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Research Article

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Plasma membrane fractions prepared from mild as well as severe cholestasis had increased alkaline phosphatase activity, and reduced 5′-nucleotidase and Mg^{2+}-ATPase activities. Co^{2+}-CMPase activity was unchanged. Kinetic analysis of 5′-nucleotidase and Mg^{2+}-ATPase activities in plasma membrane fractions demonstrated reduced V_{maz} (but unaltered K_m). Reducted V_{maz} was unrelated to addition in vitro of di-or trihydroxy bile salts or ethinyl estradiol and, therefore, suggests that reduced activities in cholestasis are due to decreased enzyme content. Cholestasis was not associated with changes in the synthesis or degradation […]

Alteration of Bile Canalicular Enzymes in Cholestasis

A POSSIBLE CAUSE OF BILE SECRETORY FAILURE

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ABSTRACT Bile secretory failure (cholestasis) may result from several possible mechanisms involved in bile secretion. We have examined the possibility that abnormalities in enzyme content, composition, and turnover of liver plasma membrane constituents are altered in cholestasis.

Severe and mild cholestasis were produced by 5 days of bile duct ligation and ethinyl estradiol administration, respectively. Bile duct ligation but not ethinyl estradiol treatments was associated with elevations of the serum bilirubin level and 5′-nucleotidase activity. However, basal bile flow and bilirubin transport maximum (T_m) were significantly reduced after ethinyl estradiol treatment. Liver plasma membrane fractions rich in canalicular membranes were prepared from groups of rats in each of three categories: normal, after bile duct ligation, or ethinyl estradiol administration, and their respective controls. Electron microscopy and enzyme marker studies demonstrated plasma membrane fractions free of significant contamination.

Plasma membrane fractions prepared from mild as well as severe cholestasis had increased alkaline phosphatase activity, and reduced 5′-nucleotidase and Mg^{2+}-ATPase activities. Co^{2+}-CMPase activity was unchanged. Kinetic analysis of 5′-nucleotidase and Mg^{2+}-ATPase activities in plasma membrane fractions demonstrated reduced V_{max} (but unaltered K_m). Reducted V_{max} was unrelated to addition in vitro of di-or trihydroxy bile salts or ethinyl estradiol and, therefore, suggests that reduced activities in cholestasis are due to decreased enzyme content. Cholestasis was not associated with changes in the synthesis or degradation rate of pulse-labeled plasma membrane proteins or alterations in the major protein bands separated on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Plasma membrane cholesterol, phospholipid, and neutral sugar content was unaltered, but sialic acid content was significantly increased in both forms of cholestasis. Alterations in specific canalicular enzymes in two forms of cholestasis suggest that these changes may be involved in the pathogenesis of bile secretory failure, or may result from cholestasis.

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INTRODUCTION

Bile secretion involves bile salt-dependent and independent fractions (1) as well as pathways for organic anions (2), cations (3), and neutral compounds (4). In addition, bile salts, the major organic anions in mammalian bile, appear to be excreted by a pathway different from that involved in biliary excretion of other organic anions such as bilirubin, various dyes, and cholecystographic agents (5, 6). Theoretically, bile secretory failure (cholestasis) can result from several mechanisms involving these pathways of bile secretion. Current hypotheses include altered bile salt metabolism and excretion (7, 8), abnormalities of the endoplasmic reticulum in the liver cell (9), increased biliary epithelial reabsorption of bile substituents (10), enhanced back-diffusion across bile canaliculi (11), and altered composition and turnover of bile canalicular components (12).

Development of techniques for isolation of liver plasma membranes, separation of membrane protein, and study of differential protein synthesis and degradation enabled us to investigate the possibility that alterations in composition, enzymatic content, and protein turnover of liver plasma membranes may contribute to the pathogenesis of cholestasis. In the present study, specific canalicular enzyme changes occurred in two models of cholestasis. The results suggest that alterations of canalicular mem-

brane proteins may be involved in the pathogenesis of bile secretory failure.

METHODS

Male Sprague-Dawley rats (200–300 g) (Marland Farms, Peekskill, N. Y.) were used in all experiments. Animals were randomly divided into three groups. The first group was untreated and is referred to as normal. The second group of animals consisted of bile duct-ligated rats and their controls which underwent sham operations. Ethinyl estradiol was administered to the third group of rats, and its vehicle, propylene glycol, to controls. Rats were fed Purina Rat Chow ad lib., and were caged in a room at constant temperature (22°C) with alternating 12 h of light and darkness. No insecticides were used.

Under ether anesthesia, extrahepatic obstruction was produced by double ligation and transection of the common bile duct close to the hilum of the liver with removal of the intervening segment. Sham-operated animals were handled in a similar manner; however, the bile duct was neither ligated nor resected. Both groups of animals were allowed to eat ad lib. after surgery. Bile duct-ligated and sham-operated rats were studied 5 and 4 days, respectively after surgery. Bile secretory failure was produced also by subcutaneous administration of ethinyl estradiol (Wyeth Laboratories, Philadelphia, Pa.), 0.5 mg/100 g body wt daily for 5 days. Ethinyl estradiol was dissolved in propylene glycol (5 mg/ml) by heating to 50°C for 10 min. Control animals were similarly injected with equal volumes of propylene glycol daily for 5 days.

Because ethinyl estradiol-treated rats had normal serum bilirubin concentration, cholestasis was confirmed by determination of the maximum capacity of the liver to excrete bilirubin (13). Randomly selected rats treated with ethinyl estradiol or propylene glycol were fasted overnight and anesthetized with intraperitoneal Nembutal (0.08 ml/100 g body wt). The common bile duct was cannulated with polyethylene tubing no. 10 and either the external jugular or femoral vein was cannulated with polyethylene tubing no. 50 and a normal saline infusion started. Body temperature was monitored with a Thermistor-Probe (Yellow Springs Instrument Co., Yellow Springs, Ohio) and kept constant at 37°±0.5°C. Two 15-min basal bile samples were collected during the period of normal saline infusion. A solution of unconjugated bilirubin (100 mg/100 ml) was infused at 125–150 μg/100 g per min for six additional 15-min collection periods. Unconjugated bilirubin was prepared fresh for infusion by dissolving recrystallized bilirubin (Eastman, Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) in a solution of 0.5 g Na_2CO_3 and 0.52 g NaCl per 100 ml at pH 7.8. Serum was obtained by cardiac puncture at completion of the experiment and stored in the dark at −20°C. Bilirubin in serum and bile was measured by the method of Malloy and Evelyn (14).

Plasma membranes were prepared from livers of normal and cholestatic rats as previously described (15, 16). With the exception of studies using double labeled isotopes, plasma membrane fractions were prepared from 30 g of liver obtained from three rats. All procedures were performed at 5°C. Rats were anesthetized with ether and the liver was quickly removed. The final membrane fraction was washed in cold 1 mM $NaHCO_3$. Plasma membrane preparations in 1 mM $NaHCO_3$ at a protein concentration of 2–4 mg/ml were either assayed immediately or stored at −20°C until examined. The purity of each preparation was monitored by phase contrast microscopy; preparations which contained more than one nucleus per low power field were discarded. Purity of each preparation was determined also by several enzyme markers and selected preparations were examined by electron microscopy.

Washed plasma membrane preparations were suspended in cold 0.25 M sucrose buffered in 0.01 M phosphate buffer pH 7.4. A pellet was prepared by centrifugation at 20,000 *g* × 30 min. Each pellet contained 6–10 mg protein, was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 (17), postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (18), and embedded in Epon (19). Thin sections were stained with lead citrate (20) and examined in a Siemens Elmiskop 1 electron microscope (Siemens Corp., Medical Industrial Div., Iselin, N. J.

Alkaline phosphatase activity (EC 3·1·3·1) was measured by the method of Bodansky (21) using 15 mM β-glycerol-phosphate as substrate in barbital buffer pH 9.8, 10 mM $MgCl_2$; 5′-nucleotidase activity (EC 3·1·3·5) by the method of Song and Bodansky (22); and glucose-6-phosphatase activity (EC 3·1·3·9) by the method of DeDuve, Pressman, Gianetto, Wattiaux, and Appelmans (23). Magnesium-stimulated ATPase activity (EC 3·6·3·5) was measured by the method of Emmelot, Bos, Benedetti, and Rumke. This activity was neither stimulated by sodium at concentrations between 50 and 105 mM nor inhibited by ouabain at 10^{-4} mM. Cobalt-stimulated cytidine monophosphatase activity (EC 3·1·3·1) was measured as described by Ma and Biempica (25) in 500 mM acetate buffer at pH 5.0 containing 0.5 mM cobalt chloride.

These five enzyme activities were assayed in 2 ml of appropriate media, and incubated at 37°C for 15–30 min depending on enzyme activity measured. Reactions were stopped with 0.5 ml of cold 30% TCA (trichloroacetic acid). The precipitate was removed by centrifugation and liberated phosphorus was determined in 0.5- to 1.0 ml-aliquots by the method of Fiske and Subba-Row (26). Activity was expressed as micromoles of phosphorus released/milligram protein per hour. All reactions were performed at two to three times substrate excess and determinations were made during the period of zero order kinetics of the reaction. Both tissue and substrate blanks were studied simultaneously and all assays were performed in duplicate. Preliminary studies demonstrated that activity was not lost on storage at −20°C for 72 h, but was decreased after storage for 1 wk. All enzyme assays were performed within 72 h of plasma membrane preparation.

Using plasma membrane fractions, Michaelis-Menten constants (K_m and V_{max}) for 5′-nucleotidase and Mg^{2+}-ATPase were determined from nonlinear plots of five or six concentrations between 1 and 2.5 mM of appropriate substrate as described by Wilkinson (27).

The effect of taurocholate, chenodeoxycholate, and ethinyl estradiol on enzymatic activities of plasma membrane preparations was determined in vitro. Conjugated, purified bile salts (28) at several concentrations were mixed with normal plasma membranes at room temperature immediately before assay of 5′-nucleotidase, Mg^{2+}-ATPase, and alkaline phosphatase activities. Ethinyl estradiol was dissolved in absolute ethanol and added to normal plasma membrane preparations at a final concentration of 5×10^{-7} M.

Studies of possible enzyme inhibitors or activators were performed by adding equal volumes of plasma membrane fractions prepared from rats with bile duct ligation or after ethinyl estradiol treatment to plasma membrane fractions prepared from normal rats. Observed activity was compared with predicted values.

Alkaline phosphatase (β-glycerolphosphate) and 5′-nucleotidase (adenosine monophosphate) activities were determined in serum obtained from the inferior vena cava immediately before death. Samples were stored at −20°C for up to 2 wk and analyzed in duplicate as described by Dixon and Purdom (29). Enzymatic activity was stable for at least 1 month.

The synthesis and relative rates of degradation for pulse-labeled liver plasma-membrane proteins were determined in normal and control rats and rats with cholestasis (16, 30). Uniformly labeled L-[^{14}C]leucine (sp act 278 Ci/mM), and L-[4,5-^{3}H]leucine (sp act 0.44 Ci/mM obtained from New England Nuclear (Boston, Mass.), were neutralized with NaOH, and diluted in normal saline for intraperitoneal injection in volumes of 1 ml or less. [^{14}C]Leucine (20 μCi) was administered intraperitoneally to rats and labeled protein radioactivity allowed to decay for 48 h. 12 h before sacrifice, [^{3}H]leucine (100 μCi) was administered intraperitoneally to the same animal. Administration of [^{14}C]leucine was begun on the 3rd day of cholestasis and the animals were killed on the 5th day. Control studies were performed to determine the range of error in the method by simultaneous administration of [^{14}C]leucine (15μCi) and [^{3}H]leucine (120 μCi) to the same animal 12 h before sacrifice. Radiolabeled animals were killed and liver plasma membranes were prepared.

Proteins (1–2 mg) were precipitated by equal volumes of cold 10% TCA and collected on glass fiber disks (Whatman). Each sample was washed serially with acetone, ether, alcohol, and cold 5% TCA. Disks were dried under negative pressure and transferred to counting vials; 0.5 ml of Protosol (New England Nuclear) was added, and proteins were digested for 12 h at room temperature (22°C). 10 ml of Toluene-Omnifluor (New England Nuclear, containing 98% 2,5-diphenyloxazole and 2% bis-MSB, 4 g/liter toluene) solution was added and radiolabeled specimens were counted in a Packard refrigerated liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Tritium was counted at 29% and ^{14}C at 70% efficiency. No significant ^{3}H activity was present in ^{14}C channel, and 22% of ^{14}C counts was present in ^{3}H channel. Counting was performed to at least 1% accuracy and quenching was corrected by external standardization. Protein synthesis was determined by ^{3}H sp act 12 h after administration of [^{3}H]leucine. Relative degradation of pulse-labeled membrane proteins was determined by the ratio of ^{3}H/^{14}C in each sample. In order to correct for differences in body weight, amount of isotope administered and free amino acid pools, a turnover index was calculated by dividing the plasma membrane ^{3}H/^{14}C ratio by its respective whole liver homogenate ^{3}H/^{14}C ratio (31).

Reagents were either analytical grade or the highest purity available. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (32) using bovine serum albumin (BSA) as standard. Organic phosphorous was measured by the method of Bartlett (33), and total cholesterol after saponification (34) was determined by the method of Zlatkis, Zak, and Boyle (35, 36) and sialic acid by the method of Warren (37) using *N*-acetylneuraminic acid as standard on membranes dialyzed overnight against 1 mM $NaHCO_3$ to remove adherent sucrose.

Freshly prepared, saline-washed plasma membrane samples from control rats and rats with cholestasis were solubilized in 2% sodium dodecyl sulfate (SDS) (wt/vol) and 5% β-mercaptoethanol (vol/vol) and were separated on 5% acrylamide gels containing 0.1% SDS; and electrophoresis was performed in neutral phosphate buffer (16).

Gels were fixed overnight (18 h) in 12% TCA and stained with fresh 0.25% coomasie blue for 2 h. In some studies, gels were fixed in TCA overnight, repeatedly washed in distilled water for an additional 24 h, and stained for carbohydrates using a periodic acid-Schiff stain as described by Zacharias, Zell, Morrison, and Woodlock (38). All gels were destained by repeated washing with 7% acetic acid. Protein and carbohydrate-stained gels were usually examined with respect to number, sequence, and intensity of staining of protein bands. Molecular weights of the seven major stained plasma membrane proteins and three periodic acid-Schiff staining bands were determined graphically as described by Shapiro, Vinuela, and Maizel (39). Immunoglobulin G (160,000), bovine albumin dimer (134,000), bovine albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and myoglobin (17,800) were obtained from Mann Research Laboratories, Inc., (New York) and electrophoresed under identical conditions as described for plasma membranes.

Students' *t* test was used for statistical analysis of results (40). *P* values equal to or less than 0.05 were considered significant. Standard error for Michaelis-Menten constants were derived from the nonlinear curves as described by Wilkinson (27).

RESULTS

5 days after bile duct ligation, serum bilirubin concentration and 5′-nucleotidase activity were significantly elevated (Table I). Serum alkaline phosphatase activity was normal at 5 days, although it was increased by 200% 18 h after bile duct ligation. Ethinyl estradiol administration did not increase serum bilirubin or 5′-nucleotidase and alkaline phosphatase activities; however, basal bile flow was reduced by 30% ($P < 0.025$) and bilirubin T_m was reduced by 50% ($P < 0.005$) when compared with controls (Fig. 1). After 5 days of bile duct ligation, rats lost 13.5±3.8% of their body weight compared with 1.8±0.6% for sham-operated animals ($P < 0.001$). Ethinyl estradiol treatment also resulted in 1.6±0.4% loss of body weight, while propylene glycol-treated animals gained 8.2±2.1% of their body weight over 5 days ($P < 0.001$).

Liver sections were stained with hematoxylin and eosin. Light microscopic examination of ethinyl estradiol-treated rats appeared normal and were similar to sections from propylene glycol-treated rats. Liver sections obtained from bile duct-ligated rats revealed portal triad inflammatory cell infiltrates, bile duct proliferation, and occasional parenchymal cell necrosis.

Each plasma membrane fraction from normal, control, and cholestatic rats was examined by phase microscopy which revealed only rare nuclei and no unbroken cells. Electron microscopic appearance of a single plasma membrane preparation from each type of cholestasis was similar to those seen with control rat plasma membrane fractions. Sheets of membranes frequently connected by intercellular junctional processes and occasional structures resembling bile canaliculi were seen (Fig. 2).

TABLE I

Serum Bilirubin, 5'-Nucleotidase and Alkaline Phosphatase in Normal Rats and Ethinyl Estradiol-Treated Rats, Bile Duct-Ligated Rats and Their Controls

	No. of rats	Serum		
		Total bilirubin	5'-Nucleotidase	Alkaline phosphatase
		mg/100 ml	*Bodansky U*	
Normal	4	<0.2	0.3±0.3	31.5±4.7
Sham operated	5	<0.2	1.2±0.8	30.9±2.0
After bile duct ligation	6	8.1±1.7*	27.2±5.5*	31.8±2.0
After administration of propylene glycol	9	<0.2	0.6±0.5	33.9±2.0
After administration of ethinyl estradiol in propylene glycol	12	<0.2	2.1±1.0	31.9±1.6

Analyses were performed 5 days after bile duct ligation or administration of propylene glycol or ethinyl estradiol in propylene glycol. See text for further details.
Figures indicate mean ±SE.
* $P = < 0.005$.

Rarely, a fragmented mitochondrion or lysosome, was present.

In normal rats the specific activities of enzymes associated primarily with the canalicular membrane (alkaline phosphatase, 5'-nucleotidase, and Mg^{2+}-ATPase) (41, 42) were increased 21- to 36-fold compared with their respective specific activities in homogenates (Table II). The specific activity of Co^{2+}-stimulated CMPase, a sinusoidal membrane enzyme (25), was increased eightfold. Glucose-6-phosphatase activity was not detected in plasma membrane fractions. Both ethinyl estradiol treatment and bile duct ligation increased the specific activity of alkaline phosphatase ($P < 0.025$) and decreased 5'-nucleotidase ($P < 0.05$) and Mg^{2+}-ATPase activities ($P < 0.025$) as compared with controls (Table III). Cobalt CMPase activity was not altered in rats with cholestasis. Alkaline phosphatase, Mg^{2+}-ATPase and 5'-nucleotidase activities were not significantly altered in sham-operated or propylene glycol-treated rats.

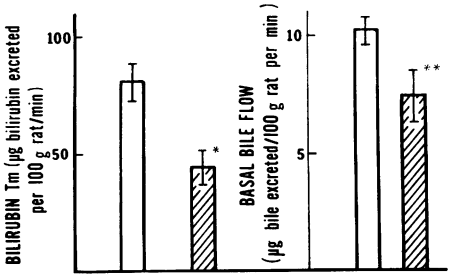

FIGURE 1 Effect of administration of ethinyl estradiol in propylene glycol (0.5 mg/100 g body wt) (hatched bars) and propylene glycol alone (clear bars) on bilirubin T_m and basal bile flow. See text for further details. Results are expressed as mean ±SE. (*)$P < 0.005$, (**)$P < 0.025$.

To determine whether decreased 5'-nucleotidase and Mg^{2+}-ATPase activities in cholestasis are related to inhibition of activity or decreased content of enzyme protein, K_m (dissociation constant), and V_{max} (the maximal reaction rate) were determined (Table IV). The V_{max} for Mg^{2+}-ATPase and 5'-nucleotidase activity in extrahepatic obstruction and ethinyl estradiol cholestasis was significantly reduced from results in control rats. The K_m for 5'-nucleotidase in both forms of cholestasis, and for Mg^{2+}-ATPase in ethinyl estradiol-treated rats was unaltered; however, the K_m for Mg^{2+}-ATPase after bile duct ligation was significantly different from values obtained in sham-operated rats. These results are compatible with noncompetitive inhibition and/or reduction in enzyme content. Possible inhibitors of enzyme activity include bile salts and ethinyl estradiol. Purified bile salts, in a range of concentrations, were added to the enzyme assay mixture (Table V). Taurocholate, the major bile salt in rats, did not inhibit 5'-nucleotidase and Mg^{2+}-ATPase activity, however, stimulation of alkaline phosphatase activity was observed. Taurochenodeoxycholate at concentrations between 0.2 and 1.0 mM caused 16% inhibition of 5'-nucleotidase activity, and enhanced Mg^{2+}-ATPase and alkaline phosphatase activities. Ethinyl estradiol in concentrations previously reported to inhibit (Na^+-K^+)-ATPase activity (29), did not affect Mg^{2+}-ATPase or 5'-nucleotidase activities; however, alkaline phosphatase activity was completely inhibited. Plasma membranes prepared from normal and bile duct-ligated or ethinyl estradiol-treated rats were also mixed in equal proportions before determination of enzyme specific ac-

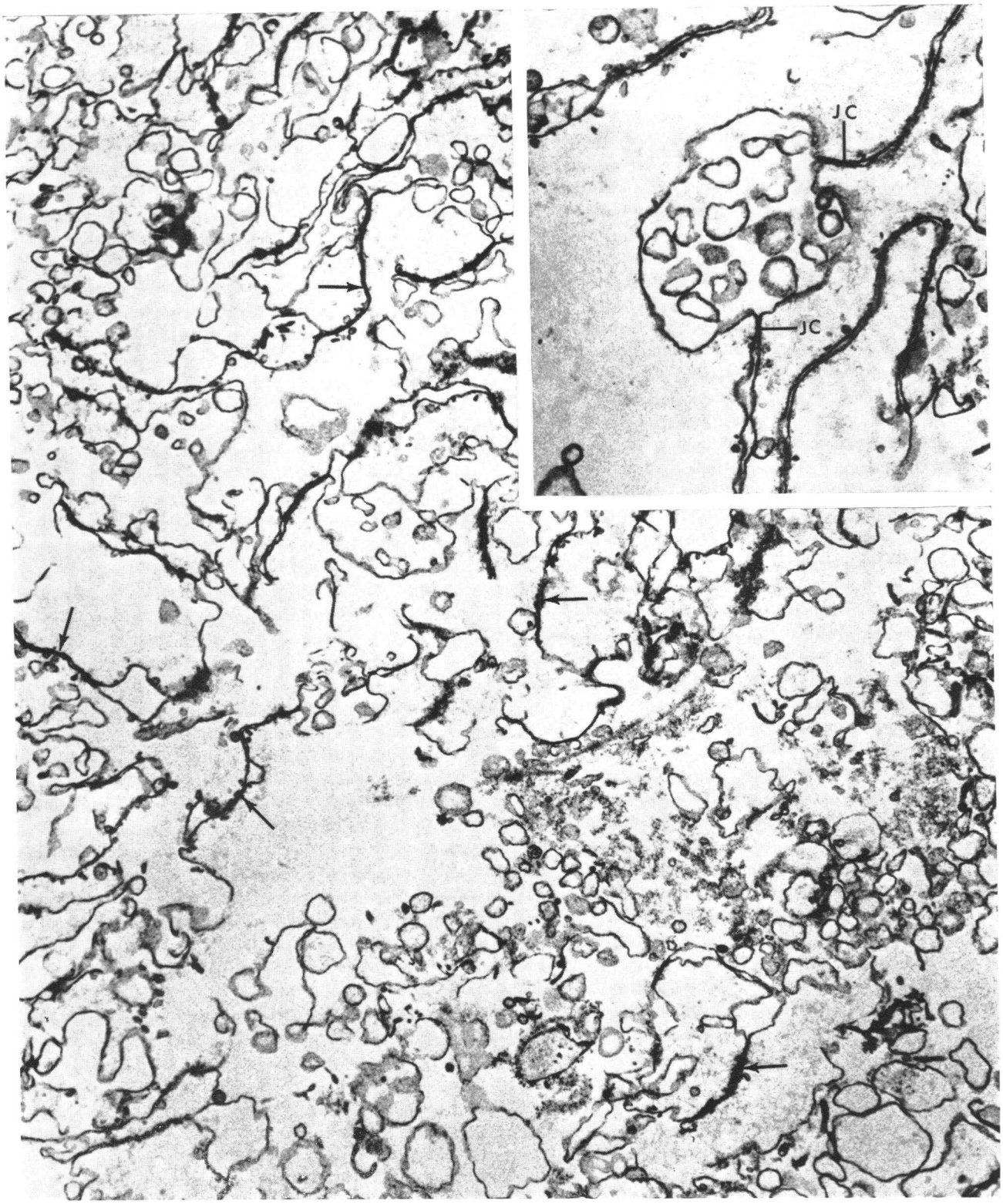

FIGURE 2 Preparation of plasma membranes from liver with cholestasis. Representative area showing a highly uniform population of plasma membranes. Arrows point to some of the numerous areas where lateral portions of plasma membranes are paired (magnification × 12,000). Inset, a relatively well-preserved bile canaliculus with microvilli and preserved junctional complexes (JC). Magnification, × 25,000. Thin sections were stained with lead citrate.

TABLE II

Enzymatic Activity of Normal Rat Liver

Enzymes	No. of rats	Specific activity		Specific activity (Membrane/Homogenate)
		Homogenate	Plasma membrane	
		μmol/mg protein per h		
Alkaline phosphatase	5	0.3±0.04	7.1±0.8	24
5′-Nucleotidase	6	2.3±0.4	83.6±3.7	36
Mg^{2+}-adenosine triphosphatase	6	3.3±0.6	70.2±6.5	21
Cobalt-cytidine monophosphatase	3	0.8±0.03	6.0±0.3	8
Glucose-6-phosphatase	6	2.7±0.3	ND	—

Enzymatic activity was determined within 72 h of membrane preparation. Plasma membranes prepared as described by Neville (12) and washed with 0.15 M NaCl. Figures indicate mean ±SE. ND, not detected.

tivity. No change from predicted results was observed in alkaline phosphatase, Mg^{2+}-ATPase, or 5′-nucleotidase activities.

Synthesis and relative degradation rates of pulse-labeled plasma membrane proteins in control rats were combined with the observations made in normal animals (Fig. 3). Neither type of experimental cholestasis has altered rates of synthesis or degradation (turnover index) of pulse-labeled membrane protein.

Liver plasma membrane content of cholesterol, phospholipid, and neutral sugars was not significantly different in control or cholestatic rats (Table VI). Sialic acid content of plasma membranes obtained from bile duct-ligated or ethinyl estradiol-treated rats was significantly elevated ($P < 0.05$).

Fig. 4 shows representative electrophoretic plasma membrane protein patterns obtained in control and cholestatic rats. Many protein bands with a wide range of molecular weights (16,000–250,000 mol wt) were observed. Although it is not possible to accurately quantitate the concentration of protein present in each band, we identified the seven qualitatively major membrane protein bands by molecular weight in each preparation and they were not altered qualitatively. Protein bands

TABLE III

Enzymatic Activity in Rat Liver Plasma Membranes Obtained after 5 Days of Ethinyl Estradiol Administration, Bile Duct Ligation, or in Control Animals

Enzyme	Specific activity			
	Sham operation	Bile duct ligation	Propylene glycol administration	Ethinyl estradiol administration
	μmol/mg protein per h			
Alkaline phosphatase	9.6±0.4 (5)	27.5±3.5*(5)	6.1±0.4 (4)	29.0±4.9‡(6)
5′-Nucleotidase	70.8±5.8 (4)	52.6±7.1§(7)	78.8±3.9 (5)	54.2±5.1‡(6)
Mg^{2+}-adenosine triphosphatase	56.6±9.7 (5)	28.6±2.9‡(8)	79.6±5.7 (4)	43.9±6.1*(6)
Cobalt-cytidine monophosphatase	4.7±0.3 (4)	5.2±0.3 (4)	3.8±0.4 (4)	3.2±0.5 (6)
Glucose-6-phosphatase	ND (2)	ND (3)	ND (2)	ND (3)

Liver plasma membranes were prepared as described by Neville (12), washed with 0.15 M NaCl and enzymatic activities determined as described in text. Ethinyl estradiol, propylene glycol, and bile duct-ligated animals were sacrified after 5 days, while sham-operated rats were studied at 4 days. Numbers in parenthesis refer to number of experiments performed on different plasma membrane fractions. Figures refer to mean ±SE. ND, not detected.

* $P < 0.005$.

‡ $P < 0.025$.

§ $P < 0.05$.

TABLE IV

Michaelis Constants for 5'-Nucleotidase and Mg^{2+}-Stimulated Adenosine Triphosphatase in Plasma Membrane Fractions Prepared from Ethinyl Estradiol, Bile Duct-Ligated, and Control Rats

Enzyme	Sham operation	Bile duct ligation	Propylene glycol treatment	Ethinyl estradiol treatment
5'-Nucleotidase				
K_m, mM	1.4±0.1	1.2±0.2	1.3±0.1	1.7±0.2
V_{max}, U	134±4.5	61±4.2*	141±5.5	88±5.2*
Magnesium-ATPase				
K_m, mM	0.9±0.2	1.5±0.1*	2.0±0.3	1.4±0.3
V_{max}, U	74±5.6	35±1.0*	107±8.3	45±4.2*

Liver plasma membranes were prepared as described in text. 5'-Nucleotidase and Mg^{2+}-ATPase were determined in duplicate as described in text for at least five different substrate concentrations. K_m and V_{max} were determined as described by Wilkinson from nonlinear hyperbolic curves (27). Results are expressed as the mean ±SE of two experiments.
* Significant difference, $P < 0.01$.

of lesser staining intensity were absent in cholestasis. A protein band of 140,000 mol wt was absent in bile duct-ligated plasma membranes; while the protein band at 100,000 mol wt was deleted in plasma membranes obtained from ethinyl estradiol-treated rats. Three quantitatively major periodic acid-Schiff staining bands were observed. The band migrating with the front represents glycolipids and the other two bands migrate with the relative rates of proteins with molecular weights of 45,000 and 120,000 (16). This pattern was unaltered in cholestasis.

DISCUSSION

The abnormal ultrastructural (43) and histochemical appearance (44) of bile canalicular membranes suggests that changes in their composition, enzyme content, and protein turnover may be involved in cholestasis. The present studies demonstrate that cholestasis is associated

TABLE V

Percent Change of Enzyme Activity in Liver Plasma Membrane Fractions Caused by Addition of Taurocholate, Chenodeoxycholate, and Ethinyl Estradiol

Inhibitor/Concentration	Percentage change in specific activity		
	5'-Nucleotidase	Mg^{++}-ATPase	Alkaline phosphatase
Taurocholate			
1 mM	+7	+16	+43
2 mM	0	+18	+14
3 mM	0	+34	+8
5 mM	+16	+10	+29
Taurochenodeoxycholate			
0.1 mM	−3	+34	+64
0.2 mM	−16	+32	+58
0.4 mM	−16	+36	+50
1.0 mM	−16	+22	+7
Ethinyl estradiol			
(5×10^{-7} M)	−7	+8	−100

Liver plasma membrane fractions were isolated and washed as described in text. Bile salts (Maybridge Company, Tintagel, Cornwall, England) were purified by thin-layer chromatography (TLC) (22) and added to incubation reaction immediately before incubation at 37°C. Enzymes were run in duplicate. Results are expressed as the mean of two experiments. (+) indicates activation and (−) indicates inhibition.

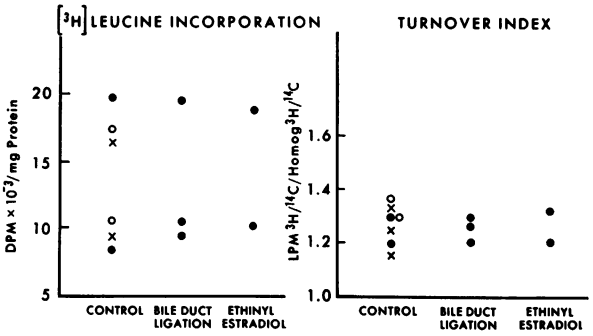

FIGURE 3 Tritium specific activity was determined in protein samples from liver plasma membrane fractions 12 h after i.p. injection of [^{3}H]leucine. The turnover index was calculated as described in text. Each point represents the result of one rat. The control group consists of untreated (●) (2), sham-operated (×) (2), and propylene glycol-treated (○) (2) rats.

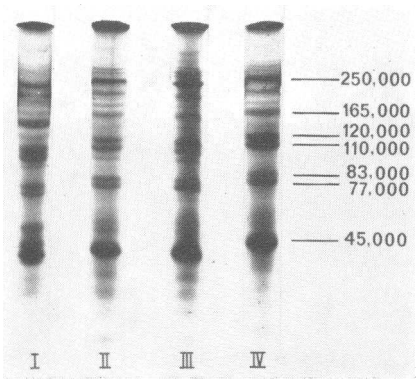

FIGURE 4 Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate (SDS). 125 μg of liver plasma membrane protein from sham-operated (I), bile duct-ligated (II), propylene glycol (III), and ethinyl estradiol-treated (IV) rats were solubilized and electrophoresed as described in text on gels 10 cm in length from anode (top) to cathode (bottom). Gels were fixed in 12% TCA and stained with commasie blue. Molecular weights were calculated as described by Shapiro, Vinuela, and Maizel (39).

with alterations in the apparent content of specific canalicular proteins.

Two models of cholestasis were investigated in order to compare changes in mild cholestasis produced by ethinyl estradiol with changes in severe cholestasis resulting from bile duct ligation. Ethinyl estradiol administration produced mild cholestasis characterized by decreased bilirubin T_m and basal bile flow. Hepatic morphology by light microscopy and serum bilirubin, bile salts (unpublished observations, Dr. Norman Javitt) and alkaline phosphatase and 5′-nucleotidase activities were normal after ethinyl estradiol treatment. Similar observations have been made by other investigations (45–47). In contrast, biliary obstruction resulted in increased serum bilirubin concentration and 5′-nucleotidase activity as well as morphologic features of cholesterasis and bile duct proliferation. It is postulated that common alterations found in two different models of cholestasis suggest that these changes may be important in the pathogenesis of bile secretory failure.

Enzymatic and electron microscopic examinations of plasma membrane fractions obtained from control and cholestatic rats reveal preferential selection of canalicular membranes. Plasma membrane fractions were greatly enriched in canalicular enzymes, (alkaline phosphatase, 5′-nucleotidase and Mg^{2+}-ATPase) as compared with Co^{2+}-CMPase activity, a sinusoidal enzyme. This conclusion was also reached by Pohl, Birnbaumer, and Rodbell using the same preparation method and adenyl cyclase as a marker enzyme for the sinusoidal membrane (48). Electron microscopic demonstration of many junctional complexes and canalicular-like structures also indicates that these preparations are rich in canalicular

TABLE VI

The Chemical Composition of Plasma Membranes Isolated from Normal Rats and Rats Treated with Ethinyl Estradiol, Bile duct ligation, and Their Controls

	No. of rats	Chemical composition			
		Cholesterol	Phospholipids	Neutral sugars	Sialic acid
		μg/mg protein			
Normal	5	237±6	532±35	73.2±10.0	21.0±1.5
Sham operation	5	237±12	575±52	67.6±12.1	18.2±2.3
Bile duct ligation	7	242±15	605±35	77.8±8.3	29.5±2.4*
Propylene glycol treatment	4	230±12	507±68	60.7±15.7	21.0±2.0
Ethinyl estradiol	7	213±15	565±40	54.6±14.7	26.4±2.1‡

Liver plasma membranes were prepared as described by Neville (22) and the chemical composition determined as described in text. The figures indicate mean ±SE.

* $P < 0.005$.

‡ $P < 0.05$.

membranes. Plasma membrane fractions in control rats and rats with cholestasis were not significantly contaminated by other organelles as shown by enzyme markers and electron microscopy.

Quantitation of plasma membrane enzymes cannot be accomplished at present, because methods for their isolation and purification to homogeneity are not available. Kinetic analysis of partially purified enzyme preparations was therefore utilized to study alterations in 5′-nucleotidase and Mg^{2+}-ATPase in liver plasma membranes. In cholestasis of both types, the V_{max} of 5′-nucleotidase and Mg^{2+}-ATPase was reduced, suggesting either reduced content or inhibition of enzyme activity. The alteration of K_m for Mg^{2+}-ATPase in extrahepatic obstruction but not after ethinyl estradiol treatment suggests part of the reduction may result from noncompetitive inhibition in severe cholestasis. Inhibition, however, does not appear to be a major cause of the reduced activity in cholestasis since mixing experiments and in vitro additions of ethinyl estradiol and bile salts did not significantly inhibit 5′-nucleotidase and Mg^{2+}-ATPase activities. The mild stimulation of alkaline phosphatase activity by taurocholate and inhibition by ethinyl estradiol in vitro suggests that accumulation of these agents is probably not an important mechanism of controlling enzyme activity. Bile salt inhibition of plasma membrane Mg^{2+}-ATPase activity, has been shown, using concentrations 20–200 times greater than those found in the livers of bile duct-ligated rats (49, 50). Cobalt-CMPase activity was unchanged in cholestasis, consistent with the hypothesis that membrane alterations in cholestasis occur primarily at the canalicular surface.

Proteins constitute 50% of liver plasma membrane dry weight, and consist of at least 20–25 species with a wide range of molecular sizes (16). Adequate solubilization of membrane proteins has been achieved only with denaturing agents such as SDS which dissociates proteins into polypeptide chains with consequent loss of enzyme activity (51, 52). SDS-polyacrylamide gel electrophoresis of plasma membrane proteins, demonstrated that the majority of proteins are unchanged in cholestasis; however, loss of qualitatively minor bands which differed in each model of cholestasis were observed. Specific membrane enzymes quantitatively contribute a minor role to the complex plasma membrane protein gel patterns; therefore, it is not possible to identify changes in SDS-polyacrylamide gel bands that represent canalicular enzymes (53). Measurement of synthesis and relative degradation rates of pulse-labeled plasma membrane proteins indicated that turnover was unchanged in cholestasis. Therefore, the alterations in alkaline phosphatase, 5′-nucleotidase, and Mg^{2+}-ATPase activities do not reflect a generalized abnormality in membrane biogenesis, but probably represent alterations in specific canalicular proteins which constitute a small fraction of total plasma membrane protein.

Plasma membrane fractions are characterized by high concentrations of cholesterol (54) and sialic acid (55) as compared with intracellular organelles. Alterations in membrane enzymes may be due to changes in the lipid or carbohydrate content of membranes. This possibility is likely since serum concentrations of cholesterol, phospholipids (56), and sialic acid (57) are increased in cholestasis. The only change in plasma membrane composition observed in cholestasis was an increase in sialic acid. This increase may reflect the rise in membrane activity of alkaline phosphatase, which is rich in sialic acid (58).

The mechanism(s) by which reduced 5′-nucleotidase and Mg^{2+}-ATPase activities are related to cholestasis is unclear. Reduced hepatic plasma membrane 5′-nucleotidase activity occurs in several experimental models of cholestasis, such as hypophysectomy (13, 59, 60), essential fatty acid deficiency (61, 62), viral hepatitis in mice (63), nutritional hepatic necrosis (64), and also in newborn animals (65, 66). This observation of reduced 5′-nucleotidase activity in many different models of cholestasis suggests that the enzyme deficit may result in reduced excretion of organic molecules across the canalicular membrane (41).

The pathogenesis of cholestasis is not known. The present studies suggest that specific canalicular enzymes are altered in cholestasis. Reduced activity of 5′-nucleotidase and Mg^{2+}-ATPase in two forms of cholestasis suggests that these alterations may be involved in the pathogenesis of bile secretory failure, or possibly may result from cholestasis.

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