homogenization medium as proposed by Ray (10). This modification did not increase microsomal contamination as judged from the relative specific activity of the microsomal marker enzyme, glucose-6-phosphatase (Table III).

The normal values for Na^+,K^+-ATPase in the present experiments are about three times higher than those reported by other workers (3, 4, 23). This could have resulted from the higher gravitational field which may have led to the precipitation of particles enriched in ATPase into the LPM fraction. It has been noted by Song et al. (9) that the pericanalicular web was lost during LPM preparation. In our modification of the Song et al. preparation, however, remaining pericanalicular web could be visualized (Fig. 1). The pericanalicular web consists of microfilaments (24) which may exhibit ATPase activity (25). Furthermore, Wisher and Evans (26) described a heavy subfraction of LPM which exhibit Na^+,K^+-ATPase activity similar to our findings. The inclusion of Ca^{2+} in the homogenization medium may have altered the ratio light/heavy LPM in our preparation, thus leading to an increase in specific Na^+,K^+-ATPase activity. Another possible explanation for the high Na^+,K^+-ATPase activity in the present study is the fact that Na^+,K^+-ATPase was assayed as ouabain-sensitive ATPase. This possibility appears improbable, however, since the values of Mg^{2+}-ATPase were similar to those reported by Layden and Boyer (4), who assayed Na^+,K^+-ATPase by omitting K^+ from the incubation medium. The failure to demonstrate significant effects of ethinyl estradiol and phenobarbital on Na^+,K^+-ATPase activity in the homogenate may have been due to dilution by enzyme activity from sources other than canalicular plasma membranes. On the average, only 48.8% of the total Na^+,K^+-ATPase activity was recovered in the canalicular-enriched LPM fraction (Tables II and IV).

Several drugs and hormones, such as ethinyl estradiol (5, 27), rose bengal (23, 28), and icterogenin (29) have inhibitory effects on both bile acid-independent bile formation in vivo and LPM Na^+,K^+-ATPase activity in vitro. Recently, Layden and Boyer (4) demonstrated that thyroid hormone increases bile acid-independent bile flow as well as canalicular Na^+,K^+-ATPase activity in the rat. Previous attempts to demonstrate an effect of phenobarbital on canalicular Na^+,K^+-ATPase activity have led to controversial results (23, 30, 31). Some of these discrepancies may be due to differences in methodology. Thus, Laperche

et al. (23) collected the surface membranes at the density 1.17–1.20 interface using a zonal rotor, whereas in the present investigation the LPM fraction was recovered at the density 1.16–1.18 interface. Simon and Sutherland (31), using Neville's procedure (32) which is similar to that of Song et al. (9), described a clear-cut increase in canalicular Na^+,K^+-ATPase activity. However, Boyer et al. (30), who used Song's method, failed to demonstrate any effect of phenobarbital on canalicular Na^+,K^+-ATPase. This discrepancy remains unexplained.

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