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Bile Salt- and Lysophosphatidylcholine-induced Membrane Damage in Human Erythrocytes

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Abstract—The interaction of bile salts and lysophosphatidylcholine (LPC) with membranes has implications both in understanding the aetiology of a number of gastrointestinal disorders, including gastritis, gastric ulcers and colonic cancer, and in enhancing drug absorption by various epithelia. The membrane toxicity of nine bile salts (the sodium (S) salts of chenodeoxycholate (CDC), deoxycholate (DC) and cholate (C) and their glycine (G) and taurine (T) conjugates) and LPC was determined using erythrocyte haemolysis as a model parameter. Washed human erythrocytes were incubated for 15–60 min at 20°C with media buffered at pH 8, 7 and 6. Bile salt toxicity was shown to be a function of type, concentration, pH and contact time with the membrane. At pH 7 toxicity decreased in the order LPC > unconjugated dihydroxy salts (SDC and SCDC) > conjugated deoxycholates (SGDC and STDC) > conjugated chenodeoxycholates (SGCDC and STCDC) > unconjugated trihydroxy salt (SC) > conjugated trihydroxy salts (SGC and STC). Incubation with equimolar combinations of bile salts (SDC + SCDC; STCDC + SGDC; SDC + STDC) indicated that the resultant damage was an additive function of the damage induced by the individual bile salts.

Bile salts and lysophosphatidylcholine (LPC) are biliary surfactants which have been employed extensively as agents to enhance the absorption of drugs by various epithelia (Murakami et al 1984; O'Hagan & Illum 1990). Undoubtedly, some of the changes in membrane permeability are as a consequence of direct physical damage induced by these surfactants (Hirai et al 1981; Hersey & Jackson 1987). Indeed, one of the predisposing factors in the pathogenesis of several upper digestive tract disorders has been reported to be the membrane damage which results after reflux of bile from the duodenum into the stomach. Such duodenogastric reflux has been particularly implicated in the aetiology of gastritis (especially postoperative alkaline gastritis) (Cheli et al 1981; Ludwig & Ippoliti 1984; Ritchie 1984) and gastric ulceration (Rhodes et al 1969; Johnson & McDermott 1974; Dewer et al 1983). However, duodenogastric reflux has been considered also as a normal phenomenon, occurring in healthy subjects during the second phase of the interdigestive period and during the postprandial phase. Attention is thus focusing on the identification of an abnormal reflux, one aspect being the alteration in the bile salt/acid and LPC composition. More deoxycholic acid has been found, for example, in the refluxate of patients suffering from atrophic gastritis than in those exhibiting either superficial or no gastritis (Masci et al 1987). Similarly, Johnson & McDermott (1974) showed patients with gastric ulcer tended to have higher concentrations of LPC in their night gastric juice than control subjects. Alteration in the bile salt and phospholipid composition of bile has also been implicated in colon carcinogenesis (Hill 1987; Morotomi et al 1990; Mullen et al 1990). Unconjugated secondary bile salts are damaging to colonic epithelial cells and cause an increase in proliferation of crypt cells (Bruce 1987).

The membrane perturbing effects of bile salts and LPC have been demonstrated on a number of membranes, including the erythrocyte (Coleman & Holdsworth 1976; Van der Meer et al 1991), erythrocyte ghosts (Coleman et al 1976), rat plasma membranes (Coleman & Holdsworth 1976), pig lymphocytes (Holdsworth & Coleman 1976), goldfish epithelium (Kellaway & Marriott 1977) and egg PC liposomes (O'Connor et al 1985). In many of the previous investigations, only representative dihydroxy or trihydroxy bile salts were selected and, in addition, no determinations of the rate of membrane induced damage have been carried out. Accordingly, given the importance of establishing the factors which influence the interaction of natural surfactants with membranes, it was the purpose of this study to carry out a comprehensive investigation of the relative toxicity at different pH of the nine predominant bile salts of human bile and LPC in one experimental model (using the erythrocyte membrane). Further aims were to determine the effects of incubation (or contact time) of the surfactant on membrane integrity and to investigate whether the damage induced to structure by mixtures was additive or synergistic.

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Materials and Methods

Materials

Sodium cholate (SC), sodium deoxycholate (SDC), sodium glycochenodeoxycholate (SGCDC), sodium glycocholate (SGC), sodium glycodeoxycholate (SGDC), sodium taurochenodeoxycholate (STCDC), sodium taurocholate (STC), sodium taurodeoxycholate (STDC), Brij 35 solution (polyoxyethylene 23 lauryl ether 30% w/v), Drabkin's reagent and trisodium citrate were all obtained from Sigma Ltd, Poole, Dorset, UK.

Lysophosphatidylcholine (Grade 1) (LPC) was obtained from Lipid Products, Nutfield, Surrey, UK. The relative molecular mass of the sample was taken to be 525.

Sodium chenodeoxycholate (SCDC) was obtained from Calbiochem-Behring, CP Laboratories Ltd, Bishop Stort-

ford, Herts, UK. Anhydrous dextrose (Analar), disodium hydrogen phosphate dihydrate (Analar), sodium chloride (Analar), sodium dihydrogen phosphate dihydrate (Analar) and tris(hydroxymethyl) methylamine (Tris) (Analar) were obtained from BDH Ltd, Poole, Dorset, UK.

Haemolysis experiments

Blood was collected from a caucasian female into heparinized tubes. The anticoagulated blood was centrifuged (Sorvall RT 600B, Du Pont) at 2200 *g* for 10 min and the plasma and buffy coat discarded. The erythrocytes were washed three times with at least five volumes of 10% McIlvaine's buffer containing 134 mM NaCl at the relevant pH and resuspended in this solution to give a 12% haematocrit. Samples of this suspension (0·2 mL) were incubated at 20°C with 0·2 mL of the McIlvaine's buffer solution, at the relevant pH, containing the appropriate amount of bile salt for 30 min. In other experiments, reaction periods of 15, 30, 45 or 60 min were employed. After incubation, the samples were spun in a microcentrifuge for 15 s. Samples of the supernatant (0·2 mL) were mixed with 3 mL Drabkin's solution and allowed to stand for 15 min before spectrophotometric (Perkin Elmer 554 spectrophotometer, Perkin Elmer Ltd) determination at 540 nm. The absorbance values for 100% haemolysis were obtained by removing samples (0·2 mL) from uncentrifuged solutions, mixing these with 3 mL Drabkin's solution and assaying as described above. All samples were prepared in triplicate and the whole experimental procedure was repeated at least once.

Results

In all experiments, the preparation of the erythrocyte suspension occurred on the day of phlebotomy and this suspension was always used either on the same day or the day after preparation. It is assumed that optimum cell stability occurs at the physiological pH of 7·4. Alteration of pH from this optimum will probably result in greater cell fragility and consequently greater relative haemolysis with the addition of bile salt and LPC solutions. For this reason, experiments which involved adjustment of pH could only be used to compare gross changes in toxicity. When no surfactant was present (control samples) haemolysis was always less than one percent. Although standard deviations have been omitted from the figures for reasons of clarity, the coefficient of variation for each set of haemolysis experiments was typically in the range 0–10% (El-Hariri 1990). The effects of LPC and bile salts on erythrocyte haemolysis at pH 8, 7 and 6 are shown in Figs 1–3, respectively. LPC was found to be more haemolytic than any of the bile salts at the same concentration, 50% haemolysis occurring at LPC concentrations as low as 0·07 mM (pH 6 and 8) and 0·09 mM (pH 7). The six dihydroxy bile salts (SDC, SCDC, SGDC, STDC, SGCDC and STCDC) were more haemolytic than the trihydroxy bile salts (SC, SGC and STC) at all three pH values.

At pH 7 (Fig. 2), the unconjugated dihydroxy salts (SDC and SCDC) showed greater haemolytic activity than the conjugated deoxycholates (SGDC and STDC) which in turn were more toxic than the conjugated chenodeoxycholates (SGCDC and STCDC). Within the trihydroxy bile salt group, the unconjugated bile salt (SC) showed greater

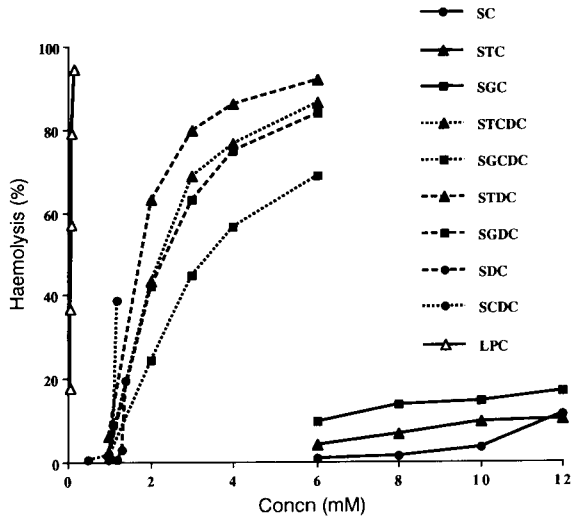

FIG. 1. The effect of bile salt and LPC concentration on the percentage haemolysis of erythrocytes at pH 8 (n = 6). See Materials and Methods for abbreviations.

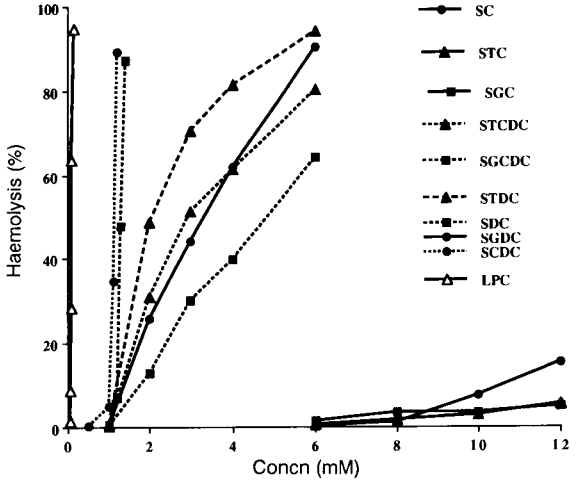

FIG. 2. The effect of bile salt and LPC concentration on the percentage haemolysis of erythrocytes at pH 7 (n = 6). See Materials and Methods for abbreviations.

membrane disrupting effects than the conjugated salts (SGC and STC) and this was particularly apparent at pH 6 (Fig. 3).

The contact time between bile salt and erythrocyte was varied up to 60 min to determine whether this led to an increase in erythrocyte haemolysis. Concentrations of bile salt were selected on the basis of the overall toxicity determined previously (Figs 1–3) so that the kinetics of haemolysis could be established. Figs 4–6 show that at pH 8, 7 and 6, respectively, an increase in haemolysis was seen with increasing contact or reaction time. In Fig. 5 (pH 7) the slopes of the plots of percentage haemolysis as a function of reaction time are clearly greater for the unconjugated bile salts SDC, SCDC and SC, than the conjugated salts. At pH 6 (Fig. 6), the increase in slope also becomes apparent for SGCDC and SGDC.

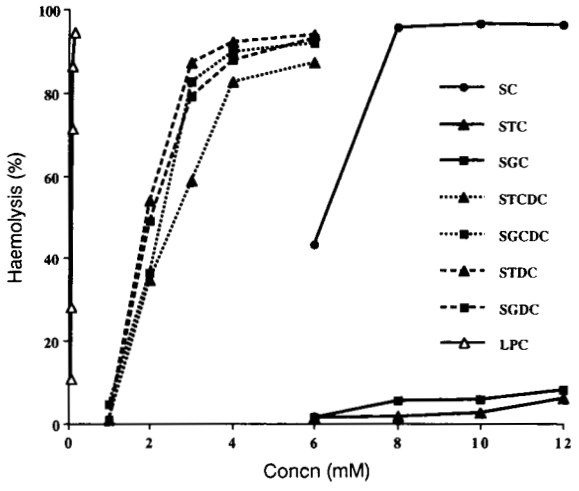

FIG. 3. The effect of bile salt and LPC concentration on the percentage haemolysis of erythrocytes at pH 6 (n = 6). See Materials and Methods for abbreviations.

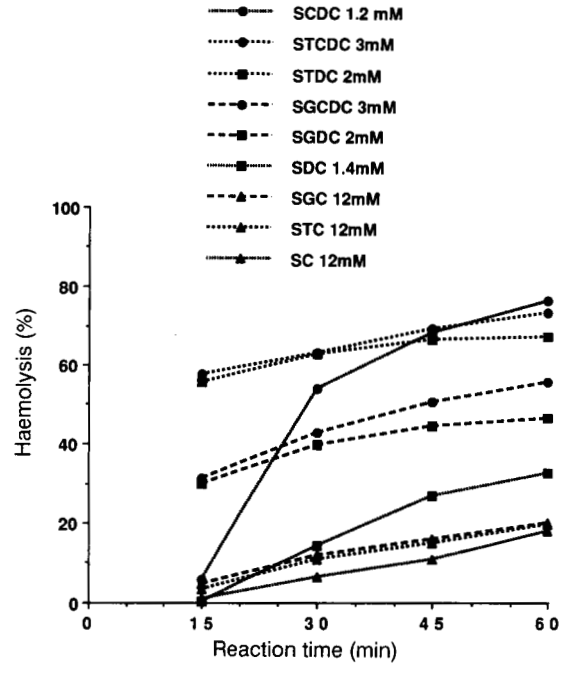

FIG. 4. The effect of time on the percentage haemolysis of erythrocytes induced by bile salts at pH 8 (n = 6). See Materials and Methods for abbreviations.

Experiments were carried out to determine whether the toxicity induced by equimolar combinations of bile salts was additive or synergistic. If haemolysis were additive, the net haemolysis expected to be induced by a combination of two bile salts would be obtained from the following equation:

$$yA + yB = \frac{2yA + 2yB}{2} \quad (1)$$

where (yA + yB) is the calculated percentage haemolysis induced by y mM bile salt A plus y mM bile salt B, 2yA is the actual percentage haemolysis induced by 2y mM bile salt A, and 2yB is the actual percentage haemolysis induced by 2y mM bile salt B.

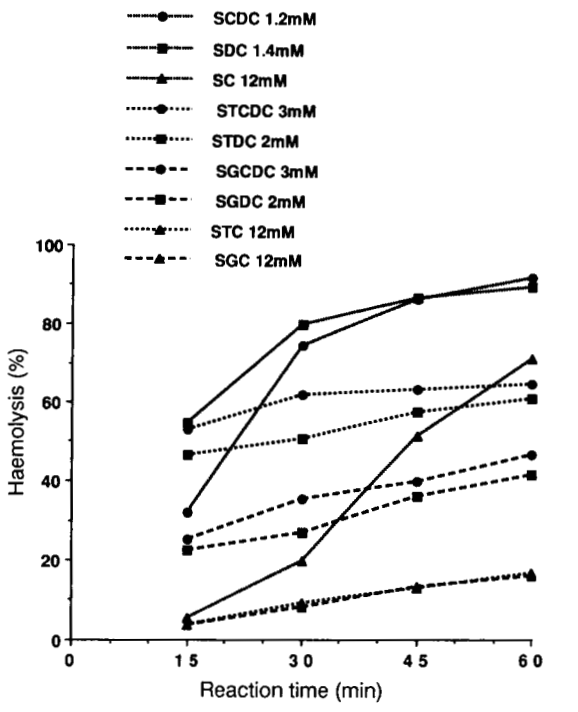

FIG. 5. The effect of time on the percentage haemolysis of erythrocytes induced by bile salts at pH 7 (n = 6). See Materials and Methods for abbreviations.

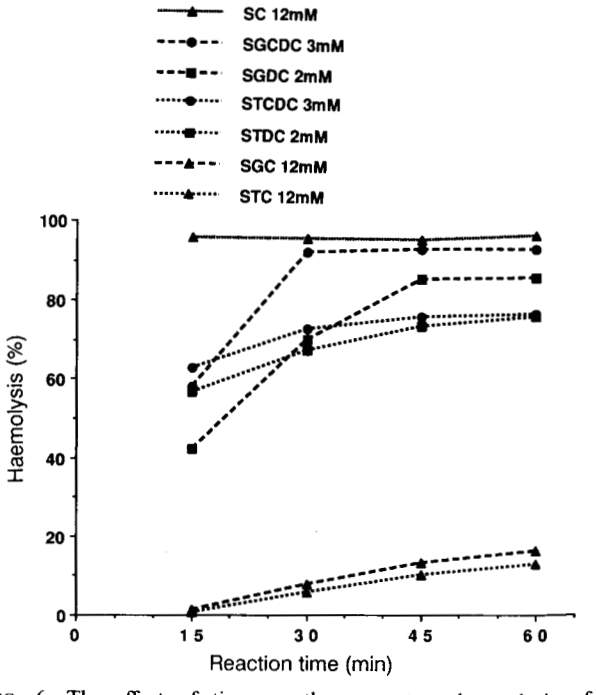

FIG. 6. The effect of time on the percentage haemolysis of erythrocytes induced by bile salts at pH 6 (n = 6). See Materials and Methods for abbreviations.

Table 1 shows the results obtained for the percentage haemolysis by individual bile and salts and bile salt mixtures, and compares the latter with the calculated percentage haemolysis values obtained using equation 1.

Table 1. Percentage haemolysis for separate bile salts and combination of bile salts at pH 7 (n = 6).

Bile salt	Concentration (mM)	Percentage haemolysis	s.d.	Calculated haemolysis*
SDC	0·6	0·2	0·2	—
SDC	1·2	24·0	6·3	—
SCDC	0·6	0·3	0·2	—
SCDC	1·2	61·6	5·4	—
SDC + SCDC	0·6 + 0·6	37·6	5·2	42·8
STCDC	1·0	0·5	0·2	—
STCDC	2·0	27·2	1·4	—
SGDC	1·0	0·5	0·2	—
SGDC	2·0	31·2	0·8	—
STCDC + SGDC	1·0 + 1·0	29·0	0·5	29·2
SDC	0·6	0·2	0·2	—
SDC	1·2	24·0	6·3	—
STDC	0·6	0·5	0·0	—
STDC	1·2	4·4	0·6	—
SDC + STDC	0·6 + 0·6	13·6	1·8	14·2

* Obtained using equation 1 (see text). Sodium deoxycholate, SDC; sodium chenodeoxycholate, SCDC; sodium taurochenodeoxycholate, STCDC; sodium glycodeoxycholate, SGDC; sodium taurodeoxycholate, STDC.

Discussion

The relative membrane damaging effects of bile salts are not constant but depend on a number of factors, including pH and contact time. As pH decreases, the pK_a of individual salts are sequentially approached and they become in turn apparently more toxic to membranes. The pK_a values of the unconjugated bile salts (SDC, SCDC and SC) are in the range 5·0–6·5. For SDC and SCDC, a large increase in toxicity was found between pH 8 and 7, but for SC the corresponding increase occurred between pH 7 and 6. This difference is probably related to the greater solubility and hydrophilicity of the trihydroxy bile salt, in comparison with the dihydroxy salts. The decreased solubility of the unconjugated dihydroxy bile salts resulted in gel formation at concentrations as low as 1 mM at pH 6 and this phenomenon precluded haemolysis studies involving these bile salts at this pH. The gel formation of these bile salts has been reported previously by Rich & Blow (1958) and Small (1971).

The pK_a values of the dihydroxy glycine conjugated salts are within the range 4·2–4·8. Between pH 7 and 6, there was a marked increase in toxicity of SGCDC and SGDC, the effect being more marked for SGCDC (Figs 2, 3).

The pH of the solution did not influence the toxicity of SGC, probably as a result of the greater solubility of the trihydroxy bile salt. The taurine conjugates and LPC, with their low pK_a values, showed no change in toxicity with decrease in pH. In no case was precipitation of bile salt observed, any un-ionized bile salt being either co-solubilized within the bile salt micelles or present in a low enough concentration to remain in solution.

These results suggest that bile salts are more membrane damaging when in the un-ionized form. However, it is possible that two separate mechanisms of membrane damage are occurring: one primarily attributable to the bile salt and one to the bile acid.

Interestingly, a difference in bile salt toxicity was seen when contact time of the bile salt with the erythrocyte membrane was studied. In Fig. 4 (pH 8) it can clearly be seen that the kinetics of SCDC-induced membrane damage differed from all the other bile salts. In Fig. 5 (pH 7) the kinetics for SDC and SC appeared to be similar to SCDC and different from the conjugated bile salts. At pH 6 (Fig. 6) SGCDC and SGDC also indicated changed kinetics of interaction. These results can be correlated with the bile salt concentration studies, i.e. as pH decreased, bile salts approaching the pK_a became more membrane damaging, which in turn induced a change in the kinetics of interaction between bile salt and membrane. Indeed, the most toxic compounds, namely LPC, SCDC and SDC, once past the haemolytic threshold, caused almost 100% haemolysis with very little increase in concentration. With less haemolytic agents, e.g. SGC and STC, an increase in haemolysis occurred gradually as a function of concentration.

Billington & Coleman (1978) have shown that SGC removes membrane components from the human erythrocyte. At concentrations below 6 mM, microvesicles were released with a phospholipid composition similar to the original membrane. These microvesicles were probably composed of both leaflets of the membrane bilayer. At higher concentrations, phospholipids were removed from the membrane in a 'soluble' form. The profile of this extracted phospholipid was similar to the externally orientated membrane lipids.

Differences in dihydroxy and trihydroxy mechanisms of action have been proposed (Coleman & Holdsworth 1975). For example, SGDC was shown to initiate cell lysis at or below the critical micelle concentration (CMC), whereas SGC only caused lysis above the CMC, although both compounds were shown to release components from the membrane before the point of lysis (Lowe & Coleman 1981).

The time course of surfactant-induced haemolysis has been found generally to follow an *S*-shaped curve, the reaction being preceded by a lag phase of a few seconds to several minutes, followed by a rapid lysis phase (Isomaa et al 1988). Reman et al (1969) found that LPC with acyl chain lengths of 16 and 18 carbon atoms were the most haemolytic and the introduction of a double bond into the acyl chain (i.e. oleoyl-LPC and linoleoyl-LPC) rendered the compounds less lytic. Decanoyl-LPC was found to have no haemolytic activity. It has been suggested that LPC can bind to membranes at concentrations well below the CMC (0·01–0·001% for egg LPC (Helenius & Simons 1975)) and that micelle formation is apparently not required for haemolysis (Weltzien et al 1977; Ferrell et al 1988).

An interesting finding from this study was that the toxicity of equimolar mixtures of bile salts appeared to be additive. This was shown to be true for equimolar mixtures of two unconjugated salts, two conjugated salts, and a combination of a conjugated and unconjugated bile salt (Table 1), the calculated value for erythrocyte haemolysis being virtually identical to the experimentally determined value in each case. No evidence of synergism between bile salts was apparent.

The disadvantage of using the isolated erythrocyte membrane is that only direct effects of bile salts on a cell membrane may be studied. It is apparent from this study that membrane disruption is directly related to hydrophobicity of the bile salt, both as a consequence of structure (i.e. degree of hydroxylation) and pK_a. The absorption-promoting effect of bile salts on drug absorption by the nasal and rectal membranes generally correlates with increasing hydrophobi-

city (Murakami et al 1984; Gordon et al 1985; Duchateau et al 1986, 1987). However, such absorption enhancement by bile salts is likely to involve other mechanisms, besides direct membrane perturbation. These are likely to include reduction in mucus structure (Martin et al 1978), effects on glycocalyx structure (Rafter et al 1986), formation of pores within the membrane (Gordon et al 1985), possible enzyme inhibition (Hanson et al 1986) and the binding of calcium ions associated with the tight junctions (Hunt 1983).

In conclusion, it would appear that on the basis of this study, the overall toxicity of bile salts to membrane is related to a number of factors, including bile salt type, bile salt concentration, pH of the milieu and bile salt contact time with the membrane. LPC damage was relatively insensitive to pH. These findings are of likely relevance to understanding the pathological involvement of bile in disease states of the gastrointestinal tract and, when considered with previous investigations, tend to confirm that absorption enhancement by such species is often a direct consequence of membrane damage.

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