

Reversal of Ethinyl Estradiol-induced Bile Secretory Failure with Triton WR-1339

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ABSTRACT The effects of Triton WR-1339 and phenobarbital on ethinyl estradiol bile secretory failure were examined to determine the mechanism responsible for decreased bile salt excretion. When administered to ethinyl estradiol-treated rats, Triton WR-1339 restored bile salt independent bile flow and maximum taurocholate transport, whereas phenobarbital corrected bile flow only. Ethinyl estradiol decreased the activities of $\text{Na}^+\text{-K}^+\text{-ATPase}$, 5'-nucleotidase, while increasing the activities of $\text{Mg}^{++}\text{-ATPase}$ and alkaline phosphatase. In contrast to these heterogeneous changes in surface membrane enzyme activities, the number and affinity of [^{14}C]cholic acid carriers were not altered. When administered in vivo or added directly to surface membrane fractions Triton WR-1339 restored the activities of $\text{Na}^+\text{-K}^+\text{-ATPase}$ and $\text{Mg}^{++}\text{-ATPase}$ of rats treated with ethinyl estradiol through a process that did not require protein synthesis (unaffected by cycloheximide). Phenobarbital also restored the activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$ to control levels, but, unlike Triton WR-1339 it did not correct the defect responsible for reduced bile salt secretion. Ethinyl estradiol increased the concentration of cholesterol esters in surface membrane fractions. When administered to ethinyl estradiol-treated rats, Triton WR-1339 restored cholesterol ester concentrations to normal, whereas phenobarbital did not. These combined data suggest that decreased or altered bile salt carriers or reduced sodium driving forces resulting from impaired activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$ are not responsible for decreased bile salt

excretion in ethinyl estradiol-treated rats. It is proposed that the diverse changes in surface membrane function, which are associated with ethinyl estradiol bile secretory failure, may be the result of a generalized alteration in membrane lipid structure.

INTRODUCTION

Drugs such as estrogens, anabolic steroids, phenothiazines, and others (1) may cause intrahepatic cholestasis by interfering with either bile salt dependent, independent, or both components of bile flow. The cause(s) of bile secretory failure (cholestasis) is unknown, but these agents have focused attention on abnormalities in the hepatocyte. Ethinyl estradiol, a synthetic estrogen, has been extensively studied as a drug which produces bile secretory failure characterized by decreases in the bile salt independent component of bile flow (2-4), the maximum capacity to excrete organic anions such as bile acids (5), bilirubin (6), and bromosulphothalein (4, 7) and decreased hepatic $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity (8, 9). It has been suggested that this diffuse abnormality in biliary secretory processes is primarily the result of changes in membrane lipid structure (9).

In these studies, we have sought to determine whether the reduction of bile acid transport in ethinyl estradiol-treated rats is also related to changes in membrane lipids; or rather is caused by alterations of bile salt receptors, or decreased driving forces for the sodium transport system secondary to reduced $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. The results indicate that alterations in liver surface membrane lipid composition may be responsible for decreased bile acid transport in ethinyl estradiol-treated rats.

METHODS

Materials and animals. Male Sprague-Dawley rats weighing 180-250 g (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) allowed free access to Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.) and water were used in all experiments. Rats were kept in cages with aspen wood chip bedding (American Excelsior Co., Denver, Colo.) and

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alternating 12 h of light and darkness. Insecticides were not used in the animal quarters.

[24-¹⁴C]cholic acid (50–60 mCi/mmol, 99% radiochemically pure) from New England Nuclear (Boston, Mass.) and [¹⁴C]leucine (348 mCi/mmol from Amersham Corp., Arlington Heights, Ill.) were used. Taurocholic acid (>95% purity) was obtained from Maybridge Research Chemicals, Cornwall, England. Purity of bile acids was confirmed by thin-layer chromatography (10). Adenosine 5'-triphosphate, adenosine 5'-monophosphate, ouabain, albumin (bovine), EGTA, and ethinyl estradiol were obtained from Sigma Chemical Co., St. Louis, Mo. Triton WR-1339 (oxyethylated tertiary octylphenol polymethylene polymer) was obtained from Ruger Chemical Company, Inc. (Irvington, N. J.).

Ethinyl estradiol was dissolved in corn oil and administered through a gastric tube, 5 mg/kg body wt daily for 5 d, or given subcutaneously in either 0.5 ml of corn oil or 0.2 ml propylene glycol. Control rats received either 0.5 ml of corn oil or 0.2 ml propylene glycol. Control and randomly selected ethinyl estradiol-treated rats were fasted overnight and injected on the fifth day of treatment with Triton WR-1339 (62.5 mg/ml dissolved in 0.9% saline so that each received 22.5 mg/100 g body wt i.p.) and killed 16 h later. In some experiments cycloheximide 15 mg/ml was dissolved in 0.9% saline and injected intraperitoneally at a dosage of 150 µg/100 g body wt, 30 min before the administration of Triton WR-1339. In an additional set of experiments phenobarbital (80 mg/kg body wt i.p.) was administered daily either alone or simultaneously with ethinyl estradiol for 5 d.

Liver surface membrane fractions were prepared through the final stage according to the procedure of Neville (11). Surface membrane fractions were washed once each with ice-cold 1 mM NaHCO₃ and 0.15 M NaCl. Microsomal fractions were prepared from the supernate by modification of the procedure of Evans and Gurd (12) as previously described (13). Microsomal fractions were washed by the method of Weihing et al. (14) to remove adsorbed and intracisternal proteins.

Enzyme and chemical assays. Na⁺-K⁺-ATPase (ATP phosphohydrolase, EC 3.6.1.3) was assayed in liver surface membrane fractions and in liver homogenates according to the method of Ismail-Beigi and Edelman (15), after overnight freezing (16). 5'-nucleotidase activity (EC 3.1.3.5) was measured by the method of Song and Bodansky (17), glucose-6-phosphatase (EC 3.1.3.9) activity according to deDuve et al. (18), alkaline phosphomonoesterase (EC 3.1.3.1) using β-glycerol phosphate as substrate according to Bodansky (19), and acid phosphomonoesterase (EC 3.1.3.2) according to Shibhe and Tappel (20). Enzyme activities were determined by the initial rate of release of phosphorous (21) from appropriate substrates at 37°C and expressed as micromoles of phosphorous released per milligram of protein per hour. Cytochrome c oxidase (EC 1.9.3.1) was measured by the method of Straus (22) and expressed as micrograms of amine hydrochloride formed per milligram protein per minute. Protein was measured by the method of Lowry et al. (23).

Cholesterol and cholesterol esters were extracted by addition of 20 times sample volume of chloroform-methanol (2:1) as previously described (9). After evaporation of the chloroform extracts under nitrogen, samples were brought up into 2-propanol and the mass of cholesterol and cholesterol esters were determined using the enzymatic fluorometric assay as described by Heider and Boyett (24). Cholesterol esters were calculated as the difference between total mass of cholesterol (after hydrolysis with microbial cholesterol esterase) and free cholesterol (no hydrolysis). All values were obtained from a standard curve using authentic free and esterified cholesterol. Repeat determinations of a representa-

tive sample were within 5% for both free and esterified cholesterol.

Binding assay. Bile acid binding to liver homogenate and surface membrane fractions were measured in an incubation media that contained 66 mM monosodium/dissodium phosphate buffer, pH 6.0, and [¹⁴C]cholic acid, as previously described (13). The binding reaction was started by adding the tissue sample to complete a reaction volume of 0.2 ml. After incubation at 4°C for 20 min the binding reaction was terminated by vacuum filtration. Filters with adsorbed membrane [¹⁴C]cholic acid complexes were counted by liquid scintillation spectrometry at 80% efficiency.

Hepatic bile salt excretory transport maximum. Studies in all groups were performed as previously described (25) under pentobarbital (Nembutal; Abbott Laboratories, North Chicago, Ill.) anesthesia (4 mg/100 g body wt i.p.). Body temperature was maintained between 36.8° and 37.5°C using a warming lamp and monitored by rectal temperatures. For collection of bile the common bile duct was cannulated with PE-10 polyethylene tubing (Clay-Adams, Div., Becton Dickinson & Co., Parsippany, N. J.) just below the bifurcation. Bile was collected over 90 min using tared tubes for individual 15-min periods. The femoral vein was cannulated with PE-50 polyethylene tubing for infusion of bile salts. Taurocholic acid (45 mM) was dissolved in 0.9% saline solution containing 3% bovine serum albumin. After two 15-min collections of bile during infusion of saline, the bile salt infusion was started using a Harvard pump (Harvard Apparatus Co., Inc., S. Natick, Mass.) at a constant rate (1.2 µmol/min per 100 g body wt). Bile acids were measured in bile by the enzymatic method of Talalay (26) using purified 3-β-steroid dehydrogenase (Worthington Biochemical Corp., Freehold, N. J.). Bile acid excretion reached a maximum value by 30 min after taurocholate infusion and did not significantly change during at least the following 30 min in both control and experimental rats. Greater infusion rates of taurocholate decreased both bile flow and bile acid secretion, and failed to raise maximum hepatic excretory capacity (T_m).¹ Taurocholate T_m was estimated from the mean of the two highest consecutive 15-min biliary bile acid outputs in each animal.

Bile salt independent bile flow (BSIBF) determination. BSIBF is assumed to be that volume of bile that is theoretically secreted in the absence of bile salt secretion. It was determined indirectly for each group of animals by extrapolating the calculated linear regression of bile flow vs. bile salt secretion to zero bile salt secretion. Although the linear assumption of this line (27) and its total independence from bile salt excretion have been questioned (28), currently it is a reasonable method to estimate this component of bile flow. Values of slope, y-intercept (BSIBF) and correlation coefficient were calculated using all the experimental values of bile flow and bile salt excretion obtained for each different group of rats.

Mathematical and statistical analysis. Specific cholic acid binding constants, apparent maximal binding capacity and dissociation constant (K_d) were determined from the plot of cholic acid binding vs. free cholic acid concentrations, as previously described (13). Best-fit lines and apparent constants were estimated by a nonlinear least squares regression program (29) on a CDC series 6400 computer (Control Data Corp., Minneapolis, Minn.). It has previously been demonstrated by Scatchard analysis that both conjugated and free cholic acid binding at equilibrium best fits a single class

¹Abbreviations used in this paper: BSIBF, bile salt independent bile flow; T_m, maximum hepatic excretory capacity.

of binding sites that do not demonstrate cooperative interactions (13).

Variances of best-fit lines were compared by an F test. Significance of differences among estimated parameters was determined by a two-sample *t* test or a Z score (30). *P* values ≤ 0.05 were considered significant.

RESULTS

Effect of ethinyl estradiol treatment on weight, liver surface membrane enzyme activities, and protein recovery. As previously shown, the average weight gain of ethinyl estradiol-treated rats ($24.5 \pm 0.5\%$) compared to corn oil-injected controls ($13.5 \pm 2.9\%$, $P < 0.05$) was significantly decreased, while liver size relative to body weight (4.1 ± 0.2 vs. $5.2 \pm 0.2\%$, $P < 0.001$) was increased (31). However, ethinyl estradiol administration did not change liver surface membrane protein recovery (0.25 ± 0.02 vs. 0.28 ± 0.01 mg protein/g liver).

Enzymatic activities from control and ethinyl estradiol-treated rats of liver homogenate and the surface membrane fraction are shown in Table I. Marker enzymes of both the canalicular (Mg⁺⁺-ATPase and alkaline phosphatase) and sinusoidal (Na⁺-K⁺-ATPase) surface membranes show equal enrichment of activities in surface membrane fractions from control and treated rats without apparent preferential purification of either canalicular or sinusoidal membranes. Ethinyl estradiol administration apparently did not alter the small degree of intracellular organelle contamination

in the surface membrane fraction. Compared to control animals, administration of ethinyl estradiol significantly decreased hepatic Na⁺-K⁺-ATPase and 5'-nucleotidase and increased alkaline phosphatase and Mg⁺⁺-ATPase in the surface membrane as well as the homogenate (Table I).

Bile acid binding to liver surface membrane fraction. We have previously shown that [¹⁴C]cholic acid specifically binds in a saturable and reversible manner to liver surface membrane fractions (13). Since in vitro binding kinetic characteristics are similar to those for bile acid transport (7, 13) and since a parallel decrease in transport and the number of bile acid binding sites are observed after cycloheximide administration, it has been proposed that the bile acid receptor represents the putative bile acid carrier (13, 25). To determine whether changes in putative bile acid carriers can account for abnormal transport, the number of [¹⁴C]cholic acid binding sites was determined in liver homogenates and in surface membrane fractions (Table II). In contrast to the heterogeneous changes in liver surface membrane enzymes, ethinyl estradiol treatment did not significantly modify the maximum number of specific [¹⁴C]cholic acid binding sites in either the surface membrane fraction or in liver homogenates from ethinyl estradiol-treated animals compared to controls. Transport of bile acids might result from an alteration in the affinity of the carrier for the ligand (*K_d*). Since, however, the dissociation constant was not significantly changed by

TABLE I
Enzymatic Activity in Livers From Control and Ethinyl Estradiol-treated Rats

	Na ⁺ -K ⁺ -ATPase	Mg ⁺⁺ -ATPase	Alkaline phosphomonoesterase	5'-Nucleotidase	Glucose-6-phosphatase	Cytochrome c oxidase	Acid phosphomonoesterase
	<i>μmol Pi/hmg protein</i>				<i>μg amine-HCl/ min/mg protein</i>		<i>μmol Pi/ h/mg protein</i>
Control							
Specific activity							
Homogenate	2.5±0.2	8.9±0.3	0.6±0.1	2.9±0.1	5.1±0.3	75.9±2.8	1.8±0.02
Liver surface membrane	63.8±4.2	133.0±16.8	8.1±0.8	87.0±5.6	0.4±0.2	62.4±6.1	ND
Relative specific activity	26	15	14	30	0.1	0.8	—
Percent recovery	5.3±1.0	4.4±0.8	2.9±0.3	6.4±1.0	0.01	0.3±0.03	—
Ethinyl estradiol							
Specific activity							
Homogenate	1.4±0.1*	14.5±2.8*	1.2±0.3*	2.4±0.1*	5.2±0.3	70.5±2.0	1.5±0.05
Liver surface membrane	41.7±2.5*	259.1±25.6*	18.4±2.4*	61.0±5.3*	1.7±0.2*	31.3±1.9	ND
Relative specific activity	29	18	15	29	0.5	0.4	—
Percent recovery	9.7±2.2*	4.0±0.8	3.1±0.2	7.2±1.2	0.08±0.02	0.21±0.01	—

Liver surface membrane fractions were prepared and enzyme activities determined as described under Methods. Ethinyl estradiol (5 mg/kg body wt) and corn oil (controls) was administered subcutaneously daily for 5 d. Six separate determinations were analyzed from the combined homogenates of 12 rats (two rat livers per determination) from each group. ND, none detectable. Results are expressed as the mean ± SEM.

* *P* < 0.01 compared to control.

TABLE II
Effect of Ethinyl Estradiol Administration on [¹⁴C]Cholic Acid Binding to Liver Surface Membrane Fractions

	[¹⁴ C]Cholic acid binding		
	Homogenate	Surface membrane fractions	
		<i>K_d</i>	N
	<i>μ</i> mol/g liver	mM	nmol/mg protein
Control	453±50 (6)	0.5±0.1 (6)	28.4±0.6 (6)
Ethinyl estradiol	488±93 (6)	0.6±0.2 (3)	26.2±2.9 (3)
Percent change	+3	+17	-8
Significance	NS	NS	NS

Control rats were given corn oil. Ethinyl estradiol (5 mg/kg body wt) was administered subcutaneously daily for 5 d. Liver surface membrane fractions were isolated according to Neville (29) and [¹⁴C]cholic acid binding determined as described under Methods. *K_d* and apparent binding capacity were determined from separate experiments by analysis of the rectangular hyperbola by nonlinear least squares regression as previously described (13, 29), for six different cholic acid concentrations from 0.2 to 2.5 mM. The number in parenthesis indicates the number of individual experiments. Results are expressed as the mean±SEM. NS, no significant difference between groups.

administration of ethinyl estradiol this possibility seems unlikely.

Effect of Triton WR-1339 and phenobarbital on bile flow and maximum bile salt excretory transport and Na⁺-K⁺-ATPase activity. The ability to generate bile flow and excrete bile acids in response to an infusion of taurocholate was examined in ethinyl estradiol-treated rats. Bile acid excretion reached maximum values between 15 and 30 min after the start of the taurocholate infusion, and its excretion did not significantly change during the next 30 min of the study. As previously shown (3-5, 31), ethinyl estradiol treatment significantly reduced bile flow in the basal state (*P* < 0.01) and attenuated the response to the infusion of taurocholate (*P* < 0.001). Similarly, the maximum capacity to excrete bile acids was reduced to 60% of control (*P* < 0.01) (Table III). Triton WR-1339, a nonionic detergent which has previously been shown to restore the abnormal physical properties of the liver surface membrane from ethinyl estradiol-treated rats (9), also restored basal bile flow, BSIBF, and the maximum capacity to excrete taurocholate to normal values (Table III). In contrast, Triton WR-1339 administered to untreated rats did not significantly alter basal bile flow (9.1±1.2), or maximum bile acid excretory capacity (1.0±0.2).

Since Triton WR-1339 restores Na⁺-K⁺-ATPase activity to control values (9) as well as bile acid T_m, we

TABLE III
Effect of Ethinyl Estradiol and Phenobarbital on Basal Bile Flow, BSIBF, Hepatic Na⁺-K⁺-ATPase, and Bile Salt T_m

	Control	Ethinyl estradiol	Ethinyl estradiol and phenobarbital	Phenobarbital alone	Ethinyl estradiol and Triton WR-1339
Basal bile flow, <i>μ</i> l/min/100 g body wt	7.6±0.6 (7)	4.6±0.6* (7)	9.7±0.7 (4)	10.2±0.6* (4)	7.8±0.8 (9)
BSIBF, <i>μ</i> l/min/100 g body wt	5.2±0.4 (5)	3.5±0.3* (6)	6.0±0.6 (4)	7.8±0.8* (4)	6.5±0.4 (9)
Na ⁺ -K ⁺ -ATPase activity, <i>μ</i> mol Pi/h/mg protein	32.6±1.8 (6)	15.7±0.8* (6)	31.6±2.6† (5)	47.9±2.6* (6)	48.9±1.6 (6)
Bile salt T _m , <i>μ</i> mol/min/100 g body wt	1.0±0.08 (5)	0.6±0.1* (6)	0.7±0.1§ (6)	1.1±0.2 (4)	1.2±0.1 (9)

Ethinyl estradiol (5 mg/kg body wt) was administered subcutaneously in corn oil, and phenobarbital (80 mg/kg body wt) was administered simultaneously with the ethinyl estradiol. Na⁺-K⁺-ATPase activity was measured in partially purified liver surface membrane fractions (29) as described in Methods. Basal bile flow, BSIBF, and bile salt T_m were measured as described in Methods. Number in parentheses refers to number of observations. Results are reported as mean±SEM.

* *P* < 0.01 (compared to controls).

† *P* < 0.01 compared to phenobarbital alone.

§ Not significantly different from ethinyl estradiol-treated rats.

administered phenobarbital, a drug also known to increase $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity (8, 16) to examine whether restoration of normal transport function may be possibly related to changes in sodium pump activity. Phenobarbital administered simultaneously with ethinyl estradiol restored basal bile flow, BSIBF, and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity to values not significantly different from control (Table III). In contrast, although phenobarbital administration restored $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity to control values, bile acid Tm was still significantly reduced (70% of control value, $P < 0.01$). When administered in the absence of ethinyl estradiol, phenobarbital increased $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity and BSIBF but did not alter the maximum hepatic transport of taurocholate (Table III).

The values for bile flow and the activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$ of rats treated with both phenobarbital and ethinyl estradiol are significantly less than those found for rats treated with phenobarbital alone (Table III). However, maximal activity was obtained by adding Triton WR-1339 directly to the surface membrane fractions of rats treated with phenobarbital and ethinyl estradiol (Fig. 1). Thus, the activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$ increased in a manner related to the amount of Triton WR-1339 added, reaching a value similar to that observed with phenobarbital administration alone. In contrast, Triton WR-1339 did not alter the activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$ when added to surface membranes from rats treated only with phenobarbital (Fig. 1). It has been previously shown that Triton WR-1339 causes a similar stimulation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ when added to surface membranes from rats treated with ethinyl estradiol alone (9). Thus, surface membranes from rats treated with ethinyl estradiol with and without phenobarbital possess latent

activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$, which can be maximally expressed in the presence of Triton WR-1339.

Effect of ethinyl estradiol, Triton WR-1339, and phenobarbital on hepatic surface membrane lipid composition. To examine whether the inability of phenobarbital to restore reduced bile acid Tm in ethinyl estradiol-treated rats was due to its failure to correct the abnormal hepatic surface membrane lipid composition, the changes in cholesterol and phospholipid were examined in surface membrane fractions from treated and control animals (Fig. 2). As previously shown for partially purified membrane fractions (9), Triton WR-1339 administration to ethinyl estradiol-treated rats restores cholesterol ester concentrations to control values. In contrast, phenobarbital given simultaneously with ethinyl estradiol failed to reduce the increased cholesterol ester concentration. As previously noted with recovery of membrane enzymes, ethinyl estradiol administration did not significantly alter the recovery of surface membrane lipids (data not shown). In addition, phenobarbital administration with ethinyl estradiol did not significantly change either the surface membrane content of total phospholipid or free cholesterol.

To examine whether the changes in lipid composition observed in heterogeneous liver surface membrane fractions with ethinyl estradiol and Triton WR-1339 treatment may account for changes in the sinusoidal as well as the canalicular surface membrane, we compared the effect of Triton WR-1339 on the enzymatic activities of $\text{Na}^+\text{-K}^+\text{-ATPase}$ (sinusoidal) and $\text{Mg}^{++}\text{-ATPase}$ (canalicular) after in vivo and after in vitro administration. The results are shown in Table IV and demonstrate that Triton WR-1339 administration restored both of these enzyme activities to control

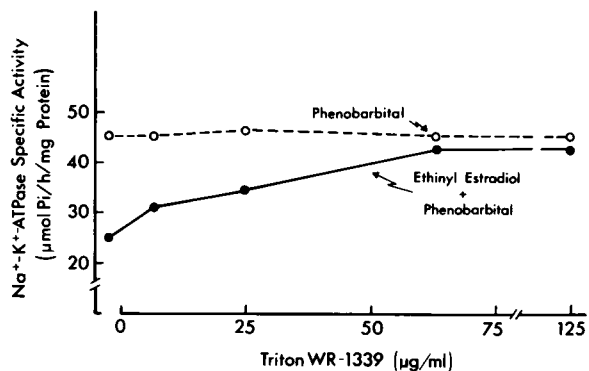


FIGURE 1 Effect of adding Triton WR-1339 to surface membrane fractions on $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in vitro. Triton WR-1339 was added to partially purified surface membrane fractions ($\sim 200 \mu\text{g}$ protein/tube) from ethinyl estradiol (5 mg/kg body wt) plus phenobarbital (80 mg/kg body wt) (●) and phenobarbital alone (○) treated rats. Each point is the mean of three separate experiments, which agreed within 10% of each other.

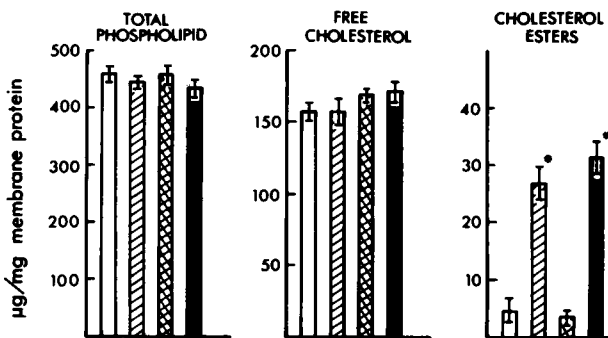


FIGURE 2 Lipid composition of liver surface membrane fractions. Lipids were measured in liver surface membrane fractions (29) after extraction in control (□), ethinyl estradiol (▨), ethinyl estradiol plus Triton WR-1339 (▩), or ethinyl estradiol plus phenobarbital (■). Values are expressed as the mean \pm SEM, and are the determination from six different sets of animals in each treatment group. (*) $P < 0.001$, compared to control determinations.

TABLE IV
In Vivo and In Vitro Reversal of Ethinyl Estradiol-induced Changes in Liver Surface Membrane Enzymes with Triton WR-1339

	Na ⁺ -K ⁺ -ATPase		Mg ⁺⁺ -ATPase	
	Saline	Triton	Saline	Triton
In Vivo*				
Control	29.5±2.8	30.2±1.7	78.7±16.1	80.4±16.4
Ethinyl estradiol	15.9±1.4	31.8±1.5†	133.4±12.6	83.8±17.3†
In Vitro§				
Control	30.3±1.8	32.1±2.8	68.8±7.8	59.5±5.5
Ethinyl estradiol	16.3±1.6	33.5±5.7†	131.8±10.2	83.2±3.3†

* Triton WR-1339 22.5/100 g body wt i.p. administered 16 h before sacrifice.

† $P < 0.05$.

§ 125 µg/ml Triton WR-1339 added to enzyme before assay, which has a final vol of 2 ml. Partially purified surface membrane fractions were prepared from untreated and ethinyl estradiol (5 mg/kg body wt)-treated rats (29). Enzyme assays were performed as described in Methods. The results are presented as the mean ± SEM of in vivo ($n = 9$) and in vitro ($n = 3$) experiments.

values, whether given to the whole animal (in vivo) or added directly to the test tube (in vitro). In contrast, addition of phenobarbital in vitro did not change enzyme activity (data not shown).

Effect of cycloheximide on hepatic Na⁺-K⁺-ATPase activity, basal bile flow, and bile salt Tm. Cycloheximide, a potent inhibitor of protein synthesis (31) was administered to untreated and ethinyl estradiol-treated rats to determine whether reversal of decreased Na⁺-K⁺-ATPase activity, BSIBF, and bile salt Tm after Triton WR-1339 administration to ethinyl estradiol-treated rats is dependent on *de novo* protein synthesis. Ethinyl estradiol treatment significantly reduced Na⁺-K⁺-ATPase activity (54%), BSIBF (67%), and bile salt Tm (56%), whereas Triton WR-1339 returned these parameters to control levels (Table V). Cycloheximide administration did not prevent Triton WR-1339 reversal of either Na⁺-K⁺-ATPase activity or BSIBF in the ethinyl estradiol-treated rat, although the dose employed (150 µg/100 g body wt) has previously been shown to inhibit protein synthesis and decrease bile acid Tm, as well as prevent phenobarbital induction of Na⁺-K⁺-ATPase and bile flow (16, 25).

Neither Na⁺-K⁺-ATPase activity nor BSIBF were significantly altered after cycloheximide administration. In contrast, inhibition of protein synthesis reduced bile salt Tm in both untreated and ethinyl estradiol-treated rats to 65 and 59%, respectively. Triton WR-1339 administration to rats treated with ethinyl estradiol and cycloheximide restores bile salt Tm to levels similar to that found for its control (cycloheximide administration alone; 89%, $P = NS$). These studies suggest that correction of bile salt transport with Triton WR-1339 in ethinyl estradiol-treated animals,

like that for Na⁺-K⁺-ATPase activity and bile flow, does not require protein synthesis.

DISCUSSION

The biochemical events and morphological components involved in the bile secretory process are poorly understood. One requirement to study biliary transport mechanisms is the ability to alter this complex process in a controlled manner. In this sense ethinyl estradiol administration has been shown to predictably reduce BSIBF, and the maximum capacity to excrete organic anions (32). In addition, this diffuse alteration in canalicular membrane transport is not associated with morphological changes in hepatocyte ultrastructure (33, 34) and thus serves as an excellent model to understand hepatic transport processes.

The transcellular movement of substances in polar epithelial cells requires asymmetry of the membrane transport elements (35). In this regard, transport of bile acids across the contraluminal membrane (sinusoidal surface) is a carrier-mediated process requiring sodium; whereas, although poorly defined, the rate-limiting luminal (canalicular) carrier-mediated excretory step is probably independent of sodium (36). The sodium gradient is maintained by Na⁺-K⁺-ATPase, whose function can be considered the biochemical equivalent of the sodium pump (37). Additionally, Na⁺-K⁺-ATPase is a transmembrane enzyme whose function is very sensitive to its lipid environment (38). Thus, its activity is a measure of both the driving forces for the sodium potential difference as well as a probe of the luminal surface membrane structure. In hepatocytes Mg⁺⁺-ATPase, whose activity is also lipid sensitive (38), is

TABLE V
Effect of Ethinyl Estradiol, Triton WR-1339, and Cycloheximide
on Na⁺-K⁺-ATPase Activity, BSIBF, and Bile Salt T_m

	Na ⁺ -K ⁺ -ATPase	BSIBF	Bile salt T _m
	μmol P _i /mg protein	μl/min/100 g body wt	μmol/min/100 g body wt
Control	44.3±3.0 (6)	5.2±0.4 (5)	1.0±0.08 (6)
Ethinyl estradiol	21.9±2.0 [49%]* (6)	3.5±0.3 [67%]* (6)	0.56±0.08* (6)
Ethinyl estradiol + Triton WR-1339	48.9±1.6 [110%] (6)	6.5±0.4 [125%] (9)	1.24±0.13 (9)
Ethinyl estradiol + cycloheximide + Triton WR-1339	40.0±2.0 [91%] (6)	4.8±0.5 [92%] (3)	0.58±0.06* (3)
Ethinyl estradiol + cycloheximide	25.2±0.9* [57%] (3)	3.9±0.4 [75%]* (4)	0.33±0.06*† (4)
Cycloheximide alone	36.2±2.7 [82%] (3)	4.6±0.6 [89%] (4)	0.65±0.07* (4)

Na⁺-K⁺-ATPase was measured in partially purified liver surface membrane fractions (29). Control animals received propylene glycol, while ethinyl estradiol (5 mg/kg body wt) was administered subcutaneously in propylene glycol. Cycloheximide was administered 30 min before Triton WR-1339, and animals sacrificed 16 h later. Values are recorded as the mean±SEM. Numbers in parenthesis represent the determinations in different animals, while those in brackets are percent of control.

* $P < 0.05$, compared to controls.

† $P < 0.05$, compared to ethinyl estradiol.

located on the luminal surface (39), and thus also provides a convenient in vitro measurement of canalicular membrane structure.

Bile acid transport in the hepatocyte may be altered at at least three possible levels. Firstly, the carrier or its affinity for bile acids may be modified at either the sinusoidal or the canalicular membrane surfaces; secondly, at the level of the driving forces, which are derived from the electrochemical potential difference of sodium across the contraluminal surface; and lastly, by changes in the membrane lipid environment through restriction of lateral mobility and/or conformational changes in the carrier.

Estrogen administration is known to alter many hepatic functions (32, 40, 41) and thus it is necessary to quantitate each of the possible steps in bile acid translocation to determine the mechanism(s) of decreased bile acid excretion in ethinyl estradiol-induced bile secretory failure. Previous studies suggested that decreased basal bile flow and Na⁺-K⁺-ATPase activity results from decreased surface membrane lipid fluidity (9). Evidence is presented in this study that changes in the lipid composition of the surface membrane may be indirectly involved in decreased bile acid transport.

This was suggested by the observation that similar to our previous study demonstrating restoration to normal of basal bile flow, reduced bile acid T_m in ethinyl estradiol-treated animals was returned to control values 16 h after Triton WR-1339 administration.

To examine the possible membrane steps involved in the pathogenesis of ethinyl estradiol-induced cholestasis and its reversal with Triton WR-1339, liver surface membrane fractions were prepared. Anatomically, the surface membrane of the liver parenchymal cell is a composite of three functionally different regions, which are a single physical entity (42). However, it has proven difficult to successfully isolate these components into fractions free of significant cross contamination from intracellular organelles or other surface membrane regions. While it is not possible to determine the relative enrichment of sinusoidal vs. canalicular membrane regions because both Mg⁺⁺-ATPase and alkaline phosphatase have intracellular isoenzymes (43), the marked enrichment of Na⁺-K⁺-ATPase activity suggests this final membrane fraction may at the very least contain equal proportions of sinusoidal and canalicular membrane elements. Furthermore, the surface membrane fractions are relatively free of intra-

cellular membranes, as judged by intracellular marker enzymes.

As previously shown (8, 16, 31), administration of ethinyl estradiol is associated with decreased activities of $\text{Na}^+\text{-K}^+\text{-ATPase}$ and 5'-nucleotidase and increased activities of $\text{Mg}^{++}\text{-ATPase}$ and alkaline phosphatase. The unaltered relative enrichment of membrane enzymes and recovery of membrane protein through purification of the surface membrane fraction from ethinyl estradiol-treated rats strongly suggests these enzyme changes are not due to alterations in the recovery of membrane fractions. This conclusion is strengthened by the findings that similar enzyme activity alterations are observed in the homogenate. Furthermore, changes in enzyme activities associated with both the sinusoidal as well as the canalicular membrane surface suggests that ethinyl estradiol produces similar changes in both membrane surfaces. Thus, although the present membrane isolation method recovers both sinusoidal as well as canalicular surfaces, their relative proportion does not change after experimental treatment. Furthermore, the enzyme changes suggest that ethinyl estradiol treatment apparently changes the contraluminal as well as the luminal surface membrane; for $\text{Na}^+\text{-K}^+\text{-ATPase}$, in addition to the activities of $\text{Mg}^{++}\text{-ATPase}$, alkaline phosphatase and 5'-nucleotidase, is altered.

The possibility that decreased bile acid transport results from a reduced number or alteration of the structure of putative bile acid carrier proteins in the liver surface membrane was examined by *in vitro* binding techniques. Specific [^{14}C]cholic binding sites are postulated to quantitate the number of putative carriers (13). This hypothesis is supported by the association of decreased bile acid binding sites changing in parallel with reduced bile acid T_m after cycloheximide administration (25). Although it is presently not possible to selectively quantitate changes in the number of luminal and contraluminal carriers, the present study failed to observe a significant reduction in the number of [^{14}C]cholic acid binding sites or an alteration in their affinity for binding. Failure to demonstrate changes in the number of bile acid binding sites is not due to selective membrane isolation, for the total number of bile acid binding sites in liver homogenates was also unchanged. Although it is possible that selective changes in the number of carriers localized to canalicular membranes could not be detected in this mixed population of surface membranes; we believe this is unlikely, for a similar reduction in bile acid T_m after administration of cycloheximide was associated with changes in the number of [^{14}C]cholic acid binding sites (25).

Transport of bile acids across membranes as with other ligands initially involves their recognition by "carrier" proteins followed by translocation. Measure-

ment of specific binding sites allows *in vitro* quantitation of this initial step independent of the final interaction with the entire process. Thus, the observation that ethinyl estradiol treatment does not alter the initial recognition step for bile acids suggests that reduced maximum transport capacity is due to abnormalities in translocation which may be mediated by alterations in the driving forces for active transport (contraluminal) or through changes in the membrane lipid environment (luminal and/or contraluminal).

It is generally believed that the rate-limiting step for bile acid transport is luminal excretion, but it is possible that in ethinyl estradiol treatment, reduced bile acid T_m is secondary to a reduced sodium-dependent uptake process, since its administration reduces hepatic sodium pump activity to 50% of control. Therefore, we attempted to increase $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity to determine if this would also correct bile acid T_m . $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity is restored to 107% of control with phenobarbital. In contrast, despite normal bile flow and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity, bile acid T_m is not significantly improved. This finding is consistent with a previous study by Gumucio and co-workers (5).

The present study confirms the striking elevation in membrane cholesterol esters in a "purified" but mixed surface membrane fraction (9), however, we did not detect an increased free cholesterol concentration in this study. In other studies we (9) and others (44) have shown that ethinyl estradiol administration increases liver surface membrane free cholesterol content and also increases microviscosity. In the present study we used a newly developed enzymatic fluorometric cholesterol assay (24), which we have found to be more sensitive and precise compared to our previous assay. It is possible that the change in the cholesterol assay may account for the differences between the results obtained in this study and those of previous studies.

In contrast to Triton WR-1339, phenobarbital administered simultaneously with ethinyl estradiol does not correct the elevated concentration of esterified cholesterol. Reversal of reduced bile acid T_m with Triton WR-1339 and failure of a similar change with phenobarbital correlates with the changes in hepatic cholesterol ester content. This association suggests that the abnormal accumulation of esterified cholesterol may account for reduced bile salt T_m , possibly through the previously shown increase in membrane viscosity (9, 44). The lipid composition of the surface membrane is complex and it is possible that the changes in the physical structure of surface membranes obtained from ethinyl estradiol-treated rats may be the result of alterations in more than a single lipid component. It is not possible in the present study to determine whether the change in the surface membrane structure is located in a specific surface membrane domain or the change is generalized. However, the observations that:

(a) the activities of both Mg^{++} -ATPase (luminal) and Na^+ - K^+ -ATPase (contraluminal) are changed with ethinyl estradiol treatment, (b) that both enzyme activities are restored to control values by in vivo Triton WR-1339 administration; and especially (c) that similar reversal of both abnormal enzyme activities with addition of Triton WR-1339 in vitro, all strongly support the suggestion that the alteration exists in both surface membrane regions.

In contrast to its failure to induce bile acid Tm, phenobarbital induces Na^+ - K^+ -ATPase activity in normal rat liver (8, 16); and restored reduced activity of ethinyl estradiol-treated rats to control values. However, phenobarbital administration to ethinyl estradiol treated rats does not increase enzyme activity or bile flow to values obtained with phenobarbital alone (Table III). Since phenobarbital induces the number of Na^+ - K^+ -ATPase enzyme units in the normal rat (16), these observations suggested the possibility that similar to ethinyl estradiol administration alone the enzyme units are present but unable to function at optimal rates. This hypothesis is strongly supported by activation of latent Na^+ - K^+ -ATPase activity in vitro with Triton WR-1339 in the ethinyl estradiol plus phenobarbital-treated animals to values similar to those found in liver surface membrane fractions from phenobarbital alone-treated rats.

Although cycloheximide has previously been shown to block phenobarbital induction of Na^+ - K^+ -ATPase (16), it did not prevent Triton WR-1339 reversal of reduced activity in ethinyl estradiol-treated animals, further suggesting that altered lipid composition is primarily responsible for reduced sodium pump activity. Similarly, protein inhibition does not inhibit restoration of bile acid transport to the level seen with cycloheximide alone.

Previous studies show that ethinyl estradiol increases hepatic cholesterol ester concentrations by activating microsomal cholesterol acyl-CoA transferase (45). Triton WR-1339 was found to inactivate this enzyme when administered in vivo or when added directly to microsomal fractions obtained from ethinyl estradiol-treated rats (45). Triton WR-1339 apparently reverses abnormal bile acid transport as well as Na^+ - K^+ -ATPase activity and bile flow through mechanisms independent of *de novo* protein synthesis. However, the specific mechanism(s) responsible for reversal with Triton WR-1339 is not clear. At least two possibilities exist. First, the concentration of hepatic cholesterol esters is reduced by Triton WR-1339. This may be due either to inhibition of the ethinyl estradiol activation of microsomal cholesterol acyl-CoA transferase, the rate-limiting step for cholesterol esterification (45); or possibly Triton WR-1339 may activate lysosomal cholesterol ester hydrolysis. These potential changes in cholesterol ester turnover may be responsible for restoration of normal

membrane lipid composition and structure, and thus functional activity. Secondly, Triton WR-1339 may insert directly into the lipid bilayer (46) and restore membrane viscosity to normal independent of lipid changes as demonstrated by the in vitro restoration of normal Na^+ - K^+ -ATPase and Mg^{++} -ATPase activities.

Since ethinyl estradiol administration decreases transport of a number of compounds with different membrane carrier processes, altered membrane function caused, in part, by changes in surface membrane lipid composition is a reasonable hypothesis to account for these diverse changes. Abnormalities in bile acid transport thus may result from either a decreased number of carrier molecules (25) or abnormal lipid composition as shown in the present communication. Further detailed examination of other models of bile secretory failure may also demonstrate abnormalities in the driving forces for translocation.

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