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HUMAN ERYTHROCYTE MEMBRANES ARE FLUID DOWN TO −5°C

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This first observation of the deuterium nuclear magnetic resonance (^{2}H-NMR) spectrum of phospholipid molecules incorporated into intact human erythrocyte ghosts shows that the liquid crystalline phase is stable down to a temperature of −5°C. The quality of the ^{3}H-NMR spectra indicate that it is now possible to carry out clinical studies of erythrocyte membranes using the techniques employed in this study.

In the last few years deuterium nuclear magnetic resonance (^{2}H-NMR) has been widely applied to the study of lipid dynamics and structure within the membrane lipidic bilayer. The orientational order associated with membrane fluidity has been measured in a non-perturbative manner with this technique in model membranes [1–4], and more recently in biological membranes [5–10] where deuterated acyl chains were incorporated into the lipids of the membranes by growing bacteria on media supplemented with deuterated fatty acids. Further results have been obtained on phospholipid-protein reconstituents [11–13].

The erythrocyte membrane is probably the most widely studied biological membrane. Moreover, it is frequently used to study the correlations between diseases and structural modifications occurring in the erythrocyte [14–16]. For these reasons the application of ^{2}H-NMR to the erythrocyte membrane should be particularly fruitful for the understanding of the structure of membranes in higher animals. At the same time it might become a valuable empirical tool to identify structural abnormalities in diseased individuals. Thus far only one study has been reported in which ^{2}H-NMR has been applied to the erythrocyte membrane. The ^{2}H-NMR spectra were obtained from a perdeuterated palmitic acid probe which had been previously incorporated into the erythrocyte membrane by incubation [17]. Fatty acids are an 'unnatural' probe and are known to produce structural modifications in membranes. Moreover to minimize these unwanted effects the authors had to use low fatty acid concentration which required the lyophilization of the membranes and the addition of small amounts of water before taking the ^{2}H-NMR spectra in order to obtain an adequate concentration of ^{2}H to provide a good signal/noise ratio.

In this paper we report the first ^{2}H-NMR study of phospholipid molecules incorporated in human erythrocyte membranes. The high level of incorporation of dipalmitoylphosphatidylcholine with perdeuterated acyl chains (DPPC-d_{62}) obtained by means of the phosphatidylcholine (PC) transfer protein from bovine liver [18,19] allowed the study of intact erythrocyte ghosts without lyophilization. The spectra show for the first time the possible existence of a transition from the characteristic liquid crystalline shape to a gel type structure

below −5°C. At the same time the liquid crystalline phase above −5°C shows a high degree of stability without any evidence of phase separations due to formations of cholesterol rich and cholesterol poor regions [20]. The presence of a phase transition at 20°C as suggested by some authors [21–25] is not observed in our study, which confirms the conclusions reached in the previous report [17] using a perdeuterated palmitic acid probe.

Ghosts were prepared from human erythrocytes according to the method of Dodge [26]. Vesicles of DPPC-d_{62} were prepared by sonication of a suspension of 114 mg of DPPC-d_{62}, 50 mg of cholesterol and a trace of egg [^{14}C]PC in 20 ml 7 mM phosphate buffer (pH 7.4). Sonication for 30 min at room temperature was followed by another 30 min at 45°C at a power setting of 70–80 W. Before use the vesicles were centrifuged at 30000 × g for 25 min to sediment the remaining multilamellar structures; the supernatant was used and contained about 100 mg of DPPC-d_{62} (recovery of 90%).

Perdeuterated DPPC was introduced into the erythrocyte membrane by incubation of the vesicles (50 mg of DPPC-d_{62}) and ghosts (40 mg of PC) with 100 μg of PC-transfer protein in a total volume of 75 ml of the hypotonic phosphate buffer containing 0.1% bovine serum albumin [30]. After 2 h of incubation at 37°C the ghosts were sedimented at 30000 × g for 20 min. Upon resuspension the ghosts were reincubated for 4 h at 37°C with another 50 mg of vesicle DPPC-d_{62} and 200 μg of transfer protein to increase the level of DPPC-d_{62} in the membrane. After washing the ghosts with hypotonic buffer twice, the amount of DPPC-d_{62} incorporated in the ghosts was calculated to be 50% of the total PC. This calculation, based on the hypothesis that egg [^{14}C]PC and DPPC-d_{62} have the same behaviour in the exchange process, was confirmed by the ^{2}H-NMR signal intensity. Spectra were taken from 1 ml of ghost pellet (spun down at 100000 × g) containing about 4–5 mg of DPPC-d_{62}.

^{2}H-NMR spectra were obtained by using the quadrupolar echo technique [27,28] on a Bruker SXP 4-100 spectrometer operating at 37.18 MHz. Details of the methods and of the moment analysis are given elsewhere [1,3].

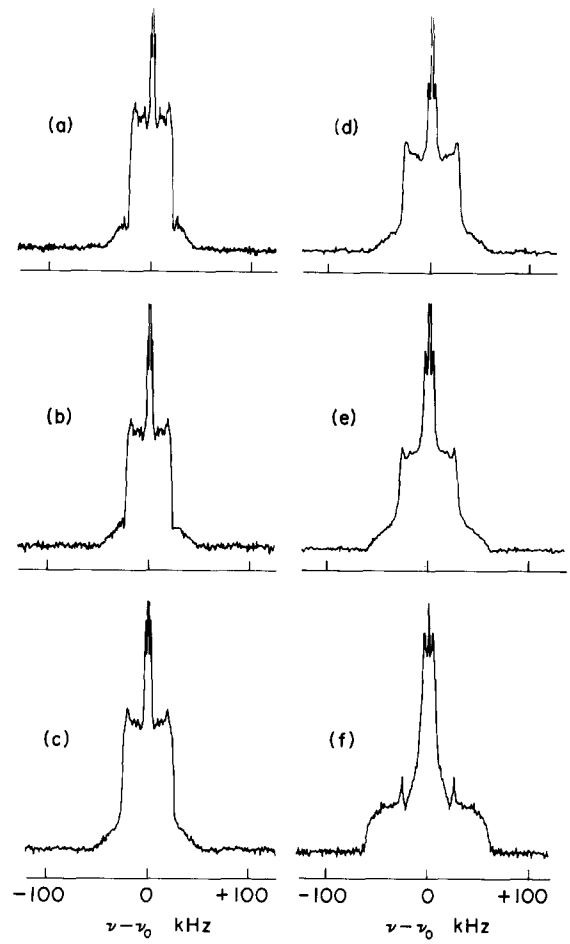

Fig. 1. The ^{2}H-NMR spectra at 37.18 MHz of DPPC-d_{62} incorporated in human erythrocyte ghosts at different temperatures. Accumulation rate was at 4 scans/s. (a) T=45°C, 80000 scans; (b) T=35°C, 100000 scans; (c) T=25°C, 154000 scans; (d) T=5°C, 65000 scans; (e) T=−5°C, 150000 scans; (f) T=−15°C, 160000 scans.

Some of the ^{2}H-NMR spectra of DPPC-d_{62} in the erythrocyte membranes at different temperatures are shown in Fig. 1. A line shape which is characteristic of the liquid crystalline phase is evident at all temperatures down to 5°C. The spectra at $T \geqslant 5$°C are very similar to those obtained in the liquid crystalline phase of pure DPPC-d_{62} [3], except for the much larger average quadrupolar splitting. In fact the frequency separation of the edges in pure DPPC-d_{62} at 36°C is about 30 kHz, which is to be compared to a value of about 45 kHz for DPPC-d_{62} in the erythrocyte membranes

at $T = 35°C$. Measurements were made on different days under conditions of increasing and decreasing temperature. No dependence on thermal history was observed.

At all temperatures investigated from 45°C down to 5°C there is no evidence of a liquid crystal to gel phase transition or a coexistence of phases as hypothesized by some authors [20–24]. The microscopic structural change, to which the transition near 20°C has been ascribed by the authors of a recent positronium study [25], must, therefore, take place in the polar head group region of the membrane.

The progressive broadening of the liquid crystalline spectrum when the temperature is lowered leads to a maximum separation of the edges of about 60 kHz at 5°C. This corresponds to an order parameter $S \simeq 0.5$ which is the value predicted for DPPC rotating about its main axis perpendicular to the bilayer surface in the all-*trans* state. More than half the deuterons contribute to the spectral feature corresponding to $S \simeq 0.5$. Thus the erythrocyte membrane is the only one studied thus far in which the hydrocarbon chains exist almost in an all-*trans* conformation without having undergone a phase transition as the temperature was decreased.

At temperatures $-5 > T > -15°C$ a distinctive change in the lineshape is observed. The spectrum broadens to 120 kHz with a substantial decrease of the area under the edges. Only a small remnant of the edges of the −5°C spectrum can still be observed in the −15°C spectrum. The line-shapes of the low temperature phase are similar to those found for DPPC in the gel state at similar temperatures [3]. The change in lineshape is accompanied by large changes in the moments of the lineshape shown plotted versus temperature in Fig. 2.

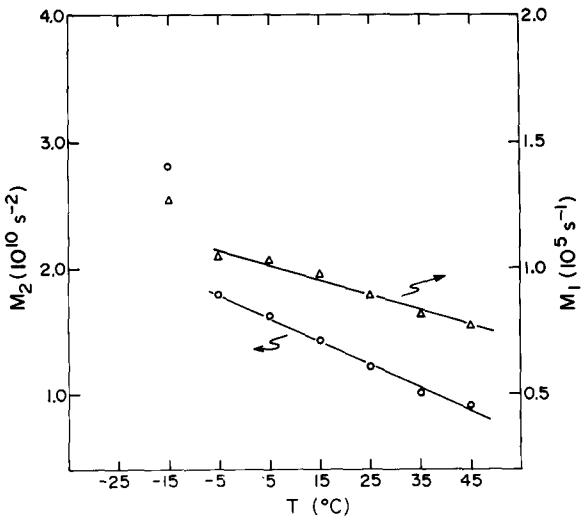

Fig. 2. The temperature dependence of the first (M_1) and second (M_2) moments of the spectra of DPPC-d_{62} incorporated in human erythrocyte membranes.

It seems clear from these result that the properties of the erythrocyte membrane are quite different from those of bacterial membranes previously investigated [6–10]. Though there is some similarity to *Acholeplasma laidlawii* having large concentrations of cholesterol [8], the acyl chains of the lipids of the erythrocytes show a higher degree of order than those of bacteria at corresponding temperatures [6–10]. In addition, the liquid crystalline phase of the *A. laidlawii* membrane where approx. 40 mol% of the lipid was cholesterol [8] is stable only down to about 20°C while that of the erythrocyte, where about 50% of the lipid is cholesterol, is stable down to −5°C. Though the addition of cholesterol to pure phospholipid systems usually gives rise to increased orientational order, the fluidity of the system, as determined from measurements of the lateral diffusion constant, is not necessarily decreased [29]. It is likely, therefore, that the translational motions of phospholipid molecules in the erythrocyte membrane remain fluid-like down to −5°C. A direct measurement of the temperature dependence of the lateral diffusion constant of phospholipid molecules in erythrocyte membranes would be very interesting.

Finally it is worth remarking that with this experiment it has been demonstrated that ^{2}H-NMR can now be applied also to the study of the erythrocyte membranes of abnormal erythrocytes. Previously, the low sensitivity of NMR and the difficulty of incorporating a sufficient amount of a deuterated 'natural' probe had inhibited the initiation of such a research program, due to the large amount of blood necessary for the ^{2}H-NMR signal to be detectable. With the use of PC transfer protein it is possible to obtain spectra of ghosts with the quality reported in this paper by using only about 20 ml of fresh blood.

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