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Cholestasis, Altered Junctional Permeability, and Inverse Changes in Sinusoidal and Biliary Glutathione Release by Vasopressin and Epinephrine

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SUMMARY

The mechanism for the vasopressin- and epinephrine-induced decrease in bile formation and increase in sinusoidal efflux of glutathione was investigated in rat livers perfused with recirculating fluorocarbon emulsion. Vasopressin and epinephrine transiently decreased bile flow and excretion of endogenous bile acids and glutathione and increased the bile/perfusate ratio of [^{14}C]sucrose, suggesting an increase in junctional permeability, but had no effect on the bile/perfusate ratio of [^{3}H]polyethylene glycol-900. The decreased biliary glutathione was balanced by an increase in sinusoidal efflux, such that total hepatic release remained unchanged. The adrenergic antagonist dihydroergotamine blocked the effects of epinephrine. To examine whether an increase in junctional permeability *per se* could account for the changes in glutathione efflux, biliary permeability was increased by either bile duct ligation, lowering of perfusate Ca^{2+} concentration with ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), or addition of taurolithocholate, a cholestatic bile acid. All three maneuvers produced a decrease in biliary glutathione excretion and a concomitant increase in sinusoidal glutathione efflux, whereas total glutathione release was largely unaffected. The effects of EGTA were partially reversed if $CaCl_2$ was reintroduced into the perfusate. Because the GSH/GSSG ratio in perfusate could not be measured in this experimental system due to the spontaneous oxidation of GSH to GSSG, additional experiments in the nonrecirculating mode examined the effects of vasopressin and bile duct ligation on sinusoidal release of GSH and GSSG. In control livers there was no detectable GSSG in perfusate (<0.5 nmol·min^{-1}·g^{-1}). After vasopressin administration, the additional sinusoidal glutathione was mainly as GSH, although there was also a significant amount of GSSG (1–2 nmol·min^{-1}·g^{-1}). The additional glutathione released into perfusate after bile duct ligation was 47% as GSSG. When vasopressin was administered to livers whose bile duct had been ligated, its ability to enhance sinusoidal glutathione release was diminished, suggesting that the effects of vasopressin and bile duct ligation are not additive. These observations support previous findings that vasopressin and epinephrine can modulate hepatocyte tight junctional permeability and demonstrate that these hormones produce cholestasis and inverse changes in sinusoidal and biliary glutathione efflux. Other maneuvers that increased biliary permeability to [^{14}C]sucrose also produced cholestasis and a redistribution of glutathione efflux from bile to perfusate, suggesting that an increase in junctional permeability may allow biliary glutathione to reflux from bile to plasma.

Hepatic glutathione turnover is achieved largely by efflux of the tripeptide across canalicular and basolateral plasma membranes. Recent studies in perfused rat liver demonstrate that approximately 50% of the glutathione released by rat liver is secreted into bile (1). Glutathione concentrations in rat hepatic bile are relatively high, between 5 and 8 mM (1–3), whereas plasma concentrations are 3 orders of magnitude lower, from 5 to 20 μM. This large chemical gradient for glutathione between bile and plasma is associated with a canalicular lumen-negative electrical potential (4, 5), thus creating a substantial electrochemical gradient for this anionic species. The bile to plasma gradient for glutathione constitutes part of the osmotic driving force for hepatic bile formation (3).

Sies and Graf (6, 7) observed that vasopressin, epinephrine, and angiotensin II, hormones whose intracellular mediators are Ca^{2+} and the inositol phosphates, elicit a pronounced increase in sinusoidal glutathione release. In contrast, dibutyryl-cAMP has no effect on glutathione efflux (6, 7). The mechanism for the hormonal stimulation of glutathione efflux has not been identified. These same hormones, but not dibutyryl-cAMP, also increase hepatic junctional permeability (8). Hepatic paracellular permeability is determined largely by the properties of the tight junctions that seal adjacent hepatocytes and separate the sinusoidal and intercellular spaces from bile canalicular spaces (9–11). This junctional barrier normally excludes solutes as

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ABBREVIATION: EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate.

large as proteins but is leaky to and is the site of entry of a large fraction of the water and small ions in bile (4, 5). Lowe, Hardison, and co-workers (8, 12, 13) have shown that hepatocyte junctional barriers are dynamic structures capable of influencing the composition of bile.

The present study examines the mechanism for the stimulation of sinusoidal glutathione efflux by vasopressin and epinephrine. The results indicate that these hormones shift the relative proportions of glutathione appearing in bile and plasma, an effect that was coincident with an increase in biliary permeability to [^{14}C]sucrose.

Experimental Procedures

Materials and analytical procedures. [^{14}C(U)]Sucrose (4.6 mCi/mmol) and [1,2-^{3}H]polyethylene glycol of molecular weight 800–1000 (4.0 mCi/g) were obtained from New England Nuclear (Boston, MA). Glutathione reductase, lactate dehydrogenase, 3-α-hydroxysteroid dehydrogenase, NAD, NADH, NADPH, lysine vasopressin, epinephrine, and taurolithocholate were obtained from Sigma Chemical Co. (St. Louis, MO), and dihydroergotamine was from Sandoz Pharmaceuticals (East Hanover, NJ). The fluorocarbon emulsion (Oxypherol FC-43) was obtained from Alpha Therapeutic (Los Angeles, CA). Liver donors were male Sprague-Dawley rats (219–268 g) from Charles River Laboratories (Kingston, NY). The animals were fed *ad libitum* until time of experimentation.

The concentrations of GSH and GSSG (14, 15), total bile acids (16), and perfusate lactate dehydrogenase activity (17) were determined as described. Glutathione release is expressed as total glutathione (i.e., GSH plus 2 × GSSG) equivalents released·min^{-1}·g of liver^{-1}. Bile volume was determined gravimetrically, assuming a density of 1.0 g/ml. [^{14}C]Sucrose and [^{3}H]polyethylene glycol-900 in perfusate and bile were measured by dual label counting in a Packard model 4530 scintillation counter, using a correction for the spillover of ^{14}C counts into the ^{3}H channel. Quenching was corrected for by using an external standard. From 50 to 100 μl of bile-sulfosalicylic acid mixture from each collection period and 50 μl of perfusate sampled in the middle of each bile collection interval were placed in 5 ml of Safety-Count (Research Products International, Mount Prospect, IL) for the determination of ^{14}C and ^{3}H activities.

All values are expressed as means ± standard deviations. Student's *t* test was used for statistical comparison of two means. Differences were considered to be statistically significant when $p < 0.05$.

Liver perfusions in the recirculating mode. The surgical isolation of the liver, the perfusion system, and the functional characterization of the isolated liver were as described previously (1, 3). Perfusions were carried out with 80 ml of a recirculating buffer prepared by mixing 40 ml of a 20% fluorocarbon emulsion (prepared in Krebs-Ringer solution, as described by the manufacturer; Green Cross, Osaka, Japan) with 40 ml of Krebs-Henseleit buffer. The final buffer contained the following: 10% (w/v) fluorocarbon emulsion, 1.5% hydroxyethyl starch, 1.28% Pluronic F-68, 111 mM NaCl, 4.6 mM KCl, 0.6 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.3 mM $MgSO_4$, 1 mM $MgCl_2$, 1.88 mM $CaCl_2$, and 5 mM D-glucose. Immediately after the liver was placed in the perfusion chamber, trace concentrations of [^{14}C]sucrose (1–2 μCi) and [^{3}H]polyethylene glycol (2 μCi) were added to the perfusate reservoir. Perfusion flow rate was adjusted at the start of each experiment to achieve an initial perfusion pressure of 3–4 cm of H_2O (between 3.36 and 4.63 ml·min^{-1}·g of liver^{-1}; mean ± SD, 4.01 ± 0.30 ml·min^{-1}·g of liver^{-1}; n = 45 perfusions) and was not changed for the remainder of the experiment. Perfusion pressure was monitored using an open-tube manometer placed just proximal to the portal vein cannula. The portal vein cannula was a 14-gauge stainless steel needle, and the in-line filter contained a Millipore prefilter (AP25) and a 1.2-μm filter (RAWP).

After a 10-min stabilization period, bile collections were initiated. Bile was collected in tared, ice-chilled microfuge tubes, containing 150 μl of 5% 5′-sulfosalicylic acid, every 10 min for the first 30 min and every 15 min for the subsequent 60 min. Perfusate samples (0.3 ml) were taken from the perfusate reservoir at each bile collection interval and immediately deproteinized with 15 μl of 20% 5′-sulfosalicylic acid. Additional perfusate samples were taken from the perfusate reservoir every 30 min for the determination of lactate dehydrogenase release. At the end of the experiment, the liver was removed from the perfusion chamber and the median lobe was removed for analysis of glutathione.

In all experiments, each liver served as its own control because treatment was initiated after the second control bile collection interval (20 min). The agents were added to the perfusate reservoir starting at 19 min, because it took approximately 1 min for the buffer in the reservoir to reach the liver. Vasopressin (15 nM, n = 5) and epinephrine (30 nM, n = 5) were added to the reservoir in three bolus injections of 0.4 and 0.8 nmol each, respectively, at 19, 24, and 29 min of perfusion. Dihydroergotamine (150 nM, n = 5) was added as a single bolus at 18 min, i.e., 1 min before the administration of the first dose of epinephrine. EGTA (2 mM, n = 7) was added over a 2-min interval, at 19–21 min of perfusion, from an 82.5 mM stock whose pH had been adjusted to 7.0 with NaOH. Calcium chloride (1.5 mM, n = 6) was administered over a 3-min interval (at 27–30 min) in a volume of 1.25 ml. Taurolithocholate was dissolved in 0.9% NaCl containing 0.3% bovine serum albumin and was administered as a bolus of 0.8 μmol at 19 min, followed by a constant infusion of 0.4 μmol/min from 19 to 44 min (n = 5). Bile duct ligation was performed at 20 min (n = 6).

Single-pass liver perfusions. For livers perfused in the nonrecirculating mode, Krebs-Henseleit buffer supplemented with 5 mM D-glucose was used as perfusate. Bile was collected in ice-chilled 1.5-ml microfuge tubes, containing 75 μl of 5% 5′-sulfosalicyclic acid, every 5 min for 50 min. Effluent perfusate samples were also collected every 5 min in beakers containing 1 ml of 20% 5′-sulfosalicylic acid. Aliquots of perfusate samples (1 ml) were mixed immediately with 5 μl of 2-vinylpyridine for derivatization of GSH.

In this experimental system, bile duct ligation was performed after the second control bile collection interval (10 min). Vasopressin was infused from 30 to 40 min of perfusion at a rate of 0.14 nmol·min^{-1}, to give a concentration of ~4 nM in the portal perfusate.

Results

Fig. 1 illustrates the effects of vasopressin, epinephrine, and dihydroergotamine plus epinephrine on bile flow (Fig. 1A), glutathione excretion into bile (Fig. 1B) and perfusate (Fig. 1C), endogenous bile acid excretion (Fig. 1D), and bile to perfusate ratios of [^{14}C]sucrose (Fig. 1E) and [^{3}H]polyethylene glycol (Fig. 1F). Data are expressed as a percentage of pretreatment values, where values observed at the second bile collection interval (20 min) were normalized to 100%. For clarity, standard deviation bars are illustrated only for the control group. Statistically significant differences ($p < 0.05$) are noted by the *filled symbols*.

Administration of vasopressin or epinephrine was associated with a decrease in bile flow (Fig. 1A) and in biliary excretion of glutathione (Fig. 1B) and endogenous bile acids (Fig. 1D). The effects of epinephrine were transient, whereas vasopressin produced sustained decreases in each of these parameters. The decreased biliary glutathione excretion was balanced by increased efflux into perfusate (Fig. 1C), such that total hepatic glutathione release remained unchanged (Fig. 2A). Neither hormone had any effect on hepatic tissue GSH or GSSG concentrations, as measured at the end of the experiment (Table 1); however, vasopressin did produce a slight increase in the rate of lactate dehydrogenase release into perfusate (Table 1). This enhanced lactate dehydrogenase release corresponds to a leakage rate of only 0.0025%/min, compared with a rate of 0.001–0.002%/min in control experiments (Table 1).

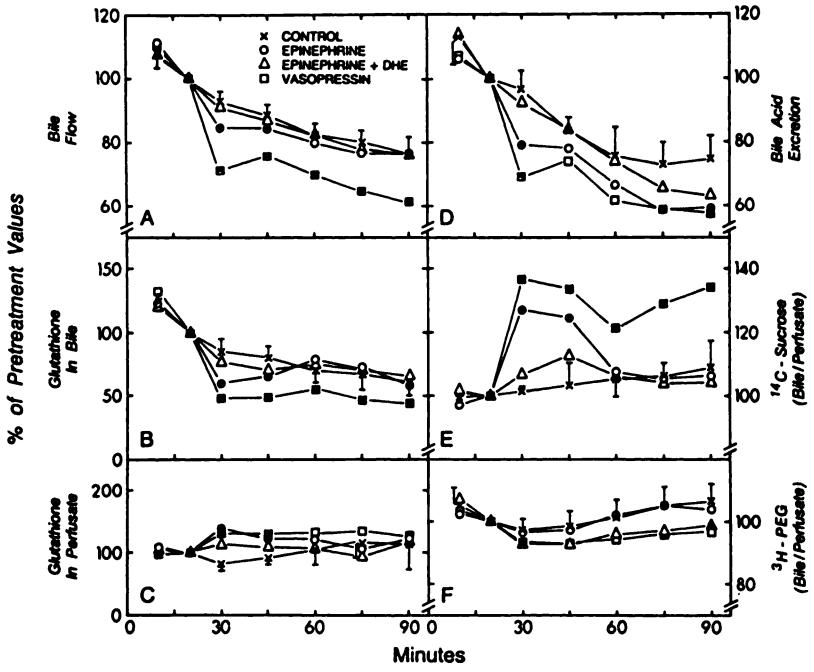

Fig. 1. Effects of vasopressin (1.2 nmol, $n = 5$), epinephrine (2.4 nmol, $n = 5$), epinephrine plus dihydroergotamine (*DHE*) (12 nmol, $n = 5$), or 0.9% NaCl for controls ($n = 6$) on bile flow (A), glutathione excretion into bile (B) and perfusate (C), endogenous bile acid excretion (D), and the bile to perfusate ratios of [^{14}C]sucrose (E) and [^{3}H]polyethylene glycol (F), in the isolated perfused rat liver. Treatment was initiated after the second control bile collection interval (20 min). Data are expressed as a percentage of pretreatment values, where values observed at the second bile collection interval were normalized to 100%. *Standard deviation bars* are illustrated only for the control group. Statistically significant differences ($p < 0.05$) are noted by the *filled symbols*. The 100% values ± SD for control, vasopressin, epinephrine, and epinephrine plus dihydroergotamine were, respectively: bile flow, 1.34 ± 0.13, 1.42 ± 0.11, 1.34 ± 0.07, and 1.26 ± 0.13 $\mu l \cdot min^{-1} \cdot g$ of $liver^{-1}$; glutathione excretion in bile, 6.8 ± 2.5, 7.1 ± 3.1, 6.5 ± 1.8, and 5.8 ± 1.4 nmol of glutathione equivalents $\cdot min^{-1} \cdot g$ of $liver^{-1}$; glutathione excretion in perfusate, 7.3 ± 2.8, 8.7 ± 1.9, 7.1 ± 2.4, and 9.1 ± 3.5 nmol of glutathione equivalents $\cdot min^{-1} \cdot g$ of $liver^{-1}$; bile acid excretion, 1.93 ± 0.34, 1.58 ± 0.45, 2.26 ± 0.97, and 2.26 ± 1.12 nmol $\cdot min^{-1} \cdot g$ of $liver^{-1}$; bile/perfusate ratio of [^{14}C]sucrose, 0.25 ± 0.06, 0.30 ± 0.02, 0.28 ± 0.03, and 0.26 ± 0.03; and bile/perfusate ratio of [^{3}H]polyethylene glycol (*PEG*), 8.0 ± 1.6, 8.4 ± 1.2, 8.9 ± 1.5, and 8.0 ± 1.3.

Assuming that an equivalent fraction of intracellular glutathione would be released nonspecifically along with lactate dehydrogenase, ~0.15 nmol of glutathione $\cdot min^{-1} \cdot g$ of $liver^{-1}$ [i.e., (0.0025% $\cdot min^{-1}$) × (6 μmol of glutathione $\cdot g$ of $liver^{-1}$)] would be released. This is only an approximation, of course, but it suggests that this pathway of glutathione release is quite small but not insignificant when compared with the specific sinusoidal efflux of 5–13 nmol $\cdot min^{-1} \cdot g^{-1}$. Perfusion pressure increased abruptly after each of the three bolus administrations of either vasopressin (0.2–0.6 cm of H_2O) or epinephrine (1–2 cm of H_2O). This increase was transient, however, inasmuch as perfusion pressure returned to basal levels, 3–4 cm of H_2O, within 2–3 min (i.e., before the next bolus administration of hormone).

Both hormones also increased the bile/perfusate ratio of [^{14}C]sucrose (Fig. 1E), suggesting an increase in paracellular permeability. In contrast, the bile/perfusate ratio of [^{3}H]polyethylene glycol was unaffected by epinephrine and only slightly decreased by vasopressin (Fig. 1F). Polyethylene glycol-900 is concentrated in rat bile up to 14-fold, presumably by a vesicular transport mechanism (18), although this marker enters bile by several different routes (18, 19). In the present study, the basal bile/perfusate ratio of polyethylene glycol was between 5.7 and 11.5 (mean ± SD, 8.4 ± 1.3; $n = 32$ livers).

The effects of epinephrine were blocked by the adrenergic antagonist dihydroergotamine (Fig. 1), suggesting a specific interaction of epinephrine with its hepatic receptors. Pretreatment with dihydroergotamine prevented the effects of epinephrine on biliary permeability to [^{14}C]sucrose, bile flow, and the excretion of glutathione and bile acids (Fig. 1).

In an attempt to rule out the hypothesis that an increase in junctional permeability *per se* can lead to an enhanced release of glutathione into perfusate, paracellular permeability was altered by three procedures that presumably exert their effects by independent mechanisms, (a) removal of perfusate Ca^{2+} by chelation with EGTA, a procedure known to disrupt junctional barriers and lead to cholestasis (5, 20–22), (b) bile duct ligation, which probably opens junctional barriers by multiple mechanisms, including increased biliary pressure and increased accumulation of bile acids and metabolic waste products (9, 10, 23), and (c) the addition of taurolithocholate, a toxic bile acid that has a variety of effects on the liver, including the ability to increase intracellular Ca^{2+} (24, 25) and biliary permeability to sucrose (26).

The effects of bile duct ligation and EGTA are illustrated in Fig. 3. After bile duct ligation, there was a marked increase in glutathione appearing in the perfusate (Fig. 3C), yet the total amount of glutathione released by the liver remained unchanged (Fig. 2B), suggesting that the glutathione normally secreted into bile was now gaining access to the sinusoidal circulation. Similarly, EGTA produced nearly complete cessation of bile flow (Fig. 3A) and biliary excretion of glutathione (Fig. 3B) and bile acids (Fig. 3D), yet the total amount of glutathione released by the liver remained relatively constant (Fig. 2B). A significant increase in total glutathione release was observed during the first bile collection interval after EGTA administration (Fig. 2B).

All of the effects of EGTA were reversed if $CaCl_2$ was reintroduced into the perfusion system (Fig. 3). Thus, as biliary excretion of bile acids and glutathione returned towards normal and as the bile/perfusate ratio of [^{14}C]sucrose declined, bile flow rates returned to basal levels. Observe also that, as biliary glutathione excretion increased after $CaCl_2$ supplementation (Fig. 3B), sinusoidal glutathione excretion declined (Fig. 3C), such that the total amount released was once again unchanged (Fig. 2B). Consistent with its role as a marker of transcellular vesicular transport (18), the bile/perfusate ratio of [^{3}H]polyethylene glycol was largely unchanged in this experiment (Fig. 3F); there was a significant decline of ~10%, but only during the last bile collection interval (Fig. 3F).

Taurolithocholate also increased the bile/perfusate ratio of [^{14}C]sucrose (Fig. 4E). This was associated with a transient cholestasis (Fig. 4A) and a lower rate of biliary glutathione excretion (Fig. 4B) but a higher rate of sinusoidal glutathione release (Fig. 4C), such that the total amount released was

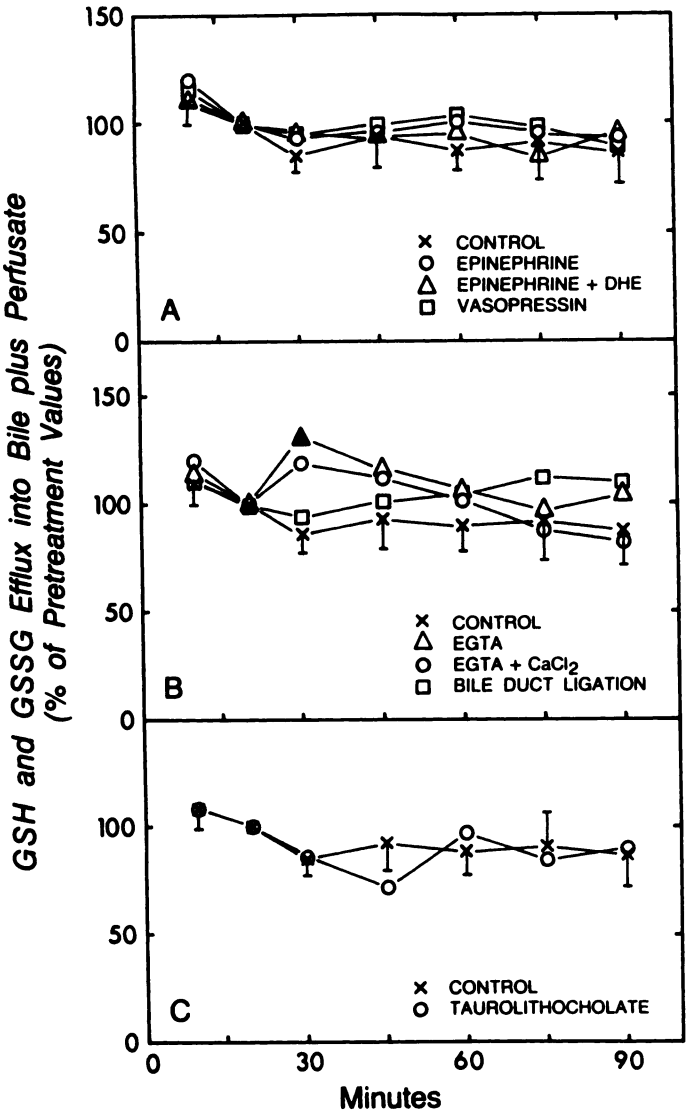

Fig. 2. Total amount of GSH and GSSG released by the isolated perfused rat liver into perfusate plus bile. Data represent the sum of biliary and perfusate excretion of GSH plus 2 × GSSG for the experiments illustrated in Fig. 1 for vasopressin, epinephrine, and epinephrine plus dihydroergotamine (*DHE*) (A); in Fig. 3 for EGTA, EGTA plus $CaCl_2$, and bile duct ligation (B); and in Fig. 4 for taurolithocholate (C). For explanatory information, see legend to Fig. 1. The 100% values ± SD were, control, 14.1 ± 4.5; vasopressin, 15.8 ± 3.3; epinephrine, 13.6 ± 1.2; epinephrine plus dihydroergotamine, 14.9 ± 3.8; EGTA, 17.6 ± 5.6; EGTA plus $CaCl_2$, 15.3 ± 2.1; bile duct ligation, 16.1 ± 3.9; and taurolithocholate, 14.8 ± 5.8 nmol of glutathione equivalents $\cdot min^{-1} \cdot g$ of $liver^{-1}$.

largely unchanged (Fig. 2C). Total glutathione release was depressed at the 45-min bile collection interval (Fig. 2C); however, this difference was not statistically significant. Note that, as biliary glutathione excretion increased near the end of the experiment, sinusoidal glutathione release decreased in inverse proportion (Fig. 4, B and C).

Bile flow was decreased by taurolithocholate, despite a large increase in total bile acid excretion (Fig. 4D). Bile acid excretion increased from a basal level of 1.63 ± 0.95 to 10–12 nmol $\cdot min^{-1} \cdot g$ of $liver^{-1}$ during the last two bile collection intervals. Coincident with the increase in bile acid excretion (Fig. 4D) and presumably in bile acid-dependent bile flow (Fig. 4A), the bile/perfusate ratio of [^{3}H]polyethylene glycol declined (Fig. 4F), consistent with the suggestion made by Berenson *et al.* (18) that polyethylene glycol-900 is a marker of transcellular vesicular transport. However, the administration of taurolithocholate was associated with a slight increase in lactate dehydrogenase release (Table 1), suggesting some hepatocellular damage.

For all experiments illustrated in Figs. 1–4, decreased biliary glutathione excretion was attributed to a decrease in both GSH and GSSG in bile (data not shown). Biliary GSH and GSSG usually changed in parallel, such that the GSH/GSSG ratio remained comparable to controls. However, one important parameter that could not be measured in these experiments performed with a recirculating perfusate was the GSH/GSSG ratio in the sinusoidal effluent, because a variable fraction of the GSH released into the recirculating perfusate was spontaneously oxidized to GSSG. To circumvent this problem, the effects of bile duct ligation and vasopressin were reexamined in livers perfused in the single-pass mode, where GSH and GSSG efflux into perfusate can be measured. Fig. 5 presents these results both in terms of total sinusoidal glutathione release (GSH plus 2 × GSSG; Fig. 5A) and as separate values for GSH and GSSG efflux (Fig. 5B).

In agreement with results presented in Fig. 3, bile duct ligation produced an increase in sinusoidal glutathione efflux (Fig. 5A). A large fraction of the additional glutathione released into perfusate was as GSSG (47%; Fig. 5B). Similarly, vasopressin produced an increase in both GSH and GSSG efflux into perfusate (Fig. 5B); however, most (81%) was as GSH. When vasopressin was administered to livers whose bile duct had been ligated, its effects on glutathione release were attenuated (Fig. 5). In control livers, vasopressin increased glutathione release by 7.5 nmol $\cdot min^{-1} \cdot g^{-1}$, whereas after bile duct ligation it increased glutathione efflux by only 3.8 nmol $\cdot min^{-1} \cdot g^{-1}$ (Fig. 5A), suggesting that the effects of vasopressin and bile duct ligation are not additive. Biliary excretion of both GSH and GSSG was diminished by vasopressin administration (Fig. 5C).

Discussion

Vasopressin and epinephrine interact with receptors on the liver cell plasma membrane to elicit a distinct set of biochemical responses (27–29). Two of the proximate intracellular signals for the activation of these processes are the concentration of ionic calcium and the inositol phosphate cycle (27–29). One response common to both hormones is an increase in hepatocellular tight junctional permeability (8). Recent studies indicate that these hormones, but not dibutyryl-cAMP, can increase junctional permeability to [^{14}C]sucrose and horseradish peroxidase (8). Because tight junctions constitute the principal barrier separating blood from bile, any change in their permeability characteristics is expected to have a profound influence on bile composition and flow (8–12). The cholestatic effects of vasopressin and epinephrine in the rat have been reported (5, 8, 30); however, the quantitative aspects of the response remain controversial. Using the isolated perfused rat liver, Lowe *et al.* (8) observed a transient decrease in bile flow, whereas Graf (5) and Krell *et al.* (30) observed a biphasic response to epinephrine, with a small transient increase (1–2 min) followed by a larger and more sustained decrease (2–20 min). The cholestatic effects of other α-adrenergic agonists have also been reported

TABLE 1

Hepatic tissue GSH and GSSG concentrations and lactate dehydrogenase release by the isolated perfused rat liver

Values are means ± standard deviations. Hepatic tissue GSH and GSSG concentrations were measured at the end of the 90-min perfusion.

	n	GSH	GSSG	Lactate dehydrogenase		
				0–30 min	30–60 min	60–90 min
		μmol of GSH equivalents · g^{-1}		units · g^{-1} · 30 min^{-1}		
Control	6	6.24 ± 0.57	0.14 ± 0.10	0.21 ± 0.07	0.17 ± 0.07	0.28 ± 0.07
Vasopressin	5	5.99 ± 1.12	0.11 ± 0.09	0.20 ± 0.16	0.31 ± 0.10[a]	0.51 ± 0.17[a]
Epinephrine	5	6.24 ± 0.25	0.09 ± 0.09	0.17 ± 0.13	0.13 ± 0.05	0.28 ± 0.16
Epinephrine + DHE[b]	5	6.27 ± 0.29	0.14 ± 0.09	0.26 ± 0.12	0.16 ± 0.03	0.27 ± 0.11
EGTA	7	6.00 ± 1.10	0.08 ± 0.03	0.23 ± 0.11	0.19 ± 0.28	0.32 ± 0.33
EGTA + $CaCl_2$	6	6.10 ± 0.74	0.09 ± 0.03	0.23 ± 0.10	0.16 ± 0.03	0.25 ± 0.05
Bile duct ligation	6	6.19 ± 0.47	0.12 ± 0.11	0.18 ± 0.27	0.26 ± 0.31	0.26 ± 0.29
Taurolithocholate	5	5.91 ± 0.69	0.20 ± 0.05	0.15 ± 0.07	0.55 ± 0.09[a]	0.49 ± 0.12[a]

[a] Significantly different from controls, using Student's *t* test ($p < 0.05$).
[b] DHE, dihydroergotamine.

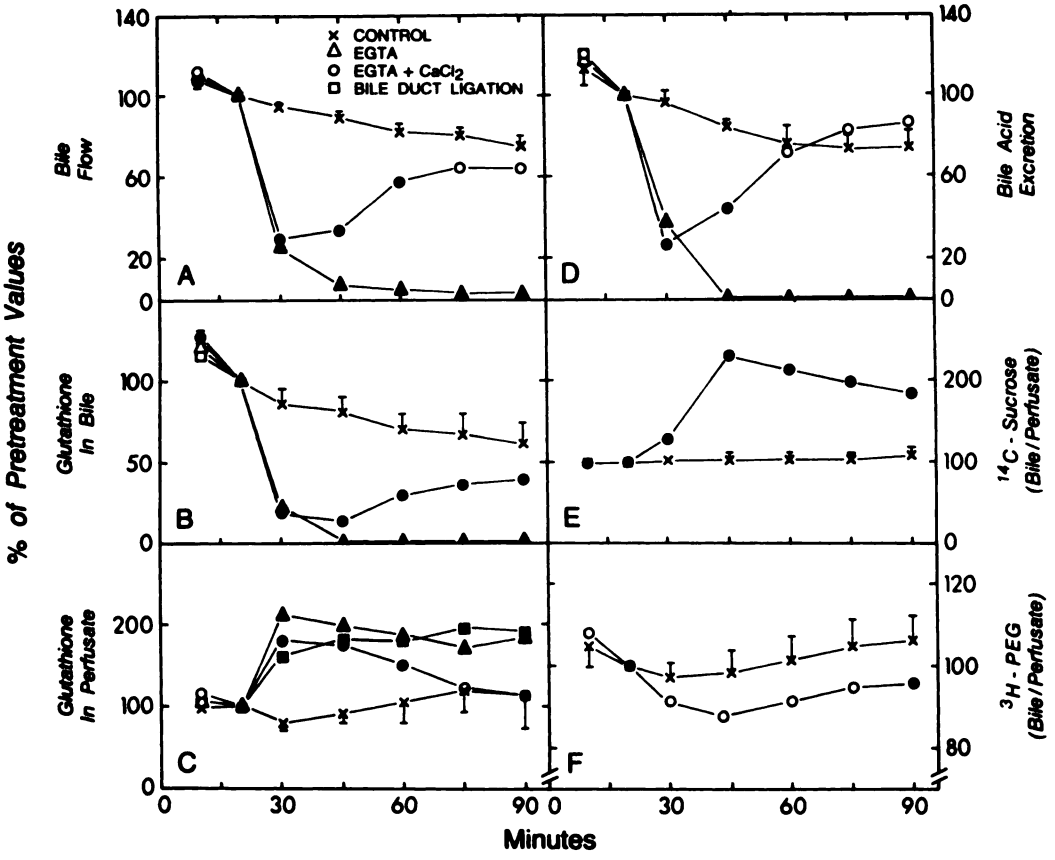

Fig. 3. Effects of EGTA (2 mM, *n* = 7), EGTA plus $CaCl_2$ (1.5 mM added from 27 to 30 min, *n* = 6), and bile duct ligation (at 20 min of perfusion, *n* = 6) on bile flow (A), glutathione excretion into bile (B) and perfusate (C), endogenous bile acid excretion (D), and the bile to perfusate ratios of [^{14}C]sucrose (E) and [^{3}H]polyethylene glycol (F). For explanatory information, see legend to Fig. 1. The 100% values ± SD for control, EGTA, EGTA plus $CaCl_2$, and bile duct ligation were, respectively: bile flow, 1.34 ± 0.13, 1.33 ± 0.14, 1.19 ± 0.12, and 1.24 ± 0.25 μl · min^{-1} · g of liver^{-1}; glutathione excretion in bile, 6.8 ± 2.5, 6.7 ± 1.4, 5.8 ± 1.4, and 7.2 ± 2.8 nmol of glutathione equivalents · min^{-1} · g of liver^{-1}; glutathione excretion in perfusate, 7.3 ± 2.8, 10.9 ± 5.0, 9.5 ± 2.3, and 9.0 ± 1.7 nmol of glutathione equivalents · min^{-1} · g liver^{-1}; and bile acid excretion, 1.93 ± 0.34, 1.48 ± 0.39, 1.24 ± 0.49, and 1.85 ± 0.77 nmol · min^{-1} · g^{-1} liver^{-1}. The [^{14}C]sucrose bile/perfusate ratios for control and EGTA plus $CaCl_2$ were 0.25 ± 0.06 and 0.24 ± 0.08, whereas the [^{3}H]polyethylene glycol (*PEG*) bile/perfusate ratios were 8.0 ± 1.6 and 8.2 ± 0.9, respectively.

(7, 31, 32). In contrast to the perfused rat liver, vasopressin is choleretic in intact dogs (33).

The present studies confirm the effects of vasopressin and epinephrine on paracellular permeability and demonstrate that this increase in permeability is associated with a decrease in bile flow and endogenous bile acid excretion but no change in the bile to perfusate ratio of polyethylene glycol. Although the mechanism of cholestasis is unknown, it is reasonable to suggest that it may be related to both the decrease in bile acid excretion and the increase in junctional permeability (Fig. 1). Bile secretion is driven by the osmotic effects of organic and inorganic solutes that are actively transported into the canalicular lumen and are impermeant (34). A decrease in bile acid excretion or an increase in junctional permeability could diminish these osmotic gradients and, consequently, the driving force for bile secretion, leading to cholestasis.

The decrease in bile flow produced by the hormones may also be related to their ability to increase portal pressure; however, this is unlikely since the doses of vasopressin and epinephrine used in the present study produced relatively small (0.2–0.6 and 1–2 cm of H_2O, respectively) and transient changes in pressure. In particular, vasopressin produced a large and sustained cholestasis even though perfusion pressure was changed only slightly and for a very brief period of time (2–3 min). Furthermore, because perfusate was forced through the liver at a constant rate throughout the experiment, perfusion flow rate remained unchanged during hormone treatment. Other investigators have used nitroprusside to minimize the vasoconstrictive effects of the hormones; however, our prelim-

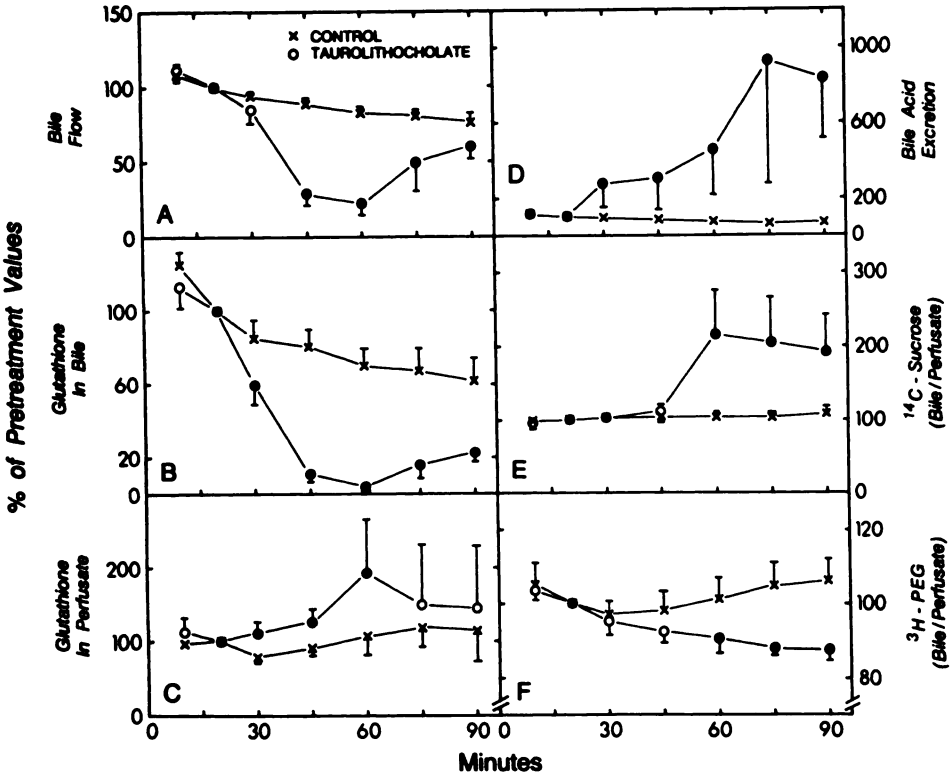

Fig. 4. Effects of taurolithocholate (bolus of 0.8 μmol at 20 min plus a constant infusion of 0.4 μmol/min from 20 to 45 min, $n = 5$) on bile flow (A), glutathione excretion into bile (B) and perfusate (C), endogenous bile acid excretion (D), and the bile to perfusate ratios of [^{14}C]sucrose (E) and [^{3}H]polyethylene glycol (F). For explanatory information, see legend to Fig. 1. The 100% values ± SD for control and taurolithocholate were, respectively: bile flow, 1.34 ± 0.13 and 1.31 ± 0.12 $\mu l \cdot min^{-1} \cdot g$ of $liver^{-1}$; glutathione excretion in bile, 6.8 ± 2.5 and 6.5 ± 2.7 $nmol \cdot min^{-1} \cdot g$ of $liver^{-1}$; glutathione excretion in perfusate, 7.3 ± 2.8 and 8.3 ± 6.3 $nmol \cdot min^{-1} \cdot g$ of $liver^{-1}$; bile acid excretion, 1.93 ± 0.34 and 1.63 ± 0.95 $nmol \cdot min^{-1} \cdot g$ of $liver^{-1}$; bile/perfusate ratio of [^{14}C]sucrose, 0.25 ± 0.6 and 0.20 ± 0.07; and bile/perfusate ratio of [^{3}H]polyethylene glycol (*PEG*), 8.0 ± 1.6 and 9.1 ± 1.2.

inary experiments suggest that nitroprusside (20 μM) itself alters hepatic glutathione release (increases biliary GSSG excretion; data not shown), precluding use of this drug in the present context. Although changes in portal pressure clearly affect blood flow distribution and biliary physiology in certain experimental conditions (31, 35–38), they are probably not solely responsible for the vasopressin- and epinephrine-induced cholestasis.

Vasopressin and epinephrine also enhanced sinusoidal while decreasing biliary glutathione excretion, such that total hepatic release remained unchanged (Figs. 1 and 2). The additional glutathione released into plasma may have originated from either the intracellular or biliary compartments. As indicated earlier, there is a large electrochemical gradient for glutathione between bile and plasma, such that an increase in paracellular permeability might allow biliary glutathione to reflux into the sinusoidal circulation. Although the present studies cannot distinguish between these two possibilities, indirect evidence suggests that both mechanisms may be operative. Estrela and co-workers (39) reported that relatively high concentrations of phenylephrine (10 μM), an α-adrenergic agonist, stimulate glutathione efflux and inhibit its bioysnthesis in hepatocytes isolated from rats fasted for 48 hr. However, in contrast to findings in the perfused rat liver (6, 7), these investigators observed that the additinal glutathione released by the hepatocytes was in the disulfide form (GSSG) and that there was an increase in intracellular GSSG concentrations, suggesting deleterious changes in the thiol-redox status. Because GSSG is excreted preferentially into bile in the intact liver, the results in isolated hepatocytes predict that phenylephrine should enhance biliary GSSG excretion. This prediction is not supported by the present findings with epinephrine or vasopressin (Figs. 1 and 5) or by the observations of Sies and Graf (6, 7) that vasopressin, epinephrine, and phenylephrine diminish biliary excretion of total glutathione (GSH plus 2 × GSSG) and stimulate sinusoidal efflux of GSH.

The present study provides evidence for the alternative hypothesis, namely that some of the glutathione that appears in the sinusoidal circulation after hormone stimulation was originally secreted into bile and enters the sinusoidal circulation by refluxing through the tight junctions. Thus, vasopressin and epinephrine increase paracellular permeability (Ref. 8 and Fig. 1) and produce inverse changes in biliary and sinusoidal glutathione efflux rates (Fig. 1). The close temporal association between changes in biliary permeability and glutathione efflux suggests a coupling between these responses. In contrast, dibutyryl-cAMP has no effect on either paracellular permeability (8) or glutathione efflux (6, 7).

To examine the hypothesis that the paracellular pathway could modulate hepatic glutathione efflux rates, experiments were performed using three different models of cholestasis that are known to increase junctional permeability, bile duct ligation (23), lowering of perfusate Ca^{2+} concentration (5, 20–22), and administration of the toxic bile acid taurolithocholate (26). Although these procedures do increase junctional permeability, each also produces a variety of other effects that may alter biliary excretion. Nevertheless, if hormonal stimulation of glutathione efflux occurs as a result of a receptor-mediated increase in basolateral glutathione transport capacity, it would seem unlikely that these models of cholestasis should mimic the effects of the hormones. The present results, however, indicate that the different models of cholestasis do mimic the effects of vasopressin and epinephrine. All three cholestatic interventions produced inverse changes in biliary and sinusoidal glutathione release, with minimal effects on net hepatic efflux. As with the hormones, changes in glutathione efflux

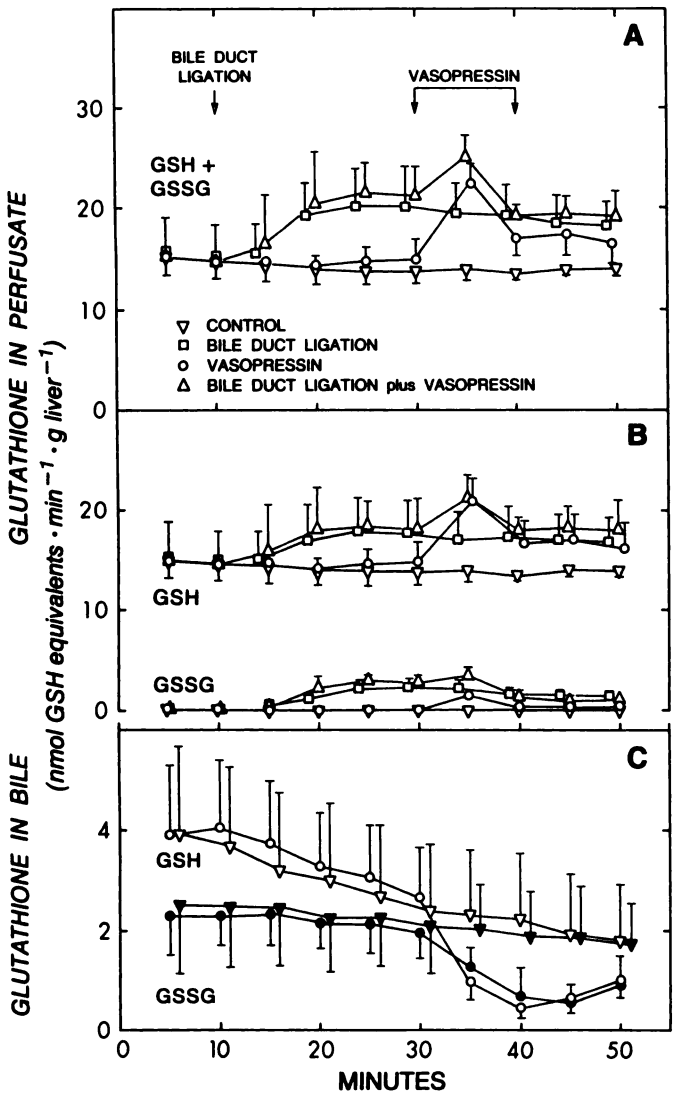

Fig. 5. Efflux of GSH and GSSG into perfusate (A and B) and bile (C) after administration of vasopressin (4 nM from 30 to 40 min, $n = 4$), bile duct ligation (at 10 min, $n = 6$), or both maneuvers ($n = 6$), in livers perfused with nonrecirculating Krebs-Henseleit buffer. Glutathione efflux is presented both in terms of total (i.e., GSH plus 2 × GSSG) release (A) and as separate values for GSH and GSSG (B and C), in GSH equivalents · min^{-1} · g of $liver^{-1}$. Values are means ± standard deviations.

occurred in parallel with changes in bile flow and junctional permeability.

Because transport of glutathione across the basolateral plasma membrane is nearly saturated at normal liver glutathione concentrations (40), transport across this membrane can be enhanced only if additional transport proteins are inserted into the membrane or if existing carriers increase their turnover rate. Furthermore, in order to produce the observed decrease in biliary glutathione excretion, the glutathione transport systems on the canalicular membrane would have to be inactivated concurrently with the activation of basolateral transport systems. Because of the rapidity and magnitude of the effects of the different cholestatic maneuvers on glutathione release, these possibilities appear remote and the effects are more consistent with the regurgitation of glutathione from bile to plasma via the paracellular pathway. The appearance of GSSG in the sinusoidal circulation after bile duct ligation or vasopressin administration is also consistent with this hypothesis. In control liver perfusions, the amount of GSSG released into the sinusoidal circulation is normally below the detection limit (<0.5 nmol · min^{-1} · g^{-1}), whereas a large fraction, 30–80%, of the glutathione in bile is as GSSG (1). The data in Fig. 5 demonstrate that sinusoidal GSSG excretion increased by 2–3 and 1–2 nmol · min^{-1} · g^{-1} after bile duct ligation and vasopressin administration, respectively. This increase in sinusoidal GSSG excretion was associated with either a complete (bile duct ligation) or partial (vasopressin) inhibition of biliary GSSG excretion (Fig. 5). Furthermore, the attenuated response to vasopressin in bile duct-ligated livers is consistent with the suggestion that at least a part of the effects of these two maneuvers is mediated via a common mechanism.

Hepatocellular tight junctions express a charge selectivity, with cations penetrating this barrier more rapidly (13, 41). However, both anionic and cationic proteins as large as horseradish peroxidase have been shown to cross the junctions (8, 13). Recent findings by Hardison and co-workers (13) indicate that paracellular entry of an anionic isozyme of horseradish peroxidase is increased nearly 8-fold by 10^{-10} M vasopressin. It is not unreasonable to anticipate that smaller anions such as glutathione (molecular weight 307), or even bile acids, could diffuse down their large electrochemical gradients from bile to plasma if the barrier properties were somehow altered. At present there is no direct evidence to support this contention.

As in previous studies on the role of the paracellular pathway in bile formation, interpretation of the present data is constrained by the limited specificity of both the experimental models of cholestasis and the probes used to measure cellular permeability. Bile to perfusate ratio of [^{14}C]sucrose has traditionally been used to assess junctional permeability; however, there are practical and theoretical limitations to its use [for discussion of these limitations see Tavoloni (42)]. Even with these shortcomings, it remains the most valid and sensitive marker of junctional permeability (42). In contrast, the use of polyethylene glycol-900 remains controversial. Polyethylene glycol-900 was initially considered a marker of canalicular bile flow (19), but more recent evidence suggests that it enters bile via several routes, with the transcellular vesicular pathway being predominant (18). Although the present findings cannot resolve this controversy, the results are more consistent with the latter interpretation. The basal bile to perfusate ratio of polyethylene glycol was 8.4 ± 1.3, suggesting a concentrative mechanism. This ratio was unaffected by vasopressin and epinephrine but was decreased by taurolithocholate administration.

The mechanism for the hormone-induced increase in junctional permeability has not been identified, although some have speculated that Ca^{2+} may somehow be involved (8, 43). A common intracellular signal for vasopressin, epinephrine, and antiogensin II is the concentration of Ca^{2+}. The calcium ionophore A23187 increases both intracellular calcium and junctional permeability (43), although its effects may not be specific. Similarly, taurolithocholate produces a variety of ill defined effects on the liver, including an increase in the bile/perfusate ratio of [^{14}C]sucrose (Fig. 4) and intracellular Ca^{2+} (24, 25), whereas noncholestatic bile acids have no effect on intracellular calcium. Additional indirect evidence comes from studies on the effects of thiol-oxidizing agents on junctional permeability and bile formation (44). Oxidant-induced hepatocellular injury

is associated with an increase in intracellular calcium (45) and junctional permeability (44). In contrast, dibutyryl-cAMP has no effect on the [^{14}C]sucrose bile/perfusate ratio or the fast entry of horseradish peroxidase into bile (8).

Calcium is the only ion in plasma that is absolutely required for bile formation (5, 21). Bile flow ceases if calcium is omitted or chelated with either citrate or EGTA (5, 21, 22) but spontaneously recovers upon the readmission of calcium chloride. Free calcium ion concentrations in plasma have to be decreased to very low levels (<100 μM) before any changes in bile flow become detectable (22). Opening of the tight junctions is the primary mechanism for this effect; however, removal of extracellular calcium also disrupts intracellular calcium homeostasis. In contrast to the calcium-mobilizing hormones, taurolithocholate, or the thiol-oxidizing agents, depletion of extracellular Ca^{2+} decreases intracellular calcium concentrations (24, 25), so its mechanism of action is probably different. Studies are needed to determine whether, in fact, intracellular Ca^{2+} is a stimulus for the opening of junctional barriers and whether changes in extracellular calcium can influence this process.

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