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A Comparative Electron Spin Resonance Study of the Erythrocyte Membrane in Myotonic Muscular Dystrophy†

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ABSTRACT : Stearic acid methyl ester spin-labels with the paramagnetic center located at the 5, 12, and 16 positions on the fatty acid alkyl chain have been used to study the erythrocyte membrane from patients with myotonic muscular dystrophy, a systemic disorder inherited as an autosomal dominant trait. It has been demonstrated that the physical state of the erythrocyte membrane from patients with this disease is different from that of normal controls. At all levels of penetration into the membrane probed by the spin-label, myotonic membranes were more fluid and less polar than control membranes. The overall average values of the order parameter, S, and the nitrogen isotropic coupling constant, a_N, obtained with the 5, 12, and 16 labels were respectively (0.65, 15.9 G), (0.59, 15.5 G), and (0.26, 13.9 G) in normal membranes while the parameters were respectively (0.61, 15.8 G), (0.58, 15.3 G), and (0.25, 13.8 G) for the three labels in myotonic erythrocytes. The fluidity difference between normal and myotonic membranes is most apparent near the surface of the membrane while the polarity difference is approximately constant at various depths within the membrane. These results support the concept of myotonic muscular dystrophy as a diffuse membrane disease.

Myotonic muscular dystrophy is a systemic disorder inherited as an autosomal dominant trait. Physiological investigations have localized the functional defect to muscle membranes (Lindsley and Curren, 1936; Denny-Brown and Nevin, 1941; Landau, 1952) although recent studies have suggested that muscle changes are secondary to neuropathic disturbances (Gallup and Dubowitz, 1973; McComas *et al.*, 1971). Biochemical data from our own laboratories have supported a more diffuse membrane involvement rather than a defect limited to the muscle or nerve. Endogenous membrane protein kinase was decreased in aged frozen erythrocyte ghosts as well as in freshly prepared nonfrozen ghosts (Roses and Appel, 1973, 1974). Similar alterations in membrane bound protein kinase activity have also been demonstrated in carefully controlled muscle membrane experiments (Roses and Appel, 1974b). These studies not only document the wide-spread membrane involvement in this disorder, but also support the usefulness of red blood cells as a readily available membrane source with which to assess the metabolic error.

Our more recent study employed electron spin resonance (esr) spectroscopy to substantiate the presence of a membrane defect in myotonic erythrocytes (Butterfield *et al.*, 1974). The spin-labeling technique is well documented (McConnell and McFarland, 1970; Jost *et al.*, 1971; Keith and Mehlhorn, 1972; Schreier-Muccillo and Smith, 1973) and has been particulary successful in understanding both model (Mukai *et al.*, 1972) and biological (Hubbell and McConnell, 1968, 1969a,b, 1971, Hubbell *et al.*, 1970; Kaplan *et al.*, 1973) membranes. The spectra of spin-labeled erythrocytes from patients with myotonic dystrophy were found to be recognizably different from those with normal erythrocytes. Using the methyl ester of stearic acid substituted with a nitroxide-containing oxazolidine ring at the 5 position (5-NMS)[1] we noted incorporation of the spin-label

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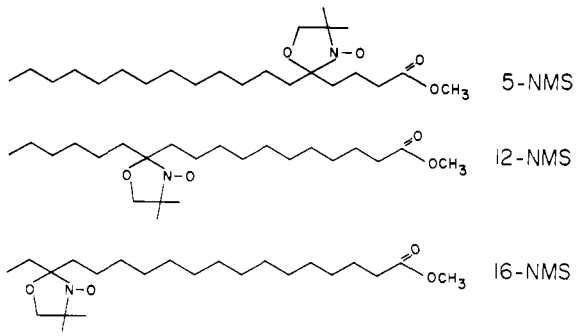
[1] Abbreviations used are: 5-NMS, 5-nitroxide methyl stearate; 12-NMS, 12-nitroxide methyl stearate; 16-NMS, 16-nitroxide methyl stearate.

in a less polar and more fluid region in myotonic erythrocytes compared to normal cells. The present study reinvestigates the effect of the methyl stearate substituted at the 5 position and extends our investigation to include the methyl stearates substituted at the 12 and 16 positions in order to determine whether the fluidity and polarity differences in myotonic membranes are present throughout the extent of the membrane. At all levels of penetration, the myotonic membrane is more fluid and less polar than the control membrane. However, the changes in both parameters were more marked near the surface rather than in the interior of the membrane.

Experimental Section

The present experiments involve chiefly the three spin-labels 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl methyl ester (5-nitroxide methyl stearate or 5-NMS), 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl methyl ester (12-nitroxide methyl stearate or 12-NMS), and 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl methyl ester (16-nitroxide methyl stearate or 16-NMS), all of which were obtained from Syva Associates, Palo Alto, Calif.



The erythrocytes used in this study were obtained from heparinized blood from patients with myotonic muscular dystrophy and from age matched controls (normal). Ratios of spectral parameters from normal and myotonic cells were made only on samples run on the same day. In comparison experiments involving 5-NMS, 7 normal and 9 myotonic patients were used, while for those experiments involving 12-NMS and 16-NMS, 15 normal and 8 myotonic patients and 10 normal and 11 myotonic patients, respectively, were used. In each experiment the intact erythrocytes were prepared by centrifuging the blood at 1570*g* for 10 min in the cold and by washing the cells twice with 0.15 M sodium chloride in 0.01 M Tris(hydroxymethyl)aminomethane-HCl buffer (Tris) (pH 7.4). The buffy coat was carefully removed (Redman, 1971) and the cells were resuspended in the isotonic Tris buffer at a hematocrit of 45–50%.

In order to spin-label the cells 0.25 ml of a 1 mM solution of the appropriate spin-label in $CHCl_3$ was measured into an aluminum foil-shielded test tube following which the chloroform was evaporated by a stream of nitrogen gas leaving a thin film of label; 0.5 ml of washed cells (hematocrit = 45–50%) was added to the test tube and the system incubated for 16 hr by gentle shaking in a 37° water bath in the dark. Recent experiments involving labeling of the cells for 10 min at room temperature show that an adequate amount of label is taken up in this short time. Once equilibrated for 10 min at room temperature no striking changes occur with time, indicating that either equilibrium of the label in the system has been attained in that time span or that, assuming that several label sites may exist, the different site spectra are very similar.

The esr measurements were performed at X-band (9.5 kMHz) on a standard Varian V-4502 12-in. system equipped with frequency and power monitoring devices. Saturation and modulation broadening were avoided by working at low microwave power and modulation amplitudes. The use of low spin-label concentrations (approximately 5×10^{-4} M) should minimize any line broadening due to spin–spin exchange providing no aggregation of label occurs in the membrane. For room temperature measurements the labeled cells are placed in a Varian V-4548 quartz aqueous sample cell (flat cell) in order to minimize dielectric loss. The previous work with the 5-NMS label (Butterfield *et al.*, 1974) involved temperature studies and employed capillaries for holding the cell solution. The flat cell yields better results and, although the two sets of 5-NMS data are essentially equivalent, we report in this paper only the flat cell results for all labels at room temperature. The esr spectra were typically recorded with a sweep range of 100 G and a scan time of 10 or 25 min.

In experiments to determine the origin of the "isotropic lines" in the 12-NMS spectrum, labeled red cells were spun on a desk-top centrifuge for approximately 10 min. The resulting supernatant was then run in the esr spectrometer while the pellet was washed two or three times with Tris buffer. After the washes the cells were spun, and the spectrum of the final supernatant was recorded. Part of the resulting "moist pellet" was taken up into a capillary tube, which was then placed in a normal quartz esr tube, and the spectrum was recorded. The rest of the "moist pellet" was resuspended in buffer at the original hematocrit and run in a flat cell within 10 min.

Results

The basic parameters measured in these experiments are the $T'_{\parallel}$ and $T'_{\perp}$ components of the motionally averaged nitrogen hyperfine tensor, the nitrogen isotropic coupling constant a_N, and the order parameter S. The rationale for the usefulness and applicability of the measurements is well established (Butterfield *et al.*, 1974; McConnell and McFarland, 1970; Jost *et al.*, 1971; Keith and Mehlhorn, 1972; Hubbell and McConnell, 1971). The order parameter S is a measure of local fluidity while the isotropic coupling constant a_N is related to the local polarity in which the paramagnetic center of the spin-label is found. S is calculated using the polarity correction factor employed by Hubbell and McConnell and the crystal T-tensor parameters of the oxazolidine label of cholestane studied by these authors (Hubbell and McConnell, 1971). The analysis of the data reported in this paper assumes that one may treat the observed spectra in terms of powder spectra. That is, any motion of the probe in the membrane is assumed to be rapid with respect to the anisotropy of the crystal parameters. This assumption of rapid motion may or may not be correct; the esr spectra of partially or fully immobilized radicals is not easily calculated although a better understanding is currently being developed (McCalley *et al.*, 1972; Kuznetsov *et al.*, 1971). However, while the physical interpretation of spectra may be ambiguous due to theoretical difficulties, nevertheless operational quantities may be defined and used to specify differences in spectra.

Spin-label spectra arising from all three spin-labels incorporated into erythrocyte membranes from myotonic patients are reproducibly and significantly different from con-

TABLE I: Magnetic Resonance Parameters Averaged Over All Experiments (Room Temperature).[a]

Label	$T'_{\parallel}$	$T'_{\perp}$	$\Delta T' = T'_{\parallel} - T'_{\perp}$	a_N[b]	S[c]	n
			Normal Erythrocytes			
5-NMS	28.2 (0.3)	9.78 (0.3)	18.4 (0.4)	15.9 (0.2)	0.65 (0.02)	7
12-NMS	26.2 (0.8)	10.1 (0.2)	16.1 (0.8)	15.5 (0.3)	0.59 (0.02)	15
16-NMS	18.1 (0.1)	11.8 (0.1)	6.3 (0.1)	13.9 (0.1)	0.26 (0.004)	10
			Myotonic Erythrocytes			
5-NMS	27.2 (0.4)	10.1 (0.2)	17.2 (0.5)	15.8 (0.1)	0.61 (0.02)	9
12-NMS	25.7 (0.7)	10.1 (0.1)	15.6 (0.8)	15.3 (0.2)	0.58 (0.02)	9
16-NMS	18.0 (0.1)	11.8 (0.1)	6.2 (0.2)	13.8 (0.1)	0.25 (0.01)	11

[a] The standard deviations are given in parentheses; n is the number of data points. [b] $a_N = {}^1/_3(T'_{\parallel} + 2T'_{\perp})$. [c] $S = [(T' - T'_{\perp})/(T_{\parallel} - T_{\perp})_{xl}][(a_N)_{xl}/a_N']$, where xl refers to single crystal parameters obtained in this case from the data of Hubbell and McConnell (1971) on the oxazolidine derivative of cholestane, and where $T'_{\perp}$ has been calculated from the apparent $2T'_{\perp}$ splitting employing the 1.6 G correction factor as shown in Figure 1.

TABLE II: Comparison of Spectral Parameters of Normal (N) and Myotonic (M) Erythrocytes.[a]

Label	$(\Delta T')_{\parallel}/(\Delta T')_M$[b]	$(a_N)_N/(a_N)_M$	$(S)_N/(S)_M$
5-NMS			
$\bar{X}$ (SD)	1.068 (0.031)	1.007 (0.012)	1.061 (0.029)
P	<0.005	<0.05	<0.0005
$n = 11$			
12-NMS			
$\bar{X}$ (SD)	1.025 (0.046)	1.005 (0.019)	1.019 (0.036)
P	<0.025	$0.1 < P < 0.15$	<0.025
$n = 19$			
16-NMS			
$\bar{X}$ (SD)	1.014 (0.017)	1.003 (0.004)	1.011 (0.018)
P	<0.005	<0.01	<0.025
$n = 15$			

[a] Mean, $\bar{X}$; standard deviation, SD; probability, P; n, number of data. [b] $\Delta T' = T'_{\parallel} - T'_{\perp}$.

trol experiments with normal volunteers. Table I gives the overall averages of the data for control and myotonic spectra while Table II gives the ratio of parameters (normal to myotonic) for each of the three labels. Representative spectra are illustrated in Figure 1.

The difference in the spectra of all three spin-labels in normal and myotonic erythrocytes is statistically significant. In Table II, the mean ratio of the spectral parameters $\Delta T'$, a_N, and S are calculated. Using the Student's t-test, the significance of each of these mean ratios has been calculated using the null hypothesis that the membranes are not different and the ratio in each case is unity.

Figure 1 illustrates typical spectra for the three ester labels used in the study. With the exception of the 12-3 ester (5-NMS) all of the spectra show the presence of a sharp set of lines in addition to the broad background powder spectrum. These lines have been referred to as "liquid lines" and are thought to be due to the label in free solution. The isotropic lines, most obvious in the case of the 12-NMS, show a hyperfine coupling of approximately 15.6 G. In order to demonstrate that these lines do result from radicals in free solution a sample of cells labeled with 12-NMS was centrifuged at 1500g and the supernatant removed leaving a moist pellet of cells. The removed supernatant shows only the three isotropic lines (with a coupling constant of 15.6 G) while the separate spectrum of the moist pellet gives a spectrum where the presence of these lines is at best marginal. Resuspension of the moist pellet in isotonic Tris buffer results again in the reappearance of the isotropic lines within minutes, thus indicating rapid exchange of the 12-NMS label between the cell membrane and the external aqueous medium. A similar experiment using 5-NMS label

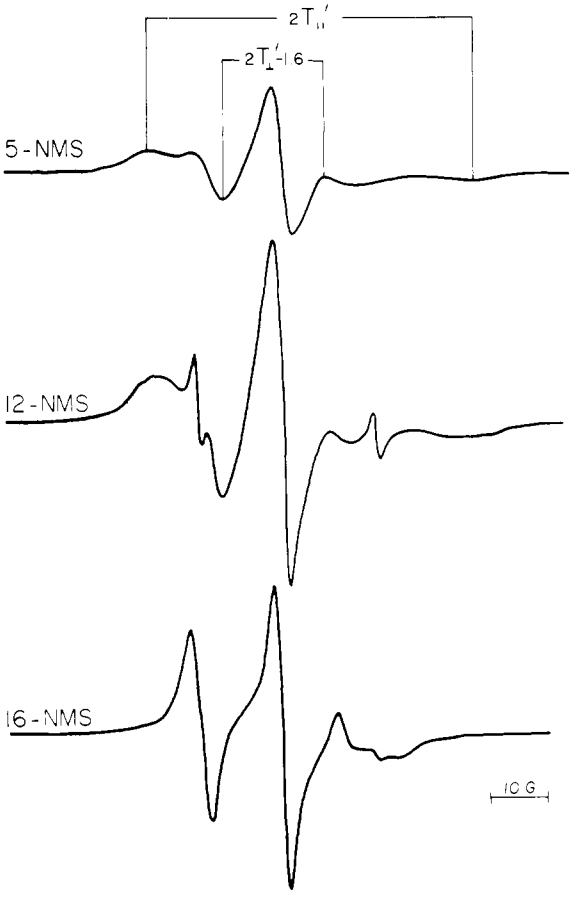


FIGURE 1: Typical spectra of erythrocytes spin-labeled with 5-NMS, 12-NMS, and 16-NMS. The extrema used in the calculation of the T-tensor components are indicated for the case of the 5-NMS spectrum.

shows that the entirety of the spectrum is due to the radical incorporated in the membrane.

In general, attempts to dissolve the label directly into the buffer solution in the absence of erythrocyte cells produce an extremely low signal-to-noise spectrum. Such an experiment involving the 5-10 label (the label showing the most apparent liquid line structure in the membrane systems) barely shows the presence of these lines, with a coupling constant of 15.6 G, confirming the notion that the "liquid lines" do result from the spin-label sampling essentially a polar water environment. Since it is to be expected that all three labels would show the same coupling constant in water, it is interesting that the liquid lines coupling constant of 15.6 G is actually smaller than the measured coupling constant for 5-NMS in the membrane. While the measure of a_N in the membrane may suffer from theoretical difficulties in the interpretation of spectra of systems such as these, this effect could very well be real and be due to the highly dipolar nature of the erythrocyte membrane leading to an environment for the paramagnetic center of the 5-NMS label which is actually more polar than that experienced in the aqueous solution.

Discussion

Our previous experiments using 5-NMS were interpreted as indicating that the spin-label was located in a less polar and somewhat more fluid region in myotonic membranes compared to normal erythrocytes. These studies have been confirmed using an improved technique of measurement and, in addition, these differences are also present using 12-NMS and 16-NMS labels. For all three labels the isotropic coupling constant is larger in the normal membrane than it is in the myotonic membrane. The order parameter, S, is also always larger in the normal membrane using all three labels.

The general model employed in the interpretation of labels incorporated into either model or biological membranes presumes that the molecular axis of the stearic ester is aligned essentially perpendicular to the membrane surface (McConnell and McFarland, 1970; Jost *et al.*, 1971; Keith and Mehlhorn, 1972; Schreier-Muccillo and Smith, 1973). As the oxazolidine ring is attached further down the molecular chain one expects the nitroxide probe to sample an environment in the membrane closer to the middle of the bilayer. The interior of the membrane is less polar and more fluid in both normal and myotonic membranes, as demonstrated by the changes in a_N and S as one goes from the 5-NMS to the 12-NMS to the 16-NMS labels (Table I). While in all cases these parameters are somewhat larger in the control membranes, the differences between normal and myotonics decrease steadily as the nitroxide group penetrates further into the membrane (see Table II). Although the differences are small, they are statistically significant and indicate that at all levels of penetration the myotonic membrane is more fluid and less polar than the control. These results suggest that the effect which causes the local environment near the label's nitroxide group to be more fluid and less polar in myotonic membranes is located near the surface of the membrane. It must be noted, however, that since the fluidity increases as one moves toward the interior of both control and myotonic membranes, it is quite conceivable that a given perturbation could be accomodated more easily further from the surface and therefore give rise to less of an experimental difference for probes located in regions of greater accomodation.

There are several possible explanations for the observed fluidity and polarity differences between control and myotonic erythrocytes. The behavior of these systems suggest that the spin-labels in the myotonic membrane may find themselves by some physical effect slightly further away or somewhat more excluded from the membrane surface so that their environment would be slightly less polar and would also demonstrate a somewhat higher degree of motional freedom. Kaplan *et al.* (1973) suggested that the spin-label (similar to those used here) was located in the outer surface of the lipid bilayer in experiments using human erythrocytes, human lymphocytes, and cultured mouse L-cells. These workers found differences between the S values in the different types of cells to be of the order of 0.02-0.03. They suggested that these fluidity differences between various cell types may be due to the gross differences in molecular composition of their membranes. It should be carefully noted that differences of this order of magnitude have been demonstrated between myotonic and control erythrocytes that have been extensively examined for measurable constitutive differences of proteins, lipids (phospholipids, gangliosides, cholesterol, fatty acids), and carbohydrates (sialic acis and inositol) (Roses and Appel, 1973). To date, only altered membrane bound endogenous protein kinase alterations have been demonstrated. Therefore it would be unexpected that large biophysical differences would be present.

It is probable that very subtle differences in the chemical composition of the membrane may be responsible for these esr differences. For example, endogenous protein kinase activity is membrane bound and may have associated with it specific boundary lipids that are necessary for enzymatic activity. It is conceivable that the alteration in protein kinase activity in myotonic dystrophy is due to an altered boundary lipid containing an excess of a highly unsaturated fatty acid. If these lipids represent a small per cent of the total population they would not have been detected by the thin layer or gas-liquid chromatographic techniques previously employed.

Another possible explanation for the altered lipid-protein interaction of myotonic red cells might be related to an altered membrane protein, although not necessarily limited to either the protein kinase enzyme or one of its substrates. If a particular protein exists in an altered conformation or if the protein organization is modified in myotonic membranes, differences in lipid-protein and protein-protein interaction may be expected. The decreased enzymatic activity of endogenous membrane protein kinase may be due to differences in either specific substrate or enzyme conformation, or to differences in proteins that regulate lipid metabolism within the membrane.

The local polarity and fluidity differences are present at all levels of penetrations. These esr meaurements are averaging techniques and it is conceivable that these changes could represent a subtle change in a small number (perhaps even only one) of membrane constituents resulting in a substantial alteration of the three-dimensional array of molecules in the membrane. Small changes in the charge of the membrane, perhaps manifested by the state of phosphorylation of endogenous protein, may materially affect structure and molecular relationships.

Many studies of quantitative analysis of membrance constituents and assays of various enzymatic activities have been performed in membrane systems from myotonic and other muscular dystrophies (Swift and Finegold, 1969; Di-

Mauro *et al.*, 1967; Radu *et al.*, 1970; Samaha and Gergely, 1969; Samaha *et al.*, 1967; Kuhn and Seiler, 1970; Kunze *et al.*, 1973). None of these attempts has yet defined the specific defect in any of these diseases. In fact, it is remarkable that no reproducible alterations have been demonstrated. Therefore, it is to be expected that any demonstrated changes would be small. Were these alterations gross, earlier studies would certainly have defined parameters of difference. The reproducible differences in polarity and fluidity in myotonic membranes found in our present study are significant and support the concept of myotonic muscular dystrophy as a diffuse membrane disease.

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