Immunoperoxidase Localization of Bile Salts in Rat Liver Cells

Evidence for a Role of the Golgi Apparatus in Bile Salt Transport

Y. Lamri, A. Roda,* M. Dumont, G. Feldmann, and S. Erlinger
*Institut National de la Santé et de la Recherche Médicale U-24, Hôpital Beaujon, 92118 Clichy, Laboratoire de Biologie Cellulaire, Faculté de Médecine Xavier-Bichat, 75018 Paris, France; and *Istituto di Scienze Chimiche, Facolta di Farmacia, Universita Degli Studi di Bologna, 40127 Bologna, Italy*

Abstract

The mechanisms of intracellular transport of bile acids from the sinusoidal pole to the canalicular pole of the hepatocyte are poorly understood. There is physiological and autoradiographic evidence for a vesicular pathway. The purpose of this study was to determine the localization of natural bile acids in the liver using antibodies against cholic acid conjugates and ursodeoxycholic acid. An indirect immunoperoxidase technique was used on rat liver sections fixed either with paraformaldehyde (PF) and saponin, a membrane-permeabilizing agent that allows penetration of antibodies into the cell, or with PF alone. Retention of taurocholate in the liver after tissue processing was 26±SD 15% of the bile acid initially present. When sections fixed with PF and saponin were incubated with the antibody against cholic acid conjugates, a granular cytoplasmic staining was observed by light microscopy in all hepatocytes. By electron microscopy, strong electron-dense deposits were observed mostly on vesicles of the Golgi apparatus (GA) and, sometimes, in the smooth endoplasmic reticulum (SER). After taurocholate infusion, the intensity of the reaction increased. When the liver was fixed with PF alone, almost no reaction was visible on light microscopy, but on electron microscopy the label was localized on the hepatocyte plasma membrane, mainly on the bile canalicular domain and to a lesser extent on the sinusoidal domain. With the antibody against ursodeoxycholic acid, no staining was observed in three of four livers, and a slight staining was observed in one. However, after infusion of ursodeoxycholic acid, staining of GA and SER vesicles was observed when the liver was fixed with PF and saponin. With PF alone, the reaction was intense on the canalicular membrane. These results support the view that, within the limits of the method, vesicles from the GA and possibly vesicles of the SER are involved in the intracellular transport of bile acids before canalicular secretion.

Introduction

Vectorial transport of bile acids from sinusoidal blood into bile is an important function of hepatocytes. Hepatic secretion of bile acids involves uptake by the sinusoidal plasma membrane, intracellular transport from the sinusoidal pole of the cell to the canalicular pole, and translocation into bile across the canalicular membrane (1). Uptake of conjugated bile acids by the sinusoidal plasma membrane involves a sodium-dependent process, stimulated by a sodium gradient (Na^+ outside > Na^+ inside) (2–7). The carrier proteins responsible for this process have been identified by photoaffinity labeling techniques (8–11) and partially purified (12). Canalicular secretion is thought to be a sodium-independent process that is probably energized, at least in part, by the membrane potential (6, 13). Putative canalicular carrier proteins have also been identified by photoaffinity labeling (11, 14).

Whereas the sinusoidal and canalicular steps of the transport process are partially understood, little is known about intracellular transfer from the sinusoidal to the canalicular membranes. Bile acid–binding proteins have been identified in the cytosolic fraction of liver cells (15–17), but their role in transport has not been established. Autoradiographic studies of the localization of the bile acid analogues ^{125}I-cholylglycyltyrosine and ^{125}I-cholylglycylhistamine have shown a preferential distribution of grains over the Golgi apparatus (GA)[1] (18, 19); grains were also associated with the smooth endoplasmic reticulum (SER). These observations have led to the hypothesis that bile acids may move from the sinusoidal plasma membrane to bile via a vesicular pathway that includes the SER and the GA.

In an effort to determine the localization of natural bile acids in the liver, we have used an indirect immunoperoxidase technique with an antibody against conjugated cholic acid. We have attempted to localize cholic acid conjugates in the liver of rats in the basal state and in the liver of rats infused intravenously with sodium taurocholate. We have also used an antibody against ursodeoxycholic acid in normal rats (which have no or minimal amounts of this bile acid) and in rats loaded with ursodeoxycholate (UDCA) by an intravenous infusion. The results show a staining of vesicular structures, mostly of the GA and the SER after incubation with the antibodies against conjugated cholic acid. They support the view of a participation of a vesicular system to the transport of bile acids by the hepatocyte.

Methods

Animals. Adult male Sprague-Dawley rats weighing between 200 and 250 g (Charles River Breeding Laboratories, Saint-Aubin-les-Elbeuf, France) and fed ad lib. were used. For the localization of cholic acid conjugates, two groups of animals were investigated: (*a*) 12 normal

Address reprint requests to Dr. S. Erlinger, INSERM U-24, Hôpital Beaujon, 92118 Clichy Cedex, France.

Received for publication 9 July 1987 and in revised form 8 June 1988.

J. Clin. Invest.

0021-9738/88/10/1173/10 $2.00
Volume 82, October 1988, 1173–1182

1. *Abbreviations used in this paper:* CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GA, Golgi apparatus; PF, paraformaldehyde; TC, taurocholate; SER, smooth endoplasmic reticulum; UDCA, ursodeoxycholate.

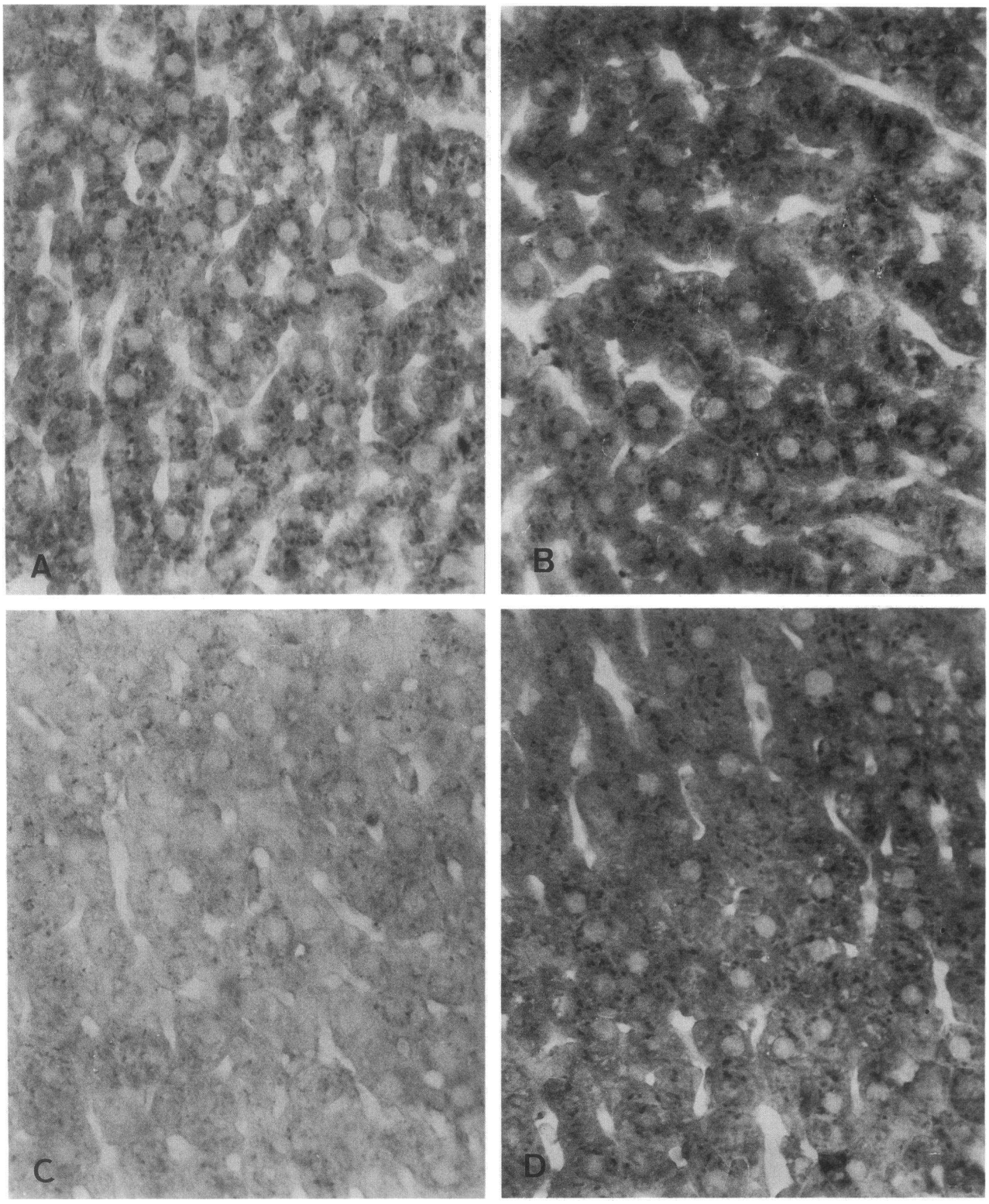


Figure 1. Light microscopy. Liver fixed by PF in the presence of saponin. *A* and *B*, antibodies against conjugated cholic acid. *C* and *D*, antibodies against ursodeoxycholic acid. (*A*) Normal rat, without bile acid infusion. The reaction indicating the presence of the conjugated cholic acid is expressed by dark granules located in the cytoplasm of every hepatocyte. (*B*) Rat infused with TC. The intensity of the reaction in hepatocytes is increased. (*C*) Normal rat, without bile acid infusion. A small number of dark granules of weak intensity is visible in the cytoplasm of some hepatocytes. (*D*) Rat infused with ursodeoxycholic acid. Dark granules are visible in the cytoplasm of every hepatocyte. ×500, *A–D*.

Table I. Experimental Groups

Group	No. of animals	Antibody used	Bile acid infusion
1	12	Conjugated cholic acid	No
2	6	Conjugated cholic acid	TC
3	4	Ursodeoxycholic acid	No
4	4	Ursodeoxycholic acid	UDCA

rats; and (*b*) 6 rats infused intravenously for 60 min with a solution of taurocholate (TC) (Sigma Chemical Co., St. Louis, MO) at a rate of 1,200 nmol/min per 100 g body wt; TC was dissolved in 0.15 M NaCl at a concentration of 125 mmol/liter and infused at a rate of 20 μl/min into a jugular vein.

Two groups of rats were investigated for the localization of UDCA: (*a*) six normal rats; and (*b*) four rats infused intravenously for 60 min with a solution of UDCA (courtesy of Roussel Uclaf, Romainville, France) at a rate of 1,200 nmol/min per 100 g body wt.; UDCA was dissolved in 0.15 M $NaCl/Na_2CO_3$, vol/vol at a concentration of 76 mmol/liter and infused at a rate of 37.5 μl/min into a jugular vein. When needed, the jugular vein was cannulated and infusions of bile acids were given through a No. 1 catheter (i.d. 0.3 mm, Biotrol, Paris, France). The experimental groups are indicated in Table I.

Preparation and specificity of antisera against bile salts. Two antisera, one against conjugated cholic acid and the other against UDCA were used. These antisera were prepared by immunizing rabbits with the bile salt linked to BSA according to a technique previously described (20). Anti-BSA antibodies were removed by incubation with aqueous BSA.

The absence of cross-reactivity between both antisera and BSA was checked by immunoelectrophoresis.

The antisera specificity was assessed by RIA as previously reported (21). Tritium-labeled glycocholic acid (5 Ci/mmol sp act) and 22-23-[^{3}H]ursodeoxycholic acid (37 Ci/mmol sp act) were used. Standard solutions with amounts ranging from 1 to 500 pmol for each bile acid were prepared and an appropriate dilution of the antiserum was cross-reacted with its specific bile acid and with other bile acids. The assay was carried out in 0.1 M phosphate buffer, pH = 7.2, at 4°C for 12 h. The bound fraction was separated by $(NH_4)_2SO_4$ precipitation and the radioactivity in the free fraction was measured by liquid scintillation counting. Results for the antiserum against cholic acid conjugates have been published elsewhere (21). For the antiserum against UDCA, cross-reactivity, expressed as percent cross-reaction at 50% of antigen-antibody bound, was 100% for unconjugated UDCA, glyco-UDCA, and tauro-UDCA, 2% for chenodeoxycholic acid (CDCA), 1% for glyco-CDCA, 0.5% for tauro-CDCA, 0.2% for deoxycholic acid (DCA), 0.2% for glyco-DCA and tauro-DCA, 0% for cholic, taurocholic, and glycocholic acid, and 7% for β-muricholic acid.

Table II. Retention of [^{14}C]Taurocholate in Liver after Tissue Processing

Exp. no.	Fixation	Retention after perfusion fixation*	Retention after tissue processing*
		%	
1	PF	93	22
2	PF	89	23
3	PF	81	39
4	PF + S	76	48
5	PF + S	47	16
6	PF + S	36	8
Mean		70±21	26±15

* Expressed as percent of the liver content before perfusion. Mean±SD. PF, paraformaldehyde. S, saponin.

Liver fixation. Rat livers were perfused with a 4% paraformaldehyde (PF) solution buffered with 0.1 M sodium phosphate buffer, pH 7.4, through the portal vein for 5 min at a flow rate of 10 ml/min per 100 g body wt according to a technique described by Pignal et al. (22). 1-mm-thick slices were made randomly in the fixed liver and immersed in the same fixative for 4 h. After being washed for 24 h in a 0.1 M sodium phosphate buffer, pH 7.4, the slices were placed overnight in a 10% glycerol solution prepared with the same buffer. Slices were then frozen in isopentane prechilled with liquid nitrogen.

Immunohistochemical procedures. 8-μm-thick cryostat sections were incubated in a 1:50 solution of the specific antiserum for 2 h at room temperature. After being washed with PBS, the sections were incubated with sheep peroxidase labeled anti-rabbit immunoglobulins (Institut Pasteur Production, Paris, France). After three washings with PBS, peroxidase activity was revealed by a specific histochemical reaction (23). When indicated, saponin (Sigma Chemical Co.), a membrane-permeabilizing agent, at a concentration of 0.1%, was added to the fixative, the washing solutions, and the first antiserum.

Some sections were observed by light microscopy. Other sections were postfixed for 30 min in 1% osmium tetroxide solution, buffered

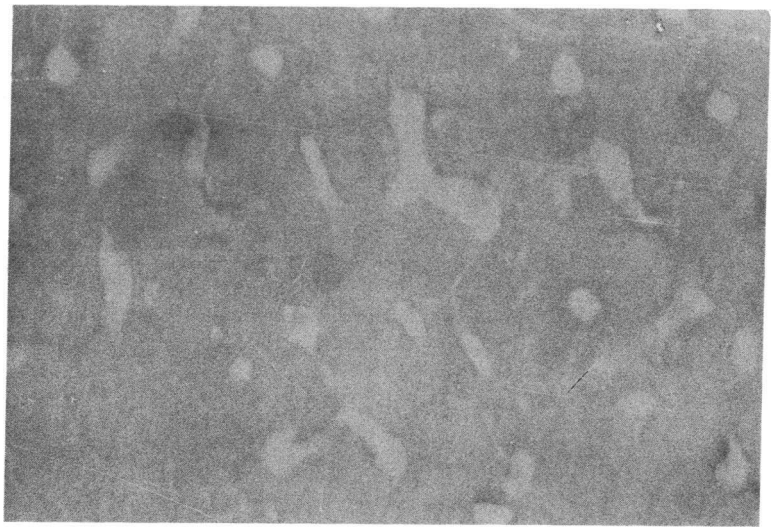
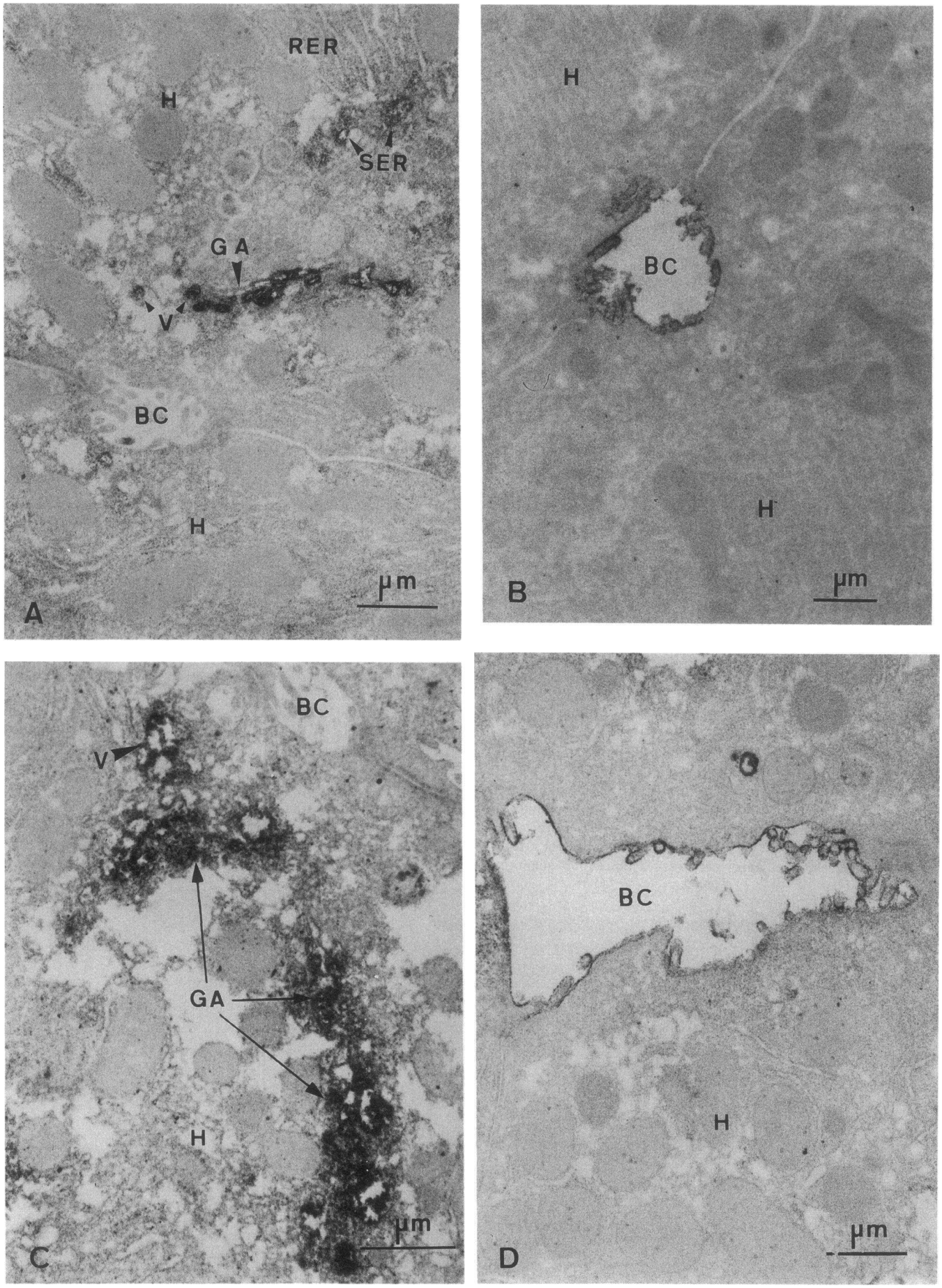


Figure 2. Light microscopy. Control reaction after saturation of the antibody against conjugated cholic acid with TC. No reaction is visible in the hepatocytes. ×600.


RER
H
SER
GA
V
BC
H
μm
A
H
BC
H
μm
B
BC
V
GA
H
μm
C
BC
H
μm
D

with veronal buffer pH 7.2, dehydrated in graded ethanol solutions, and embedded in epoxy resin. Ultrathin sections were made and observed with a Siemens Elmiskop IA electron microscope without further staining.

Three control reactions were made: (*a*) incubation of the sections with the peroxidase-labeled antiserum only; (*b*) immersion of the sections in the medium specific of the peroxidase activity (22); and (*c*) incubation of the sections with the antibody against conjugated cholic acid that had first been saturated with TC in vitro.

To determine the loss of bile acids from the liver during fixation and tissue processing, in six experiments, 10 μmol/100 g body wt TC with 4 μCi of [^{14}C]TC (New England Nuclear, Boston, MA) were injected into the jugular vein. 45 s later, a liver biopsy specimen was taken to measure initial radioactivity, before perfusion fixation. Perfusion of PF (or PF + saponin) was then started and another biopsy specimen was taken at the end of the perfusion to measure radioactivity after perfusion fixation. The specimen was then processed as indicated above, and all processing fluids were collected and counted to determine the loss of radioactive bile acids during processing. The liver specimens were weighed and digested in 1 ml soluene 350 (Packard Instruments Co., Inc., Downers Grove, IL); 10 ml of liquid scintillation fluid was added and the specimen was counted in a liquid scintillation spectrometer. Aliquots of the processing fluids were counted in the same way.

Results

The retention of bile acids after injection of labeled TC is indicated in Table II. On the average, the retention after perfusion fixation was 70%, and the retention after tissue processing was 26%.

Light microscopy

Group 1. In normal rat livers fixed by PF and when saponin was added, incubation with the antibody against conjugated cholic acid resulted in staining of dark-brown granules located in the cytoplasm of every hepatocyte, throughout the hepatic lobule (Fig. 1 *A*). No obvious difference in the intensity of the reaction was observed between the different zones of the lobule. In each hepatocyte, a variable number of granules was visible and their volume differed slightly from cell to cell. Granules were located either at the periphery of the hepatocyte and mostly near the bile canaliculus, or randomly distributed in the cytoplasm of the cell. No reaction was observed in the nonparenchymal cells.

In rat livers fixed by PF without saponin, granules were not visible. A slight brownish reaction was only visible on the sinusoidal plasma membrane of hepatocytes.

Group 2. In rats infused with TC and when the liver was fixed in presence of saponin, the intensity of the reaction observed in the hepatocytes increased, but the cytoplasmic granule distribution was similar to that observed in the normal state (Fig. 1 *B*). Again, there was no difference between the zones of the hepatic lobule. In rat livers fixed by PF alone, as in the normal animals, cytoplasmic granules were not visible.

Group 3. In three of the four rats, no staining was observed. In one of the four rats, when the liver was fixed with PF and saponin, the staining after incubation with the antibody against UDCA was visible only in < 1% of hepatocytes randomly distributed in the hepatic lobule. The reaction was expressed by a limited number of dark-brown cytoplasmic granules of weak intensity (Fig. 1 *C*). No reaction was visible in the other hepatic cells.

In rat livers fixed by PF alone, no reaction was observed.

Group 4. In rats infused with UDCA and when the liver was fixed with PF and saponin, granules were observed in the cytoplasm of most hepatocytes (Fig. 1 *D*). In rat livers fixed with PF alone, granules were not detected in the hepatocytes.

Control reactions were negative in all groups of rats. In particular, almost no reaction was observed after incubation with the antibody, which had first been saturated with TC (Fig. 2).

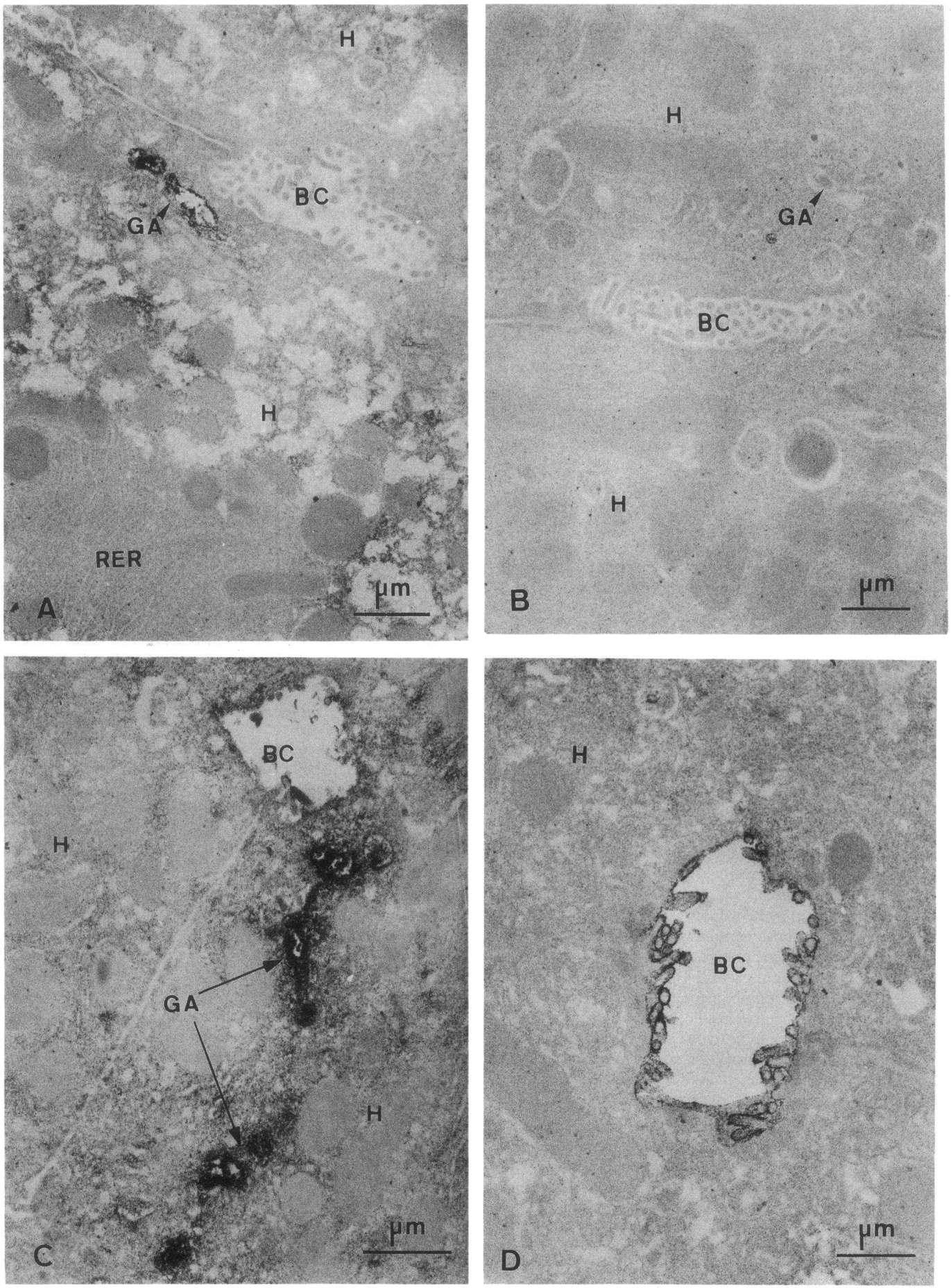
Electron microscopy

Group 1. When the liver was fixed by PF and saponin, the reaction with anti–conjugated cholic acid antibody was expressed as electron-dense deposits, mainly located in the GA and sometimes in the SER. In the GA, the labeling of the saccules was homogeneous in some organelles; in other organelles, *trans* and medial saccules presented a strong labeling, with no labeling visible in the *cis* saccule (Fig. 3 *A*). Most often, electron-dense deposits were present on the membranes of the saccules; however, some deposits were also visible in the lumina of the dilated parts of the Golgi saccules. Vesicles around the saccules and between the *trans* saccule and the plasma membrane of the bile canaliculus were sometimes labeled. Deposits were also visible on some vesicles belonging to the SER (Fig. 3 *A*). No reaction was detected on the membranes and in the cisternae of the rough endoplasmic reticulum. The other hepatocyte organelles were consistently negative. No deposit was observed in nonparenchymal hepatic cells.

In rat livers fixed by PF alone, a strikingly different pattern was observed. The reaction was located on the plasma membrane and almost no reaction was visible in the cytoplasm of hepatocytes. Staining was mainly present on the bile canalicular membrane, and rather homogeneously distributed on the microvilli of the bile canaliculus (Fig. 3 *B*). There were also some electron-dense deposits on the hepatocyte sinusoidal membrane but the distribution was irregular, some microvilli being devoid of labeling. The lateral plasma membrane was not labeled. The GA was never positive. In a small number of hepatocytes, some cytoplasmic vesicles were labeled.

Group 2. In rats infused with TC and when the liver was fixed by PF and saponin, an intense reaction was observed in the GA and the SER of the hepatocytes. The reaction was more strongly positive than in group 1 rats, which had not been infused with TC. The GA was more voluminous than in normal rats. Numerous labeled vesicles were observed around

Figure 3. Electron microscopy. *A* and *C*, Liver fixed with PF in the presence of saponin. *B* and *D*, liver fixed with PF alone. Antibodies against conjugated cholic acid. (*A*) Normal rat, without bile acid infusion. The reaction indicating the presence of conjugated cholic acid is expressed as electron-dense deposits located mainly in the GA at the level of the medial and *trans* saccules. A positive reaction is also visible in some vesicles (V) of the GA and the SER. No reaction is present on the rough endoplasmic reticulum (RER). (*B*) Normal rat, without bile acid infusion. The electron-dense reaction is limited to the membrane of the bile canaliculus. No reaction is visible in the hepatocyte. (*C*) Rat infused with TC. A voluminous GA with numerous labeled vesicles (V) is visible. (*D*) Rat infused with TC. Dilated bile canaliculus with a labeling of its membrane. BC, bile canaliculus; H, hepatocyte. ×15,000, *A;* ×12,000, *B;* ×18,000, *C;* ×15,000, *D*.


H
BC
GA
H
RER
A
μm
H
GA
BC
H
B
μm
BC
H
GA
H
C
μm
H
BC
D
μm

the Golgi saccules. The labeling was also more intense, homogeneous, and visible on every GA saccule (Fig. 3 *C*). In the GA and the SER, staining was mainly present on the membranes of these organelles but sometimes visible inside their lumina. The other hepatocyte organelles were not labeled. A dilatation of the bile canaliculi was visible but their plasma membrane was not labeled.

In rats fixed by PF alone, the plasma membrane was labeled particularly on the bile canaliculi, which were dilated (Fig. 3 *D*); the microvilli of the bile canaliculi were almost absent. On the remaining microvilli the labeling was more intense than that observed in group 1 rats. Sinusoidal plasma membrane labeling was also visible. In the cytoplasm there was no labeling except for a few vesicles.

Group 3. When the liver was fixed by PF and saponin, the reaction with anti-ursodeoxycholic acid antibody was visible only in the GA of some hepatocytes from one animal out of the four examined (Fig. 4 *A*). Some SER vesicles were also labeled. The hepatocytes of the three other animals were consistently negative. In rat livers fixed by PF alone, no staining was observed in the cytoplasm of hepatocytes (Fig. 4 *B*). No reaction was visible on the membrane of the bile canaliculus.

Group 4. In rat livers fixed with PF and saponin, the bile canalicular plasma membrane was generally negative. A positive reaction was observed in the GA (Fig. 4 *C*) and in some vesicles belonging to the SER.

In rat livers fixed with PF alone, the labeling of the bile canalicular membrane was intense (Fig. 4 *D*). Bile canaliculi also were dilated. No labeling was observed in the cytoplasm of the hepatocytes.

Control reactions were negative in all groups of rats.

Discussion

In this work, two different methods for liver fixation were used; (*a*) perfusing the liver with PF alone; and (*b*) using saponin and PF. Saponin is a membrane-permeabilizing agent widely used in immunoenzymatic techniques (24). When PF was used alone, almost no intracellular labeling was recognized in the hepatocytes, whereas the plasma membrane was strongly labeled, particularly the canalicular membrane. This was probably due to a lack of penetration of antibodies into the hepatocytes: in the absence of plasma membrane permeabilization, it is well known that peroxidase-labeled antibodies cannot penetrate the cells easily (24, 25). The labeling of the plasma membrane may possibly be explained by a localization of conjugated cholic acid on the external face of the membrane during the steps of sinusoidal uptake and canalicular secretion. When saponin was added to the fixative, labeling of the plasma membrane was no longer apparent, possibly because of changes in the structure of the membrane; saponin is a cholesterol-dissolving agent (26) and produces holes in the plasma membrane (27). Such changes may modify bile acid localization or recognition by the antibodies. It should be pointed out, however, that after the use of saponin, intracellular membranes remain morphologically and functionally intact (28). The loss of bile acids during the procedure was, on the average, 75% (Table II). This is of the same order of magnitude as that reported by Suchy et al. (18). It may be speculated that the bile acids lost during processing were mostly those not associated with intracellular organelles. This raises the possibility that the conclusions of the study may only apply to the fraction of bile acids retained in the liver during the procedure.

In the experiments with the antibody against conjugated cholic acid, light microscopy showed staining in the cytoplasm of all hepatocytes throughout the lobule; the intensity of the staining clearly increased when the animals had previously been loaded with TC by an intravenous infusion, and the reaction disappeared almost completely when an antibody first saturated with TC was used. These observations strongly suggest that the staining was due to recognition of conjugated cholic acid itself by the antibody and not artifactual. No lobular gradient in the staining was observed, in contrast to previous studies using labeled bile acids or bile acid analogues and autoradiography (29,30). However, such a lobular gradient is only observed with tracer doses of the labeled bile acid, and the gradient disappears when a relatively high dose (8 μmol) is injected (30). Therefore, in our experimental conditions, i.e., with a high infusion rate (1,200 nmol/min per 100 g body wt), one would expect a homogeneous distribution of taurocholate throughout the lobule. No staining was observed in the liver of normal rats incubated with the antibody against ursodeoxycholic acid, except for a weak staining of a small number of Golgi vesicles in one animal. This, again, is expected because normal rats synthesize no or minimal amounts of ursodeoxycholic acid (31). The weak staining in one animal may be explained either by small quantities of ursodeoxycholic acid in this animal, or by cross-reactivity with rat bile acids, in particular, muricholate.

By electron microscopy, the intracellular staining was clearly localized in the Golgi apparatus and, to a lesser extent, the smooth endoplasmic reticulum. Again, the labeling was more intense in animals infused with taurocholate. In these animals, the Golgi apparatus appeared dilatated. Note that an increase in the amount of Golgi-rich area and Golgi membranes has previously been reported after TC infusion (32, 33). These observations strongly suggest that one step of the intracellular transport of TC involves an interaction with the vesicles of the GA and of the endoplasmic reticulum. The hypothesis of the participation of vesicles in the intracellular transport of bile acids is consistent with several other previous observations. Firstly, the autoradiographic studies of Suchy and associates (18), using ^{125}I-cholylglycyltyrosine, have shown a predominant labeling of the plasma membrane and the SER 30 s after portal injection, and a sevenfold increase in the labeling of the GA 300 s after injection. In the same experiments, labeling of the pericanalicular area was also noted 300 s after injection. Secondly, subcellular distribution studies have

Figure 4. Electron microscopy. *A* and *C*, liver fixed with PF in the presence of saponin. *B* and *D*, liver fixed with PF alone. Antibodies against ursodeoxycholic acid. (*A*) Normal rat, without bile acid infusion. In a small number of hepatocytes of one animal, a weak electron-dense reaction is visible on the GA. No reaction is visible on the rough endoplasmic reticulum (RER). (*B*) Normal rat, without bile acid infusion. No reaction is visible on the membrane of the bile canaliculus and in the hepatocytes. (*C*) Rat infused with ursodeoxycholic acid. A strong reaction is visible in the GA. (*D*) Rat infused with ursodeoxycholic acid. Dilated bile canaliculus with a labeling of its membrane. BC, bile canaliculus; H, hepatocyte. ×13,000, *A;* ×14,500, *B;* ×17,000, *C;* ×15,000, *D*.

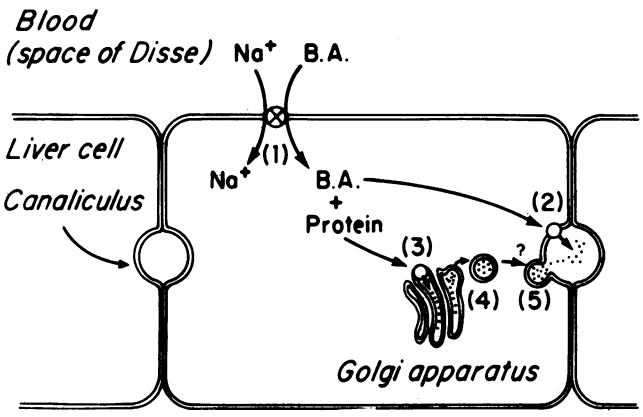


Figure 5. Proposed hypothesis for transport of bile acids by the hepatocyte. After uptake by the sinusoidal membrane (1), the bile acid may be bound to protein, transported through the cytoplasm to the canalicular carrier (2), and secreted into bile by carrier-mediated transport. Alternatively, it may be transported into the GA (3) and transferred to the bile canaliculus in Golgi-derived vesicles (4). Secretion into the canalicular lumen would occur by exocytosis (5). B.A., bile acid.

shown high-affinity binding sites for bile acids on the GA (34). Furthermore, an uptake mechanism for TC, distinct from that of the sinusoidal plasma membrane, has been identified in the Golgi and smooth microsomal fractions of the hepatocyte (35), a finding consistent with a role of smooth vesicles and of the Golgi apparatus in taurocholate transport. This system has been postulated to translocate TC into the lumen of the GA (35). Thirdly, colchicine, which interferes with microtubule-dependent vesicular transport processes, has been shown to inhibit bile acid secretion in the rat after a bile acid load (36–39) and, to a lesser extent, bile acid uptake by isolated rat hepatocytes (40). This effect is observed when a load of bile acid is given, and not when a tracer dose is given (41). This suggests that a tracer dose can be secreted via a colchicine-insensitive mechanism. Fourthly, as indicated above, infusions of bile acids were associated with an increase in Golgi-rich area and vesicles in the pericanalicular area (33, 42). In our experiments, we observed a marked dilatation of some Golgi vesicles during infusions of bile acids and choleresis. In time-lapse movies of isolated hepatocytes, an extensive traffic, especially between the Golgi region and the bile canaliculus, was observed (43); this activity became more intense in the presence of bile acids (44). Staining of *trans* Golgi vesicles was more intense than that of other parts of the GA. It is now well established that the *trans* Golgi network plays an essential role in vesicular secretory processes (45).

When antibodies against ursodeoxycholic acid were used in animals infused with this bile acid, in livers fixed with paraformaldehyde and saponin, vesicles of the SER and the GA were stained. However, the staining was less intense than that obtained with antibodies against cholic acid conjugates in animals infused with TC. In livers fixed without saponin, an intense labeling of the canalicular membrane was observed.

It may be speculated that the staining of the membrane could correspond to binding of the bile acid to the canalicular carrier responsible for excretion into the bile canaliculi. By autoradiography, grains were also observed in the canalicular area and over the canalicular membrane (18). Note that some staining of the sinusoidal membrane was also observed: this could possibly correspond to binding of the bile acid to the sinusoidal carrier. Such staining of the plasma membrane was observed mostly in animals infused with bile acids (TC or UDCA); in these animals, the concentration of the bile acid within the hepatocyte plasma membrane was presumably higher than in animals not receiving exogenous bile acids.

One cannot completely exclude a redistribution of bile acids during fixation and tissue processing. If this were the case, the specific redistribution of TC on the GA would directly confirm the presence of high-affinity binding sites for TC on the GA, as demonstrated by Simion et al. (34).

The participation of vesicles of the Golgi complex in bile acid transport by the hepatocyte does not exclude nonvesicular mechanisms, such as binding to cytosolic proteins. The absence of any labeling of the cytoplasm in our experiments does not rule out this possibility. The absence of labeling could be due to a concentration of bile acids in the cytosol too low to be detected. It is possible that small amounts of bile acids, for example bile acids present as monomolecular species, may be transported in the cell cytoplasm and secreted into bile by the canalicular carrier (14). However, a model including vesicular transport, as proposed by Simion et al. (35) and Goldsmith et al. (19), would be most compatible with many observations, in particular with those of this study. According to this model, taurocholate would first be taken up by a sodium-dependent, secondary active process; it could then either be bound to cytosolic proteins and/or directly taken up by Golgi-associated vesicles; the bile acid would be transferred into the lumen of the Golgi vesicles by a specific transport system; the vesicles would then move to the canalicular area and bile acids would be secreted into bile, possibly by exocytosis (Fig. 5). It should be pointed out that exocytosis, at this stage, is under speculation: we have not observed any morphological picture indicating that such a process was indeed operative. Another possibility could be incorporation into the Golgi membrane itself. Carey and Cohen (46) have recently suggested that bile salt/lipid vesicles could be formed under certain experimental conditions. Taurocholate, in this case, could be transported by membrane/membrane interaction, or again, by exocytosis after membrane fusion.

The speed of vesicular transport of a substrate that needs no biotransformation by the liver cell after being taken up may be fast enough to account for the kinetics of biliary secretion of taurocholate. It is known that substrates may move within cells by vesicular transport at a rate of up to 5 μms^{-1}, as demonstrated in neurons (47); this fast transport is microtubule dependent and may be related to a microtubule-associated ATPase named kinesin (48), which is present in liver cells (49). At rates of this order of magnitude, taurocholate could move from the sinusoidal pole to the canalicular pole of the hepatocyte (a distance of 10–20 μm) in less than a minute. Its time of appearance in common duct bile (including transit time in the bile ducts) is of the order of 65 s (50). A vesicular transport of this kind thus may be faster and more efficient than random diffusion of a protein-bound substrate throughout the hepatocyte to direct bile acids from the sinusoidal pole to the canalicular pole of the hepatocyte.

Acknowledgments

The authors thank Roger Vranckx for his help in the immunoelectrophoresis studies.

References

1. Erlinger, S. 1987. Physiology of bile secretion and enterohepatic circulation. *In* Physiology of the Gastrointestinal Tract. 2nd ed. L. R. Johnson, editor. Raven Press, New York. 1557–1580.

2. Scharschmidt, B. F., and J. E. Stephens. 1981. Transport of sodium, chloride, and taurocholate by cultured rat hepatocytes. *Proc. Natl. Acad. Sci. USA.* 78:986–990.

3. Inoue, M., R. Kinne, T. Tran, and I. M. Arias. 1982. Taurocholate transport by rat liver sinusoidal membrane vesicles: evidence of sodium cotransport. *Hepatology (Baltimore).* 2:572–579.

4. Ruifrok, P. G., and D. K. F. Meijer. 1982. Sodium ion-coupled uptake of taurocholate by rat-liver plasma membrane vesicles. *Liver.* 2:28–34.

5. Duffy, M. C., B. L. Blitzer, and J. L. Boyer. 1983. Direct determination of the driving forces for taurocholate uptake into rat liver plasma membrane vesicles. *J. Clin. Invest.* 72:1470–1481.

6. Meier, P. J., A. S. Meier-Abt, C. Barrett, and J. L. Boyer. 1984. Mechanisms of taurocholate transport in canalicular and basolateral rat liver plasma membrane vesicles. *J. Biol. Chem.* 259:10614–10622.

7. Suchy, F. J., J. C. Bucuvalas, A. L. Goodrich, M. S. Moyer, and B. L. Blitzer. 1986. Taurocholate transport and Na^+-K^+-ATPase activity in fetal and neonatal rat liver plasma membrane vesicles. *Am. J. Physiol.* 251:G665–G673.

8. Kramer, W., U. Bickel, H. P. Busher, W. Gerok, and G. Kurz. 1982. Bile-salt-binding polypeptides in plasma membranes of hepatocytes revealed by photoaffinity labelling. *Eur. J. Biochem.* 129:13–24.

9. Von Dippe, P., and D. Levy. 1983. Characterization of the bile acid transport system in normal and transformed hepatocytes. *J. Biol. Chem.* 258:8896–8901.

10. Wieland, T., M. Nassal, W. Kramer, G. Fricker, U. Bickel, and G. Kurz. 1984. Identity of hepatic membrane transport systems for bile salts, phalloidin, and antamanide by photoaffinity labeling. *Proc. Natl. Acad. Sci. USA.* 81:5232–5236.

11. Fricker, G., S. Schneider, W. Gerok, and G. Kurz. 1987. Identification of different transport systems for bile salts in sinusoidal and canalicular membranes of hepatocytes. *Biol. Chem. Hoppe-Seyler.* 368:1143–1150.

12. Von Dippe, P., M. Ananthanaryanan, P. Drain, and D. Levy. 1986. Purification and reconstitution of the bile acid transport system from hepatocyte sinusoidal membrane. *Biochim. Biophys. Acta.* 862:352–360.

13. Inoue, M., R. Kinne, T. Tran, and I. M. Arias. 1984. Taurocholate transport by rat liver canalicular membrane vesicles. Evidence for the presence of an Na^+-independent transport system. *J. Clin. Invest.* 73:659–663.

14. Ruetz, S., G. Fricker, G. Hugentobler, K. Winterhalter, G. Kurz, and P. J. Meier. 1987. Isolation and characterization of the putative canalicular bile salt transport system of rat liver. *J. Biol. Chem.* 262:11324–11330.

15. Sugiyama, Y., T. Yamada, and N. Kaplowitz. 1983. Newly identified bile acid binders in rat liver cytosol. Purification and comparison with glutathione S-transferases. *J. Biol. Chem.* 258:3602–3607.

16. Stolz, A., Y. Sugiyama, J. Kuhlenkamp, and N. Kaplowitz. 1984. Identification and purification of a 36 kDa bile acid binder in human hepatic cytosol. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 177:31–35.

17. Stolz, A., Y. Sugiyama, J. Kuhlenkamp, B. Osadchey, T. Yamada, W. Belknap, W. Balistreri, and N. Kaplowitz. 1986. Cytosolic bile acid binding protein in rat liver: radioimmunoassay, molecular forms, developmental characteristics and organ distribution. *Hepatology (Baltimore).* 6:433–439.

18. Suchy, F. J., W. F. Balistreri, J. Hung, P. Miller, and S. A. Garfield. 1983. Intracellular bile acid transport in rat liver as visualized by electron microscope autoradiography using a bile acid analogue. *Am. J. Physiol.* 245:G681–G689.

19. Goldsmith, M. A., S. Huling, and A. L. Jones. 1983. Hepatic handling of bile salts and protein during intrahepatic cholestasis. *Gastroenterology.* 84:978–986.

20. Roda, A., and G. F. Bolelli. 1980. Production of a high-titer antibody to bile acids. *J. Steroid Biochem.* 13:449–454.

21. Roda, A., E. Roda, R. Aldini, D. Festi, G. Mazzella, C. Sama, and L. Barbara. 1977. Development, validation and application of a single-tube radioimmunoassay for cholic and chenodeoxycholic conjugated bile acids in human serum. *Clin. Chem.* 23:2107–2113.

22. Pignal, F., M. Maurice, and G. Feldmann. 1982. Immunoperoxidase localization of albumin and fibrinogen in rat liver fixed by perfusion or immersion: Effect of saponin on the intracellular penetration of labeled antibodies. *J. Histochem. Cytochem.* 30:1004–1014.

23. Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291–301.

24. Feldmann, G., M. Maurice, D. Bernuau, E. Rogier, and A. M. Durand. 1983. Penetration of enzyme-labelled antibodies into tissues and cells: a review of the difficulties. *In* Immunoenzymatic Techniques. S. Avrameas, P. Druet, R. Masseyeff, and G. Feldmann, editors. Elsevier Science Publishers B.V., Amsterdam. 3–15.

25. Sternberger, L. A. 1979. Immunocytochemistry. 2nd ed. John Wiley & Sons, New York. 99.

26. Ohtsuki, I., R. M. Manzi, G. E. Palade, and J. D. Jamieson. 1978. Entry of macromolecular tracers into cells fixed with low concentrations of aldehydes. *Biol. Cell.* 31:119–126.

27. Seeman, P. 1967. Transient holes in the erythrocyte membrane during hypotonic hemolysis and stable holes in the membrane after lysis by saponin and lysolecithin. *J. Cell Biol.* 32:55–70.

28. Burgess, G. M., J. S. McKinney, A. Fabiato, B. A. Leslie, and J. W. Putney, Jr. 1983. Calcium pools in saponin-permeabilized guinea pig hepatocytes. *J. Biol. Chem.* 258:15336–15345.

29. Jones, A. L., G. T. Hradeck, R. H. Renston, K. Y. Wong, G. Karlaganis, and G. Paumgartner. 1980. Autoradiographic evidence for hepatic lobular gradient of bile acid derivative. *Am. J. Physiol.* 238:G233–G237.

30. Groothuis, G. M. M., M. J. Hardonk, K. P. T. Keulemans, P. Nieuwenhuis, and D. K. F. Meijer. 1982. Autoradiographic and kinetic demonstration of acinar heterogeneity in taurocholate transport. *Am. J. Physiol.* 243:G455–G462.

31. Haslewood, G. A. D. 1967. Bile Salts. Methuen, Inc., London. 99.

32. Jones, A. L., R. H. Renston, D. L. Schmucker, J. S. Mooney, R. K. Ockner, and D. Adler. 1979. A morphologic evaluation of the pericanalicular cytoplasm in the rat hepatocyte: demonstrating possible components of the bile secretory apparatus. *In* The Liver. Quantitative Aspects of Structure and Function. R. Preisig and J. Bircher, editors. Editio Cantor, Aulendorf, FRG. 63–70.

33. Jones, A. L., D. L. Schmucker, J. S. Mooney, R. K. Ockner, and R. D. Adler. 1979. Alterations in hepatic pericanalicular cytoplasm during enhanced bile secretory activity. *Lab. Invest.* 40:512–517.

34. Simion, F. A., B. Fleischer, and S. Fleischer. 1984. Subcellular distribution of bile acids, bile salts, and taurocholate binding sites in rat liver. *Biochemistry.* 22:6459–6466.

35. Simion, F. A., B. Fleischer, and S. Fleischer. 1984. Two distinct mechanisms for taurocholate uptake in subcellular fractions from rat liver. *J. Biol. Chem.* 259:10814–10822.

36. Gregory, D. H., Z. R. Vlahcevic, M. F. Prugh, and L. Swell. 1978. Mechanism of secretion of biliary lipids: role of a microtubular system in hepatocellular transport of biliary lipids in the rat. *Gastroenterology.* 74:93–100.

37. Dubin, M., M. Maurice, G. Feldmann, and S. Erlinger. 1980. Influence of colchicine and phalloidin on bile secretion and hepatic ultrastructure in the rat. Possible interaction between microtubules and microfilaments. *Gastroenterology.* 79:646–654.

38. Barnwell, S. G., P. J. Lowe, and R. Coleman. 1984. The effects of colchicine on secretion into bile of bile salts, phospholipids, choles-

terol and plasma membrane enzymes: bile salts are secreted unaccompanied by phospholipids and cholesterol. *Biochem. J.* 220:723–731.

39. Crawford, J. M., A. B. Clifford, and J. L. Gollan. 1988. Role of the hepatocyte microtubular system in the excretion of bile salts and biliary lipid: implications for intracellular vesicular transport. *J. Lipid Res.* 29:144–146.

40. Reichen, J., M. D. Berman, and P. D. Berk. 1981. The role of microfilaments and of microtubules in taurocholate uptake by isolated rat liver cells. *Biochim. Biophys. Acta.* 643:126–133.

41. Kacich, R. L., R. H. Renston, and A. L. Jones. 1983. Effects of cytochalasin D and colchicine on the uptake, translocation, and biliary secretion of horseradish peroxidase and [^{14}C]sodium taurocholate in the rat. *Gastroenterology.* 85:385–394.

42. Boyer, J. L., M. Itabashi, and Z. Hruban. 1979. Formation of pericanalicular vacuoles during sodium dehydrocholate choleresis: a mechanism for bile acid transport? *In* The Liver. Quantitative Aspects of Structure and Function. R. Preisig and J. Bircher, editors. Editio Cantor, Aulendorf, FRG. 163.

43. Oshio, C., and M. J. Phillips. 1981. Contractility of bile canaliculi: implications for liver function. *Science (Wash. D.C.).* 212:1041–1042.

44. Phillips, M. J., S. Poucell, and M. Oda. 1986. Mechanisms of cholestasis. *Lab. Invest.* 54:593–608.

45. Griffiths, G., and K. Simons. 1986. The trans Golgi network: sorting at the exit site of the Golgi complex. *Science (Wash. DC).* 234:438–443.

46. Carey, M. D., and D. E. Cohen. 1987. Biliary transport of cholesterol in vesicles, micelles and liquid crystals. *In* Bile Acids and the Liver with an Update on Gallstone Disease. G. Paumgartner, A. Stiehl, and W. Gerok, editors. MTP Press Limited, Lancaster, England. 287–300.

47. Grafstein, B., and D. S. Forman. 1980. Intracellular transport in neurons. *Physiol. Rev.* 60:1167–1283.

48. Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell.* 42:39–50.

49. Vale, R. D., J. M. Scholey, and M. P. Sheetz. 1986. Kinesin: possible biological roles for a new microtubule motor. *Trends Biol. Sci.* 11:464–468.

50. Häcki, W., and G. Paumgartner. 1973. Determination of the biliary dead space using ^{14}C-taurocholate as a marker. *Experientia (Basel).* 29:1091–1093.