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Mitigation of Surfactant Erythrocyte Toxicity by Egg Phosphatidylcholine

LARISSA A. GOULD, ALISON B. LANSLEY, MARC B. BROWN, BEN FORBES AND GARY P. MARTIN

Department of Pharmacy, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 8WA, UK

Abstract

Polyoxyethylene alkyl ether surfactants have been shown to have excellent penetration enhancing abilities although they are associated with a high level of local toxicity. We have compared the toxicity of a range of polyoxyethylene alkyl ethers (Brij 96, Brij 76, Brij 56, 10 lauryl ether and 9 lauryl ether) to an anionic surfactant (sodium dodecyl sulphate (SDS)), an ampholytic surfactant (lysophosphatidylcholine) and a cationic surfactant (tetradecyltrimethylammonium bromide (TTAB)), in the presence and absence of egg phosphatidylcholine.

The toxicity of the surfactants or phospholipid/surfactant mixtures was assessed by measuring haemolytic activity. The test samples were incubated with a suspension of red blood cells for 30 min and Drabkin's reagent was used to indicate the amount of haemoglobin released.

All of the polyoxyethylene alkyl ethers, SDS, TTAB and lysophosphatidylcholine exhibited haemolytic activity at concentrations between 0·10 and 0·25 mM. The addition of egg phosphatidylcholine reduced the toxicity for all of the surfactants, with the toxicity of Brij 96 being mitigated to a greater extent than the toxicity of the other polyoxyethylene surfactants examined. The rate of haemolysis induced by Brij 96 or 10 lauryl ether was also reduced by increasing concentrations of phosphatidylcholine. As the phosphatidylcholine content of a mixed surfactant system comprising egg phosphatidylcholine : Brij 96 was replaced by lysophosphatidylcholine and fatty acid, the haemolytic action of the mixture increased markedly.

The results from this study show that the toxicity of surfactants to erythrocytes can be mitigated by the addition of egg phosphatidylcholine. Synthetic surfactants combined with phosphatidylcholine may generate drug delivery systems worthy of more extensive investigation.

A fundamental consideration in the use of surface-active materials to enhance the transport of drugs across epithelial membranes is their potential toxicity. The application of penetration enhancers at mucosal sites may damage the epithelial membrane and affect its barrier function, including its ability to exclude bacterial toxins and pathogens present on the mucosa.

Consequently, relatively few surfactants have been approved for use as absorption promoters. In addition, toxicity considerations may limit the incorporation of surfactants in formulations of drugs intended for intravenous use, since some surfactants may lyse the cells in the blood and damage the endothelial cells of the blood vessels. Nevertheless a number of surfactants have been utilized successfully as emulsifying or solubilizing agents for poorly soluble drugs (Petrowski 1975), or to prevent precipitation of a drug upon its injection into the blood, e.g. taxol (Alkan-Onyuksel et al 1994) and teniposide (Alkan-Onyuksel & Son 1992).

The toxic effects of bile salts, which are naturally occurring surfactants, have been studied extensively (Swenson et al 1994; Heuman et al 1996). It has been reported that their toxicity can be mitigated by the addition of egg phosphatidylcholine (Gjone 1961; Martin & Marriott 1981; Newbery et

Correspondence: G. P. Martin, Department of Pharmacy, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 8WA, UK.
E-Mail: gary.martin@kcl.ac.uk

al 1984; Martin et al 1985; El-Hariri et al 1992; Sagawa et al 1993). The addition of an egg phosphatidylcholine liposome preparation to bile salts has been shown to decrease their haemolytic activity (Sagawa et al 1993), while the toxicity of bile salts to goldfish epithelia was reduced by the addition of egg phosphatidylcholine (Martin & Marriott 1981). The effect of bile salts on the rat gastric mucosa was mitigated by egg phosphatidylcholine (Newbery et al 1984; Martin et al 1985) and Gjone (1961) demonstrated that haemolysis induced by lysophosphatidylcholine could be decreased by human serum phosphatidylcholine. On the basis of results derived from a more detailed study of the mitigating effects of egg phosphatidylcholine on the haemolytic potential of bile, it was postulated that the mitigating effects of the egg phosphatidylcholine and other phosphatidylcholines on the toxicity of sodium taurodeoxycholate was due to the formation of mixed micelles between the bile salt and phospholipid (El-Hariri et al 1992). Such a hypothesis was supported more recently by data confirming a similar protection conferred by phosphatidylcholine against the haemolysis induced by taurine conjugates of ursodeoxycholate, cholate and chenodeoxycholate (Narain et al 1997). Later Narain et al (1999) proposed that the presence of cholesterol reversed the toxicity-mitigating effect of egg phosphatidylcholine by raising the concentration at which these mixed micelles were formed.

Polyoxyethylene alkyl ether surfactants have been shown to have excellent penetration enhancing abilities (Walters et al 1981, 1982; Hirai et al 1981) although they, like many of the bile salts, are associated with a high level of local toxicity. Consequently such non-ionic surfactants are not used clinically. However, the effect of egg phosphatidylcholine on the toxicity of these and other synthetic surfactants has not been reported to date.

Various models exist for the examination of the membrane-toxicity of molecules including in-situ animal models where the damage to the mucosa can be visualized by electron micrographs. However, the effect of surfactants on the cell membrane is more conveniently examined with single cell models using erythrocytes, erythrocyte ghosts, or liposomes. The erythrocyte model has been widely used (Helenius & Simons 1975) as it gives a direct indication of the toxicity of formulations used as injectables as well as a general indication of membrane toxicity. Another advantage to the use of erythrocytes is that blood is readily available and that the cells are easy to isolate from the blood.

The aim of this study was to compare the toxicity of the polyoxyethylene alkyl ethers (Brij 96, Brij 76, Brij 56, 10 lauryl ether and 9 lauryl ether) with the toxicity induced by an anionic (sodium dodecyl sulphate (SDS)), an ampholytic (lysophosphatidylcholine) and a cationic (tetradecyltrimethylammonium bromide (TTAB)) surfactant and to determine the effect of combining egg phosphatidylcholine with each of these surfactants. In addition, since phosphatidylcholine is degraded in-vivo by phospholipase A to yield lysophosphatidylcholine and the appropriate fatty acid, an attempt was made to assess the toxicity of the mixed aggregates of Brij 96 and the potential breakdown products of egg phosphatidylcholine.

Materials and Methods

Materials

All materials were of reagent grade unless otherwise stated. Chloroform, di-sodium hydrogen orthophosphate (dihydrate), citric acid (monohydrate) and sodium chloride were supplied by BDH Ltd (Poole, Dorset, UK). Grade 1 egg phosphatidylcholine and grade 1 lysophosphatidylcholine were purchased from Lipid Products (Nutfield, Surrey, UK). Brij 35 solution 30% w/v, Drabkin's agent, oleic acid, polyoxyethylene 10 oleyl ether (Brij 96), polyoxyethylene 10 stearyl ether (Brij 76), polyoxyethylene 10 cetyl ether (Brij 56), polyoxyethylene 10 lauryl ether, polyoxyethylene 9 lauryl ether, SDS, and TTAB were acquired from Sigma Chemical Co. Ltd (Poole, Dorset, UK).

Buffer and reagent preparation

McIlvaine's buffer was prepared as follows: solution 1 containing citric acid (2·1 g) and sodium chloride (7·83 g) made up to 1000 mL with Ultrapure water, was mixed with solution 2, containing disodium hydrogen orthophosphate (3·56 g) and sodium chloride (7·83 g) made up to 1000 mL with Ultrapure water, to produce the required pH of 7·0.

Drabkin's reagent, as used in this study, was made by mixing 1·25 g Drabkin's agent with 0·5 mL Brij 35 solution (30% w/v) in 1000 mL Ultrapure water.

Preparation of the surfactant and egg phosphatidylcholine mixtures

The required amount of egg phosphatidylcholine dissolved in chloroform was added to 5 mL chloroform in a 50-mL round-bottomed flask. The solvent was removed by rotary evaporation (Rotavapor RE111, Buchi Ltd, Switzerland) at 40°C to

leave a thin film of phospholipid in the flask. The phospholipid was resuspended in the required volume of surfactant in McIlvaine's buffer to give egg phosphatidylcholine : surfactant molar ratios in the range of 0·01 : 1·0 to 4·0 : 1·0. The preparations were thoroughly mixed for 5 min until no phospholipid was apparent on the wall of the flask, after which they were left to stand at 4°C for 18 h.

Preparation of egg phosphatidylcholine : surfactant : lysophosphatidylcholine : oleic acid mixtures
The egg phosphatidylcholine film was prepared as described above, but the required amount of oleic acid was included before rotary evaporation. The required amount of lysophosphatidylcholine and surfactant, dissolved in McIlvaine's buffer (pH 7·0) was added. The mixtures were left to stand at 4°C for 18 h.

Erythrocyte haemolysis
Human blood was collected from an individual female Caucasian subject and added to heparinized tubes (Sarstedt Ltd, Beaumont Leys, Leicester, UK). The plasma and buffy coat were removed by centrifuging at 2200 *g* for 10 min and the erythrocytes were washed three times, in at least five times their volume of McIlvaine's buffer, pH 7·0. The erythrocytes were adjusted to approximately 12% haematocrit by resuspending them in buffer to 3·33-times their original weight. The erythrocyte suspension was stored on ice at 4°C and was used within 48 h of collection.

A suspension of red blood cells (0·2 mL) was then incubated for the required time with an equal volume of the test sample of surfactant or phospholipid mixture, prepared in McIlvaine's buffer, at 20 or 37°C. The incubation time was 30 min in all experiments, apart from the study examining the effect of incubation time on haemolysis, where the incubation time was varied between 15 and 60 min. After incubation, the mixtures were spun in a microcentrifuge for 15 s, and 0·2 mL of the resulting supernatant was added to 3 mL Drabkin's reagent to assay for the amount of haemoglobin released. Positive controls consisted of 0·2-mL samples, taken from uncentrifuged mixtures of erythrocyte suspensions (0·2 mL) and buffer (0·2 mL), which were added to 3 mL Drabkin's reagent to obtain a value for 100% haemolysis. A negative control, included to assess the levels of spontaneous haemolysis, comprised 0·2 mL buffer mixed with 0·2 mL erythrocytes. After centrifugation for 15 s, a 0·2-mL sample of supernatant was added to 3 mL Drabkin's reagent. The absorbance (540 nm) of the samples was determined spectrophotometrically and the values expressed as a percentage of the maximum haemolysis.

Statistical analysis
A two-tailed Mann-Whitney U-test was used to analyse the data.

Results and Discussion

Sigmoidal relationships were observed when percentage haemolysis was plotted against concentration of surfactant (Figure 1) and are typical of those reported previously (Kondo & Tomizawa 1968; Azaz et al 1981). All of the polyoxyethylene ethers showed haemolytic activity at concentrations between 0·1 and 0·25 mM, which are above the critical micelle concentrations (CMC) for each of the surfactants (Walters et al 1981).

When the experiments were performed at 37°C (Figure 1a), the concentration of the surfactants needed to induce 50% haemolysis was generally

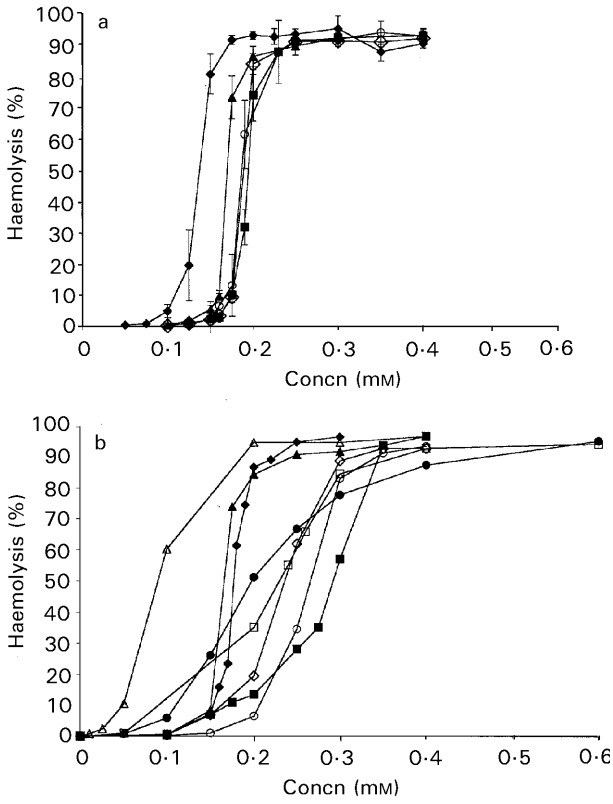

Figure 1. a. Haemolysis induced by surfactants after 30 min at 37°C, pH 7·0 (mean ± s.d., n = 9). b. Haemolysis induced by surfactants after 30 min at 20°C, pH 7·0 (n = 9, CV ≤ 10% at values of haemolysis greater than 25%). Brij 96 (◆), Brij 76 (■), Brij 56 (▲), 10 lauryl ether (◇), 9 lauryl ether (○), SDS (●), TTAB (□) and lysophosphatidylcholine (△).

lower than when the experiment was performed at 20°C (Figure 1b). Brij 56 was an exception with the same concentration (0·17 mM) inducing 50% haemolysis at 20 and 37°C. The concentration range over which the haemolysis induced by any surfactant increased from 0 to 100% was much narrower at 37°C than when the experiment was performed at 20°C. This was especially true for Brij 76, 10 lauryl ether and 9 lauryl ether, where, at 20°C, the gradient of the haemolytic profile was less steep than that observed for Brij 96 and 56. Consequently, experiments were performed at the lower temperature (20°C) when compared with other studies (Florence et al 1978; Zaslavsky et al 1978; Hirai et al 1981; Walters et al 1981) to examine more closely differences between the structure–activity relationships of the surfactants.

A decrease in the alkyl length from C18 (Brij 76) to C16 (Brij 56) resulted in increased haemolysis at the same surfactant concentration. However, a further decrease in chain length from C16 to C12 (10 lauryl ether) resulted in a reduction of haemolysis. A decrease in the number of ethylene oxide residues from 10 (10 lauryl ether) to 9 (9 lauryl ether) also resulted in a decrease in toxicity. The haemolytic profiles of SDS and TTAB were similar to that of 10 lauryl ether. Brij 96 was more toxic than SDS, TTAB and 10 lauryl ether at concentrations of 0·18 mM and above, but was generally of lesser or similar toxicity at lower concentrations.

Inter-laboratory comparisons of the absolute values of the haemolytic concentrations of surfactants is hard to perform since the experimental conditions are often dissimilar in different studies. For example, it has been shown that the concentration of the erythrocytes is important in determining the haemolytic concentration of surfactant (Helenius & Simons 1975). The concentration of surfactant needed to induce haemolysis increased when higher concentrations of red blood cells were used and the total amount of surfactant needed to induce X% haemolysis was linearly related to the cell count.

Even though the haemolytic concentrations of surfactants cannot be compared directly, it is surprising that the trends observed in a homologous series have not been consistent. For example, 9 lauryl ether and Brij 56 have been reported to be equivalently potent and more haemolytic than Brij 76 (Hirai et al 1981). Other studies have demonstrated that the C12 chain has the greatest effect on the disruption of biological membranes (Florence et al 1978; Zaslavsky et al 1978; Walters et al 1981). In this study the surfactant containing predominantly 16 carbon atoms in the alkyl chain was found to be more toxic than the C12 analogues (9 and 10 lauryl ether). The anomaly between the results from this study and those of other groups may be due to the inherent impure nature of commercially available Brij surfactants which are supplied based on the predominant chain length, although the proportions of other alkyl chain lengths present may vary between batches.

Egg phosphatidylcholine had no haemolytic activity at the concentrations used in this study (data not shown). The addition of egg phosphatidylcholine to the surfactant solutions reduced the haemolytic action of all of the surfactants studied (Figure 2). Egg phosphatidylcholine was combined with the concentration of surfactants that induced approximately 80% haemolysis to give lipid : surfactant molar ratios ranging between 0·01 and 2·0. Concentrations of surfactant that induced approximately 80% haemolysis were chosen so that any decrease in haemolytic activity could be easily observed. Surfactant concentrations inducing 100% haemolysis were not used as the concentration of surfactant required to induce exactly 100% haemolysis was difficult to determine.

The ratio of egg phosphatidylcholine : surfactant required to reduce the haemolytic activity of the ionic surfactants was greater than the ratio that was necessary to mitigate the haemolysis of the polyoxyethylene surfactants. The toxicity of Brij 96 was mitigated to a greater extent than that of the other surfactants examined and Brij 96 was the only polyoxyethylene alkyl ether where the toxicity was reduced to zero. Such an observation could be explained by Brij 96 having a greater affinity for egg phosphatidylcholine than the other surfactants tested. The combination of egg phosphatidylcholine with the other surfactants tested resulted in

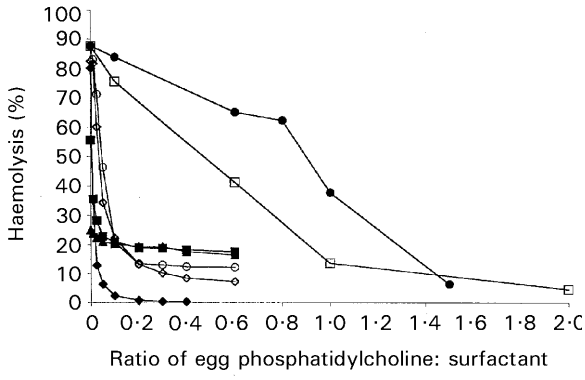

Figure 2. Haemolysis induced by surfactants combined with egg phosphatidylcholine after 30 min at 20°C, pH 7·0 (n = 9, CV ≤ 12% at values of haemolysis greater than 25%). 0·2 mM Brij 96 (◆), 0·32 mM Brij 76 (■), 0·2 mM Brij 56 (▲), 0·26 mM 10 lauryl ether (◇), 0·3 mM 9 lauryl ether (○), 0·4 mM SDS (●) and 0·3 mM TTAB (□).

mixtures that still contained some residual toxicity at all the ratios examined.

At higher concentrations (1·0 mM) of 10 lauryl ether and Brij 96, higher lipid : surfactant ratios were needed to reduce the toxicity of the surfactants to erythrocytes (Figure 3). When the lower concentrations of 0·2 mM Brij 96 and 0·26 mM 10 lauryl ether were combined with egg phosphatidylcholine the toxicity of Brij 96 was reduced to a greater extent than 10 lauryl ether (Figure 2). However, when the higher concentrations of the two surfactants (well above that required to produce 100% haemolysis) were combined with egg phosphatidylcholine the differences in the extent of the reduction of toxicity were less apparent. Nevertheless, the differences between the haemolysis induced by egg phosphatidylcholine : surfactant ratios of $\geq$1·0 for 1·0 mM Brij 96 and 10 lauryl ether were significant ($P < 0.001$), with egg phosphatidylcholine : 10 lauryl ether mixtures being the more toxic.

Although the ability of egg phosphatidylcholine to mitigate the toxicity of non-ionic surfactants has not been demonstrated previously, this effect has been observed with egg phosphatidylcholine : bile salt combinations. The percent haemolysis induced by 1·4 mM sodium deoxycholate and 2·0 mM sodium taurodeoxycholate was reported to be approximately 80 and 60%, respectively, after a 30-min incubation. Such values were similar to those observed in this study for surfactants alone. The ratio of egg phosphatidylcholine : bile salt needed to achieve mitigation of the toxicity of sodium deoxycholate and sodium taurodeoxycholate to approximately 2% was 0·3 (El-Hariri et al 1992). This ratio of egg phosphatidylcholine : bile salt was similar to the ratio of egg phosphatidylcholine : Brij 96 needed to achieve mitigation of Brij 96-induced haemolysis, and to reduce the toxicity of the other polyoxyethylene alkyl ether surfactants to a minimum (Figure 2). A reduction in bile salt haemolytic activity by the addition of egg phosphatidylcholine was also demonstrated by Sagawa et al (1993) and Narain et al (1997). In those studies the amount of haemolysis was comparable with that induced by 9 and 10 lauryl ether, combined with egg phosphatidylcholine at the same ratio.

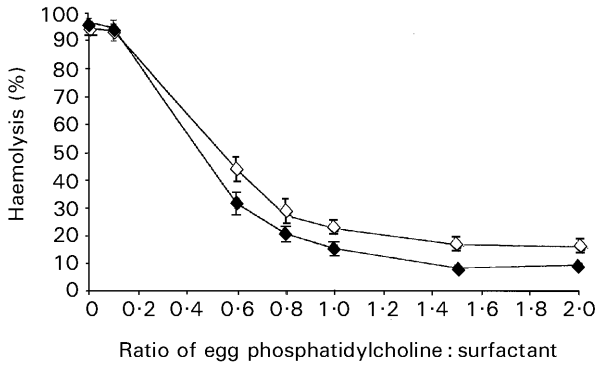

Figure 3. Haemolysis induced by 1·0 mM Brij 96 (◆) and 10 lauryl ether (◇) combined with egg phosphatidylcholine after 30 min at 20°C, pH 7·0 (mean ± s.d., n = 9).

The present work has demonstrated that egg phosphatidylcholine was able to reduce the toxicity of structurally different surfactants, since the haemolytic activity of the anionic surfactant SDS and cationic surfactant TTAB was mitigated in addition to the alleviation of the toxicity of the non-ionic polyoxyethylene alkyl ether surfactants. A previous study showed that the toxicity of SDS to goldfish membranes could be reduced by the addition of egg phosphatidylcholine (Martin & Marriott 1981), but this protective effect had not previously been shown to extend to erythrocytes. Interestingly, egg phosphatidylcholine was less protective in combination with the ionic surfactants than when combined with the non-ionic polyoxyethylene alkyl ethers. Egg phosphatidylcholine may interact with polyoxyethylene alkyl ethers to a greater extent than SDS and TTAB, reducing the amount of 'free' surfactant available to induce haemolysis. Such a theory is supported by electron microscopy and photon correlation spectroscopical studies (Gould et al 1997) in which the haemolytic effects of the mixed phosphatidylcholine : surfactant aggregates were compared with their physical characteristics. It was observed that aggregates with reduced toxicity were of a larger particle size ($\geq$61 nm) which appeared to correlate with a change in the type of mixed aggregate from a micellar to a lamellar system. Such a transition might lead to a reduction in the availability of 'free' surfactant.

The kinetics of the erythrocyte haemolysis induced by Brij 96 and 10 lauryl ether and associated lipid surfactant mixtures were determined (Figure 4). It was found that the higher ratios of egg phosphatidylcholine : Brij 96 were less toxic, with a longer lag time and a slower rate of haemolysis than the lower egg phosphatidylcholine : surfactant ratios (Figure 4a). A similar effect on the rate of haemolysis was observed with ratios of egg phosphatidylcholine : 10 lauryl ether although no lag time was observed (Figure 4b). The rate of haemolysis decreased as egg phosphatidylcholine content increased, although there was no significant difference ($P > 0.05$) between the haemolysis induced by 0·26 mM 10 lauryl ether alone and the same concentration in the presence of the lowest concentration of phosphatidylcholine (0·01 mM) at all time points examined. In contrast, the difference between the haemolysis induced by 0·2 mM Brij 96

and when combined with 0·01 mM egg phosphatidylcholine was significant ($P < 0.001$) after 30-min incubation.

The toxicity of mixtures containing different proportions of egg phosphatidylcholine : Brij 96 : lysophosphatidylcholine : oleic acid are shown in Figure 5, where each point on the graph represents 1·0 mM Brij 96 combined with egg phosphatidylcholine, oleic acid and lysophosphatidylcholine. Decreasing the proportion of egg phosphatidylcholine and thus increasing the relative amounts of lysophosphatidylcholine and oleic acid increased the percentage haemolysis induced. The lysophosphatidylcholine itself was confirmed to be highly haemolytic (Figure 1b) as previously demonstrated (Reeman & Van Deenan 1967; Weltzein et al 1977; Bierbaum et al 1979; Isomaa et al 1988). The concentration of 0·2 mM lysophosphatidylcholine required to induce 100% haemolysis in this study was consistent with reports of the haemolytic activity of lysophosphatidylcholine under similar experimental conditions (Martin et al 1992). Oleic acid also has penetration enhancing properties, although much higher concentrations than those used in this study have been required to produce these effects (Tengamnuay & Mitra 1990). Different ratios of egg phosphatidylcholine : Brij 96 : lysophosphatidylcholine : oleic acid were used to model what might be expected to occur should the mixed micelles come into contact with phospholipases which break down the egg phosphatidylcholine into lysophosphatidylcholine and a fatty acid e.g. oleic acid. The increase in toxicity observed as the concentration of egg phosphatidylcholine was decreased (and the concentration of fatty acid and lysophosphatidylcholine was increased) was probably due to two effects. Firstly, as the amount of egg phosphatidylcholine was decreased more Brij 96 was made available to the erythrocyte membrane, resulting in an enhancement of haemolysis. Secondly, the increasing proportion of lysophosphatidylcholine in the mixtures contributed to the haemolytic activity.

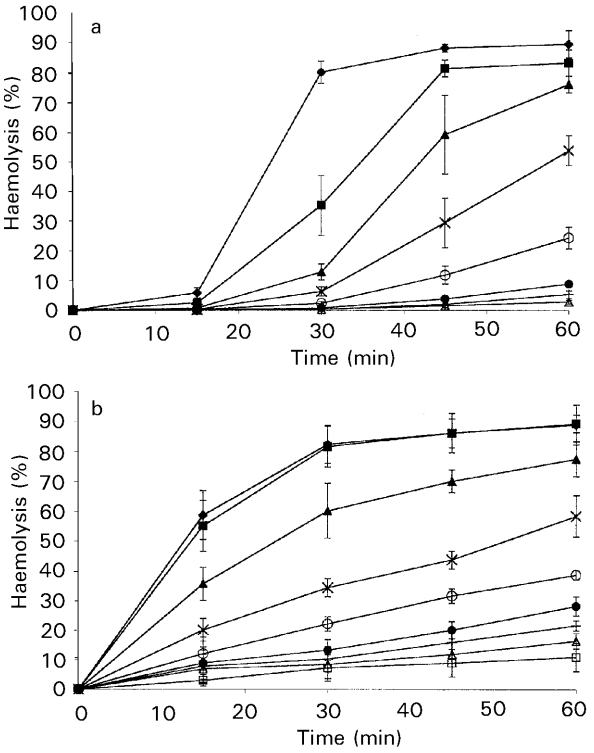

Figure 4. a. Effect of incubation time on haemolysis induced by various egg phosphatidylcholine and 0·2 mM Brij 96 mixtures at 20°C, pH 7·0 (mean ± s.d., n = 9). Brij 96 alone (◆), 0·01 mM egg phosphatidylcholine : Brij 96 (■), 0·025 mM egg phosphatidylcholine : Brij 96 (▲), 0·05 mM egg phosphatidylcholine : Brij 96 (×), 0·1 mM egg phosphatidylcholine : Brij 96 (○), 0·2 mM egg phosphatidylcholine : Brij 96 (●), 0·3 mM egg phosphatidylcholine : Brij 96 (+) and 0·4 mM egg phosphatidylcholine : Brij 96 (△). b. Effect of incubation time on haemolysis induced by various egg phosphatidylcholine and 0·26 mM 10 lauryl ether mixtures at 20°C, pH 7·0 (mean ± s.d., n = 9). 10 lauryl ether alone (◆), 0·01 mM egg phosphatidylcholine : 10 lauryl ether (■), 0·025 mM egg phosphatidylcholine : 10 lauryl ether (▲), 0·05 mM egg phosphatidylcholine : 10 lauryl ether (×), 0·1 mM egg phosphatidylcholine : 10 lauryl ether (○), 0·2 mM egg phosphatidylcholine : 10 lauryl ether (●), 0·3 mM egg phosphatidylcholine : 10 lauryl ether (+), 0·4 mM egg phosphatidylcholine : 10 lauryl ether (△), and 0·4 mM egg phosphatidylcholine : 10 lauryl ether (□).

Conclusions

The haemolysis of erythrocytes can be used to determine the interaction of mixed surfactant systems with membranes. It has been shown that the toxicity of non-ionic, cationic and anionic surfactants to erythrocytes can be mitigated by the addition of egg phosphatidylcholine. These results

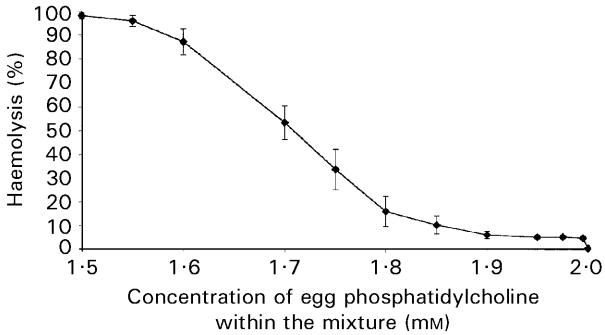

Figure 5. Haemolysis induced by egg phosphatidylcholine : Brij 96 : lysophosphatidylcholine : oleic acid after 30 min at 20°C, pH 7·0 (mean ± s.d., n = 9). The Brij 96 concentration was maintained constant at 1 mM and the total concentration of the components in all mixtures at 3 mM. The phosphatidylcholine concentration was varied between 1·5 and 2 mM, with the balance (0–0·5 mM) being equimolar concentrations of oleic acid and lysophosphatidylcholine. For example, the composition of the mixture showing 98·1% haemolysis was 1·5 mM phosphatidylcholine, 1 mM Brij 96, 0·5 mM oleic acid : 0·5 mM lysophosphatidylcholine.

illustrate how mixed aggregates of egg phosphatidylcholine and surfactant could function as drug delivery systems; acting as drug solubilizers and carriers with the added advantage of containing a penetration enhancer. If mixed micelles of a penetration enhancer and a phospholipid were administered to the body upon encountering phospholipases the mixed micellar structure might be expected to break down, releasing the drug and the penetration enhancer simultaneously.

Acknowledgements

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