Effects of Ethinylestradiol-Induced Cholestasis on Bile Flow and Biliary Excretion of Estradiol and Estradiol Glucuronide by the Rat* (33944)

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(Introduced by Vincent P. Dole)
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The phenolic steroid hormones estrone and estradiol, are excreted in bile and undergo an enterohepatic circulation in the rat and in man. It has been shown that high doses of exogenous estrogen may impair hepatic excretory function in human females and in the rat (1, 2). There is also evidence that endogenous hormones may cause abnormalities of liver function or cholestasis during late pregnancy (3–5), and that in those individuals who have suffered from cholestatic jaundice of pregnancy or pruritus gravidarum, moderate or low dose of estrogen administered in the nonpregnant state will reproduce the symptoms and liver function test abnormalities that occurred during pregnancy (4, 5). The present study was carried out to determine whether administered estrogen impairs bile flow and biliary excretion of labeled estrogen in the rat.

Methods and Materials. Female Sprague-Dawley rats, weighing from 150 to 180 g were fed ethinylestradiol (0.5 mg in saline suspension, by oral tube) daily, for 9 days. Litter-mate control animals were similarly saline sham-fed. On the tenth experimental day common bile duct cannulation and femoral vein catheterization were performed with the rats under light ether anesthesia. Each animal was then confined to a modified plastic restraining cage. Constant intravenous hydration with 5% dextrose and saline solution administered by a Harvard infusion apparatus at a rate of 0.0764 ml/min, and constant room temperature conditions, 25–26°, were maintained throughout the entire study. On the eleventh experimental day, 18–20 hr after surgery and 40–48 hr after the last dose of ethinylestradiol, a tracer dose of radioactive estrogen (estradiol or estradiol glucuronide) and bromsulfophthalein (10 mg) were administered intravenously via the femoral catheter, in 0.4 ml of 50% ethanol-saline solution, followed by a 1.0-ml saline flush. Bile was collected in 10-min fractions during the first hour, 15-min fractions during the second hour, 30-min fractions during the next 2 hr, and at 1-or 2-hr intervals up to a total of 9 hr. At the end of the collection periods, the animals were killed, the livers were examined grossly and under light microscopy with hematoxylin and eosin sections.

Radioisotopes, estradiol-17β-6, 7-^{3}H, 5.6 Ci/mmole, estradiol-17β-4-^{14}C, 8.18 mCi/mmole and estradiol-6, 7-^{3}H, 17β-D-glucuronide, 1.0 Ci/mmole, were obtained from the New England Nuclear Corporation and were repurified by paper chromatography prior to use. The activity of the radioactive estrogen solutions to be administered was measured at the time of each injection. Bile samples were measured for volume. Qualitative analysis for the appearance of bromsulfophthalein in the bile was made in all control and estrogen-treated animals. Aliquots were taken for measurement of radioactivity. To each bile aliquot, 50 μl or less, was added 2 ml of ethanol and 10 ml of toluene–POPOP phosphor solution. Quench correction was carried out by the addition of an appropriate internal standard to each sample. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter model 3000 or in a Nuclear Chicago 720 series liquid scintillation counter.

Results. Bile flow in the estrogen-treated animals was strikingly less than that in the

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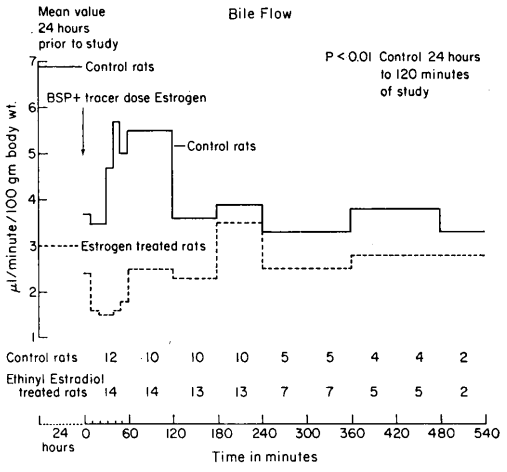

FIG. 1. Bile flow (μl/min/100 g of body wt) in control and ethinylestradiol-treated rats: mean value for 24 hr following bile duct cannulation and values for each bile fraction taken during excretion studies are charted.

control animals throughout study (see Fig. 1). During the 24 hr following cannulation and prior to bromsulfophthalein and estradiol or estradiol glucuronide excretion study, the mean bile flow rate was 6.9 μl/min/100 g of body weight in the control animals and 3.0 μl/min/100 g of body weight in the ethinylestradiol treated group, a significant difference ($p<0.01$). Similarly in the nine hours of the study during each time period the mean flow rates in the control animals exceeded those in the estrogen treated animals (3.3–5.5 μl/minute/100 grams body weight in the controls and 1.5–3.5 μl/minute/100 g body weight in estrogen treated animals ($p<$ 0.01, 0–120 minutes of study).

Bromsulfophthalein appeared in the bile during the first collection period (0–10 min) in the control animals and during the third collection period (20–30 min) in the ethinylestradiol-treated group. Similarly the appearance of radioactivity in the bile after administration of estradiol was also delayed in the estrogen-treated animals, appearing at 30–40 min as opposed to within 10 min in the controls.

Estradiol-17β-4-^{14}C or estradiol-17β-6,7-^{3}H was more completely excreted into the bile in the control animals than in the ethinylestradiol-treated animals (see Fig. 2). Forty-two percent of the administered dose of estradiol was excreted during the first 4 hr (6 animals), 50% by the end of 6 hr (4 animals) and 52% (4 animals) by the end of the 9-hr study period in the control animals. In the estrogen-treated animals, 18% of the administered estradiol was excreted during the first 4 hr (10 animals), 28% by the end of 6 hr (6 animals) and 32% by the end of 9 hr (3 animals). The differences between the control and treated groups were significant at all time periods ($p < 0.01$, 0–240 min; $p <$ 0.02, 240–480 min; $p<0.05$, 480–540 min).

Similarly, the biliary excretion of estradiol-6, 7-^{3}H–17β-D-glucuronide was imparied in the estrogen-treated animals, although the excretion of this compound was more rapid and more complete in both groups than that of the unconjugated estradiol (Fig. 3). Sixty-eight percent of the administered dose of estradiol glucuronide was excreted in the first 4 hr in the control group (4 animals) and 81% by the end of a 6-hr study period (4 animals). In the estrogen-treated group, 25% of the administered dose was excreted in 4 hr (3 animals) and 40% by the end of a 6-hr study period (2 animals). The differences between the control and treated groups were significant at all time periods ($p<0.01$, 0–90 min; $p<0.02$, 90–120 min; $p<0.05$, 120–360 min).

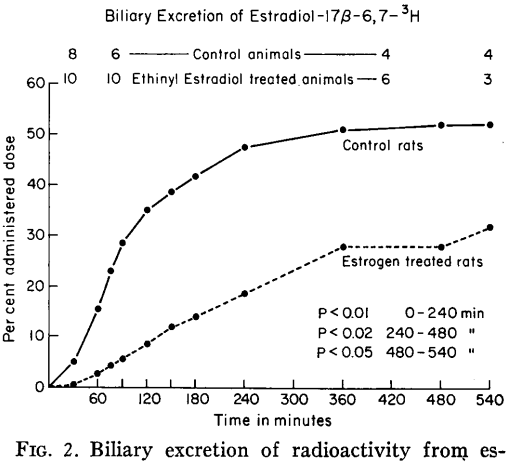

FIG. 2. Biliary excretion of radioactivity from estradiol-17β-6, 7-^{3}H in control and ethinylestradiol-treated rats during 9 hr following intravenous administration of tracer dose of estrogen and 10 mg of bromsulfophthalein.

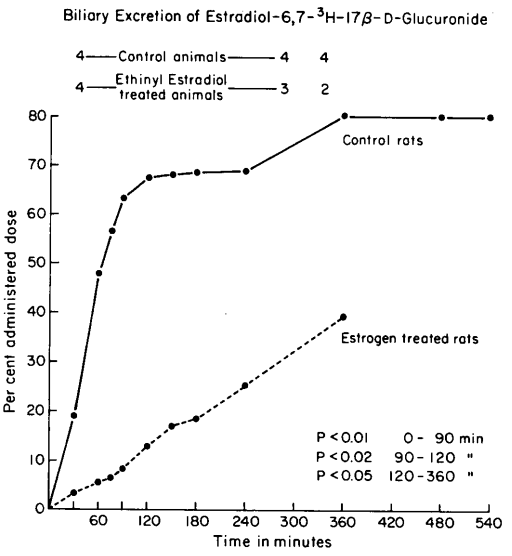

FIG. 3. Biliary excretion of radioactivity from estradiol-6, 7-^{3}H-17β-D-glucuronide in control and ethinylestradiol-treated rats during 9 hr following the intravenous administration of tracer doses of estrogen and 10 mg of bromsulfophthalein.

Examination of the rat livers at postmortem and by light microscopy with hematoxylin and eosin sections revealed no significant changes in gross pathology nor of microscopic pathology with no morphologic evidence of cholestasis in livers of the control or estrogen treated groups.

Discussion. The observation that the bile flow rate in ethinylestradiol-treated animals was markedly less than that of control rats, both during the initial 24 hr of bile drainage and during the 9-hr study period was of great interest. Since bile flow rate has been shown to be predominantly dependent upon bile salt secretion (6–9), this suggests that primary bile acid secretion may be impaired by estrogen. In light of these studies documenting estrogen-induced impairment of estradiol and estradiol glucuronide excretion into the biliary tract, a similar estrogen-induced impairment of bile acid excretion could be suspected when one considers the structural similarities of the two groups of steroids. This marked diminution of bile flow in the setting of high dose estrogen administration also raises the question of whether the high levels of estrogens in mid and late pregnancy may not decrease similarly bile flow rate and thus increase the possibility of biliary tract disease and stone formation.

Temperature changes can influence bile flow rate (10); this parameter was controlled in these studies. Permanent diversion of circulating bile salts with a chronic bile fistula preparation produces a marked depression of bile flow (8). The elimination of bile salts by chronic diversion exceeds the stimulated hepatic synthesis of bile acids. It also was shown that even when biliary excretion rate of taurocholate is kept at a constant level by infusion, spontaneous fluctuations in bile flow will occur (8). In these studies, bile flow in control and in ethinylestradiol-treated rats did decrease during the 24 hr following bile fistulization. The average bile flow rate during the excretion studies thus was less in both groups than during the previous 24 hr. Minor fluctuations in bile flow rate were seen in the later periods of study in both groups, after the acute changes observed following the injection of bromsulfophthalein and estrogen in ethanol–saline solution.

Colorless bile was obtained in the estrogen-treated animals during the 10-min period immediately preceding the appearance of bromsulfophthalein, when bile flow rate was minimal. This might have been similar to the bile fraction, independent of bile salt content, observed by Preisig *et al.* (9) in the dog. Bromsulfophthalein appearance in the bile was delayed in the estrogen-treated animals as had been previously reported (2). It is of interest that in earlier work of Brauer and Pessotti, a more rapid bromsulfophthalein clearance *in vivo* was observed in male than in female rats (11).

Estrogens are the only group of steroids of physiologic significance that undergo a substantial enterohepatic circulation in man (12–14). Sandberg and Slaunwhite have shown that 50–60% of an administered dose of labeled estradiol will appear in the bile in man when a bile fistula is present, but only 7–10% will be present in the feces of intact man, implying a large enterohepatic circulation (12). In man the corticosteroids and androgens are initially excreted predomi-

nantly in the urine, whereas in the rat, the neutral steroids are excreted initially primarily by biliary route (12–15). In a recent study Sandberg *et al.* (16) showed that in the rat phenolic steroids similary undergo a biliary excretion and enterohepatic circulation. It has been well documented that estradiol is converted primarily into estrone and thus the results obtained here using labeled estradiol may be compared with those in which estrone was administered (17).

Sandberg *et al.* (16) found that 58.1% of an administered tracer dose of estrone was excreted into rat bile within 4 hr. In these studies 42% of the administered tracer dose of estradiol was recovered in bile within 4 hr and 52% in 9 hr. In the ethinylestradiol-treated animals, 40–48 hr after estrogen therapy discontinuation and 24 hr after constant bile drainage via cannula, the biliary excretion of the tracer dose of estradiol was markedly reduced from the control group, 18% in 4 hr and 32% in 8 hr. This suggests that estrogen-induced hepatocellular and/or biliary changes which result in a diminished T_m for bromsulfophthalein (1, 2) and, as observed here, reduced bile flow, also impair the hepatobiliary excretion of estrogen. Analysis of the excretion pattern and the radioactivity per unit volume of bile suggests that the diminution in biliary excretion of estradiol and its metabolites exceeds the diminution of the bile flow. The total dose of estrogen used in studies was very large (a total of 4.5 mg of ethinylestradiol over a 9-day period). No attempt was made to determine the minimal dose required to induce cholestasis. However, in previous studies Gallagher *et al.* (2) showed that significant bromsulfophthalein retention in the rat occurred after the administration of 1.0 mg of estradiol for 4–5 days. It is unlikely that the large dose directly influenced the delayed biliary clearance of estradiol by expanding the estrogen pool size and thus competing for hepatobiliary transport. In loading experiments, Sandberg *et al.* (16) showed that the acute administration of up to 10.0 mg of estrone or estriol did not alter the biliary excretion of labeled estrogens in the rat. However, since estrogen-induced cholestasis caused marked alterations in estrogen metabolism, it is certainly possible that metabolites of ethinylestradiol were accumulated.

The specific metabolites and conjugates of estradiol appearing in rat bile have not been studied. It was suggested by Sandberg *et al.* (16) that after estrone administration, glucuronide, sulfate, and perhaps mixed conjugates appear in rat bile. Estradiol-17β-D-glucuronide is probably not a natural metabolite in rat bile. In these studies, glucuronide or other polar conjugate formation did not appear to be the primary or limiting step in biliary excretion of tracer dose estrogen, impaired by estrogen-induced cholestasis. When estradiol glucuronide was administered, a greater proportion of radioactivity appeared in the early bile fractions of both control and treated rats than had appeared after unconjugated estradiol administration and the total excretion over a 6-hr period (81% of administered done in controls and 40% in ethinylestradiol-treated animals) also exceeded the excretion of estradiol during the same period of time. However, the biliary excretion of radioactivity from the administered estradiol glucuronide by the estrogen-treated rats again was far less than that by control animals. Thus it is suggested that the major estrogen-induced defect is one of impaired passage of the conjugated steroid from the hepatocyte into the bile canaliculi.

Taylor (15) suggested that steroid glucuronides, in addition to being secreted primarily from liver parenchymal cells to the bile canaliculi, may also, in part, be readsorbed back into blood after conjugation and then secreted directly across the epithelium of bile ductules into bile. Thus an alternative mechanism of action of estrogen leading to the diminished excretion of both the metabolites of estradiol and estradiol glucuronide would be one of altering the permeability of bile ductules leading to a back diffusion of the conjugated steroids into blood. Further studies will be needed to define the mechanism of action of estrogen in causing cholestasis and impairing the biliary excretion of estrogens.

If estrogen induced cholestasis in the human leads to a similar alteration in estrogen metabolism with diminished hepatobiliary excretion, accumulation of various metabolites of estrogen could occur, unless urinary excretion of the atypical metabolic products became enhanced. Such an accumulation of estrogen could further provoke the primary problem. It is of interest that Adlercreutz *et al.* (18), in a study of patients with cholestatic jaundice of pregnancy, showed that the total urinary excretion of estrone, estradiol, and estriol is normal during the icteric phase of the disorder, but that the pattern of estrogen metabolites and conjugates is altered. In one patient with cholestatic jaundice of pregnancy, he demonstrated a diminished biliary excretion of estrogens during the last trimester of pregnancy which, however, was accompanied by reduced blood and urinary levels with an altered pattern of metabolites as compared with controls.

Summary. The effects of ethinylestradiol treatment on bile flow and biliary excretion of bromsulfophthalein, estradiol, and estradiol glucuronide in the rat were studied. One day following 9 days of 0.5 mg of ethinylestradiol treatment, bile duct cannulation and femoral vein catheterization were carried out. From that time until the end of study, bile flow was strikingly reduced in the estrogen-treated animals to less than 50% of that in the control group. One day following surgery, studies of biliary excretion were performed revealing a markedly diminished biliary excretion of radioactivity from intravenously administered tracer doses of both estradiol and estradiol glucuronide in the treated animals. The effect of estrogen treatment on bile flow and estrogen excretion in the bile is similar to that previously observed for bromsulfophthalein clearance. Possible mechanisms of estrogen action and implications in human estrogen-induced cholestasis are discussed.

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