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MEMBRANE LIPID COMPOSITION AND SUSCEPTIBILITY TO BILE SALT DAMAGE

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Summary

Erythrocyte membranes with low sphingomyelin : choline-containing phospholipid ratios haemolyse at low concentrations of the bile salt, glycocholate. Erythrocytes with higher sphingomyelin : choline-containing phospholipid ratios require progessively greater concentrations of the bile salt for lysis.

Sublytic concentrations of glycocholate remove phospholipid and acetylcholinesterase from the membranes. Membranes with low sphingomyelin : choline-containing phospholipid ratios lose both particulate (microvesicles of distinct composition) and 'solubilized' material, the particulate form predominating. The proportion of particulate material falls with increase of the membrane sphingomyelin : choline-containing phospholipid ratio and those membranes of highest sphingomyelin : choline-containing phospholipid ratio lose material predominantly in 'solubilized' form.

Sheep erythrocytes treated to increase their content of phosphatidylcholine (and thereby reduce their membrane sphingomyelin : choline-containing phospholipid ratio) become more susceptible to lysis by glycocholate.

These observations indicate a correlation between membrane lipid composition and the perturbation of membranes with bile salt; they also point to possible features of membranes capable of surviving exposure to the high bile salt concentrations of the biliary tract.

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

TABLE I

CHOLINE-CONTAINING PHOSPHOLIPIDS OF ERYTHROCYTE MEMBRANES FROM VARIOUS MAMMALIAN SPECIES

The relative amounts of sphingomyelin and phosphatidylcholine are expressed as a percentage of the membrane total phospholipids and the sources of these values are given in square brackets.

Species	% of total phospholipids		Ratio, sphingomyelin:total choline-containing phospholipids
	Sphingomyelin	Phosphatidyl-choline	
Guinea-pig [6]	11	41	0.21
Rat [6]	13	48	0.21
Human [2]	25	30	0.45
Pig [6]	26	23	0.53
Sheep [3]	49	1	0.98
Ox [6]	46	0	1.00

Introduction

In a series of recent experiments studying the effects of bile salts upon membranes it was shown, using human erythrocytes, that (i) the concentration at which cell lysis occurred depends upon the nature of the bile salt [1]; (ii) membrane phospholipids and proteins are released in the form of microvesicles of unique composition at low, sublytic concentrations of glycocholate [2]; (iii) at higher, but still sublytic, concentrations of glycocholate membrane phospholipids and proteins are released in 'solubilized' form [2].

Studies with sheep erythrocytes, however, show, in comparison with human erythrocytes, that (i) a much higher concentration of glycocholate is required to effect lysis [3,4], and (ii) the material released from the membrane at sublytic concentrations of glycocholate contains only 'solubilized' material [2]. There is thus a substantial difference in the response of these two membranes to the same bile salt; this in its turn must reflect some differences in composition or structure between these two membranes. One significant difference lies in their choline-containing phospholipids. In both membranes these make up about half the total phospholipid complement but in sheep erythrocytes this is almost exclusively in the form of sphingomyelin whereas human erythrocytes possess almost equal amounts of sphingomyelin and phosphatidylcholine (Table I).

The influence of this aspect of lipid composition upon the behaviour of membranes to bile salts has now been investigated with a series of erythrocytes, in pairs of species, selected to represent significant variations in choline-containing phospholipid content (Table I). A preliminary account of some of this work has appeared [5].

Materials and Methods

Materials. Ox, sheep and pig blood from local slaughterhouses, and guinea-pig (female, approx. 450 g) and rat (male, approx. 250 g) blood were taken

into 0.33 vol. acid/citrate/dextrose solution. Human blood in acid/citrate/dextrose solution was obtained by courtesy of a local transfusion service and was used within seven days of donation. Erythrocytes were washed (three times, 5 vols.) and finally resuspended in 0.154 M NaCl/1.5 mM Hepes adjusted to pH 7.4, to a final concentration of approx. 2 μmol phospholipid phosphorus/ml. Glycocholate (sodium salt, A grade, more than 98% pure) was obtained from Calbiochem. Ltd., Bishops Stortford, Herts., U.K.

Methods. 1 vol. of erythrocyte suspension was incubated at 37°C for 10 min with 3 vols. of 0.14 M NaCl/15 mM Hepes (pH 7.4) containing appropriate concentrations of bile salts. Incubations were terminated by centrifugation at 14 000 × *g* for 1 min (Jobling 320 microcentrifuge) or 3500 × *g* for 4 min (MSE bench centrifuge). In some experiments aliquots of these supernatants were further centrifuged at 150 000 × *g* for 60 min.

The percentage haemolysis was determined by comparing the absorbance at 525 nm of appropriate dilutions of the low-speed supernatant with that of a corresponding uncentrifuged control totally haemolysed by dilution with 24 vols. water.

Supernatants were assayed for phospholipid phosphorus, phospholipid profile and for acetylcholinesterase activity (uncorrected for inhibition by the different concentrations of bile salts) as described previously [2]. The critical micellar concentration of glycocholate in 0.14 M NaCl, 15 mM Hepes (pH 7.4) was determined by a dye method [7].

The technique for increasing the phosphatidylcholine content of sheep erythrocyte membranes was essentially that of Borochov et al. [8]. The phosphatidylcholine-enriched cells, and their controls, were washed and suspended to a concentration of approx. 2 μmol phospholipid phosphorus/ml prior to incubation with bile salt.

Results

Susceptibility to lysis by glycocholate

Rat and guinea-pig erythrocytes (sphingomyelin : choline-containing phospholipid ratio 0.21) showed lysis at low concentrations of glycocholate (5—10 mM); approx. 10% lysis was achieved at approx. 10 mM in both cases (Fig. 1). Human and pig erythrocytes (sphingomyelin : choline-containing phospholipid ratio approx. 0.5) required greater amounts of glycocholate (15—20 mM for initial lysis, and 25—30 mM for 10% lysis) for equivalent degrees of lysis. Ox and sheep erythrocytes (sphingomyelin : choline-containing phospholipid ratio approx. 1.0 required progressively more glycocholate (30—40 mM for initial lysis, and 40—50 mM for 10% lysis) (Fig. 1).

The critical micellar concentration of glycocholate under these conditions was 6.1 mM and thus rat and guinea-pig erythrocytes lysed approximately at, or just beyond, the critical micellar concentration, whereas erythrocytes with a higher sphingomyelin : choline-containing phospholipid ratio lysed well above it.

All cells released membrane phospholipid at sublytic concentrations of glycocholate; rat, guinea-pig, human and pig cells released approx. 15% of total whereas the release from ox and sheep cells was lower, approx. 10% (Fig. 1).

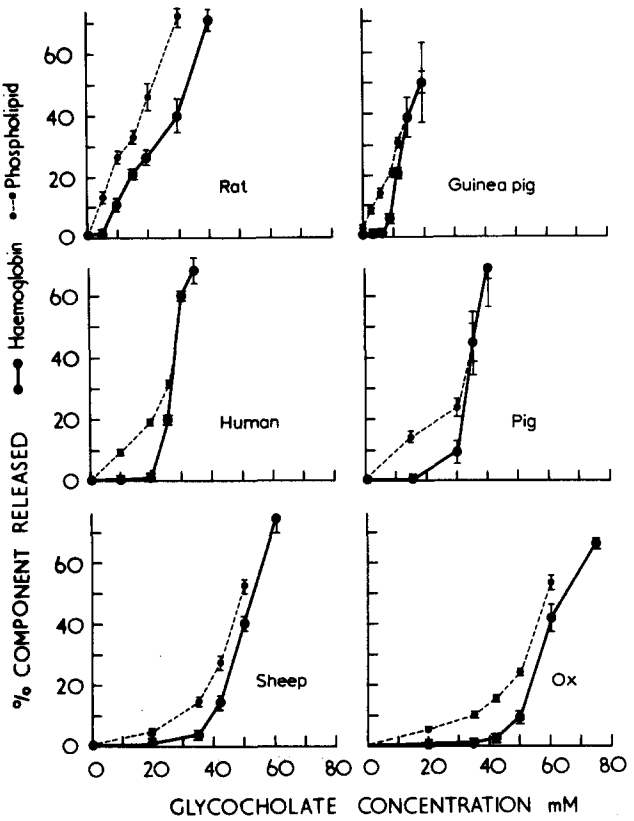

Fig. 1. The release of materials from erythrocytes by glycocholate. Erythrocytes were incubated with glycocholate at 37°C for 10 min and the incubation terminated by centrifugation at 14 000 × g_{min}. Supernatants were assayed for haemoglobin (●————●), and phospholipid (●- - - - -●). Values are means of three or four experiments ± S.E.

Form of material released at sublytic concentrations of glycocholate

Concentrations of glycocholate were selected prior to, and approximately at, the lysis point. 'Low speed' supernatants (14 000 × g_{min}) were sampled for haemoglobin, acetylcholinesterase and phospholipid and the remainder centrifuged at 150 000 × g for 60 min. This sedimented any particulate material leaving material which had been 'solubilized' in the 'high-speed' supernatant.

There was a marked difference between the distribution of particulate and solubilized material in the sublytic extracts. Phospholipid and acetylcholinesterase activity was mostly sedimented in extracts from cells of lower sphingomyelin : choline-containing phospholipid values; at intermediate sphingomyelin : choline phospholipid levels there were appreciable amounts of both 'solubilized' and particulate materials, whereas cells with the highest sphingomyelin : choline-containing phospholipid values released both acetylcholinesterase and phospholipid predominantly in 'solubilized' form (Fig. 2). In all cases the amount (and proportion) of solubilized material was greater at the higher glycocholate concentration.

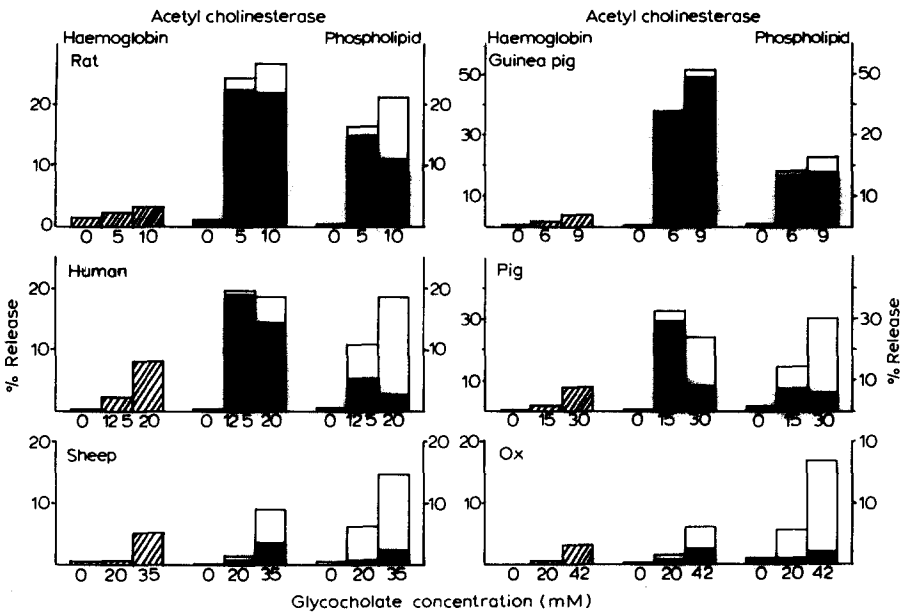

Fig. 2. Centrifugation of low-speed supernatants. After incubation with glycocholate at the concentrations indicated at the foot of each column, the cells were centrifugated at 14 000 X g_{min} to obtain a low-speed supernatant (total height of column). These supernatants were then centrifuged at 150 000 X g for 60 min to obtain particulate material (solid column) and a high-speed supernatant (open column). Low and high-speed supernatants were assayed for acetylcholinesterase, phospholipid and haemoglobin. The extent of cell lysis was denoted by the hatched column. 100% values for rat, guinea-pig, human, pig, sheep and ox are: acetylcholinesterase, 0.6, 0.8, 5.2, 1.7, 0.9, 2.3 μmol/min per ml final erythrocyte suspension; phospholipid, 2.1, 1.9, 2.2, 2.1, 2.3, 1.9 μmol/ml final erythrocyte suspension. All values on histograms and in the legend represent means of three or four (mostly) experiments. S.E. have been omitted for clarity of presentation.

The proportion of particulate acetylcholinesterase released exceeded that of particulate phospholipid by factors of 1.5—4-fold; this indicated that the particulate form comprises not merely fragments of the original membrane, since this would have produced an unchanged ratio of acetylcholinesterase to phospholipid. Glycocholate therefore has released material with a composition distinct from the original membrane.

Effect of phosphatidylcholine incorporation into sheep erythrocyte membranes

Borochov et al. [8] have been able to increase the amount of phosphatidylcholine in sheep erythrocyte membranes by incubation with human plasma lipoproteins (to supply phosphatidylcholine) and 2 mM EGTA (to inhibit phosphatidylcholine hydrolysis by a constituent phospholipase of the membranes). The incorporation of phosphatidylcholine by these techniques brought about a fall in the sphingomyelin : choline-containing phospholipid ratio from 0.97 to 0.87; phospholipid chromatography confirmed the presence of phosphatidylcholine in the membrane preparations.

These phosphatidylcholine-enriched erythrocytes showed a significant increase in susceptibility to lysis by glycocholate, when compared to appropri-

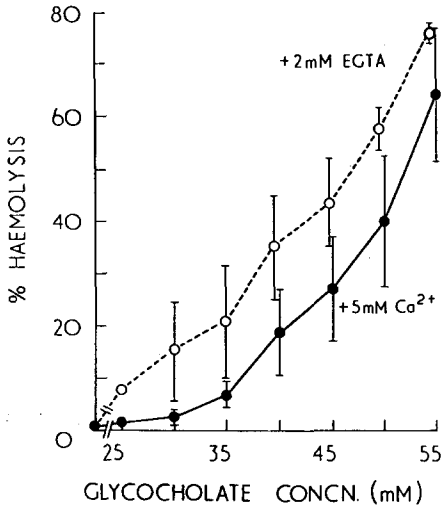

Fig. 3. Lysis of sheep erythrocytes by glycocholate after incubation with plasma lipoproteins. Sheep erythrocytes were incubated with shaking at 37°C for 48 h with human plasma and either 2 mM EGTA (○) or 5 mM $CaCl_2$ (●). The cells were then washed and incubated at 37°C for 10 min with glycocholate. Incubations were terminated by centrifugation at 14 000 × g_{min} and the percent lysis determined by measurement of released haemoglobin in the supernatants. Values are means of three experiments ± S.E.

ate controls (Ca^{2+} substituted for EGTA during the incubation with plasma lipoproteins) (Fig. 3).

Discussion

These results show a good correlation between the lipid composition of the membrane and the susceptibility to bile salt-induced lysis. Whether it is more appropriate, however, to view this as a lower susceptibility to lysis in erythrocytes with a high sphingomyelin content, or as a higher susceptibility in those of increasing phosphatidylcholine content, remains to be resolved. There also appears to be a correlation of membrane lipid composition with the membrane perturbation leading to loss of particulate material into the supernatant. Membranes possessing both phosphatidylcholine and sphingomyelin appear to lose particulate material in addition to that appearing in 'solubilized' form. The 'solubilized' material from human and sheep erythrocytes has been shown to contain mostly those lipids derived from the outer leaflet of the membrane [1—3].

From studies with human erythrocytes, the particulate material has been shown to be predominantly in the form of small vesicles of distinct composition [2]. For the remaining species, the form of the released particulate material has not been established, but also appears similar for the following reasons: (i) preliminary experiments indicate that the majority of the particulate material cannot be sedimented at 15 000 × g for 15 min, i.e. it is of small-sized vesicles, and (ii) an enrichment in the acetylcholinesterase : phospholipid ratio is observed in the particulate material, as compared to the original membrane, in every case. This implies selectivity in the formation of the released

material and the differential migration of membrane components (see also Refs. 2 and 9); it is interesting that this ability to differentiate a distinct vesicular material shows a correlation with the lipid composition of the parent membrane.

Other differences in the properties of erythrocyte membranes of various species have been observed to correlate with lipid composition. The permeability of erythrocyte membranes to a series of small molecules (urea, thiourea, ethylene glycol and glycerol) appears to decrease with increasing membrane sphingomyelin (and decreasing phosphatidylcholine)content [6,10,11]. In most cases the permeability observed is probably simple diffusion through the lipid bilayer and therefore the chemical composition (and therefore physical properties) of the bilayer will influence permeation rate; in a few of these cases the results are complicated, however, by the superimposition of a transport system (e.g. for glycerol in some species).

Bile salts are secreted by hepatocytes; their concentration in bile may rise to 20—30 mM and they may be concentrated further in the gall bladder. In vivo, therefore, high concentrations of these potentially membrane-damaging agents are in contact with the surface membranes of biliary tract cells [4,12]. How do such cells survive? The relative resistance of erythrocyte membranes with a high sphingomyelin : choline-containing phospholipid ratio may be a good indicator to this, and it is therefore interesting that liver plasma membrane preparations rich in bile canalicular membranes have relatively high contents (18—25% of total phospholipid [13,14]) of sphingomyelin which may contribute to their resistance to bile salt attack [4,14].

Acknowledgements

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