Biochem. J. (1979) **178**, 201–208
Printed in Great Britain

Membranes and Bile Formation

COMPOSITION OF SEVERAL MAMMALIAN BILES AND THEIR MEMBRANE-DAMAGING PROPERTIES

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(*Received* 26 *July* 1978)

The total content and profile of bile salts and phospholipids are reported for several mammalian biles. Rabbit and guinea-pig biles are characterized by high proportions of conjugated dihydroxy bile salts with respect to trihydroxy bile salts, but contain relatively little phospholipid. Both rabbit and guinea-pig biles exhibit little evidence of hepatic cell damage, even though they are able to cause membrane damage (as evidenced by lysis of human erythrocytes) at low (2–3 mM) concentrations of bile salts; this lytic behaviour is also a property of their predominant bile salts. Addition of phosphatidylcholine to the bile or bile salt is able to decrease the lytic behaviour. Perhaps the most significant observation is that these biles, and their predominant bile salts, are dramatically less lytic towards sheep erythrocytes, indicating that some factor(s) in membrane composition and structure may partly explain the resistance of membranes of the biliary tract to the presence of high concentrations of potentially membrane-damaging bile salts.

Mammalian biles contain high concentrations of potentially membrane-damaging detergents, the bile salts. Experiments with intact cells (Coleman & Holdsworth, 1976; Holdsworth & Coleman, 1976) and with isolated membrane preparations (Coleman *et al.*, 1976; Vyvoda *et al.*, 1977) have shown that the dihydroxy bile salt, deoxycholate (and its conjugate, glycodeoxycholate), is more damaging to membranes than the trihydroxy bile salt, cholate (and its conjugates, glycocholate and taurocholate). Our earlier work, together with supporting observations from other laboratories, prompted the suggestion that potential damage to the membranes of the hepatobiliary system is decreased by secretion of biles containing a high proportion of the less membrane-damaging bile salts (Coleman *et al.*, 1977).

Phospholipids have been reported in the biles of several mammalian species (Adams & Heath, 1963; Spitzer *et al.*, 1964; Illingworth & Glover, 1968; Heaton, 1972; Evans *et al.*, 1976) and occur as mixed micelles together with bile salts and cholesterol. The characteristics of phospholipid removal from intact erythrocytes by the less membrane-damaging bile salts (Coleman & Holdsworth, 1976; Billington *et al.*, 1977; Billington & Coleman, 1978) prompted suggestions for the origin of biliary lipids and the possible protection of the hepatobiliary system by the formation of mixed micelles with lipid (Coleman *et al.*, 1977).

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In order to explore further the relationship between the composition of mammalian bile and the effects of bile salts on biological membranes, we have examined bile from different species for their content and major distribution of bile salts, for their phospholipid content and, in some cases, for the presence of certain cellular enzymes. In addition, we have tested the biles from several species, and their predominant bile salts, for their ability to cause membrane damage (as judged by erythrocyte lysis) and the possible protection against membrane damage by added phospholipid. A preliminary account of some of this work has appeared (Coleman & Billington, 1978).

Materials and Methods

Materials

Ox, sheep and pig biles were obtained fresh from local slaughterhouses, and rabbit (New Zealand White, male, 2.5–3.5 kg) and guinea-pig (Dunkin–Hartley strain, female, 300–600 g) biles were obtained from freshly killed animals. In all cases bile samples were removed from the gall bladder and animals were used without regard to previous food intake. Rat bile was a gift from Dr. A. Hackett, Department of Biochemistry, Birmingham University, and was collected for 16 h after cannulation of the bile duct. A sample of human hepatic bile, from a patient undergoing biliary drainage after cholecystectomy, was kindly provided by Dr. H. G. Sammons, East Birmingham Hospital. All bile samples were either

used fresh or stored at −20°C for up to 2 weeks until required. Where appropriate, liver samples were obtained from the donor animals immediately after bile collection and homogenized (10%, w/v) in 1 mM-$NaHCO_3$, pH 7.4, in a tightly fitting Potter–Elvehjem homogenizer. The homogenates were filtered through a coarse nylon mesh and either used fresh or stored at −20°C until required. Human blood was obtained through the courtesy of the Midland Blood Transfusion Service and was used within 7 days of donation. Sheep blood was obtained fresh from local slaughterhouses and collected into acid citrate/glucose.

All bile salts (sodium salt, A grade) were obtained from Calbiochem, Bishop Stortford, Herts., U.K., except glycohyodeoxycholate, which was a gift from Professor G. A. D. Haslewood, Department of Biochemistry, Guy's Hospital Medical School, London. Hydroxy steroid dehydrogenase (grade II, from *Pseudomonas testosteroni*, containing both 3α- and 3β- activities) and other fine chemicals were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Other reagents were from Fisons, Loughborough, Leics., U.K., and were of the highest grade available.

Methods

Bile salt assay. Total bile salt concentrations were assayed by using hydroxy steroid dehydrogenase essentially as described by Talalay (1960). Bile samples were prepared for analysis by diluting 10–100 μl of bile to a final volume of 1 ml with methanol. The reaction mixture contained 33 mM-sodium pyrophosphate, pH 9.5, 0.33 M-hydrazine sulphate, 0.33 mM-NAD^+ and 0.1 ml of methanolic bile to give a final volume of 2.8 ml. The reaction was started by the addition of 0.5 unit of 3α-hydroxy steroid dehydrogenase (in 0.2 ml of 10 mM-potassium phosphate buffer, pH 7.4) and the change in A_{340} after 1 h at 20°C was recorded.

Bile salt chromatography. Bile salts were separated by t.l.c. on silica-gel H plates by an adaptation of the method of Ganshirt *et al.* (1960). Bile (1 vol.) was mixed with 4 vol. of methanol/acetone (1:1, v/v) and heated at 37°C for 2 min in a stoppered tube. After centrifugation at 1000*g* for 5 min various samples of the supernatant were loaded and the plate was developed with butan-1-ol/acetic acid/water (10:1:1, by vol.) as solvent. Spots were located with I_2 vapour and identified by reference to known standards. After the spots were marked, the plates were warmed in an oven to remove I_2 and the spots scraped off into stoppered tubes. The silicic acid was extracted for 30 min with 4 ml of methanol and, after centrifugation at 1000*g* for 5 min, 3 ml of this extract was evaporated to dryness. The residue was taken up in 0.1 ml of methanol and assayed for bile salts as described above.

Phospholipid analysis. Lipid extracts (Bligh & Dyer, 1959) of bile were assayed for phospholipid phosphorus basically by the method of Bartlett (1959), except that samples were digested with 72% (w/v) $HClO_4$ (Galliard *et al.*, 1965). Phospholipid chromatography was carried out on silica-gel H t.l.c. plates. Plates were developed with chloroform/methanol/acetic acid/water (75:45:12:2, by vol.) as solvent (Skipski *et al.*, 1964). Spots were detected with I_2 and identified by reference to known standards.

Isolation of phosphatidylcholine. Phosphatidylcholine was prepared from hen's-egg yolks by chromatography on alumina (Mangiapane *et al.*, 1973); it gave a single spot on t.l.c. in the same position as authentic phosphatidylcholine.

Enzyme assays. In experiments to compare the enzyme composition of bile with liver-homogenate samples from the same animal, the following assays were used: alkaline phosphatase (EC 3.1.3.1) (Kinne & Kinne-Saffran, 1969); alkaline phosphodiesterase I (EC 3.1.4.1) (Brightwell & Tappel, 1968); 5′-nucleotidase (EC 3.1.3.5) (Michell & Hawthorne, 1965) modified by determination of released P_i by the method of Baginski *et al.* (1967), and L-leucine-β-naphthylamidase (EC 3.4.11.1) (Goldbarg & Rutenberg, 1958) modified by inclusion of 1 mM-$CoCl_2$ in the assay system (Lewis, 1974). All these enzymes were assayed at 37°C. Owing to the low activities of some of these enzymes in bile, extended incubation times (up to 4 h) were sometimes required. Lactate dehydrogenase (EC 1.1.1.27) was assayed at 20°C by the method of Stolzenbach (1966). Possible inhibition of these enzymes by biliary constituents was investigated by mixing equal volumes of bile and liver homogenate at 0°C before assay, and comparing these with controls containing either bile or homogenate alone. Protein was determined (Lowry *et al.*, 1951) with bovine serum albumin as standard.

Acetylcholinesterase (EC 3.1.1.7) was assayed at 20°C as described previously (Billington & Coleman, 1978).

Membrane-damaging effects of various biles and purified bile salts. The membrane-damaging properties of mammalian biles were assayed by determining the extent of lysis of washed erythrocytes treated with increasing concentrations of bile or bile salts. Blood samples were centrifuged at 2500*g* for 10 min and the plasma and buffy coat removed by aspiration. The packed erythrocytes were washed with 3 × 5 vol. of 0.154 M-NaCl/1.5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], adjusted to pH 7.4 with NaOH and resuspended in an equal volume of this medium to give a final phospholipid concentration of approx. 2 mM. Then 1 vol. of the erythrocyte suspension was incubated at 37°C for 10 min with 3 vol. of 0.14 M-NaCl/15 mM-Hepes, pH 7.4, containing appropriate amounts of either

bile or bile salts. Incubations were terminated by centrifugation at 14000*g* for 1 min and the percentage haemolysis was determined by comparing the A_{525} of appropriate dilutions of the supernatant (with water) with that of an uncentrifuged incubation mixture totally haemolysed by dilution with 24 vol. of water. Haemolysis in the absence of bile or bile salts was always less than 0.5%.

When phosphatidylcholine was used, appropriate amounts were obtained by evaporation of chloroform solution on to the side of an incubation tube. Bile or bile salts and 0.14M-NaCl/15mM-Hepes, pH 7.4, were then added and the tube was preincubated at 37°C for 10 min before addition of erythrocytes.

Results

Bile salt and phospholipid content of mammalian biles

The bile salt content of gall-bladder bile from sheep, ox and pig was very high, concentration approx. 200mM (10%, w/v), and in the rabbit was even higher, approx. 350mM (17%, w/v) (Table 1). Gall-bladder bile from guinea pig was more dilute, approx. 25mM (1%, w/v), and resembled more the concentration of bile salts in bile from the rat (21 mM), which has no gall bladder, and human hepatic bile (14 mM) (Table 1).

All the above biles contained phospholipid, but the concentration of phospholipid did not follow that of the bile salts; this is best seen from the phospholipid/bile salt ratios. Thus sheep, ox, pig, rat and man appear as species with a relatively high content of phospholipid in relation to bile salts, whereas rabbit and guinea pig have a much lower phospholipid/bile salt ratio (Table 1).

Bile salt and phospholipid profiles of mammalian biles

The various biles showed considerable differences in their bile salt profile, with respect both to predominant conjugation type and to relative amounts of di- and tri-hydroxy bile salts. Taurine conjugates predominated in sheep, glycine conjugates predominated in rabbit and guinea pig, whereas ox and pig possessed significant amounts of both conjugation types (Table 2). Trihydroxy bile salts predominated in sheep and ox biles, whereas the biles of pig, rabbit and guinea pig were almost exclusively composed of dihydroxy bile salts (Table 2).

Lipid extraction and phospholipid chromatography (results not shown) showed that in every case phosphatidylcholine was the predominant (greater than 80%) phospholipid. In sheep bile an appreciable amount of lysophosphatidylcholine was found.

Table 1. *Bile salt and phospholipid content of biles from several mammalian species*

Determination of total bile salt and phospholipid concentrations were as described in the Materials and Methods section. Values are means ± S.D. for the numbers of observations given in parentheses. Only single samples of rat and human biles were available.

	Guinea pig (5)	Rabbit (6)	Sheep (5)	Ox (3)	Pig (4)	Rat	Human
Total bile salt concn. (mM)	25.8 ± 7.6	358 ± 92	191 ± 44	177 ± 25	208 ± 17	20.7	14.0
Total phospholipid concn. (mM)	0.11 ± 0.11	4.8 ± 1.8	10.8 ± 3.0	14.5 ± 3.9	22.8 ± 4.4	1.97	2.8
Phospholipid/bile salt molar ratio	0.004	0.013	0.057	0.082	0.110	0.095	0.200

Table 2. *Relative proportions of the main classes of bile salts in the biles of several mammalian species*

Bile salt chromatography and assay were as described in the Materials and Methods section. Values are mean percentages of the total material recovered from the t.l.c. plate ± S.D. for the numbers of observations given in parentheses. In some cases the values add up to less than 100%; this represents material at the origin or solvent front of the plate rather than other bile salts running at intermediate positions.

	Sheep (2)	Ox (3)	Pig (3)	Rabbit (3)	Guinea pig (3)
Taurotrihydroxy	55 ± 2.5	27 ± 10	6 ± 5	0	0
Taurodihydroxy	39 ± 0.5	5 ± 5	21 ± 4	0	5 ± 5
Glycotrihydroxy	1 ± 0.5	38 ± 9	2 ± 2	3 ± 2	1 ± 1
Glycodihydroxy	5 ± 0.5	17 ± 11	65 ± 12	96 ± 5	94 ± 6
Glycine/taurine ratio	0.07	1.7	2.5	100	19
Dihydroxy/trihydroxy ratio	0.78	0.33	11	32	99

Table 3. *Enzyme profile of rabbit and guinea-pig biles*

Enzyme activities were assayed as described in the Materials and Methods section and are expressed in nmol/min per mg of protein. Values are uncorrected for inhibitions and are means ± S.D. for the numbers of observations given in parentheses; inhibitions were determined in at least three samples and are expressed as percentages of control values. Protein values were as follows: guinea-pig bile, 0.64 ± 0.24 mg/ml (12); rabbit bile, 12.3 ± 3.1 mg/ml (15) (means ± S.D.).

	Alkaline phosphatase	5'-Nucleotidase	Alkaline phosphodiesterase I	L-Leucine β-naphthylamidase	Lactate dehydrogenase
Rabbit					
Sp. activity in bile	20.0 ± 6.2 (10)	0.81 ± 0.75 (6)	0.46 ± 0.26 (8)	0.012 ± 0.015 (6)	5.0 ± 3.9 (4)
Sp. activity in liver	7.5 ± 3.7 (10)	2.9 ± 0.55 (6)	9.2 ± 2.5 (8)	12.7 ± 1.7 (6)	1100 ± 110 (4)
Bile/liver ratio	2.7	0.28	0.05	0.001	0.005
Inhibition (%)	0–25	0–25	60–90	80–90	0–40
Guinea pig					
Sp. activity in bile	2.8 ± 0.9 (10)	0 (4)	4.2 ± 2.0 (12)	2.4 ± 1.5 (7)	13.5 ± 15.0 (4)
Sp. activity in liver	1.0 ± 0.4 (10)	4.2 ± 2.3 (4)	5.2 ± 2.3 (12)	18.6 ± 2.3 (7)	440 ± 60 (4)
Bile/liver ratio	2.8	0	0.80	0.13	0.031
Inhibition (%)	0–10	0–30	0–20	70–90	0–10

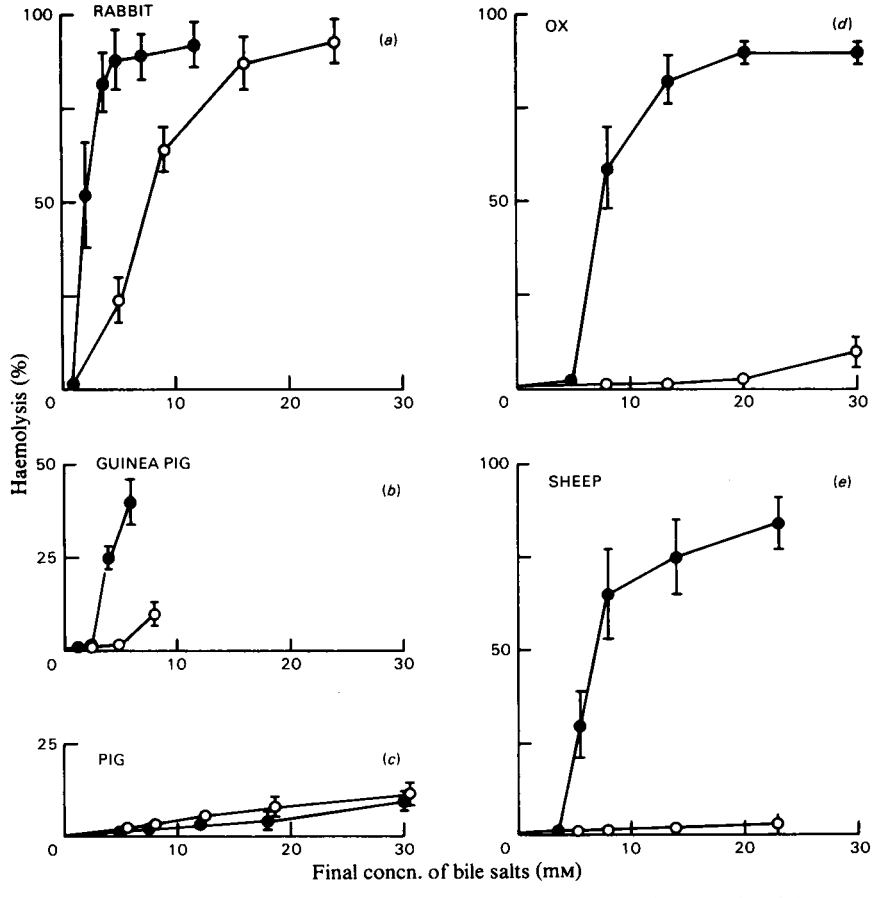

Fig. 1. *Haemolysis of human and sheep erythrocytes by several mammalian biles*

Human (●) or sheep (○) erythrocytes were incubated at 37°C for 10 min with bile from (*a*) rabbit, (*b*) guinea pig, (*c*) pig, (*d*) ox or (*e*) sheep. Values are means ± S.D. for either three or four duplicate observations.

Enzyme activities in rabbit and guinea-pig biles

The enzyme profiles of rabbit and guinea-pig biles were examined in order to assess whether cellular damage had occurred within the liver during their formation; the enzyme profiles of sheep, ox, pig, rat and human biles have been reported from this laboratory (Holdsworth & Coleman, 1975).

Perhaps the best indication of general cell damage is release of a cytosolic enzyme (e.g. lactate dehydrogenase). Table 3 shows that the lactate dehydrogenase activity of the bile is very low compared with its activity in the liver of the donor animal. In both cases lactate dehydrogenase was little inhibited by biliary constituents (Table 3), and thus its low specific activity in rabbit and guinea-pig biles probably indicates a relative absence rather than an inhibition of activity.

In spite of the apparent resistance of rabbit and guinea-pig liver to membrane damage resulting in enzyme leakage, the enzyme profiles of their biles show an appreciable content of plasma-membrane enzymes. Alkaline phosphatase was enriched 3-fold in both rabbit and guinea-pig bile compared with its specific activity in liver (Table 3). Alkaline phosphodiesterase I and L-leucine β-naphthylamidase were present in smaller amounts, but their apparent activities in bile must be greatly decreased as a result of inhibition caused by other biliary components; in the rabbit, 1 vol. of bile added to 1 vol. of liver homogenate inhibited both activities by about 80% (Table 3). 5'-Nucleotidase was present in rabbit bile, but was not detected in guinea-pig bile (Table 3).

Lysis of erythrocytes by several mammalian biles

Rabbit and guinea-pig biles caused extensive lysis of human erythrocytes at a bile salt concentration of 2–3 mM (Figs. 1*a* and 1*b*), whereas sheep and ox biles did not cause extensive lysis until 6–8 mM (Figs. 1*d* and 1*e*). Pig bile caused little lysis of human erythrocytes even at bile salt concentrations up to 30 mM (Fig. 1*c*).

Similar results were obtained with sheep erythrocytes in that rabbit and guinea-pig biles were more lytic than sheep, ox and pig biles (Fig. 1). Sheep erythrocytes were dramatically more resistant to haemolysis by these biles than were human erythrocytes (Fig. 1).

Lysis of erythrocytes by some dihydroxy bile salts

The effects of conjugated trihydroxy bile salts on human (Coleman & Holdsworth, 1976) and sheep (Billington *et al.*, 1977) erythrocytes have been previously reported. In this study, the effects of three glycine-conjugated dihydroxy bile salts (glycodeoxycholate, glycochenodeoxycholate, glycohyodeoxycholate) were compared; these represent the predominant bile salts of rabbit, guinea-pig and pig biles.

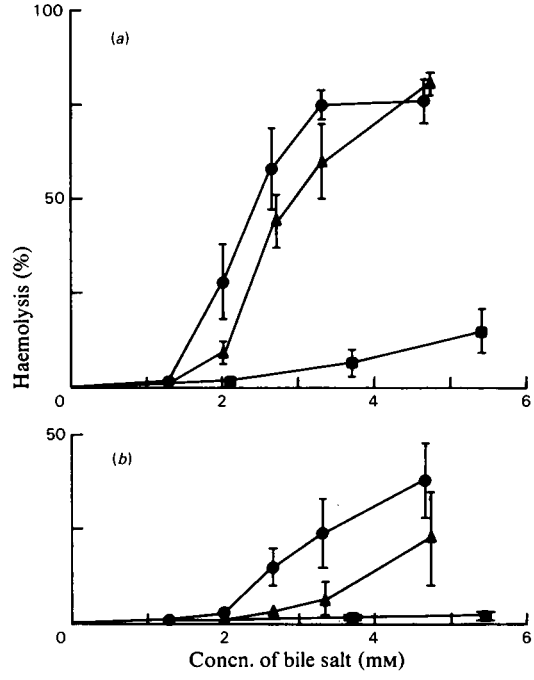

Fig. 2. *Haemolysis of human and sheep erythrocytes by dihydroxy bile salts*

Human (*a*) or sheep (*b*) erythrocytes were incubated at 37°C for 10 min with glycodeoxycholate (●), glycochenodeoxycholate (▲) or glycohyodeoxycholate (■). Values are means ± S.D. of 3 experiments.

Glycodeoxycholate and glycochenodeoxycholate caused approx. 50% haemolysis of human erythrocytes at 2–3 mM, whereas glycohyodeoxycholate was less membrane-damaging and caused only 10–15% haemolysis at 5.5 mM (Fig. 2*a*). Glycohyodeoxycholate was also less lytic than glycodeoxycholate and glycochenodeoxycholate when incubated with sheep erythrocytes (Fig. 2*b*). In every case, sheep erythrocytes were much more resistant to attack by these bile salts than were human erythrocytes (Fig. 2).

Effect of phospholipid on the lysis of human erythrocytes by rabbit bile and glycodeoxycholate

Phosphatidylcholine was used in this series of experiments since it is the major (greater than 80%) phospholipid of all the mammalian biles tested. A concentration of rabbit bile (2.5 mM), or glycodeoxycholate (1.37 mM), was chosen to give approx. 20% haemolysis. When the phospholipid/bile salt ratio was increased from its initial value of 0.01 in rabbit

bile to 0.1, by addition of phosphatidylcholine, haemolysis of human erythrocytes was dramatically decreased (Fig. 3*a*). Similarly, the haemolysis caused by glycodeoxycholate was decreased by added phosphatidylcholine (Fig. 3*b*). Also, the release of the externally orientated erythrocyte membrane protein, acetylcholinesterase (Steck, 1974), by both rabbit bile and glycodeoxycholate was decreased by added phosphatidylcholine, although to a lesser extent (Fig. 3).

Discussion

Bile salts, particularly deoxycholate, are frequently used at concentrations of up to 1% (w/v) to solubilize various components from isolated membrane preparations (see Coleman, 1974; Helenius & Simons, 1975). The concentration of bile salts in gall-bladder bile from guinea pig and in hepatic bile from rat and man is in this range, but in gall-bladder bile from sheep, ox, rabbit and pig it is considerably higher (10–15%, w/v) (Table 1). The gall bladder concentrates hepatic bile by as much as 10-fold by reabsorbing NaCl and water in iso-osmotic proportions (Diamond, 1965), and therefore the concentration of bile salts in hepatic bile of sheep, ox, rabbit and pig is probably of the order of 1% (w/v). Thus the ability of the liver to secrete such high concentrations of detergents without killing itself presents a problem which is biologically analogous to the survival of the pancreas, which secretes membrane-damaging enzymes, and to the survival of intestinal epithelial cells during extracellular digestion.

On the basis of some aspects of the composition of bile and of studies of the effects of different bile salts on isolated membranes and intact cells, an explanation for the above paradox was attempted by Coleman *et al.* (1977) in the following terms. (1) Mammalian liver secretes a bile salt pattern which contains a high proportion of 'mild' (i.e. less membrane-damaging) bile salts. (2) The bile salts are present in extremely low concentrations inside the hepatocyte and are pumped out by an active-transport system. (3) The bile salt concentration rises to micellar values in the canalicular lumen and materials are then removed from the outer leaflet of the plasma membrane without resulting in sufficient damage to cause leakage of intracellular materials; the individual phospholipid and enzyme profiles of the bile may be accounted for partly by the topography of plasma-membrane components and partly by the profile of bile salts being secreted. (4) The health of the membranes of the bile canaliculus is maintained by continuous biosynthetic repair mechanisms.

The biles of the species investigated in this study showed considerable differences in the relative amounts of the different classes of bile salts (Table 2). In sheep and ox biles, trihydroxy bile salts make up an important proportion of the bile salt complement; this pattern is basically similar to that reported for rat and man (Heaton, 1972). On the other hand, the biles of guinea pig, rabbit and pig contain almost

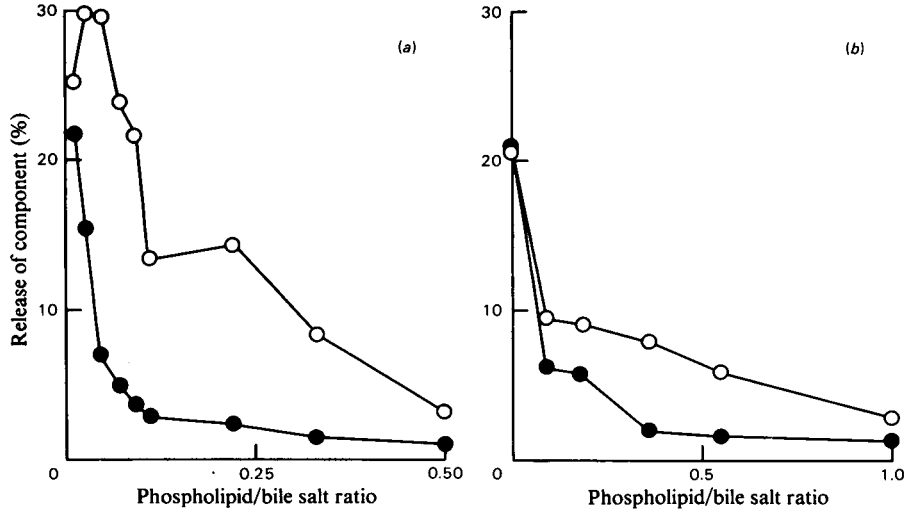

Fig. 3. *Effect of phosphatidylcholine on the release of haemoglobin and acetylcholinesterase from human erythrocytes by rabbit bile or by glycodeoxycholate*

Human erythrocytes were incubated at 37°C for 10 min with various amounts of phosphatidylcholine and either (*a*) rabbit bile (final bile salt concentration, 2.5 mM) or (*b*) 1.37 mM-glycodeoxycholate. ●, Haemoglobin release; ○, acetylcholinesterase release.

exclusively dihydroxy bile salts, and it is difficult to explain the composition of these biles in terms of the proposals outlined above.

It was therefore of immediate interest to examine rabbit and guinea-pig biles for evidence of cellular damage during their formation (the enzyme profile of pig bile has been reported by Holdsworth & Coleman, 1975). The cytosolic marker enzyme, lactate dehydrogenase, was present in rabbit and guinea-pig biles only at extremely low activities compared with its activity in the liver, indicating absence of extensive cell lysis. Also, alcohol dehydrogenase (EC 1.1.1.1) was not detected in these biles (P. P. Godfrey & S. Iqbal, unpublished work). However, putative plasma-membrane enzymes were present in rabbit and guinea-pig biles, and these enzymes may originate from the membrane of the bile canaliculus and/or the luminal surface of other cells lining the biliary tract (Coleman *et al.*, 1977). The overall enzyme composition of rabbit and guinea-pig biles shows many similarities to the biles of other species previously investigated (Holdsworth & Coleman, 1975). Thus intracellular enzymes are virtually absent, whereas several plasma-membrane marker enzymes are present in bile, and often at specific activities representing a considerable enrichment over their corresponding values in liver homogenates (Holdsworth & Coleman, 1975).

The biles of all species showed a spectrum of ability to elicit membrane damage, as evidenced by lysis of human and sheep erythrocytes. Previous experiments with purified bile salts have shown that dihydroxy bile salts are generally more membrane-damaging than are trihydroxy bile salts (Coleman & Holdsworth, 1976). This may partly explain why rabbit and guinea-pig biles (containing almost exclusively dihydroxy bile salts) are more haemolytic than sheep and ox biles (containing predominantly trihydroxy, but some dihydroxy, bile salts), which in turn are more haemolytic than is glycocholate alone (in similar experiments, greater than 20mM-glycocholate was required to cause extensive haemolysis of human erythrocytes; Billington & Coleman, 1978). The relatively non-haemolytic behaviour of pig bile is apparently anomalous in that the bile contains predominantly dihydroxy bile salts. An explanation for this lies in the relative lytic properties of the predominant bile salts of pig, rabbit and guinea pig. Thus glycohyodeoxycholate, the predominant bile salt of pig bile (Matschiner, 1971), was far less lytic than glycodeoxycholate, the predominant bile salt of rabbit bile (Gregg, 1966; Haslewood, 1967; Taylor, 1977), and glycochenodeoxycholate, the predominant bile salt of guinea-pig bile (Haslewood, 1967). In glycodeoxycholate (3α-OH, 7α-OH) and glycochenodeoxycholate (3α-OH, 12α-OH) the hydroxy groups lie on the same face as the glycine moiety, giving overall hydrophilic and hydrophobic regions to the molecule (Small, 1971). In glycohyodeoxycholate (3α-OH, 6α-OH) the 6α-hydroxy group may be located nearer to the hydrophobic region of the molecule, thereby decreasing its amphiphilicity.

The biles of all the species tested contained phospholipid, and in every case phosphatidylcholine was the predominant phospholipid. However, in the sheep there was also an appreciable amount of lysophosphatidylcholine present. This was also noticed by Adams & Heath (1963), who reported that approx. 40% of sheep bile phospholipid was lysophosphatidylcholine, whereas only trace amounts were detected in 2-week-old lambs. A striking anomaly, however, is the relative absence of phospholipid from the biles of rabbit and guinea pig, compared with those of sheep, ox, pig, rat and man (Table 1). Added phosphatidylcholine decreased the membrane-damaging effects of rabbit bile, and of its principle bile salt glycodeoxycholate. This occurred at phospholipid/bile salt ratios similar to those occurring *in vivo* in sheep, ox, pig, rat and human biles (see Table 1 and Fig. 2), and therefore implies that biliary phospholipid in some way attenuates the membrane-damaging effects of the bile salts. Indeed, the introduction of micelles containing only bile salts into the gall bladder of the dog has been reported to cause extensive inflammation of the epithelial mucosa (see Kappas & Palmer, 1963).

It is clear that the working hypothesis presented previously (see Coleman *et al.*, 1977, and above), in an attempt to link the properties of bile salts with biliary composition and physiology, is not able to account adequately for the situation in rabbit and guinea-pig biles, i.e. a highly membrane-damaging bile salt present in moderate (guinea pig) or high (rabbit) concentrations in the relative absence of phospholipid. Some further factor(s) are required to be taken into account and these may also be operating in other species. Thus there may be specific resistance of the plasma membranes lining the lumen of the biliary tract, including the bile canaliculus, to bile salt attack. Support for this idea is provided by the greater resistance to lysis of sheep erythrocytes than of human erythrocytes; this is seen with glycocholate (Billington *et al.*, 1977), with dihydroxy bile salts (Fig. 2) and with various biles (Fig. 1). What specific features of membrane composition or organization might dictate this difference are not yet known. It is noteworthy that sheep erythrocytes are smaller than those of human (erythrocyte volumes: sheep 30μm^3, human 87μm^3; Whittam, 1964), and, whereas human erythrocytes contain approx. 30% phosphatidylcholine, this is almost entirely replaced by sphingomyelin in sheep erythrocyte membranes (Nelson, 1972). Preparations rich in bile-canalicular membranes have been reported to have relatively high contents (18–25%) of sphingomyelin (Yousef *et al.*, 1975; Kremmer *et al.*, 1976), and this may contribute

to the resistance of membranes to bile salt attack (see Kremmer *et al.*, 1976). However, this amount of sphingomyelin is only equivalent to that in the human erythrocyte membrane (Coleman & Holdsworth, 1976; Billington & Coleman, 1978), but is less than that in membranes of sheep erythrocytes (Billington *et al.*, 1977).

The membrane-damaging effects reported here have mainly been explored with model systems on the assumption that they are relevant to the situation *in vivo* in liver. Clearly of more relevance would be the effects of bile salts studied directly in the lumen of the canaliculus, in which they are exposed to the outer face of the plasma membrane. However, owing to the technical inaccessibility of this structure, direct experiments are not feasible. Studies with related systems clearly have many of the objections that can be raised to studies with erythrocytes. Thus exposure of isolated liver cells to bile salts presents regions of the plasma membrane not normally exposed to these agents (e.g. the sinusoidal and contiguous faces), which may have a different susceptibility to bile salt attack from that of the canalicular membrane. Studies with isolated plasma membranes, as carried out by ourselves (Coleman *et al.*, 1976; Vyvoda *et al.*, 1977) and by Yousef & Fisher (1976), have the drawback that not only are different regions of the plasma membrane presented, but also both faces of the membrane. However, in spite of these drawbacks, these studies have shown that intact membrane-like structures can still be observed after exposure to quite high concentrations of some bile salts.

We thank Mrs. L. Woodhead for skilled technical assistance and the Medical Research Council for financial support.

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