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Abnormal secretion of proteins into bile from colchicine-treated isolated perfused rat livers

Stephen G. BARNWELL and Roger COLEMAN
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

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The microtubule poison, colchicine, caused an abnormal output of a variety of proteins into rat bile. After 3h of exposure to the drug, livers were isolated and perfused with media of defined protein composition. There was no essential change in permeability of the hepatobiliary system to proteins (e.g. bovine serum albumin) entering bile from the perfusion fluid. The rat (serum) albumin and fibrinogen that were secreted into bile from colchicine-treated livers were probably derived from the hepatocytes. Disruption of the microtubular system reduces the secretion of proteins at the sinusoidal face of the hepatocyte and results in an accumulation of secretory vesicles in the cytoplasm. It is suggested that under these conditions some of the vesicles discharge their contents into the bile canaliculus.

There are now known to be a considerable variety of proteins present in mammalian bile; some of these are derived from the serum (Mullock & Hinton, 1978), whereas others are derived from the hepatocyte (Holdsworth & Coleman, 1975; Coleman *et al.*, 1979; La Russo & Fowler, 1979; Godfrey *et al.*, 1981). Biliary proteins show diversity not only in their origin and character but also in the mechanisms by which they enter bile.

A proportion of hepatocyte proteins have been suggested to enter bile by the action of bile salts on the bile canaliculus (Holdsworth & Coleman, 1975; Coleman *et al.*, 1977). Administration of micelle-forming bile salts into humans (Bode *et al.*, 1973), bile-fistula rats (Layden & Poppe, 1977; Reichen *et al.*, 1979; Hatoff & Hardison, 1982; Billington *et al.*, 1983) or isolated perfused rat livers (Barnwell *et al.*, 1983*a*,*b*) has been shown to cause an increased output of plasma-membrane enzymes into bile.

Some serum proteins such as serum albumin are found in bile at a much lower concentration than in blood serum. Dive & Heremans (1974) and Dive *et al.* (1974) showed that the amount of a given serum protein in bile was inversely proportional to its molecular weight and suggested that these proteins entered bile by a paracellular pathway (i.e. limited diffusion through tight junctions) or by non-specific pinocytosis through endothelial cells at the head of the bile ducts. Thomas (1980) found that exogenous protein entered bile without passing through hepatocytes, and later showed (Thomas *et al.*, 1982) that the transit time of exogenous proteins increased with molecular weight. Barnwell *et al.* (1983*a*) found that bovine serum albumin present in the perfusion fluid of isolated livers appeared in bile within 20min of isolation; increasing the concentration of this protein in the perfusate from 1 to 3% (w/v) caused a proportional increase in the amount appearing in bile, supporting the suggestion that a non-specific mechanism (diffusion through tight junctions or pinocytosis through endothelial cells) was operating.

Immunoglobulin A in the polymeric form is present in rat bile at a very much higher concentration than in rat serum. This protein, bound to secretory component (Lemaitre-Coelho *et al.*, 1977; Mullock & Hinton, 1981), traverses the hepatocyte in a series of receptor-containing endocytotic vesicles. This system has been shown to be sensitive to microtubular poisons both in the isolated perfused liver (Mullock *et al.*, 1980) and in the fistula rat (Godfrey *et al.*, 1982). Similarly the secretion, via the sinusoidal face of the hepatocyte, of serum proteins, such as albumin, fibrinogen and very-low-density lipoprotein, into blood is inhibited by the microtubular disrupting agents colchicine, vincristine and vinblastine (Orci *et al.*, 1973; Le Marchand *et al.*, 1973; Feldmann *et al.*, 1975; Redman *et al.*, 1975).

Godfrey *et al.* (1982) found abnormally large amounts of rat serum albumin in bile from animals treated with colchicine and vinblastine. Two explanations were offered: either the paracellular pathway by which albumin is normally thought to enter bile had become more permeable, or a

proportion of the secretory vesicles containing rat serum albumin normally directed to the sinusoidal face of the hepatocyte were emptying their contents into bile by mistake.

Using the advantage of the isolated perfused liver system, whereby rat blood is replaced by a simplified medium containing exogenous proteins, the present study aims to determine the mechanism by which colchicine enhances biliary secretion of serum albumin and also investigates the effect of this microtubular disrupting agent on the fate of fibrinogen, a serum protein normally absent from bile.

Materials and methods

Materials

Antisera to rat immunoglobulin A, rat serum albumin, rat fibrinogen and bovine serum albumin were purchased from Nordic Immunological Laboratories, Maidenhead, Berks., U.K. Sagatal was obtained from May and Baker, Dagenham, Essex, U.K., cannulation tubing PP10 was manufactured by Portex, Hythe, Kent, U.K., and heparin was made by Weddel Pharmaceuticals, London E.C. 1, U.K. Colchicine and other fine chemicals were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Methods

Male Wistar rats weighing approx. 250g were used throughout; these had been allowed free access to standard laboratory diet and had been maintained under a constant light cycle. Colchicine (20μmol/100g body wt.) in 1ml of iso-osmotic NaCl or 1ml of iso-osmotic NaCl alone (controls) was administered by intravenous injection while the rats were under pentobarbital (Sagatal) anaesthesia. Bile-duct cannulations were performed 2h after injection.

Isolated perfused livers. At 20min after bile-duct cannulation the livers were isolated by the method of Barnwell *et al.* (1983*a*). The liver was initially flushed free of rat blood using calcium-free Krebs–Ringer bicarbonate, pH 7.4, and then switched to a recirculating perfusion with 100ml of Krebs–Ringer bicarbonate buffer, pH 7.4, containing 2mM-$CaCl_2$, 10mM-glucose, 1% (w/v) bovine serum albumin, a physiological amino acid mixture and 10% (v/v) washed human red cells. This solution was gassed continuously with O_2/CO_2 (19:1) and maintained at a constant 37°C.

After collecting two 20min samples, subsequent bile collections were increased to 30min for the remainder of the experiment. At $t = 100$min (i.e. 80min after isolation) the perfusion fluid was changed to replace substrates used by the liver. At $t = 190$min the liver was perfusion-fixed with 0.2% (w/v) glutaraldehyde in chilled perfusion fluid and processed for viewing by electron microscopy. Bile samples obtained through the experiment were collected on ice and then stored at −20°C until required; no deterioration of components in the samples was noticed during storage.

Specific protein determination. Rat immunoglobulin A, rat serum albumin, rat fibrinogen and bovine serum albumin in bile were determined by quantitative radial immunodiffusion by the method of Mancini *et al.* (1965) with specific antisera, using authentic rat serum albumin and bovine serum albumin for standardization; immunoglobulin A and fibrinogen (for which no standards were available) are expressed in arbitrary units relating to the diameter of the precipitation zone.

Results

Bile flow

Initial bile flow from colchicine-treated rats ($t = 0$–20min) was similar to controls and agrees with the findings of Godfrey *et al.* (1982), whereby 20μmol of colchicine/100g body wt. had little effect on basal bile flow. Perfusion of livers with bile-salt-free media resulted in a decline in bile flow (Barnwell *et al.*, 1983*a*); livers pretreated with colchicine produced less bile (approx. 50%) than controls even though the livers were shown to be viable (i.e. high O_2 consumption, even perfusion and low intracellular enzyme release; see Barnwell *et al.*, 1983*a*,*b*) (Fig. 1).

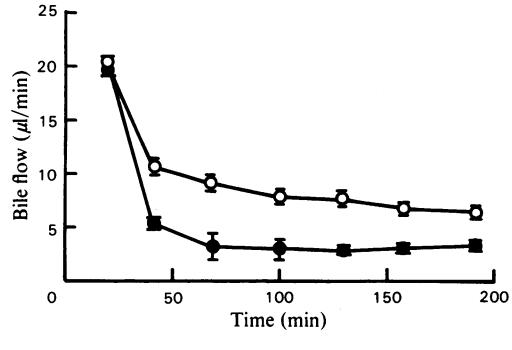


Fig. 1. *Bile flow as a function of time*
Bile ducts were cannulated at $t = 0$. Isolation and perfusion were commenced at $t = 20$min. The perfusion fluid was removed at $t = 100$min. Bile was collected at the time intervals shown and the output was obtained by dividing the total amount collected in a given time interval by the number of minutes in the collection period. Values are means ± S.E.M. ($n = 3$–18). Symbols: ○, controls, ●, rats treated with colchicine (20μmol/100g body wt.) 2h before cannulation of the bile duct.

Immunoglobulin A

Interference with an intact microtubular network has been shown to prevent the biliary secretion of dimeric immunoglobulin (Mullock *et al.*, 1980; Godfrey *et al.*, 1982). In the present experiments measurement of dimeric immunoglobulin A in the bile from control and treated livers was used as an indication of the extent to which colchicine had affected the hepatic microtubular system (see Fig. 2). In all colchicine-treated livers, biliary secretion of immunoglobulin A was reduced by at least 60%.

Bovine serum albumin

Bovine serum albumin entered bile within 20 min of isolation and reached a concentration of about 0.35 mg/ml (Fig. 3*a*). The results indicated that there was little difference between the concentration of bovine serum albumin in bile collected from either control or treated livers. The results suggest that colchicine does not increase the permeability of the hepatobiliary tract to albumin.

Bovine serum albumin is of similar size but immunologically distinct from rat serum albumin; it is likely that they are cleared from blood or perfusion fluid into bile by the same mechanism (see Barnwell *et al.*, 1983*a*). Presenting the results in terms of bovine serum albumin output (μg/min) shows that the reduction in colchicine-treated livers compared with controls was related to the decreased bile flow (50%) (Fig. 3*b*). If serum proteins usually enter bile by a non-specific pathway, the maintenance of a constant concentration ratio between serum and bile would cause a reduced output of any given serum protein (μg/min) if bile flow is reduced.

Rat serum albumin

The biliary output of rat serum albumin in control animals declined virtually to zero by 130 min (Figs. 4*a* and 4*b*), presumably reflecting the lack of supply from the perfusion fluid (the amount in the perfusion fluid during these experiments was barely detectable) and the inability of hepatocytes to secrete newly synthesized albumin directly into bile under normal circumstances.

Before isolation at $t = 20$ min, bile from colchicine-treated livers had a rat serum albumin concentration 2-fold greater than controls (similar to

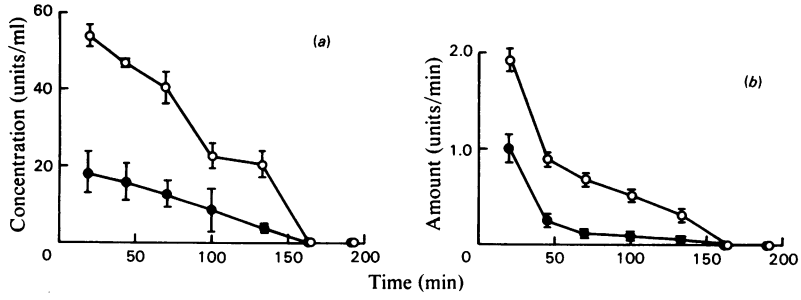


Fig. 2. *Concentration (a) and output (b) of immunoglobulin A in bile as a function of time*
For details see the legend to Fig. 1 and the Materials and methods section. Immunoglobulin A is expressed in arbitrary units. In (*a*) concentration of immunoglobulin A is expressed as units per ml; in (*b*) output is expressed as units per min and is obtained by dividing the total amount collected in the given time interval by the number of minutes in the collection period. Values are means ± S.E.M. ($n = 3$–18) of triplicate determinations. Symbols: ○, controls; ●, rats treated with colchicine (20 μmol/100 g body wt.) 2 h before cannulation of bile duct.

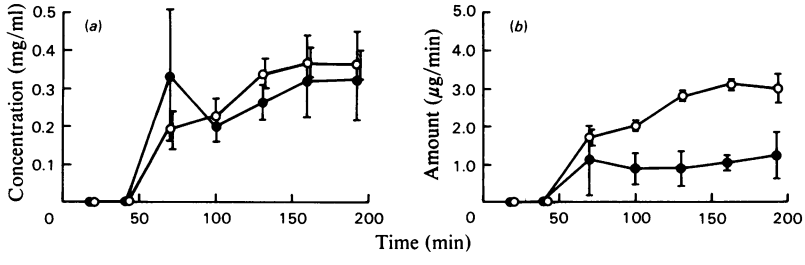


Fig. 3. *Concentration (a) and output (b) of bovine serum albumin in bile as a function of time*
For conditions, symbols etc. see the legend to Fig. 2. Bovine serum albumin concentrations were obtained in relation to purified bovine serum albumin used as a standard.

Godfrey *et al.*, 1982) (Fig. 4*a*). When the livers were isolated the concentration of this protein in bile increased substantially, reflecting the reduction in bile flow. Reconsideration of the same data, however, in terms of protein output (μg/min) (Fig. 4*b*) revealed that at $t = 20$ min the excess output of rat serum albumin over controls was still about 30%. Beyond 130 min the decline in rat serum albumin output from colchicine-treated livers stopped, leaving a continued and constant output of about 2.2 μg/min, at a time when the output from control livers had subsided to zero (Fig. 4*b*).

Disruption of the microtubular system [which normally directs newly synthesized serum proteins to the sinusoidal face of the hepatocyte (Le Marchand *et al.*, 1974; Redman *et al.*, 1975)] had therefore caused rat serum albumin to appear in bile in considerable amounts even when the supply from the circulation had been removed (Fig. 4*b*). The origin of this rat serum albumin in the bile from colchicine-treated livers, both after 130 min and in excess of controls before this, must therefore be newly synthesized material from within the hepatocyte.

Fibrinogen

Fibrinogen, another serum protein normally carried in secretory vesicles to the sinusoidal face of the hepatocyte, has a molecular weight of approx. 330000 and therefore, unlike serum albumin, is too large to enter bile via the paracellular route to any detectable extent. Feldmann *et al.* (1975) found that colchicine caused an accumulation of fibrinogen inside liver cells and therefore suggested that secretion at the sinusoidal face was reduced. The results featured in Figs. 4(*c*) and 4(*d*) show that the bile from colchicine-treated livers contained significant quantities of fibrinogen, whereas it is completely absent from controls, implying that this protein originates from the hepatocytes themselves, and is put out into the bile after colchicine treatment of the livers and the subsequent disruption of microtubular function.

Electron microscopy

Inspection of colchicine-treated livers by transmission electron microscopy revealed that most (approx. 80%) of the hepatocytes contained an accumulation of small vesicles; these vesicles were far less numerous in cells not exposed to colchicine. The general morphology of other organelles in the colchicine-treated cells appeared essentially normal. The vesicles were especially numerous in the canalicular pole of the cell and, in a few cases, fusion of vesicles with the canalicular membrane was observed (Plate 1).

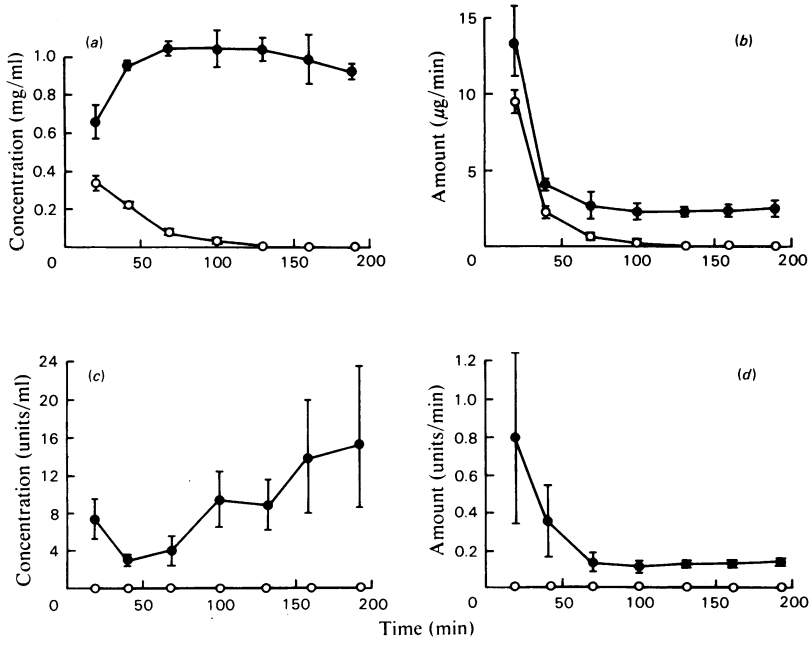
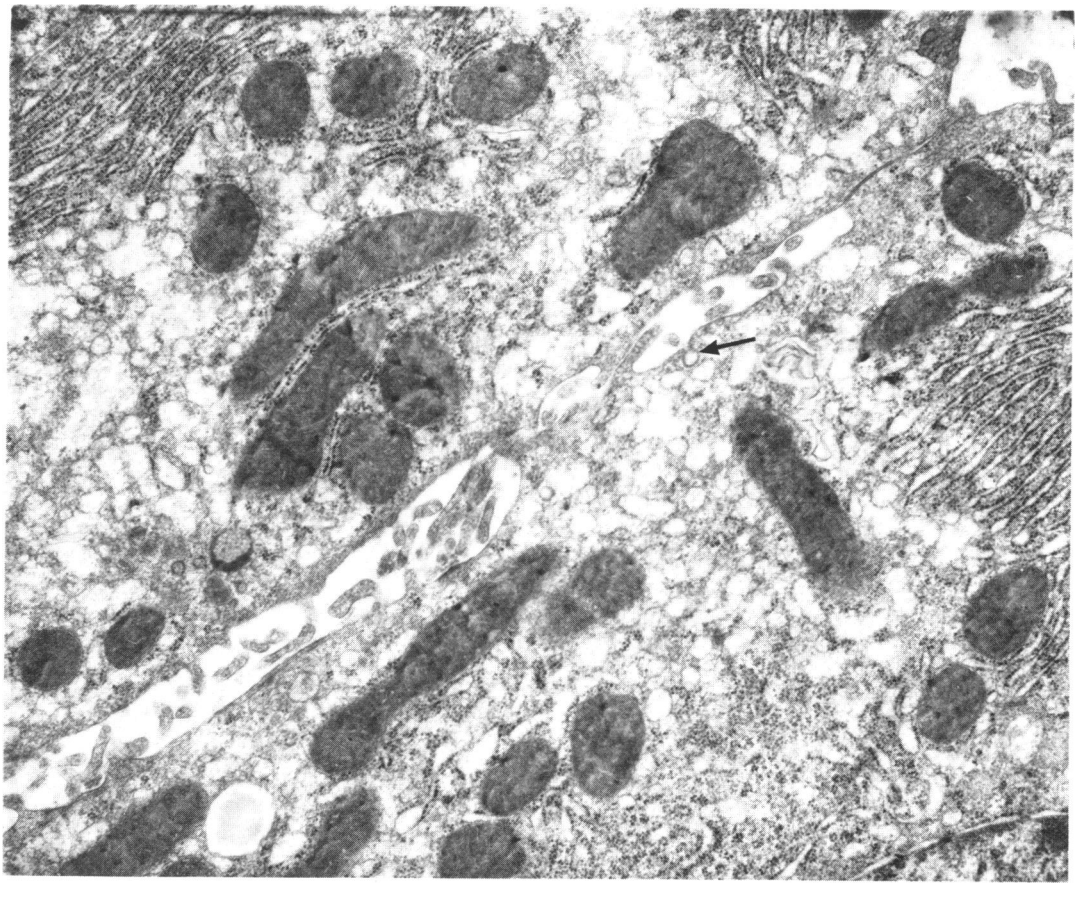


Fig. 4. *Concentration (a and c) and output (b and d) of rat serum albumin (a and b) and of fibrinogen (c and d) in bile as a function of time*

For conditions, symbols etc. see the legend to Fig. 2. Rat serum albumin concentrations were obtained in relation to purified rat serum albumin used as a standard. Fibrinogen is expressed in arbitrary units.



EXPLANATION OF PLATE 1

Transmission electron micrograph of an example of hepatocytes from colchicine-treated rats

Cells showing the characteristics illustrated were prominent (approx. 80%) in the livers of colchicine-treated rats, but were not observed in the livers of control rats. The cytoplasm contains many small vesicles, one of which is seen (arrow) in the act of fusing with the membrane of a bile canaliculus. Mitochondria and endoplasmic reticulum show essentially similar morphology to untreated cells. Livers were perfusion-fixed with 0.2% glutaraldehyde in chilled perfusion fluid before processing for electron microscopy. Magnification × 20000.

Discussion

Peters *et al.* (1973) determined the rate at which rat livers were able to synthesize serum albumin under a variety of conditions. For the size of rats used in the present study the liver synthesizes and then secretes approx. 25 μg of serum albumin per min. The results given in Fig. 4(*b*) show the output of rat serum albumin into bile derived from the hepatocyte to be in the order of 10–15% of the rat's serum albumin secretory capacity under colchicine treatment.

Microtubular disrupting agents such as colchicine, vinblastine and vincristine interfere with the transport of secretory vesicles containing very-low-density lipoprotein (Orci *et al.*, 1973; Stein *et al.*, 1974; Le Marchand *et al.*, 1973), high-density lipoprotein (Stein *et al.*, 1974), albumin (Redman *et*

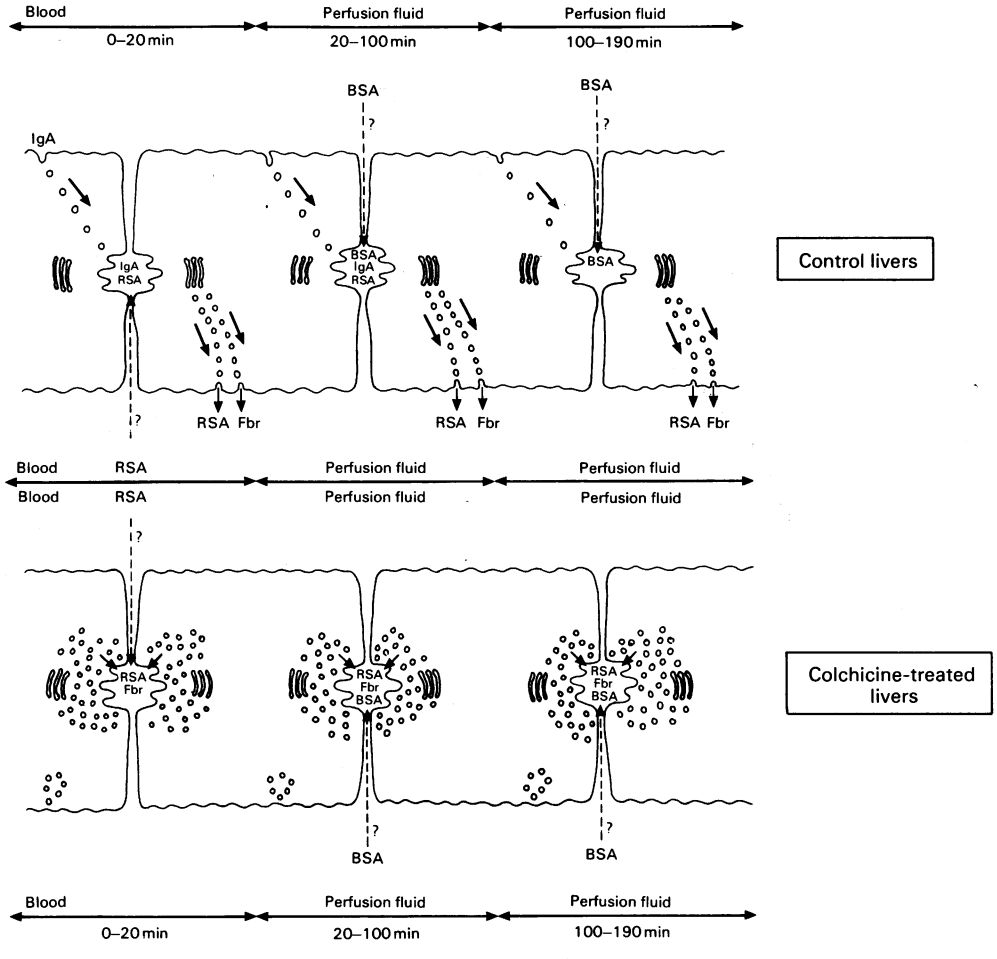


Fig. 5. *Diagrammatic representation of possible phenomena contributing to protein patterns in the bile of control and colchicine-treated rat livers*

In control livers (upper series) immunoglobulin A (IgA) enters bile via a receptor-mediated vesicle transport; there is none in the perfusion fluid and thus the amount in bile declines as the amount in passage through the liver is reduced. Bovine serum albumin (BSA), which is present in the perfusion fluid after isolation of the liver (20 min), enters bile through a non-specific mechanism (e.g. limited penetration through tight junctions between cells); its concentration builds up to a maximum. Rat serum albumin (RSA) enters bile from blood probably by a similar mechanism; its concentration in bile falls after 20 min, since no rat serum albumin is added to the perfusion fluid. There is a continuous flow of secretory vesicles carrying rat serum albumin and fibrinogen (Fbr) to the sinusoidal face of the hepatocytes. In colchicine-treated livers (lower series) the transport of immunoglobulin A into bile is interrupted. There is no contribution of rat serum albumin into bile from the perfusion fluid. The transport of rat serum albumin and fibrinogen to the sinusoidal face is interrupted; a few of the secretory vesicles accumulating then discharge into bile. The entry of bovine serum albumin from the perfusion fluid is little different from the control.

al., 1975), fibrinogen (Feldmann *et al.*, 1975), alkaline phosphatase (Oda & Ikehara, 1981) and insulin receptors (Whittaker *et al.*, 1981) to the sinusoidal plasma membrane.

Many of these workers noticed newly synthesized material had accumulated in vesicle-like structures inside hepatocytes similar to those observed in the present study (Plate 1) (Stein *et al.*, 1974; Redman *et al.*, 1975), whereas others detected accumulated activity of fibrinogen (Feldmann *et al.*, 1975), labelled serum albumin (Le Marchand *et al.*, 1974) and alkaline phosphatase (Oda & Ikehara, 1981), particularly in the Golgi region of the cell. Le Marchand *et al.* (1974) and Redman *et al.* (1975) pointed out that only transport and not synthesis was disrupted by microtubular poisons, and in the case of alkaline phosphatase synthesis was induced by colchicine treatment (Oda & Ikehara, 1981). The results from the present study suggest further that a substantial proportion of the vesicles, normally destined to empty their contents at the sinusoidal face of the liver cell, may then empty their contents at the biliary pole of the hepatocyte when the microtubular network is disrupted (see Fig. 5). The possibility that these serum proteins enter bile after internalization by and subsequent biliary emptying of lysosomes seems unlikely, as the serum proteins rat serum albumin and fibrinogen remain antigenically distinct and therefore not substantially degraded. Whether the secretory vesicles simply fuse with the bile-canalicular membrane by mistake owing to its close proximity to the Golgi (Evans *et al.*, 1980), or whether the presence of fusogens takes a directing role, is still unclear.

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