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Effects of Bile Salts on the Plasma Membranes of Isolated Rat Hepatocytes

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The conjugated trihydroxy bile salts glycocholate and taurocholate removed approx. 20–30% of the plasma-membrane enzymes 5'-nucleotidase, alkaline phosphatase and alkaline phosphodiesterase I from isolated hepatocytes before the onset of lysis, as judged by release of the cytosolic enzyme lactate dehydrogenase. The conjugated dihydroxy bile salt glycodeoxycholate similarly removed 10–20% of the 5'-nucleotidase and alkaline phosphatase activities, but not alkaline phosphodiesterase activity; this bile salt caused lysis of hepatocytes at approx. 10-fold lower concentrations (1.5–2.0mM) than either glycocholate or taurocholate (12–16mM). At low concentrations (7mM), glycocholate released these enzymes in a predominantly particulate form, whereas at higher concentrations (15mM) glycocholate further released these components in a predominantly 'soluble' form. Inclusion of 1% (w/v) bovine serum albumin in the incubations had a small protective effect on the release of enzymes from hepatocytes by glycodeoxycholate, but not by glycocholate. These observations are discussed in relation to the possible role of bile salts in the origin of some biliary proteins.

The liver secretes bile, a fluid rich in potentially membrane-damaging detergents, the bile salts, which occur as mixed micelles together with phospholipid (predominantly phosphatidylcholine) and cholesterol (Heaton, 1972). In addition, the bile of many mammalian species contains significant amounts of alkaline phosphatase, alkaline phosphodiesterase I, 5'-nucleotidase and L-leucyl-β-naphthylamidase (Holdsworth & Coleman, 1975*a*; Evans *et al.*, 1976; Coleman *et al.*, 1979); these enzymes are glycoproteins (Holdsworth & Coleman, 1975*b*; Evans *et al.*, 1976) and are normally associated with the plasma membranes of liver cells (De Pierre & Karnovsky, 1973; Misra *et al.*, 1974). These enzymes are released into bile in the absence of significant cell damage, as evidenced by the relative absence from bile of intracellular enzymes such as lactate dehydrogenase (Holdsworth & Coleman, 1975*a*; Coleman *et al.*, 1979). Mouse and rat bile 5'-nucleotidase have been shown to be immunologically identical with the plasma-membrane forms (Evans *et al.*, 1976; Mullock *et al.*, 1977). Also, mouse bile and liver plasma-membrane alkaline phosphodiesterase I activities show similar pH profiles (Evans *et al.*, 1976), suggesting that the bile and membrane activities are due to the same enzyme.

Model experiments on the effects of various bile salts on human (Coleman & Holdsworth, 1976; Billington & Coleman, 1978) and sheep (Billington *et al.*, 1977) erythrocytes and pig lymphocytes (Holdsworth & Coleman, 1976) have demonstrated the release of plasma-membrane materials from intact cells before the onset of significant cell lysis. Cell lysis was judged by release of the appropriate intracellular proteins haemoglobin or lactate dehydrogenase. As a result of these studies, it has been suggested that bile salts are instrumental in the release of plasma-membrane components into bile (Coleman *et al.*, 1977, 1979).

In the present study we report the effects of several bile salts on the plasma membranes of isolated rat hepatocytes as a closer model of what may be happening in the hepatobiliary system *in vivo*. A preliminary account of some of this work has appeared (Billington *et al.*, 1979).

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Materials and Methods

Materials

Albino Wistar rats (200–250g) of either sex, maintained on a standard laboratory diet, were used throughout without previous regard to food and water intake.

All bile salts (sodium salts, A grade) were obtained from Calbiochem, Bishop Stortford, Herts., U.K. Fine chemicals were from Sigma (London) Chemical Co., Poole, Dorset, U.K., and other reagents were from Fisons, Loughborough, Leics., U.K., and were of the highest grade available. Bovine serum albumin (Sigma, fraction V), defatted by the method of Chen (1967), was a gift from M. Wakelam, University of Birmingham.

Methods

Preparation and incubation of hepatocytes. Hepatocytes were isolated essentially by the method of Berry & Friend (1969). Rats were anaesthetized by the intraperitoneal administration of 0.3ml of Sagatal and the liver was cannulated via the portal vein. The liver was first freed of blood by perfusion with Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH7.4 (Krebs & Henseleit, 1932), containing 0.1% (w/v) glucose. The perfusion was then continued cyclically with 100ml of the same buffer containing 30mg of collagenase; in some experiments 2.5mM-$CaCl_2$ was also included (Seglen, 1976). The perfusion was at 37°C and all solutions were continually gassed with O_2/CO_2 (19:1, v/v). After perfusion for 20–30min, the liver was gently stirred in a plastic beaker with a plastic rod, and the mixture was filtered through a nylon mesh (100μm diameter). Hepatocytes were freed of smaller cell types and damaged parenchymal cells by centrifugation at 400g for 2min followed by three to four washes in Krebs–Ringer bicarbonate buffer, pH7.4, containing 0.1% (w/v) glucose and 2.5mM-$CaCl_2$. The cells were approximately 90% viable as judged by Trypan Blue exclusion and when prepared from 24h-starved animals were capable of glucose synthesis from 10mM-pyruvate or 10mM-L-alanine at rates of approx. 150μmol/h per g dry wt., which are comparable with those reported by other workers (for example, Berry & Friend, 1969; Clark *et al.*, 1974; Gatley *et al.*, 1975).

The dry weight of cells per ml of final suspension was in the range 10–50mg dry wt./ml, with most experiments being carried out in the range 15–40mg dry wt./ml. In our early experiments carried out at higher dry weights, the background release of lactate dehydrogenase (15–20%) was routinely greater than in our more recent experiments carried out at lower dry weights (8–15%); this may reflect difficulties in maintaining full gaseous exchange. However, these differences in cell concentration did not quantitatively affect the extent of release of plasma-membrane enzymes by bile salts and the values reported in the present paper are means of all experiments.

One vol. of this hepatocyte suspension was incubated in an atmosphere of O_2/CO_2 (19:1) with shaking at 37°C for 10min with 3vol. of Krebs–Ringer bicarbonate buffer, pH7.4, containing 2.5mM-$CaCl_2$ and 0.1% (w/v) glucose. The final incubation volume was 2.0ml and various bile salts were added at different concentrations. In experiments to investigate the effect of protein on the release of components by bile salts, bovine serum albumin was included in the incubations at a final concentration of 1% (w/v). Incubations were terminated by centrifugation at approx. 2500g-min; supernatants were immediately removed and examined for the presence of enzymes. In experiments to examine the physical form of released enzymes, a portion of this supernatant was further centrifuged at 150000g for 60min at 4°C in an MSE model 50 ultracentrifuge.

Enzyme assays. Supernatants were assayed for the plasma-membrane enzymes 5′-nucleotidase (EC 3.1.3.5), alkaline phosphatase (EC 3.1.3.1) and alkaline phosphodiesterase I (EC 3.1.4.1); these enzymes were assayed at 37°C as described previously (Coleman *et al.*, 1979). Lactate dehydrogenase (EC 1.1.1.27) was assayed at 20°C by the method of Stolzenbach (1966). Background release of enzymes was determined in incubations in the absence of bile salts; this activity was subtracted from all other activities. Results are expressed as a percentage of the total enzyme activity, which was determined in corresponding uncentrifuged control incubations treated with 0.5% (v/v) Triton X-100 to minimize latency. Possible inhibition of these enzymes by bile salts was investigated; at the concentrations of bile salts used, lactate dehydrogenase and 5′-nucleotidase were consistently unaffected, whereas alkaline phosphatase and alkaline phosphodiesterase I were inhibited by up to 20%.

Electron microscopy. Samples were prepared for electron microscopy essentially as described by Simionescu & Simionescu (1976). Hepatocytes, incubated with or without glycocholate as described previously, were fixed in suspension with an equal volume of 6.25% (v/v) glutaraldehyde followed by 1% (w/v) OsO_4 and then 1% (w/v) tannic acid to increase and diversify contrast. The fixed cells were dehydrated in ethanol and embedded in Epon resin. Thin sections were stained with lead citrate (Reynolds, 1963) before examination in a Philips EM 301 transmission electron microscope.

Critical micellar concentration. The critical micellar concentrations of glycocholate, taurocholate and glycodeoxycholate were determined in Krebs–Ringer bicarbonate buffer, pH7.4, containing 2.5mM-

$CaCl_2$ and 0.1% (w/v) glucose and gassed with O_2/CO_2 (19:1), by the dye method of Benzonana (1969).

Results

Release of enzymes from isolated hepatocytes by bile salts

As judged by release of the cytosolic marker enzyme lactate dehydrogenase, the conjugated trihydroxy bile salts glycocholate and taurocholate caused lysis of isolated hepatocytes at 12–16mM (Figs. 1 and 2). This was preceded by the release of significant amounts (20–30% of the plasma-membrane enzymes 5′-nucleotidase, alkaline phosphatase and alkaline phosphodiesterase I; Figs. 1 and 2). Glycodeoxycholate, a conjugated dihydroxy bile salt, also caused the prelytic release of significant, but smaller, amounts (10–20%) of 5′-nucleotidase and alkaline phosphatase (Fig. 3). However, there was no prelytic release of alkaline phosphodiesterase I (Fig. 3). Although alkaline phosphodiesterase I is inhibited by glycodeoxycholate (up to 20% at the concentrations used), it is doubtful whether this can totally explain why the release of

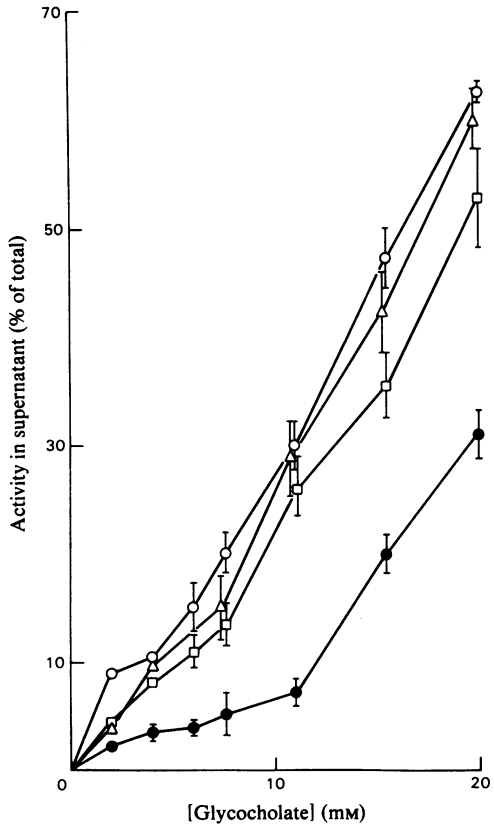


Fig. 1. *Release of enzymes from isolated rat hepatocytes by glycocholate*

Hepatocytes were incubated at 37°C for 10min with glycocholate; incubations were terminated by centrifugation at approx. 2500*g*-min and supernatants were assayed for lactate dehydrogenase (●), 5′-nucleotidase (○), alkaline phosphatase (□) and alkaline phosphodiesterase (△). Values are means (± S.E.M.) for four to ten observations and are corrected for background release of enzymes. Background release (expressed as a percentage of total activity) was as follows: lactate dehydrogenase, 16.1 ± 1.6; 5′-nucleotidase, 4.1 ± 0.9; alkaline phosphatase, 10.9 ± 1.1; alkaline phosphodiesterase I, 6.9 ± 1.1.

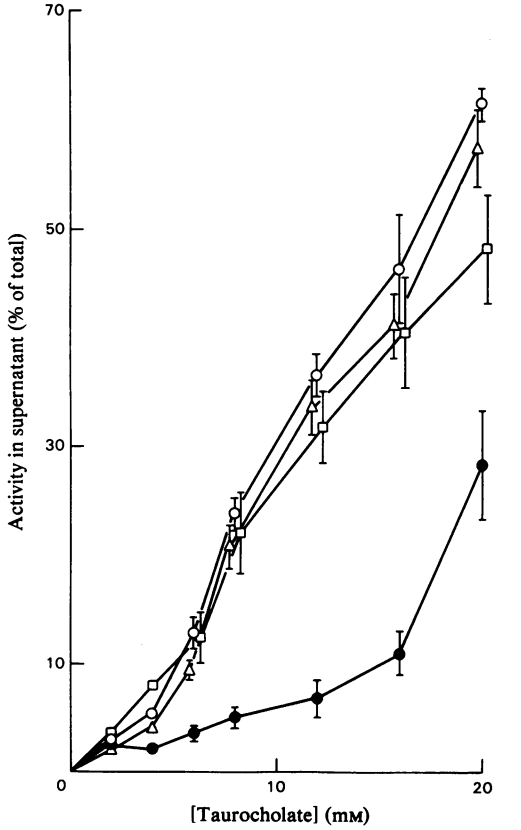


Fig. 2. *Release of enzymes from isolated rat hepatocytes by taurocholate*

Methods and symbols are as described in the legend to Fig. 1. Values are means (± S.E.M.) for four observations and are corrected for background release, which was as follows: lactate dehydrogenase, 10.4 ± 1.6; 5′-nucleotidase, 3.7 ± 1.7; alkaline phosphatase, 4.6 ± 2.4; alkaline phosphodiesterase I, 2.9 ± 0.4.

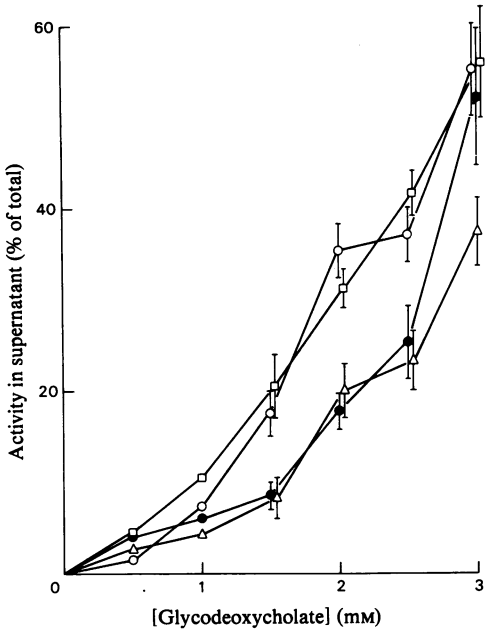


Fig. 3. *Release of enzymes from isolated rat hepatocytes by glycodeoxycholate*

Methods and symbols are as described in the legend to Fig. 1. Values are means (± S.E.M.) for four to nine observations and are corrected for background release, which was as follows: lactate dehydrogenase, 19.5 ± 1.9; 5′-nucleotidase, 7.0 ± 1.3; alkaline phosphatase, 9.0 ± 1.5; alkaline phosphodiesterase I, 7.0 ± 1.0.

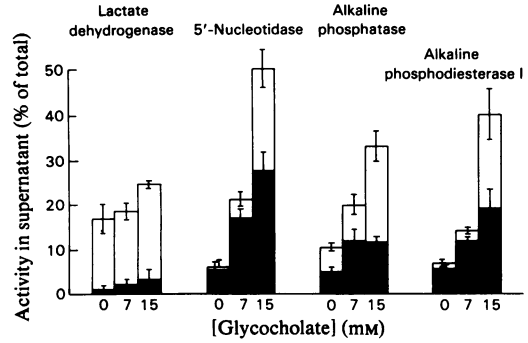


Fig. 4. *Sedimentation of released enzymes on high-speed centrifugation*

Hepatocytes were incubated at 37°C for 10min with glycocholate and incubations were terminated by low-speed centrifugation (2500 *g*-min). Portions of the supernatant were assayed for enzyme activity and another portion was further centrifuged at 150000 *g* for 60min at 4°C; the supernatant from this was assayed for enzyme activity. 'Soluble' activity (i.e. that remaining in the supernatant on high-speed centrifugation) is represented by open bars, and particulate activity was calculated from the difference in the activities in the low- and high-speed supernatants and is represented by filled bars. Results are expressed as a percentage of the total enzyme activity in the incubations and values are means for five observations ± S.E.M.

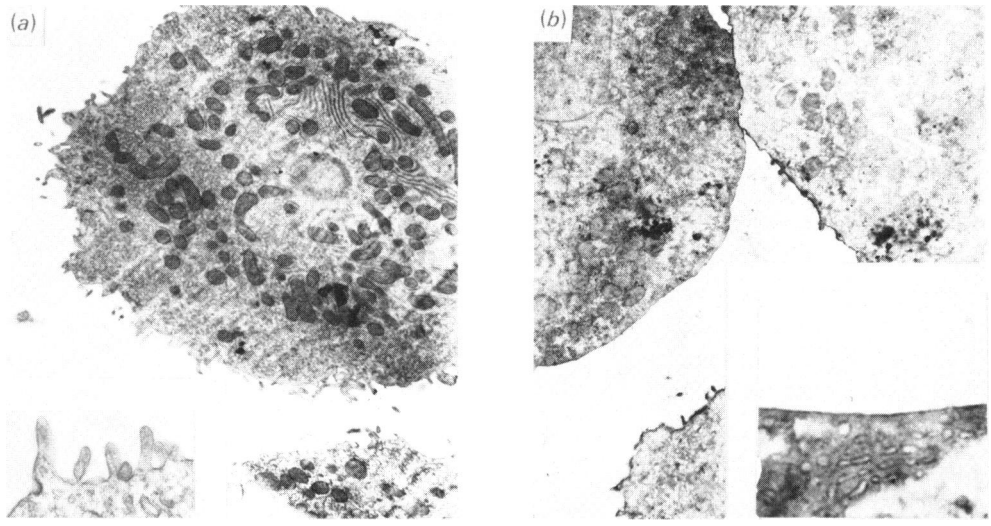
alkaline phosphodiesterase I parallels that of lactate dehydrogenase.

Glycodeoxycholate caused lysis of hepatocytes at 1.5–2.0mM (Fig. 3). This is approx. 10-fold lower than the concentration of either glycocholate or taurocholate required to cause lysis and is in agreement with previous studies on human erythrocytes (Coleman & Holdsworth, 1976) and pig lymphocytes (Holdsworth & Coleman, 1976). The critical micellar concentrations of glycocholate, taurocholate and glycodeoxycholate were found to be 6.2, 6.5 and 1.6mM respectively. Therefore, in the case of glycodeoxycholate, lysis occurred close to its critical micellar concentration, but hepatocytes resisted concentrations of the trihydroxy bile salts well above their critical micellar concentrations.

Physical form of the released enzymes

To examine the physical form of the enzymes released by glycocholate treatment of hepatocytes, a portion of the low-speed supernatant (2500 *g*-min) was further centrifuged at 150000 *g* for 60min. At the concentrations of glycocholate studied, the released lactate dehydrogenase was predominantly in a 'soluble' form (i.e. remained in the supernatant on high-speed centrifugation), very little existing in a particulate form (i.e. disappeared from the supernatant on high-speed centrifugation); in addition, the release of lactate dehydrogenase above the background value was only small (approx. 7% at 15mM-glycocholate) (Fig. 4). In contrast, glycocholate released plasma-membrane enzymes from hepatocytes in two ways. First, at low concentrations (7mM), glycocholate caused the release of 5′-nucleotidase, alkaline phosphatase and alkaline phosphodiesterase I in a predominantly particulate form, whereas at higher concentrations (15mM) further release of these enzymes was in a predominantly 'soluble' form (Fig. 4).

Transmission electron micrographs of hepatocytes incubated in the absence of bile salts showed the presence of several microvillus-like projections from the plasma membrane of every cell in the preparation (Plate 1). The surfaces of cells treated with 15mM-glycocholate exhibited a mixed morphology; a substantial proportion of the cells (60–70%) showed a smooth profile over the surface of the cell, whereas the remainder of the preparation showed populations of microvilli varying from small rudi-



EXPLANATION OF PLATE 1

Transmission electron micrographs showing the effect of glycocholate on hepatocyte plasma-membrane morphology
Hepatocytes were incubated at 37°C for 10min in the presence of (*a*) 0mM and (*b*) 15mM-glycocholate. Details of fixation etc. are given in the Materials and Methods section. Magnification ×3800 (insets ×13000).

mentary projections at infrequent intervals to projections similar in size and relative frequency to the control cells (Plate 1). In most cases, however, these latter types of cell had distinct microvilli in only one region of their surface, and smooth areas still predominated (Plate 1). The morphology of the surface of cells treated with 7mM-glycocholate was intermediate between these two extremes; the majority of cells (greater than 70%) showed small infrequent projections. Some cells possessed more prominent microvilli and others exhibited smooth areas of plasma membrane. It is possible therefore that the loss of the microvillus-like projections from hepatocytes after treatment with glycocholate may be equated with the pinching-off of these projections from the plasma membrane (via membrane fusion at the cytoplasmic face) leading to the formation of microvesicles. Similar prelytic concentrations of glycocholate have been shown to cause human erythrocytes to shed particulate material in the form of microvesicles (Billington & Coleman, 1978). In this case, the vesicles were enriched in the plasma-membrane ectoenzyme acetylcholinesterase, but were depleted in several other membrane proteins (Billington & Coleman, 1978). Vesiculation would account for the particulate nature of the material released from hepatocytes by low concentrations of glycocholate in these experiments, but the electron micrographs do not exclude loss of material as membrane fragments.

Effect of protein on the release of enzymes from hepatocytes by bile salts

Bovine serum albumin, at a final concentration of 1% (w/v), had a small, but significant, protective effect on the release of lactate dehydrogenase and 5′-nucleotidase from hepatocytes by glycodeoxycholate (Fig. 5); similar observations were made with alkaline phosphatase and alkaline phosphodiesterase I (results not shown). In contrast, similar concentrations of bovine serum albumin had little or no effect on the release of both plasma membrane and cytosolic marker enzymes from hepatocytes by glycocholate.

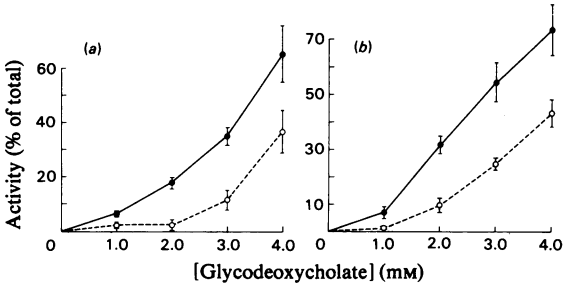


Fig. 5. *Effect of endogenous protein on the release of (a) lactate dehydrogenase and (b) 5′-nucleotidase from isolated rat hepatocytes by glycodeoxycholate*

Hepatocytes were incubated at 37°C for 10min with glycodeoxycholate in the absence (●) or the presence (○) of 1% (w/v) bovine serum albumin. Incubations were terminated by centrifugation at 2500*g*-min and supernatants were assayed for enzyme activity. Values are means for four observations ± S.E.M. and are corrected for background release of enzymes.

Discussion

5′-Nucleotidase is thought to be an intrinsic membrane protein having its active site on the extracytoplasmic face of the plasma membrane (Misra *et al.*, 1974) and, by analogy with other cells (De Pierre & Karnovsky, 1973; Trams & Lauter, 1974), alkaline phosphatase and alkaline phosphodiesterase I might similarly be expected to be externally orientated plasma-membrane enzymes. However, Stanley & Luzio (1979) have recently shown that only 48% of the total 5′-nucleotidase is available to exogenous substrate in hepatocytes prepared by collagenase dispersion of rat livers, and that the remaining activity is membrane bound and probably represents internalized plasma-membrane vesicles (Stanley *et al.*, 1980). In agreement with this, intact hepatocytes exhibited only approx. 60% of the 5′-nucleotidase activity shown by hepatocytes treated with 0.5% (w/v) Triton X-100; similarly intact cells exhibited only 60–70% of the alkaline phosphatase and alkaline phosphodiesterase I activities of hepatocytes treated wtih Triton X-100 (P. P. Godfrey, R. Coleman & D. Billington, unpublished work). Since total cellular enzyme activity was assayed in the presence of Triton X-100, the prelytic release of these plasma-membrane enzymes from the exposed surface of hepatocytes by conjugated trihydroxy bile salts may be substantially greater than the 20–30% shown in Figs. 1 and 2.

In an attempt to explain the unique phospholipid and enzyme composition of mammalian bile, Coleman *et al.* (1977) proposed that as the total bile salt concentration in the canalicular lumen increases to exceed micellar values, plasma-membrane components are removed from the outer leaflet of the bile canalicular membrane in such a manner as not to cause gross cellular damage (i.e. leakage of intracellular components); the integrity of the bile canalicular membrane is then maintained by continuous biosynthetic repair. In support of this, 5′-nucleotidase of mouse (Evans *et al.*, 1976) and rat (Mullock *et al.*, 1977) bile has been shown to be immunologically identical with the corresponding plasma-membrane forms. The results presented here lend further support to these proposals since they

show that bile salts can release, in the absence of lysis, significant proportions of plasma-membrane enzymes from intact hepatocytes. However, these enzymes were released in both 'soluble' and particulate (vesicular) forms. It is difficult to assess the possible contribution of each of these forms to the overall biliary enzyme profile. Any released particulate enzyme activities would be expected to be 'solubilized' with increasing total bile salt concentration as the bile passes down the biliary tract towards the duodenum, or, in several species other than rat, where bile is concentrated in the gall bladder by reabsorption of iso-osmotic proportions of NaCl and water (Diamond, 1965). Surface-area-to-volume considerations dictate that, even if plasma-membrane vesiculation forms a major contribution to the overall biliary enzyme profile, plasma-membrane enzymes would occur in far greater relative proportions than any cytosolic enzymes that may be entrapped during vesiculation.

Studies have indicated that the working hypothesis of Coleman *et al.* (1977) cannot explain satisfactorily the total protein composition of mammalian bile. Kakis & Yousef (1978) have shown that, although biliary bile salt secretion fluctuates markedly over a 48h period after cannulation of the rat bile duct, bile flow and total biliary protein secretion remain relatively constant, suggesting that gross biliary protein secretion is unrelated to bile salt secretion. Mullock *et al.* (1978) reported that, of 16 polypeptide species identified in rat bile by two-dimensional gel electrophoresis, 13 are immunologically identical with rat serum proteins, and these workers suggested that a selective transport of proteins from serum to bile must occur. Also, rat bile contains much larger amounts of immunoglobulin A than rat serum (Lemaitre-Coelho *et al.*, 1977), and intravenously injected immunoglobulin A is rapidly transferred from blood to bile in rats (Jackson *et al.*, 1978; Orlans *et al.*, 1978). It would seem likely, therefore, that although the quantitatively major proportion of biliary protein arises from the serum, the unique enzyme composition of mammalian bile (i.e. the presence of plasma-membrane enzymes and the relative absence of intracellular enzymes) can be best explained by the release of plasma-membrane components from the bile canalicular membrane as a result of bile salt attack. Indeed, the output of both 5′-nucleotidase and alkaline phosphatase is increased when hepatic bile salt output is increased (Javitt, 1965; Bode *et al.*, 1973) and there is no parallel increase in the output of intracellular enzymes (Bode *et al.*, 1973).

It must be remembered that in the experiments reported in the present paper, the canalicular, sinusoidal and contiguous faces of the hepatocyte plasma membrane are exposed to bile salt attack, whereas *in vivo* only the canalicular membrane is normally exposed. In spite of this, isolated hepatocytes can withstand a concentration of 10–15mM of the conjugated trihydroxy bile salts, concentrations above the critical micellar concentration, without substantial lysis. Rat cannula bile often contains in excess of 20mM bile salts (see for example, Paumgartner, 1977; Coleman *et al.* 1979), mainly as taurocholate with some glycocholate and conjugated dihydroxy bile salts (Haslewood, 1967). Therefore, additional factors are probably involved in determining the resistance of the liver to gross membrane damage during bile formation.

One possibility is the presence of protein in bile. Rat bile contains appreciable amounts of various proteins, many of which are immunologically identical with serum proteins (Mullock *et al.*, 1978). Serum albumin (bovine; 1%, w/v) had a small protective effect on the release of components from hepatocytes by glycodeoxycholate, but not by glycocholate (Fig. 5). Since albumin binds bile salts (Makino *et al.*, 1973; Reichen *et al.*, 1977), it is possible that protein can significantly decrease the free bile salt concentration at the concentrations of glycodeoxycholate used (up to 3mM), but is less effective at the concentrations of glycocholate used (up to 20mM). Thus, protein binding of bile salts may be more important as a protective effect in biles rich in dihydroxy, rather than trihydroxy, bile salts.

The possibility that some special physicochemical features of the canalicular region of the hepatic plasma membrane may render it especially resistant to bile salt attack is perhaps of more interest. It is now known that the composition of the bile canalicular membrane differs substantially from other regions of the hepatocyte plasma membrane. Liver plasma-membrane preparations rich in canalicular membranes have been reported to contain greater proportions of sphingomyelin when compared with preparations rich in either sinusoidal or contiguous membranes (Yousef *et al.*, 1975; Kremmer *et al.*, 1976), and canalicular membranes have been shown to contain greater specific activities of 5′-nucleotidase, alkaline phosphoidesterase I and other hydrolytic enzymes (Wisher & Evans, 1975). The possible relationship of the sphingomyelin content of membranes to bile salt attack has recently been studied directly by Coleman & Billington (1979), who, in a series of model experiments with mammalian erythrocytes, have shown that membranes rich in sphingomyelin are more resistant to bile salt attack.

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