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BILAYER RIGIDITY OF THE ERYTHROCYTE MEMBRANE ^{2}H-NMR OF A PERDEUTERATED PALMITIC ACID PROBE

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Summary

Perdeuterated palmitic acid was intercalated into the human erythrocyte membrane and its motion studied by deuterium nuclear magnetic resonance (^{2}H-NMR). From analysis of temperature dependent changes in the ^{2}H-NMR spectra and from an analysis of derived moments we conclude that the acyl chains of the erythrocyte lipids do not exhibit a detectable phase transition.

The human erythrocyte membrane is probably the most extensively studied biological membrane. This is not only because erythrocyte membranes can be prepared in large quantities and in homogeneous form by simple hypotonic lysis procedures, but also because they represent a readily accessible tissue for studies of the molecular basis of human disease states with generalized membrane involvement [1—3]. There is some controversy, however, as to whether the lipids of the erythrocyte membrane undergo a phase transition. Viscosity measurements [4], X-ray diffraction [5], laser-Raman spectroscopy [6], ^{31}P nuclear magnetic resonance (^{31}P-NMR) [7] and fluorescence [8] have yielded contradictory and inconclusive results.

We have therefore used deuterium nuclear magnetic resonance (^{2}H-NMR) to study the erythrocyte membrane. This technique has given fruitful and unambiguous results in studies on model membranes [9,10] and recently these studies have been extended to biological membranes [11].

Erythrocyte membranes were prepared by standard methods [12]. 200 ml of erythrocyte membranes (2.9 mg protein/ml) in 10 mM Tris-Cl (pH 7.4) was incubated with 0.72 ml of 50 mM perdeuterated palmitic

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acid (Merck) in methanol for 15 min at room temperature. The suspension was then centrifuged in a Spinco Type 30 rotor at 30 000 rev./min for 30 min. The pellet was washed twice by resuspension in 10 mM Tris-Cl and recentrifugation. The washed pellet was resuspended in water and lyophilized. NMR spectra were obtained using approximately 0.5 g of lyophilized membrane hydrated with 1—2 ml H_2O. It has been shown from NMR studies on deuterated membranes of *Escherichia coli* that lyophilization does not alter the state of the lipid bilayer as determined by deuterium NMR (unpublished observations). A quadrupolar spin-echo technique which allows one to obtain undistorted spectra [13] was used for our measurements.

The ratio of deuterated palmitic acid probe to total erythrocyte membrane lipid was less than 5% to minimize structural modification of the membrane by the free fatty acid. We found that this level of palmitic acid did not alter the motion of the spin probe, 5-doxyl stearic acid, in the erythrocyte membrane as detected by electron spin resonance (data not shown).

Some typical ^{2}H-NMR spectra are shown in Fig. 1. The ^{2}H-NMR spectrum of the perdeuterated palmitic acid in the erythrocyte membrane results from a superposition of powder patterns. The features of the line shape are the same at the different temperatures and are somewhat typical

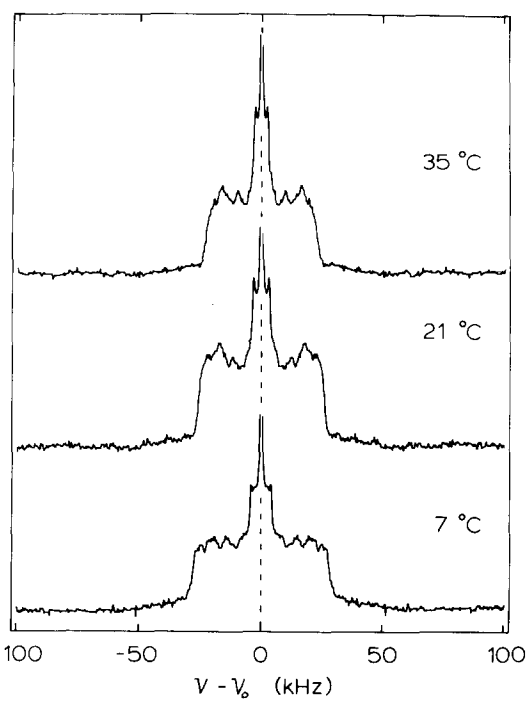

Fig.1. Three typical deuterium NMR spectra of perdeuterated palmitic acid incorporated into the human erythrocyte membrane. The spectra, from top to bottom, were taken at 35°C, 21°C, and 7°C respectively, at 34.4 MHz. The number of scans for the three spectra were 165 000, 178 000 and 129 000 respectively. The signals are recorded at the ^{2}H Larmor frequency, ν_0 = 34.4 MHz, thus the low field half of the symmetric spectrum is folded back and superimposed on the high field half. This increases the signal to noise ratio by a factor $\sqrt{2}$. These spectra have been plotted after reflection about ν_0 to render the visual analysis more immediate. For this reason the spectra show a false perfect symmetry.

of a liquid-crystalline state, even though the overall spectrum width decreases with increasing temperature.

The shape of the spectra is similar to that of the spectra of perdeuterated dipalmitoyl phosphatidylcholine in the liquid-crystalline phase [13], except that the erythrocyte spectra are systematically broader indicating a higher degree of rigidity, presumably due to the high cholesterol content. The spectrum also resembles that of *Acholeplasma laidlawii* membranes where the deuterated fatty acids were incorporated biosynthetically into the bacterium during growth [11]. The sharp edges of the spectra present over the whole temperature range of our measurements, as shown in the examples of Fig. 1, are quite typical of systems having a plateau in the plot of the order parameter versus the position on the chain [9,14]. The splitting between the edges varies from about 56 kHz at 2°C to about 40 kHz at 45°C and corresponds to an approximate change of the order parameter of the plateau from 0.45 to 0.32. These values can be compared to the liquid-crystalline plateau order parameters of 0.2 for *A. laidlawii* at 42°C [11] and 0.23 for dipalmitoyl phosphatidylcholine at 37°C (Davis, J.H., unpublished data).

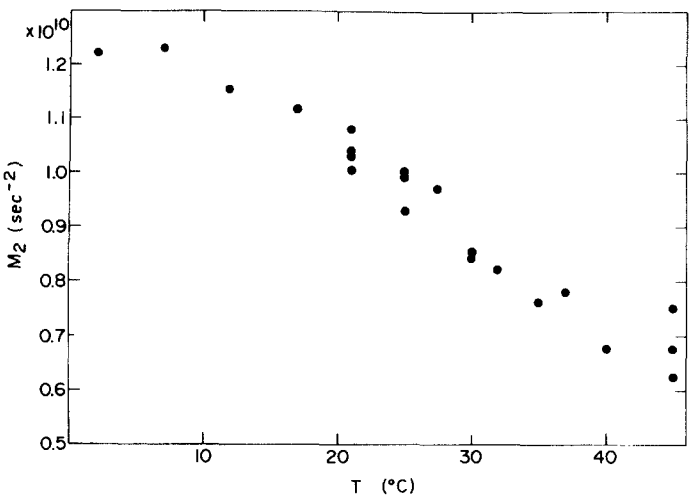

Fig.2. The second moment, M_2, of the deuterium NMR spectrum versus temperature. Some temperatures were repeated several times throughout the course of the measurements.

Due to the complexity of the spectrum envelope resulting from the superposition of powder pattern spectra for each position on the palmitic chain, it is not yet possible to relate the spectrum envelope to the order parameter dependence on position. An analysis of the spectrum in terms of moments*, however, can provide some important information. The second moment, M_2, of the spectrum shows a gradual decrease when plotted versus temperature (Fig. 2) which reflects the increased motion of the whole

*The moments are defined as $M_n = \int_{\nu_0}^{\infty} d\nu f(\nu - \nu_0)(\nu - \nu_0)^n / \int_{\nu_0}^{\infty} d\nu f(\nu - \nu_0)$ where $f(\nu - \nu_0)$ is the lineshape function, i.e., the spectrum as shown in Fig.1.

chain with increasing temperature. The change in M_2 between 2 and 45°C is somewhat less than that observed for dipalmitoyl phosphatidylcholine, *A. laidlawii* and *E. coli* (unpublished observations) suggesting a less distinct phase transition. This is confirmed by an analysis of M_2/M_1^2. For a line-shape whose analytical representation $f(\nu - \nu_0)$ does not change with temperature, the ratio of the second moment to the first moment squared, M_2/M_1^2, is independent of temperature. This is true even when the line width changes. In particular, for a single powder pattern spectrum it can be shown that $M_2/M_1^2 = 1.35$ (Bloom, M., Dahlquist, F.W. and Davis, J.H., unpublished). The value of M_2/M_1^2 for the erythrocyte membrane is 1.55 ± 0.04 throughout the temperature range. The higher value for this ratio is to be expected as the spectrum is not a single powder pattern, but rather a superposition of many powder patterns. The parameter

$$\Delta_2 = \{M_2/(1.35M_1^2)\} - 1$$

which is more sensitive to phase coexistence than M_2/M_1^2, is plotted versus temperature in Fig. 3. Throughout the entire temperature range Δ_2 is constant within experimental error and equal to 0.15 ± 0.03. This result can be compared to a variation of Δ_2 from about 0.085 at 36°C to about 0.340

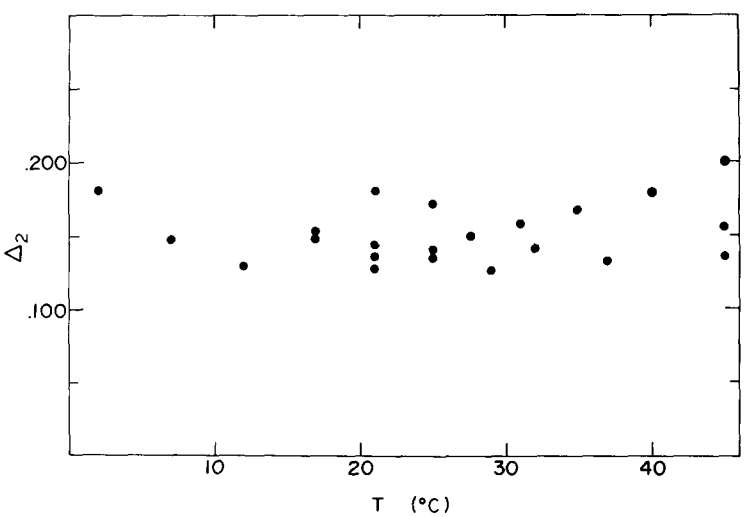

Fig.3. The parameter $\Delta_2 = M_2/1.35M_1^2 - 1$ versus temperature.

at 34.5°C for perdeuterated dipalmitoyl lecithin, a system that exhibits a sharp phase transition, and to a two fold variation over the broad phase transition exhibited by the membranes of *E. coli* and *A. laidlawii* (unpublished observations). The fact that Δ_2 does not change with temperature means not only that the line-shape does not change but also that there is no distinct phase transition from the liquid crystal to the gel state in agreement with the results of ^{31}P-NMR studies of normal erythrocyte ghosts [7]. In fact the coexistence of phases with different degrees of order of the acyl chains would cause an increase of Δ_2 even though each of the phases has a

powder pattern spectrum and, consequently, identical Δ_2 (Davis, J.H., unpublished data).

Lateral diffusion, e.g., in egg yolk lecithin [15], is still quite rapid at high cholesterol concentrations. Since deuterated fatty acids are not bulky probe molecules, this diffusion may insure that the fatty acids sample all lipid regions of the membrane. However, this has never been conclusively demonstrated. On the other hand it is conceivable that the diffusion rate of the palmitic acid label in the membrane is higher than that of the natural lipids. If diffusion is sufficiently fast to produce enough line narrowing for the spectra of two possibly coexisting phases to be averaged, the expected increase in Δ_2 might be eliminated. While it is not possible to identify or distinguish the coexistence of different environments of the fatty acid label from a single spectrum, the temperature dependence of the spectra indicates that all of the probe molecules are in environments which experience the same temperature variation. This lack of temperature variation of Δ_2 in erythrocyte membranes also tends to contradict the hypothesis of the coexistence of cholesterol rich and cholesterol poor regions. [7].

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