

Dissociation of bile flow and biliary lipid secretion from biliary lysosomal enzyme output in experimental cholestasis¹

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Abstract Although the cellular mechanisms controlling bile flow and biliary lipid secretion are unclear, morphologic data suggest that intracellular vesicles may be involved. Therefore, to investigate the role of hepatocyte lysosomes in bile flow and biliary lipid secretion, we studied the effect of cholestasis on biliary lipid output and on lysosomal enzyme activities and total protein concentration in liver and bile. Castrated male rats were treated with ethinyl estradiol at 0.5 or 5 mg/kg per day for 5 days; bile was collected through a complete bile fistula hourly for 4 hours, and then liver homogenates were prepared. Bile acids, cholesterol, and phospholipid were measured in bile, and three lysosomal glycosidases (β -glucuronidase, β -galactosidase, and *N*-acetyl- β -glucosaminidase) and total protein were measured in bile and liver. Ethinyl estradiol inhibited bile flow in a dose-dependent fashion; it also inhibited bile acid and phospholipid outputs. In contrast, a marked and parallel increase in the biliary outputs of all three lysosomal hydrolases was observed after high-dose ethinyl estradiol; no change in the biliary concentration of total protein was found. Our data suggest that bile flow and biliary lipid secretion involve cellular mechanisms other than vesicular transport by lysosomes.—**Lopez del Pino, V. H., and N. F. LaRusso.** Dissociation of bile flow and biliary lipid secretion from biliary lysosomal enzyme output in experimental cholestasis. *J. Lipid Res.* 1981. **22**: 229–235.

Supplementary key words lysosomes · vesicular transport · acid hydrolases · biliary proteins

The specific cellular mechanisms whereby bile acids, cholesterol, and phospholipid are excreted into bile remain unclear. Theoretically, vesicular or non-vesicular mechanisms could be involved. Vesicular transport might involve sequestration of lipids into vesicles at intracellular sites of synthesis (2), degradation, or uptake. Transcellular movement of lipid-filled vesicles to the canaliculus and release of lipids into bile could then occur by membrane fusion and exocytosis (3). Vesicular transport probably accounts for hepatocyte secretion of lipoproteins (4, 5), glycoproteins (6, 7), and albumin (8) into plasma; it may also explain the biliary output of lysosomal enzymes (9) and other proteins (10). Alternatively,

lipid transport and biliary excretion may involve nonvesicular mechanisms, such as intracellular soluble transport proteins (11) or solubilization of canalicular membranes by bile salts (12–14).

Existing morphologic data suggest that bile flow and biliary lipid secretion involve vesicles. For example, cholestatic and choleric agents alter the density and distribution of hepatocyte organelles, including Golgi complexes and lysosomes (15–17). Published biochemical data relevant to this question are conflicting. Stein, Sanger, and Stein (18) interpreted the lack of effect of colchicine on the biliary secretion of cholesterol and phospholipid as evidence against vesicular transport of biliary lipids. On the other hand, lysosomotropic agents can reverse ethinyl estradiol-induced cholestasis, suggesting that lysosomes may be involved in bile secretion (19). Also, phospholipid accumulates in hepatocyte lysosomes after administration of lysosomotropic agents (2), implying that lysosomes can sequester endogenous lipids, possibly for transport. Thus, available data permit no definite conclusions regarding the role of vesicles, particularly lysosomes, in the processes of bile flow and biliary lipid secretion.

Therefore, to provide biochemical information on a possible role for lysosomes in bile flow and biliary lipid output, we studied the effect of cholestasis induced in rats by ethinyl estradiol (17 α -ethinyl- Δ -1,3,5-estratriene-3,17 β -diol) on biliary lipid secretion, hepatic and biliary lysosomal enzyme activities, and total protein concentrations in liver and bile.

¹ A part of this work has been presented at the 1979 meeting of the American Gastroenterological Association and published in abstract form (1).

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METHODS

General experimental procedure

Twenty prepubertal male Sprague-Dawley rats (100–200 g) were castrated to eliminate the effects of endogenous estrogen from the testes (20). Twenty-one days later, the rats were divided into three groups: control (N = 6), low-dose ethinyl estradiol (N = 7), and high-dose ethinyl estradiol (N = 7). Ethinyl estradiol (Sigma Chemical Co., St. Louis, MO) dissolved in propylene glycol was administered subcutaneously daily for 5 days at 0.5 mg/kg per day (low dose) or 5 mg/kg per day (high dose); controls received propylene glycol. The rats were fasted for 24 hr and, at 0700, were weighed after anesthesia with intraperitoneal sodium pentobarbital (40 mg/kg). Bile ducts were cannulated with PE-10 tubing, tracheostomies were performed to facilitate respiration, and the rats were placed in a constant

temperature apparatus with an adjustable heat lamp. Body temperature was monitored with a rectal thermometer and maintained at approximately 37°C. After a 2-hr equilibration period, bile was collected on ice for 4 hr into preweighed vials at 1-hr intervals. Anesthesia was maintained by intraperitoneal sodium pentobarbital administered intermittently in a standard fashion, and the volumes of collected bile samples were determined by weight. The rats were then killed by exsanguination; their livers were removed and immersed in ice-cold 250 mM sucrose in preweighed beakers. Total liver weight was determined, and then 6 g of liver was homogenized and centrifuged to separate nuclei, unbroken cells, and tissue debris (pellet or N-fraction) from other organelles and cell sap (supernate or E-fraction) as described (9, 21). Studies were performed on aliquots of bile or liver E- and N-fractions diluted in sucrose.

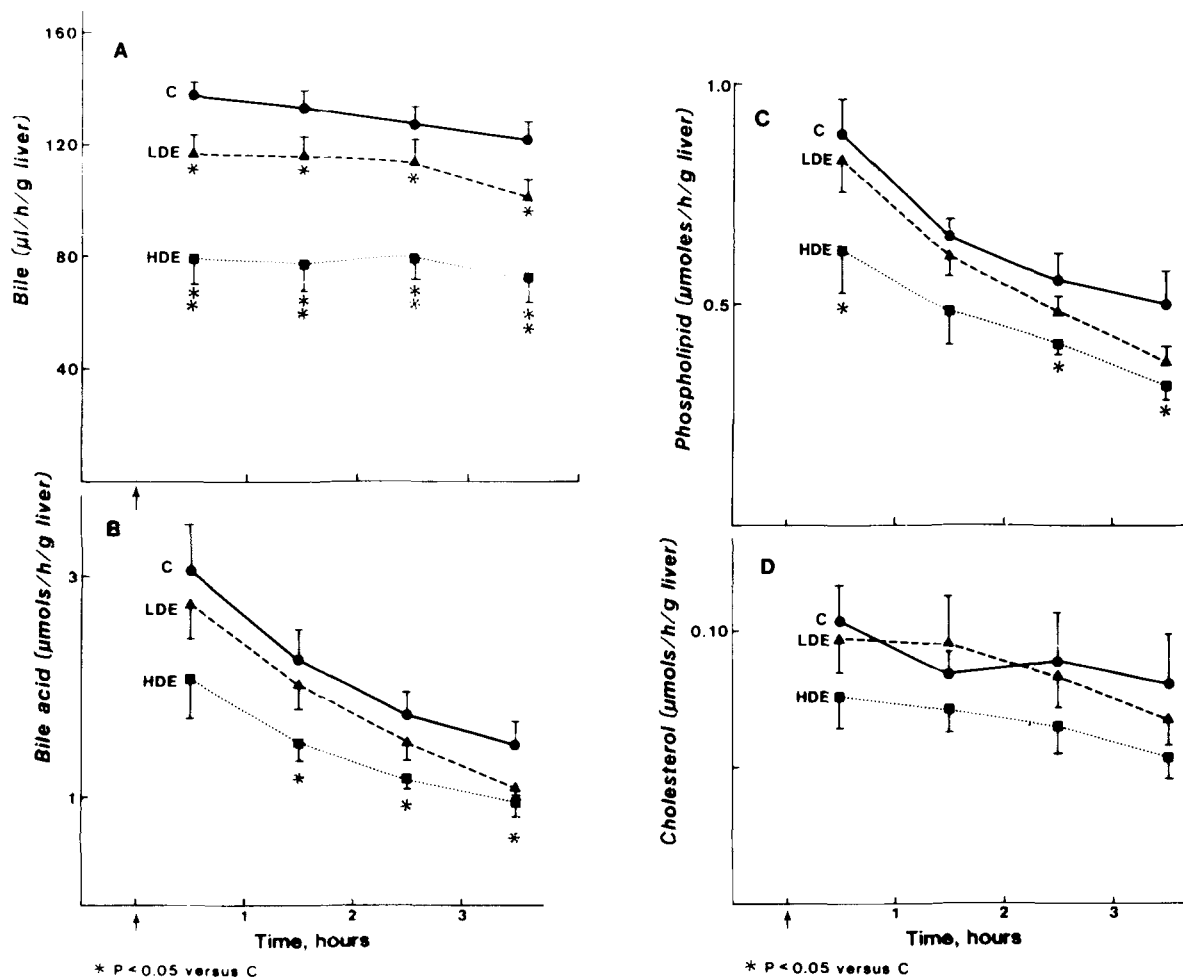


Fig. 1. Hourly outputs of bile and biliary lipids. A, bile flow; B, bile acid output; C, phospholipid output; D, cholesterol output. Data (mean \pm SEM) are given as μ l or μ mols per hr per g of liver for control (●—●), low-dose ethinyl estradiol (▲---▲), and high-dose ethinyl estradiol (■—■). Asterisk (*) indicates $P < 0.05$ compared with control values. C, control; LDE, low-dose ethinyl estradiol; HDE, high-dose ethinyl estradiol.

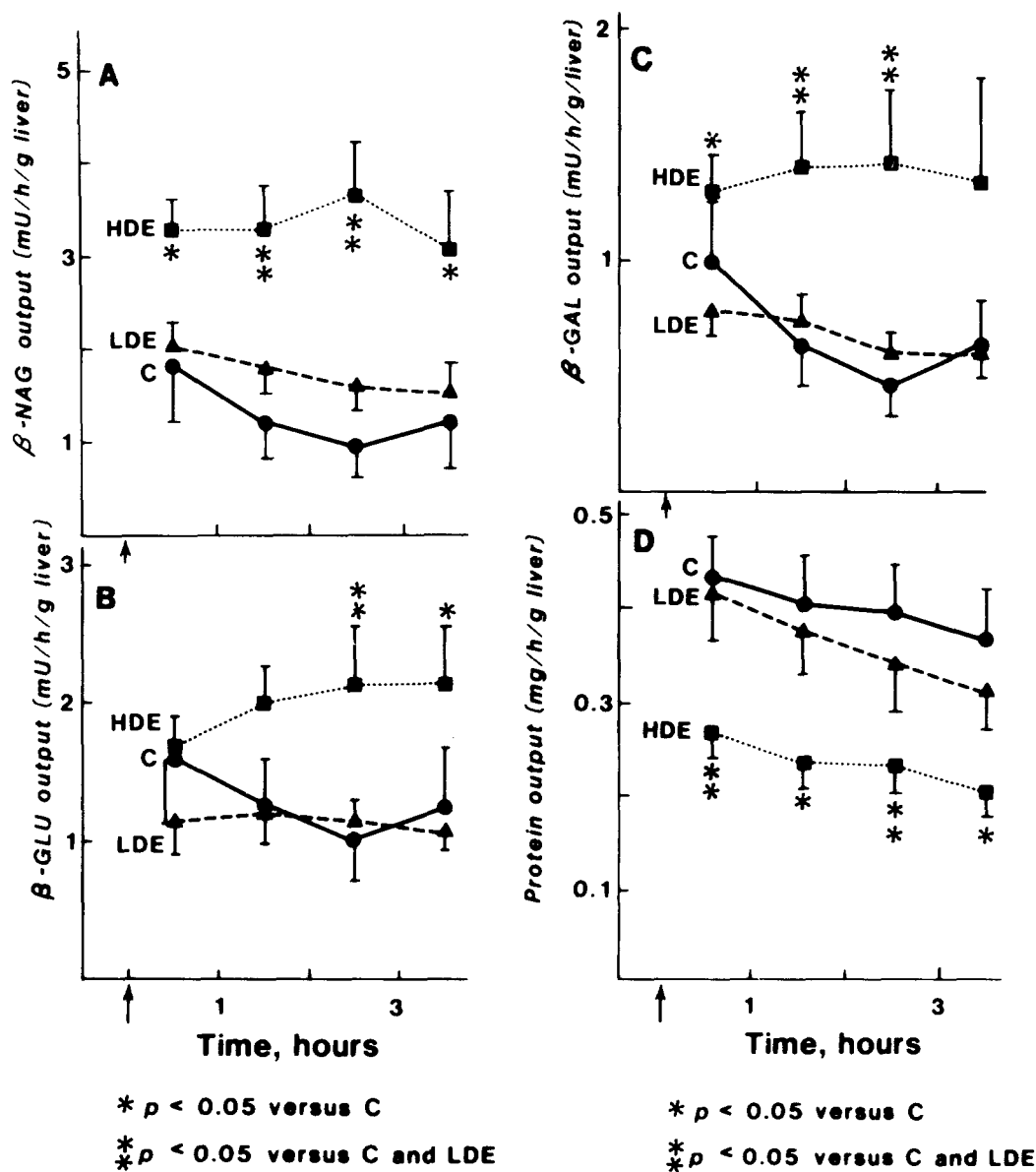


Fig. 2. Hourly biliary outputs of acid hydrolases and total protein. A, *N*-acetyl- β -glucosaminidase; B, β -glucuronidase; C, β -galactosidase; D, total protein. Data (mean \pm SEM) are given as milliunits or milligrams per hr per g of liver for control (\bullet — \bullet), low-dose ethinyl estradiol (\blacktriangle — \blacktriangle), and high-dose ethinyl estradiol (\blacksquare — \blacksquare). β -NAG, *N*-acetyl- β -glucosaminidase; β -GLU, β -glucuronidase; β -GAL, β -galactosidase. C, control; LDE, low-dose ethinyl estradiol; HDE, high-dose ethinyl estradiol.

Analytic procedures

Three lysosomal glycosidases, β -glucuronidase (EC 3.2.1.31), β -galactosidase (EC 3.2.1.23), and *N*-acetyl- β -glucosaminidase (EC 3.2.1.30), were assayed fluorometrically with 4-methyl umbelliferyl substrates (22) using assay conditions previously established by us (9). Latent acid hydrolase activity in E-fractions was utilized as an index of lysosomal membrane integrity (3) and was determined by subtracting free activity (activity in the absence of 0.1% Triton X-100 in the assay mix) from total activity (activity

in the presence of 0.1% Triton X-100) (3). Bile acids were measured by modification of the method of Talalay (23), cholesterol, by gas-liquid chromatography using the extraction method of Abell, Levy, and Brodie (24), and phospholipid, by the method of Chen, Toribara, and Warner (25). Protein determinations were done by the procedure of Lowry et al. (26).

For all hydrolases, 1 U of activity corresponds to the hydrolysis of 1 μ mol substrate per minute.

Statistical analysis was done by the paired *t* test for within-group comparisons and by the unpaired

TABLE 1. Biliary outputs of lysosomal enzymes and total protein^a

Treatment	4-hr Outputs of Lysosomal Enzymes						Total Protein <i>mg/g liver</i>
	<i>N</i> -Acetyl- β -glucosaminidase		β -Glucuronidase		β -Galactosidase		
	Absolute	Relative	Absolute	Relative	Absolute	Relative	
Control (6)							
Propylene glycol	5.27 \pm 3.67	0.45 \pm 0.36	5.12 \pm 3.44	0.39 \pm 0.34	2.74 \pm 1.91	0.42 \pm 0.32	1.61 \pm 0.60
Low-dose (7)							
Ethinyl estradiol	6.96 \pm 2.48	0.44 \pm 0.13	4.56 \pm 1.86	0.35 \pm 0.13	2.74 \pm 0.90	0.42 \pm 0.15	1.37 \pm 0.38
High-dose (7)							
Ethinyl estradiol	13.14 \pm 5.37 ^b	0.90 \pm 0.43 ^b	8.05 \pm 2.69 ^b	0.61 \pm 0.29	5.46 \pm 3.02 ^b	0.98 \pm 0.59 ^b	0.95 \pm 0.20 ^b

^a Cumulative 4-hr biliary outputs were calculated from data presented in Fig. 2 and Table 2. Sample sizes are indicated in parentheses, and values are given as mean \pm SD. Enzyme data are expressed as milliunits per gram of liver (absolute) or as a percentage of the total liver content (relative).

^b $P < 0.05$ versus control value or low-dose ethinyl estradiol value or both.

t test for between-group comparisons. *P* values of 0.05 or less were considered statistically significant.

RESULTS

Body and liver weight

After 5 days of treatment, control rats lost $4.3 \pm 3.0\%$ (M \pm SD) total body weight; weight loss was significantly greater at both low-dose ($10.9 \pm 4.8\%$) and high-dose ($10.5 \pm 2.8\%$) regimens. Liver weights of animals treated with high-dose ethinyl estradiol (7.7 ± 1.2 g) were significantly greater than those in the control (6.2 ± 0.5 g) and low-dose (5.8 ± 0.9 g) groups.

Bile flow and biliary lipid secretion

Patterns of bile flow and biliary lipid excretion are shown in Fig. 1. A dose-dependent inhibition of bile flow of approximately 10% and 40% occurred after low- and high-dose ethinyl estradiol treatment, respectively. The total 4-hr output of bile after high-dose treatment (310 ± 5 μ l/g liver) was significantly less than the outputs for the control (509 ± 5 μ l/g liver) and the low-dose treatment (459 ± 5 μ l/g liver) groups. Bile acid and phospholipid outputs were also significantly diminished in a dose-dependent fashion (Fig. 1). A 23% decrease in cholesterol output occurred after high-dose treatment, but this decrease was not statistically significant.

Biliary outputs of lysosomal enzymes and total protein

The biliary excretion of the lysosomal glycosidases and total protein are shown in Fig. 2 and in Table 1. A significant increase in the biliary outputs of the three lysosomal enzymes occurred after high-dose

ethinyl estradiol; the increases were apparent whether outputs were expressed in absolute terms or relative to hepatic enzyme activity (Table 1). The excretion of β -galactosidase in each group was less than the excretion of the other two glycosidases; however, when total biliary enzyme outputs were related to total hepatic enzyme activities, no difference was apparent (Table 1). There were linear correlations among rates of biliary outputs of individual lysosomal enzymes in all studies, a result suggesting coordinate release of lysosomal protein into bile (Fig. 3). Total protein output into bile was significantly decreased after high-dose ethinyl estradiol treatment when compared with results in controls and low-dose treatment (Fig. 2). This decreased output reflected diminished bile flow, since total protein concentrations in bile were not different.

Hepatic lysosomal enzyme activities and total protein content

Hepatic glycosidase activities and total protein concentrations are presented in Table 2. There were no significant differences among the three groups in the total or specific activities of *N*-acetyl- β -glucosaminidase and β -glucuronidase; a slight but significant decrease in the activity of β -galactosidase after high-dose ethinyl estradiol was noted. The latency of *N*-acetyl- β -glucosaminidase measured in liver E-fractions was not altered by either dose of ethinyl estradiol (Table 2). Ethinyl estradiol administration did not affect total hepatic protein content (Table 2). When ethinyl estradiol dissolved in propylene glycol was added directly in vitro to liver E-fractions in concentrations from 0.05 to 5 μ M, no alteration of lysosomal enzyme activity was observed compared to control assays with or without propylene glycol.

DISCUSSION

The main finding recorded in this paper is that ethinyl estradiol administration causes a dose-dependent inhibition of bile flow and biliary lipid secretion while simultaneously stimulating the release of lysosomal enzymes into bile. These results

suggest that bile flow and biliary lipid secretion, at least in the rat, involve mechanisms other than vesicular transport by lysosomes. Thus, we have now demonstrated that bile flow and biliary lipid secretion are dissociated from lysosomal enzyme output during experimental cholestasis as well as under basal conditions (9). Although we have not done

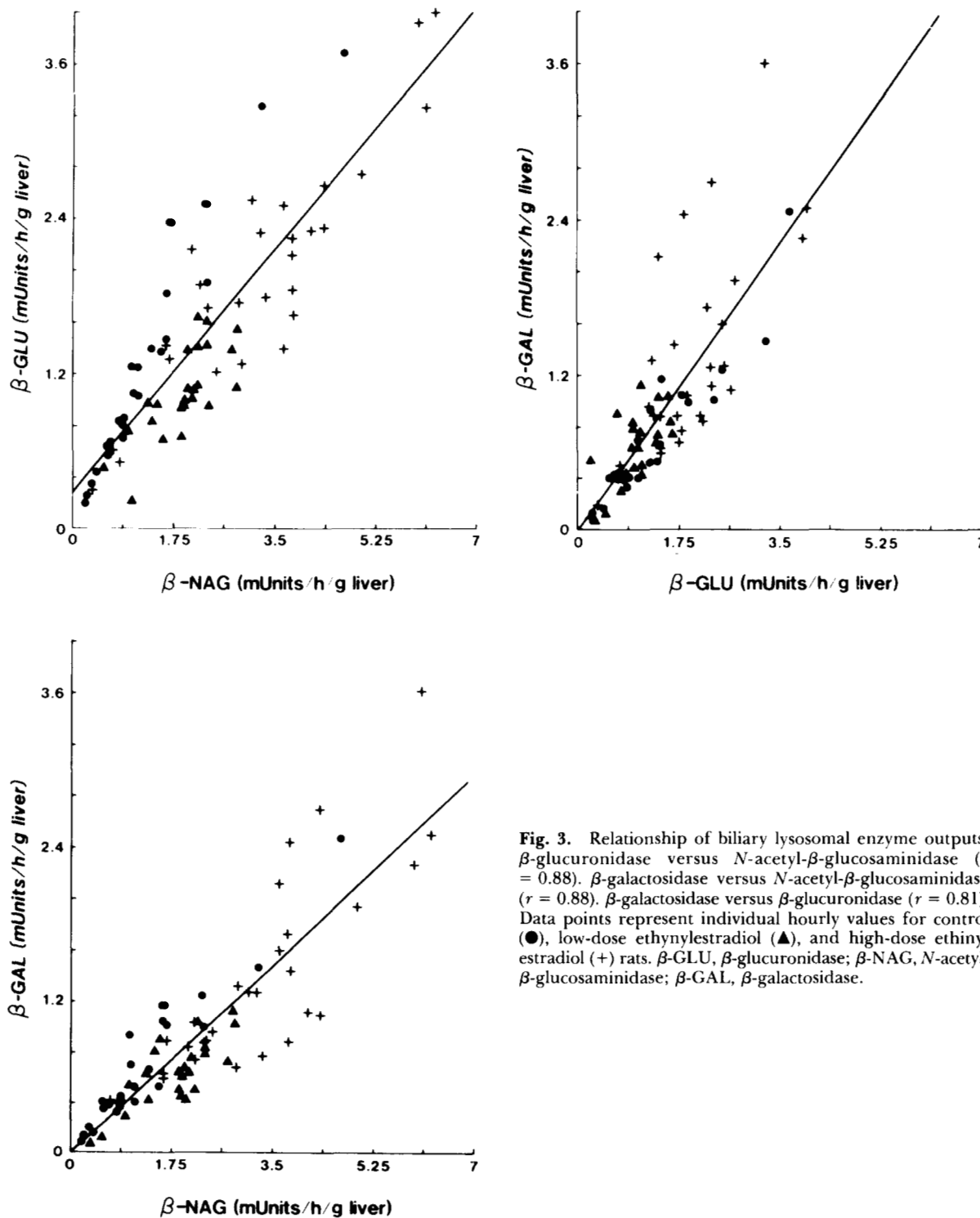


Fig. 3. Relationship of biliary lysosomal enzyme outputs. β -glucuronidase versus *N*-acetyl- β -glucosaminidase ($r = 0.88$). β -galactosidase versus *N*-acetyl- β -glucosaminidase ($r = 0.88$). β -galactosidase versus β -glucuronidase ($r = 0.81$). Data points represent individual hourly values for control (●), low-dose ethinylestradiol (▲), and high-dose ethinyl estradiol (+) rats. β -GLU, β -glucuronidase; β -NAG, *N*-acetyl- β -glucosaminidase; β -GAL, β -galactosidase.

TABLE 2. Rat liver total and latent glycosidase activities, and total protein content

Treatment	Lysosomal Enzymes ^a				Total Protein mg/g liver
	N-Acetyl- β-glucosaminidase	β-Glucuronidase	β-Galactosidase	N-Acetyl-β-glucosaminidase Latent Activity, % of Total Activity ^b	
Control (6)					
Propylene glycol	1.37 ± 0.34	1.61 ± 0.51	0.70 ± 0.10	83.5 ± 3.0	206.6 ± 6.3
Low-dose (7)					
Ethinyl estradiol (7)	1.59 ± 0.22	1.39 ± 0.51	0.66 ± 0.10	83.0 ± 2.8	200.9 ± 25.6
High-dose (7)					
Ethinyl estradiol (7)	1.51 ± 0.21	1.40 ± 0.23	0.58 ± 0.06 ^c	88.0 ± 1.8	206.9 ± 14.0

^a Data are expressed as units per gram liver.

^b Percent latent activity was calculated by subtracting the free from the total activity as described in Methods and expressing this difference as a percent of the total activity.

^c $P < 0.05$ versus control value.

parallel morphologic studies, our biochemical data suggest little if any role for lysosomes in bile and biliary lipid secretion. However, our results do not exclude a role for other cell organelles in bile secretion.

Our data provide no direct information on the mechanism whereby release of lysosomal enzymes into bile is increased after ethinyl estradiol treatment. We have previously suggested exocytic bulk discharge of hepatocyte lysosomes as the most likely mechanism (9). The excellent correlations we observed among rates of release of individual lysosomal enzymes support this interpretation. It is known that steroid hormones alter lysosomal membrane integrity in and facilitate the release of lysosomal enzymes from certain tissues (27–29). In our study, lysosomal latency was unaffected by ethinyl estradiol, suggesting that increased lysosomal membrane fragility was not a major factor in the increase in biliary lysosomal activity we observed. Whether estrogen-induced changes in plasma membrane fluidity (30) or in proliferation of microfilaments (31) can account for increased lysosomal enzyme output into bile, possibly by stimulating intracellular movement of lysosomes and membrane fusion, remains to be determined. Also, alterations in the rates of endocytosis (32), catabolism (33, 34), and release into plasma (35) of lipoproteins occur after estrogen administration: these may also be related to the increased biliary output of lysosomal enzymes since lysosomes participate in lipoprotein degradation (36). Finally, we cannot completely exclude the possibility that the increase in biliary lysosomal enzyme activities might reflect changes in unidentified enzyme inhibitors or activators in bile rather than alterations in the amount of biliary lysosomal protein. Results of our previously reported mixing experiments (9), the lack of effect in vitro of ethinyl estradiol on biliary acid hy-

drolase activities, and the parallel changes in bile of the three hydrolases make this possibility unlikely. Clearly, additional studies are necessary to determine if the estrogen-induced increase in biliary lysosomal enzyme output is a result of cholestasis, hormone administration, or a combination of these two factors.

An additional observation from our studies was the dissociation between total protein output and lysosomal enzyme release into bile. We have previously observed such a dissociation under non-cholestatic conditions (9). Taken together, our studies suggest that lysosomal enzymes may be released into bile by a different process and possibly from a different source than the bulk of biliary proteins, many of which originate from plasma (37) or from the canalicular membrane of the hepatocyte (13).^{□□}

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