Effects of fusogenic agent on membrane structure of erythrocyte ghosts and the mechanism of membrane fusion

MEMBRANE fusion clearly requires that participating lipids assume some transitory non-bilayer configuration during the intermediate stages. Previous workers have suggested that intermediate micellar[1] or inverted micellar[2,3] structures may occur, but the precise nature of possible intermediates and their relation to the physical properties of membrane lipids are obscure. In this regard, Lucy and co-workers[4-8] have shown that 'fusogenic' agents such as fatty acids and their derivatives induce erythrocytes to fuse. Such agents might possibly promote fusion by enabling endogenous lipids to assume non-bilayer configurations. We have therefore investigated the influence of two such fusogens on the structure of the erythrocyte (ghost) membrane using ^{31}P NMR techniques, which have been found to be sensitive to phospholipids in non-bilayer phases[9,10]. We show that the incorporation of oleic acid and glycerol mono-oleate into the ghost membrane, at concentrations similar to those needed to induce cell fusion between erythrocytes *in vitro*, produce a well-defined transition of a variable portion of the membrane phospholipids from the bilayer phase to an hexagonal (H_{II}) phase. These results lead us to propose a model for membrane fusion induced by oleic acid, which we suggest may also apply to fusion events *in vivo*.

The phases available to hydrated liquid crystalline phospholipids, and the corresponding ^{31}P NMR spectra obtained are illustrated in Fig. 1. Briefly, the phospholipids in (large) bilayer structures give rise to broad asymmetric ^{31}P NMR spectra with a low field shoulder[9-14]. Alternatively, phospholipids in the hexagonal (H_{II}) phase (ref. 15) exhibit narrower spectra with a high field shoulder[10]. Finally, phospholipids in other available phases exhibit much narrower symmetrical ^{31}P NMR spectra[10].

The 36.4 MHz ^{31}P NMR spectra obtained from erythrocyte ghosts incubated in the presence of increasing amounts of oleic acid are shown in Fig. 2. A progressive conversion of the membrane phospholipids from the bilayer phase to the hexagonal (H_{II}) phase is observed. Similar effects were also observed for glycerol mono-oleate. Such spectra cannot arise from lipids in

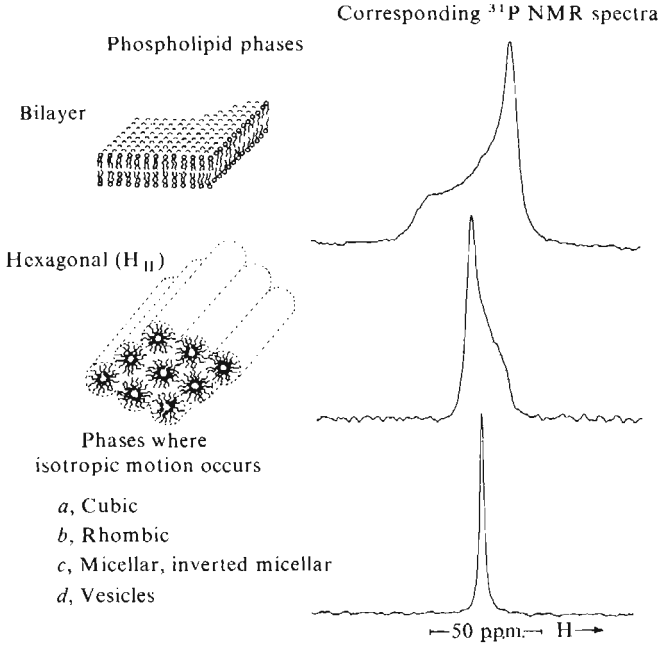

Fig. 1 Phospholipid phases and corresponding (36.4 MHz) ^{31}P NMR spectra observed. The 36.4 MHz ^{31}P NMR spectra were obtained from (unsonicated) aqueous dispersions of egg yolk lecithin at 30 °C (bilayer phase), soya phosphatidylethanolamine at 30 °C (hexagonal H_{II} phase) and a mixture of soya phosphatidylethanolamine and 15 mol% egg yolk lecithin at 30 °C (cubic, rhombic or inverted micellar phase, for fuller details see ref. 10). All aqueous dispersions contained 25 mM Tris-HAc (*p*H 7.1) and 2 mM EDTA. All spectra were obtained in the presence of high power (18 W) broad band proton decoupling.

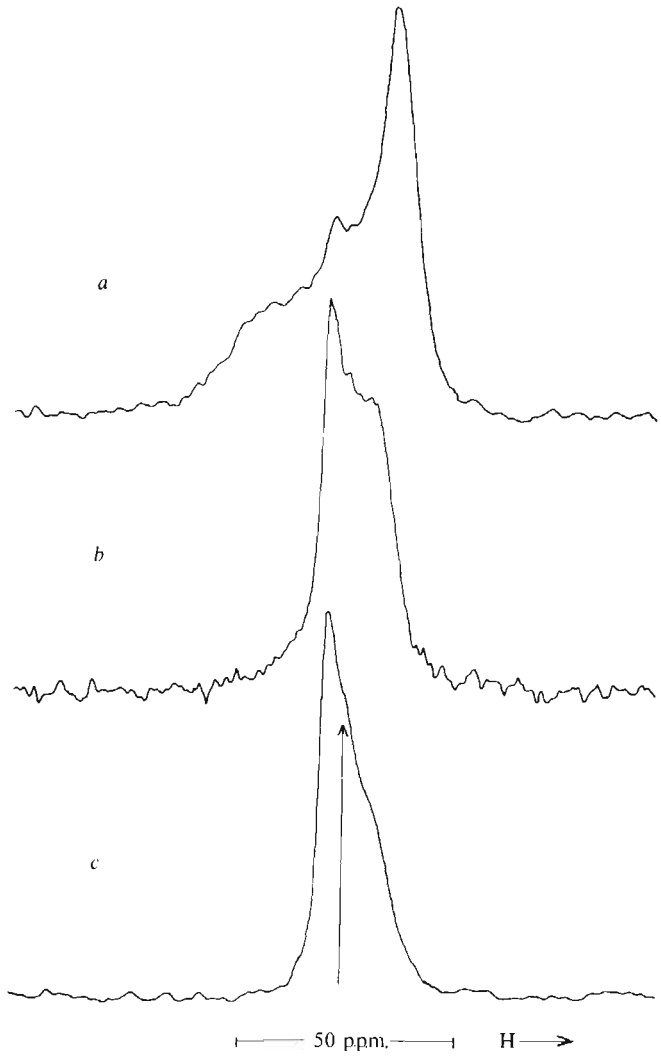

Fig. 2 36.4 MHz ^{31}P NMR spectra obtained at 37 °C from *a*, 150 mg (dry weight) erythrocyte ghost incubated in medium A (for details of incubation conditions and composition of medium A, see below) in the absence of oleic acid; *b*, 150 mg (dry weight) erythrocyte ghost incubated in medium A containing 35 mg oleic acid; *c*, 150 mg (dry weight) erythrocyte ghost incubated in medium A containing 50 mg oleic acid. The arrow indicates the position of ^{31}P NMR spectra arising from sonicated vesicles. Erythrocyte ghosts were prepared from human bank blood as detailed previously[24] and were subsequently lyophilised and stored under N_2 at −20 °C before use. Medium A contained NaCl (0.12 M), KCl (6 mM), $MgSO_4.7H_2O$ (5 mM), $CaCl_2$. H_2O (2 mM) and Tricine (20 mM), and the *p*H adjusted to 6.8. The oleic acid was added drop by drop to 40 ml of medium A and subsequently sonicated (5 min) to obtain a milky suspension which was added to the (dry) ghost material. The resulting mixture was incubated at 37 °C for 20 min while stirring continuously. The material was subsequently centrifuged (50,000*g*) for 10 min at 0 °C and 0.1 ml D_2O (25 mM Tris-HAc, *p*D 7.0) added to the pellet, which was then transferred to the NMR tube. Spectra were obtained from up to 40,000 transients in the presence of high power (20 W) proton decoupling, using a 45° resonance frequency pulse and a 0.18 s interpulse time. Fatty acid determinations (see text) indicated an oleic acid to phospholipid ratio (mol/mol) of 1.3 for sample (*b*) and 2.4 for sample (*c*).

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micellar or inverted micellar phases, or from bilayer phase lipids in rapid exchange with such micellar phases, as either situation would give rise to a narrow spectral component ($\simeq$20 Hz linewidth) with a chemical shift characteristic of isotropic averaging. Freeze fracture electron micrographs obtained from the sample of Fig. 2*c* reveal (Fig. 3) that the protein is segregated away from regions of the H_{II} phase, which exhibit a characteristic striated pattern consistent with previous freeze fracture visualisations of the H_{II} phase in model systems[16].

The formation of the H_{II} phase was observed to be strongly dependent on the presence of Ca^{2} in the incubation medium. Incubations equivalent to that performed for the sample of Fig. 2*c* but where Ca^{2+} was omitted resulted in broad, rather featureless ^{31}P NMR spectra which could not be clearly identified with either the bilayer or H_{II} phases. Control experiments (see below) showed that similar concentrations of oleic acid were associated with the ghost membrane in both situations. ^{31}P NMR studies were also performed on liposomes composed of the total lipids extracted from the erythrocyte membrane (to which various amounts of fusogen had been added prior to hydration) in the presence and absence of Ca^{2+}. Addition of Ca^{2+} to these systems promoted the formation of the H_{II} phase (above a fusogen/phospholipid ratio of 1 mol/mol). This provides strong evidence that Ca^{2+} directly facilitates the formation of the H_{II} phase in the ghost membrane. This apparent ability of Ca^{2+} to induce bilayer to hexagonal (H_{II}) phase transitions may be related to the previously noted[17,18] ability of Ca^{2+} to cause lateral phase separation of charged lipid species in mixed lipid systems.

If the H_{II} phase formed in the fusogen-treated erythrocyte ghost membranes is relevant to the mechanism by which fusogens facilitate fusion between erythrocytes *in vitro*, similar amounts of fusogen should be associated with ghosts and erythrocytes when significant hexagonal (H_{II}) phase or fusion, respectively, is observed. Thus fusion was induced between erythrocytes, employing oleic acid, in the manner detailed previously[7]. The cells were centrifuged on observation of initial fusion events (10–20% fusion, as observed in a phase contrast microscope), and a fatty acid analysis of the pellet allowed a determination of the oleic acid associated with the cells. Excess fatty acid floated on the supernatant after centrifugation. Approximately 1 mol of oleic acid per mol of erythrocyte phospholipid was required before significant fusion occurred. This may be compared to an oleic acid/phospholipid ratio of 1.3 (mol/mol) obtained for the sample of Fig. 2*b* where significant formation of the H_{II} phase is observed. Assuming that a similar proportion of the membrane associated fusogen is contained in the ghost and erythrocyte bilayers respectively, these results indicate that membrane concentrations of fusogen sufficient to initiate fusion in erythrocytes are also sufficient to promote formation of the H_{II} phase in the ghost membrane. This suggests that observation of the H_{II} phase in the ghost system corresponds to ghost fusion (although this has not yet been observed by other techniques) and also suggests that formation, or a tendency to formation, of the H_{II} phase in the erythrocyte membrane itself is vital to subsequent fusion events.

On the basis of this information we propose a model of cell fusion induced by oleic acid which is illustrated in Fig. 4. The fusion event is envisaged to proceed through (at least) four stages; these involve aggregation of protein to produce areas of (protein free) lipid bilayer[19], which subsequently make contact (Fig. 4*b*). It is then suggested that the two outer monolayers combine to form an intermediate hexagonal H_{II} phase (Fig. 4$_c$) which is subsequently restabilised to bilayer structure (Fig. 4*d*) to complete the fusion process. The presence of Ca^{2+} is probably important at each stage, indeed Ca^{2+} has already been implicated in the processes of protein aggregation and cell–cell contact[20]. We emphasise, however, that the presence of Ca^{2+} would seem to be particularly important for the formation of the H_{II} phase of Fig. 4*c*. Further, the restabilisation event may be envisaged to occur by way of an active (ATP-dependent) removal of Ca^{2+} from the cytoplasm of the partially fused cells of Fig. 4*c*, thus producing a strong chemical potential between the aqueous pores of the H_{II} phase and the cytoplasm. This would encourage the removal of Ca^{2+} and subsequent reversion of the H_{II} lipid to the bilayer phase.

Fig. 3 Freeze-etch electron micrograph obtained from erythrocyte ghost sample giving rise to the spectra of Fig. 2*c*. The sample was quenched from 20 °C. Other conditions were as detailed previously[25]. Magnification $\times$250,000.

There are strong indications that the mechanism of membrane fusion shown in Fig. 4 may also apply to naturally occurring membrane fusion *in vivo*. In particular, many species of naturally occurring lipids found in significant concentrations in biological membranes prefer the hexagonal (H_{II}) phase or other inverted phases (intermediate formation of inverted micelles would be equally acceptable to the fusion model of Fig. 4) over bilayer structure. Notable examples include unsaturated phosphatidylethanolamines[9,10,21,22] and cardiolipin[23]. Moreover, the formation of H_{II} phases for both these lipid species may be facilitated by the presence of Ca^{2+} (ref. 23 and P. R. Cullis, unpublished). Cholesterol also tends to favour the formation of hexagonal (H_{II}) phases in certain situations[10]. Thus 'natural fusogens' abound, and segregation of such non-bilayer lipids into particular regions of the membrane (in the presence of Ca^{2+}) would be expected to facilitate fusion events. Cell–cell contact in the form of Fig. 4*c* may also correspond to 'quasi-fusion' events[20] associated with tight junctions.

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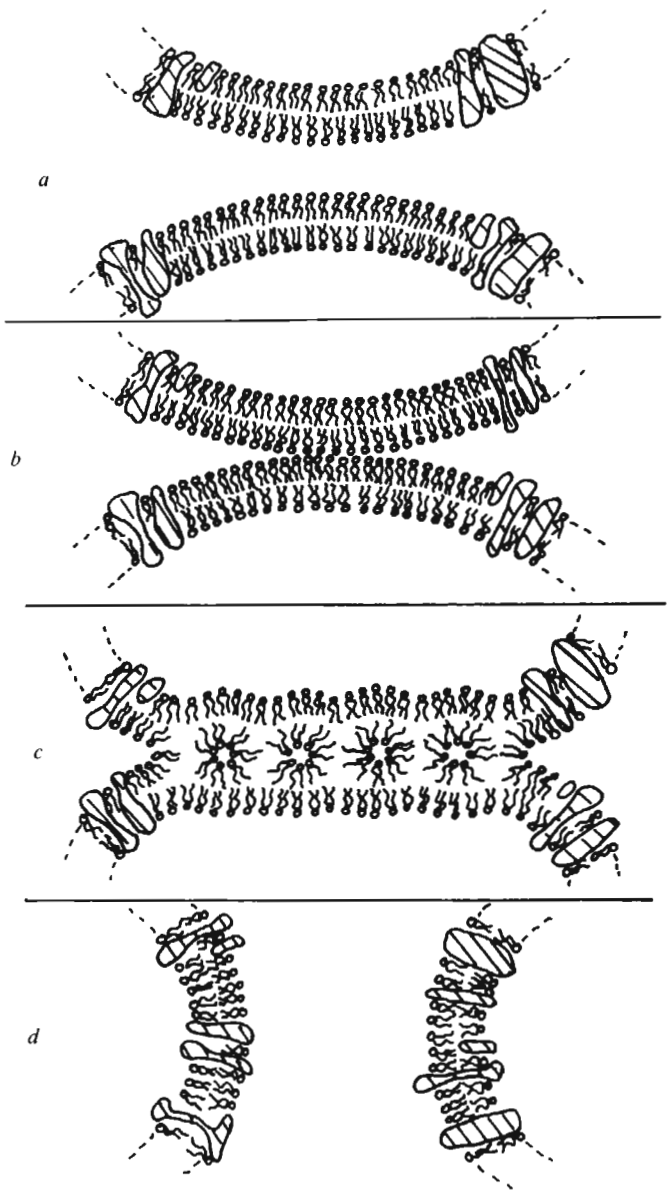

Fig. 4 Proposed mechanism of membrane fusion. The slashed areas traversing the membrane indicate integral membrane protein. Note that the diameter of the aqueous channels of the hexagonal phase component of part *c* are drawn approximately to scale with respect to the thickness of the bilayer.

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Distribution of sialic acids on the red blood cell membrane in β thalassaemia

SIALIC acids (SA) of the red blood cell (RBC) membrane are considered to play an important part in the physiology of the RBC[1]. There are indications that the amount of membrane sialic acid is a major factor in distinguishing young from aged RBC. Thus, ageing of circulating RBC is associated with a reduction of 20–30% SA which may be an important determinant of RBC survival[2,3] in the eventual recognition and sequestration of the latter cells by the reticuloendothelial system[2]. During studies of thalassaemic RBC we observed, among other alterations, that the average level of SA was approximately 25% less than in normal RBC membranes obtained from healthy donors[4]. We, therefore, investigated the comparative ultrastructural distribution of SA on the surface of thalassaemic RBC membranes. We found that the SA residues on the surface of thalassaemic RBC are distributed in an uneven manner and are less abundant than those present on normal RBC surfaces.

We used three cytochemical methods in conjunction with transmission electron microscopy, in order to detect SA on membranes of intact RBC. One method is based on the production of a biotinyl derivative of SA which can be visualised by ferritin–avidin conjugates as described previously[5,6]. In addition, two other techniques were used, cationised ferritin (CF) (ref. 7) and ruthenium red[8], both of which interact with negatively charged sites on the membrane. The latter techniques were used because membrane-based sialyl residues are known to contribute most of the negative charge on the surface of the human RBC[9].

The results obtained from all three techniques are illustrated in Fig. 1. The distribution of the above membrane probes from representative thalassaemic RBC samples was compared with that of normal RBC. In the former cases, only cells with characteristic thalassaemic morphology[10,11] were evaluated. Both normal and thalassaemic RBC stained with ruthenium red, and a continuous layer of electron-dense material was observed. The deposit was much less dense, however, on the surface of thalassaemic RBC (Fig. 1*a*) than on normal cell membranes (Fig. 1*b*).

Unlike the ruthenium red stain, which results in an amorphous layer of reaction products, ferritin-based stains are characterised by a particulate marker of uniform size. The ferritin label can be quantitatively evaluated by enumeration and enables a finer resolution of the given surface site. Thus, quantification of cationised ferritin (CF) on micrographs of tangentially sectioned membrane (Figs 1*c* and *d*) revealed less attached CF particles per μ^2 on thalassaemic cells ($1{,}200 \pm 275\,\sigma$) than on normal RBC ($2{,}030 \pm 150\,\sigma$). In some instances the CF label on the former cell membranes was interrupted by unlabelled gaps (Fig 1*c*, arrows), in contrasts to the more even distribution of the label on normal RBC.

Additional details of the distribution of membrane SA were obtained by a more specific technique using ferritin–

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