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Enzymes and proteins in bile

Variations in output in rat cannula bile during and after depletion of the bile-salt pool

Philip P. GODFREY, Martin J. WARNER and Roger COLEMAN*
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

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The protein concentration in bile from several species is reported. The changes in output of protein, bile salts and several enzymes have been followed in rat bile over a 48h cannulation period. Bile-salt concentration dropped rapidly owing to interruption of the enterohepatic circulation but the output of protein, lysosomal enzymes [acid phosphatase (EC 3.1.3.2) and β-D-glucuronidase (EC 3.2.1.31)] and plasma-membrane enzymes [5′-nucleotidase (EC 3.1.3.5) and phosphodiesterase I (EC 3.1.4.1)] was maintained. Liver cell damage, monitored by output of lactate dehydrogenase, was very low throughout. Protein, lysosomal enzymes and plasma-membrane enzymes showed different patterns of output with time, but all showed a net increase between 12 and 24h. The output of lysosomal and plasma-membrane enzymes was between 1 and 5% of the total liver complement over the first 24h; if inhibition by biliary components is taken into account the output of some of these enzymes, particularly acid phosphatase, may be greater. Ultracentrifugation of bile showed that as the concentration of bile salts decreases the proportion of plasma-membrane enzymes in a sedimentable form increases. The results are discussed in relation to other studies of biliary proteins and to studies of the perturbation of membranes and cells with bile salts.

In contrast to the extensive studies that have been made of the content and output of bile salts, cholesterol, phospholipids and pigments in mammalian bile, biliary proteins have received much less attention. Electrophoretic and immunological studies of human (Russell & Burnett, 1963; Wales *et al.*, 1969; Englert, 1970; Dive & Heremans, 1974) and rat (Lemaitre-Coelho *et al.*, 1977; Mullock *et al.*, 1978) bile indicate the presence of many protein species. Proteins derived from serum contribute a substantial proportion of total protein, but most of these occur at lower concentrations and in different relative proportions to their contributions in serum (Mullock *et al.*, 1978). IgA, in polymeric form bound to secretory component, occurs at a higher concentration in bile than in serum and this, together with free secretory component, account for another substantial contribution to the biliary-protein profile (Lemaitre-Coelho *et al.*, 1977; Mullock *et al.*, 1978).

A number of hepatocyte enzymes have been identified in normal bile; these appear to be derived largely from the plasma membrane and lysosomes, with other characteristic intracellular enzymes being present in much lower relative concentrations (Holdsworth & Coleman, 1975; Evans *et al.*, 1976; La Russo & Fowler, 1979; Coleman *et al.*, 1979); this indicates that there is little damage to the hepatocyte during bile formation, even though bile contains high concentrations of potentially membrane-damaging bile salts (Coleman *et al.*, 1977, 1979). The presence of lysosomal enzymes, in the absence of other intracellular enzymes, indicates a specific discharge of lysosomal contents into bile (De Duve & Wattiaux, 1966; Mullock *et al.*, 1978; La Russo & Fowler, 1979).

The activities of several plasma-membrane enzymes have been identified in the bile of a large number of mammalian species (Holdsworth & Coleman, 1975; Evans *et al.*, 1976; La Russo & Fowler, 1979; Coleman *et al.*, 1979) and, where examined, the biliary and membrane forms have been shown to be identical (Pope & Cooperband, 1966; Price *et al.*, 1972; Evans *et al.*, 1976; Mullock *et al.*, 1977). These enzymes have been shown to be released from the plasma membranes of isolated hepatocytes by the action of bile salts (Billington *et al.*, 1980), and it has been suggested that the presence of plasma-membrane enzymes in bile may

* To whom correspondence should be addressed.

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be the result of bile-salt action on the plasma membrane in the bile canaliculus (Coleman *et al.*, 1977).

In the present study we report the changes seen in a number of proteins, especially the plasma-membrane (5′-nucleotidase and phosphodiesterase I) and lysosomal enzymes (acid phosphatase and β-D-glucuronidase) in rat bile, at various times after interruption of the enterohepatic circulation and consequent depletion of the bile-salt pool, to learn more of the factors involved in the secretion of the various biliary proteins.

Materials and methods

Materials

Male Wistar rats, weighing approx. 350g, were used throughout. These had been maintained on standard laboratory diet and under a constant light cycle; all were cannulated at approx. 10.00h. Bile-duct cannulation was performed while the rat was under pentobarbital anaesthesia. After cannulation the rats were kept in restraining cages with free access to food and water. Bile was collected on ice and, for most experiments, was then stored at −20°C until analysed. At the end of the experiment the animal was killed and the liver removed and homogenized in 9vol. of 0.14M-NaCl/0.015M-Hepes, pH7.4, in a tightly fitting Potter–Elvehjem homogenizer. The homogenate was filtered through coarse nylon mesh and was frozen at −20°C until required; storage at −20°C did not affect the activities of either homogenate or biliary enzymes.

In some experiments, before freezing the bile, samples were taken for centrifugation. These were centrifuged initially at 2500*g* for 10min. A portion of the supernatant was then further centrifuged at 150000*g* for 60min. Any pellets obtained were suspended in 0.14M-NaCl/15mM-Hepes (pH7.4) and pellets and supernatants were stored at −20°C until required. Similar centrifugations were performed on samples of normal sheep gall-bladder bile obtained from a local slaughterhouse and transported to the laboratory on ice. Ox and pig gall-bladder biles were obtained in a similar fashion. Gall-bladder bile was obtained from rabbits (males, New Zealand White, approx. 3kg body wt.), laboratory frogs and guinea pigs (males, Dunkin–Hartley strain, approx. 500g body wt.) by syringe, immediately after death. A sample of human T-tube bile, from a patient undergoing biliary drainage after cholecystectomy, was kindly provided by Dr. H. G. Sammons, East Birmingham Hospital.

Hydroxysteroid dehydrogenase (grade II from *Pseudomonas testosteroni*) 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

The following enzyme assays were used: 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 inorganic phosphate by the method of Baginski *et al.* (1967); acid phosphatase (EC 3.1.3.2) (Hübscher & West, 1965); β-D-glucuronidase (EC 3.2.1.31) (Gianetto & De Duve, 1955); lactate dehydrogenase (EC 1.1.1.27) (Stolzenbach, 1966). All enzymes were assayed at 37°C, apart from lactate dehydrogenase which was assayed at 20°C. Each enzyme was assayed under conditions where activity was proportional to protein concentration; suitable enzyme and substrate blanks were incorporated in all assays. Activities are expressed as μmol of substrate hydrolysed/h.

Total bile salt was assayed with 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) (Coleman *et al.*, 1979). Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The addition of known amounts of protein to bile samples gave quantitative estimation; controls for the contributions of bile pigment and small-molecular-weight biliary components were investigated and found to be of minimal importance.

Results and discussion

Output of protein

It is clear from Table 1 that protein occurs in the bile of all the species studied. Rabbit gall-bladder bile (approx. 350mM-bile salts; for values see Coleman *et al.*, 1979) has a higher protein concentration than ox, sheep and pig gall-bladder biles (approx. 200mM-bile salts), whereas guinea-pig gall-bladder bile (approx. 25mM-bile salts) has the lowest protein concentration. This correlation of protein level with bile salt concentration cannot be taken too far, however, since the level of protein in rat bile, a hepatic bile and therefore dilute (approx. 20mM-bile salt), is substantial; this indicates in the rat, at least, the majority of protein output is not directly related to bile-salt output.

This can be seen more strikingly in Fig. 1, which shows that overall protein output in these bile-duct-cannulated rats is maintained in spite of a substantial fall in bile-salt concentration to a level maintained by biosynthesis *de novo*; moreover, protein levels fall after 36h at a time when bile-salt output is beginning to rise again. These patterns of overall protein output are essentially similar to those observed by Kakis & Yousef (1978) and by La Russo & Fowler (1979).

There is quite a large discrepancy in the values

obtained for protein concentration in rat cannula bile; these range between 1 and 2mg/ml (Elias & Boyer, 1978; Reichen *et al.*, 1979), 2–5mg/ml (La Russo & Fowler, 1979), 8–15mg/ml (present work) and 16–20mg/ml (Kakis & Yousef, 1978). The reasons for these differences are not known, since many workers have used similar measurement techniques, and our controls (see the Materials and methods section) indicated little extra contribution to, or interference with, colour development. Amongst factors that may contribute to these differences are (i) the time of collection (all the low value results are from within the first hour of cannulation, all the higher values are from extended studies) and (ii) sex, age, body weight and feeding patterns etc.

Table 1. *Total protein concentration in bile of various species*

Values represent means ± S.D. (no. of observations are given in parentheses) of duplicate determinations. * Over 48h cannulation period; ** pooled sample from five animals; g.b., gall bladder.

Animal	Source	Total protein (mg/ml)
Sheep (14)	g.b.	8.5 ± 2.6
Ox (6)	g.b.	3.8 ± 0.9
Pig (8)	g.b.	4.7 ± 1.6
Rabbit (7)	g.b.	14.2 ± 5.4
Guinea pig (8)	g.b.	0.64 ± 0.11
Rat (5)	cannula	11.5 ± 2.3
Human (1)	T-tube	5.2
Frog (5)**	g.b.	12.5

Output of lysosomal enzymes

The output of acid phosphatase and β-D-glucuronidase, enzymes normally found in lysosomes, is low initially, rises dramatically to a maximum at about 24–30h, then falls (Fig. 1); this pattern can be seen in the data of La Russo & Fowler (1979), but these authors did not investigate the fall in output on

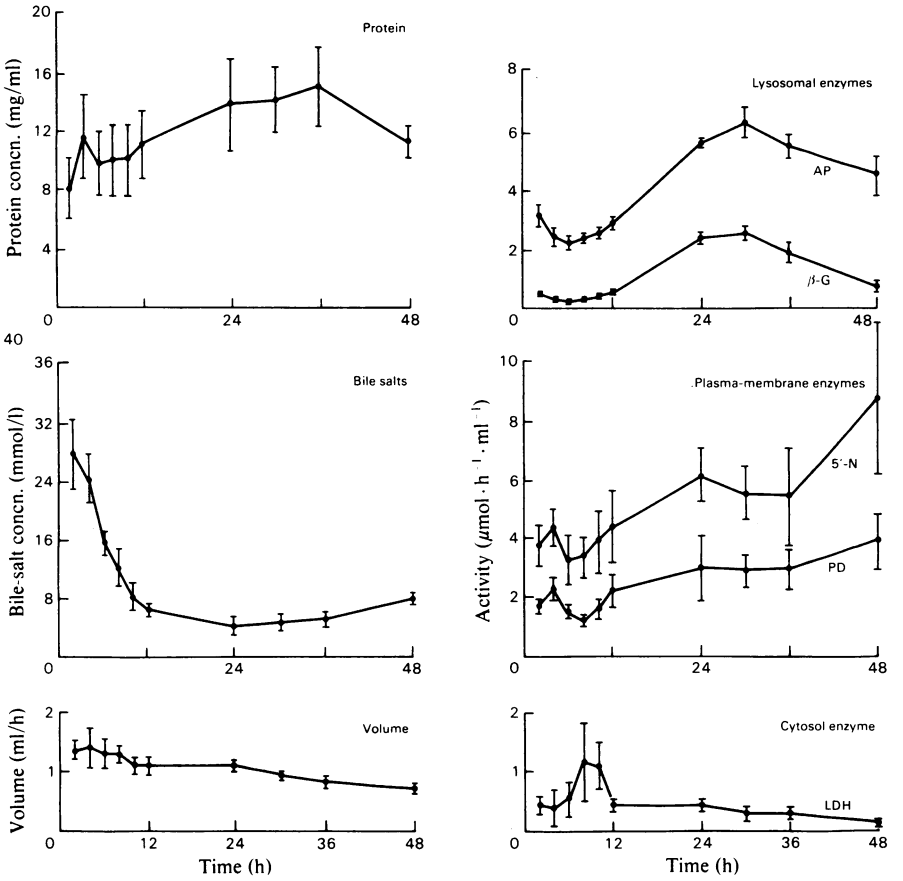

Fig. 1. *Composition of bile as a function of time after cannulation*

Values represent means ± S.E.M. ($n = 5$ or $n = 4$: AP, β-G, LDH) for duplicate determinations. Abbreviations used: AP, acid phosphatase; β-G, β-D-glucuronidase; 5'-N, 5'-nucleotidase; PD, phosphodiesterase I; LDH, lactate dehydrogenase.

the second day. The pattern of lysosomal-enzyme output somewhat parallels that of total protein, but the amount of lysosomal protein contributing to the overall protein content is not known. There is no parallelism with bile-salt output and thus it is likely that the two phenomena are unrelated; the peak of lysosomal-enzyme output is also clearly not the result of cellular damage since cytosol-enzyme release (lactate dehydrogenase) is low at this time (Fig. 1). It has been suggested that lysosomes may be involved in the transport of some proteins from serum to bile (Mullock *et al.*, 1978) and it is therefore possible that the increase in overall biliary protein at around 24h may be the result of increased lysosomal transfer activity.

Comparison of the amount of lysosomal enzymes put out in the first 24h with their total amount in liver (Table 2) shows that this output is substantial; it is much greater than the relative release of lactate dehydrogenase (0.02%) and further indicates that lysosomal-enzyme output is much more specific than can be accounted for by cell damage. Both of the lysosomal enzymes are inhibited (in liver homogenates) by some component(s) of bile (Table 2) and their apparent activity in bile may therefore be lower than their actual content. This is confirmed by their activation during gel exclusion chromatography of bile (Table 3), a phenomenon noted for other lysosomal enzymes by Toyoda *et al.* (1977). The nature of the inhibitory compound(s) is not known but the pattern of inhibition/activation does not bear any simple relationship to bile-salt concentration. When these activations on gel exclusion chromatography are taken into account the output of lysosomal enzymes may lie between 5 and 10% of the total liver content per day. Biliary protein output amounts to about 15% of the amount of protein in the liver per day but, since most of this protein may be derived from serum (Mullock *et al.*, 1978), this value cannot be as readily compared with material derived directly from liver cells.

Output of plasma-membrane enzymes

The output of plasma-membrane enzymes over the first 24h is also substantial, amounting, after allowance for inhibitions (Tables 2 and 3), to about 2–3% of the total liver complement per day for both 5′-nucleotidase and phosphodiesterase I, once again far more than can be accounted for by cell damage (lactate dehydrogenase output). The concentrations of both plasma-membrane enzymes in the bile increased slightly over the first 4h, declined and then rose slowly overall for the remainder of the cannulation period (Fig. 1). This pattern is distinct

Table 2. *Output of biliary components in 24h as a fraction of total liver content*

Total liver content was assessed from homogenates [10% (w/v) in 0.14M-NaCl/15mM-Hepes (pH7.4)]. Protein is expressed as mg/liver or mg/24h. Inhibition (in two experiments) was assessed by incubation of 1vol. of liver homogenate with 5vol. of bile (0–2, 24–30h) for 10min at 25°C: portions were then sampled for activity and compared with an equivalent amount of untreated homogenate. Inhibition was not allowed for in the 24h bile values. Bile and liver values are means ± S.E.M. ($n = 5$).

	Content					
	Protein	5′-Nucleotidase	Phosphodiesterase I	β-D-Glucuronidase	Acid phosphatase	Lactate dehydrogenase
Total activity in 0–24h bile (μmol of substrate hydrolysed/h)	300 ± 71	142 ± 20	65.1 ± 15.6	41.8 ± 7.6	122 ± 14	65 ± 15
Total activity in liver (μmol of substrate hydrolysed/h)	2160 ± 70	5690 ± 1110	6800 ± 560	794 ± 114	10100 ± 2000	270000 ± 7250
Secretion in 0–24h (%)	13.9	2.5	1.0	5.2	1.2	0.02
Inhibition (% of control)	—	0	5,5	10,10	50,10	0

Table 3. *Gel exclusion chromatography of rat bile*

Samples (1ml) of bile were applied to Sephadex G-50 columns (1.5cm diam. × 8cm) and the columns eluted with 140mM-NaCl/15mM-Hepes, pH7.4. Enzyme activities were recorded as the total enzyme activity recovered from the column, expressed as a percentage of the total activity applied (assayed directly on untreated bile samples). Bile samples used represent collections pooled between the times shown. Values are means ± S.E.M. ($n = 4$).

	Activity recovered (%)			
Bile sample applied	5′-Nucleotidase	Phosphodiesterase I	β-D-Glucuronidase	Acid phosphatase
0–10h	107 ± 15	197 ± 38	210 ± 18	404 ± 14
11–24h	117 ± 18	187 ± 40	220 ± 23	750 ± 28

from that of lysosomal, cytosol or total proteins and is also different from the pattern of total bile-salt secretion except that the general increase in concentration after 24h is shared by both bile salts and the plasma-membrane enzymes (Fig. 1).

In trying to arrive at a mechanism for the appearance of plasma-membrane enzymes in bile, attention has long been focused on the possible involvement of bile salts, thus infusion of taurocholate increased the biliary output of 5'-nucleotidase (Javitt, 1965; M. Eakins, personal communication) and alkaline phosphatase (Bode *et al.*, 1973), whereas secretin (a stimulator of bile salt-independent flow) and dehydrocholate (which does not form micelles), were much less effective (Bode *et al.*, 1973). Consideration of the role of bile salts has mainly been focused on their detergent properties that might enable them to solubilize the enzymes from the canalicular membrane at high concentration (Song *et al.*, 1967; Holdsworth & Coleman, 1975; Coleman *et al.*, 1976; Evans *et al.*, 1976; Yousef & Fisher, 1976; Vyvoda, *et al.*, 1977). In other studies a comparison has been made of the membrane-perturbing effects of bile salts at lower concentrations and these have shown that the various bile salts differ in their membrane-perturbing ability and that the bile salts could cause the removal of plasma membrane material from cells without causing cell lysis (Coleman & Holdsworth, 1976; Billington & Coleman, 1978; Billington *et al.*, 1980).

The maintenance, and even increase, of plasma-membrane output in the face of decreased output of total bile salts requires comment. Kakis & Yousef (1978) have shown that during biliary drainage the composition of the bile-salt output changes from one containing 16% deoxycholate conjugates and 40% cholate conjugates to one containing no deoxycholate conjugates and 60% cholate conjugates; thus, although the overall concentration of bile salts is falling (Fig. 1), the proportion of cholate conjugates is increasing. In the experiments of Billington *et al.* (1980), on isolated hepatocytes, it was found that glycocholate released a higher proportion of each enzyme at low bile-salt concentrations than did glycodeoxycholate. The effectiveness of cholate conjugates at low concentrations may therefore be a reason for the maintenance of plasma-membrane enzyme output.

The experiments with hepatocytes (Billington *et al.*, 1980) have also shown that enzymes released from the plasma membrane by low concentrations (7mM) of bile salts were predominantly (50–95%) in sedimentable form; these are probably identifiable with small vesicles of unique composition pinched off from the cell surface (See Billington & Coleman, 1978). As the concentration of bile salts to which the cells were exposed was increased a greater proportion of the membrane material was released in 'solubilized' form (Billington & Coleman, 1978; Billington *et al.*, 1980), the membrane phospholipids being taken into mixed micelles with the bile salt.

These findings may be highly relevant to the results on the centrifugation of bile (Table 4). Although only small amounts of total protein were sedimented (4–9%), this sedimented material was greatly enriched in the two plasma-membrane enzymes, 5'-nucleotidase and phosphodiesterase I, and, to a lesser extent, with β-D-glucuronidase, whereas the other lysosomal enzyme acid phosphatase remained largely soluble. The relative amounts of the two plasma-membrane enzymes were high (48–67%) in the later stages of bile production, when the bile-salt concentration was low (<8mM), whereas the proportion of sedimentable material was lower (27–39%) in the earlier stages of bile production, when the bile-salt concentration was higher; at higher bile-salt concentrations the bile also contained appreciable quantities (approx 1.5mM) of phospholipid. Sheep bile, which has only a small proportion (<10%) of these enzymes in sediment-

Table 4. *Ultracentrifugation of bile*

Bile was centrifuged at 2500*g* for 10min. The supernatant was centrifuged at 150000*g* for 60min. The pellet then obtained was suspended in a volume of 0.14M-NaCl/15mM-Hepes, pH 7.4, equal to the volume of the original bile sample and recentrifuged. The washed pellet was resuspended in the NaCl/Hepes buffer. Bile samples represent collections pooled between the times shown (rat) or gall-bladder bile (sheep). Values are recorded as the total activity or amount in the final pellet expressed as a percentage of the total activity or amount recovered (the initial pellet at 2500*g* for 10min contained <2% of all parameters); values are means ± S.E.M. (no. of observations are given in parentheses).

	Content in 150000*g*, 60min pellet (%)				
Bile sample	Protein	5'-Nucleotidase	Phosphodiesterase I	β-D-Glucuronidase	Acid phosphatase
Rat 0–10h (8)	4.0 ± 0.4	27.7 ± 3.1	39.1 ± 2.3	30.5 ± 5.4	9.0 ± 2.5
Rat 11–24h (8)	4.7 ± 1.1	55.9 ± 3.6	50.5 ± 3.5	27.9 ± 5.6	6.5 ± 2.3
Rat 31–48h (3)	8.8 ± 2.0	67.1 ± 13.8	48.4 ± 10.4	42.7 ± 8.9	6.6 ± 1.6
Sheep (9)	10.2 ± 0.6	8.1 ± 1.9	9.4 ± 2.2	—	5.0 ± 1.4

able form (Table 4), has both a high bile-salt (approx. 190mM) and a high phospholipid (approx. 10mM) concentration (Coleman *et al.*, 1979). One interpretation for this data is that the plasma-membrane material may be released initially in the form of small vesicles pinched off the tips of the microvilli of the bile canaliculus: this could occur at low bile-salt concentrations. If more bile salts were secreted, e.g. before depletion of the bile-salt pool or when the bile salts become concentrated in a gall gladder, this initial particulate material would become 'solubilized' until, as in the case of the sheep, at very high concentrations of bile salts most of the material would be in solubilized form.

Total protein secretion

The elaboration of the protein complement of bile is clearly complex and may possibly involve transport via vesicles, membrane perturbation and even leakage via tight junctions (see Hinton *et al.*, 1980). Studies of cholestasis, caused by a variety of compounds of different action (Elias & Boyer, 1979), may prove to be a method for the manipulation of the various processes in the elaboration of the biliary-protein profile; such studies are our current interest.

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