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# The Paracellular Pathway and Bile Formation[1]

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Choleretic infusions of taurocholate (40 μmoles for one hour) result in a significant increase in the number of lateral cell surface invaginations observed by scanning electron microscopy adjacent to the junctional complex of bile canaliculi in rat liver. Transmission electron microscopy indicates that these invaginations resemble "blisters" induced by osmotic gradients across epithelial tissues, a morphologic change which correlates with increases in ionic and hydraulic conductivity of the paracellular "shunt" pathway in such tissue. Since taurocholate infusions result in localization of ionic lanthanum chloride within hepatocyte junctional complexes, bile acids may also stimulate the movement of fluid and electrolytes across paracellular pathways during the process of bile formation.

## INTRODUCTION

Bile acids are major stimulants of bile production in a variety of species including man [1], and are transported into bile in high concentration relative to plasma. Sperber originally proposed that their osmotic activity passively moves fluid and electrolytes into bile following concentrative transport into the bile canalicular lumen [2]. While this theory is indirectly supported by Hanzon's vital fluorescent microscopic studies which visually demonstrate concentration of the organic anion fluorescein in canalicular bile [3], neither the mechanisms of bile acid transport into bile nor the pathway by which fluid or electrolytes enter bile is clearly understood. Carrier transport of bile acids across the canalicular membrane has been the assumed mechanism for their biliary transport but recent evidence suggests that bile acid excretion may also involve vesicle or vacuole transport, perhaps from the Golgi apparatus to the canalicular lumen [4,5].

While fluid and electrolytes may also enter bile by this process, other studies have directed attention to the paracellular shunt pathway between hepatocytes as a site for movement of fluid and small ions into bile. Junctional complexes between hepatocytes have traditionally been regarded as "tight" but choleretic infusions of taurodehydrocholate, a synthetic triketo bile acid analogue, increase the penetration of the hepatocyte junctional complex by the electron dense ionic lanthanum chloride,



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increase the biliary permeability to $^{14}$C-sucrose and produce morphologic changes in the intercellular space known as "blisters" [6]. These focal dilations of the intercellular space lie adjacent to the canalicular lumen and are analogous to "blisters" which form in the intercellular space of epithelial tissues which are exposed to hypertonicity [7–11]. Such intercellular blisters are associated with increases in junctional permeability to a variety of inert solutes and to an increase in ionic conductance and a decrease in electrical resistance. Since similar electrical measurements cannot be performed across the hepatocyte junctional complex because of the obvious technical difficulties in puncturing the canaliculus with micro-electrodes, the phenomenon of blister formation following bile acid induced choleresis is of particular interest since it seems possible that comparable changes in electrical resistance and ionic conductivity occur in the paracellular pathway during the production of bile by osmotic choleretics. In this report we present evidence that taurocholate, the predominant bile acid found in rat bile, also produces parajunctional blister formation in the lateral cell membrane and localizes ionic lanthanum in junctional complexes of rat hepatocytes when infused at 40 $\mu$moles per hr. Although the findings are less pronounced than when 120 $\mu$moles of dehydrocholate are infused [6], they suggest that paracellular movement of fluid occurs under more physiologic conditions in response to endogenous bile acids.

## METHODS

Male Sprague Dawley rats were obtained from Charles River Laboratories, Inc., Wilmington, MA, and weighed between 224 and 275 g. Taurocholate was purchased from Calbiochem, San Diego, CA, and was greater than 98% pure by thin layer chromatography. Fed rats were utilized for scanning and transmission electron microscopic studies of parajunctional blisters while studies with ionic lanthanum chloride were performed after the animals were fasted overnight. Three to five animals were used in each group including controls. On the morning of study rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and the common duct was cannulated above the entrance of the pancreatic ducts. Body temperature was maintained at 37°C by a heating lamp and was regulated by a constant temperature regulator from Yellow Springs Instrument Co., Yellow Springs, Ohio. Bile was collected for a 30 min control period and then animals were infused intravenously with 1% albumin–0.9% sodium chloride with or without the addition of taurocholate (40 $\mu$moles for one hour). Infusions were delivered with a Harvard pump (Harvard Apparatus Co., Millis, MA) at 2.6 ml per hr, and bile was collected at 10 min intervals and measured for volume. At the end of the hour the abdomen was reopened and the portal vein was cannulated with a #16 gauge intracatheter while the infusion continued. The liver was first perfused free of blood for 30 sec with Ringer's-lactate containing 10 units heparin per ml at 4°C at a hydrostatic pressure of 15 cm and was then followed by an infusion of cold 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Specimens were then prepared for scanning or transmission electron microscopy as previously described [6,12]. When studies were performed for localization of ionic lanthanum chloride, the animals were heparinized by injection of heparin (1,000 units per 100 g.bw) into the vena cava after the one hour bile acid infusion. The portal vein was then cannulated, and the liver was perfused as noted above but with a 310 mosmolar solution containing 5 mM lanthanum ($LaCl_{3\text{-}6}H_2O$, Fisher Scientific, St. Louis, MO) in 5 mM Tris pH 7.4 for 3 min, followed by the glutaraldehyde infusion, as previously described [6].

Scanning electron microscopy was performed at the Enrico Fermi Institute using a Coates-Welter scanning electron microscope (Coates and Welter Instrument Corp., Sunny Vale, CA) without knowledge of treatment and photographs were obtained at 2000 × magnification in periportal zones of the lobule, where bile acids would theoretically be in highest concentration [13]. These photographs were reviewed blindly and the number of invaginations or "blisters" adjacent to the bile canaliculi on the lateral cell membrane were counted from the surface of more than 100 hepatocytes in each group.

Transmission electron microscopy of these blisters was performed with a RCA-EMU-4 transmission electron microscope (RCA Solid State, Somerville, NJ) after staining specimens in uranyl acetate and lead citrate. Sections of liver from 8 studies with lanthanum were treated similarly but were viewed with a Siemens Elmskop 1A electron microscope (Siemen's Corp., Islein, NJ), without knowledge of the treatment. Fifteen to 30 bile canaliculi were examined randomly from each specimen and the number of junctions containing lanthanum was recorded.

## RESULTS

The intravenous infusions of 40 $\mu$ moles of taurocholate for 1 hour resulted in a 40% increase in bile production and a significant increase in the number of intercellular diverticuli or "blisters" observed by scanning electron microscopy (Table 1). These "blisters" were variable in size and were consistently viewed on the smooth-surfaced portion of the lateral surface membrane facing the intercellular space. They were always adjacent to the region of the junctional complex rather than near the sinusoid (Fig. 1, A and B). These structures were indistinguishable from those previously observed when bile flow was doubled by larger choleretic infusions of dehydrocholate (120 $\mu$moles) and differed only in that they were observed less frequently with this more physiologic stimulus to bile production [6]. As in previous studies, the increase in number of blisters was observed if tissue was fixed while bile acids were being infused and were not frequently noted if either the bile acid infusion was stopped a few minutes prior to fixation or if the tissue was removed and immersion fixed rather than fixed in situ. These findings suggest that the blisters were not a "toxic" effect or artifact but represent a response to a rapidly dissipated osmotic gradient produced by the bile acids.

Transmission electron micrographs serve to confirm both the location and the size of these intercellular structures (Fig. 2). Some blisters also contained membranous material within their lumen, a finding reported in tissues such as the toad bladder following induction of fluid movement across junctional barriers by osmotic gradients [7].

TABLE 1

Effect of taurocholate infusions on bile flow and lateral cell surface invaginations ("blisters")

| | Control | Taurocholate |
|---|---|---|
| Bile Flow ($\mu$l min$^{-1}$g$^{-1}$ liver) | | |
| Basal | 2.00 ± .07 | 2.04 ± .06 |
| Post-infusion | 2.08 ± .16 | 2.67 ± .08* |
| "Blisters" (no. per cell surface) | 0.62 ± .09 (129) | 1.44 ± .16* (105) |

"Blisters" equal the number of paracanalicular invaginations per lateral cell surface in portal regions of control or taurocholate infused animals. Number of surfaces measured is represented in parentheses. Four animals were used for control and 3 for the taurocholate infused group.

*Significant difference from control group, $p < .01$. Data is represented as the mean ± SD.

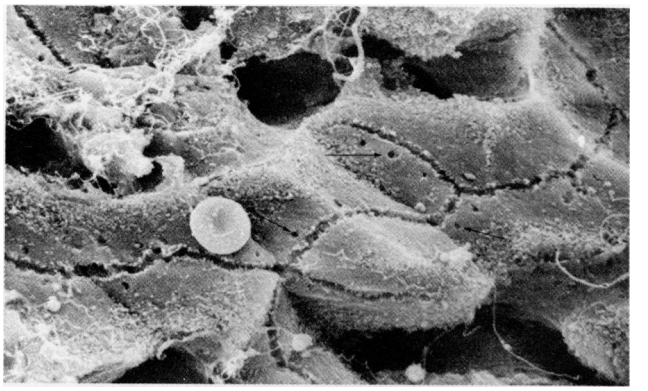

FIG. 1A. Scanning electron micrograph of lateral surface of rat hepatocyte demonstrating several invaginations on the smooth surface adjacent to the bile canaliculus (*arrows*) following an infusion of taurocholate (40 $\mu$moles for 1 hr). During the fracturing of the specimen the liver cells separate from one another at the junctional complex revealing the hepatocyte surfaces and hemi-bile canaliculi. Original magnification × 2000.

In fasted animals one hour infusions of taurocholate (40 $\mu$moles) also resulted in junctional penetration of ionic lanthanum and was observed in 29% of the junctional complexes viewed randomly (Fig. 3). Since lanthanum was not observed in the junction of the fasted control animals, it seemed likely that taurocholate either induced a change in junctional permeability to this small solute or that lanthanum was moved into the junction as a result of a net increase in paracellular fluid movement from the intercellular space into bile.

## DISCUSSION

Junctional complexes which border the bile canaliculus between hepatocytes have traditionally been regarded as "tight" and the production of bile has been thought to result from transport of solute and fluid across the canalicular membrane where osmotic equilibrium presumably occurs. However, freeze facture replicas of hepatocyte junction anatomy indicate that the number of junctional barriers or filaments is

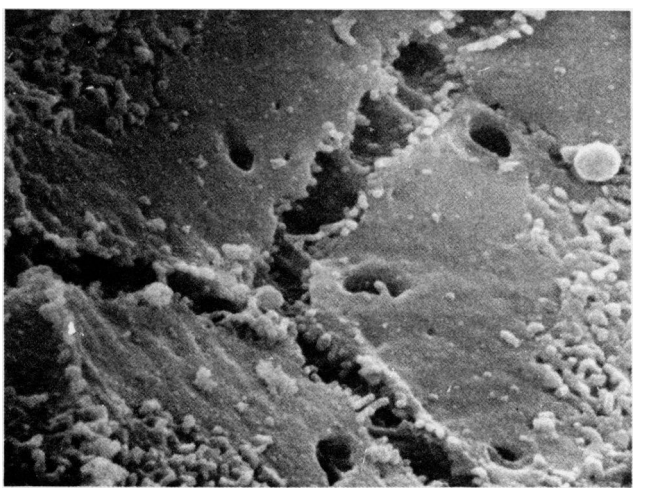

FIG. 1B. Scanning electron micrograph (original magnification × 10,000) demonstrating details of the smooth surface of the lateral cell surface membrane and several invaginations or "blisters" near the canalicular lumen.

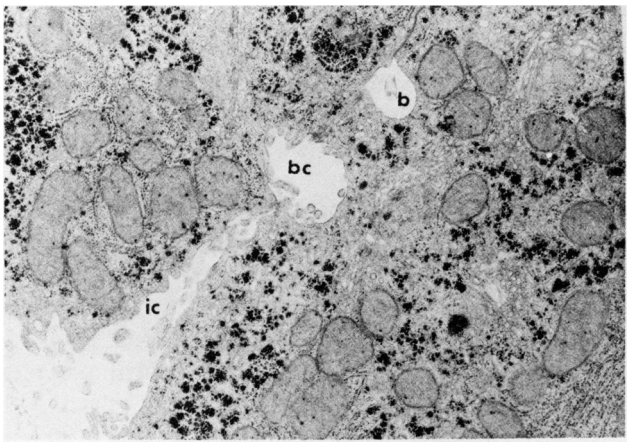


FIG. 2. Transmission electron micrograph of two adjacent hepatocytes demonstrating a bile canaliculus (bc), the intercellular space (ic) and a "blister" (b).

intermediate between classic "tight" and "leaky" epithelia [14]. Furthermore, bile is an iso-osmotic fluid which is characteristically a product of "leaky" epithelia, and the rapid equilibrium of solutes such as inulin or sucrose in bile when compared to hepatocyte water suggests that they may enter bile through paracellular pathways [15,16].

Many previous studies have attempted to assess the permeability of this barrier by injection of electron dense tracers into plasma or by retrograde injection by way of the biliary tree, and evidence sought for their passage across the junctional barrier. Interpretation of these studies has been complicated by the use of tracers such as horseradish peroxidase [17] or colloidal lanthanum [18] which have relatively large molecular radii when compared to sodium, or because of the use of retrograde injection which increases the pressure within the biliary tree in excess of the intercellular space and which may impair the integrity of the barrier. The use of

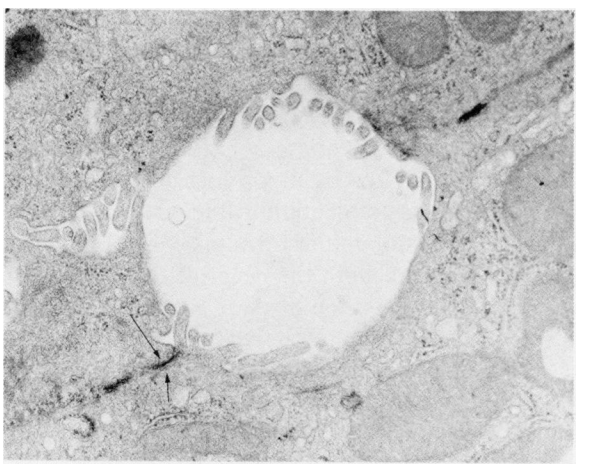

FIG. 3. Ionic lanthanum chloride localized within zonula occludens ("tight-junction") of two adjacent hepatocytes (*arrows*). Lanthanum was observed in an average of 29% of junctional complexes following a 1 hr infusion of taurocholate in 5 different studies but was not seen in fasted controls. Lanthanum is also visualized within the intercellular space to the right of the canaliculus.

intraportal infusions of ionic lanthanum chloride provides a better approach to the problem since lanthanum is similar to sodium in size when in its ionized state and localizes within the junctional complex in tissues where colloidal lanthanum does not penetrate [19,20]. Localization of ionic lanthanum also correlates well with measurements of transepithelial electrical resistance which is an accepted method to assess junctional permeability [21]. Our previous demonstration that choleretic infusions of taurodehydrocholate result in both blister formation within the intercellular space, and localization of ionic lanthanum within hepatocyte junctional complexes is consistent with the concept that these junctional barriers are permeable to salt and water, and that bile acids may alter the permeability characteristics of the junctions when they create an osmotic gradient across this junctional barrier [6]. It is unlikely that these bile acid induced changes in the junctional complex and adjacent intercellular space represent toxic changes or artifact related to dehydrocholate (DHC) or taurodehydrocholate or their hepatic metabolites since infusions of taurocholate, the primary bile acid in rat bile, at nearly physiologic rates also causes similar phenomena. However, both the number of junctions containing lanthanum and the number of intercellular "blisters" was greater following DHC infusions which is consistent with the interpretation that these changes result from osmotic effects of bile acids.

Intercellular parajunctional "blisters" have been described extensively in epithelial tissues such as the toad bladder or frog skin in response to artificially induced osmotic gradients, and it is reasonably clear that such osmotic gradients may not only move fluid and electrolytes passively across the junction but may also increase ion conductivity and decrease electrical resistance [7–11]. While such osmotic gradient induced changes in electrical properties of junctions have been demonstrated without simultaneous production of "blisters," the finding of "blisters" has been associated with a decrease in electrical resistance whenever these measurements could be made. For this reason it seems likely that the increased localization of ionic lanthanum in junctional complexes of rat hepatocytes reflects an increase in junctional permeability as well as an increase in ion flux. Since taurocholate infusions (40 $\mu$moles hr) produce intercellular "blisters" and move lanthanum into junctions, it is likely that the findings have physiologic significance and that water and electrolytes may flux across these intercellular barriers in response to a physiologic bile acid concentration gradient.

What then is the significance of these observations for our understanding of bile formation? The most compelling hypothesis is that the volume of secretion from a given hepatocyte may be controlled by modifying both the osmotic gradient within the canaliculus, and junctional permeability. Bile acids are transported into bile by mechanisms still poorly understood, and form micelles. Since the junctional barriers probably are negatively charged, both the negative charge of the bile acid monomer and the larger size of the micelles should retard movement of bile acids through the junctions and out of bile. The osmotic activity of the transported bile acids would therefore augment the movement of water and other electrolytes into bile both from the hepatocyte and from the intercellular space. Small increases in permeability of the junction might be associated with an increase in flux of water and electrolytes into bile while larger increases in permeability should have the reverse effect, since the osmotically active solutes would reflux from bile to the intercellular space and dissipate the osmotic gradient between bile and plasma. Thus factors which regulate hepatocyte paracellular permeability would not only change the rate of bile formation but could also play a critical role in the pathogenesis of bile secretory failure.

The observation that taurocholate infusions increase the permeability of the biliary tree in the Rhesus monkey to $^{3}$H-inulin in association with an increment in water flux per $\mu$mole of excreted bile acid might be explained by small changes in the permeability barriers of the junctional complex [22]. Similarly, the increase in permeability of the biliary tree to inulin or sucrose that has been observed when bile secretion is impaired by estrogens [23] or the cholestatic bile acid taurolithocholate [24] could also be explained if the paracellular pathway between hepatocytes was structurally altered. However, none of these studies has determined the precise location of the observed permeability change and much further work on the fine structure of hepatocyte junctional complexes in these experimental models will be necessary to fully understand their role in bile formation and the pathogenesis of cholestasis.

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