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Membrane damage by bile salts: the protective function of phospholipids

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The direct toxicity of sodium deoxycholate (SDC) and lysophosphatidylcholine (LPC) to biological membranes was assessed by measurement of goldfish overturn time. When phosphatidylcholine (PC) was incorporated into the aqueous media, the toxicity of both SDC and LPC was reduced, as indicated by increased overturn time. Fish were also pretreated for various times in media containing (a) 1 mM SDC and (b) 1 mM SDC with 1 mM PC. Subsequent transfer to solution, 100 mg litre^{-1} quinalbarbitone sodium showed that reciprocal overturn times for fish treated using method (a) increased linearly with duration of pretreatment up to a limiting value, obtained after 20 min exposure; 40 min exposure to 1 mM SDC was directly toxic. Fish pretreated using regimen (b) survived longer when challenged with barbiturate, and the reciprocal overturn times were a linear function of time of pretreatment up to at least 40 min. PC also provided protection against membrane damage caused by the synthetic surfactant sodium dodecyl sulphate. Mixed micelle formation between PC and surfactant is thought to account for the protective effects. The results are of significance in the consideration of reflux hypotheses for the aetiology of gastric ulceration and also to the possible formulation of drug delivery systems intended to enhance absorption whilst minimizing gastrointestinal damage.

Bile salts and phosphatides are the two major types of surface active compounds occurring naturally within the intestine. It has been reported that poorly soluble drugs are better absorbed following the stimulation of bile production (Crounse 1961; Giannina et al 1966) and that the co-administration of bile salts enhances the absorption of a number of drugs in both the rat (Kakemi et al 1970; Feldman et al 1970; Kimura et al 1972) and the goldfish (Gibaldi & Nightingale 1968; Nightingale et al 1969; Marriott & Kellaway 1976). Mayerson et al (1969) demonstrated that the absorption of riboflavine in man was increased by 50–80% by prior dosing with sodium deoxycholate (SDC). Bile salts, beside increasing drug solubility (Martin et al 1978a) and dissolution rate (Bates et al 1966) also increase absorption by altering the permeability of biological membranes (Feldman & Gibaldi 1969a; Feldman et al 1970). Indeed, elevated levels of bile acids (Rhodes et al 1969) and lysophosphatidylcholine (LPC) (Johnson & McDermott 1974) in the stomach, as a result of bile reflux, have been implicated in the initiation of gastric ulceration by causing leakage of hydrogen ions from the gastric lumen (Davenport 1968, 1970). These naturally occurring surfactants have also been shown to break down mucus structure (Martin et al 1978b) and to be directly toxic to membranes (Kellaway & Marriott 1977). Low concentrations of bile salt cause pronounced changes in the gross appearance of the mucosal surface (Gibaldi & Feldman 1970; Yonezawa 1977; Gullikson et al 1977) and accelerate the release of phospholipids and protein from the membrane (Whitmore et al 1979).

Although bile salts and LPC are apparently toxic to the gastric mucosa, as well as to in vitro preparations, no extensive disruption of the intestinal mucosa occurs. The purpose of this study was, therefore, to examine whether phosphatidylcholine (PC) (the major phospholipid in bile) would reduce the toxicity of bile salts and lysophospholipids to biological membranes, by combining with them to form mixed micelles.

The goldfish was selected as a convenient model membrane system, since it provided an easily determinable end point for toxicity studies (Kellaway & Marriott 1977) and has been used extensively for drug absorption investigations.

MATERIALS AND METHODS

Goldfish, *Carassius aurata*, common variety, 3–5 g were used. Overturn times were obtained at 18–20 °C and 3–9 fish were used at each surfactant concentration. Sodium deoxycholate (Sigma (London) Chemical Co. Ltd., Poole) and sodium dodecyl sulphate, specially purified for biochemical work

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(BDH Chemicals Ltd., Poole), were used as supplied.

Egg phosphatidylcholine (BDH) was purified by elution from an alumina column followed by recrystallization, as described previously (Martin et al 1978a). After a single spot was obtained by thin-layer chromatography, the phospholipid was stored at 5 °C under dry acetone until required. Sols were prepared by adding dry PC to buffered surfactant solution and using a 20 kHz sonic probe (Dawe Instruments) for 30–60 min until a transparent dispersion was obtained.

LPC was prepared from purified egg PC using the method of Hanahan et al (1954). The product was recrystallized from hot butan-2-one and stored under dry acetone at 5 °C.

Toxicity experiments

A range of surfactant concentrations was made in 0·1 M Tris buffer (pH 7·4) and an overturn time (T) determined for individual fish immersed in 100 ml of solution. The reciprocal of overturn time was used as an index of toxicity.

Absorption experiments

Pretreatment solutions were prepared by dissolving 1 mmol litre^{-1} of SDC in 500 ml Tris buffer (pH 7·4). PC 1 mM was also included in some solutions, sonication being employed, as described previously, to obtain a clear sol. Fish were immersed in the pretreatment solution for time periods ranging from 5–40 min, rinsed, and subsequently transferred to a buffered (Tris, pH 6·8) solution of quinalbarbitone sodium 100 mg litre^{-1} (EliLilly & Co. Ltd, Basingstoke). The time taken (min) for overturn (T_Q) was obtained and used as a measurement of drug absorption rate.

Determination of critical micelle concentration (cmc)

The cmc of sodium dodecyl sulphate was determined from the inflection of the log molar concentration versus surface tension plot. Surface tensions were determined using a Du Noüy tensiometer (Cambridge Instruments, London) at 20 °C.

RESULTS

Toxicity experiments

A plot of SDC concentration against T^{-1} (Fig. 1) produced a similar curve to that previously described by Kellaway & Marriott (1977) and a minimum was confirmed to occur at a concentration of 5 mmol litre^{-1}. Whilst linearity existed at higher concentrations, the minimum effective concentration of SDC

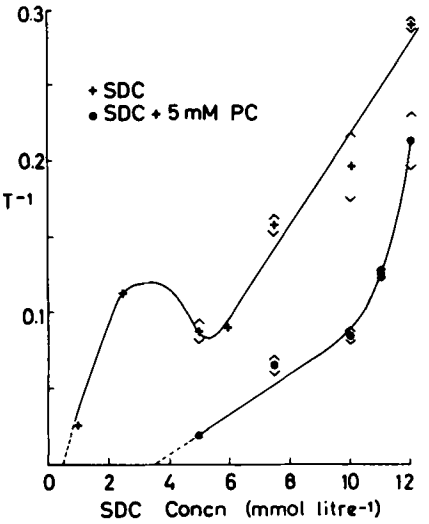

FIG. 1. The effect of sodium deoxycholate concentration (mmol litre^{-1}) on the reciprocal of overturn time, T^{-1} (min^{-1}) for the bile salt alone (+) and the bile salt in the presence of 5 mM PC (●). Standard error bars are included.

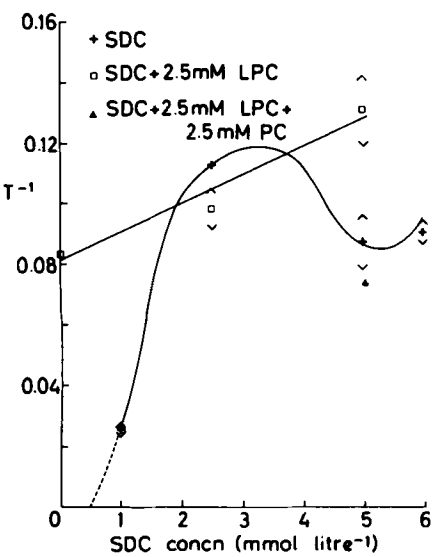

FIG. 2. The effect of sodium deoxycholate concentration (mmol litre^{-1}) on the reciprocal of overturn time, T^{-1} (min^{-1}) for the bile salt alone (+), in the presence of 2·5 mM LPC (□) and 2·5 mM LPC combined with 2·5 mM PC (▲). Standard error bars are included.

necessary to produce overturn was found to be approximately 0·5 mmol litre^{-1}. The presence of 5 mmol litre^{-1} PC significantly increased overturn times ($P < 0.01$) at all of the deoxycholate concentrations examined. The minimum effective concentrations of bile salt in the presence of PC was increased to over 3 mmol litre^{-1}.

LPC was directly toxic to the goldfish membrane, 2·5 mmol litre^{-1} causing overturn in 12 min (Fig. 2). When this concentration of LPC was present with deoxycholate, then the toxicity increased additively as SDC concentration increased. When PC was added to the LPC/SDC solution then overturn times were increased by a factor of between 2 and 3.

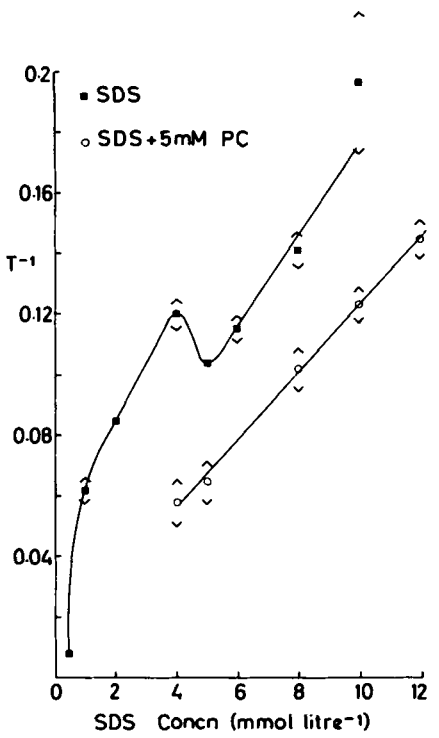

FIG. 3. The effect of sodium dodecyl sulphate concentration (mmol litre^{-1}) on the reciprocal of overturn time, T^{-1} (min^{-1}) for SDS alone (■) and in the presence of 5 mM PC (○). Standard error bars are included.

To examine whether PC reduced the toxicity of other anionic surfactants (as well as SDC) the effect of increasing concentrations of SDS on overturn times was investigated. Fig. 3 shows that overturn times in SDS were remarkably similar to those obtained in the same molar concentrations of SDC (Fig. 1). A minimum was again found to exist in the curve, 5 mM SDS being less toxic than 4 mM SDS. When PC was included with SDS, overturn times were lengthened and a good linear relationship (linear correlation coefficient, 0·999) was found to exist between 1/T and SDS concentration. The presence of PC did not appear to increase the minimum effective concentration of SDS (Fig. 3) as it did when incorporated with SDC (Fig. 1).

Absorption experiments

The influence of bile salt induced membrane damage on drug absorption was examined by pretreating the goldfish for various times and subsequently transferring them to a solution containing quinalbarbitone sodium. The overturn time T_Q was found to decrease with increasing SDC exposure time up to 20 mins (Fig. 4). When exposure was lengthened to 30 min, no further increase in T_Q was noted whereas 40 min exposure to 1 mM SDC was directly toxic to the goldfish membrane (Fig. 1) and

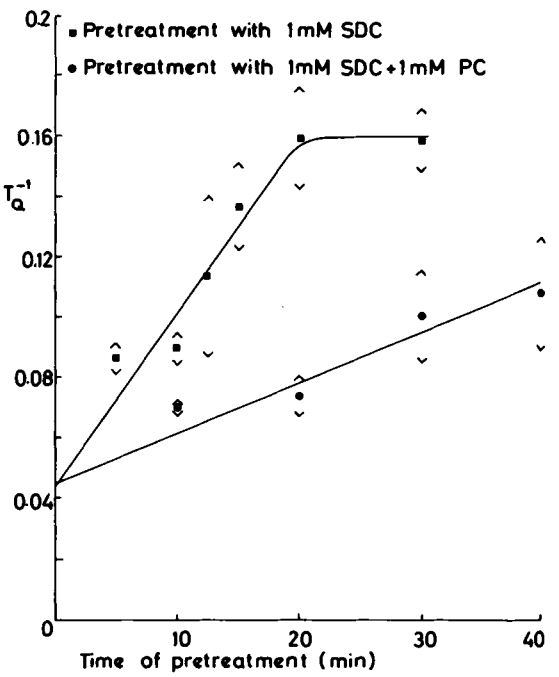

FIG. 4. The influence of time of pretreatment (min) with 1 mM SDC (■) and 1 mM SDC combined with 1 mM PC (●) on the reciprocal of overturn time, T_Q^{-1} (min^{-1}), in 100 mg litre^{-1} quinalbarbitone sodium. Standard error bars are included.

overturn occurred before immersion in the barbiturate solution. When 1 mM PC was included in the pretreatment solution, then T_O values were increased and the curve of T_O^{-1} against exposure time proved to be linear (linear correlation coefficient, 0·973) up to 40 min (Fig. 4).

DISCUSSION

The relationship between bile salt toxicity and concentration is complex, as shown by the shape of the curve in Fig. 1. Although gross histological changes have been reported in intestinal tissue treated with bile salts (Yonezawa 1977; Gibaldi & Feldman 1970), the exact mechanisms by which the effects are exerted on biological membranes remain unclear. The minimum in the curve when SDC concentration is plotted against 1/T (Fig. 1) may reflect a change in mechanism of bile salt damage to the membrane. At lower concentrations the bile salt may be incorporated into membranes with subsequent alteration of the permeability whilst higher concentrations of bile salt extract and solubilize both protein and lipid components. The possibility that submicellar and postmicellar concentrations of bile salt may affect biological membranes in a significantly different manner is supported by permeability studies with salicylate (Feldman & Gibaldi 1969a). The amount of membrane protein and lipid released at different bile salt concentrations has been shown to correlate well with altered drug permeability characteristics (Feldman et al 1973; Whitmore et al 1979). Considering the membrane altering effects of different surfactant concentrations in relation to a cmc is probably of dubious value in the case of bile salts. Bile salts have been shown not to possess the distinct cmc of the long chain hydrocarbon surfactants, but instead associate in a complex stepwise manner (Mukerjee & Cardinal 1976). SDC forms dimers and higher oligomers in different proportions dependent upon concentration, and some association occurs even in dilute solution (Chang & Cardinal 1978). If this model of association is correct, then the wide variation in literature values for the cmc of SDC (Small 1971) may be explained. A previous attempt by one of us to obtain cmc of SDC in Tris buffer, produced an inflection in conductivity and surface tension curves corresponding to a bile salt concentration of 4–5 mmol litre^{-1} (Marriott & Kellaway 1976). Such a value appears to correspond to the region of the minimum in Fig. 1.

PC reduces the toxicity of the bile salt, as evidenced by the increased overturn times (Fig. 1). Mixed micelles are formed between SDC and PC where disc-shaped bimolecular leaflets of the phospholipid are surrounded on the hydrophobic parts by bile salt molecules (Small 1971). A more recent model (Zimmerer & Lindenbaum 1979) suggests that bile salt may also be incorporated within the phospholipid interior. The mixed micellar aggregates reduce the rate at which the intact membrane is damaged because of the reduced solubilizing capacity of bile salt micelles for membrane components. Since 1 mole of bile salt can solubilize up to a maximum of 2 moles of PC (Small 1971), 5 mM PC should be completely solubilized by 2·5 mM SDC. The new minimum effective concentration of SDC necessary to produce turnover in the presence of 5 mM PC is approximately 3 mmol litre^{-1} (Fig. 1).

The integrity of the intestinal mucosa may, therefore be maintained in vivo partially because of mixed micelle formation between the bile salts and PC. Within the gastrointestinal tract hydrolysis of PC will proceed to give the lysophospholipid, LPC, a process which is catalysed by bile salts and trypsin (Johnson & McDermott 1974). The mixed bile salt/PC micelles of the type described previously will, therefore, break down as hydrolysis proceeds. Fig. 2 shows that LPC is directly toxic to the goldfish membrane and that toxicity is directly additive to that caused by SDC. It is perhaps significant that no extensive disruption of the intestinal mucosa is apparent until the bile salts reach the site of active absorption in the ileum, where mucosal cells have the shortest life span (Fry & Staffeldt 1964). These results would also appear to be of significance in vivo when, as a result of a faulty pyloric sphincter, reflux of duodenal contents into the stomach initiates the process that may ultimately lead to gastric ulceration (Rhodes 1972). After reflux, hydrolysis of PC continues, the mixed PC/bile salt micelles break down and the LPC and bile salts are released to attack both mucus structure (Martin et al 1978b) and the underlying mucosa (Davenport 1968, 1970). PC reduces the toxicity of the LPC/SDC system to the membrane (Fig. 2) by again decreasing the solubilizing capacity for membrane protein and lipid components.

The synthetic anionic surfactant, SDS was found to be as toxic to the goldfish membrane as SDC (Fig. 3). The toxicity is partially attributable to the lysis of the gill cells induced by direct action of the surfactant (Abel 1976). SDS causes gross histological damage to the intestinal mucosa (Nadai et al 1972; Yonezawa 1977;), protein and lipid components are released (Feldman & Reinhard 1976; Whitmore et al 1979) and permeability to diffusing species is increased (Nadai et al 1975; Whitmore et al 1979). Surface

tension data indicated that the cmc of SDS in the Tris buffer system was 0·92 mmol litre^{-1}. Although 0·5 mmol litre^{-1} proved directly toxic to the goldfish, overturn times were found to decrease rapidly immediately above the cmc. A minimum was found in the plot of SDS concentration against reciprocal of overturn time (Fig. 3) at approximately 5 mmol litre^{-1}, which is well above the determined cmc. The inflection in the curve may again be indicative that the toxicity of SDS to the goldfish, as with SDC, is mediated via two different mechanisms which operate at surfactant concentrations above and below 4–5 mmol litre^{-1}. If this is the case, the altered pattern of toxicity would appear to be unrelated to the cmc of the surfactant. It is apparent that the formation of mixed micelles between SDS and PC, reduces the overall toxicity of the synthetic surfactant (Fig. 3). However, the increase in the minimum effective concentration which was observed when PC was incorporated with SDC was not apparent with SDS. This presumably reflects differences in structure of the mixed micelles formed, SDS being a long hydrocarbon chain surfactant, while SDC possesses a steroidal structure.

Pretreatment of the fish with SDC potentiated the effects of subsequent exposure to quinalbarbitone sodium. The degree of potentiation was found to be a function of the duration of pretreatment (Fig. 4) up to a time of 20 min. These results are in contrast to those reported by Gibaldi & Nightingale (1968) when sodium taurodeoxycholate was found to mediate the maximum effect on membrane permeability to sodium pentobarbitone within 5 min. However, those authors used bile salt concentrations that were 5 to 10 times lower and which had no visually observed toxic action on the fish. In the current investigation exposure to 1 mM SDC alone produced overturn in 39 min. These results would appear to confirm that bile salts modify membrane permeability by two different mechanisms; one is operative at lower concentrations, the effects being mediated quickly within the membrane, and the other at higher concentrations when more permanent damage is caused over a longer period. Perhaps significantly, in this context, fish pretreated with 1 mM SDC for time periods in excess of 5 min were killed by subsequent barbiturate exposure, whereas complete recovery within 24 h was reported for low pretreatment concentrations (Gibaldi & Nightingale 1968).

The increased membrane permeability to quinalbarbitone, brought about by SDC pretreatment, was reduced when PC was incorporated in the aqueous medium. Subsequent barbiturate overturn times were reduced (Fig. 4) because membrane integrity was preserved for a longer period as a result of mixed micelle formation. Membrane barbiturate and salicylate (Feldman & Gibaldi 1969b) transfer rates increase therefore, after bile salt treatment but decrease towards those obtained in the untreated, intact membrane when PC is included.

In conclusion, it would appear that the deleterious effects of bile salts, LPC and synthetic anionic surfactants to biological membranes are reduced in the presence of PC. The presence of PC in bile may therefore be of importance in maintaining intestinal integrity in vivo.

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