Biochimica et Biophysica Acta, 509 (1978) 33—47

BBA 78002

EFFECTS OF BILE SALTS ON HUMAN ERYTHROCYTES

PLASMA MEMBRANE VESICULATION, PHOSPHOLIPID SOLUBILIZATION AND THEIR POSSIBLE RELATIONSHIPS TO BILE SECRETION

DAVID BILLINGTON and ROGER COLEMAN

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (U.K.)

(Received August 22nd, 1977)

Summary

Glycocholate removed approximately 25% of the membrane acetylcholinesterase and 10% of the membrane phospholipid from intact human erythrocytes prior to the onset of cell lysis. At low concentrations (up to 6 mM), glycocholate caused human erythrocytes to become echinocytic and to pinch off microvesicles, whereas at higher concentrations glycocholate also specifically released components from the outer leaflet of the plasma membrane in a 'soluble' form (as defined by their presence in a 150 000 × *g*/60 min supernatant) and caused the cells to become stomatocytic. Whilst the phospholipid profile of the 'soluble' material differed from that of the whole membrane, the profile of the microvesicle fraction was similar. The microvesicles were depleted in several membrane proteins with respect to phospholipids. These observations are discussed in relation to the possible role of bile salts in the origins of biliary phospholipid and protein.

Introduction

Bile salts are detergents which have found extensive use in biochemistry as tools for the dissection of membrane components [1]. Mammalian liver secretes large quantities of bile salts; the combined concentration of these may reach 0.5—1.0% (10—20 mM) in hepatic bile, and, if concentrated in the gall bladder, may rise to as high as 7% [2,3]. Conjugated cholate derivatives (glycocholate and taurocholate) compose approximately half the bile salt pool of human bile [4—6]. The membrane-damaging properties of bile salts are in the order deoxycholate > cholate > glycocholate and taurocholate [7,8] and this secretion bias towards the less membrane-damaging bile salts has been interpreted [9] as a means of minimising damage to the membranes of the hepatobiliary system.

Bile contains significant amounts of the plasma membrane enzymes alkaline phosphatase, alkaline phosphodiesterase I, L-leucyl-β-naphthylamidase and 5'-nucleotidase, whilst intracellular enzymes (for example lactate dehydrogenase) are virtually absent [10]. These enzymes are glycoproteins [11] and are known to be externally orientated in the plasma membrane [12—14]. Glycocholate and taurocholate have been shown to remove a proportion of these enzymes from intact pig lymphocytes prior to the release of significant amounts of intracellular proteins [15]. Further, glycocholate will remove from intact human erythrocytes a proportion of the membrane acetylcholinesterase and phospholipid before the onset of lysis [16]. Acetylcholinesterase is an externally orientated erythrocyte protein [17] and the phospholipids released also represent externally orientated types [18,19]. Recently, glycocholate and taurocholate have been shown to remove outer leaflet phospholipids from intact sheep erythrocytes before lysis [20].

These observations led Coleman et al. [9] to propose that biliary phospholipid and some of the biliary proteins arise from the specific removal of externally orientated components of the bile canalicular membrane in the form of, for phospholipids, mixed micelles. We have further tested this hypothesis by examining the structure and composition of the lipid and protein components released by glycocholate from human erythrocytes.

Materials and Methods

Materials. Human blood was obtained through the courtesy of the Midland Blood Transfusion Centre, Birmingham. It was stored at 4°C in citrate/phosphate/dextrose solution and used within 7 days of donation. Sheep and pig blood were obtained fresh from local slaughterhouses and collected in glucose/citrate solution.

Glycocholate and taurocholate (Grade A, more than 98% pure) were obtained from Calbiochem Ltd., Hereford, U.K. and 1,5-bis-(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51) was obtained from Wellcome Research Laboratories, Beckenham, BR3 3BS, U.K. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (from rabbit muscle) and various fine chemicals were obtained from Sigma Chemical Co., Kingston upon Thames, KT2 7BH, U.K.

Methods. Blood samples were centrifuged at 2500 × *g* for 10 min and the plasma and buffy coat removed. The packed erythrocytes were extensively washed at 4°C in 154 mM NaCl/1.5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), pH 7.4, and resuspended in this medium to an approximate concentration of 2 μmol of phospholipid per ml (assayed in lipid extracts by the method of King [21]). One volume of this suspension was incubated at 37°C for 10 min with 3 volumes of 140 mM NaCl/15 mM HEPES (pH 7.4) containing different concentrations of bile salts. Supernatants were obtained by centrifuging at 14 000 × *g* for 1 min in a Jobling model 320 microcentrifuge. In some experiments the supernatants were further centrifuged at 150 000 × *g* at 4°C for 60 min in a MSE model 50 ultracentrifuge.

The extent of erythrocyte lysis was assayed by comparing the absorbance at 525 nm of supernatants with dilutions (in water) of corresponding un-

centrifuged controls. Lipid extracts [22] of supernatants were assayed for phospholipid phosphorus essentially by the method of Bartlett [23] except that samples were digested with 72% $HClO_4$ [24]. The phospholipid profile of lipid extracts was determined by quantitative thin-layer chromatography as described by Skipski et al. [25]. Neutral lipids were separated on silica gel G plates using benzene/diethyl ether/ethanol/acetic acid (50 : 40 : 2 : 0.2, v/v) as described before [26].

Total protein was determined by the method of Lowry et al. [27] using bovine serum albumin (fraction V, fatty acid free) as standard. Allowance was made for the contribution of HEPES to the developed colour. Haemoglobin was assayed by the pyridine haemochromogen method of Dodge et al. [28]. Non-haemoglobin protein was estimated by subtracting the value obtained for haemoglobin from that obtained for total protein.

Acetylcholinesterase (EC 3.1.1.7) activity was determined essentially as described by Ellman et al. [29]. The assay mixture contained 0.33 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 1.67 mM acetylthiocholine and 100 mM sodium phosphate, pH 8.0, in a final volume of 3.0 ml at 37°C. The reaction was followed spectrophotometrically by the increase in absorbance at 412 nm in a Uvichem mark 2 spectrophotometer equipped with a Servoscribe recorder. Observed activities were corrected for inhibition by high concentrations of glycocholate and the spontaneous hydrolysis of acetylthiocholine in the absence of enzyme. The acetylcholinesterase activity released from human erythrocytes by glycocholate was completely inhibited by 30 μM BW284C51 which is specific for 'true' cholinesterase at this concentration.

Sodium dodecyl sulphate gel electrophoresis was performed according to Weber and Osborn [30]. Protein samples were solubilised at 37°C overnight in 8 M urea/4% sodium dodecyl sulphate/1% β-mercaptoethanol/10 mM phosphate buffer (pH 7.0) containing bromophenol blue as tracking dye. (Identical polypeptide profiles were obtained when solubilisation was performed at 100°C for 2 min suggesting minimal proteolysis at 37°C). The electrophoresis buffer was 0.1% sodium dodecyl sulphate in 0.1 M phosphate buffer, pH 7.0. Each 6.25% polyacryalamide gel (0.4 × 14 cm) was run for 30 min at 2 mA/gel, loaded with the protein sample and run at room temperature for 1 h at 1 mA/gel and then for 7—8 h at 4 mA/gel; this method was found to give the best resolution of the polypeptide bands. Gels were stained for protein with Coomassie Blue and after destaining were scanned at 570 nm in a Unicam SP500 spectrophotometer fitted with a Gilford gel-scanning attachment. Bands were numbered according to the convention proposed by Steck [17]. Glyceraldehyde-3-phosphate dehydrogenase (2 μg per gel) was included as an internal standard (see Results).

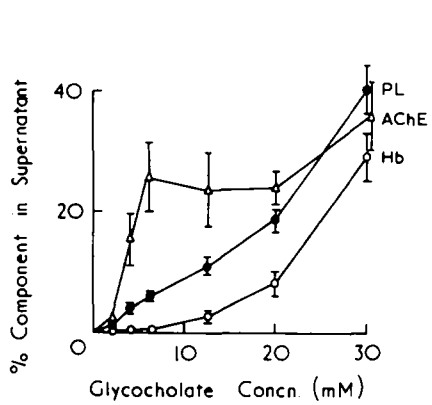
Samples for electron microscopy were fixed in 4% glutaraldehyde followed by 1% OsO_4. For transmission microscopy, thin sections of the material were embedded in Epon resin and stained with Reynolds lead citrate stain [31] before examination in a Phillips EM301 electron microscope. For scanning microscopy, the fixed samples were dehydrated in suspension with acetone, coated with gold and examined in a Cambridge Steroscan 600.

Results

The release of erythrocyte membrane components by glycocholate

When intact human erythrocytes were incubated at 37°C for 10 min with increasing concentrations of glycocholate, approx. 25% of the membrane acetylcholinesterase and 10% of the membrane phospholipid were released into the medium prior to the onset of lysis (Fig. 1). The further release of acetylcholinesterase and phospholipid was paralleled by haemolysis (Fig. 1). In these experiments supernatants were obtained by centrifugation at 14 000 × *g* for 1 min. However, on further centrifuging this low speed supernatant at 150 000 × *g* for 60 min, a proportion of the acetylcholinesterase and phospholipid was sedimented (Fig. 2) and a small red pellet was observed at the bottom of the tube. At low glycocholate concentrations (up to 6 mM) all the released acetylcholinesterase and phospholipid was sedimented whilst at higher glycocholate concentrations increasing amounts of these membrane components remained in the supernatant (Fig. 2). Although the pellet obviously contained haemoglobin, the sensitivity of the assay used did not allow its disappearance from the supernatant on further centrifugation at 150 000 × *g* for 60 min to be accurately determined.

Thus, low concentrations (up to 6 mM) of glycocholate release from intact human erythrocytes acetylcholinesterase and phospholipid which can be sedimented by high speed centrifugation. This material can be partially solubilised; increasing the glycocholate concentration in a 6 mM extract of human erythrocytes caused progressively more acetylcholinesterase and phospholipid to remain in the supernatant after further centrifugation at 150 000 × *g* for 60 min (Fig. 3).



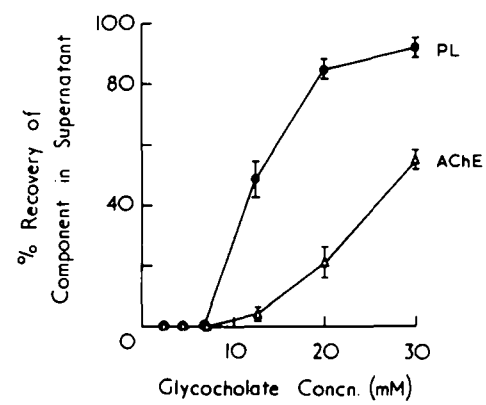


Fig. 1. The release of acetylcholinesterase and phospholipid from intact human erythrocytes by glycocholate. Erythrocytes were incubated at 37°C for 10 min at the final glycocholate concentration shown. Supernatants were obtained by centrifugation at 14 000 × *g* for 1 min. Values are means of three experiments ± S.E. △, Acetylcholinesterase (AChE); ●, phospholipid (PL); ○, haemoglobin (Hb).

Fig. 2. The sedimentation of released acetylcholinesterase and phospholipid on high speed centrifugation. Human erythrocytes were incubated at 37°C for 10 min at the final glycocholate concentrations shown. Supernatants were obtained by centrifugation at 14 000 × *g* for 1 min and assayed for acetylcholinesterase and phospholipid. The supernatant was further centrifuged at 150 000 × *g* for 60 min and the acetylcholinesterase and phospholipid recovered in this supernatant is expressed as a percentage of that in the low speed supernatant. Values are means of three experiments ± S.E. △, Acetylcholinesterase (AChE); ●, phospholipid (PL).

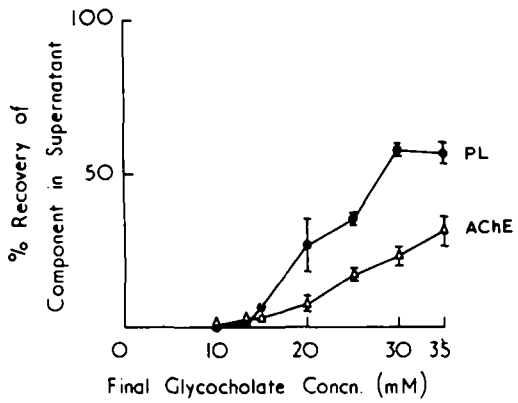


Fig. 3. 'Solubilisation' of acetylcholinesterase and phospholipid released from erythrocytes. Human erythrocytes were incubated at 37°C for 10 min in the presence of 6 mM glycocholate. Supernatant was obtained by centrifugation at 14 000 × *g* for 1 min. Glycocholate was added to aliquots of this supernatant to give the final concentrations shown. The mixtures were then incubated at 37°C for 10 min and centrifuged at 150 000 × *g* for 60 min. Acetylcholinesterase and phospholipid recovered in each supernatant are expressed as a percentage of that present in the low speed supernatant from the original 6 mM glycocholate incubation. Values are means of two experiments ± S.E. △, Acetylcholinesterase (AChE); ●, phospholipid (PL).

A similar series of experiments with pig erythrocytes showed that at low glycocholate concentrations (6 mM) all the acetylcholinesterase and phospholipid released into low speed supernatants could be sedimented by further centrifugation at 150 000 × *g* for 60 min. In contrast, none of the phospholipid released from sheep erythrocytes by glycocholate and taurocholate could be sedimented at 150 000 × *g* for 60 min. (Acetylcholinesterase activity could not be detected in sheep erythrocytes and therefore its release was not studied).

Morphological changes induced by glycocholate

Incubation at 37°C for 10 min in the presence of 6 mM glycocholate caused human erythrocytes to become crenated spheres (echinocytes) of a smaller diameter (Fig. 4b) when compared to the essentially biconcave discoid shape of control incubated cells (Fig. 4a). Incubations at 20 mM glycocholate gave rise to predominantly cup-shaped cells (stomatocytes) (Fig. 4d) whilst incubations at 12.5 mM glycocholate gave a mixture of echinocytes and stomatocytes together with intermediate forms (Fig. 4c). The results presented in Fig. 4 were obtained by scanning electron microscopy; similar observations were made by both transmission electron microscopy and phase contrast microscopy.

The high speed pellet from a 6 mM glycocholate extract of erythrocytes showed, by transmission electron microscopy, a population of small spheres (microvesicles) approx. 80—120 nm in diameter, together with some cylindrical forms (Fig. 5). The size and shape of the membranous material of the microvesicular fraction suggested that this was derived by a pinching-off process from the projections seen in echinocytic erythrocytes at the same glycocholate concentration (Fig. 4b).

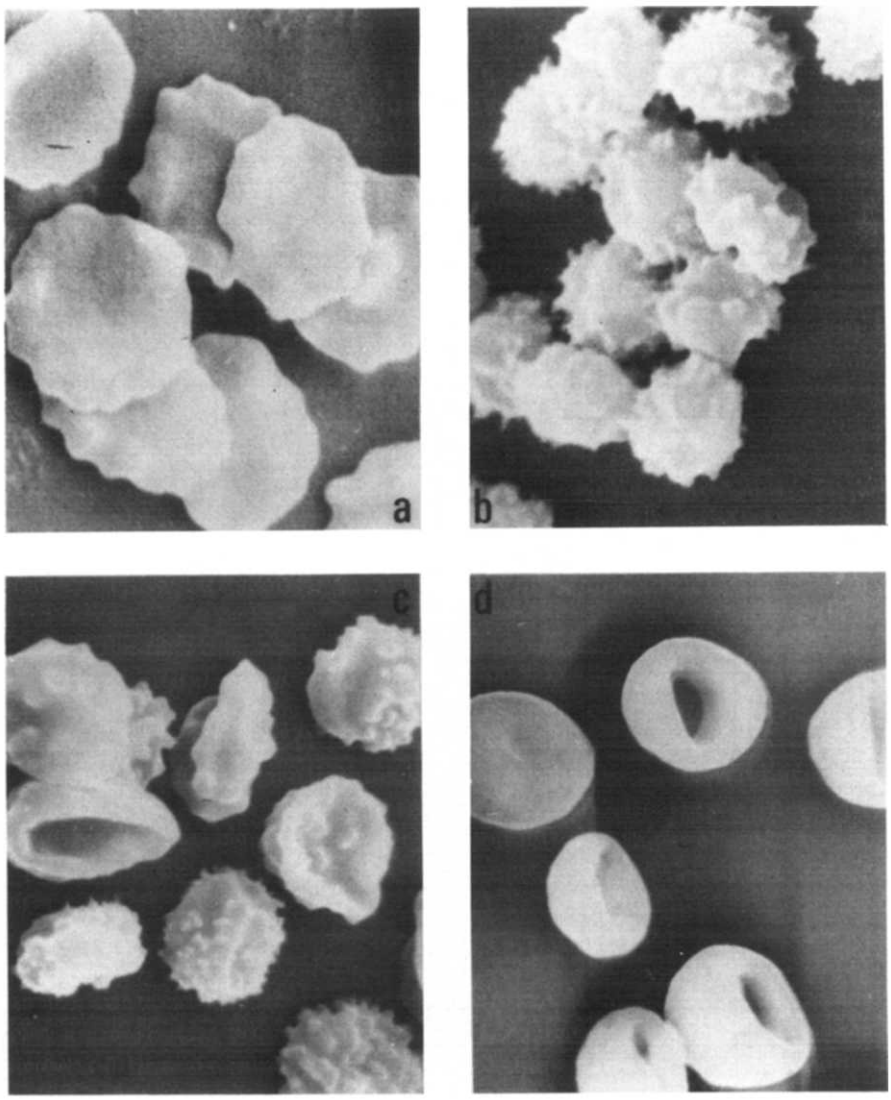


Fig. 4. Scanning electron micrographs showing the effect of increasing concentrations of glycocholate on human erythrocyte morphology. Erythrocytes were incubated at 37°C for 10 min in the presence of (a) 0 mM; (b) 6 mM; (c) 12.5 mM; (d) 20 mM glycocholate. Details of fixation etc. are given in Methods. Magnification ×2700.

Composition of the microvesicle fraction

Table I gives the relative amounts of acetylcholinesterase, phospholipid, total protein, haemoglobin and non-haemoglobin protein present in the microvesicle fraction, derived by high speed centrifugation of 20 ml of a 6 mM glycocholate extract, and in a similar volume of an uncentrifuged control incubation. Because the microvesicles were isolated in isoionic saline solution, which would have removed surface-adsorbed material [32], the haemoglobin content of the fraction must represent entrapped material.

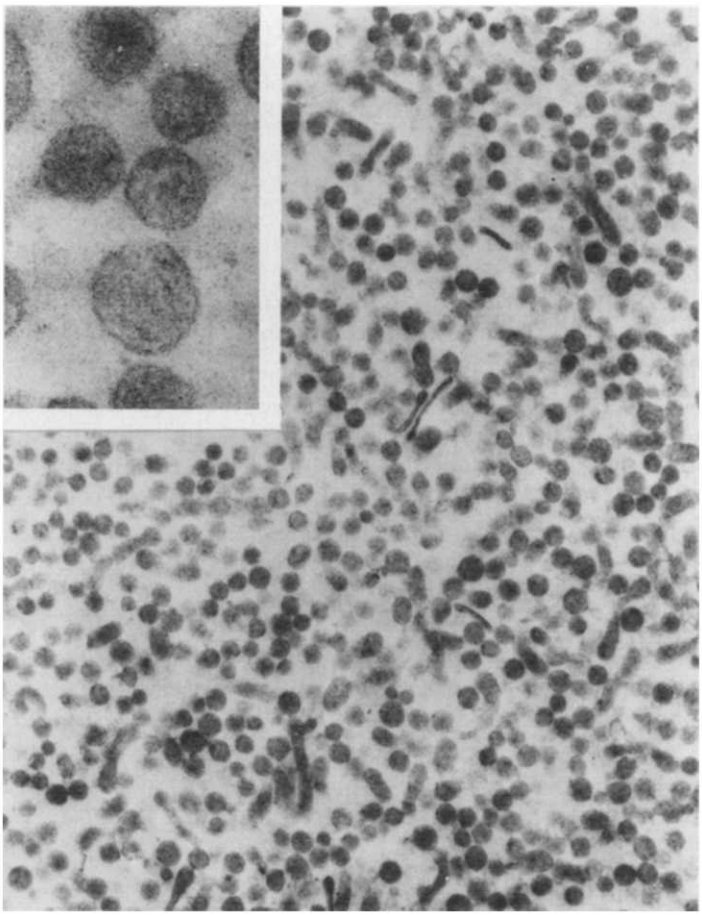


Fig. 5. Transmission electron micrographs of the microvesicle fraction from human erythrocytes treated with glycocholate. Erythrocytes were incubated at 37°C for 10 min in the presence of 6 mM glycocholate. Supernatant was obtained by centrifugation at 14 000 × *g* for 1 min. The microvesicular fraction was sedimented from this supernatant by centrifugation at 40 000 × *g* for 30 min (centrifugation at 150 000 × *g* for 60 min gave a material in which individual microvesicles could not be identified due to the extremely dense packing) and the pellet was fixed and embedded as described in Methods. Magnification ×26 000; inset ×116 000.

TABLE I

COMPOSITION OF THE MICROVESICLE FRACTION

Human erythrocytes were incubated at 37°C for 10 min in the presence of 6 mM glycocholate. Supernatant was obtained by centrifugation at 14 000 × *g* for 1 min. The microvesicle fraction was obtained by further centrifuging 20 ml of this supernatant at 150 000 × *g* for 60 min. Haemoglobin and total protein were assayed directly in the resuspended microvesicle fraction; acetylcholinesterase and phospholipid were estimated as the difference in their occurrence in the two supernatants. Values are the amounts present in the microvesicle fraction or in 20 ml of a corresponding uncentrifuged control incubation of intact erythrocytes and are means of four experiments ±S.D.

	Microvesicle fraction	Control cells	Percent of component in microvesicle fraction
Acetylcholinesterase (μmol/min)	26.7 ± 6.7	101 ± 11.9	26.3 ± 5.7
Phospholipid (μmol)	0.436 ± 0.085	7.02 ± 0.70	6.3 ± 1.7
Total protein (mg)	0.722 ± 0.141	801 ± 59	0.097 ± 0.023
Haemoglobin (mg)	0.447 ± 0.046	760 ± 40	0.060 ± 0.008
Non-haemoglobin protein (mg)	0.275 ± 0.12	41.0 ± 36.5	0.67 ± 0.31

The ratio of non-haemoglobin protein to phospholipid for erythrocyte membranes is best obtained from haemoglobin-free ghosts rather than from intact erythrocytes. This is because the value for non-haemoglobin protein determined in intact erythrocytes is grossly inaccurate due to the large errors introduced in a subtraction involving two values which differ by less than 5% (see Table I). In our laboratory the non-haemoglobin protein to phospholipid ratio for haemoglobin-free erythrocyte ghosts prepared under isoionic conditions has been shown to be 1.4 mg protein per μmol phospholipid [33]. The corresponding value for the microvesicle fraction was 0.63 mg protein per μmol phospholipid (see Table I). This is likely to be an inaccurate estimate for reasons similar to those outlined above for intact erythrocytes. However, it can be concluded that the microvesicles are considerably depleted in membrane protein when compared to the original membrane. In contrast, the acetylcholinesterase to phospholipid ratio was increased 4-fold in the microvesicle fraction (see Table I) showing selectivity in the depletion process.

Using the data in Table I, the number of microvesicles formed per erythrocyte was calculated in two different ways from either surface area or volume considerations. In both cases the diameter of the microvesicles has been taken as 100 nm and that of a sphered erythrocyte to 7000 nm. (1) Assuming phospholipid in the membrane of the microvesicles and whole erythrocytes is similarly distributed (see below), from surface area ratios the number of microvesicles formed was 308 ± 81 per erythrocyte (mean value ± S.D. of four experiments). (2) Assuming haemoglobin is present inside the microvesicles and whole erythrocytes at the same concentration, from volume ratios the number of microvesicles formed was 203 ± 26 (mean value ± S.D. of four experiments).

Phospholipid profile of the microvesicle fraction

Table II shows that the phospholipid profile of the microvesicle fraction released from human erythrocytes by 6 mM glycocholate was essentially similar to that of the whole membrane with the exception of a small, but statistically significant, increase in phosphorus-containing material at the solvent front. This similarity in phospholipid profile between the microvesicle fraction and the erythrocyte membrane supports the assumption made earlier when calculating

TABLE II

COMPARISON OF THE PHOSPHOLIPID PROFILE OF THE MICROVESICLE FRACTION WITH THAT OF CONTROL ERYTHROCYTES

Human erythrocytes were incubated at 37°C for 10 min with 6 mM glycocholate. Supernatant was obtained by centrifugation at 14 000 × *g* for 1 min and the microvesicle fraction obtained by further centrifugation at 150 000 × *g* for 60 min. Values are mean percentage phospholipid composition for five experiments ±S.D. The results were statistically assessed using a paired *t*-test and the significant differences ($P < 0.05$) designated*.

	Whole erythrocytes	Microvesicle fraction
Origin	0.5 ± 0.3	1.5 ± 1.3
Sphingomyelin	25.3 ± 1.8	20.6 ± 4.0
Phosphatidylcholine	29.9 ± 0.7	28.0 ± 2.2
Phosphatidylserine + phosphatidylinositol	10.6 ± 2.4	13.1 ± 2.8
Phosphatidylethanolamine	30.9 ± 2.4	30.7 ± 2.7
Solvent front	2.4 ± 2.0	6.0 ± 2.0 *

the number of microvesicles formed per erythrocyte from surface area considerations.

When human erythrocytes were incubated with 6 mM glycocholate, all the released phospholipid was sedimented by centrifugation at 150 000 × *g* for 60 min, whereas at a higher concentration of glycocholate (12.5 mM) only approx. 50% of the released phospholipid was sedimented under these conditions (Fig. 2). The phospholipid profile of this sedimented microvesicle fraction was also similar to that of the whole membrane (Table III), but the phospholipid profile of the supernatant after low speed centrifugation, and more particularly after high speed centrifugation, was significantly different (Table III). These supernatants were enriched in sphingomyelin and phosphatidylcholine and depleted in phosphatidylserine plus phosphatidylinositol and phosphatidylethanolamine (Table III). An earlier report from this laboratory [16] had studied this latter, soluble material but was unaware of the sedimentable component.

Therefore, at low concentrations (up to 6 mM), glycocholate releases from human erythrocytes microvesicles whose phospholipid profile is similar to that of the whole membrane, whereas at higher concentrations, glycocholate also releases phospholipid in a 'soluble' form whose profile is markedly different to that of the whole membrane.

Polypeptide profile of the microvesicle fraction

In order to estimate quantitatively the amounts of individual polypeptides in the erythrocyte ghost [33] and microvesicle membranes, an internal standard was included in the sodium dodecyl sulphate gel electrophoreses. This allowed for variations in staining and destaining and enabled the intensity of bands in different gels to be meaningfully compared. Glyceraldehyde-3-phosphate dehydrogenase (polypeptide molecular weight 40 000) was found suitable since it did not overlap with any endogenous polypeptides present in the microvesicle fraction; it gave a single band at the concentration used and the

TABLE III

COMPARISON OF THE PHOSPHOLIPID PROFILE OF A 12.5 mM GLYCOCHOLATE EXTRACT OF HUMAN ERYTHROCYTES WITH THAT OF CONTROL CELLS

The method is as described in the legend to Table II except that erythrocytes were incubated with 12.5 mM glycocholate. The low speed supernatant was obtained by centrifugation at 14 000 × *g* for 1 min. Aliquots of this supernatant were further centrifuged at 150 000 × *g* for 60 min to give a pellet (microvesicle fraction) and a high speed supernatant. Values are mean percentage phospholipid composition for five experiments ±S.D. The results were statistically assessed using a paired *t*-test and the significant differences ($P < 0.05$) designated *.

	Whole erythrocytes	Low speed supernatant	Microvesicle fraction	High speed supernatant
Origin	0.7 ± 0.5	1.7 ± 0.8	1.0 ± 1.4	1.2 ± 1.3
Sphingomyelin	26.1 ± 2.2	33.5 ± 1.4 *	21.7 ± 3.7	33.3 ± 2.5 *
Phosphatidylcholine	30.7 ± 3.0	39.2 ± 2.8 *	27.5 ± 4.1	49.8 ± 4.6 *
Phosphatidylserine + phosphatidylinositol	8.6 ± 1.5	6.9 ± 2.1	12.2 ± 3.7	4.0 ± 1.9 *
Phosphatidylethanolamine	31.8 ± 1.2	14.0 ± 1.4 *	33.8 ± 3.6	5.9 ± 3.0 *
Solvent front	1.9 ± 0.3	4.5 ± 0.7 *	3.7 ± 0.8 *	5.2 ± 1.5 *

intensity of stain with Coomassie Blue was proportional to the amount of polypeptide added. Thus, in gels of microvesicles and erythrocyte ghosts 2 μg of glyceraldehyde-3-phosphate dehydrogenase per gel was included as internal standard (Fig. 6). Similar gels (but without glyceraldehyde-3-phosphate dehydrogenase) were run for comparison (Fig. 6).

The polypeptide profile of the microvesicle fraction was markedly different to that of erythrocyte ghosts prepared under isoionic conditions [33] (Fig. 6). All the polypeptides were depleted with respect to membrane phospholipid (Table IV). This would be expected from the low non-haemoglobin protein to phospholipid ratio in the microvesicle fraction. In addition, this depletion appeared to be selective in that bands 1 plus 2 (spectrin), band 5 (actin) and bands 4.1 plus 4.2 were particularly depleted whilst other polypeptides (bands 3, 4.5 and 7) were depleted to a lesser extent (Table IV). By summing the normalised peak areas given in Table IV, it is apparent that there is approx. 20 times less membrane protein per unit of phospholipid in the microvesicle fraction when compared to the erythrocyte membranes. This contrasts with our earlier estimate (see above) that the non-haemoglobin protein to phospholipid ratio was decreased only 2—3-fold in the microvesicle fraction. This discrepancy is almost certainly due to inaccuracies in determining the non-haemoglobin protein content of the microvesicle fraction and the reasons for these errors have been discussed earlier.

Therefore, the microvesicle membranes are not only depleted in protein but the balance of the remaining polypeptides, as seen from the percentage composition in Table IV, is entirely different from the erythrocyte membrane. Bands 1 plus 2 were the most abundantly stained polypeptides in the

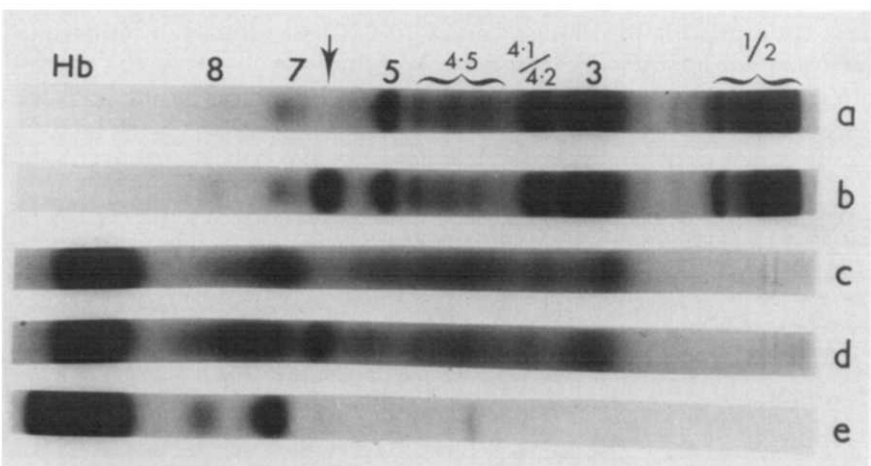


Fig. 6. Sodium dodecyl sulphate gel electrophoreses of human erythrocyte ghosts prepared under isoionic conditions (a, b) [33]; microvesicle fraction released from human erythrocytes incubated with 6 mM glycocholate at 37°C for 10 min (c, d), and supernatant (soluble fraction) after centrifuging at 20 000 X *g* for 10 min erythrocytes lysed under isoionic conditions (e). Gels a and b contained 45 nmol of membrane phospholipid, gels c and d contained 170 nmol of membrane phospholipid and gel e contained 250 μg of protein. Gels b and d also contained 2 μg of glyceraldehyde-3-phosphate dehydrogenase as internal standard and this is indicated by the arrow. Bands were designated according to Steck [17]. Hb, haemoglobin.

TABLE IV

COMPARISON OF THE MAJOR POLYPEPTIDES OF HUMAN ERYTHROCYTE GHOSTS AND THE MICROVESICLE FRACTION

Gels b and d, as in Fig. 6, were scanned at 570 nm. These gels contained 2 μg of glyceraldehyde-3-phosphate dehydrogenase as internal standard. Peak areas were estimated by cutting out and weighing and normalised to the peak area of internal standard and to a load of 1 mmol of phospholipid per gel. In addition, the peak areas of the bands are expressed as a percentage of the total endogenous stained polypeptides. Values are means ± S.D. with the number of observations in parentheses.

Band	Approximate molecular weight	Percent total endogenous polypeptides		Peak area of band / Peak area of internal standard × (per mmol phospholipid)	
		Ghosts (6)	Microvesicles (4)	Ghosts (6)	Microvesicles (4)
1 + 2	240 000–215 000	51.3 ± 3.1	2.6 ± 1.9	148 ± 22	0.3 ± 0.1
3	88 000	29.9 ± 1.1	40.2 ± 4.5	79 ± 15	6.2 ± 3.4
4.1 + 4.2	78 000– 72 000	8.9 ± 1.6	5.2 ± 0.8	26 ± 7	0.8 ± 0.4
4.5	55 000	5.7 ± 1.4	22.1 ± 2.3	17 ± 6	3.3 ± 1.3
5	43 000	5.4 ± 0.4	3.1 ± 0.9	16 ± 3	0.5 ± 0.2
7	29 000	1.8 ± 0.3	23.3 ± 3.5	5 ± 0.5	3.5 ± 1.6
8	23 000	—	3.5 ± 1.7	—	0.6 ± 0.4

erythrocyte membrane preparation whereas band 3 was the most abundantly stained polypeptide in the microvesicle fraction, and together with bands 4.5 and 7 comprised more than 80% of the membrane protein. Some of the components of bands 7 and 8, and possibly band 4.3 (one of the components of the broad zone designated band 4.5) are, however, cytoplasmic (see Fig. 6e). The possibility therefore exists that a proportion of these components, like haemoglobin, were trapped in the interior of the microvesicles on their formation and may therefore contribute to the polypeptide profile of the microvesicle membrane fraction.

Discussion

Previous studies from this laboratory have indicated that glycocholate and taurocholate specificially remove from human erythrocytes [16], sheep erythrocytes [20] and pig lymphocytes [15] externally orientated membrane phospholipid and protein before the release of intracellular components. It has been proposed [9] that, in the case of phospholipids, these membrane components are removed from the outer leaflet of the membrane to form mixed micelles with the detergent. However, the results obtained in this study show that, at least in the case of human erythrocytes, this may be a more complex process.

Glycocholate removed membrane components from human erythrocytes in two ways. Firstly, at low concentrations (up to 6 mM), glycocholate caused microvesicles to be released from erythrocytes; the phospholipid profile of this microvesicle fraction was similar to that of the original membrane suggesting that this material contained both leaflets of the lipid bilayer. Secondly, at higher concentrations, glycocholate removed from human erythrocytes phospholipid in a 'soluble' form (as judged by its appearance in the supernatant

after centrifugation at 150 000 × *g* for 60 min). The profile of this phospholipid was significantly different from that of the whole membrane in that it was enriched in the choline-containing phospholipids; these are known to be externally orientated in the erythrocyte membrane [18,19].

Whilst the phospholipid profile of the microvesicle fraction was similar to that of the erythrocyte membrane, the polypeptide profile was markedly different. The most abundant membrane polypeptides of the microvesicle fraction were bands 3, 4.5 and 7; bands 3 and 4.5 are the major intrinsic spanning proteins of the erythrocyte membrane [34], whereas band 7 consists of both cytoplasmic and membrane components [35]. In addition there was an enrichment in acetylcholinesterase activity. Compared to the balance of intrinsic proteins in the erythrocyte membrane, the polypeptides of the microvesicle fraction showed a higher relative proportion of band 4.5 to band 3. Although bands 3, 4.5 and 7 comprised greater than 80% of the microvesicle membrane protein, these polypeptides were substantially depleted with respect to phospholipid. However, the most notably depleted polypeptides of the microvesicle fraction were bands 1 and 2 (spectrin) and band 5 (actin); these are extrinsic proteins which are known to form a meshwork covering the cytoplasmic surface of the lipid bilayer [36]. The net result of the depletion of all these polypeptide species was to yield a microvesicle membrane with a many-fold decreased protein to phospholipid ratio than the original membrane. Severely protein-depleted microvesicles are also shed from human erythrocyte ghosts in conditions causing spectrin aggregation [37] and from intact human erythrocytes treated with Ca^{2+} and the divalent cation ionophore (compound A23187) [38] or on ageing [39].

Outward vesiculation must involve membrane fusion at the cytoplasmic surface of the projections of echinocytic erythrocytes. Ahkong et al. [40] have proposed that before fusion can occur, the area of membrane involved must become more fluid with the removal of all intrinsic proteins. However, glycocholate-induced microvesicles do contain some intrinsic proteins. It is possible that the incorporation of glycocholate into the membrane and the depletion of intrinsic membrane proteins may sufficiently increase membrane fluidity for fusion to occur without the complete removal of these proteins. Since the microvesicles are almost devoid of extrinsic proteins (spectrin and actin) it is likely that their removal is necessary before membrane fusion can occur.

Diacylglycerol is a fusogenic lipid [41,42] which has been shown to accumulate during vesiculation of human erythrocytes in several conditions [38,39,43–46]. However, diacylglycerol was not detected (by thin-layer chromatography) in the microvesicle fraction. In addition, glycocholate-induced vesiculation was not blocked by 1 mM ethyleneglycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) (Billington, D. and Coleman, R., unpublished observations) whereas the accumulation of diacylglycerol and subsequent vesiculation of human erythrocytes caused by treatment with Ca^{2+} and A23187 was blocked by EGTA [44]. Therefore, glycocholate-induced membrane fusion is unlikely to be mediated through diacylglycerol production but more likely by the detergent directly increasing membrane fluidity as discussed above. Although the mechanisms for glycocholate- and Ca^{2+}-induced

vesiculation are different, both processes yield microvesicles of apparently similar morphology, size and composition.

Low concentrations (6 mM) of glycocholate caused human erythrocytes to change from the normal biconcave discoid shape to crenated spheres (echinocytes) whilst higher concentrations (20 mM) caused them to become cup-shaped (stomatocytes). Bile salts, together with several other anionic amphiphilic compounds, are known to be echinocytogenic agents whereas stomatocytogenic agents are, in general, cationic amphiphilic substances [47,48]. Thus, glycocholate is an amphiphilic compound which can act as both an echinocytogenic and stomatocytogenic agent. It seems probable that the transformation in shape of human erythrocytes to echinocytes by low concentrations (6 mM) of glycocholate is due to the bile salt being sufficiently lipophilic to be incorporated into the outer leaflet of the lipid bilayer (and displace membrane protein); this causes the outer leaflet to expand in area such that projections are formed with pinch off to give microvesicles. In contrast, at higher concentrations, glycocholate specifically removes outer leaflet components such that the outer leaflet then occupies a smaller surface area than the inner leaflet and the most favourable shape is a stomatocyte. This explanation is in accord with the proposal of Sheetz and Singer [49] that incorporation and depletion of materials from one leaflet of the bilayer will result in changes in membrane curvature.

It is of interest that both human and pig erythrocytes shed microvesicles when incubated with glycocholate, whereas sheep erythrocytes did not. Contributing factors to this may be that sheep erythrocytes contain little or no phosphatidylcholine whereas approx. 30% of human and pig erythrocyte phospholipid is phosphatidylcholine [49]; also sheep erythrocytes are smaller (erythrocyte volumes: sheep 30 μm^3, pig 58 μm^3, human 87 μm^3 [50]) and more resistant to glycocholate attack [20]. Although glycocholate does not cause sheep erythrocytes to shed microvesicles, the bile salt does, however, remove significant amounts of membrane phospholipid before the onset of lysis and these phospholipids represent externally orientated types [20].

Bile salts are known to be actively transported out of the hepatocyte into the canaliculi where their concentration is sufficiently high to form micelles [2]. Coleman et al. [9] have proposed that these micelles then incorporate phospholipids and cholesterol from the outer leaflet of the bile canalicular membrane. In support of this, the choline-containing phospholipids (predominantly phosphatidylcholine) are the major phospholipids of mammalian bile [2,3], and, by analogy with studies on the sidedness of phospholipids in the plasma membrane of human erythrocytes [18,19], these are probably externally orientated in the bile canalicular membrane. The results obtained in this study add a further aspect to this hypothesis in that the profile of the 'soluble' phospholipids released from human erythrocytes by glycocholate (see also ref. 16), and from sheep erythrocytes by both glycocholate and taurocholate [20], reflects the composition of the outer leaflet of these cells. The possibility that the choline containing-phospholipids were preferentially solubilised by glycocholate was discounted since the phospholipid profiles of glycocholate and taurocholate extracts of unsealed sheep erythrocyte ghosts [20] and human erythrocyte ghosts (Coleman, R., un-

published observations, cited in ref. 1) were similar to that of the whole membrane.

The phospholipid profile of the microvesicles derived from human erythrocytes by the action of low levels of glycocholate does not, however, fit in with this general hypothesis in that it resembled both leaflets of the bilayer rather than the outer leaflet. In view of the specific phospholipid composition of mammalian bile [2,3], microvesicles derived from the canalicular membrane containing a phospholipid profile similar to the whole membrane could not be expected to make a major contribution to the biliary phospholipid profile. Bouchier [52] has suggested that microvesicles may be the form in which phospholipid is released into bile, prior to subsequent reassembly to form micelles, and has attempted to circumvent the problems of phospholipid composition by suggesting that the membrane of the bile canaliculus has a different composition from the rest of the hepatocyte plasma membrane. Whilst available preparations of bile canalicular membranes show some uniqueness in phospholipid profile, in no case has a preparation been presented with a phospholipid composition almost exclusively phosphatidylcholine (see, for example, refs. 53 and 54). Thus, the contribution of microvesicles to the biliary output of phospholipid may be minor and other mechanisms probably are quantitatively more important, e.g. by removal of outer leaflet components into mixed micelles.

The potential role of bile salt-induced membrane vesiculation may lie more in relation to the protein content of bile. Many mammalian biles contain low concentrations (about 5 mg/ml) of protein whose profile is characterised by the presence of significant amounts of plasma membrane enzymes and the relative absence of intracellular enzymes [10], suggesting that biliary protein does not arise as a result of general cell damage. The microvesicles which were shed from human erythrocytes by glycocholate were selectively depleted in protein. By analogy, if microvesicles were shed from the canalicular membrane this may partly explain the unique, but low, protein content of mammalian bile.

Acknowledgements

We thank Mrs. L. Woodhead for excellent technical assistance and Mr. J. Berriman for the electron microscopy. We thank the Medical Research Council for financial support.

References

1 Coleman, R., Holdsworth, G. and Vyvodá, O.S. (1977) in Membranous Elements and Movement of Molecules (Reid, E., ed.), pp. 163—169, E. Horwood Ltd., Chichester, U.K.
2 Heaton, K.W. (1972) Bile Salts in Health and Disease, pp. 82—97, Churchill Livingstone, London
3 Coleman, R. (1973) in Form and Function of Pholpholipids (Ansell, G.B., Dawson, R.M.C. and Hawthorne, J.N., eds.), pp. 345—375, Elsevier, Amsterdam
4 Sjövall, J. (1960) Clin. Chim. Acta 5, 33—41
5 Izumi, K. (1965) Fukuoka Acta Med. 56, 488—523
6 Dam, H., Kruse, I., Prange, I., Kallegauge, H.E., Fenger, H.J. and Jensen, M.K. (1971) Z. Ernährungswiss. 10, 160—177
7 Coleman, R., Holdsworth, G. and Finean, J.B. (1976) Biochim. Biophys. Acta 436, 38—44
8 Vyvoda, O.S., Coleman, R. and Holdsworth, G. (1977) Biochim. Biophys. Acta 465, 68—76
9 Coleman, R., Holdsworth, G. and Vyvoda, O.S. (1977) in Membrane Alterations as a Basis of Liver Injury (Popper, H., Bianchi, L. and Reutter, W., eds.), pp. 59—70, M.T.P. Press, Lancaster, U.K.
10 Holdsworth, G. and Coleman, R. (1975) Biochim. Biophys. Acta 389, 47—50

11 Holdsworth, G. and Coleman, R. (1975) Biochem. Soc. Trans. 3, 746—747
12 De Pierre, J.W. and Karnovsky, M. (1973) J. Cell Biol. 56, 275—303
13 Trams, E.G. and Lauter, C.J. (1974) Biochim. Biophys. Acta 345, 180—197
14 Misra, D.N., Gill, T.J. and Estes, L.W. (1974) Biochim. Biophys. Acta 352, 455—461
15 Holdsworth, G. and Coleman, R. (1976) Biochem. J. 158, 493—495
16 Coleman, R. and Holdsworth, G. (1976) Biochim. Biophys. Acta 426, 776—780
17 Steck, T.L. (1974) J. Cell Biol. 62, 1—19
18 Zwaal, R.F.A., Roelofsen, B. and Colley, C.M. (1973) Biochim. Biophys. Acta 300, 159—182
19 Renooij, W., van Golde, L.M.G., Zwaal, R.F.A. and van Deenen, L.L.M. (1976) Eur. J. Biochem. 61, 53—58
20 Billington, D., Coleman, R. and Lusak, Y.A. (1977) Biochim. Biophys. Acta 466, 526—530
21 King, E.J. (1932) Biochem. J. 32, 292—302
22 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—919
23 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
24 Galliard, T., Michell, R.H. and Hawthorne, J.N. (1965) Biochim. Biophys. Acta 106, 551—563
25 Skipski, V.P., Peterson, R.F. and Barclay, M. (1964) Biochem. J. 90, 374—378
26 Freeman, C.P. and West, D. (1966) J. Lipid Res. 7, 324—327
27 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
28 Dodge, J.T., Mitchell, O.D. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
29 Ellman, G.L., Courtney, D., Andres, D. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—98
30 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
31 Reynolds, E.S. (1963) J. Cell Biol. 17, 208—212
32 Coleman, R. (1973) Biochim. Biophys. Acta 300, 1—30
33 Billah, M.M., Finean, J.B., Coleman, R. and Michell, R.H. (1976) Biochim. Biophys. Acta 433, 54—62
34 Steck, T.L., Ramos, B. and Strapzon, E. (1976) Biochemistry 15, 1154—1161
35 Billah, M.M. (1977) Ph.D. dissertation, University of Birmingham
36 Tilney, L.G. and Detmers, P.J. (1975) J. Cell Biol. 66, 508—520
37 Elgsaeter, A., Shotton, D.M. and Branton, D. (1976) Biochim. Biophys. Acta 426, 101—122
38 Allan, D., Billah, M.M., Finean, J.B. and Michell, R.H. (1976) Nature 261, 58—60
39 Rumsby, M.G., Trotter, J., Allan, D. and Michell, R.H. (1977) Biochem. Soc. Trans. 5, 126—128
40 Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. (1975) Nature 253, 194—195
41 Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. (1973) Biochem. J. 136, 147—155
42 De Boer, E. and Loyter, A. (1971) FEBS Lett. 15, 325—327
43 Allan, D., Low, M.G., Finean, J.B. and Michell, R.H. (1975) Biochim. Biophys. Acta 413, 209—316
44 Allan, D. and Michell, R.H. (1975) Nature 258, 348—349
45 Allan, D., Watts, R. and Michell, R.H. (1976) Biochem. J. 156, 225—232
46 Allan, D. and Michell, R.H. (1976) Biochim. Biophys. Acta 455, 824—830
47 Deuticke, B. (1968) Biochim. Biophys. Acta 163, 494—500
48 Weed, R.I. and Chailley, B. (1973) in Red. Cell Shape (Bessis, M., Weed, R.I. and Leblond, P.F., eds.), pp. 55—68, Springer Verlag, New York
49 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4457—4461
50 Nelson, G.J. (1972) in Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism (Nelson, G.J., ed.), pp. 317—388, Wiley Interscience, New York
51 Whittam, R. (1964) Transport and Diffusion in Red Blood Cells, p. 2, Edward Arnold, London
52 Bouchier, I.A. (1971) Lancet 1, 711—715
53 Evans, W.H., Kremmer, T. and Culvenor, J.G. (1976) Biochem. J. 154, 589—595
54 Fisher, M.M., Bloxam, D.L., Oda, M., Phillips, M.J. and Yousef, I.M. (1975) Proc. Soc. Exp. Biol. Med. 150, 177—184