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Electron Spin Resonance Studies of Erythrocytes from Patients with Myotonic Muscular Dystrophy

(membrane/spin label/autosomal dominant trait)

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ABSTRACT **Electron magnetic resonance experiments have demonstrated that spin-labeled myotonic erythrocyte membranes have spectra that are recognizably different from those of normal erythrocytes. The spin label incorporated in the erythrocyte membranes of patients having myotonic muscular dystrophy is apparently located in a less polar and somewhat more fluid region than the label in a normal membrane. Although the mechanisms of molecular interaction and their relationship to enzymatic differences is unclear, the results lend confirmation to the suggestion that myotonic muscular dystrophy may be a disease resulting from a basic membrane abnormality.**

Myotonic muscular dystrophy is a systemic disorder inherited as an autosomal dominant trait. Although physiological studies have suggested a membrane abnormality as the underlying metabolic defect, it was the work by Roses and Appel (1) that demonstrated a reproducible metabolic abnormality in the membrane-bound protein kinase of erythrocytes from patients with myotonic dystrophy. They compared results from control and myotonic patients and found a significant difference in the phosphorylation of endogenous membrane protein of erythrocytes present in aged frozen cells. Possible explanations included an intrinsic change in the enzyme protein, a change in the state of the membrane associated with a less stable configuration of the enzyme protein, or an altered reactivity with specific substrates.

Myotonic muscular dystrophy is a disorder of humans that involves many organ systems. Although muscle is one of the principal target organs, the delineation of the primary biochemical defect is made extremely difficult on minimal biopsy material by the presence of atrophy, fibrous tissue, and the changes of denervation that may result in multiple secondary biochemical changes, and by the small yield and impurity of isolated sarcolemma. Erythrocytes offer an easily accessible source of membrane preparation that has no known pathological abnormality in myotonic dystrophy. In addition, a suitable sample size is critical for the spin-labeling technique since one is usually forced to work with low concentrations of the paramagnetic probe.

The spin-labeling technique is used to elucidate both static and dynamic effects of the environment in which the label is found (2–4). It has been used to study both model and biological membranes (5, 6) and perturbations of these membranes (7, 8) by external agents. It has been used to show anisotropic rotation of spin labels oriented perpendicular to membrane surfaces, to demonstrate the enhanced fluidity of the membrane as one moves further away from the membrane surface, and to confirm the lipid bilayer model of such membranes (9–12).

Based on the assumption that subtle structural changes may be associated with subtle biochemical differences in myotonic membranes, we have attempted to define differences in the spin-label signals from intact normal and myotonic erythrocytes. A small but significant and reproducible difference has been found in the magnetic resonance spectra in the two cases as well as evidence that indicates that a difference exists in the polarity of the domain in which the spin label finds itself. Measurements on the local fluidity in the two cases suggest that it may also differ, although the statistical significance is borderline.

EXPERIMENTAL

Erythrocytes were obtained from heparinized blood from patients with myotonic muscular dystrophy and from age- and sex-matched controls (normal). Ten myotonic patients from five different families were used. In each experiment 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 NaCl in 10 mM Tris·HCl, pH 7.4. The buffy coat was carefully removed (13).

The present experiments involve the spin label 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl methyl ester (5-nitroxide methyl stearate or 5-NMS) obtained from Synvar Associates, Palo Alto, Calif. 500 μl of a 1 mM solution of the solubilized spin label in chloroform were measured into an aluminum foil-shielded test tube. The chloroform was then evaporated by a stream of nitrogen gas leaving a thin film of label. One milliliter of washed cells (hematocrit 50%) was added to the test tube; the tubes were incubated for 16 hr by gentle shaking in a 37° water bath in the dark.

Several other spin labels were involved in attempts to label the erythrocyte membranes. The piperidine ester of octanoic acid (2,2,6,6-tetramethylpiperidine-1-oxyl-4-octanoate) and the oxazolidine derivative of cholesterol (4′,4′-dimethylspiro-[5α-cholestane-3,2′-oxazolidin]-3′-yloxyl) did not enter the erythrocyte membranes under the conditions used. It was possible to have the oxazolidine derivative of androstane (17β-hydroxy - 4′,4′ - dimethylspiro[5α - androstane - 3,2′ - oxazolidin]-3′-yloxyl) penetrate the membrane (data not given). Because of the relative ease of using the 5-NMS label we have initially concentrated on studies involving this label.

Electron spin resonance (ESR) measurements were performed on a standard Varian V-4502 12-inch system equipped with frequency and power-monitoring devices. Saturation and

Abbreviations: 5-NMS, 5-nitroxide methyl stearate; ESR, electron spin resonance.

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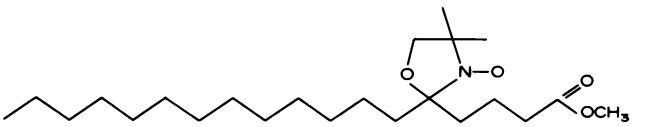

5-NMS

modulation broadening were avoided by working at low microwave power and small modulation amplitudes. The use of low concentrations of spin label (about 0.5 mM) should avoid any line broadening due to spin–spin exchange providing no aggregation of label occurs in the membrane. A Varian V-4540 temperature control unit maintained the desired temperature to within ±2° as measured by a copper–constantan thermocouple in thermal contact with the sample container. The labeled intact cells were placed in small capillary tubes which were in turn held inside a normal quartz ESR tube. Spectra were typically recorded with a sweep width of 100 G with a sweep time of 25 min.

RESULTS AND DISCUSSION

Our basic result is that there is a distinct and reproducible difference in the ESR spectra of cells from myotonic patients and those from controls. This difference shows up in several characteristic amplitude ratios, as well as in the value of the isotropic hyperfine coupling constant of the nitrogen atom. A fluidity change may also be involved, but the statistical significance of this result is borderline. We shall discuss the experimental results, the statistical inferences that may be made, and the physical effects that the results imply.

The spin label used in this study is the methyl ester of stearic acid in which the oxazolidine ring is separated by three carbon atoms from the polar carboxyl ester group. Labels of this type orient themselves in the cell membrane with the long molecular axis on the average perpendicular to the membrane surface. The polar head group is thought to be held rather firmly to the polar portion of the lipid–protein bilayer, while the hydrophobic tail of the molecule is less restricted and can undergo rotational motion in the interior of the bilayer (9, 10). Not only is the oxazolidine ring rigidly bound to the hydrocarbon chain of the molecule so that its motion reflects the rotational motion of the adjacent segment of the molecule, but also the "parallel" axis of the T-tensor is also approximately parallel to the long axis of the molecule (9). This simplifies the analysis of the spectral results.

Although the present experiments deal with intact erythrocytes, it is clear that the label is bound to the membrane portion of the cell. This hypothesis is based on the current ideas of the structure of erythrocyte membranes (14) and on the fact that similar studies of erythrocyte ghosts yield similar spectra (9). In addition, Kaplan *et al.* (11) have shown that the ESR signal of spin-labeled cells in which the label has been chemically reduced can be regenerated by the action of ferricyanide, which does not penetrate the cell membrane. Finally, and perhaps most pertinent, are the spectra observed in the present study, examples of which are given in Fig. 1. This type of spectrum is characteristic of this type of label undergoing anisotropic rotation about its long axis in an otherwise stationary or immobilized environment. Ordinarily, the spectra of strongly immobilized labels (that is, a powder spectrum) of the typical nitroxide type is such that it does not show significant "perpendicular" structure near the central portion of the spectrum. Such structure does appear, however, in cases where rapid anisotropic rotational motion results in new effective values of the T-tensor leading to a larger effective $T_\perp$. Fig. 1 shows the measurements that are used to yield the pertinent parameters involved in this study.

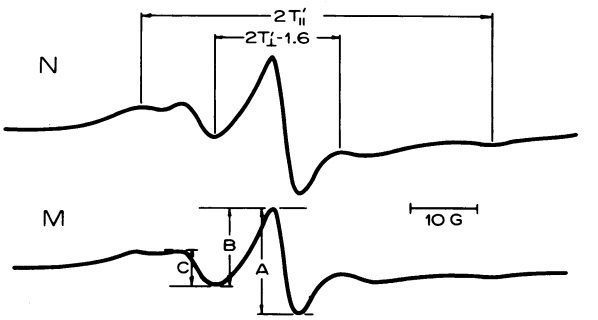

FIG. 1. Representative spectra of normal (N) and myotonic (M) systems. $T_{||}'$ and $T_\perp'$ are determined from the distances indicated in the top spectrum; the A/B and A/C amplitude ratios are defined in the lower figure.

Hubbell and McConnell (10) have given a very clear exposition of the effects of anisotropic rotation on the T and g tensors for nitroxide spin labels. They present equations that relate the values of $T_{||}'$ and $T_\perp'$ that one observes experimentally in a situation in which the molecule is undergoing rapid rotation about an axis that makes appropriate angles with the principal axis of the molecule. When axial T-tensor symmetry is present, such as in the present case, the equations simplify to

$$(T_{||}' - T_\perp')/(T_{||} - T_\perp)_{xl} \cdot a_{xl}/a' = \tfrac{1}{2}\langle 3\cos^2\theta - 1\rangle_T \equiv S_T \quad [1]$$

where S is defined as an order parameter and the expectation value involves the mean square angle that the axis of rotational motion makes with the parallel (principal) axis of the molecule. The measured $T_{||}' - T_\perp'$ value is compared to the crystal value $(T_{||} - T_\perp)_{xl}$. In order to compensate for polarity differences in the two cases, it is normalized by the factor a_{xl}/a' (10), a' being the isotropic coupling constant for the label appropriate to the system being studied. Similar equations may be derived for the component of the g-tensor although relative polarity effects are usually ignored. In this work the values for the crystal g- and T-tensors were taken to be those of the oxazolidine label of cholestane studied by McConnell and Hubbell (10). In addition, $T_\perp'$ was determined by adding 0.8 G to the apparent value (see Fig. 1), a procedure suggested by these authors to yield results in better agreement with calculated spectra.

In real systems, the angle that the principal axis makes with the rotational axis undoubtedly varies during motion of the molecule. In the present case, since the principal axis of the nitroxide-containing oxazolidine ring is parallel to the chain axis of the molecule and since the chain axis is believed to be essentially perpendicular to the membrane surface, one would expect an average value of θ in the vicinity of zero. Undoubtedly, the chain axis wobbles considerably during the motion of the molecule so that S is really a measure of the degree of wobble about a probable mean of 0° during the molecular motion. The closer S is to unity, the more rigid is the molecule held; the smaller S is, the greater is the local fluidity of that segment of the molecule containing the oxazolidine ring.

TABLE 1. *Spectral amplitude ratios A/B for 5-NMS in normal (N) controls and myotonic (M) patients and the ratio R of normal controls to myotonic patients*

A. Detailed data at 20°C. Standard deviations of all possible ratios for a particular experiment are given in parentheses.

Exp.	$(A/B)_N$	$(A/B)_M$	$R = (A/B)_N/(A/B)_M$
1	1.28	1.26	1.02
2	1.36	1.30	1.05
3	1.68	1.37	
	1.83	1.35	1.24(0.08)
	1.66	1.47	
4	1.37	1.32	1.04
5	1.89	1.67	1.13
6	1.52	1.51	1.02(0.01)
		1.47	
7	1.25	1.24	1.01
8	1.48	1.47	1.01
9	1.33	1.18	1.13
10	1.36	1.30	1.04(0.02)
	1.38	1.34	
		$\bar{x}$ (mean)	1.12
		SD	0.11
		SEM	0.024
		P	<0.0005
		n	22

B. Averaged R data at selected temperature. The total sample size at each temperature is denoted by *n*.

T(°C)	mean R	SD	*n*	SEM	*P*
20	1.12	0.11	22	0.024	<0.0005
30	1.09	0.09	19	0.020	<0.0005
40	1.06	0.06	19	0.015	<0.0005
50	1.06	0.06	13	0.017	<0.0005

TABLE 2. *Magnetic resonance parameters of normal (N) controls and myotonic (M) patients measured at 20°C. T and g refer to T-tensor and g-tensor parameters, while S_T and S_g represent the order parameter as calculated separately from the T and g data*

A. Detailed T, g, and S parameters for each experiment.

Exp.	Type	$\Delta T' = T_{\|\|}' - T_{\perp}'$	TrT'	-10^3 $(g_{\|\|}' - g_{\perp}')$	S_T	S_g
1	N	16.4	46.7	3.2	0.60	0.61
	M	15.5	47.0	2.8	0.56	0.53
2	N	17.2	47.6	3.5	0.61	0.67
	M	17.1	47.5	3.2	0.61	0.61
3	N_1	16.8	47.7	3.7	0.60	0.70
	N_2	17.0	47.6	3.9	0.61	0.74
	M_1	16.8	46.8	3.8	0.61	0.72
	M_2	15.9	46.9	2.8	0.58	0.54
	M_3	16.8	46.1	—	0.62	—
4	N	16.9	47.4	3.4	0.60	0.64
	M	16.5	47.1	3.4	0.60	0.64
5	N	15.8	46.5	—	0.57	—
	M	15.9	45.6	—	0.59	—
6	N	16.1	46.7	3.9	0.59	0.74
	M_1	15.5	45.9	3.1	0.57	0.60
	M_2	15.7	46.1	2.6	0.58	0.49
7	N	18.6	48.1	4.4	0.66	0.83
	M	18.1	48.4	3.5	0.63	0.67
8	N	17.6	48.1	4.2	0.62	0.80
	M	17.2	47.6	3.3	0.61	0.61
9	N	18.3	48.0	—	0.64	—
	M	18.2	47.8	—	0.64	—

B. Parameters averaged over all experiments at 20°C. Standard deviations are shown in parentheses.

Type	$T_{\|\|}'$	$T_{\perp}'$	$T_{\|\|}' - T_{\perp}'$	TrT	S_T	S_g
N	27.2	10.1	17.1	47.4	0.61	0.72
	(0.8)	(0.1)	(0.9)	(0.6)	(0.02)	(0.08)
M	26.7	10.1	16.6	46.9	0.60	0.60
	(0.9)	(0.2)	(0.9)	(0.8)	(0.02)	(0.07)

The ESR spectra of partially or fully immobilized radicals are not easily calculated, although a better understanding is being developed (15, 16). While the interpretation of subtle spectral changes may be hard to analyze in terms of physical effects, nonetheless differences in spectra can be observed and used to differentiate two different types of systems. This is the first principal point to be made in the present study; there is a definite, significant, and reproducible difference in the spin-label spectra of cells from myotonic patients compared to those of normal controls. Fig. 1 shows the ratios A/B and A/C, which have been used to characterize this difference. Table 1 contains the detailed data of the ratios at 20°C and a comparison of ratios taken at other temperatures. We have chosen to report the difference in the spectra as given by the ratio A/B although either amplitude ratio shows basically the same effect; the ratio of the amplitude ratio of normal to myotonic patients is denoted as R (Table 1*A*). In only 10 of a total of 73 individual determinations at the four stated temperatures (Table 1*B*) was the observed R ratio equal to or less than unity.§ Note that (Table 1*A*) the comparison of amplitude ratios is taken for each experiment, each experiment having been run on a particular day with a particular set of samples. This is a standard procedure in treating biological systems due to variations in the prepared cells that can often occur from one day to another. At the bottom of Table 1*A* is given the mean value of R, the standard deviation (SD), the standard error of the mean (SEM), the sample size (*n*), and the quantity *P*. *P* here represents the probability in a one-tailed Student's *t*-test that one makes an error in rejecting the null hypothesis that normal and myotonic cells are identical (parameter ratios of unity) and accepting the alternate hypothesis that they do indeed differ (parameter ratios greater than unity). Statisticians often use a *P* value of 0.05–0.01 to indicate that results are "probably significant," and values of *P* of 0.01 or smaller to indicate that they are "highly significant." The results in Table 1*A* show that the ratio of the amplitude parameters of normal to myotonic patients is highly significant in a statistical sense.

Table 1*B* shows the R-data averaged over all experiments for various temperatures. In each case the *P*-value indicates that the null hypothesis can be rejected. There is, however, no real basis for indicating that R changes with temperature. The main point at this juncture is to indicate the significant difference that is observed in the two types of systems. A

§ For the spectral parameter A/C, in only three of 79 determinations at all temperatures was the observed ratio of normal controls to myotonic patients equal to or less than unity.

TABLE 3. *Ratios of magnetic resonance parameters of normal controls (N) to myotonic patients (M) at 20°C*
The isotropic coupling constant of the nitrogen atom is given by a = 1/3 TrT. Standard deviations are shown in parentheses.

Exp.	$(a)_N/(a)_M$	$(\Delta T')_N/(\Delta T')_M$	$(S_T)_N/(S_T)_M$	$(Sg)_N/(Sg)_M$
1	0.994	1.059	1.066	1.16
2	1.003	1.008	1.005	1.10
3	1.022(0.008)	1.025(0.030)	1.003(0.034)	1.18(0.20)
4	1.005	1.020	1.016	1.01
5	1.021	0.994	0.973	—
6	1.016(0.003)	1.034(0.010)	1.018(0.008)	1.36(0.18)
7	0.994	1.028	1.034	1.24
8	1.010	1.023	1.013	1.30
9	1.004	1.004	1.000	—
Mean	1.013	1.023	1.011	1.20
SD	0.0116	0.023	0.029	0.16
SEM	0.003	0.006	0.007	0.05
P	<0.0005	<0.0005	$0.10 < P < 0.05$	<0.005
n	15	15	15	11

discussion of the physical implications of the spectral differences is delayed until we discuss the polarity and fluidity effects.

In Table 2 are given the magnetic resonance parameters for the normal and myotonic cells as measured at 20°C. While both $T_{||}'$ and $T_{\perp}'$ were observable at 20°, $T_{||}'$ could not be adequately measured at higher temperatures, so that we concentrate on the results at room temperature. The data as recorded on separate experiments (separate days) are given in detail in Table 2*A*, the overall averages of the parameters in Table 2*B*, and the ratio of parameters in Table 3. The ratio of the trace of the T-tensor (three times the isotropic nitrogen coupling constant) for normal to myotonic patients shows that this ratio differs significantly from one, having a *P* value of less than 0.0005. The ratio of $\Delta T'$ (Table 3) indicates the same degree of significance, but an examination of the column involving the ratio of the order parameters as determined from the T-tensor measurements (S_T) shows that the deviation from unity of the S_T ratio is of dubious significance, having a *P* value between 0.1 and 0.05. Although we report the order parameters as determined from the g-tensor measurements, we do not consider them as accurate and report them more for completeness.

The results indicate that there is a significant change in the polarity of the spin label in the two environments (that is, the normal and myotonic erythrocyte membranes) but that there is little if any change in fluidity. This conclusion is reflected in the fact that the S_T parameter is apparently unchanged in the two systems but that the TrT = 3a parameter does change. This view is reinforced by the fact that so long as there is essentially no change in the fluidity of the system, the ratio of the $\Delta T'$ values should also be a measure of the ratio involving the parameter a; that is, a change in the polarity of the environment will cause both $T_{||}'$ and $T_{\perp}'$ to change in approximately the same fractional amount.

Examination of Table 2*B*, however, indicates another piece of evidence that must be considered. One sees that the overall average of $T_{||}'$ is indeed larger for the normal cells as compared to the myotonic cells but that $T_{\perp}'$ is virtually unaffected in the two cases. While a decrease in the local polarity in which the spin label finds itself will cause a decrease in *both* $T_{||}'$ and $T_{\perp}'$, an increase in the fluidity would cause a decrease in $T_{||}'$ but an *increase* in $T_{\perp}'$. The fact that $T_{\perp}'$ remains virtually unchanged (when the two types of cells are compared) leads one to suggest that compensating changes have occurred; that is, a decrease in polarity causing $T_{\perp}'$ to move in one direction has been equalized by an increase in fluidity which results in a shift in the opposite direction, the two effects cancelling each other out. Such an occurrence is compatible with the changes in the measured parameters. We conclude, therefore, that the polarity change is the more significant occurrence but that the decrease in polarity may also be accompanied by a very slight increase in fluidity. The spin label in both cases is located near the membrane surface where the polar parts of the phospholipids and proteins tend to be. It is extremely reasonable to expect that any modification of the surface near the label in which a difference in the charge distribution or polarizability occurs would also result in a change in the fluidity of the nearby region. It is not immediately obvious, however, which direction these effects should take. The behavior of the systems under study suggests that the spin label in the myotonic membranes may find itself by some physical effect slightly further away or somewhat more excluded from the membrane surface so that its environment would be slightly less polar and where at the same time it would have a slightly higher degree of motional freedom. It is also possible that simply a decrease in charge of the membrane reduces the polarity seen by the label.

The most striking feature showing a difference in the normal compared to the myotonic cells is the ratio A/B (or A/C) (Table 1). Jost and coworkers (3) use the ratio A/B to detect phase changes in dipalmitoylphosphatidylcholine–H_2O dispersions, a process studied also by Hubbell and McConnell (10) by observing changes in S at the transition temperature. The physical process is clearly one in which the fluidity is increased so that, although no data are given for possible polarity changes, in this example a decrease in A/B is noted with an increase in fluidity. Eleter *et al.* (12) have recently studied the motional freedom or fluidity of lipids in microsomal preparations treated with phospholipase A. Such a treatment would be expected to affect the fluidity of the lipid region, and indeed they conclude that the fluidity is enhanced. It is significant to note that the spectra exhibited in their Fig. 4 show an *increase* in the ratio A/B with the fluidizing treatment, in direct contrast to the previous examples and to the effect observed in the present study. The decrease in the ratio

A/B observed in our study of myotonic systems apparently reflects both a decrease in polarity and a small increase in fluidity.

Myotonic erythrocytes have been demonstrated in the present study to have ESR spectra that are recognizably different from those of normal cells. Although it is premature to attempt to relate the spin-label spectra to the previously demonstrated differences in the endogenous protein kinase activity in myotonic erythrocytic ghosts, we can make several statements regarding the physical differences in normal and myotonic membranes.

It was previously suggested (1) that the endogenous protein kinase differences could be the result of differences in the state of the membrane. However, one must conservatively interpret these demonstrated differences here in lipid–lipid or lipid–protein interactions, since information concerning the specific location of the probe in the membranes is unavailable. Although several of the effects one observes are statistically significant, they are still very small and may result from very subtle changes in the general membrane structure or in particular parts of it. In the spin-label experiments by Kaplan and coworkers (11) involving human erythrocytes, human cultured lymphocytes, and cultured mouse L-cells, it was suggested that the spin label (similar to ours) was located in the outer surface of the lipid bilayer. These workers found the differences between the S values in the different types of cells to be about 0.02–0.03. They suggested that differences in the S values might represent differences in the molecular composition of the membranes in each different type of cell. However, the protein, lipid, and carbohydrate membrane constituents in myotonic and control erythrocytes have been extensively examined and found to have no gross constituitive differences (1). We are led to conclude, therefore, that the changes in the isotropic coupling constant and the possible change in fluidity observed in the present study demonstrate that either myotonic erythrocytes reflect a local difference at the specific sites involved with endogenous protein kinase activity or that a subtle change occurs in a small number (perhaps even only one) of constituents resulting in a substantial alteration of the three-dimensional array of molecules in the membrane.

The state of the erythrocytic membranes in myotonic dystrophy is clearly different from that of controls. Although the mechanisms of molecular interaction and their relationship to enzymatic differences is unclear, the present results lend confirmation to the suggestion that myotonic muscular dystrophy may be a disease resulting from a basic membrane abnormality.

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