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Phalloidin-induced cholestasis: A microfilament-mediated change in junctional complex permeability

(bile flow/tight junctions)

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ABSTRACT **Phalloidin, administered to male rats for 7 days (500 μg per kg/day), increased the mean hepatic content of filamentous actin. Both bile flow and bile acid excretion diminished proportionally, whereas the bile-to-plasma ratios of [^{3}H]inulin and [^{14}C]sucrose increased significantly from 0.08 and 0.16 in controls to 0.37 and 0.69, respectively, in phalloidin-treated animals. Simultaneously, junctional permeability was altered as noted by the free penetration of ionic lanthanum into the zonula occludens and bile canaliculus. Freeze-fracture replicas of the junctional complex revealed rearrangements of the junctional elements and regions in which only a single element separated the canaliculus from the lateral intercellular space. These findings suggest that microfilaments influence the permeability of "tight junctions" between hepatocytes and that bile constituents might reflux from the canaliculus to the intercellular space in phalloidin-induced cholestasis.**

Phalloidin, a cyclic peptide derived from the poisonous mushroom *Amanita phalloides*, specifically binds to purified muscle F-actin and prevents its depolymerization (1–3). Phalloidin increases the quantity of F-actin when added to isolated hepatocytes (4) or to isolated liver plasma membranes (2, 5, 6) and produces a marked increase in microfilaments in the pericanalicular region of hepatocytes when the alkaloid is administered chronically in low doses to rats (7). In the intact rat, chronic phalloidin administration, like other microfilament poisons, is associated with a diminution in bile production (7–9) and an extensive rearrangement of hepatocyte junctional elements observed by freeze-fracture electron microscopy (10). These changes in junctional structure suggest that microfilaments help regulate junctional development and support the notion that the junctional barrier may in turn influence the formation of bile. Because accumulating evidence suggests that the paracellular pathway is an important site for water, electrolyte, and solute entry into bile (11–14), the phalloidin-treated rat provides a useful experimental model to examine more closely the relationship among microfilament function, junctional structure, and bile secretion.

In this study, we measure the effects of phalloidin on bile formation and biliary permeability to inulin and sucrose, two large molecular weight solutes that rapidly reach equilibrium between plasma and bile before liver water, suggesting that their entry into bile is via a paracellular route. Junctional structure and permeability were also examined by freeze-fracture studies and by the infusion of ionic lanthanum chloride at a time when increases in hepatic content of polymerized actin were also observed.

METHODS

Male Sprague–Dawley rats (Charles River), initially weighing 175–225 g, were treated for 7 days with either phalloidin at 500 $\mu g\ kg^{-1}\ day^{-1}$ in saline (16 animals) or saline alone as a control (16 animals) by intraperitoneal injection as described by Gabbiani *et al.* (7). Phalloidin was obtained from Boehringer Mannheim. On the eighth day, the rats were anesthetized by intraperitoneal injection of sodium phenobarbital at 50 mg (kg of body weight)$^{-1}$, and the bile duct was cannulated with polyethylene tubing (PE10, Clay Adams). Body temperature was monitored by rectal probe and maintained at 37°C by a heating lamp regulated by a constant temperature regulator (Yellow Springs Instrument). After 60–80 min, the abdomen was reopened and heparin (10,000 units kg^{-1}) was injected into the inferior vena cava before fixation of the liver by portal vein perfusion.

Bile Flow and Bile Acid Excretion. Bile was collected for 80 min at 20-min intervals and expressed as $\mu l\ min^{-1}$ (g of body weight)$^{-1}$ and as $\mu l\ min^{-1}$ (g of liver)$^{-1}$. Bile acids were analyzed by the hydroxysteroid dehydrogenase method (15). Nine control animals and 10 phalloidin-treated animals were used.

[^{3}H]Inulin and [^{14}C]Sucrose Clearance. [^{3}H]Inulin and [^{14}C]sucrose were obtained from New England Nuclear. After ligation of the renal pedicles, 2.5 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{14}C]sucrose and 10 μCi of [^{3}H]inulin were injected intravenously into five control and six phalloidin-treated animals. Forty minutes later, bile was collected for two 20-min periods. Plasma samples were obtained at the midpoint of each bile collection period and at the end of the experiment. One hundred microliters of either bile or plasma was added to 10 ml of Beckman Ready Solve HP scintillation fluid, and ^{14}C and ^{3}H were assayed in a Beckman LS-255 liquid scintillation counter. Samples were assayed in duplicate to an error of less than 2%. Quenching was corrected by use of an external standard. Bile-to-plasma concentration ratios (B/P) for [^{14}C]sucrose and [^{3}H]inulin were determined for each collection period. In addition, in three phalloidin-treated and three control animals, 100–500 mg of liver was dissolved in Beckman BTS tissue solubilizer, and [^{3}H]inulin and [^{14}C]sucrose were assayed to determine isotope liver/plasma ratios (L/P).

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Abbreviations: B/P, bile-to-plasma concentration ratio(s); L/P, liver-to-plasma concentration ratio(s).

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Quantitation of Actin in Liver. Unfixed liver from five pairs of animals (five controls and five phalloidin-treated) were perfused free of blood, minced in 2 vol of buffer containing 10 mM imidazole chloride (pH 7.0), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM ATP, 1 mM dithiothreitol, and 0.34 M sucrose at 4°C, and homogenized in a loose Dounce homogenizer. The homogenate was centrifuged for 90 min at 100,000 × *g* in a Beckman L5-65 ultracentrifuge.

Protein concentrations in whole homogenate, pellet, and supernatant were measured by Biuret reactions (16).

DNase Affinity Chromatography. Six-tenths of a milliliter of the 100,000 × *g* supernatant liver extract was passed over a 2-ml column containing DNase (Worthington) in buffer (3 mM imidazole/0.5 mM ATP/0.1 mM $CaCl_2$/0.75 mM 2-mercaptoethanol/0.01% NaN_3). The column was washed with 0.1 M KCl followed by 0.75 M guanidine and finally by 3 M guanidine to elute bound actin as described by Lazarides and Lindberg (17). The eluate was dialyzed against buffer and lyophilized and redissolved in electrophoresis sample buffer for electrophresis on a $NaDodSO_4$/9% polyacrylamide gel, and the recovery of 42,000-dalton proteins was measured by spectrophotometric scanning of the gel at 546 nm in a Gilford spectrophotometer after staining with Coomassie blue. Protein concentration of eluate fractions was measured according to Lowry (18).

Polyacrylamide Gels. $NaDodSO_4$/9% polyacrylamide gels of whole homogenate, pellet, and supernate were obtained by electrophoresis according to Laemmli (19). The gels were scanned at 546 nm in a Gilford spectrophotometer. Quantitation was by paper weighing.

Ionic Lanthanum Studies. Four rats (two control and two phalloidin-treated) were studied after an overnight fast. The portal vein was cannulated with a 16-gauge needle and perfused with an isoosmotic Ringer solution containing 5 mM lanthanum chloride (Fisher) in 5 mM Tris·HCl, pH 7.4/Na^+ (133 mM)/K^+ (3.6 mM)/Cl^- (156 mM)/Ca^{2+} (4.5 mM) for 3 min at 15 cm of pressure as described (11). This was followed by immediate perfusion–fixation with cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Sections were stained with uranyl acetate and lead citrate and viewed with a Siemens Elmskop electron microscope (Siemens, Iselin, NJ).

Freeze-Fracture Electron Microscopy. Liver from four controls and five phalloidin-treated rats were fixed by portal vein perfusion as described above. Prior to freezing, tissue was soaked in 25% glycerol/0.1 M sodium cacodylate. Tissue was frozen in Freon 22 cooled by liquid nitrogen. A Balzers freeze etch unit BAF 301 (Balzers High Vacuum, Lichtenstein) was used with an electron beam evaporation device (EVM 052) and a quartz crystal thin-film monitor (QSG 201). Replicas were examined with a Zeiss 10B electron microscope and quantitation was performed without prior knowledge of tissue treatment.

Table 1. Body and liver weights

Treatment	Body weight, g		Liver weight*
	Day 1	Day 8	
Saline	235 ± 32	291 ± 14	3.55 ± 0.60
	(6)	(6)	(5)
Phalloidin	239 ± 11	250 ± 72	4.74 ± 0.83
	(7)	(7)	(6)
	$P > 0.35$	$P < 0.02$	$P < 0.025$

Numbers in parentheses refer to number of animals.
* Grams per 100 g of body weight.

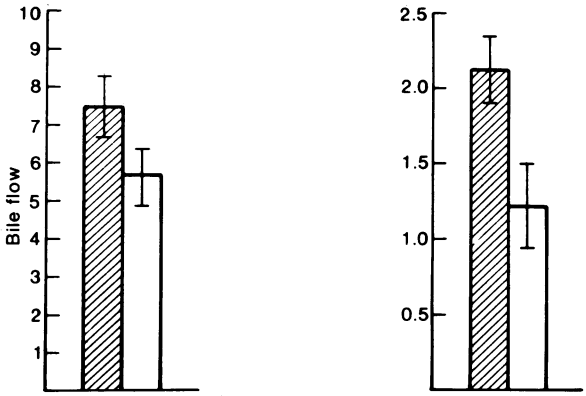

FIG. 1. Effect of phalloidin on bile flow. Nine controls (hatched bars) and 10 phalloidin-treated animals (clear bars) secreted 7.5 ± 0.82 and 5.38 ± 0.98 μl min^{-1} (100 g of body weight)$^{-1}$, respectively (*Left*). $P < 0.005$. When expressed as μl min^{-1} (g of liver)$^{-1}$ (*Right*), these values were 2.12 ± 0.27 and 1.18 ± 0.28, respectively. $P < 0.001$. The bars, mean ± SD.

RESULTS

Body Weight, Liver Weight, and Bile Flow. There was no significant difference in body weights between phalloidin-treated and saline- (control) rats at the beginning of the experiment but weight gain after 8 days was less in phalloidin-treated animals (Table 1). Liver weight was significantly increased in phalloidin-treated animals ($P < 0.025$).

Bile flow was significantly decreased in phalloidin-treated animals compared to controls whether expressed in terms of body weight or liver weight (Fig. 1).

Bile Acid Excretion. Bile acid excretion rates were consistently lower in phalloidin-treated animals than in controls (Table 2), although differences achieved statistical significance only during the later collection periods. The decline in bile flow was proportional to the decrease in bile acid excretion.

(B/P) of [^{14}C]Sucrose and [^{3}H]Inulin. B/P for [^{14}C]sucrose ($P < 0.001$) and [^{3}H]inulin ($P < 0.001$) were significantly greater in phalloidin-treated animals than in controls (Fig. 2). The increase in B/P was much greater than could be accounted for solely by a decline in flow (Fig. 3), so that the biliary clearance of these solutes was increased. The analysis in Fig. 3 indicates that the permeability coefficients for inulin and sucrose entry into bile is also increased in phalloidin-treated animals. (For a more detailed discussion of these theoretical considerations, see refs. 20–24.)

Hepatic Levels of [^{3}H]Inulin and [^{14}C]Sucrose. Eighty minutes after intravenous administration, [^{14}C]sucrose L/P

Table 2. Bile acid excretion

	Rate of excretion		
	0–20 min	20–40 min	40–60 min
	μmol min^{-1}		
Saline	0.80 ± 0.24	0.61 ± 0.14	0.52 ± 0.12
Phalloidin	0.68 ± 0.13	0.51 ± 0.08	0.34 ± 0.10*
	μmol min^{-1} (g liver)$^{-1}$		
Saline	0.08 ± 0.02	0.06 ± 0.02	0.05 ± 0.01
Phalloidin	0.05 ± 0.01†	0.04 ± 0.01*	0.03 ± 0.01‡
	μmol min^{-1} (100 g body weight)$^{-1}$		
Saline	0.26 ± 0.08	0.20 ± 0.04	0.17 ± 0.03
Phalloidin	0.23 ± 0.05	0.17 ± 0.03	0.12 ± 0.04*

Values represent the mean ± SD of seven saline- and six phalloidin-treated animals.
* $P < 0.02$.
† $P < 0.05$.
‡ $P < 0.001$.

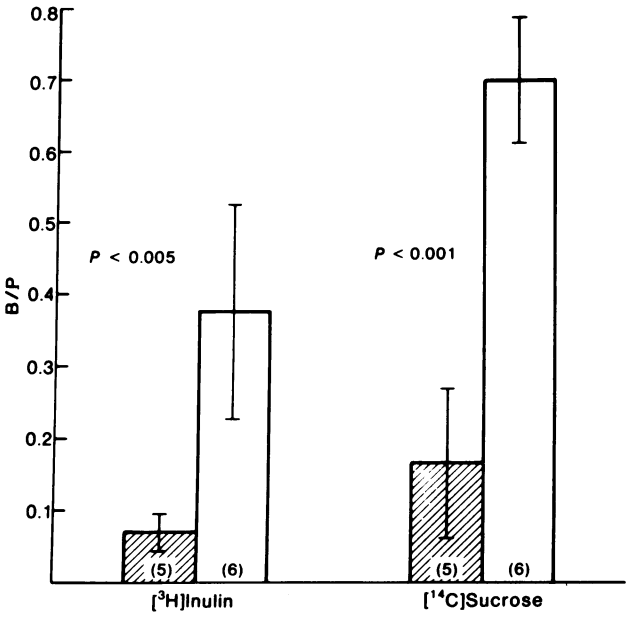

FIG. 2. B/P for [^{3}H]inulin and [^{14}C]sucrose. B/P averaged 0.08 ± 0.02 and 0.16 ± 0.10, respectively, in controls (hatched bars) and 0.37 ± 0.15 and 0.69 ± 0.08, respectively, in phalloidin-treated animals (open bars). Bars, mean ± SD. Numbers in parentheses indicate number of animals.

averaged only 0.002 ± 0.001 in three control animals, whereas [^{3}H]inulin L/P (0.058 ± 0.035) approached B/P. Phalloidin treatment did not alter L/P ratios of [^{14}C]sucrose (0.003, 0.001, and 0.03) and increased [^{3}H]inulin L/P only slightly (0.078, 0.056, and 0.15). Thus, the large increase in solute B/P in phalloidin animals must occur through extracellular routes.

Quantitation of Actin. After 7 days of phalloidin administration, there was a relative increase in filamentous actin (Fig. 4). No differences were detectable in the proportion of the 42,000-dalton band present in supernatant extract between the two groups, either by quantitating peaks on NaDodSO$_4$ gel scans or as measured more specifically by DNase affinity chromatography. Thus, the total content of liver actin increased with the increment accounted for by a rise in filamentous actin.

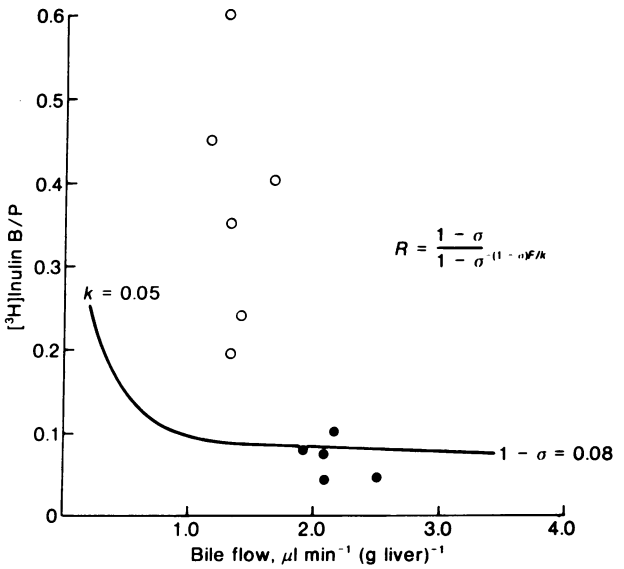

FIG. 3. Relationship of [^{3}H]inulin B/P to bile flow in saline- (●) and phalloidin-treated (○) rats. A diffusion-permeability coefficient of 0.05 is used to construct the solid curved line, which defines the theoretical relationship between B/P and inulin and bile flow, where R = inulin B/P, F = bile flow, k = diffusion permeability coefficient, and σ = the Staverman reflection factor (also see ref. 20); a sieving coefficient of 0.08 was also used ($1 - \sigma = 0.08$). Note that in each phalloidin experiment the observed value of R fell above the theoretical line, indicating that the diffusion permeability coefficient must have increased above that observed in control animals whose values for R fell on the line.

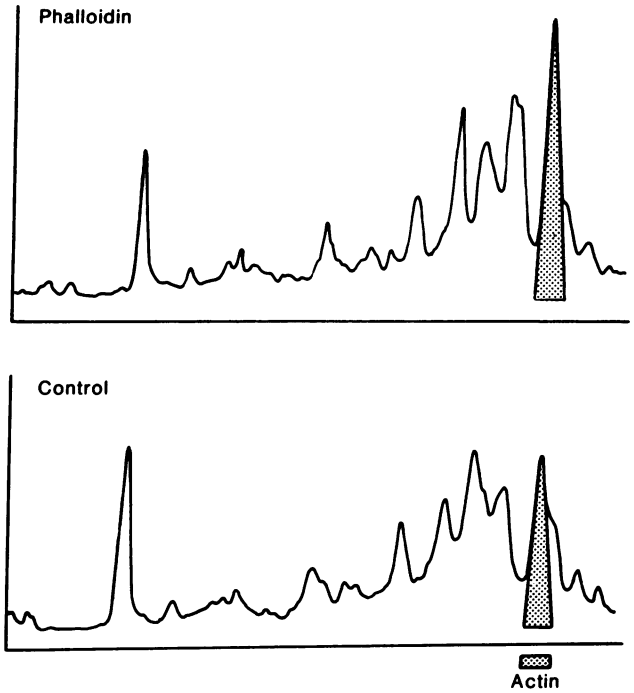

FIG. 4. Densitometric tracings of representative NaDodSO$_4$ gels (9%) containing sedimented protein (100,000 × g; 90 min) from homogenates of both phalloidin- and saline-treated animals. A 42,000-dalton peak migrated with the actin standard and was consistently increased in the phalloidin-treated animal compared to its paired saline control. Although there was considerable variation within individual experiments, values averaged 11.3 ± 3.8% and 9.8 ± 1.0% for six phalloidin- and six saline-treated controls, respectively. The differences were significant at the $P < 0.025$ value by the Student paired t test.

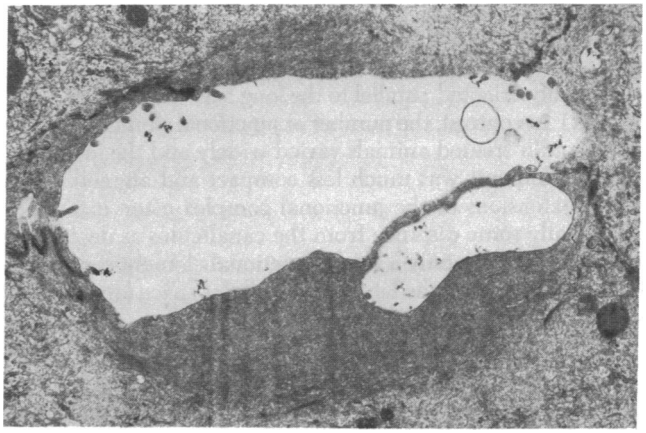

FIG. 5. Transmission electron micrograph of a dilated bile canaliculus, demonstrating a marked increase in pericanalicular microfilaments and loss of surface microvilli. Electron-dense material (lanthanum) may be seen within the intercellular space and junctional complex and within the bile lumen itself. Lanthanum fails to penetrate the junctional complex and enter the bile canalicular lumen in fasted control animals. (×4700.)

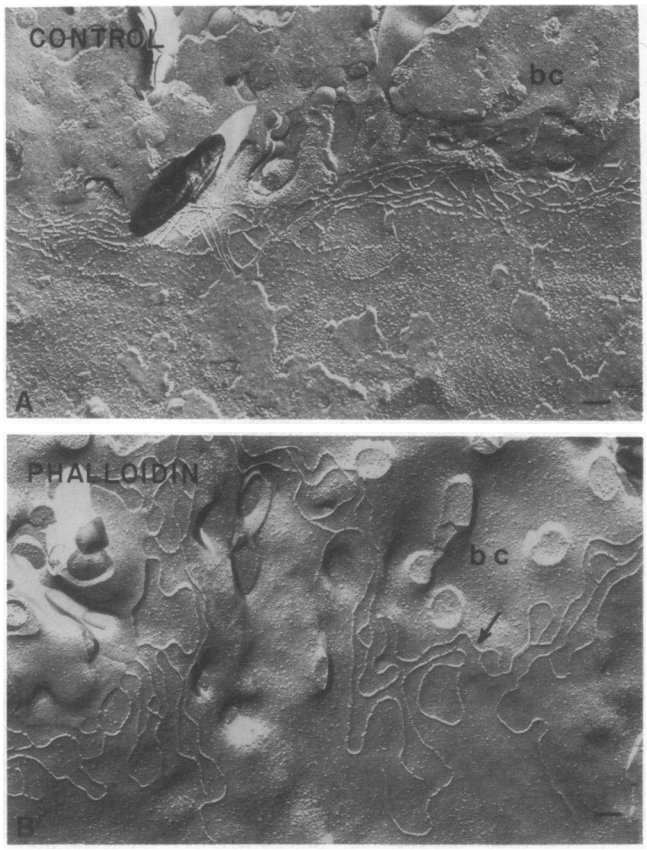

FIG. 6. Freeze-fracture replica of the junctional complex bordering the lumen of a bile canaliculus in a control animal (*A*) and in a phalloidin-treated animal (*B*). (*A*) Note the parallel and close arrangement of the junctional elements. (×11,200.) (*B*) Junctional strands are much less regularly arranged in phalloidin-treated animals; they have lost their parallel array and in focal areas the number of strands is diminished to as few as one (arrow) separating the lumen of the bile canaliculus (bc) from the intercellular space. (×11,200.)

Ionic Lanthanum Studies. As noted (11), very little ionic lanthanum penetrated junctional complexes in fasted control animals and none was seen within the canalicular lumen. In contrast, in phalloidin-treated animals (Fig. 5), widespread heavy lanthanum penetration of junctional complexes was seen, and moderate accumulations of lanthanum were present within the canalicular lumen.

Freeze-Fracture Studies. In saline controls, the number of junctional elements ranged from three to seven and elements were usually oriented parallel to the long axis of the canaliculus (Fig. 6*A*). In contrast, the number of junctional elements found in phalloidin-treated animals varied widely and the arrangement of elements was much less compact and aligned. Prolonged extensions of the junctional complex often extended abluminally some distance from the canaliculus as described (10). In focal areas only a single junctional element separated the canaliculus from the lateral intercellular space (arrow, Fig. 6*B*).

DISCUSSION

Although the morphological effects of chronic administration of low doses of phalloidin on rat liver are now well described and consist initially of an increase in microfilaments that are particularly prominent in the submembranous region of the bile canaliculus, the functional counterparts of this striking morphological change are less well understood. The response to phalloidin seems reasonably specific for a primary effect on the equilibrium in the hepatocyte between soluble G-actin and the polymerized F-actin to which phalloidin specifically binds. Our finding that the 42,000-dalton-band protein is increased in the pelletable protein from livers of phalloidin-treated animals compared to controls is confirmation of the electron microscopic evidence that microfilaments are increased. Because there was no decrease in the 42,000-dalton band in the supernatant from the phalloidin-treated rats, soluble or G-actin content appears to be unchanged, a qualitative finding that was confirmed by the more specific affinity chromatography studies with DNase, which specifically binds G-actin (17). Thus, actin synthesis was presumably increased. Although we were unable to detect any ultrastructural change in hepatocyte organelles, dilation of bile canaliculi and loss of canalicular microvilli were noted. In addition, prominent microtubules and 100-Å filaments could be observed trapped within the striking accumulation of pericanalicular microfilaments. Thus, it is likely that the shift in equilibrium from G- to F-actin in the hepatocyte had effects on transport functions that were not measured in the present study but might well influence bile formation. However, the changes in junctional structure and permeability were of particular interest because there is suggestive evidence in epithelial tissues that microfilaments can influence the permeability of junctional complexes (25), and the present findings are consistent with this interpretation.

Direct evidence for increased permeability of the junctional complexes was provided by the ionic lanthanum studies in which the degree of penetration of the electron-dense tracer into junctional complexes and canalicular lumina was grossly different from that seen in saline-treated controls.

Furthermore, morphologic studies of hepatocyte junctional complexes by freeze-fracture revealed striking changes in junctional element organization in phalloidin-treated animals. Montesano *et al.* (10) and Gabbiani *et al.* (7) have reported reorganization of junctional elements after phalloidin treatment, but the finding of a decreased number of strands has not been previously emphasized. Although a close correlation between the number of junctional elements and junctional permeability is not observed in all circumstances (26, 27), a relationship between junction structure and junctional permeability has been noted for some tissues (28, 29). In phalloidin-treated animals the number of junctional elements was decreased in focal regions to a single element, and in regions where many elements were present they were loosely arranged.

Because our previous studies (11, 12) suggest that osmotic gradients between bile and plasma stimulate the flux of water and ions such as ionic lanthanum across the junctional barrier, the finding that structural alterations in this barrier are associated with a striking increase in penetration of ionic lanthanum in the present investigation strongly suggests that these junctional barriers influence movement of solutes between plasma and bile.

Finally, phalloidin treatment resulted in a diminution of bile flow in association with a marked increase of biliary permeability to both [^{14}C]sucrose and [^{3}H]inulin. The several-fold increase in the appearance of these solutes in bile cannot be accounted for by decreased bile flow alone, and implies a marked increase in permeability at the level of the hepatocyte. Theoretically, such increased permeability may be associated with the plasma membrane of the hepatocyte or occur via the paracellular pathway (9). In view of the rapidity with which both solutes entered bile in relatively high concentrations and of the low permeability of cell membranes to these solutes, the transcellular route seems unlikely, particularly because we were unable to detect significant increases in inulin or sucrose in hepatocytes after phalloidin treatment. Because the paracellular

pathway is the likely route for entry of these solutes into bile, this evidence provides further support linking changes in the junctional complex barrier to the action of phalloidin.

How then would these findings, which suggest that the entry of solutes from blood to bile is increased, account for a decrease in biliary secretion. Back diffusion of bile has frequently been postulated as a mechanism underlying various forms of bile secretory failure (cholestasis), and the results of this study would also be consistent with the hypothesis that cholestasis is a consequence of diffusion of bile solutes out of the canaliculus, a process that would diminish the osmotic gradient and the flow of bile. Normally solutes such as bile acids that are secreted into the canalicular lumen and stimulate secretion are associated in large aggregates or micelles (whose molecular weight has been estimated at around 10,000) and have their exit from bile prevented by the "tight" junctions that demarcate the boundary between canalicular and lateral intercellular spaces. Bradley and Herz (30) provide evidence that the junctional barrier is negatively charged, which would further retard the egress of biliary bile acid monomers that are in equilibrium with the bile micelle. The finding that bile acid secretion is decreased in phalloidin-treated animals in association with a proportional decline in bile flow could be explained by "back diffusion" of bile acids via leaky junctions, although a defect in hepatic transport could produce this result as well. Further studies will be necessary to distinguish between these two possibilities. However, if bile acids reflux out of bile, the osmotic gradient that normally results in net movement of fluid into the biliary canaliculi would be dissipated, and bile production would diminish. Alterations in the freeze-fracture appearance of junctional elements between hepatocytes following mechanical obstruction of the common bile duct in rats suggests that paracellular reflux of bile may occur in other experimental models of cholestasis (31, 32). However, direct evidence of paracellular reflux of bile still remains to be established for each of these models.

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