^{31}P NMR STUDIES OF THE EFFECT OF METHEMOGLOBIN ON ERYTHROCYTE AND MODEL MEMBRANES

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I. INTRODUCTION

The importance of protein-lipid interactions [1] in modulating biological activity is becoming increasingly recognized. Among the many facets of this broad area of study, the interaction of different forms of hemoglobin with artificial lipid membranes [2] has been reported to influence both structure and dynamics of membranes [3,4]. In the light of the above studies we have used ^{31}P NMR to investigate whether methemoglobin alters the phase behaviour or structure of erythrocyte membranes and model membranes. This could be of importance in diseases such as malaria where the intracellular parasites degrade hemoglobin and red cell lysis occurs in the process of parasite propagation [5,6].

II. MATERIALS AND METHODS

NMR spectra were obtained on a Bruker CXP-300 spectrometer using phase-cycled Hahn echoes [7]. Dry lipid samples (100-200 mg) were hydrated with 10 mM Tris HCl, with or without hemoglobin or methemoglobin, vortexed, and freeze-thawed.

III. RESULTS

Membranes of PC:PE

Mixtures of egg phosphatidyl-choline:egg phosphatidylethanolamine, PC:PE (2:1), were rehydrated with buffer or with methemoglobin in a 2:1 (w/w) protein/lipid ratio. These mixtures both showed doubling of peaks in the phospholipid powder pattern originating from the spectra of the individual lipid components. The widths of the phospholipid powder patterns were identical with or without protein (48 ppm) (Figure 1a and 1b), at 27 and 40°C.

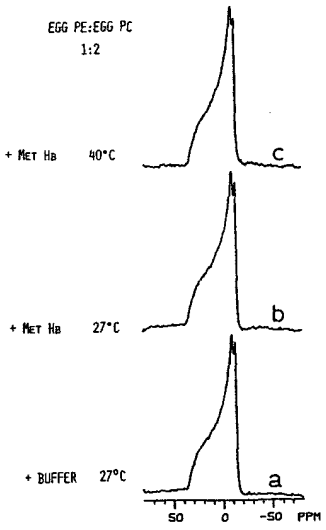

Fig. 1a ^{31}P NMR spectra at 121.47 MHz of Egg PE:Egg PC mixture (720 scans, 5 μs 90° pulse, 1 s recycle delay).

The spectra of 1:1 (w/w) mixtures of dimyristoyl PE (DMPE) and dimyristoyl PC (DMPC) show bilayer patterns between 10 and 50°C. The spectral widths at half-height, total spectral widths and second moments of the spectra all indicate an abrupt change between 28°C and 38°C (Figure 2a). Differential scanning calorimetry on these mixtures showed a peak at 40°C (Figure 2b). The narrowing of the NMR spectra (midpoint 33°C) is thought to arise from axial rotation of the phospholipid which occurs well below the acyl chain phase transition (at 40°C). None of these properties was perturbed by methemoglobin.

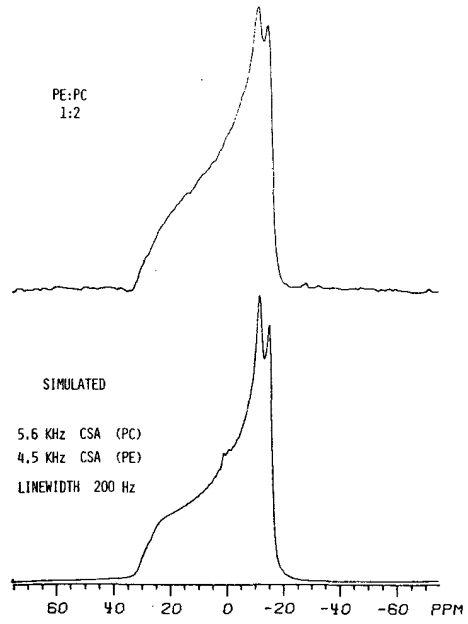

Fig. 1b Lower Trace: Simulated ^{31}P NMR spectrum of a PE:PC (1:2) dispersion, chemical shift anisotropies 5.6 kHz (46 ppm; PC) and 4.5 kHz (37 ppm; PE), 200 Hz linewidth of the 90° orientation, P_2 (cos θ) angular dependence of the linewidth, Lorentzian lineshape for individual resonances. The isotropic peak of PC was chemically shifted 1 ppm from that of PE.

Membranes of Egg PE:

Studies on egg PE were performed to determine whether protein perturbed the transition from bilayer to hexagonal phase occurring between 35°C and 45°C. We found that methemoglobin and hematin raise the midpoint of the transition (Figures 3,4). Raising the temperature from 25°C to 50°C in presence of methemoglobin caused the appearance of an isotropic peak which did not disappear at 30°C. Similar effects were observed with native hemoglobin, as well as heat-treated methemoglobin and hematin (Figure 5). All these effects were reversed upon freezing and thawing the samples.

Membranes of DLPE:

Although methemoglobin affects the bilayer to hexagonal phase transition of PE it does not perturb the gel-liquid crystal transition of dilauroyl PE (DLPE) at 31°C. Relaxation measurements do however show interaction between DLPE and methemoglobin. The T_2 values at 40°C show a decrease from 1.5 msec to 1.0 msec (90° orientation in the powder pattern) in the presence of methemoglobin (2:3

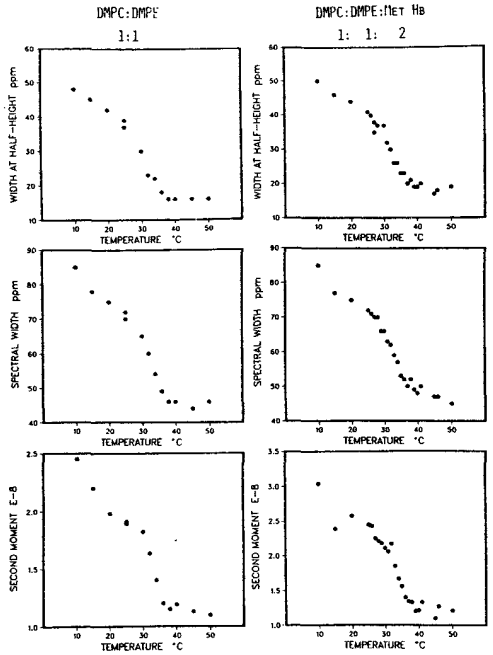

Fig. 2a Temperature dependence of the linewidth at half-height, the spectral width of the powder pattern and the second moment of the ^{1}H-^{31}P dipolar coupled ^{31}P NMR spectrum (121.47 MHz) of DMPC:DMPE (1:1) mixtures (left column) and DMPC:DMPE:MetHb (1:1:2) mixtures (right column).

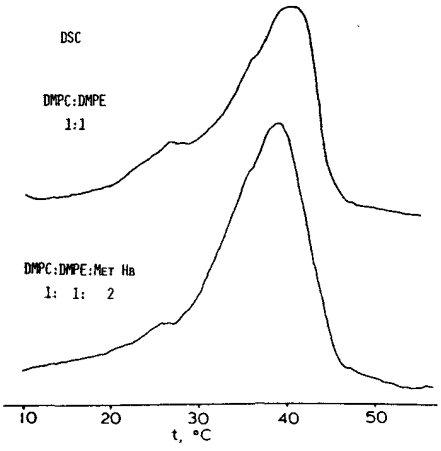

Fig. 2b Differential scanning calorimetry scans of dispersions of DMPC:DMPE 1:1 in 10 mM Tris HCl buffer (pH 7.4) (upper trace) and dispersions of DMPC:DMPE:MetHb 1:1:2 in 10 mM Tris HCl buffer (pH 7.4) (lower trace).

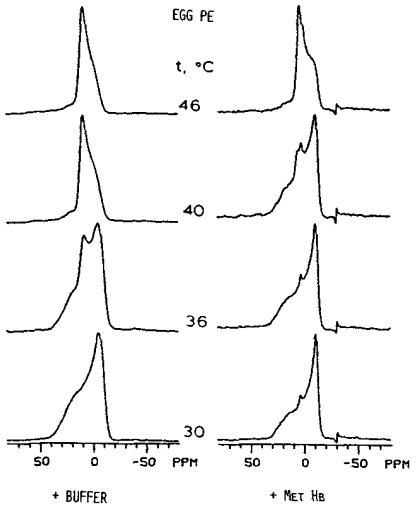

Fig. 3 Temperature dependence of ^{31}P NMR spectra at 121.47 MHz of egg-PE (7200 scans, 5 μsec 90° pulse, 1 s recycle delay).

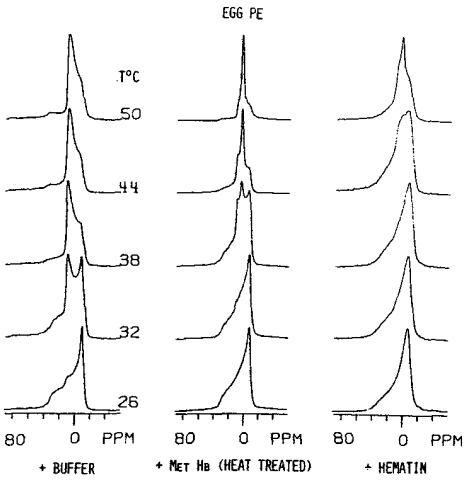

Fig. 5 ^{31}P NMR spectra at 121.47 MHz of egg phosphatidylethanolamine observed as a function of temperature. Other conditions are as described in Figure 3.

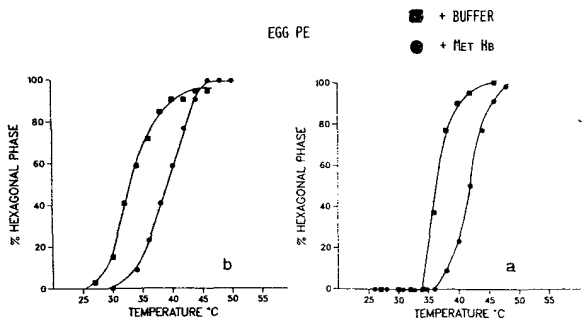

Fig. 4 Percentage hexagonal phase lipid observed as a function of temperature in dispersions of egg PE in buffer or in the presence of methemoglobin. Data from two individual experiments on different samples of egg PE are shown in a and b.

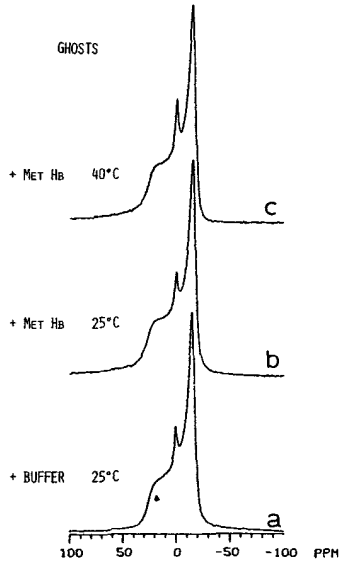

Fig. 6 ^{31}P NMR spectra at 121.47 MHz of hemoglobin - free erythrocyte ghosts (^1H-decoupled, 6,000 scans, 5 μsec 90° pulse, 5 second recycle delay).

w/w) in ^1H-coupled spectra. Similarly, the T_1 values decrease from 0.8 sec to 0.3 sec in the presence of methemoglobin.

Erythrocyte ghosts

Erythrocyte ghosts (500 mg) dispersed in buffer, or in methemoglobin solution (260 mg), show a typical bilayer pattern 48 ppm in width (Figure 6). Extracted lipids also yield a bilayer pattern. There is no visible perturbation of the bilayer phase of the membrane lipids at 25° or 40°C in the presence of methemoglobin (Figure 7).

IV. DISCUSSION

Chupin et al. [4] have reported interaction of methemoglobin with model membranes of egg PC and egg PC:egg PE (1:1 and 2:1). They found that methemoglobin added to dispersions of these lipids at 40°C over a period of time led to the appearance of an isotropic peak indicating polymorphic

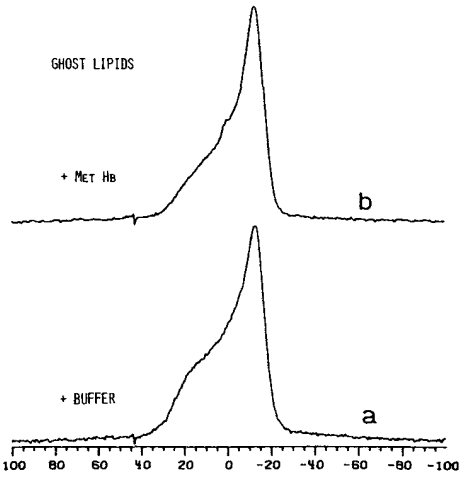

Fig. 7 ^{31}P NMR spectra of lipids extracted from hemoglobin-free ghosts. Conditions are those shown in Figure 6.

rearrangements in the liposome under the action of methemoglobin. The time dependence (a few hours) of the phenomenon was concluded to result from the time required for methemoglobin to distribute to all the bilayers in the liposome.

Our results show that egg PC:egg PE liposomes are stable in the presence of MetHb at 40°C. In our protocol all bilayers are exposed to protein immediately upon hydration of the sample since the dry lipid is hydrated with a solution containing metHb. We obtain identical spectra of egg-PE dispersion whether the buffered methemoglobin solution is simply added to the dry lipid and subject to Vortex mixing or multiple freeze-thaw cycles are performed to insure penetration of the protein to all the bilayers in the liposome.

We have demonstrated the appearance of an isotropic peak in egg-PE samples which were heated to 50°C in the presence of metHb, heat-treated metHb, hemoglobin and hematin. This was not observed in egg PE dispersions alone. The temperature of the bilayer to hexagonal phase transition in egg PE is raised by 5°C in the presence of metHb and heat-treated metHb. The presence of hematin in egg PE dispersions also raises this temperature. In all these samples, the isotropic component observed at 50°C remained present when the sample was cooled to 30°C. In all cases freeze-thawing the sample gave rise to spectra characteristics of pure lipid bilayers at 30°C. Thus, it would appear that the heat treatment causes no permanent change in the ability of the lipids in the system to form bilayers. An explanation consistent with all the above results is that methemoglobin stabilizes the bilayer. At temperatures where hexagonal phase is formed in pure lipid dispersions the presence of metHb (or hematin) could cause formation of vesicles giving rise to isotropic peaks, as is the case with glycophorin [8].

V. CONCLUSIONS

Using ^{31}P NMR we have shown that hemoglobin-free erythrocyte ghosts and total lipid extracts of these ghosts form stable bilayers in the presence of methemoglobin at physiological temperature. Model membranes of egg PC:egg PE as well as membranes made of DMPC:DMPE show chemical shift anisotropy patterns characteristic of bilayers. Doubling of the main peak of the bilayer pattern is observed in both cases. Differential scanning calorimetry of the samples containing DMPC:DMPE show a major endothermic transition at 40°C. The peak doubling observed in the ^{31}P NMR spectra is thus attributed to different intrinsic CSA values for DMPE and DMPC within a single phase. The powder patterns of egg PC:egg PE as well as DMPC:DMPE and DLPE in the presence of methemoglobin solutions are characteristic of bilayers.

Dispersions of egg PE in buffer or in buffered solutions containing methemoglobin undergo bilayer to hexagonal phase transitions. The presence of methemoglobin exerts a stabilizing effect on the bilayer as monitored by a delay in transition to the hexagonal phase of 5°C. Heating samples of egg PE containing methemoglobin to 50°C causes the appearance of "isotropic" peaks in the bilayer spectra observed at 30°C. This effect is not specific to

methemoglobin as it has been observed with hemoglobin, heat-treated metHb and hematin as well. Freeze-thawing the samples causes the spectra observed at 30°C to revert to bilayer type patterns in all cases.

The above results which demonstrate no disruptive effect of metHb on lipid structures of red cell ghosts or on model membranes at physiological temperatures are consistent with the reported observation that erythrocytes from a patient with methemglobinemia survived normally in both a normal recipient as well as the subject's own circulation [9]. The presence of methemoglobin did not increase the spontaneous hemolysis of human erythrocytes *in vitro* [9]. Methemoglobin *per se*, is thought to have little deleterious effect on the integrity of human erythrocytes [9]. Furthermore, we have shown that methemoglobin exerts a stabilizing effect on lipids which undergo bilayer to hexagonal phase transitions, and leads to the presence of an isotropic phase at high temperatures. The latter could be the result of formation of small vesicles of lipid as observed with glycophorin and PE [8]. We have also shown addition of metHb to lipid bilayers of DLPE causes a decrease in T_2 of ^{31}P, reflecting lipid-protein interaction at the lipid headgroup but with no concomitant change in the orientation of headgroup (as reflected in the CSA) and no change in the temperature of the gel-liquid crystal phase transition.

The above studies lead us to postulate that the autoxidative breakdown of polyunsaturated lipids, which is thought to be responsible for cell lysis, results from the autoxidation of HbO_2 leading to formation MetHb with the release of superoxide. The $O_2^{\cdot}$ radicals can give rise to other oxidants such as H_2O_2 and hydroxyl ions ($OH^{\cdot}$). If the defense mechanisms of the erythrocytes against these oxidants prove inadequate, irreversible damage to the cell membrane may occur. Using electron spin resonance (ESR) spin-trapping methods [10] we are currently investigating the relative importance of HbO_2, MetHb and haematin in the generation of free radicals thought to be responsible for the oxidtion of unsaturated fatty acids. This should give a better understanding of the process of cell lysis process in diseases such as malaria.

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