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Research Article

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A substantial fraction (75%) of basal bile flow in the isolated hamster liver was estimated to be independent of bile acid secretion. Cholestasis occurred after taurolithocholate, whereas bile acid secretion was maintained. The results indicate that the most likely mechanism for acute cholestasis induced by taurolithocholate in isolated hamster liver was interference with the bile acid–independent fraction of canalicular or ductular bile flow or both.

INTRODUCTION

Lithocholic acid causes cholestasis and hepatic injury in experimental animals and has been implicated in the initiation and perpetuation of cholestatic liver disease in man (1). It is a secondary bile acid formed in the intestinal tract by bacterial dehydroxylation of chenodeoxycholic acid (2). Lithocholic acid differs from most other bile acids in that its solubilization at body temperature in aqueous solution requires protein or micellar bile acid (3, 4). Hepatic synthesis, increased intestinal absorption, or decreased biliary excretion of lithocholic acid enhances the exposure of the liver to this agent, which is normally present in only trace amounts (5, 6). Studies in animals, however, have not fully elucidated the mechanism of acute or chronic cholestasis induced by lithocholic acid (7).

Bile flow and composition are determined by canalicular and ductular functions. In several species (8–11), the canalicular component of bile flow is comprised of one fraction that is dependent on bile acid secretion and another that is independent of bile acid secretion. Structural or physicochemical alterations of the bile secretory apparatus (12), competition for transport sites (13), or inhibition of energy processes (14, 15) may lead to decreased secretion of bile salts or the agent that controls

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the fraction of bile flow that is independent of bile acid secretion. Precipitation of the cholestatic agent or its metabolites within the canaliculus (16), back diffusion from bile ducts to blood (17), and decreased hepatic blood flow and anoxia (18) also have been proposed as mechanisms for cholestasis.

Previously, our group studied the secretion of bile acids and sulfobromophthalein (BSP) [1] in isolated hamster liver, using this species because the bile acids in hamster bile are the same as those in man (19). The aim of the current study was to investigate the mechanism of acute cholestasis induced by taurolithocholate in isolated hamster liver. The effects of taurolithocholate on bile flow and on the secretion of bile acid and BSP, anions secreted at the canaliculus, were determined.

METHODS

Preparation of isolated liver and perfusion technique. The apparatus and general perfusion procedures were based on the method developed by Brauer, Pessotti, and Pizzolato (20) for isolated rat liver and are the same as described by us using isolated hamster liver (19). The perfusate consisted of 70 ml of hamster blood (supplied by Con Olson Co., Madison, Wis.) and 25 ml of normal saline. Female golden syrian hamsters served as liver donors. Liver weights were not significantly different among groups.

With the hamster under ether anesthesia, an abdominal incision was made, and the esophagus was tied and transected below the diaphragm. The common duct was cannulated with polyethylene tubing (0.024 in. o.d., grade PE-10; Clay-Adams, Parsippany, N. J.), and the cystic duct was ligated. The gastric vein was ligated, and 0.5 ml of heparin (1.5 mg) was injected into inferior vena cava, which was then ligated. The portal vein and superior portion of the inferior vena cava were then cannulated (0.075 in. o.d., PE-200).

The liver was freed from its attachment and transferred to the perfusion apparatus where it was placed with its diaphragmatic surface on a sheet of siliconized rubber in an evaporating dish. The dish contained a 5 mm aperture in the bottom through which the outflow cannula in the inferior vena cava protruded. The liver was covered with an inverted Petri dish. The entire procedure took 20–25 min; the time from cannulation of the portal vein to establishment of the perfusion was 4–6 min.

The enclosing cabinet provided a constant temperature at 37°C and a humidity greater than 50%; oxygenation of blood was accomplished with a mixture of 95% oxygen and 5% carbon dioxide. Blood entered the liver through the portal vein at 12.5 cm of blood pressure.[2] Blood flow began at 4–9 ml/min immediately after connection of the cannulas to the circulatory system. There was a transient decrease in flow and then a gradual increase to a maximal of 11 ±4.7 ml/min (mean ±SD) at 4 hr. Bile flow commenced within several minutes. Cultures of bile for aerobic and anaerobic microorganisms were negative. After 4 hr of perfusion, livers were immediately fixed in formalin and were examined microscopically by a pathologist who had no knowledge of which agents were administered during the experiments.

Experimental design. 60 isolated liver preparations were utilized. Experiments included a Latin square–controlled experimental design wherein there were the following four groups: (*a*) injection and continuous infusion of BSP, (*b*) injection of taurolithocholate, (*c*) injection of both taurolithocholate and BSP, and (*d*) injection of neither (basal). A continuous infusion of taurocholate (1.7 μM/hr) was administered to all perfusions to maintain bile acid output.

Administration of taurocholate or BSP was begun 30 min after the initiation of perfusion when bile that had been in the biliary tract and cannula (dead space approximately 0.1 ml) was eliminated. After an additional 30 minute equilibration period, bile was collected hourly in calibrated, tapered test tubes.

In BSP experiments, a bolus injection of this dye (10 mg) was administered at the start of its infusion (4.4 mg/hr), establishing a BSP transport maximum (Tm). Preliminary experiments with BSP showed that both lower and higher doses led to lower biliary concentrations and outputs of BSP.

Sodium taurolithocholate (1–3 ml of a 2 mM solution in 10% albumin) was injected as a bolus after the 1st hr of bile collection. A dose-response relationship between taurolithocholate and the degree of cholestasis was examined. Taurolithocholic acid was synthesized from lithocholic acid (Mann Research Labs. Inc., New York), by the method described by Norman (21). The product was more than 95% pure (calculated by thin-layer and gas-liquid chromatography) and had a melting point of 211–213°C. Sodium taurolithocholate solution was administered in 10% human serum albumin to facilitate solubilization and to minimize hemolytic effects. Solubility was enhanced by warming for 30–60 min at 37°C just before injection.

Analysis of bile. Total bile acids were quantified by the enzymatic method of Talalay (22), using concentrated steroid dehydrogenase (specific activity 0.73 U/mg andosterone and 0.87 U/mg testosterone; Worthington Biochemical Corp., Freehold, N. J.). By this method recovery of taurolithocholate, added (1 μmole/ml) to hamster bile, was 95% ±4% (mean ±SE) in six experiments. Recovery of other bile acids including glycine and taurine conjugates of cholic, chenodeoxycholic, and deoxycholic acids was also virtually complete. Pure bile acid reference standards were supplied by Dr. Alan F. Hofmann. The ammonium salt of taurolithocholate sulfate, supplied by Dr. R. H. Palmer, was converted to the sodium salt with Dowex 50 cation-exchange resin.

Determination of total bile acid in bile using the steroid dehydrogenase method is complicated by the presence of BSP. The steroid dehydrogenase enzyme has optimal activity in alkaline medium (pH > 9.0). This pH at which reduced nicotine adenine dinucleotide is determined results in a violet BSP solution. The maximal light absorption peak of BSP occurs at 580 mμ; however, a minor peak occurs at 340 mμ and coincides with the maximal absorbance of reduced nicotine adenine dinucleotide. BSP caused a decrease in the optical densities expected from differing concentrations of bile acids. Statistical analysis of data from experiments using known concentrations of BSP and bile acids in bile allowed formulation of a simple multiple regression equation for estimating total bile acid in the presence of BSP, as follows:

$$BA = 0.0397 + 0.000236\ BSP + 0.530\ OD,$$

[1] *Abbreviations used in this paper:* BSP, sulfobromophthalein; Tm, transport maximum.

[2] Blood flow was measured by determining the rate at which a 6 ml calibrated bulb became filled with blood after occlusion of the polyethylene tubing just distal to the bulb.

in which

BA = total bile acid concentration (μmoles/100 μl),
BSP = concentration of sulfobromophthalein (mg/100 ml), and
OD = final optical density after steroid dehydrogenase − optical density of blank containing bile with BSP and nicotine adenine dinucleotide in a buffered alkaline medium (pH 9.5).

The equation describes a plane that approximates the mean bile acid concentration for given values of BSP and optical density. There is only a small variation (SD = 0.01046) of observed values of bile acids about this plane. The coefficient of variation was 2.9% at low levels of bile acid concentration (0.36 μmoles/0.1 ml) and 2.1% at high levels (0.51 μmoles/0.1 ml). The BSP concentration had a statistically significant effect in determining the bile acid concentration.

BSP hepatic removal rate, hepatic content, and transport maximum. Hepatic removal rate was calculated using the following equation:

$$R = I - (\Delta P/\Delta t \cdot PV),$$

in which,

R = hepatic removal rate (mg/hr per g wet liver weight),
I = infusion rate of BSP (mg/hr),
$\Delta P/\Delta t$ = change in plasma concentration of BSP (mg/100 ml) per hour, and
PV = plasma volume (ml).

Hepatic content was calculated using the following equation:

$$C = I_t - P_t - B_t,$$

in which

C = hepatic content of BSP (mg/g wet liver weight),
I_t = total amount of BSP infused by time t (mg),
P_t = plasma content of BSP in mg at time t (plasma concentration in mg/100 ml × PV in ml), and
B_t = total amount of BSP excreted in bile by time t (mg).

Transport maximum (Tm) was measured directly as milligrams of BSP excreted in bile per hour (23).

BSP in bile and plasma was determined as previously described (24, 25). An amount of 0.01 ml of bile was added to 10.99 ml of alkaline saline (pH 9.0) and read at 580 mμ in a Beckman junior spectrophotometer using 0.01 ml of bile in normal saline (pH 5.0) as the blank. Plasma BSP concentrations were determined using 0.1 ml of plasma in 10.9 ml of alkaline saline with normal saline and plasma from each individual sample as the blank. Standards were prepared in hamster bile or plasma. BSP conjugates in bile were analyzed by the technique of Meltzer, Wheeler, and Cranston (26) using ascending paper chromatography and the solvent system *n*-butanol: glacial acetic acid (4:1), saturated with water.

Identification and quantification of individual bile acids. The three major bile acids in perfused hamster liver bile were previously identified by the peak shift technique as chenodeoxycholic, deoxycholic, and cholic acids; trace quantities of lithocholic acid were found (19). Gas-liquid chromatography quantification of individual bile acids was done after deconjugation by the amidase enzyme method of Nair (27). Recovery of taurolithocholate added (1 μmole/ml) to hamster bile was 85 ±2% (mean ±SE) in eight experiments. Recovery of bile acids (mean per cent ±SE), determined by addition of taurine- and glycine-conjugated bile acids to bile and quantification by enzymatic deconjugation and gas-liquid chromatography of the free bile acids, were: cholic 86.9 ±2.2 (n = 10), chenodeoxycholic 96.2 ±2.1 (n = 5), lithocholic 94.2 ±2.4 (n = 5), and deoxycholic acid 85.6 ±1.4 (n = 10).

Quantification of individual trifluoroacetate derivatives of bile acids was performed by use of gas-liquid chromatography, with a hydrogen flame detector and glass columns packed with acid-washed, silanized Gas Chrom P, 100–120 mesh, coated with 3% QF 1 (Applied Science Laboratories, Inc., State College, Pa.). The areas of bile acid peaks on the chromatograms were measured with a planimeter or disc integrator and were compared for quantification with those of known amounts of appropriate pure reference compounds in a range in which a linear detector response was obtained (28).

Thin-layer chromatography on silica gel was utilized to identify sulfated taurolithocholate in bile using three solvent systems: (*a*) butanol, 50:0.01 M Tris buffer, 9.25: proprionic acid, 0.75 (pH 3.0); (*b*) butanol, 50: acetic acid, 5: water, 5 (pH 1.0); and (*c*) proprionic acid, 30: propanol, 20: water, 10: isoamylacetate, 40.

Bile acid–independent fraction of bile flow. Bile acid secretion was correlated with bile flow, and the resultant regression line was extrapolated to zero bile acid secretion, as described by Preisig, Cooper, and Wheeler (8) to estimate the fraction of bile flow that is independent of bile acid secretion. Since active secretion of sodium has been suggested as the driving force for the bile acid–independent fraction (15), sodium and potassium were determined in bile samples by flame photometry.

RESULTS

Administration of taurolithocholic acid. Fig. 1 shows a significant linear correlation between the dose of taurolithocholate and the resultant decrease in bile flow

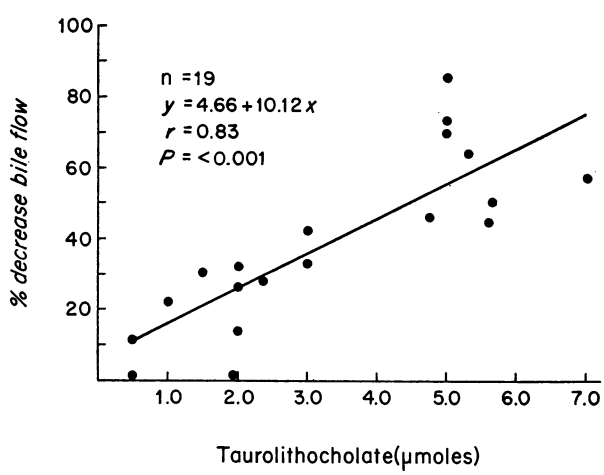

FIGURE 1 Percentage decrease in bile flow from 1st to 2nd hr in response to varied doses of taurolithocholic acid. The percentage represents a decrease from that which would have been expected in the 2nd hr had taurolithocholic acid not been given.

in the subsequent hour. Blood flow was not changed by taurolithocholate. Cholestasis was induced within 15 min. After high doses of taurolithocholate (> 5 μmoles), cholestasis persisted, whereas after lower doses, the cholestasis was reversible and bile flow returned to control values by the 4th hour.

During cholestasis, bile acid output was maintained by a significantly ($P < 0.05$) increased concentration of bile acid (Fig. 2). All of the bile acids in bile were conjugated; cholic and chenodeoxycholic acids comprised 75–89% and lithocholic acid 11–25% of the total bile acids; only traces of deoxycholate were detected. Of the taurolithocholic acid injected, 40–50% was recovered in bile as lithocholic acid, 30% was presumably converted to chenodeoxycholic acid, and only traces of lithocholic acid were in the perfusate at the end of 4 hr; the liver was not assayed. The percentage of lithocholic acid converted to chenodeoxycholic acid was calculated from the difference between chenodeoxycholic acid output with and without injection of taurolithocholic acid, on the assumption that endogenous secretion of chenodeoxycholic acid remains constant as has been previously reported (19).

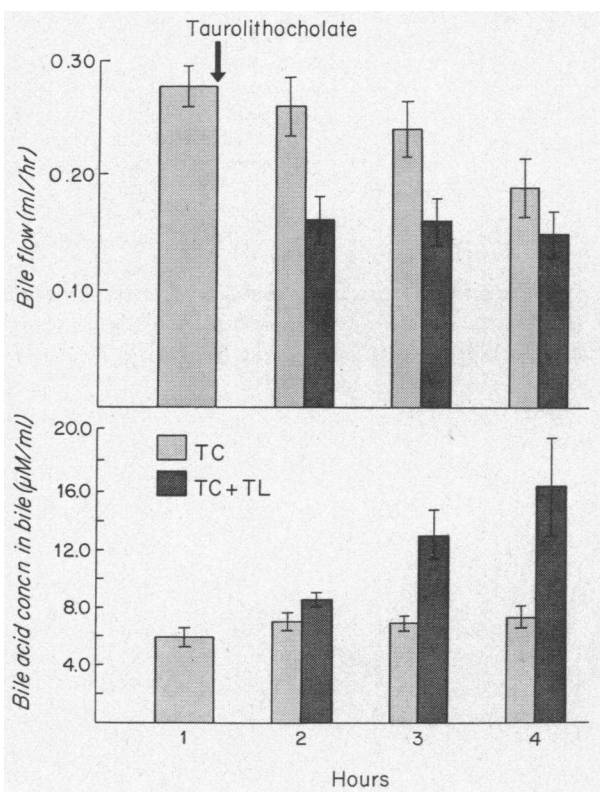

FIGURE 2 Bile flow (*upper panel*) and bile acid concentration (*lower panel*) in hourly bile under basal conditions (n = 5) and after 2–6 μmoles of taurolithocholate (TL, n = 15). Taurocholate (TC) was infused continuously in all basal perfusion experiments. Bile flow decreased gradually during basal perfusions although only the 4th hr was significantly less than the 1st ($P < 0.05$). Histograms in this and subsequent figures show the mean ± SE.

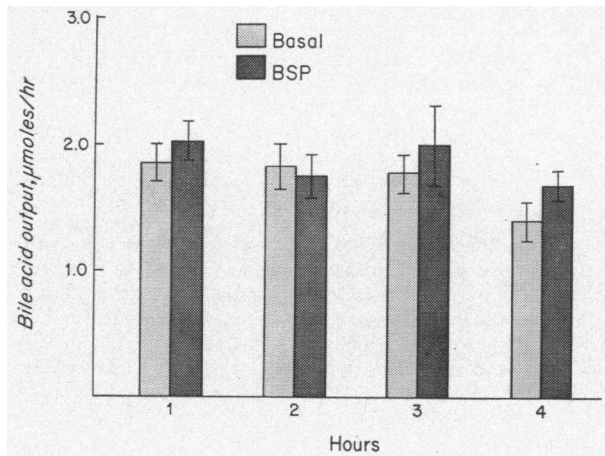

FIGURE 3 Bile acid output during basal conditions (continuous taurocholate infusion, n = 5) compared with that during simultaneous administration of BSP (n = 5).

Small amounts of taurolithocholate sulfate were detected by thin-layer chromatography of bile that contained taurolithocholate.

Administration of BSP and taurolithocholic acid. BSP induced a persistent and pronounced choleresis during which BSP concentrations were maintained. Bile acid outputs were not significantly different from basal experiments (Fig. 3).

Addition of taurolithocholic acid (Fig. 4) resulted in significant cholestasis ($P < 0.05$). During cholestasis, bile BSP concentrations were maintained and BSP output decreased significantly ($P < 0.05$). Bile acid concentration increased significantly ($P < 0.01$), resulting in sustained bile acid output. The relative proportions of cholic, chenodeoxycholic, and lithocholic acids were not different from experiments without BSP.

Administration of BSP resulted in increasing plasma concentration of BSP. Addition of taurolithocholate caused significantly greater increase ($P < 0.01$) in BSP plasma concentration during each hour (Fig. 5). Hepatic removal rate and transport maximum of BSP decreased significantly ($P < 0.001$), while hepatic content remained unchanged during the hour after taurolithocholate injection. The proportion of conjugated BSP in bile (40–60%) was unaltered by taurolithocholate.

Bile acid–independent fraction of bile flow. There was a significant correlation between bile acid secretion (1.56–8.64 μmoles/hr) and bile flow (Fig. 6, *upper panel*). For each micromole/hour increase of bile acid secretion, an increase of 0.04 ml/hr bile flow occurred.

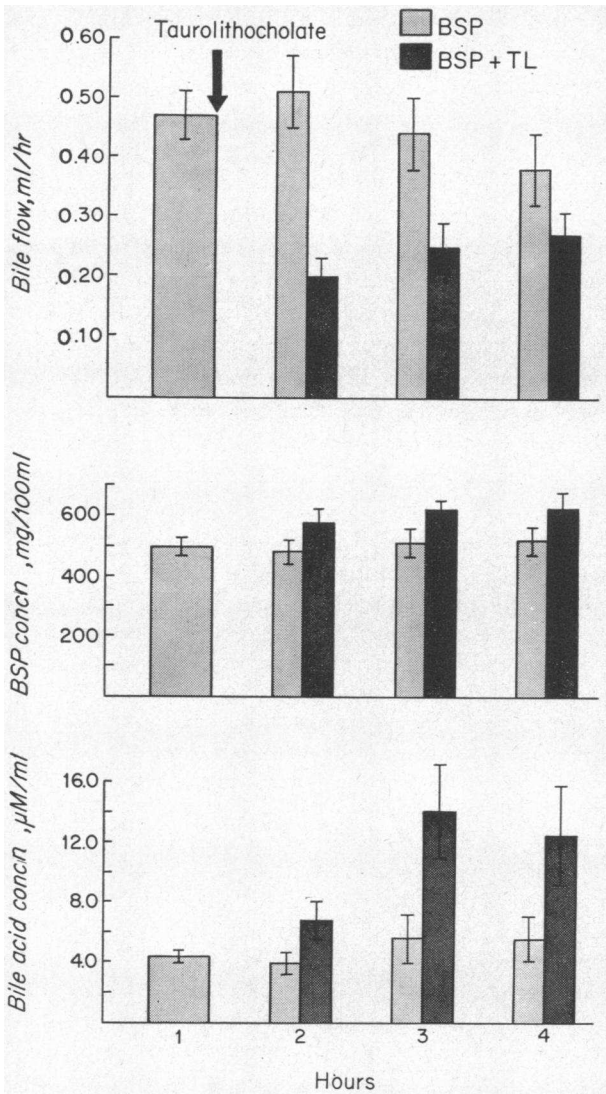

FIGURE 4 Bile flow (*upper panel*), BSP concentrations (*middle panel*), and bile acid concentrations (*lower panel*) during BSP administration with (n = 6) and without (n = 5) taurolithocholate (TL). The mean bile flow (milliliters/hour) during the 1st hr of BSP infusion was 0.48 ±0.04 SE, significantly greater ($P < 0.01$) than that during the 1st hr of basal perfusion, 0.28 ±0.02 SE.

The fraction of bile flow that was independent of bile acid secretion was extrapolated to be 0.21 ml/hr or 75% of the 1st hr basal bile flow (0.28 ml). In our previous experiments without taurocholate infusion (19), bile acid secretion ranged from 0.12 to 2.0 μmoles/hr; at these low rates of bile acid secretion, bile flow was maintained at 0.24 ml/hr. Thus, 75% should be an accurate reflection of the actual bile acid–independent fraction. A significant decrease of sodium and potassium output in bile ($P < 0.01$) occurred during the hour after taurolithocholate injection (Fig. 6 *lower panel*).

Histologic features. No significant hepatic morphologic abnormalities were observed after taurolithocholate administration.

DISCUSSION

A general concept of intrahepatic cholestasis has been proposed previously wherein disturbed secretion of bile acids is the common denominator, and lithocholic acid is afforded a central role in pathogenesis (29). However, more direct investigations, exemplified by the experiments of Javitt and Emerman (16), are needed to test the validity of this hypothesis. Acute cholestasis induced by taurolithocholate theoretically may result from morphologic or physicochemical alterations of the hepatocyte, bile canaliculus, or ducts.

The isolated hamster liver provided a suitable model with demonstrated functional and anatomic integrity (19) for study of cholestasis induced by lithocholic acid.

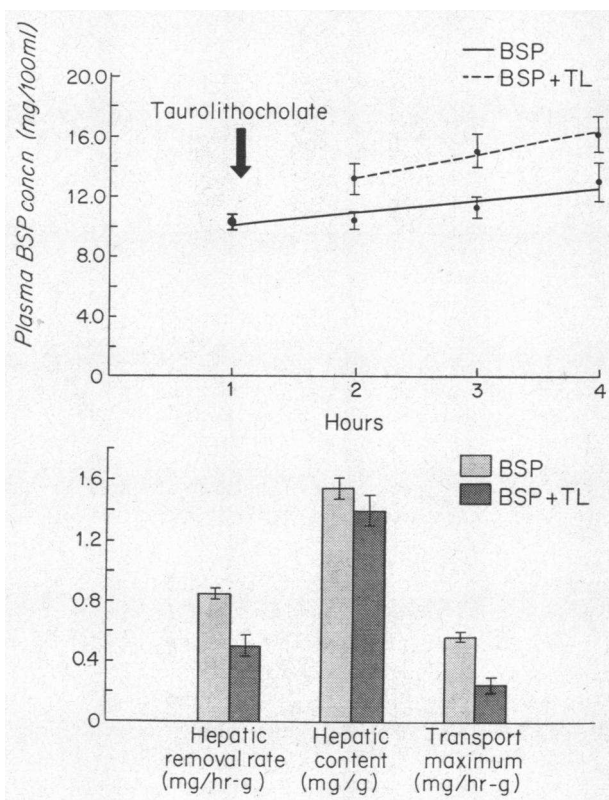

FIGURE 5 *Upper panel,* plasma BSP concentrations during BSP administration (n = 5) and after taurolithocholic injection (n = 6). Regression equations were for BSP alone: $y = 9.29 + 0.816\ x$ (n = 26, $r = 0.49$, $P < 0.01$), and after addition of TL: $y = 10.12 + 1.54\ x$ (n = 18, $r = 0.45$, $P < 0.05$). Lower panel, hepatic removal rate, content, and transport maximum of BSP during BSP administration and after taurolithocholic injection.

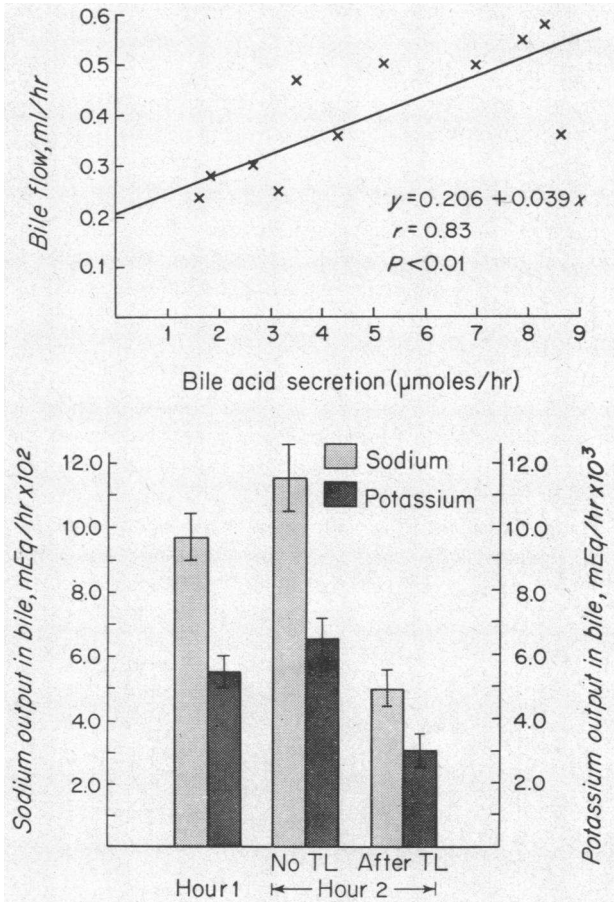

FIGURE 6 *Upper panel,* significant linear correlation between bile acid secretion and bile flow when taurocholate infusion was increased. Fraction of bile flow that is independent of bile acid secretion was estimated to be 0.21 ml/hr or 75% of basal bile flow by extrapolation of bile acid secretion to zero. *Lower panel,* sodium and potassium output during hr 1 and 2 in five basal experiments and during hr 2 in five experiments after injection of taurolithocholate. Outputs of both cations were decreased during cholestasis.

Further evidence for viability in the present experiments with taurolithocholate is found in the return to normal or the recovery of bile flow after cholestasis induced by the lower doses of taurolithocholate in which the findings were qualitatively the same as after the larger doses. The hamster was chosen because this species has the same bile acids as man and because of the importance of bile acids in bile secretion (9). The calculation that 30% of injected taurolithocholate is converted to chenodeoxycholic acid agrees with the results noted for hamsters with bile fistula that were given taurolithocholate-^{14}C (16). Bile flow rates were comparable (about 1 μl/min per g liver) with those reported for isolated rat liver (30), expressed in terms of liver weight. The isolated liver avoids problems encountered in the intact animal related to peripheral uptake, urinary losses, and hormonal and neurogenic influences on bile flow and composition.

A BSP-induced choleresis has been noted in other species (31) and is presumably due to the osmotic force generated by active canalicular secretion of BSP. Bile acid output remained constant during these experiments, so that bile acid did not contribute to the BSP-induced choleresis. The decrease in hepatic removal rate of BSP after taurolithocholate probably reflected the decrease in transport maximum during cholestasis. The decreased transport maximum of BSP was associated with a maintained concentration maximum and, therefore, was determined by the rate of bile flow. Thus, the concentrating capacity may be the rate-limiting factor in transport of BSP. Boyer, Scheig, and Klatskin (30) found the same alteration of BSP transport during cholestasis due to hypothermia in the isolated rat liver.

Precipitation of taurolithocholate within the canaliculi or bile ducts has been suggested as a mechanism of cholestasis (16). However, in our experiments, this explanation is less tenable for several reasons. Bile acid output was maintained during cholestasis, and more than 75% of the total bile acids were conjugates of cholic and chenodeoxycholic acids so that micellar solubilization of the taurolithocholate in bile rather than precipitation would be expected. During cholestasis, the molar ratio of the solubilizing bile acids to taurolithocholate was 3:1, sufficient for the solubilization of taurolithocholate. The 20–30% of injected taurolithocholate that was not recovered in bile or perfusate presumably remained in the hepatocytes rather than in the canaliculi because, when cholestasis reverted to normal flow, an increase in the secretion of taurolithocholate or chenodeoxycholic acid did not occur. Finally, microscopic morphologic evidence of cholestasis, which might occur if precipitate were blocking bile flow, was absent.

An abnormality or lesion in the bile ducts alone that increases water reabsorption is not compatible with the disparity found between increased bile acid and maintained BSP concentrations during cholestasis. If ductular bile formation were decreased or ductular absorption increased, then BSP concentration should have increased during cholestasis. Moreover, ductular bile flow in isolated liver preparations when hormonal and neural influences are absent, should comprise a much smaller fraction of total bile flow than in the intact animal (19). Just recently, Boyer and Klatskin found that clearance of mannitol-^{14}C closely approximated total bile flow in isolated rat liver and concluded that almost all bile can be assumed to be secreted by the canaliculus (32). The degree of cholestasis observed after high doses of taurolithocholate in our experiments corresponded with the

estimated size of the bile acid–independent fraction in isolated hamster liver.

Recently, Erlinger, Dumont, Dhumeaux, and Berthelot (15) have suggested that the bile acid–independent fraction is dependent on the active secretion of sodium. This group of investigators showed that rose bengal in the rabbit, like hypothermia in isolated rat liver (30), induced a decrease in bile flow by inhibiting the bile acid–independent fraction of bile flow. Hydrocortisone choleresis in the dog (33) and phenobarbital choleresis in the rat (34) are mediated by canalicular bile formation unrelated to osmotic effects or bile acid secretion. Our finding of reduced outputs of sodium and potassium during cholestasis with maintained bile acid output was to be expected with cholestasis, regardless of cause.

Osmotic forces were not measured directly in our experiments. However, the decrease in bile flow after taurolithocholate is probably not related to a decrease in the osmotic power of bile acids because (*a*) bile acid output was maintained, 75% as micellar bile acids, (*b*) there is no evidence that taurolithocholate decreases the effective osmotic force of other bile acids, and (*c*) the diminution of bile flow (50%) was greater than the bile acid–dependent fraction of bile flow (25%).

We conclude that the major mechanism of taurolithocholate-induced cholestasis in isolated hamster liver is a reduction in the canalicular or ductular fractions (or both) of bile flow that are independent of bile acid secretion and postulate from available evidence that a canalicular effect is more likely. Some additional effect on the bile acid–dependent fraction has not been entirely ruled out.

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