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INTERACTIONS OF MEMBRANE PHOSPHOLIPIDS WITH FUSOGENIC LIPIDS

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SUMMARY

The negative-staining technique of electron microscopy has been used to investigate interactions between phospholipids and a number of substances that induce hen erythrocytes to fuse into multinucleated cells. Of the fusogenic compounds investigated, only the phospholipid lysolecithin was observed to cause bilayers of lecithin to become micellar. Treatment of lecithin with fusogenic lipids, e.g. glycerylmonooleate, gave characteristic lamellar, vesicular and hexagonal assemblies. Comparable changes were observed in experiments with sphingomyelin, phosphatidyl serine, erythrocyte ghosts, and extracted erythrocyte lipids. No new structures were seen on treating either phosphatidylethanolamine or cholesterol with glycerylmonooleate. These findings are discussed in relation to possible molecular mechanisms of membrane fusion.

INTRODUCTION

Lysolecithin and a number of other lipids can induce hen erythrocytes to fuse [1–4]. It has been proposed [5] that lysolecithin may cause cell fusion by producing localized micellar structures in the plasma membrane. Unlike lysolecithin, fatty acids and other lipids that cause cell fusion transform concentric bilayers of lecithin into undulating lamellae [7]. Both these undulating lamellae, and a less commonly occurring hexagonal array, closely resemble the structures seen in negatively-stained preparations of phosphatidylethanolamine. These and other related findings were interpreted as indicating an increase in fluidity of the organisation of lecithin bilayers on adding unsaturated fatty acids and esters that cause cell fusion [4, 7].

In the present paper, the effects of several fusogenic lipids [8] on dispersions of sonicated lecithin have been studied in more detail. We have also investigated the interactions of fusogenic lipids with other phospholipids known to occur in the erythrocyte membrane, with preparations of mixed lipids extracted from human erythrocytes, and with sonicated, erythrocyte ghosts.

MATERIALS AND METHODS

Solutions of purified egg lecithin [9], phosphatidylserine (sodium salt), egg

yolk phosphatidylethanolamine, or bovine sphingomyelin (grade 1, at least 99% pure), Lipid Products, Redhill, Surrey, or cholesterol (B.D.H., Poole, Dorset) in chloroform were evaporated to dryness, under nitrogen, and the phospholipids were suspended in distilled water or 2% (w/v) potassium phosphotungstate at pH 5.6 or 7.4 unless otherwise stated. Total lipid from human erythrocytes was extracted by the procedure of Rose and Oklander [10]. The lipid was allowed to swell for 15–30 min, and then sonicated for 30 s to 2 min in an M.S.E. ultrasonic disintegrator, while being gassed with nitrogen and cooled in ice.

Glyceryl mono-, di-, and trioleate, capric, palmitic, stearic, oleic and linoleic acids (grade 1, 99% pure) were from Sigma (London) Chemical Company Limited, selachyl alcohol was from Koch-Light, (purest grade), Colnbrook, Bucks., synthetic crystalline retinol was from Roche Products Limited, Welwyn Garden City, Herts., U.K., and lysolecithin was from Lipid Products (at least 99% pure), Redhill, Surrey. Aqueous dispersions of most of these substances were prepared as above, mixed in equal volume with the appropriate dispersed phospholipid immediately after sonication, and brought to the relevant temperature before removing a sample for electron microscopy. In some experiments the lecithin or sphingomyelin was mixed with the test substances in the organic solvent. The solvent was then evaporated, and the lipid–phospholipid mixture was sonicated in aqueous buffer as above. Lysolecithin was dispersed by mechanical agitation using a Rotamixer (Hooke and Tucker Ltd. from Jencons, Hemel Hempstead, U.K.). Retinol was dissolved in ethanol under nitrogen to give 100 mg/ml stock solution which was stored in the cold, in darkness. Dilutions in ethanol were made immediately before use and the diluted solution was then injected directly into a dispersion of the appropriate phospholipid using a Hamilton syringe (Micromeasure N.V., The Hague, Netherlands). The final concentration of ethanol in the lipid mixture was 2.5%.

For electron microscopy, a small drop of the lipid dispersion in negative stain was rapidly dried at room temperature on a specimen grid. For the experiments of Figs 10 and 11, lipid or ghost dispersions in water or buffer were added to an equal volume of potassium phosphotungstate or ammonium molybdate at the appropriate pH before drying. Electron micrographs were obtained with an EM 6b (A.E.I.) electron microscope at initial magnifications of 15000–72000.

RESULTS

Interactions between lysolecithin and phospholipids

The addition of lysolecithin in aqueous dispersion to lecithin liposomes has been reported to cause a breakdown of the lamellar structure of the liposomes, with the formation of micelles 7–8 nm in diameter [6]. We have repeated these experiments and have made the same observations. Similar results were also obtained when lysolecithin and lecithin at 37 °C were taken up directly in potassium phosphotungstate solution, at pH 5.6 or 7.4 (Fig. 1). Lysolecithin (Fig. 2) and lecithin (Fig. 3) alone were in the form of globular micelles (7–7.5 nm) and bimolecular lipid spherules respectively, as originally described by Bangham and Horne [6].

Uni- to multilayered liposomes of phosphatidylserine and sphingomyelin gave rise to micelles on treatment with lysolecithin; the micellar size being of the same order as that of lecithin–lysolecithin micelles. Extracted human red cell lipid formed

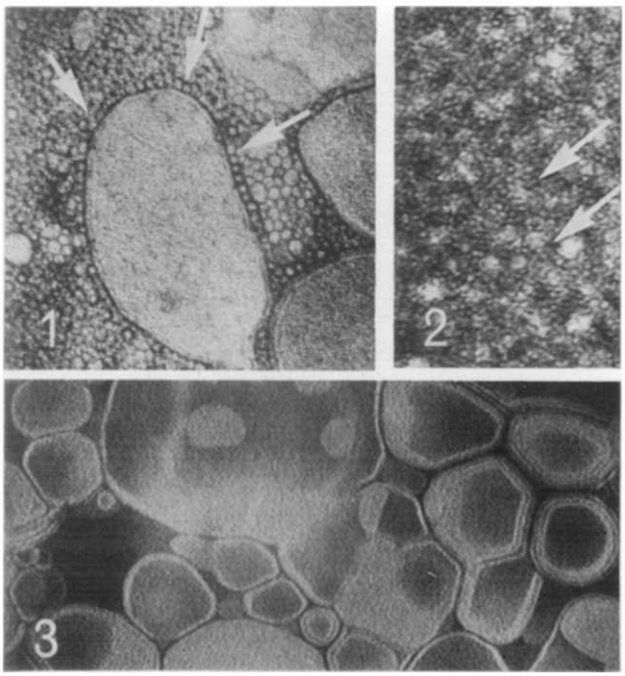


Fig. 1. Electron micrographs of phosphospholipids (final concentration: 1.25 mg/ml) dispersed by sonication in 2% potassium phosphotungstate solution. Fusogenic lipids (final concentration: 1.25 mg/ml) were dispersed, as described in Materials and Methods in 2% phosphotungstate solution, and mixed with the phospholipid dispersions. All experiments were at pH 5.6 and preparations were brought to 37 °C prior to electron microscopy, unless otherwise stated. All micrographs are × 150 000 unless otherwise stated. Sonicated egg lecithin treated with lysolecithin. The lecithin bilayer structure has broken down to form globular subunits (arrows).

Fig. 2. Lysolecithin treated as in Fig. 1, but with no added lecithin. Globular micelles can be seen (arrows).

Fig. 3. Egg lecithin at 37 °C (1.25 mg/ml) suspended by sonication in potassium phosphotungstate solution at pH 5.6. Uni- and multilamellar vesicles can be seen.

unilayered vesicles on sonication and these also appeared to form micelles on addition of lysolecithin.

Interaction of fusogenic lipids with lecithin

In order to see whether other substances, which are less haemolytic but are nevertheless capable of causing cell fusion in hen erythrocytes [8, 11], also cause micelle formation with bilayers of lecithin, similar experiments were undertaken with a variety of fusogenic lipids. No globular micelles were seen when dispersed lecithin was mixed with dispersions of glyceryl monooleate, oleic, capric or linoleic acids, selachyl alcohol, or retinol. Instead, new structures were observed that were remarkably consistent in experiments with differing fusogenic lipids. These new macromolecular assemblies of lipid were less regular in arrangement than those found in dispersions of lecithin alone, and several different types of structure were often present on the same grid (Figs 4–6).

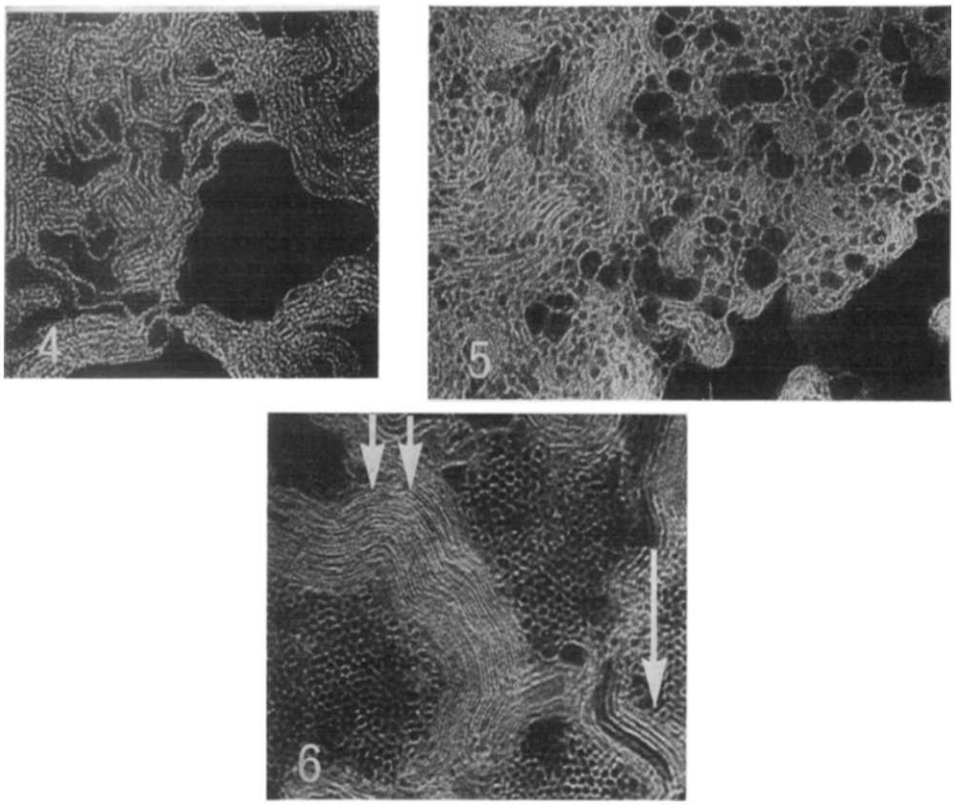


Fig. 4. Egg lecithin treated with oleic acid. Undulating lamellae can be seen.

Fig. 5. Egg lecithin treated with glyceryl monooleate. Vesicular structures are present, interspersed with the lamellar arrays.

Fig. 6. Egg lecithin treated with glyceryl monooleate. Hexagonal areas are visible and also lamellae showing a wide (arrow) and narrow (double arrow) spacing.

Undulating membranes with a repeat distance of approximately 5 nm are seen in Fig. 4. In other areas, lamellar structures were interspersed with vesiculations (Fig. 5). Rarely, a hexagonal phase was seen (Fig. 6; repeat distance approximately 8 nm) that closely resembled the appearance of negatively-stained dispersions of phosphatidylethanolamine (Fig. 7). Narrow lamellar spacings of 3.5 and 6.5 nm were seen in the hexagonal regions that may possibly be inclined views, corresponding to $D/2$ and $D\sqrt{3}/2$ of the hexagonal phase.

None of the organised structures of Figs 4–6 were observed in experiments in which lecithin was treated with glyceryl di- and trioleate, palmitic or stearic acid which have little or no ability to cause cell fusion [8]. Instead, only intact liposomes and amorphous masses of globules of lipid were seen. Since the lack of solubility of the inactive lipids might possibly be responsible for the absence of interaction with lecithin, these substances were sonicated together with lecithin. Fewer liposome structures resulted, indicating that some interaction had taken place but the same, unstructured masses of lipids were seen as before.

The characteristic structures of Figs 4–6 were also absent from negatively-stained preparations of oleic, linoleic and capric acids, selachyl alcohol, and from glyceryl monooleate alone; only amorphous aggregates, interspersed with vesiculated structures were seen. Retinol alone formed numerous large globules.

In an attempt to establish the course of events leading to the formation of the macromolecular assemblies shown in Figs 4–6, dispersions of glyceryl monooleate and lecithin were incubated at room temperature for up to 15 min. Identical structures were seen at all times, however, even in samples taken immediately after mixing. The ratio of glyceryl monooleate to lecithin was also reduced in the hope of slowing down the interactions between the two lipids. With a constant concentration of lecithin (1.25 mg/ml), the concentration of glyceryl monooleate was progressively reduced to 0.15 mg/ml. This resulted in fewer but similar structures to those seen in Figs 5 and 6. With 0.1 mg/ml of glyceryl monooleate, only lecithin liposomes were observed. Using solutions of potassium phosphotungstate at pH 5.6 and 7.4 as the suspending media, the characteristic structures of Figs 4–6 were seen at both pH 5.6 and 7.4 with glyceryl monooleate, selachyl alcohol and retinol. With oleic, capric and linoleic acids, however, the structures were seen only at pH 5.6. These structures did not form when lecithin was treated with glycerol di- or trioleate, palmitic or stearic acids at the two pH values.

Hen erythrocytes fuse into multinucleated cells on heating to 50 °C in the absence of exogenous lipids [4]. It therefore seemed possible that heating lecithin liposomes might give rise to structures resembling those observed when lecithin interacts with fusogenic lipids. In order to test this idea, lecithin liposomes were incubated immediately prior to electron microscopy at 37, 48 and 60 °C in the absence of fusogenic lipids. The only observed change was the production of blebs and protrusions from some of the liposomes. Many liposomes were unchanged in appearance from those incubated at 37 °C.

While this work was in progress, Galliard [12] reported that, on mixing sonicated dispersions of lecithin and linoleic acid at a concentration of 0.5 μequiv of each lipid, structures similar to those seen in Fig. 4 were observed, whereas assemblies similar to those of Fig. 5 were seen when 3 μequiv of these lipids were mixed. We have confirmed these findings using 3 μequiv of linoleic acid, and we have made similar observations with capric acid and selachyl alcohol. The lipids were suspended in 0.1 M ammonium acetate (pH 5.5) and negatively-stained with ammonium molybdate (pH 5.2), as described by Galliard. Our confirmation of his findings indicates that neither the suspending medium nor the negative stain used are critical to the appearance of the macromolecular assemblies present in Figs 4–6.

The repeat spacing of untreated lecithin bilayers was found to be approximately 5.9 nm. This value agrees well with that (5.9 nm) obtained with negatively-stained preparations of lecithin by Chapman and Fluck [13]. The periodicity of lecithin bilayers after treatment with oleic acid (pH 5.6), glyceryl monooleate (pH 5.6), and selachyl alcohol (pH 7.5) was only slightly reduced, to approximately 5.0 nm. In some cases it was as much as 5.5 nm. It is interesting, however, that measurements on the thickness of individual lipid layers indicated that the lipid bilayer apparently becomes thinner after treatment with oleic acid, being reduced from 3.5 nm in thickness to as little as 2.0 nm. It must nevertheless be emphasized that the latter value should be

regarded with caution in view of the difficulties of applying quantitative electron microscopy to the irregularly-layered structures under study.

Fusogenic lipids and sphingomyelin

Aqueous dispersions of sphingomyelin exhibit structures (Fig. 8) that are similar in appearance to lecithin liposomes (Fig. 3). Several different new structures were noted after treatment of sphingomyelin with the fusogenic lipids, and Fig. 9 shows irregular undulating lamellae of sphingomyelin–glyceryl monooleate which resemble the lecithin–oleic acid assemblies of Fig. 4.

Sphingomyelin gave more reproducible results when mixed with fusogenic

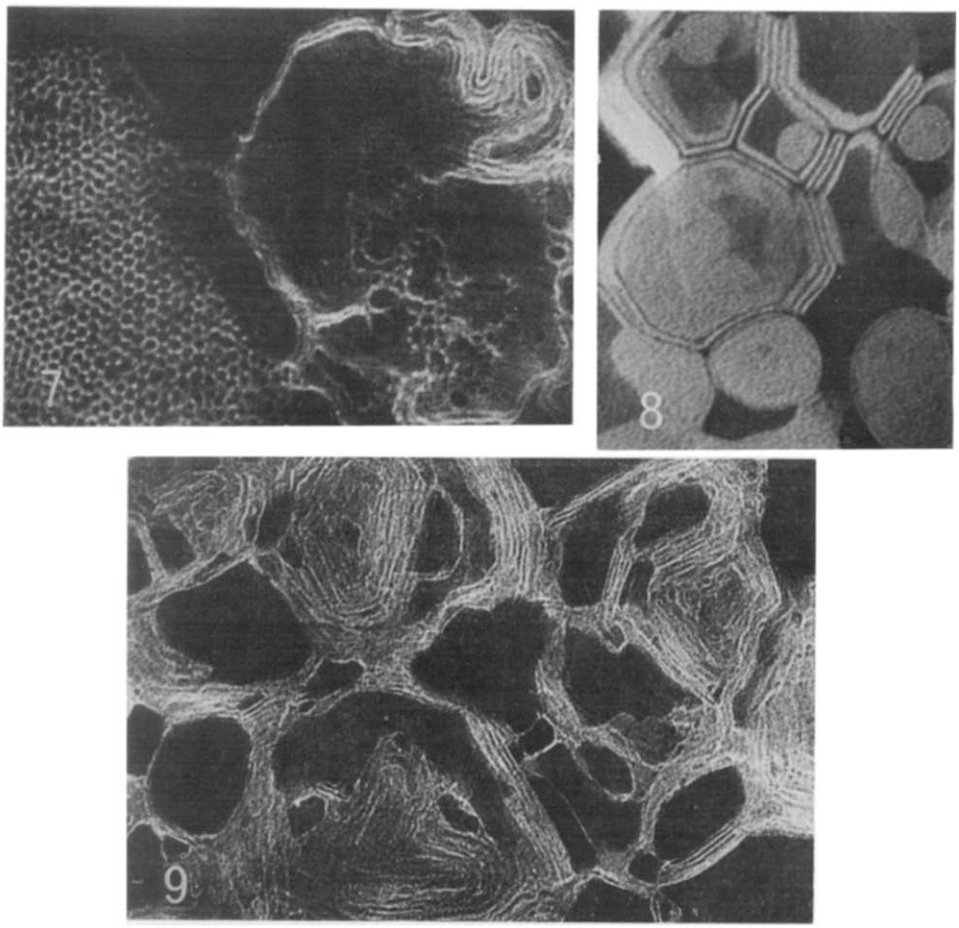


Fig. 7. Sonicated phosphatidylethanolamine. Hexagonal and lamellar phases are visible, similar in appearance to those in Fig. 6.

Fig. 8. Sonicated sphingomyelin (1.25 mg/ml) suspended in potassium phosphotungstate at pH 5.6 and brought to 45 °C. Angular liposomes are bounded by one or more bilayers.

Fig. 9. Irregular, undulating lamellae seen with sphingomyelin that has been sonicated with glyceryl monooleate. Conditions as in Fig. 8.

lipids at 45 than at 37 °C. This phospholipid is known to give rise to a lamellar, liquid-crystalline phase at 45 °C, while it is crystalline in water at 25 °C [14].

Sonication of sphingomyelin together with glyceryl monooleate gave more consistently reproducible structures than when the lipids were sonicated separately and mixed subsequently; significantly, however, glyceryl trioleate and stearic acid did not form any organised, macromolecular structures when sonicated with sphingomyelin. A relevant finding has been reported by Lester et al. [15] who found that, although brain gangliosides and lecithin form mixed micelles, pre-formed lecithin "myelins" resist solubilization by gangliosides.

Phosphatidylethanolamine

As mentioned above, the appearance of aqueous dispersions of phosphatidylethanolamine (Fig. 7) is indistinguishable from that of lecithin treated with the various fusogenic lipids (Figs 4 and 5).

Preliminary experiments with phosphatidylethanolamine indicated that the structure normally adopted by this phospholipid in aqueous dispersions was unaltered by the addition of, for example, glyceryl monooleate. Further studies with this and other lipids have now shown that this is not so. The hexagonal array that is characteristic of phosphatidylethanolamine is greatly reduced in extent, or sometimes completely absent, when the phospholipid interacts with a fusogenic lipid. However, we have not observed any new, ordered macromolecular assembly to be formed, in these experiments.

Phosphatidylserine

Sonicated dispersions of phosphatidylserine at 37 °C are predominantly in the form of concentric bilayers, closely resembling lecithin liposomes as described by Papadjopoulos and Miller [16]. In addition, we have sometimes noted blebs and protrusions on these spherules that were similar to those seen when egg lecithin was heated to 48 °C or above.

Treatment of phosphatidylserine with glyceryl monooleate or oleic acid at 37 °C gave rise to undulating lamellae, and rarely a hexagonal phase, as observed with lecithin.

Cholesterol

Since cholesterol is a major lipid component of erythrocyte membranes, sonicated cholesterol was also treated at 37 °C with glyceryl monooleate and oleic acid. Cholesterol alone exhibited amorphous masses, which remained apparently unchanged on treatment with the fusogenic lipids.

Effect of Ca^{2+}

Ca^{2+} is necessary for cell fusion by glyceryl monooleate, oleic acid and retinol [8, 11]. It was therefore interesting to see whether the 1.8 mM Ca^{2+} used in experiments on the fusion of hen erythrocytes affected the formation of the macromolecular assemblies reported in this paper.

$Ca(OH)_2$ was added to the ditilled water, or to potassium phosphotungstate at pH 5.6: alternatively, a modified Eagle's basal salt buffer (pH 5.6), which contains Ca^{2+}, was used as the suspending medium as with hen erythrocytes [8]. Lecithin

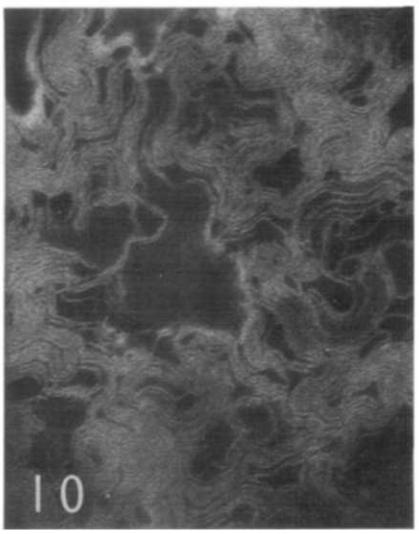
liposomes treated with glyceryl monooleate or oleic acid in these calcium-containing, aqueous media formed the usual "phosphatidylethanolamine-like" structures at 37 °C. Since phosphatidylserine is known to have a particular affinity for Ca^{2+} [17], the influence of Ca^{2+} on the interaction of this phospholipid with oleic acid and with glyceryl monooleate was investigated. A concentration of 1.8 mM Ca^{2+} in potassium phosphotungstate had no apparent effect on the interaction of these two lipids with phosphatidylserine.

Extracted erythrocyte lipids and human erythrocyte ghosts

Total lipids extracted from human erythrocytes [10] and sonicated human erythroctye ghosts that were prepared by a modification of the Dodge method [18], were treated with glyceryl monooleate in distilled water, or in potassium phosphotungstate solution (pH 5.6), or in modified Eagle's basal salt buffer (pH 5.6), and then incubated at 37 °C. Macromolecular assemblies were observed that were essentially similar to those seen when lecithin, sphingomyelin and phosphatidylserine were allowed to interact with glyceryl monooleate (Figs 10 and 11), but were not seen after treatment with stearic acid or glyceryl trioleate.

DISCUSSION

The technique of negative staining has often been criticized when applied to



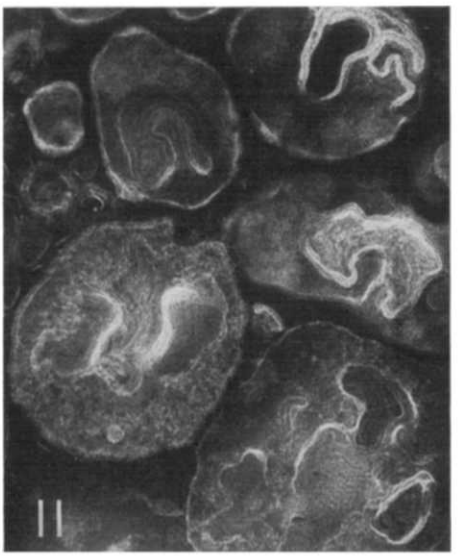


Fig. 10. Human red cell lipid (1.25 mg/ml) sonicated in Eagle's basal salt buffer, pH 5.6 (Ahkong et al. [8]) brought to 37 °C, and negatively stained with an equal volume of potassium phosphotungstate solution (pH 5.6) after the addition of glyceryl monooleate (1.25 mg/ml). Undulating lamellae are seen. × 90000.

Fig. 11. Human red cell ghosts (1.25 mg/ml) sonicated in distilled water, brought to 37 °C, and negatively stained with an equal volume of potassium phosphotungstate solution (pH 5.6) after the addition of glyceryl monooleate (1.25 mg/ml). Undulating lamellae are observable within the membranous vesicles. × 90000.

the study of lipids on the grounds that, as the stain dries, the salts will become concentrated causing structural changes [19 20]. There is no doubt that the technique needs to be used with caution since variations in pH and negative stain are capable of influencing the appearance of macromolecular lipid assemblies [21,22]. Nevertheless, Junger and Reinauer [23] have reported a rather good agreement of dimensions obtained from negatively-stained preparations of phosphatidylethanolamine with X-ray results. In addition, Johnson and Horne [24] have produced evidence that the negative stain sets rigidly into an amorphous glass-like substance while the specimen is still wet. Howell et al. [25] observed macromolecular assemblies of lipid in bile that were consistent with the disc-shaped micelles postulated to occur in bile by Small et al. [26] on the basis of nuclear magnetic resonance studies. Nevertheless Rand et al. [27], using electron microscopy and X-ray diffraction, have commented that comparisons of the dimensions of negatively-stained lipid structures with those of hydrated lipids must be done with great caution.

The precise interpretation of the structural changes observed here is therefore problematic. Most fusogenic lipids are either unsaturated: oleic acid, linoleic acid, glyceryl monooleate, selachyl alcohol and retinol, or have a short saturated fatty acid chain: capric acid. It is relevant that De Gier et al. [28], who demonstrated that the introduction of double bonds into lecithin or shortening the chain length of the constituent fatty acids increases the permeability of lecithin liposomes, have suggested that this could be explained by an increase in the fluidity of the lipid barrier.

Phillips et al. [29] have obtained evidence from X-ray diffraction data on phospholipid dispersions that the polar group of phosphatidylethanolamine lies perpendicular to the axis of the hydrocarbon chains, while the larger polar group of phosphatidylcholine has a more parallel orientation. The observed ultrastructural change in the appearance of phosphatidylcholine treated with a fusogenic lipid might thus result from a reduced electrostatic repulsion due to a reorientation of the polar groups. If a change in orientation of phospholipid head groups occurs in the intact red cell, simultaneously with a lateral movement of surface glycoprotein that exposes the lipid bilayer, it would greatly facilitate the interaction and fusion of adjacent erythrocyte membranes treated with a fusogenic lipid. Papahadjopoulos and Miller [16] noted that the inter-lamellar repeat of phosphatidylethanolamine is less than that of lecithin; they suggested that this might result from a reduction of electrostatic interactions between adjacent bilayers. On our hypothesis the repeat distance in bilayers of lecithin treated with glyceryl monooleate, for example, should be narrower than that of untreated lecithin. In fact, measurements on lecithin bilayers after treatment show only a slight reduction in period. Before treatment lecithin had a repeat period of approximately 5.9 nm; after treatment with different lipids the period generally varied from 5.0 to 5.5 nm. However, the lipid bilayer itself appeared to be thinner after treatment: with lecithin and oleic acid, it was reduced from approximately 3.5 to as little as 2.0 nm.

The most significant feature of the present work is the production of new macromolecular assemblies, seen with two different negative stains, when lecithin, sphingomyelin and phosphatidylserine interact with fusogenic lipids. A similar change in organisation is observed when extracted red cell lipids or sonicated ghosts are treated with glyceryl monooleate. In contrast, lipids that are closely related chemically, but that do not cause red cells to fuse, do not induce these structural changes. Taken

together these observations indicate that a primary effect of low-melting lipids like glyceryl monooleate and oleic acid in causing cell fusion is on the organisation of the phospholipids of the erythrocyte membrane. Changes in membrane proteins during the fusion process are, however, not excluded. These observations are consistent with a previous suggestion that membrane fusion may be induced by directly favouring (cf. lysolecithin) a localized micellar organization for the membrane lipids or lipoproteins rather than a bimolecular leaflet structure, while other perturbing molecules (cf. fusogenic lipids) would be anticipated to produce comparable changes in the organization of membranes by differing mechanisms [5].

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