J. Membrane Biol. 52, 165–172 (1980)

The Journal of
Membrane Biology

Electron Paramagnetic Resonance and Saturation Transfer Electron Paramagnetic Resonance Studies on Erythrocytes from Goats with and without Heritable Myotonia

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Summary. Erythrocytes from myotonic goats, an animal model of heritable myotonia, and normal goats were studied using electron paramagnetic resonance (EPR) and saturation transfer electron paramagnetic resonance (ST-EPR) spin labeling techniques. Three fatty acid spin labels with the nitroxide moiety at progressively greater distances from the carboxyl group were used to monitor different regions within the erythrocyte membrane. Since spin labels have been shown to induce hemolytic and morphologic alterations in erythrocytes, conditions for minimizing these alterations were first defined by hemolysis studies and scanning electron microscopy. Using these defined conditions for our studies we observed no significant differences in any of the EPR or ST-EPR parameters for normal and myotonic goat erythrocytes with any of the fatty acid spin labels used. Our results do not support the theory that myotonia is the result of a generalized membrane defect characterized by increased membrane fluidity as determined by fatty acid spin labels.

Myotonia is defined as a prolonged contraction of skeletal muscle fibers following a voluntary or induced stimulation and is associated with repetitive depolarization of the muscle cell membrane. In humans myotonia can be a manifestation of a variety of muscle diseases, including myotonia congenita, myotonia dystrophica, paramyotonia congenita, Duchenne dystrophy, and certain forms of periodic paralysis. Myotonia also occurs as a heritable disorder in the goat and has many of the features of myotonia congenita in the human.

We have described previously compositional and functional defects in the sarcoplasmic reticulum isolated from muscle of myotonic goats (Swift, LeQuire & Olson, 1974; Swift, Atkinson & LeQuire, 1979). We postulated that these defects might be related to the repetitive depolarization and prolonged contraction characteristic of myotonia. Other investigators have found defects in erythrocyte membranes from patients with myotonia dystrophica and myotonia congenita, and from animals with drug-induced myotonia, which suggest that these disorders may have generalized membrane involvement (Roses & Appel, 1973; Butterfield et al., 1976; Roses, 1977; Plishker, Gitelman & Appel, 1978).

One technique which has been used to study the organization of a membrane and its lipid environment is electron paramagnetic resonance (EPR)-spin labeling (Griffith & Jost, 1976). Using the EPR-spin labeling technique, Butterfield and coworkers reported that erythrocyte membranes from patients with myotonia congenita and myotonia dystrophica were more fluid and less polar than controls at all levels of penetration of the spin label into the membrane (Butterfield et al., 1974*a–b*; Butterfield et al., 1976; Butterfield & Watson, 1977). However, the observed changes in polarity can introduce ambiguity in the analysis of fluidity by standard EPR techniques. Butterfield concluded that in erythrocytes from patients with myotonic dystrophy, the polarity change was probably the more significant change, but it was accompanied by a very slight increase in fluidity (Butterfield et al., 1974*a*).

We have studied erythrocytes from normal and myotonic goats using conventional EPR and a new complementarily sensitive technique known as saturation-transfer electron paramagnetic resonance (ST-EPR) to investigate the concept of a generalized mem-

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0022-2631/80/0052-0165 $01.60
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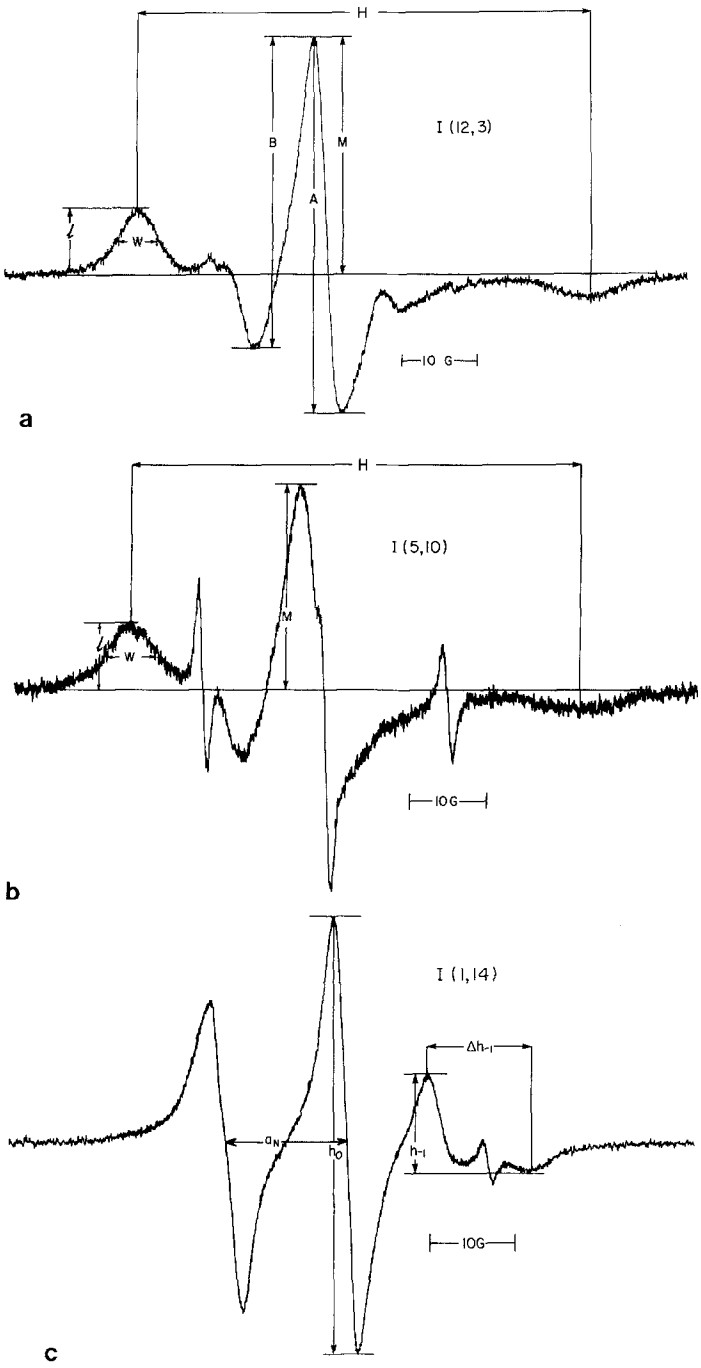


Fig. 1. EPR spectra at 24 °C of normal goat erythrocyte membranes spin labeled with: (*a*) *I*(12,3), (*b*) *I*(5,10), and (*c*) *I*(1,14). The concentration of spin label was 5×10^{-5} M. The parameters used to interpret the spectra are defined for each spin label. Spectra obtained with myotonic goat erythrocytes were identical to those shown

brane abnormality in myotonia. We have used three different fatty acid spin labels at different concentrations to monitor the environment in different regions of the membrane. It has been shown previously that fatty acid spin labels can induce morphologic and hemolytic changes in human erythrocytes (Bieri, Wallach & Lin, 1974) which can be correlated with changes in ST-EPR spectra (Wilkerson et al., 1978). Therefore, experiments were designed to define the conditions of labeling in order to minimize these alterations in the goat erythrocyte.

Materials and Methods

Animal Model and Blood Collection

The myotonic goats came from a herd that we have line bred over the past twenty years. These animals exhibited the characteristic manifestations of myotonia which were confirmed by electromyographic measurements. Normal goats of a different genetic background which had no evidence of myotonia were purchased from a local breeder. The goats were allowed to graze in the same pasture and were free of other diseases. Normal and myotonic goats were studied in pairs which were age and sex matched.

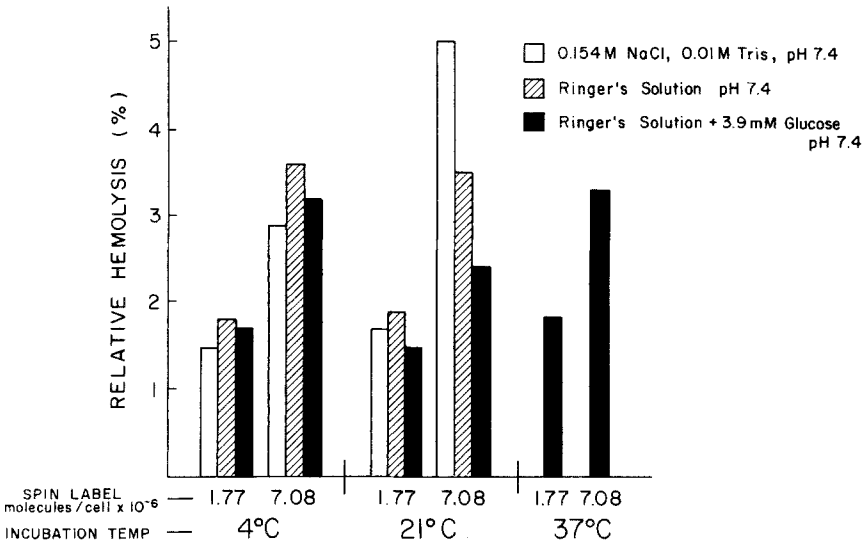
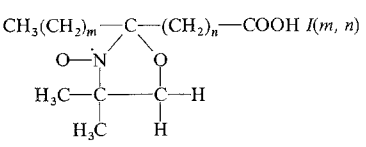


Fig. 2. Relative hemolysis of normal goat erythrocytes produced by spin labeling with *I*(12,3). Cells were labeled under the specified conditions of buffer, temperature, and spin label concentration for 10 min and then pelleted by centrifugation. The optical density at 540 nm of the supernatant was determined and related to the optical density of the supernatant from erythrocytes hemolyzed with distilled water

Blood was collected by venipuncture from the jugular vein into sodium heparin (Venoject vacuum blood collection tubes, Kimble-Terumo, Inc., Elkton, Md.). Erythrocytes were separated from the plasma by centrifugation at $1500 \times g$. The plasma and buffy coat were removed, and the cells were washed two times with ten volumes of one of the following buffers: (i) 0.154 M NaCl, 0.01 M Tris, pH 7.4; (ii) Ringer's buffer (0.154 M NaCl, 5.6 mM KCl, 6 mM $NaHCO_3$, 0.68 mM $CaCl_2$), pH 7.4; or (iii) Ringer's buffer plus 3.9 mM glucose, pH 7.4. The cells were then resuspended in the buffer to a 50% hematocrit.

Spin Labeling and EPR Measurements

The spin labels used in these studies were N-oxyl-4′ dimethyloxazolidine derivatives of stearic acid with the general formula *I*(*m*,*n*):



The labels *I*(12,3), *I*(5,10), and *I*(1,14) were used to monitor regions within the bilayer at increasing distances from the lamellar plane that is formed by the polar end of the phospholipids. Each spin label was dissolved in methanol to a concentration of 1 mM and was stored in the dark at 4°C. For labeling, an aliquot of the stock solution was added to an aluminium foil-shielded tube, and the methanol was evaporated to dryness at 40 °C under nitrogen. An aliquot (0.5 or 1.0 ml) of the erythrocyte suspension was added to the tube. The suspension was mixed gently for 10 min at ambient temperature unless otherwise indicated. In one experiment the erythrocytes were labeled by the bovine serum albumin method (Sato et al., 1978).

Spectra were recorded with a Varian E-109 Century Series EPR Spectrometer (Varian Associates, Palo Alto, CA) modified for second harmonic detection and equipped with an E-102-04 microwave bridge and a Varian E-231 rectangular TE_{102} cavity. For EPR measurements (first harmonic-in-phase absorption) the instrument was operated at 10 mW microwave power, 1.0 G modulation amplitude (peak to peak), and 100 kHz modulation frequency, while ST-EPR measurements (second harmonic out-of-phase absorption) were made at 63 mW microwave power, 5.0 G modulation amplitude, 50 kHz modulation and 100 kHz detection frequency. For temperature studies a Varian E-257 temperature controller modified for introduction of gas flow into the cavity through the radiation slots was used, and the temperature was measured with a copper constantan thermocouple in contact with the sample.

Samples were placed in a Scanlon S-812 flat cell (James F. Scanlon, Solvang, CA). The same flat cell was used for all studies. The cell was placed in the cavity in the same position for each sample with the flat cell perpendicular to the static magnetic field. Spectra were recorded at 24 °C within two hours of labeling. Some samples were studied throughout a 24-hr period. For the *I*(1,14) label the spectral temperature dependence was investigated over the range of 0.5 to 42 °C.

The EPR spectra were characterized in a manner consistent with the type of spectra recorded. Spectra of strongly immobilized labels, *I*(12,3) and *I*(5,10), were analyzed in terms of the separation of the outer extrema, *H*, as previously defined (McCalley, Shimshick & McConnell, 1972), the half width at half height of the low field peak, *W*, (Mason, Giavedoni & Dalmasso, 1977) and the relative heights of spectral lines, *A*/*B* and *M*/*l*, as shown in Fig. 1*a*. Spectral intensity was assessed by peak *A* in spectra of *I*(12,3) and peak *M* in spectra of *I*(5,10). The EPR spectra of the weakly immobilized *I*(1,14) label were consistent with a more rapid motion of the label as compared to *I*(12,3) or *I*(5,10). These spectra were analyzed in terms of the ratio of the peak height of the midfield line to the peak height of the high field line (h_0/h_{-1}), the width in gauss of the high field peak, (Δh_{-1}), and the isotropic nitrogen-nuclear hyperfine coupling constant, a_N, as defined in Fig. 1*c*. Spectral intensity was assessed by the peak height of the midfield line, h_0. ST-EPR spectra of the *I*(12,3) label were analyzed in terms of the parameter C'/C as previously described (Thomas, Dalton & Hyde, 1976), and as shown in Fig. 5.

Comparative data for normal and myotonic erythrocytes were statistically analyzed in terms of the Student's *t* test.

Hemolysis Studies

To evaluate hemolysis as a result of spin labeling, labeled cells were pelleted by centrifugation at $1500 \times g$ for 10 min, and the optical density at 540 nm of the supernatant was recorded. The supernatant from erythrocytes hemolyzed with distilled water was used as a standard.

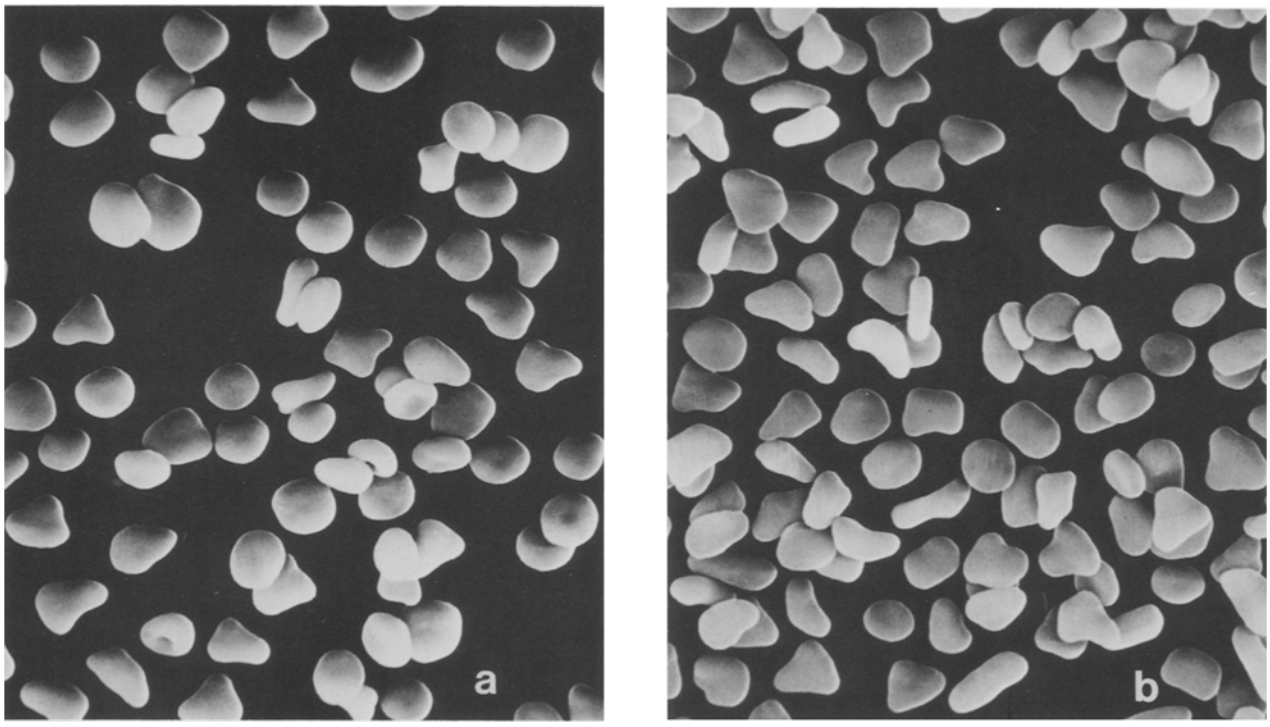


Fig. 3. Scanning electron micrographs of (*a*) normal goat erythrocytes, (*b*) normal goat erythrocytes incubated with 5×10^{-5} M stearic acid, (*c*) normal goat erythrocytes incubated with 5×10^{-5} M *I*(1,14) and (*d*) normal goat erythrocytes incubated with 5×10^{-5} M *I*(12,3). (Magnification, ×2800)

Table 1. EPR spectral parameters of *I*(12,3) in erythrocyte membranes from normal and myotonic goats

	Spin label (molecules/cell $\times 10^{-6}$)	*H* (G)	*A*/*B*	*M*/*l*	*W* (G)	*A*
Normal	1.77	59.7 ±0.4	1.183 ±0.033	3.513 ±0.073	5.5 ±0.3	3.8 ±0.4
(*n*=5)	3.54	59.5 ±0.3	1.211 ±0.019	3.488 ±0.041	5.9 ±0.1	6.2 ±0.7
Myotonic	1.77	59.3 ±0.1	1.210 ±0.018	3.848 ±0.245	5.5 ±0.2	4.2 ±0.7
(*n*=5)	3.54	59.5 ±0.5	1.182 ±0.035	3.625 ±0.142	5.8 ±0.3	6.6 ±0.5

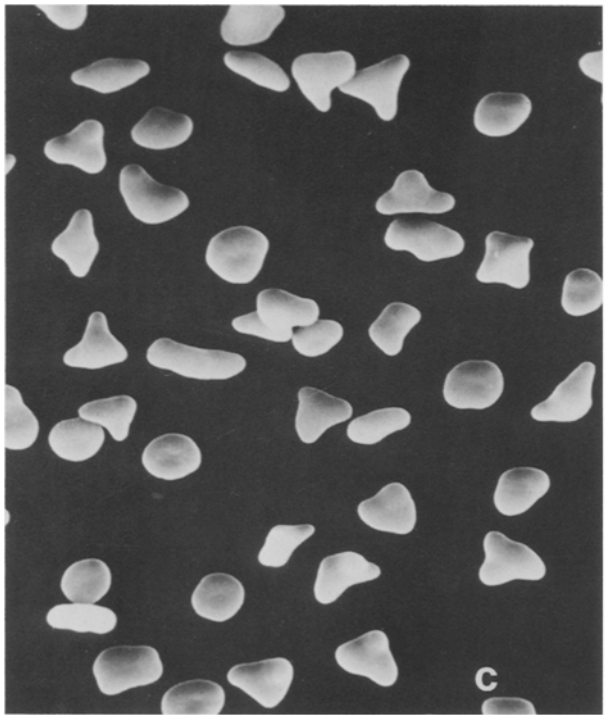
Scanning Electron Microscopy

Erythrocytes were fixed and dehydrated for scanning electron microscopy according to the technique of Bessis and Weed (1972). Fixed cells were attached to glass coverslips using 1 mg/ml of poly-L-lysine (Sigma, St. Louis, MO), dehydrated in a graded series of alcohols, and air dried. Coverslips attached to specimen mounts were sputter coated, and the samples were studied with a Hitachi S-500 scanning electron microscope.

Results

The hemolytic effect of the *I*(12,3) spin label on goat erythrocytes was studied as a function of various buffer solutions, incubation temperatures, and spin label concentrations (Fig. 2). Goat erythrocytes incubated in Ringer's glucose buffer at room temperature for 10 min with no spin label showed slight hemolysis (0.4%), while cells incubated with stearic acid (1×10^{-4} M) under identical conditions hemolyzed to a greater extent (1.0%). Hemolysis as a result of spin labeling was minimized by incubating cells in a Ringer's glucose buffer at room temperature for 10 min using spin label concentrations of 2.5×10^{-5} M to 1×10^{-4} M (1.77 to 7.08×10^{6} spin label molecules per cell). Labeling the cells at 4 °C did not change the degree of hemolysis.

Scanning electron microscopy was used as another means to evaluate the effects of fatty acid spin labels on the erythrocyte membrane. Goat erythrocytes nor-



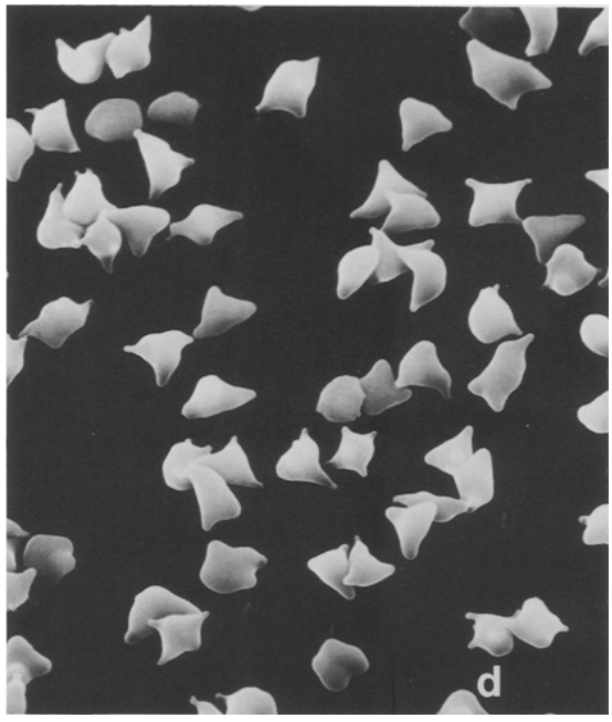


Table 2. EPR spectral parameters of *I*(5,10) in erythrocyte membranes from normal and myotonic goats

	Spin label (molecules/cell $\times 10^{-6}$)	*H* (G)	*M*/*l*	*W* (G)	*M*
Normal	1.77	59.0 ±0.9	3.062 ±0.120	7.0 ±0.8	1.7 ±0.3
(*n*=5)	3.54	58.2 ±0.5	2.977 ±0.065	7.5 ±0.7	2.3 ±0.5
Myotonic	1.77	58.2 ±1.3	3.030 ±0.176	7.1 ±0.6	1.8 ±0.2
(*n*=5)	3.54	58.3 ±1.3	2.975 ±0.172	7.5 ±0.7	2.5 ±0.6

Table 3. EPR spectral parameters of *I*(1,14) in erythrocyte membranes from normal and myotonic goats

	Spin label (molecules/cell $\times 10^{-6}$)	a_N (G)	Δh_{-1} (G)	h_0/h_{-1}	h_0
Normal	1.77	14.1 ±0.1	12.2 ±0.2	4.46 ±0.24	7.2 ±1.2
(*n*=5)	3.54	14.4 ±0.2	12.2 ±0.4	4.59 ±0.19	10.7 ±2.2
Myotonic	1.77	14.1 ±0.1	12.2 ±0.6	4.62 ±0.37	7.3 ±1.6
(*n*=5)	3.54	14.4 ±0.1	12.2 ±0.6	4.49 ±0.19	11.2 ±1.3

mally resemble spherocytes and are approximately 4 μm in diameter, as compared to human cells which are 7 μm in diameter. However, the procedures of isolating and washing the cells induce moderate poikilocytosis, as seen in Fig. 3*a*. Incubation of the cells with 5×10^{-5} M stearic acid did not alter the morphology of the normal or myotonic goat erythrocytes (Fig. 3*b*). Labeling the erythrocytes with the oxazolidinyl stearic acid derivatives *I*(5,10) or *I*(1,14) at concentrations of 5.0×10^{-5} M resulted in minimal, if any, morphologic changes (Fig. 3*c*). In contrast, labeling the erythrocytes with *I*(12,3) at a concentration of 2.5×10^{-5} M resulted in shape changes in nearly all cells, including the appearance of echinocytes having 2–3 spicules per cell. At a spin label concentration of 5×10^{-5} M these changes were more marked (Fig. 3*d*), with the presence of increased numbers of echinocytes which had 4–6 spicules per cell. In previously reported EPR studies on human cells, the spin label concentrations used were 5×10^{-4} M (Butterfield et al., 1974*a*), and the morphologic changes were more marked than those observed in our studies (Bieri et al., 1974).

A typical EPR spectrum of *I*(12,3) incorporated into either normal or myotonic goat erythrocytes is shown in Fig. 1*a*. The measured spectral parameters, shown in Table 1, reveal no significant differences between normal and myotonic erythrocytes. The spectral intensity was not linearly related to spin label concentration for either normal or myotonic erythro-

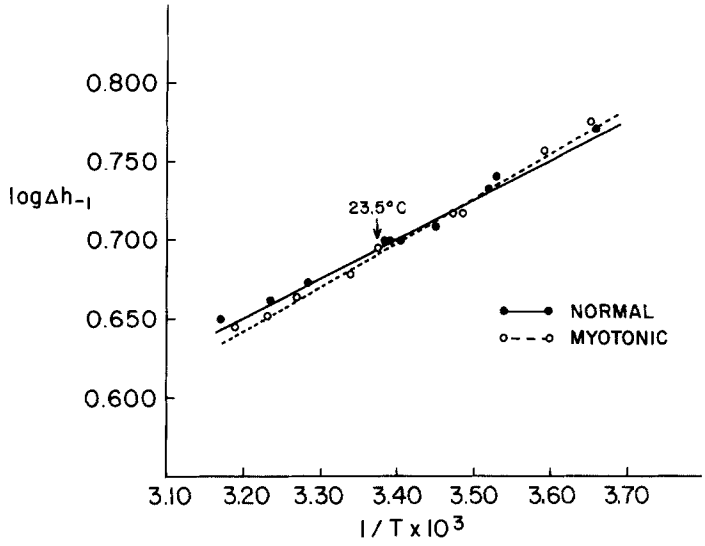


Fig. 4. Effect of temperature on the width in gauss of the high field peak (Δh_{-1}) obtained from spectra of $I(1,14)$ (5×10^{-5} M) incorporated into normal and myotonic goat erythrocytes

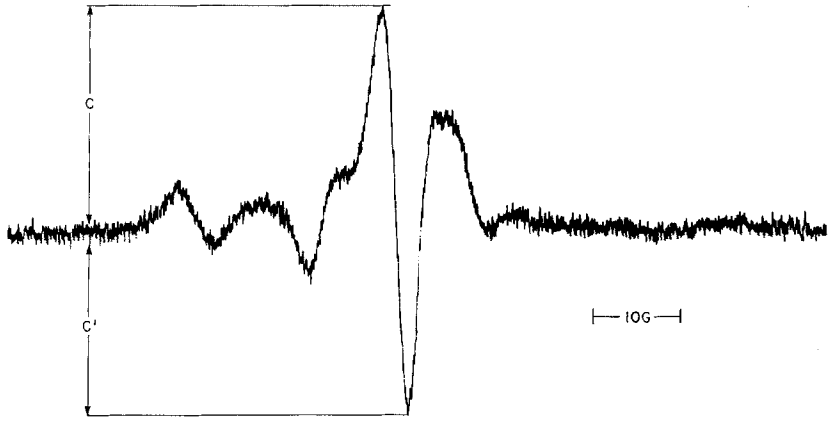


Fig. 5. ST-EPR spectrum at 24 °C of normal goat erythrocytes labeled with 5×10^{-5} M $I(12,3)$. The parameter C'/C is defined

Table 4. C'/C ratios from ST-EPR spectra of $I(12,3)$ in normal and myotonic goat erythrocyte membranes

Animal pair	Normal	Myotonic
1	0.9185	0.8837
2	0.9680	0.9163
3	0.9111	0.8462
4	0.8128	0.9341
5	0.8564	0.8363
Mean	0.8934	0.8833
SD	0.0599	0.0426

cytes. The spectra did not change throughout the 24-hr period of observation, and albumin-assisted labeling did not alter the EPR parameters.

The EPR spectra of $I(5,10)$ were identical for normal and myotonic erythrocytes (Fig. 1*b*, Table 2). As observed with the $I(12,3)$ label, the intensity of the spectra, as measured by peak M, was not linearly related to spin label concentration. Unlike spectra of $I(12,3)$, the spectra of $I(5,10)$ increased in intensity by 50% over a 24-hr period.

The $I(1,14)$ spin label yielded identical spectra for normal and myotonic erythrocytes (Fig. 1*c*). No significant differences in Δh_{-1}, h_0/h_{-1}, or h_0 were observed (Table 3). As with the $I(12,3)$ and $I(5,10)$ labels, spectral intensity, h_0, of $I(1,14)$ was not linearly related to spin label concentration. Furthermore, the intensity of $I(1,14)$ increased by 50% over a 24-hr period in a manner similar to that for the $I(5,10)$ label.

The EPR spectra of erythrocytes labeled with $I(1,14)$ from one normal and one myotonic goat were recorded at temperatures from 0.5 to 42 °C. No hysteresis in the spectral shape was noted upon recycling between these temperatures. The logarithms of Δh_{-1} were plotted against $1/T$ (°K) (Fig. 4). The values for normal and myotonic cells were very close at all temperatures with no significant difference at any

point. Statistical analysis of the points by least squares gave correlations between $1/T$ and log (Δh_{-1}) of 0.98 and 0.99 for normal and myotonic samples, respectively.

Figure 5 shows a typical ST-EPR spectrum of $I(12,3)$ incorporated into either normal or myotonic erythrocytes. These spectra were analyzed in terms of the ratio C'/C and the data are summarized in Table 4. In 4 pairs of the goats the C'/C ratio was smaller for myotonic erythrocytes. In one pair (No. 4) C'/C was higher for myotonic than for normal. This pair was repeated on two other occasions with the same results. Analysis of the data revealed no statistical difference between normal and myotonic erythrocytes.

Discussion

Based on EPR studies of erythrocytes from patients with myotonic dystrophy and congenital myotonia and from animals with drug-induced myotonia, Butterfield and coworkers concluded that increased membrane fluidity may be the common expression of a generalized membrane abnormality in various types of myotonia (Butterfield & Watson, 1977).

The goat with hereditary myotonia, similar to congenital myotonia in humans, provides an excellent experimental model to test this hypothesis. We have previously shown differences in the fragmented sarcoplasmic reticulum (FSR) isolated from skeletal muscle of normal and myotonic goats (Swift et al., 1974, 1979). When compared to normal, FSR from the myotonic goat had increased levels of membrane-bound calcium, increased sialic acid, and an increased content of saturated fatty acids. We have postulated that these compositional abnormalities may result in a stiffer, less fluid membrane which would promote increased calcium sequestration and increased calcium permeability, as was found in myotonic goat FSR. These studies formed the basis for our studies on the erythrocyte.

The EPR-spin labeling technique has been found to be a useful tool in studying the environment in membranes. Various spin labels can be introduced into model membranes, natural membranes, or membrane components. The resulting EPR spectra are sensitive to the orientation and motion of the label, the polarity of the environment, and the proximity of other labels. However, the effects of each of these factors on EPR spectra are not easily separable or quantitated. The development of saturation transfer electron paramagnetic resonance (ST-EPR) has greatly aided in the resolution of these effects since the motionally dependent parameters are not particularly sensitive to environmental polarity. Nevertheless, caution must be used in interpreting EPR or ST-EPR spectra since there are many factors which alter the EPR and ST-EPR response. In this study we first investigated the conditions of spin labeling not only to minimize morphologic and hemolytic effects, but also to minimize artifactual changes in EPR and ST-EPR parameters.

The results of our hemolysis and scanning electron microscopy studies of goat erythrocytes are similar to those of Bieri et al. (1974) on human erythrocytes. They observed morphologic changes over the range of 1.8×10^4 to 1.2×10^{10} molecules/cell and osmotic effects between 1.8×10^6 and 1.2×10^{10} molecules/cell. Using the $I(12,3)$ spin label, we found slight hemolysis with spin label concentrations of 1.77×10^6 molecules/cell. As might be predicted, increasing spin label concentrations increased the relative hemolysis. Incubation of the cells with identical concentrations of stearic acid produced minimal hemolysis, indicating that the oxazolidinyl moiety was probably responsible for the hemolytic nature of the spin label. By varying the incubation buffers and temperature of incubation we could alter the hemolytic nature of $I(12,3)$. We found that minimal hemolysis resulted when the cells were labeled in Ringer's glucose buffer at room temperature.

We observed morphologic changes in the red blood cells with $I(12,3)$ using spin label concentrations of 1.77 and 3.54×10^6 molecules/cell. The alterations were more marked at the higher spin label concentration. We chose 1.77×10^6 molecules/cell as our lowest spin label concentration for these studies since we were unable to collect EPR and ST-EPR data with acceptable signal to noise ratios at lower concentrations. Morphologic alterations using either $I(5,10)$ or $I(1,14)$ were minimal and similar to those observed when using stearic acid alone.

The EPR data corroborate the scanning electron microscopy data which indicate that the labels act as disruptive monitors of the lipid environment. For all labels used, the spectral intensity of the membrane-associated label was not linearly related to the spin label concentration. This would be expected if the label acted as a nonintrusive monitor. Furthermore, the spectral parameters of all three labels depended on the spin label concentration. Caution must therefore be employed in designing such comparative studies to minimize artifactual measurements.

We were unable to find significant differences in any of the EPR spectral parameters obtained by incorporating $I(12,3)$, $I(5,10)$, or $I(1,14)$ into erythrocytes from normal and myotonic goats. The temperature dependence of spectra from $I(1,14)$ was also identical. Butterfield et al. (Butterfield et al., 1974*a–b*,

1976; Butterfield & Watson, 1977) employed the ΔS analysis of EPR spectra to determine correlation time differences. This method of analysis is predicated on the assumption that the magnetic interactions are invariant. However, Butterfield et al. (1974*a*) observed changes in the isotropic nitrogen hyperfine interaction between erythrocytes from normal and myotonic humans, probably reflecting changes in the polarity of the membrane environments. Such variation from sample to sample would suggest caution in using the ΔS method to determine rotational correlation times. ST-EPR avoids this particular difficulty since small changes in magnetic anisotropy do not affect C'/C. However, there were no significant differences in the C'/C ratios for $I(12,3)$ in normal and myotonic goat erythrocytes.

Our studies do not support the theory that myotonia is the result of a generalized membrane defect characterized by increased membrane "fluidity" as measured by fatty acid spin labels. The difference between our results and those of Butterfield could be related to the model used. Myotonia in the goat, although very similar in many respects to congenital myotonia in humans, may be different with respect to membrane abnormalities. However, if the repetitive membrane depolarization and/or prolonged contraction characteristic of myotonia are due to changes in membrane "fluidity", similar changes would be expected in the goat model of myotonia.

Our studies do indicate that the EPR-spin labeling technique affords significant information which can help define the physical characteristics of membranes. However, the conditions of labeling – including the buffers used, the temperature, and the spin label concentration – must be carefully controlled to minimize artifactual measurements. In addition, the use of other means to monitor the disruptive effects of spin label incorporation on membrane structure and function (e.g., scanning electron microscopy) are important in order to minimize these effects.

This work was supported by grants from the Muscular Dystrophy Association (MDA) and NSF (GP-42998X, CHE7701018). RCP gratefully acknowledges support from MDA as a post-doctoral fellow. LRD is the recipient of an NIH Research Career Development Award.

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Received 5 June 1979; revised 13 September 1979