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Effect of taurochenodeoxycholate or tauroursodeoxycholate upon biliary output of phospholipids and plasma-membrane enzymes, and the extent of cell damage, in isolated perfused rat livers

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Isolated perfused rat livers were used to study the effects of taurochenodeoxycholate (TCDC) and tauroursodeoxycholate (TUDC) upon some aspects of biliary composition. After depletion of the endogenous bile salt pool of the liver, introduction of either bile salt brought about increases in bile flow, bile salt output and biliary phospholipid output. Taurochenodeoxycholate needed a lower biliary concentration to produce phospholipid output than did tauroursodeoxycholate. TCDC perfusion caused a substantial output of plasma-membrane enzymes (5′-nucleotidase and alkaline phosphodiesterase) into the bile, whereas TUDC caused little output of either enzyme; this may represent a characteristic difference between the effects of the two bile salts on the hepatobiliary system. The results from TUDC perfusion indicate also that much of the output of biliary phospholipid promoted by bile salts, may be independent of the output of plasma-membrane enzymes promoted by bile salts.

It has been suggested that bile salts are responsible for the existence of plasma-membrane material in bile (Holdsworth & Coleman, 1976; Coleman *et al.*, 1979; Godfrey *et al.*, 1981). Erythrocytes and isolated hepatocytes have been shown to release plasma-membrane enzymes and phospholipid at prelytic concentrations of bile salts (Coleman & Holdsworth, 1976; Billington & Coleman, 1978; Billington *et al.*, 1980). Similarly, administration of micelle-forming bile salts to humans (Bode *et al.*, 1973), and whole animals (Layden & Poppe, 1977; Reichen *et al.*, 1979; Billington *et al.*, 1983), increased the output of plasma-membrane enzymes and phospholipid into bile without intracellular enzyme leakage. Isolated perfused rat livers depleted of their own bile salt pool, and subsequently perfused with individual bile salts, produced bile enriched in plasma-membrane enzymes at a much lower concentration of GDOC than of TC (Barnwell *et al.*, 1983). Bile from livers treated with TDHC (a non-micelle-forming bile salt analogue) showed no enrichment in plasma-membrane material.

The bile acids chenodeoxycholate and its 7β-isomer ursodeoxycholate have, in recent years, been used as an effective means of treating gallstones (see, for example, reviews by Bouchier, 1980; Tokyo Co-operative Gallstone Study Group, 1980; Iser & Sali, 1981; Bachrach & Hofmann, 1982*a*,*b*). These bile salts reduce the cholesterol saturation of bile, allowing the cholesterol gallstones to redissolve. The rate-limiting enzymic step in cholesterol synthesis is hydroxymethylglutaryl-CoA reductase (Bucher *et al.*, 1960). Patients with gallstones usually have an increased hydroxymethylglutaryl-CoA reductase activity (Nicolau *et al.*, 1974; Coyne *et al.*, 1976; Maton *et al.*, 1980), which can be suppressed by TCDC (Coyne *et al.*, 1976; Maton & Dowling, 1979; Maton *et al.*, 1979, 1980) and TUDC (Maton *et al.*, 1977, 1979; Nakagawa *et al.*, 1977). [Cholesterol absorption from the gut has been shown to be unaffected by either bile salt (La Russo & Thistle, 1983).]

This investigation reports the contrasting effects of TCDC and TUDC upon the release of other materials into bile, especially phospholipids and enzymes probably originating from the plasma membrane, and compares these effects with those of other bile salts, TC and TDHC, used previously (Barnwell *et al.*, 1983).

Abbreviations used: GDOC, glycodeoxycholate; TC, taurocholate; TCDC, taurochenodeoxycholate; TUDC, tauroursodeoxycholate; TDHC, taurodehydrocholate.

Materials

The bile salts were obtained from Calbiochem–Behring Corporation, Bishop's Stortford, Herts.,

U.K. Hydroxy-steroid dehydrogenase (grade II, from *Pseudomonas testosteroni*) and the other fine chemicals were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Sagatal was obtained from May and Baker, Dagenham, Essex, U.K. Cannulation tubing pp10 was made by Portex, Hythe, Kent, U.K., and heparin was made by Weddel Pharmaceuticals, London, U.K.

Methods

Isolated perfused livers

Male Wistar rats, weighing 220–280g, were used throughout. These had been maintained on a standard laboratory diet and under a constant light cycle. Bile-duct cannulations were performed with pp10 tubing while the rats were under pentobarbital (Sagatal) anaesthesia. Bile was collected on ice throughout the experiment. At 20min after cannulation, the livers were isolated using the method previously described by Barnwell *et al.* (1983).

At 100min after initial cannulation (80min after isolation), the perfusion medium was changed to replenish substrates used by the liver. It was at this point, when the endogenous bile salt concentration in the collected bile had fallen to approx. 2mM, that exogenous bile salts were introduced into the new perfusion medium.

Bile collection was terminated at 190min, whereupon the liver was removed and homogenized as described in Barnwell *et al.* (1983). Samples of the perfusate were taken at 100min and 190min to test for liver damage after first removing erythrocytes by centrifugation at 400*g* for 5min. All samples were stored at −20°C until required and were found to be stable during the analytical period.

Addition of bile salts

At 100min, bile salts (dissolved in a minimal volume of Krebs–Ringer bicarbonate buffer, pH 7.4) were added to the perfusion medium to rapidly bring the concentration to 200μM. The total amount of TC, TDHC, TUDC or TCDC added over the period 100–190min was 56μmol in each case. Initially 20μmol of bile salt was added to the perfusate at 100min followed by another 36μmol added by means of an infusion pump during the period 100–190min.

Assays

Bile, perfusates and homogenates from TCDC- and TUDC-treated livers were analysed for the following enzymes: 5′-nucleotidase (EC 3.1.3.5); phosphodiesterase I (EC 3.1.4.1); as described by Godfrey *et al.* (1981). Aspartate aminotransferase (EC 2.6.1.1) activity was measured by the method of Karmen (1955).

All determinations were carried out at 37°C. Total bile salt concentrations in bile were determined using 3α-hydroxy-steroid dehydrogenase (EC 1.1.1.50) as used by Coleman *et al.* (1979). Total protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The amount of phospholipid found in bile was estimated after lipid extraction by the method of Bligh & Dyer (1959) and phospholipid phosphate determination by the method of Bartlett (1959).

Enzyme inhibition by bile salts

Aspartate aminotransferase, from homogenates, was not found to be inhibited by either TCDC or TUDC up to a concentration of 50mM; these results agree with those of Schmidt *et al.* (1982).

5′-Nucleotidase activity was not inhibited (±10%) in plasma-membrane preparations (prepared as in Lowe & Coleman, 1982) incubated with various concentrations of TUDC or TCDC up to 40mM. Passage of bile samples containing TCDC or TUDC (in high concentrations) down Sephadex G-50 columns to separate protein from micelles and from low-molecular-weight materials resulted in no (±10%) change in 5′-nucleotidase activity.

Alkaline phosphodiesterase activity in plasma-membrane preparations was inhibited up to 30% by 40mM-TUDC but was not (±10%) affected by TCDC. The activity of the inhibited enzyme could be partially restored on passage of the membranes down Sephadex G-50 columns. Passage of bile samples containing TCDC and TUDC down Sephadex columns did not (±10%) result in any change in activity of TCDC-containing biles, but brought about variable activation up to 2-fold in bile samples containing TUDC; thus the amounts put out may be up to 2-fold higher than those recorded in Fig. 1.

Results and discussion

During the period 20–100 minutes, when the isolated liver was perfused with bile-salt-free media, the output of biliary phospholipid declined as shown in Fig. 1, together with the other parameters as reported by Barnwell *et al.* (1983). The general health of perfused livers was indicated by even perfusion, high O_2 consumption (shown as a sharp colour difference between the inflow and outflow cannulas) and the ability to produce bile, a feat that requires the integrated metabolism of the liver cell. In addition, samples of the perfusion fluid were taken at 100min and 190min, and together with all the bile samples, analysed for aspartate aminotransferase, an intracellular enzyme, which would quantify the extent of liver cell damage. This cytosolic marker has two advantages over lactate dehydrogenase (EC

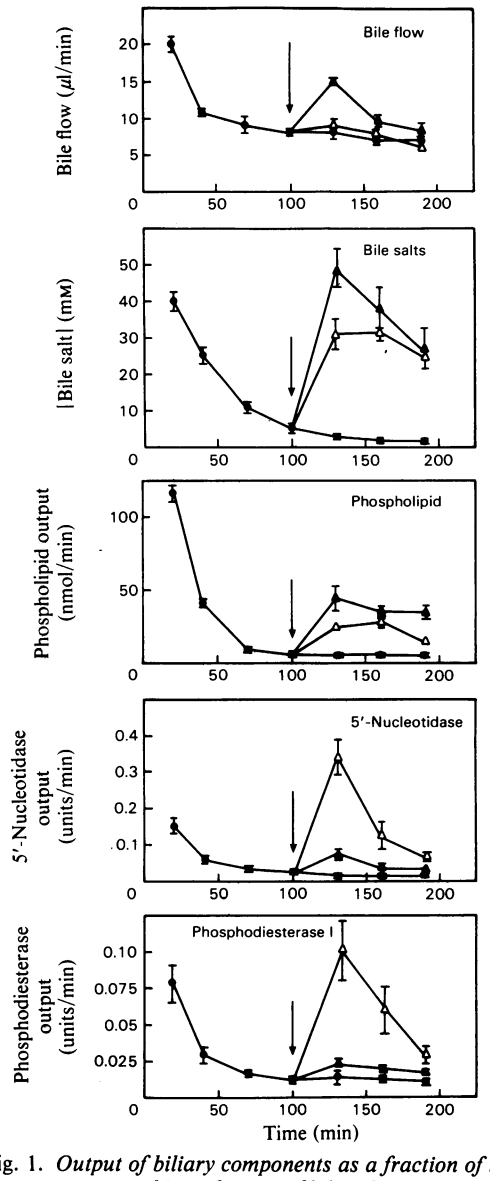

Fig. 1. *Output of biliary components as a fraction of time and introduction of bile salts*

Bile ducts were cannulated at $t = 0$. Isolation and perfusion were commenced at $t = 20$ min. The perfusion fluid was renewed and bile salts introduced at $t = 100$ min (indicated by the arrow). Bile was collected between the time intervals shown and the analyses were recorded as 0–20 min at ($t = 20$ min) etc. Enzyme units are μmol of substrate hydrolysed/h at 37°C. Output is obtained by dividing the total amount collected in the time interval by the number of minutes in the collection period. Values are means ± S.E.M. ($n = 4$) of duplicate determinations. Symbols: ●, control with no added bile salt; ▲, TUDC; △, TCDC. These bile salts were introduced thus: 20μmol at $t = 100$ min and a further 36μmol infused between $t = 100$ and $t = 190$ min.

1.1.1.27): (1) it is not inhibited by bile salts; (2) it is absent from red blood cells and therefore independent of their lysis in the perfusion medium. Analyses for this enzyme leaking from damaged cells into the perfusate showed that it remained at low levels (0.3–0.4% of total liver content) in each collection period (0–100 min, 100–190 min) in all cases (see also Barnwell *et al.*, 1983).

Bile salt concentration, bile salt output and bile flow

When either TCDC or TUDC were added to the perfusate at 100 min, the bile salt concentration increased rapidly to the level shown in Fig. 1. The final bile salt concentration in the bile produced by TUDC-treated livers (approx. 50 mM) greatly exceeded that produced by TCDC-treated livers (approx. 30 mM). The mean (±S.D.) recovery of TUDC in bile from four experiments was 69 ± 4%, whereas that from four TCDC experiments was only 35 ± 3% (mean ± S.D.). The total infused dose in both cases was 56μmol. These results suggest that the rat liver has a greater capability for transporting TUDC than TCDC. Previous studies by Hardison *et al.* (1981) reported that in rats the maximum secretory rate of TUDC was 5.0μmol/100g per min, whereas that of TCDC was only 1.0μmol/100g per min. They suggested that the maximum secretory rate for a given bile salt was dependent upon its specific toxicity for the bile salt transport system. On the basis of these measurements they predicted the order of toxicity for bile acid transport would be TCDC > TC > TUDC.

Bile flow stimulation with TUDC is much more apparent than with TCDC, possibly due to a cholestatic effect of high concentrations of TCDC. The ability of TUDC to stimulate bile flow is less than for a comparable TC concentration; this is probably due to a higher monomer concentration of TC, which has a higher critical micellar concentration (approx. 5 mM) (Barnwell *et al.*, 1983), than does TUDC (approx. 1 mM) (Carey *et al.*, 1981).

Output of plasma-membrane enzymes 5'-nucleotidase and alkaline phosphodiesterase I

Previous work has shown that the activity of these two enzymes in bile obtained from isolated perfused livers declines in a fashion similar to bile salt concentration. Subsequent introduction of the micelle-forming bile salts, TC and GDOC, increased the activity of these enzymes in bile (Barnwell *et al.*, 1983). The present study has shown how, in the same system, TCDC, at approximately 30 mM concentration, caused an increase in biliary output of both enzymes. However, the flux of enzymes was less than that observed after introduction of TC and GDOC (Barnwell *et al.*, 1983).

In contrast with TCDC, little alkaline phosphodiesterase or 5'-nucleotidase was released by TUDC even at biliary concentrations of up to 50mM. In the case of alkaline phosphodiesterase a partial inhibition of the enzyme of up to 50% (see the Methods section) by TUDC could contribute to the apparent low release, but this is not sufficient to account for the magnitude of the difference (6-fold) between the effect of the two bile salts. Moreover, in the case of 5'-nucleotidase, there is no significant inhibition of the enzyme by TUDC and yet the release of this enzyme is markedly lower than that provoked by TCDC. Hatoff & Hardison (1982), using bile-fistula rats, have observed little release of alkaline phosphatase into bile after infusion of TUDC, whereas the response to TCDC infusion was a very substantial release of the enzyme into bile. Their results and those reported above may represent a characteristic difference between the effects of the two bile salts on the hepatobiliary system.

Phospholipid output

Bile salts are thought to determine biliary phospholipid secretion (Hardison & Francis, 1969; Schersten, 1970; Schersten *et al.*, 1971; Layden & Poppe, 1977). A possible mechanism to explain this phenomenon was proposed by Coleman *et al.* (1977), whereby bile salts solubilize phospholipid directly from the outer leaflet of the canalicular membrane, the integrity of the membrane being maintained by biosynthetic repair. This idea has been supported by the results obtained from model systems (Coleman & Holdsworth, 1976; Billington *et al.*, 1977; Billington & Coleman, 1978) and from isolated liver plasma-membrane fractions (Yousef & Fisher, 1976). The phospholipid removed was found to be mainly phosphatidylcholine (Coleman & Holdsworth, 1976; Yousef & Fisher, 1976) and therefore similar to that found in normal bile. Radioactive experiments using [^{3}H]glycerol have shown that phosphatidylcholine present in the canalicular membrane has a much quicker turnover rate than elsewhere in the cell (Yousef *et al.*, 1975; Kawamoto *et al.*, 1980). The mechanism of transport of phospholipid from the endoplasmic reticulum, where it is synthesized (Gregory *et al.*, 1975), to the canaliculus is still unclear.

The present investigation showed that during the period 0–100min phospholipid output declined in parallel with biliary bile salt concentration and that phospholipid secretion into bile from perfused livers then showed different characteristics depending on the properties of the individual bile salts infused. Thus when TC was infused into the perfused liver system a bile salt concentration of approx. 40mM in bile brought about a peak output of phospholipid of 82nmol/min (results not shown). Similar infusion of TUDC, although producing a concentration of approx. 50mM, gave only 40nmol of phospholipid/min. TCDC produced even less phospholipid with only 25nmol/min. Livers to which no bile salts has been added to the perfusate secreted little phospholipid into bile. The amount of phospholipid secreted during TDHC infusion was not significantly greater than the control with no added bile salt.

A relationship between phospholipid secretion and the micellar properties of bile salts is demonstrated by these results in that TC, TUDC and TCDC, all of which form micelles, cause an output of phospholipid, whereas TDHC, which does not form micelles, is ineffective in promoting phospholipid output.

Differences in the effects of the bile salts

The relationship between the various bile salts and the output of plasma-membrane enzymes is, however, more complex. There is a clear distinction between the output of enzymes promoted by the micelle-forming bile salts, TC (Barnwell *et al.*, 1983) and TCDC (Fig. 1), and the ineffectiveness of the non-micelle-forming TDHC (Barnwell *et al.*, 1983). TUDC causes little output of enzymes, even though it forms micelles and can promote phospholipid output.

The results obtained with TUDC are therefore of much interest for two reasons since (i) they indicate that the biliary outputs of enzymes and phospholipid are distinct and can be separated and (ii) they indicate that the potential effects of TUDC in releasing membrane proteins are less than other micelle-forming bile salts including the other gallstone-reducing bile salt TCDC. This, coupled with the small amounts of any cell-lysis, demonstrates the potentially low toxicity to the membranes of the hepatobiliary system of TUDC. This potentially low toxicity is further emphasized in a cytolytic assay (Coleman & Holdsworth, 1976) using intact human erythrocytes; at a TUDC concentration of 60mM only 1% erythrocyte lysis was present, whereas TCDC caused 80% lysis at 6mM (P. J. Lowe, M. Dixey and R. Coleman, unpublished work). The present study, with isolated perfused livers, compared the acute toxic effects of TCDC and TUDC directly and, unlike feeding experiments, is less likely to be influenced by any contribution of secondary metabolites e.g. lithocholate and its sulphate, which may modify the toxicity in different species. In the rabbit, TUDC has been shown to be less hepatotoxic than TCDC by Miyai *et al.* (1982), using a combination of electron microscopy and serum enzyme profiles, and in man the Tokyo Co-operative Gallstone Study Group (1980) has shown that TUDC caused neither the increased serum aspartate aminotransferase levels nor the diarrhoea some-

times found with TCDC treatment. Since TUDC is less readily converted into lithocholate by gut microflora than is TCDC (Federowski & Salen, 1978) the toxicity of the original compound is therefore of particular importance and the direct studies reported here indicate the low toxicity of the original compound.

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