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CHARACTERISTICS OF INTERACTIONS BETWEEN SURFACTANTS AND THE HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY

1. A new equation is introduced which describes the rate of haemolysis as being '*n*th' order with respect to surfactant concentration. The fit of the experimental results to this equation is good, and *n* has a value of about 3 for sodium dodecyl sulphate and Triton X-100. Deviations from the predicted time course are explained in terms of interactions between sodium dodecyl sulphate and haemolysate and of permeability barriers preventing adsorption of Triton X-100.

2. Solubilisation of erythrocyte ghosts in surfactants is found to be also dependent upon a high order of the surfactant concentration. Binding measurements enable this to be calculated as about 8, and a similar result is obtained for the solubilisation of suspensions of lecithin.

3. In suspensions of ghosts solubilised in Triton X-100, phospholipid–surfactant micelles are separated from protein constituents. Acetylcholine esterase is solubilised more readily than the bulk of the protein and may be conveniently isolated. Washing precipitates, prepared by the de-ionisation of solubilised ghosts, with surfactant solution removes phospholipid from protein but the depleted material takes up phospholipid from lecithin suspensions.

4. An overall mechanism for haemolysis and solubilisation is postulated which involves laminar–micellar transitions in lipid regions of the membrane. The implications for membrane structure are discussed.

INTRODUCTION

Although haemolysis and the solubilisation of the red cell membrane by surfactants have been the subject of several studies[1–10], no comprehensive mechanism for either process has been postulated in terms of acceptable models of membrane structure. In order that this might be done a new approach to the problem, involving the study of both the kinetics of the interaction and the nature of the products, was necessary. The results of earlier work on haemolysis[1,3,4] could not be fitted to an overall kinetic equation and this may have been partly attributable to secondary

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interactions between the haemolysate and the anionic surfactant employed. The possibility that such confusions might arise has been pointed out by PONDER[2] and, accordingly, this study is mainly concerned with the nonionic surfactant Triton X-100 which has little tendency to interact with proteins[11]. Some previous use has been made of Triton X-100 in the solubilisation of the red cell membrane[5,6,9,10] and in particular it has been found that certain enzymes, such as acetylcholine esterase, normally closely associated with the membrane, remain fully active in the solubilisate.

Haemolysis, defined as the loss of haemoglobin from the erythrocyte, need only involve the formation of holes in the membrane such as have been described by SEAMAN[8]. The kinetics can be studied, therefore, by measuring the rate of loss of haemoglobin, or the consequent decrease of turbidity of red cell suspensions, in different concentrations of surfactant. The consideration of solubilisation is more complex and there is, as yet, insufficient data on the nature of the primary products of the process, particularly where nonionic surfactants have been used, for any tentative mechanism to be proposed. Nevertheless, an approach may be made through the consideration of a better documented system, the suspension of lecithin and surfactant in water, as a model. In this case solubilisation corresponds to a laminar–micellar transition occurring at a well defined ratio of lipid to surfactant[12]. The equilibrium position of the transition will then be dependent on the surfactant concentration and the order of dependence will be determined by the number of surfactant molecules involved. Thus the solubilisation of the erythrocyte ghost, whilst not necessarily being a laminar-micellar transition process, may be usefully studied by measuring its dependence on surfactant concentration and also by determining the binding curves of surfactant to ghost.

In this paper simple equations are developed to describe both haemolysis and solubilisation and the results of experiments designed to test them are reported. In these experiments solubilisation of erythrocyte ghosts was estimated both turbidometrically and by gel filtration, and surfactant binding was measured by surface tension determinations and by gel filtration. Comparisons were made with the solubilisation of lecithin suspensions. The products of the solubilisation of the erythrocyte membrane were studied by iso-electric focusing and by chromatography on agarose gels, and the results are discussed here in relation to an overall mechanism for the disruption of the red cell membrane by surfactants.

THEORETICAL

The simultaneous binding to a cell of the n molecules of surfactant required for hole formation may be expressed by an equilibrium constant 'K' such that

$$K = c \cdot s^n / cs_n \qquad (1)$$

where 'c', 's' and 'cs_n' are the concentrations of cells, surfactant and complex, respectively.

Now, the cell–surfactant complex is considered to decay with a rate constant 'k' to a ghost–surfactant complex with the release of haemoglobin, so that

$$-\mathrm{d}(cs_n)/\mathrm{d}t = k \cdot cs_n = k \cdot c \cdot s^n / K \qquad (2)$$

If the adsorption is much faster than the hole formation and s is constant, integrating Eqn. 2 with respect to time 't' gives

$$\ln (c_0/c) = k' \cdot s^n \cdot t \tag{3}$$

where $k' = k/K$ and c_0 is the initial cell concentration, and, for the same values of c_0/c

$$n \cdot \log s \overset{\infty}{\approx} \log (1/t) \tag{4}$$

The approach to the description of the equilibrium of a postulated laminar–micellar transition in the lipid–surfactant system is to consider the concentration dependence of the equilibrium between the two forms. If a laminar structure 'L' breaks down into 'm' micelles 'US_n' which contain 'n' molecules of surfactant 'S' at the transition point, then

$$L \leftrightharpoons m \cdot n \cdot S \leftrightharpoons LS_{m.n} \leftrightharpoons m(US_n)$$

and the overall equilibrium is expressed by

$$K = l \cdot s^{m \cdot n}/(us_n)^m \tag{5}$$

where 'l', 's' and 'us_n' are concentrations of L, S and US_n, respectively. Taking logs,

$$\log K = \log l + m \cdot n \cdot \log s - m \cdot \log us_n \tag{6}$$

Similarly, for the equilibrium between the cell membrane, C, and micelles, US_n, the expression for the equilibrium becomes:

$$\log K = \log c + m \cdot n \cdot \log s - m \cdot \log us_n \tag{7}$$

Now, since both 'm' and 'n' are likely to be much larger than unity, $\log l$ and $\log c$ in Eqns. 6 and 7 are effectively constant for small changes of 's' so that both equations reduce to

$$n \cdot \log s = \text{constant} + \log us_n \tag{8}$$

This equation may be tested by determining the relationship between free and bound surfactant concentrations.

MATERIALS AND METHODS

Lubrol 'W' (cetyl alcohol–polyoxyethylene condensate) *ex* ICI Ltd. Mol. wt. approx. 848.

Sodium dodecyl sulphate ex Koch Light Ltd. Critical micellar concentration 8.1 mM (see ref. 13).

Triton X-100 (isooctyl phenyl polyoxyethylene condensate) supplied through the courtesy of Lennig Chemicals Ltd. Average mol. wt. 680 while the critical micellar concentration is 0.9 mM (see ref. 14). (Where Triton X-100 molarities are mentioned they refer to the given value of average mol. wt.)

Erythrocytes. Whole human blood in acid–citrate–dextrose medium was centrifuged at 3000 × *g* for 30 min (MSE Major, 4 × 700 ml head, 2500 rev./min, 2°). Plasma and 'buffy coat' were removed and the erythrocytes were washed twice in 5 vol. of 1 % NaCl in 30 mM Tris–HCl, pH 7.4.

Erythrocyte stroma. Ghosts were prepared by a method adapted from that of DODGE *et al.*[15]. Washed red cells were rapidly mixed with 10 vol. of hypotonic buffer, 10 mM Tris–HCl, pH 8.0, and left for 30 min at 2° before centrifugation at 23000 × *g* for 40 min (MSE High Speed 18, 6 × 250 ml head, 12000 rev./min, 2°). Four subsequent washes in 10 vol. of hypotonic buffer were required to produce 'white' ghosts.

The entire preparation was carried out between 0 and 2° and any heavy red pellet formed was discarded. Ghost suspensions were stored for up to 2 days at 2° at the concentration of the pellet obtained in the final washing, and the term 'per cent ghost concentration' refers to a dilution of this suspension.

Egg lecithin ex British Drug Houses Ltd. Suspensions were prepared in 5 mM Tris–HCl, pH 8.0, by 20 strokes in a hand-held homogeniser and were diluted to give a final concentration of 1.5 mg/ml. The suspension was left for 30 min before use and 'per cent lecithin suspension' refers to dilutions of this stock suspension.

Turbidometric measurements of the rate of haemolysis. The intensity of 546 nm light transmitted by erythrocyte suspensions was measured on a Brice–Phoenix light scattering photometer coupled to a linear potentiometric recorder. The recorder was set at zero with the photometer shutter closed and at full scale for light transmitted by buffer alone so that the transmittance of the suspensions could be recorded on a time base. To start the haemolysis 5 ml erythrocyte suspension were blown into 35 ml of surfactant solution in a cubic light scattering cell. This procedure minimises the possibility of misleading results arising from contact of the red cells with local high concentrations of surfactant before complete mixing. Half-life times for the haemolysis were measured at the point where half of the difference between the absorbance of the intact cell suspension and that of a comparable concentration of osmotically lysed cells had been lost.

Surfactant binding experiments. Two methods were used to estimate the amounts of surfactant bound to ghost or lecithin suspensions.

(*i*) *Gel filtration.* The surfactants were included in Sephadex G-50 (*ex* Pharmacia Ltd.), whilst ghosts and solubilisate were excluded, so that it was possible to use gel filtration on this medium to study surfactant binding below the critical micellar concentration. Coarse grade Sephadex G-50 was packed into columns measuring 15 mm × 450 mm and was equilibrated with surfactant solution. 5 ml of ghost suspension in the surfactant solution was applied to the top of the column and was eluted at 2 ml/min with surfactant solution. The eluate was continuously monitored at 253 nm in order to detect the ghost material and to measure the concentration of the surfactant, Triton X-100, which, being phenolic, absorbs strongly in this region. The binding is expressed as the weight of Triton X-100 represented by the trough in the absorbance of the surfactant at the included elution volume.

(*ii*) *Drop weight analysis.* It is assumed that any rise in the surface tension of a surfactant solution when it is mixed with a ghost suspension arises from the binding of surfactant. Thus, by comparing the drop weights of ghost-surfactant mixtures with those of standard surfactant solutions, a measurement of the amount of binding may be made. Samples were made up by blowing 1 ml of ghost or lecithin suspension into 9 ml of surfactant solution. After a period of 30 min for equilibration 20 drops of each sample, produced from a melting-point tube at a constant rate of 0.33 ml/min with an LKB 'Perpex' pump, were weighed and comparisons were made with the drop weights of standard surfactant solutions by graphical means.

Protein assay. Protein concentrations were estimated by the method of LOWRY *et al.*[16] modified by the use of sodium dodecyl sulphate to prevent the formation of the water-immiscible Triton X-100–phosphomolybdate complex.

1 ml of sample solution was mixed with 5 ml of alkaline reagent (2 % sodium carbonate, 0.02 % sodium potassium tartrate, 0.0002 % $CuSO_4$ in 0.1 M NaOH) and

left at room temperature for 20 min. 0.5 ml of 0.5 % sodium dodecyl sulphate and 0.5 ml Normal Folin–Ciocalteu reagent (*ex* British Drug Houses Ltd.) were blown in and the mixture was immediately and thoroughly agitated. After 30 min the absorbance was read at 740 nm. Standard deviation was about 2 %.

Since sucrose interferes with the assay when present in the high concentrations used in the isoelectric focusing experiments, proteins in such solutions were first precipitated with 10 % trichloroacetic acid. The precipitates, which included the Triton X-100, were then treated by the method of LOWRY *et al.*[16] except for the addition of sodium dodecyl sulphate.

Phospholipid assay. Phospholipid concentrations were estimated as total phosphate in ashed samples by a method adapted from that of FISKE AND SUBBAROW[17].

3 ml of sample were ashed in 0.4 ml of 5 M H_2SO_4 and oxidised with concentrated HNO_3. To this were added 2 ml distilled water, 3.0 ml 0.75 % ammonium molybdate and 1.0 ml 0.5 % 'Amidol' (2,4-diaminophenol hydrochloride, *ex* British Drug Houses) in 8 % sodium metabisulphate. The absorbance at 700 nm was measured after 5 min and standard deviation was about 1 % in the range 0.2–1.0 μmole phosphate.

Acetylcholine esterase assay. The method of ELLMAN *et al.*[18] depends upon estimating the concentration of thiocholine, liberated by the esterase from substrate acetylthiocholine, by colorimetry of its yellow complex with bis-dithionitrobenzoic acid. The procedure was modified by the use of 0.5 ml 0.5 % sodium dodecyl sulphate to stop the reaction after 1-min and results were expressed as the change in absorbance at 412 nm per min. Standard deviation was determined as about 2 % in the experimental range.

Gel filtration. 'Sepharose' (*ex* Pharmacia Ltd.) and 'Sagavac' (*ex* Seravac Ltd.) agarose gels were packed into columns measuring 25 mm × 450 mm. Elution rates of about 0.33 ml/min were maintained with an LKB 'Perpex' pump and the eluate was continuously monitored at 253 nm (LKB 'Uvicord'). Fractions of 4–5 ml each were collected on a time base and all experiments were carried out at room temperature.

Iso-electric focusing. An LKB 8101 iso-electric focusing column was fitted with a stepwise sucrose density gradient containing a 1 % solution of pH 3–6 range of ampholytes ('Ampholine' *ex* LKB) according to the manufacturer's directions. The sample was added in the middle layers of the 100 ml gradient. At the end of each separation (24–48 h) the column contents were pumped out and collected in 3 ml fractions. The pH of each fraction was determined immediately on an extended scale meter.

Buffers. All experiments with intact erythrocytes were carried out in 30 mM Tris–HCl, pH 7.4, 1 % NaCl and those with ghost suspensions were in 10 mM Tris–HCl, pH 8.0 unless specified otherwise.

RESULTS

Haemolysis

Typical time courses of haemolysis in sodium dodecyl sulphate and Triton X-100 solutions are shown in Fig. 1 plotted as $-\log A$ versus t to test Eqn. 3. In both cases there was a marked deviation from the predicted linearity. The initial 'lag time' for

haemolysis in sodium dodecyl sulphate, about ten seconds, is probably of the same order as the mixing time and the time required for the outward diffusion of haemoglobin. The progressive slowing down of the rate of haemolysis in sodium dodecyl sulphate is expected in a system where the surfactant interacts with the haemolysate.

TABLE I

INACTIVATION OF SURFACTANTS BY HAEMOLYSATE

Half-times for the haemolysis of 0.0125% haematocrit erythrocyte suspensions were measured in surfactant solutions in which a similar suspension had haemolysed and are compared with the half-times for haemolysis in solutions of surfactant alone.

	Half-time (min) for haemolysis in:	
	11.1 μM Sodium dodecyl sulphate	*131 μM Triton X-100*
With haemolysate	3.00	2.60
Without haemolysate	0.39	2.40

The inactivation of the sodium dodecyl sulphate is shown by the results (Table I) of an experiment in which the rates of haemolysis of erythrocytes in surfactant solutions were compared with the rates of haemolysis in the same concentrations of surfactant in which a similar quantity of red cells had previously haemolysed. Triton X-100 was not inactivated in this way. Since interactions between ionic surfactants and opposite charges on proteins are almost stoichiometric[11] a factor, $m(c_0-c)$, where 'm' is the amount of surfactant bound per haemolysed cell, may be introduced into Eqn. 3 which becomes

$$\mathrm{d}(cs_n)/\mathrm{d}t = k\cdot c\cdot(s - m(c_0 - c))^n/K \qquad (9)$$

and now expresses the progressive slowing down of haemolysis in sodium dodecyl sulphate solutions.

The initial deviation ('lag time') in the time course of haemolysis in Triton X-100 (Fig. 1) is very much more marked than is that in sodium dodecyl sulphate. Triton is a bulkier molecule than sodium dodecyl sulphate and is, therefore, likely to be influenced to a much greater extent by any permeability barrier around the erythrocyte such as might be composed of the protein and carbohydrate constituents of the membrane. Water molecules are bound to the oxygens of the apolar tail of Triton X-100 which has as a result an effective mass much greater than that of sodium dodecyl sulphate.

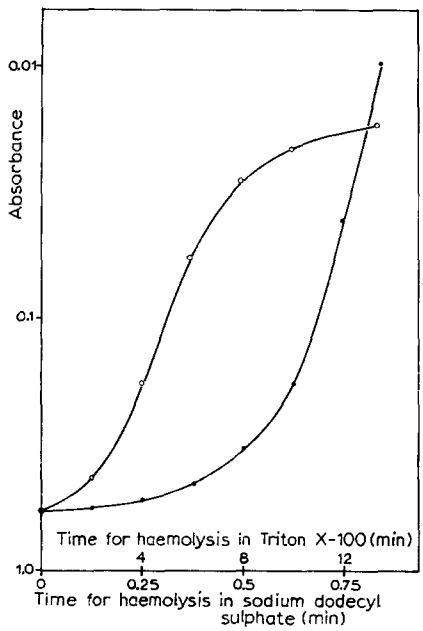
For the simplest case, the effective surfactant concentration, s_i, within the permeability barrier, is taken to be much smaller than the concentration in the medium, so that

$$s_i = D\cdot s\cdot t \qquad (10)$$

where 'D' is the diffusion constant. Eqn. 3 thus becomes

$$\mathrm{d}cs_n/\mathrm{d}t = k\cdot c\cdot(D\cdot s\cdot t)^n/K \qquad (11)$$

Since the results of the experiment to determine inactivation of surfactant with haemolysate (Table I) showed no significant change in the haemolysing activity of



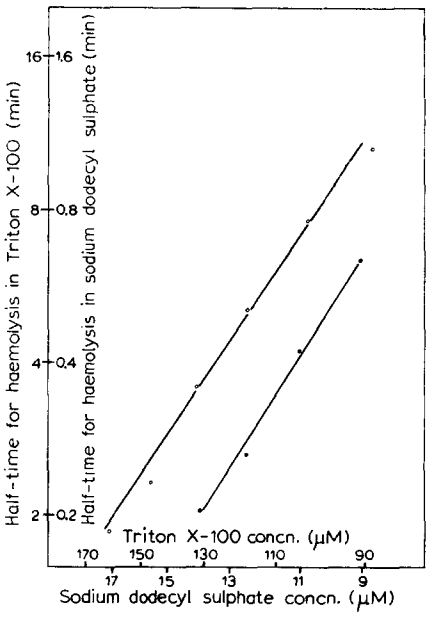


Fig. 1. Time courses for haemolysis of 0.0125% haematocrit erythrocyte suspensions in 88.2 μM Triton X-100 (●) and 13.9 μM sodium dodecyl sulphate (○) solutions. Absorbance is plotted on a log scale so that the results may be compared with the predictions of Eqn. 3.

Fig. 2. Relationship between surfactant concentration and the time taken for half the initial absorbance of 0.0125% haematocrit erythrocyte suspensions to be lost in Triton X-100 (●) and sodium dodecyl sulphate (○) solutions. Both scales are logarithmic to conform with Eqn. 4.

Triton X-100, 's' and 't' in Eqn. 11 are independent variables and the rate of haemolysis is seen to be separately dependent on both s^n and t^n. It can be seen from this equation that if 'n' is large there will be a distinct 'lag time' such as was found experimentally.

In order to estimate the value of 'n', the half life time of red cells in Triton X-100 solutions was measured and plotted on log scales against the surfactant concentration according to Eqn. 4 (Fig. 2). The points give a satisfactory fit to a straight line with a gradient of 3.0 giving the value of 'n'. A similar result was obtained for haemolysis in sodium dodecyl sulphate (Fig. 2) provided that the half life times were measured under conditions in which the ratio of surfactant to cell concentration was large so that deviation from the predicted linear time course relationship at the half time was not significant. In terms of Eqn. 9 this means that '$m(c_0-c)$' was small compared with 's' and for small changes in 's', '$(s-m(c_0-c))^n$ almost becomes a linear function of 's^n'. The value of 'n' for the haemolysis by sodium dodecyl sulphate was 3.3 which is close to that for haemolysis by Triton X-100. Sodium dodecyl sulphate, however, was nearly 15 times the more efficient lysin under these conditions and this may be at least partly due to the protection of the erythrocyte against the nonionic surfactant by an almost impermeable outer layer.

Surfactant binding. At the Triton X-100 concentrations used in the haemolysis studies (70–170 μM) no binding of the surfactant to ghost suspensions could be de-

TABLE II

BINDING OF TRITON X-100 BY GHOST SUSPENSIONS

The effect of surfactant concentration on the binding of Triton X-100 to 5 ml of a 50% ghost suspension was determined by the gel filtration technique (see text).

Triton X-100 concentration (μM)	*Surfactant bound* (*nmoles*)
73	0
147	0
220	76
294	132
368	240
441	341

tected by the gel filtration method (Table II). Surfactant binding occurred at 227 μM and increased rapidly above this concentration. This finding was confirmed by the results of the surface tension measurements and these are plotted as log (surfactant bound) *versus* log (free surfactant), according to Eqn. 8, in Fig. 3. Straight lines showed a good fit to the points and the gradients of the lines were 7.8 in each case. When similar experiments were performed on suspensions of egg lecithin a similar result was obtained (Fig. 3) and the gradient of the fitted straight line was 7.9.

Solubilisation

The high order dependence of solubilisation on surfactant concentration should be manifested as a critical concentration requirement whatever criterion of solubilisation is used. In experiments designed to investigate this, 6 series of 10 ghost–surfactant mixtures were made up and the apparent absorbance of each was read at 600 nm after a period of 30 min allowed for equilibration. Within each series the ghost concentration was held constant whilst the surfactant concentrations were varied within the range 0–600 μM. Ghost concentration was increased from 1.25% in the first series to 15% in the sixth. The results have been plotted (Fig. 4) so that the points at which half the initial absorbance had been lost in each series, could be graphically related. Microscopic examination of ghost–surfactant mixtures at these points showed that all the ghosts had fragmented, and it is assumed that they represent equivalent degrees of solubilisation in each series. Fig. 4 shows the characteristics of a high-order dependence on surfactant concentration. A close analogy may be drawn between this result and the increase in the number of micelles with total surfactant concentration above the critical micellar concentration, except that the critical micellar concentration for Triton X-100 is 900 μM and the critical concentration for 50% absorbance loss was 129 μM. Similar results were obtained for ghosts in sodium dodecyl sulphate and lecithin suspensions in Triton X-100 except that the critical concentrations were 111 μM and 276 μM, respectively. The closeness of the critical concentrations for Triton X-100 and sodium dodecyl sulphate is in contrast with the difference in the haemolytic efficiencies, as would be expected if a permeability barrier were responsible for the protection of the intact cell against the non-ionic surfactant. The larger critical concentration found for the lecithin–Triton X-100 system is not necessarily indicative of any fundamental difference. The turbidity of

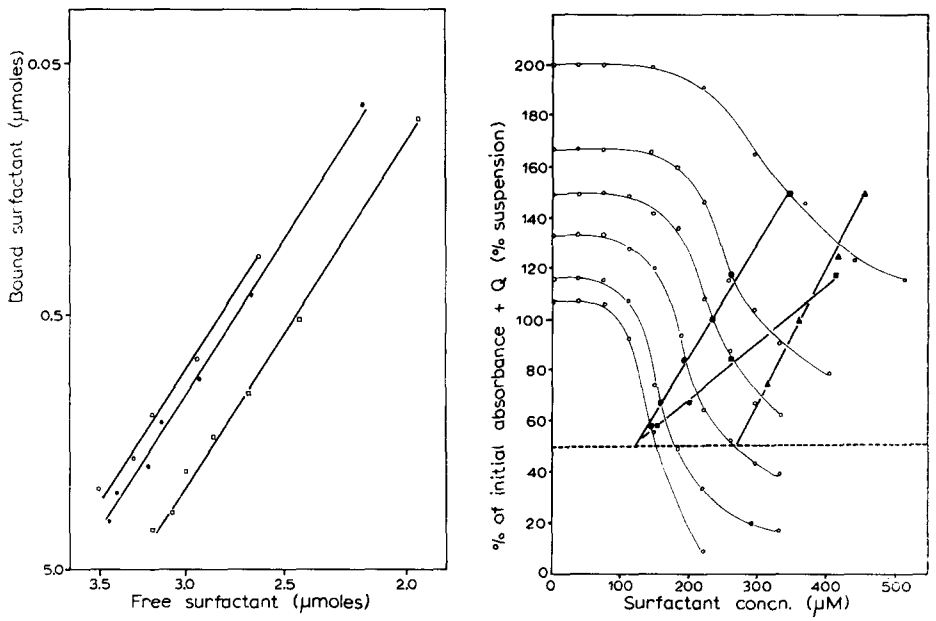


Fig. 3. Graph of free Triton X-100 against the amount of sufactant bound by 10 ml of: (i) a 10% ghost suspension (□), (ii) a 5% ghost suspension (●) and (iii) a suspension of 100 μg of lecithin, as determined by the drop-weight technique (○). Both scales are logarithmic to conform with Eqn. 8.

Fig. 4. The clarification of ghost and lecithin suspensions in Triton X-100 and sodium dodecyl sulphate. Curves only for ghost suspensions (1.25–15%) in Triton X-100 are shown (○) and are plotted in this form so that the relationship between surfactant concentration and the points (●) at 50% loss of initial absorbance in different suspension concentrations may be seen. Other points were similarily derived from curves for ghost suspensions in sodium dodecyl sulphate solutions (■) and lecithin suspensions in Triton X-100 (▲). Lines through these points are extrapolated to 'zero suspension concentration' at 50% of initial absorbance (– – – –) to obtain a critical concentration unaffected by non-clarifying interactions between the surfactant and the suspension. Scale expander 'Q' is set at 6.67 for ghost suspensions and 20 for lecithin suspensions.

ghost suspensions must decrease sharply as fragments are formed from the initial vesicular structure, whilst the turbidity of lecithin 'liposomes', made up of stacked lipid bilayers, would change more slowly.

The critical concentration requirement for solubilisation of ghost suspensions and lecithin suspensions in Triton X-100 were also investigated by chromatography on agarose gels. Ghosts and 'liposomes' are excluded from Sepharose 2B so that the amount of material included from suspensions in surfactant solution is a measure of solubilisation. Samples were made up in surfactant solution and pumped through columns of Sepharose 2B pre-equilibrated and eluted with buffers containing the same surfactant concentration. The results (Table III), expressed as the percentage of the total absorbance in the included volume above the surfactant baseline, show that no material was included from either ghosts or lecithin suspensions in 152 μM Triton X-100. Also, the elution volume of solubilised lecithin increased with surfactant concentration. A reduction in the size of a mixed phospholipid–surfactant micelle is expected as the proportion of surfactant is increased[12].

TABLE III

EFFECT OF SURFACTANT CONCENTRATION ON GEL FILTRATION CHARACTERISTICS OF GHOST AND LECITHIN SUSPENSIONS

The absorbance of material included in 2% agarose columns was determined in different concentrations of Triton X-100 and is expressed as a percentage of the total.

Ghost suspension (2.5%)		*Lecithin suspension (0.1 mg/ml)*		
Surfactant concentration (mM)	*% A included*	*Surfactant concentration (mM)*	*% A included*	*Elution vol. of included peak (ml)*
0.147	0	0.147	0	—
0.920	71	0.735	91	124
1.840	85	1.470	93	135
3.680	92			

Characterisation of solubilisate

Gel filtration was also used to investigate the elution properties of different components of solubilised ghosts and Fig. 5 shows the distribution of phospholipid, protein and acetylcholine esterase activity in the eluate of 5 ml of 50% ghost suspension in 3.8 mM Triton X-100 from a 4% agarose (Sagavac 4C) column pre-equilibrated and eluted with 3.8 mM surfactant. The enzyme activity, unlike the protein and phospholipid, formed a symmetrical, included peak, and whilst there was a difference in distribution of the components, no single constituent was completely separate. Yet when the 100–105-ml fraction was re-run under identical conditions the phospholipid peak was further retarded to give a maximum in the 120–125-ml fraction whilst the protein peak did not shift. Moreover, a similar re-run of the 70–75 ml fraction of the original eluate resulted in protein shifting to an elution volume of 100–105 ml. The probable reason for this behaviour is that, because molecular and micellar surfactant are included in the gel whilst unsolubilised material is not, there could be a progressive interaction between ghosts and Triton X-100 throughout the elution. The shift in the phospholipid peak is then analogous to the gel filtration characteristics of lecithin in Triton X-100 and also demonstrated that the surfactant separated phospholipid from protein. The shift in the protein peak indicates that initial large protein particles were further disaggregated by the surfactant during the elution. The elution volume of the final protein corresponds to a particle with a Stokes' radius of about 75 Å.

The solubilisation characteristics of the acetylcholine esterase were of interest since they were different from those of the bulk of the protein. This was shown more clearly by a comparable gel filtration experiment in which a 600 μM Lubrol 'W' solution was substituted for the 3.8 mM Triton X-100. Under these conditions acetylcholine esterase again formed a symmetrical included peak though the phospholipid and all the measurable amounts of protein were excluded. This suggests that the attachment of the enzyme to the membrane is different from that of the bulk of the protein.

The solubilisate of ghosts in Triton X-100 was further characterised by isoelectric focusing. In these experiments 100 ml gradients were made up containing 2 ml of ghost suspension, 3.8 mM Triton X-100 and ampholytes in the pH range 3–6.

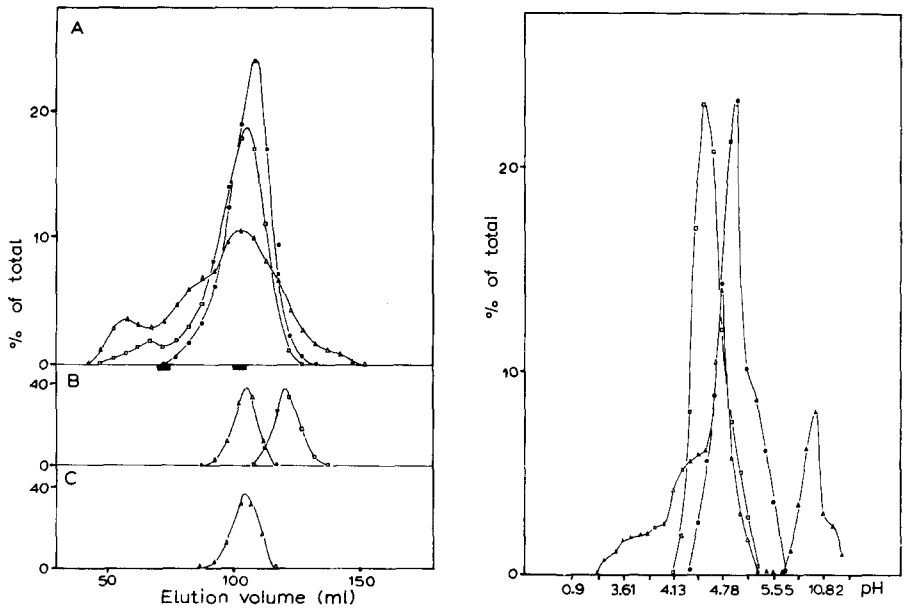


Fig. 5. A. Distribution of protein (△), phospholipid (□) and acetylcholine esterase activity (●) in the eluate of 5 ml of a 50% ghost suspension in 3.68 mM Triton X-100 from a 150 ml bed volume column of 'Sagavac 4C' 4% agarose beads equilibrated and eluted with 3.68 mM Triton X-100. B. Separation of phospholipid from protein when the 100–105 ml fraction of the original eluate (marked with a bar) was re-chromatographed on the same column. C. Distribution of protein in the eluate of the 70–75 ml fraction of the original eluate (marked with a bar) which was re-eluted from the same column.

Fig. 6. Distribution of protein (△), phospholipid (□) and acetylcholine esterase (●) in a pH 3–6 gradient containing 3.68 mM Triton X-100 after the isoelectric focusing of 2 ml of ghost suspension.

A large band of precipitated material was formed between pH 4.02 and pH 4.8 with a maximum at pH 4.7 (Fig. 6). This contained all the phospholipid and most of the protein. A second band of protein formed in the alkaline region beyond the linear region of the pH gradient. The phospholipid peak was symmetrical with a maximum at pH 4.5 but was not resolved from the asymmetric protein peak which had a maximum at pH 4.8 and extended down to the acid end of the gradient. Acetylcholine esterase formed a symmetrical peak around pH 5.0. Apart from the resolution of the alkaline protein and the enzyme activity these results do not provide evidence for the separate existence in Triton X-100 solubilisates of the large number of different proteins which have been found in erythrocyte stroma by other electrophoretic techniques[9,19]. The diffuse protein band, however, would be expected if the protein particles present contained varying proportions of peptides with different iso-electric points.

Iso-electric precipitation of ghosts and material from ghosts solubilised in Triton X-100 occurs around pH 4.5. Precipitates were formed at a higher pH by exhaustive dialysis against distilled water, and these readily redissolved when the ionic strength of the medium was raised. The material precipitated from ghosts in 750 μM Triton solution after three successive dialyses against 200 vol. of distilled water or

TABLE IV

RELEASE OF PHOSPHOLIPID FROM PRECIPITATES OF GHOST MATERIAL IN TRITON X-100 SOLUTIONS

Precipitates were prepared by the exhaustive dialysis of a 10% ghost suspension in 750 μM Triton X-100, 10 mM Tris–HCl, pH 8.0, against water and were washed twice in equal volumes of water. This material was then taken up in 10 vol. of an unbuffered Triton X-100 solution. Phospholipid release was determined as the total phosphate in the supernatant after centrifugation at 38000 × *g* (M.S.E. High Speed 18, 16 × 15 ml head, 18000 rev./min) and is expressed as μmoles/mg of total protein in the original preparation.

Phosphate released (*μmoles/mg protein*)	*Surfactant concentration* (*mM*)
0	0.147
0.048	0.294
0.097	0.441
0.128	0.588
0.188	0.735
0.239	0.882
0.260	1.030
0.306	1.175
0.320	1.321

TABLE V

UPTAKE OF LECITHIN BY PRECIPITATES OF GHOST MATERIAL DEPLETED OF PHOSPHOLIPID

The binding of lecithin to material precipitated from ghost suspensions in 7.5 mM Triton X-100 was carried out as described in the text. Results are expressed as μmoles phosphate per μg total protein in the preparation.

Phospholipid added	*Pellet phospholipid*	*Supernatant phospholipid*
0	0.212	0
0.057	0.265	0.005
0.114	0.320	0.016
0.172	0.372	0.012
0.220	0.399	0.033
0.334	0.406	0.140
0.448	0.405	0.255

Triton X-100 in distilled water contained all the acetylcholine esterase activity and ninety percent of the protein from the original ghost suspension. Washing this precipitate with Triton X-100 solutions in distilled water released amounts of phospholipid in proportion to the surfactant concentration above a minimum of 150 μM (Table IV). No detectable protein was released by a single wash but prolonged washing was found to decrease the amount of protein in the pellet, a finding perhaps analogous to that of MAZIA AND RUBY[20].

A similar precipitate, prepared by dialysing 50 ml of a 50% ghost suspension in 7.5 mM Triton X-100 against three changes of 5 l of distilled water, was twice washed with 50 ml distilled water and centrifuged at 40000 × *g* for 15 min (MSE High Speed 18, 6 × 100 ml head, 18000 rev./min) to remove surfactant. The final pellet was taken up in 16 ml distilled water. 10-ml mixtures were prepared containing 1 ml of the precipitate suspension with increasing concentrations of an egg lecithin

suspension in distilled water. Controls were made up without ghost precipitate. After 1 h at room temperature the preparations were centrifuged at 38000 × *g* for 10 min (MSE High Speed 18, 16 × 15 ml head, 18000 rev./min). Supernatants were retained for phosphate analysis and each pellet was taken up in 10 ml distilled water and centrifuged again. Final pellets were analysed for phospholipid and protein. There was no pellet in any of the control tubes. The results (Table V) were expressed as the phosphate content of the pellet per mg of protein and represent mean values from two determinations in each of two experiments. Not only was there uptake of phospholipid into the pellet but an abrupt saturation value was attained at about 0.405 μM phosphate per mg protein and this compared with a value of about 0.530 μM phosphate per mg protein measured in untreated ghosts. This demonstration that ghosts depleted of phospholipid by washing with Triton X-100 solution can take up added lecithin may justify the assumption of the reversibility of solubilisation made in the theoretical treatment. Furthermore, when the reconstituted material was suspended in 10 mM Tris–HCl, pH 8.0, a cloudy suspension was obtained which could be clarified with Triton X-100, but only at concentrations greater than 150 μM.

DISCUSSION

The construction of mathematical models, such as those developed here for haemolysis and for the solubilisation of ghosts, allows more precise statements to be made about the process. Thus it was found that the rate of haemolysis in both sodium dodecyl sulphate and Triton X-100 was approximately third order with respect to surfactant concentration and this suggests that a common mechanism was involved. On the other hand, deviations in the time courses of haemolyses in each surfactant could be explained in terms of secondary interactions of sodium dodecyl sulphate and a diffusion barrier for Triton X-100, and these required that the fundamental equation be modified to fit the characteristics of the individual surfactant. Whilst these explanations have not been proved, the overall third order relationship is established and it would be of value to study a wider range of surfactants in order to assess its universality.

The similarities in the solubilisations of erythrocyte ghosts and of lecithin suspensions and the observation that mixed lipid–surfactant micelles containing no protein are products of both processes suggests that solubilisation could result from a laminar–micellar re-arrangement occurring in lipid regions of the membrane. Furthermore the protein components appear to offer no protection to the lipids against solubilisation by a non-ionic surfactant and this is inconsistent with the existence of widespread ionic bonding between protein and lipid. Yet it has not been disproved that a minute proportion of the lipids remained bound to the proteins, perhaps ionically.

The finding that both haemolysis and solubilisation were high order processes with respect to surfactant concentration suggests that a common mechanism might have been involved. LUCY[21] has proposed that a laminar–micellar transition can occur within the membrane lipids without total breakdown of the structure and that this is involved in the fusion of avian erythrocytes promoted by the surfactant, lysolecithin. If the same phenomenon underlies haemolysis, and the formation of micelles in a lipid region is sufficient to allow the escape of haemoglobin, an overall mechanism

emerges for the disruption of the membrane by surfactants. The size and properties of mixed lipid–surfactant micelles are dependent on the proportions of the constituents[12] and it is possible to envisage a transient micelle, formed within the membrane at low surfactant concentration, being responsible for haemolysis, and a more stable micelle in a higher surfactant concentration being responsible for solubilisation. Yet, though it has been assumed that haemolysis occurring at low surfactant concentrations is a different process from solubilisation and, indeed, the different dependence on surfactant concentration supports this, it has not been shown that no solubilisation is involved in haemolysis. On the other hand, haemolysis in sodium dodecyl sulphate solutions occurred rapidly at one tenth of the critical concentration required for solubilisation and it would be necessary to use a much more sensitive technique to detect any release of phospholipid at that level.

Whilst the solubilisation of membrane lipids appears to occur without interference from the other components, protein is also taken into solution by Triton X-100. The chromatographic and electrophoretic properties of these proteins in the low concentrations of Triton X-100 used here indicate that the particles are large and are probably aggregates of a polydisperse nature but of a quite well-defined size. Furthermore, the de-ionised precipitate of these proteins took up phospholipid from a lecithin suspension in a saturable manner. If it can be shown that the protein aggregates in Triton X-100 solution are not artefacts and that the re-constituted material resembles the original membrane in essential structural aspects, then physical studies of this material would be valuable.

Until the conformation of these proteins and their orientation within the membrane is known there is insufficient information to build a model of the ghost membrane from these results. All that is indicated at present is that there are lipid regions within the membrane not ionically bonded to proteins but large enough to undergo a postulated laminar-micellar transition. The size of these regions is perhaps determined by the protein–protein bonding such as that in the de-ionised precipitate and, if this is so, these interactions are important in maintaining the structural integrity of the membrane.

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