# THE BILAYER STABILITY OF INNER MONOLAYER LIPIDS FROM THE HUMAN ERYTHROCYTE

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## 1. Introduction

An increase in the intracellular $Ca^{2+}$ concentration of human erythrocytes results in profound morphological changes and loss of plasma membrane in the form of small vesicles [1,2]. It is of interest to determine whether this behaviour could be related to a $Ca^{2+}$-induced instability in the bilayer structure of the membrane lipid. We have therefore examined the influence of $Ca^{2+}$ on model membrane systems with lipid compositions approximating those of the outer and inner monolayer of the erythrocyte membrane. We show that whereas the outer monolayer lipids are unaffected by $Ca^{2+}$, a large proportion of the inner monolayer lipids adopt the hexagonal ($H_{II}$) phase in the presence of $Ca^{2+}$. We suggest that this tendency of the inner monolayer to adopt non-bilayer configurations could be directly related to the fusion event involved in vesicle ejection following $Ca^{2+}$ influx.

## 2. Methods

Phospholipids were extracted from freeze dried erythrocyte ghosts [3]. The individual classes of phospholipid were isolated and purified using low pressure liquid chromatography. Silicic acid and carboxymethyl cellulose were used as stationary phases, and lipids were eluted with mixtures of chloroform and methanol. Purity was assessed by two-dimensional thin-layer chromatography [4] of ~50 μg phospholipid, followed by phosphorus determinations [5] on spots revealed by iodine vapour. The phosphatidylserine preparation was converted to the sodium salt [6] and the ratio of amino groups [7] to phosphorus was determined as 1.0. All the phospholipid preparations were 99+% pure with respect to phosphorus. The erythrocyte phosphatidylethanolamine underwent a bilayer to hexagonal ($H_{II}$) phase transition (as detected by $^{31}P$ NMR) between 5°C and 10°C, in agreement with an earlier report [8].

$^{31}P$ NMR was employed to study the polymorphic phase behaviour of the phospholipids [9,10] employing a Bruker WP 200 NMR spectrometer. Typically, 30 μmol phospholipid mixture representative of the outer or inner monolayer was taken to dryness under $N_2$ and kept under high vacuum for ~2 h prior to hydration. Chloroform mixtures of outer monolayer phospholipid consisted of 44 mol% phosphatidylcholine, 44 mol% sphingomyelin and 12 mol% phosphatidylethanolamine, whereas inner monolayer preparations contained 47 mol% phosphatidylethanolamine, 28 mol% phosphatidylserine, 15 mol% phosphatidylcholine and 10 mol% sphingomyelin [11]. Samples were hydrated in 0.6 ml 30% $D_2O$ containing 140 mM NaCl, 5 mM KCl, 10 mM sodium cacodylate and 2 mM EDTA, at pH 7.4.

## 3. Results

The molar ratio of cholesterol to phospholipid in the human erythrocyte membrane is ~1.0, however, the transbilayer distribution of cholesterol remains obscure. The observation that cholesterol is able to rapidly traverse the membrane [12] would suggest

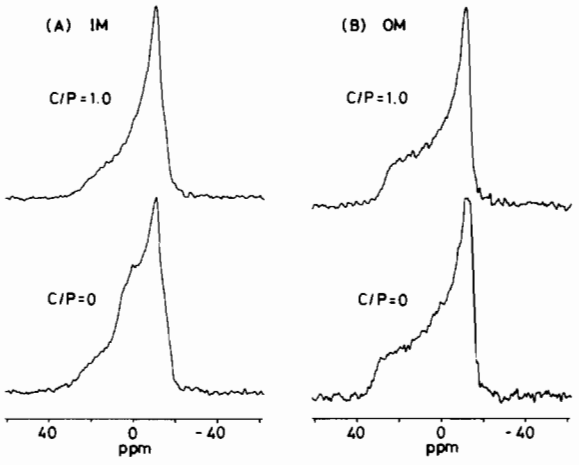


Fig.1. 81.0 MHz $^{31}$P NMR spectra of (A) inner monolayer (IM) and (B) outer monolayer (OM) in the absence and presence of equimolar cholesterol (cholesterol to phospholipid molar ratio of 0 and 1.0, respectively). Spectra were collected at 40°C from 2000 transients employing a 0.8 s repetition time, a 11 μs 90° pulse and 20 kHz sweepwidth.

that it experiences less asymmetry than phospholipids, which 'flip–flop' more slowly [13]. Consequently, the results presented here are for outer or inner monolayer phospholipids in the presence of equimolar cholesterol (unless otherwise stated). Similar results, however, were observed for samples prepared with 0.5 or 1.5 mol cholesterol/phospholipid.

Hydration of 'inner monolayer' phospholipids, in the absence of cholesterol, results in structures with a bilayer configuration. Figure 1A shows the $^{31}$P NMR spectra obtained from inner monolayer phospholipid at 40°C, in the absence and presence of equimolar cholesterol. The spectrum from the phospholipids alone has a line-shape characteristic for a bilayer, with an additional peak at 0 ppm, which indicates that a small component of the lipid experiences isotropic motion. Titration of cholesterol into the inner monolayer phospholipids results in a gradual decrease in size of this isotropic component, and in the presence of equimolar cholesterol, at 40°C, it is not detected. The equivalent spectra for the outer monolayer lipids are shown in fig.1B. Both phosphatidylcholine and sphingomyelin form bilayers in the presence and absence of cholesterol [14], which is consistent with the fact that equimolar cholesterol has little effect on the $^{31}$P NMR spectrum obtained from an aqueous dispersion of the outer monolayer lipids.

The spectra shown in fig.2 were obtained from inner monolayer phospholipid (equimolar cholesterol) in the presence of increasing concentrations of $Ca^{2+}$, at 37°C. A small hexagonal ($H_{II}$) component is induced by the addition of 0.5 mol $Ca^{2+}$/mol phosphatidyl-

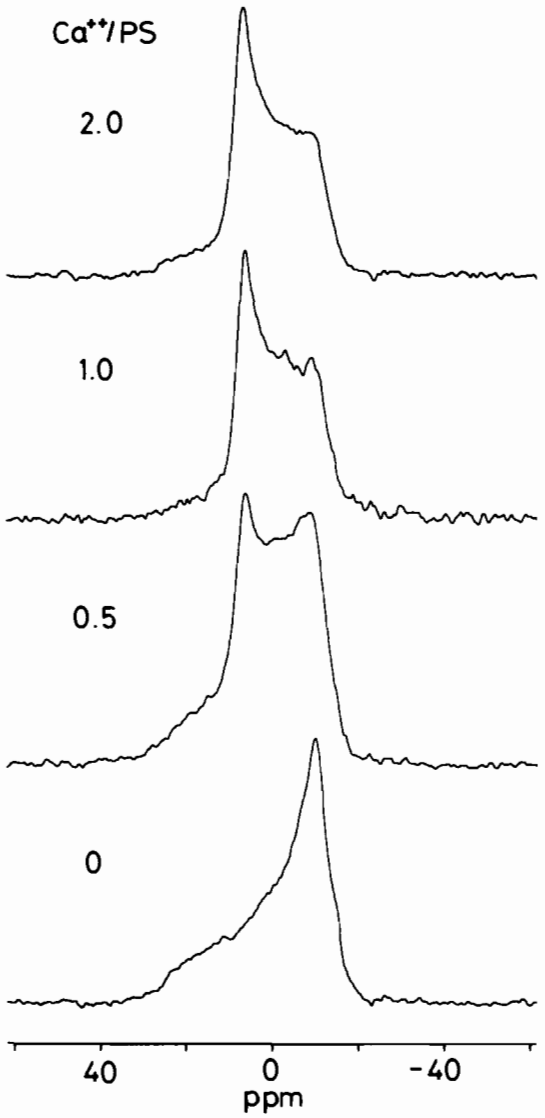


Fig.2. 81.0 MHz $^{31}$P NMR spectra of inner monolayer phospholipid, equimolar with respect to cholesterol, at 37°C. Spectra were collected from samples in the presence of $Ca^{2+}$/phosphatidylserine (PS) mole ratios of 0, 0.5, 1.0 and 2.0 under the conditions in the legend in fig.1.

serine. Raising the $Ca^{2+}$ concentration to a mole ratio of 1 induces 50–60% of the phospholipid to enter the hexagonal ($H_{II}$) phase, however, higher concentrations of $Ca^{2+}$ do not increase the size of this component further. $Mg^{2+}$ was less effective than $Ca^{2+}$, as it induced the hexagonal ($H_{II}$) phase to a maximum of 20–30% of the total phospholipid, also at an equimolar concentration with respect to phosphatidylserine.

The ability of $Ca^{2+}$ to trigger a bilayer–hexagonal ($H_{II}$) transition for a portion of the model 'inner monolayer' phospholipids is an intriguing, but not unexpected, result. In [15] we have shown that in bovine brain phosphatidylserine–egg phosphatidylethanolamine systems at 37°C, the presence of phosphatidylserine stabilizes the bilayer arrangement for the phosphatidylethanolamine, which would otherwise adopt the $H_{II}$ phase [8]. The addition of $Ca^{2+}$ to this system also triggers hexagonal ($H_{II}$) phase formation, which was attributed to a $Ca^{2+}$-induced lateral segregation of the phosphatidylserine component, allowing the phosphatidylethanolamine to revert to the $H_{II}$ phase. This $Ca^{2+}$-induced lateral segregation of phosphatidylserine is indicated by results obtained [16,17] in phosphatidylcholine–phosphatidylserine model systems.

It may be suggested that an analogous situation pertains to the model inner monolayer systems. The majority phospholipid, phosphatidylethanolamine prefers the hexagonal configuration above 14°C [8] indicating that in the absence of phosphatidylserine the neutral phospholipids may prefer a non-bilayer arrangement. That this is the case is indicated in fig.3A, which shows the $^{31}P$ NMR spectrum obtained for an inner monolayer system in the absence of phosphatidylserine. A major hexagonal ($H_{II}$) component is observable, in contrast to the situation when phosphatidylserine is present (fig.2), which establishes the bilayer stabilizing role of phosphatidylserine. An effective removal of this stabilization by $Ca^{2+}$ is then consistent with the equivalent $^{31}P$ NMR spectra of the inner monolayer in the presence of $Ca^{2+}$ (fig.2) and the phosphatidylserine depleted inner monolayer (fig.3A).

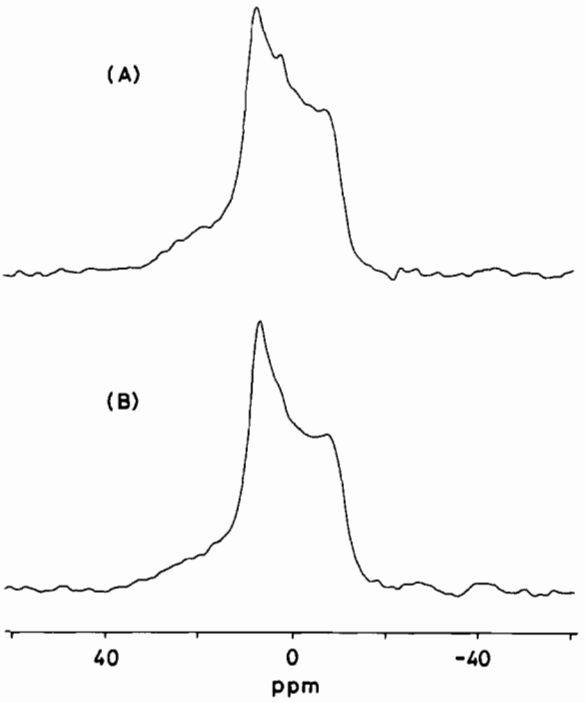


Fig.3. 81.0 MHz $^{31}P$ NMR spectra of inner monolayer phospholipid, equimolar with respect to cholesterol, at 37°C, reconstituted without phosphatidylserine. (A) is the spectrum obtained in the absence of $Ca^{2+}$ and (B) the same lipid composition in the presence of 12 mM $Ca^{2+}$. Spectra were collected under the conditions in the legend to fig.1.

## 4. Discussion

The results presented here clearly establish that the inner monolayer lipids in the human erythrocyte membrane will become unstable, in the sense that they will have a tendency to adopt the hexagonal ($H_{II}$) configuration, when intracellular levels of $Ca^{2+}$ are raised. The concept that inner and outer monolayers of a biological membrane may have differing affinities for the bilayer configuration due to different lipid compositions is certainly novel. It is of interest to first establish why such behaviour is observed and secondly to explore possible consequences. The ability of $Ca^{2+}$ to induce hexagonal ($H_{II}$) structure in model systems simulating the inner monolayer, is consistent with the work in [15], discussed in section 3. Lateral segregation of phosphatidylserine in the erythrocyte inner membrane model system would allow the residual lipid to (partially) adopt the hexagonal ($H_{II}$) phase. The phosphatidylserine may therefore be considered to stabilize the bilayer for this lipid mix-

ture and its removal, on complexing with $Ca^{2+}$, triggers $H_{II}$ phase formation.

A consequence of this inner monolayer instability could be involved with the fusion event that must be associated with the 'blebbing off' process observed for erythrocytes when intracellular levels of $Ca^{2+}$ are raised [2]. In particular, vesiculation is always preceded by morphological changes resulting in formation of echinocytes. The microvilli-like projections observed contain regions within which the inner monolayers are closely opposed (see electron micrographs in [1]) and appear to be the sites from which vesicles are pinched off. As we have indicated elsewhere there is strong evidence that membrane fusion proceeds via intermediate 'inverted cylinder' ($H_{II}$ configuration) or inverted micellar lipid configurations [18], structures which the inner monolayer lipids are not averse to assuming. Consequently we suggest that when regions of inner monolayer lipid, destabilized by the lateral segregation of phosphatidylserine, are able to make contact non-bilayer intermediates can form resulting in membrane fusion vital to subsequent ejection of a vesicle.

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