

order of magnitude faster than that of free DHAP (as is evident from the fact that, under conditions where less than 5% of the total DHAP is enzyme bound, the ratio of L- to D-glycerol phosphate [i.e., *sn*-glycerol 3-phosphate to *sn*-glycerol 1-phosphate] is 2:1²⁸), which suggests that the substrate's carbonyl group is polarized on binding to the enzyme, facilitating enolization and (incidentally) accelerating the reduction by borohydride.

It appears, therefore, that the enolizations that constitute catalysis by TIM are effected by proton abstraction by a carboxylate base, which is accelerated by general acid catalysis³³ at the oxygen of the carbonyl group. Mechanistic economy (or the minimal number rule of Hanson and Rose³⁴) would favor a formulation such as that shown in Figure 8.

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Envoi

It has proved possible to define the energetics of an enzyme-catalyzed reaction by using a combination of isotopic methods. It is clear from the free-energy profile that the enzyme has reached the end of its evolutionary development, the maximum flux of substrate being determined by a nonerodable diffusion limitation. The synthesis of this kinetic information with particularly promising structural studies (crystallographic,⁷ spectroscopic, and chemical) should allow a more intimate description of an enzyme's catalytic act than has proved feasible hitherto.

We are greatly indebted to Mark Fisher, Sue Fletcher, Alan Hall, Julia Herlihy, Peter Leadlay, Selwyn Maister, Chris Pett, and Martin Webb, who did most of the experiments reported here. Support from the Science Research Council and the National Science Foundation is gratefully acknowledged.

Electron Spin Resonance Studies of Erythrocyte Membranes in Muscular Dystrophy

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Background to the Various Disease States

Muscular dystrophy is a term which describes a class of diseases characterized by an inherited progressive muscle weakness and degeneration (dystrophy). Two common dystrophic conditions are Duchenne (DMD[†]) and myotonic (MyD) muscular dystrophy.¹ A third condition is not a dystrophic state; rather, congenital myotonia (CM) is a disease whose primary symptom is myotonia,¹ defined as a prolonged muscle contraction upon stimulation.

All three of these diseases are differentially inherited, and the clinical symptoms are expressed differently. DMD is inherited in males through a sex-linked recessive gene carried by the mother.¹ Dystrophy of proximal muscles (those near the torso) often appears in the first 5 years of life and rapidly progresses to complete immobilization in a relatively few years. In contrast, MyD, inherited as an autosomal (non-sex-linked) dominant trait by both males and females, initially demonstrates slowly progressive dystrophy of the distal musculature.¹ In addition to weakness, myotonia, and progressive dystrophy of striated muscle, many other symptoms are often present in this latter disease.¹ For example, malformation of cranial bones, testicular atrophy, presence of cataracts, abnormal electrocardiograms associated with cardiac symptoms,

various endocrine abnormalities, and other symptoms are often observed.¹ CM is a much rarer disease and can be inherited as an autosomal recessive or autosomal dominant gene.²

Myotonia is measured electrically by electromyographic techniques.¹ When the electric output of the electrode in myotonic muscle is connected to an audio amplifier, a characteristic "dive-bomber" sound is heard due to the repetitive membrane depolarization. Should a myotonic patient exert a muscle he has not recently used, the muscle will remain involuntarily contracted for a longer time than would be the case for a person without myotonia. Myotonia in CM and MyD persists even in the presence of curare, an agent which blocks the electrochemical contact between nerve and muscle.¹ This fact suggests that the defect is localized to the muscle tissue itself. More recently, physiological experiments have localized the defect responsible for myotonia in these diseases to the muscle surface membrane.³

Much effort has been undertaken to define the primary biochemical alteration in each of these diseases.⁴ No definite alterations in the proteins inti-

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† Abbreviations: DMD, Duchenne muscular dystrophy; MyD, myotonic muscular dystrophy; CM, congenital myotonia; ESR, electron spin resonance; 5-NS, 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolindinyloxy (stearic acid 5-nitroxide or 5-NS); 5-NMS, 12-NMS, 16-NMS, methyl esters of 5-NS, 12-NS, or 16-NS; MAL-6, 2,2,6,6-tetramethylpiperidinyl-1-oxy-4-maleimide; ³H-NEM, tritiated *N*-ethylmaleimide; DPH, diphenylhydantoin; RBC, red blood cell.

(1) R. D. Adams, D. Denny-Brown, and C. M. Pearson, "Diseases of Muscle", 2nd ed, Harper and Row, New York, N.Y., 1965.

(2) P. E. Becker in "New Developments in Electromyography and Clinical Neurophysiology", Vol. 1, J. E. Desmedt, Ed., Karger Press, Basel, 1973, pp 407-412.

(3) R. J. Lipicky and S. H. Bryant, in ref 2, pp 451-463.

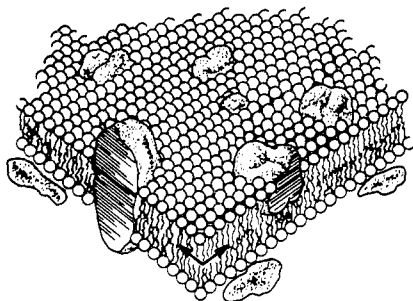


Figure 1. Schematic of the fluid mosaic model of the membrane.

mately involved in muscle contraction⁵ and oxygen storage⁶ have been found in DMD. However, alterations in muscle-derived creatine phosphokinase,¹ in serum aldolase,⁷ and calcium transport⁸ and activation of adenyl cyclase by epinephrine and sodium fluoride⁹ among other enzymes¹⁰ have been described in DMD. Still the primary biochemical defect remains unknown.

There are three important reasons why in our laboratory red blood cell membranes and not muscle membranes are studied in these apparent muscle diseases. First, finding the primary biochemical defect in muscle is made quite difficult on minimal biopsy material. The presence of atrophy, fibrous tissue, secondary biochemical changes which may occur as a result of denervation, and the small yield and impurity of the isolated muscle surface membrane are serious experimental problems.¹¹ Second, to obtain muscle from a dystrophic patient (who may have a relatively small amount of functional muscle in an advanced case) and from a control is not an innocuous procedure to the patient. Third (and this is the hypothesis under which the ESR work to be described was performed), tissue outside the nervous and neuromuscular systems may manifest the same defects which cause muscle to function abnormally even though the former tissue does not clinically manifest the myotonic and dystrophic symptoms. Such a hypothesis may explain the systemic nature of MyD and the diverse enzymatic alterations in DMD. If this hypothesis is correct, the tissue of choice to study should be easily obtainable in pure form and in abundant supply. The erythrocyte (or red blood cell) meets these requirements. Related alterations in the erythrocyte membrane, though not pathologically

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(5) (a) A. S. Penn, R. A. Cloak, and L. P. Rowland, *Arch. Neurol.*, **27**, 159 (1972); (b) F. J. Samaha, *ibid.*, **28**, 405 (1973); (c) T. Furukawa and J. B. Peter, *Neurology*, **21**, 920 (1971); (d) T. Furukawa and J. B. Peter, *Arch. Neurol.*, **26**, 385 (1972); (e) F. J. Samaha, *ibid.*, **26**, 547 (1972).

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involved with muscular dystrophy, may be the same as those found in muscle which do give rise to dystrophy and myotonia. Alterations in erythrocyte membranes would suggest that MyD, DMD, and CM may not be simply muscle diseases, but rather diseases associated with general membrane abnormalities.

Brief Review of the Fluid-Mosaic Model of the Membrane

Figure 1 is a schematic of the fluid-mosaic model of the membrane proposed by Singer.¹² The membrane is postulated as being a discontinuous lipid bilayer composed of the amphiphilic phospholipids, cholesterol, sphingolipids, and other amphipathic moieties into which the membrane proteins are incorporated to varying degrees. The primary stabilization for this membrane is a consequence of thermodynamic considerations. Especially important among these are the entropy increase of the surrounding water which results when hydrophobic moieties are sequestered from the aqueous environment (the so-called hydrophobic effect) and electrostatic interactions. Two principal kinds of membrane proteins are postulated: integral and peripheral. These proteins are distinguished by the degree of penetration into the lipid bilayer and the type of interactions by which they are stabilized in the membrane. In the erythrocyte membrane, "spectrin" is an important peripheral protein¹³ while band III is a diffuse group of integral proteins including Component a that actually span the bilayer.¹⁴

Spin Labeling as a Probe of Membrane Structure and Function

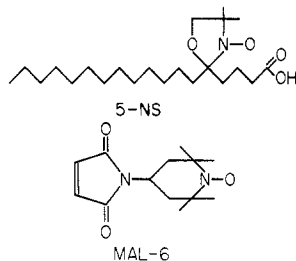
The research on erythrocyte membranes (also known as "ghosts") carried out in this laboratory has centered on the hypothesis that DMD, MyD, and CM may be diffuse membrane diseases. Any functional abnormality found in the erythrocyte (and presumably muscle) membrane may result from an altered enzyme, an altered substrate, or an altered environment within the membrane. It is this last possibility that has been studied by the use of the ESR technique of spin-labeling. No attempt to thoroughly review the theory of ESR or the application of the spin-labeling method to biological membranes will be made here. Interested readers are directed to a recent comprehensive review.¹⁵ Rather, some of the information obtainable from such studies will be presented. Spin-labels are stable, paramagnetic molecules whose structure results in an attachment to or a physical relationship with biological macromolecules such as those found in membranes. Nitroxide spin-labels can be incorporated into the system of interest to provide information concerning, among other things, structure, function, relative polarity, fluidity, conformational change, response to pharmacological agents, and chemical reactions.¹⁵ Two such spin-labels commonly used in membrane studies are nitroxide derivatives of stearic acid (5-NS) and *N*-ethylmaleimide (MAL-6).

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A typical ESR spectrum of a nitroxide radical undergoing rapid isotropic tumbling in solution consists of three equivalent lines which result from the isotropic electron-nitrogen nuclear hyperfine interaction only, the anisotropic electron-nuclear dipolar contribution to the hyperfine coupling having been averaged to zero as a result of the motion of the spin-label.¹⁶ Also, the anisotropic g tensor is averaged by the rapid isotropic motion to the isotropic g value (which defines the center of the ESR spectrum). As the temperature of the solvent is decreased, its viscosity is increased, and the tumbling rate of the spin probe is consequently decreased. As a result, two random local fields are experienced by the electron spin: one from variations in the anisotropic g tensor and another from the anisotropic hyperfine coupling.

The observed ESR line widths are a function of the relative orientations of the g and hyperfine tensors. However, the random fluctuations in these two tensors are correlated because they both vary as a consequence of the same molecular motion. Therefore, anisotropics in the g tensor and in the hyperfine tensor and their coupling lead to asymmetric line broadening of nitroxide ESR spectra as a result of decreased motion. Consider the ($M_I = +1$) low-field line. As the tumbling rate is decreased, its amplitude is decreased and its linewidth correspondingly increased until the limit of the powder spectrum is reached, whereafter no subsequent spectral changes are observed upon further cooling. The above is a brief but qualitatively correct description of the effect of motion on nitroxide ESR spectra.

In contrast to the isotropic motion demonstrated by nonoriented spin probes in a liquid, spin-labels like 5-NS are thought to orient in the lipid bilayer part of cell membranes with their long alkyl chains on the average parallel to the alkyl chains of the membrane lipids and with their polar acid groups near the polar head groups of the lipid molecules.¹⁷ The polar part of 5-NS is thought to be held rather firmly to the polar portion of the lipid-protein-water interface, while the hydrophobic tail of the molecule is less restricted and can undergo rapid anisotropic rotational motion in the interior of the bilayer.¹⁷ The oxazolidine ring is rigidly bound to the alkyl chain of 5-NS so that the motion of the nitroxide group reflects the rotational motion of the adjacent segment of the molecule. In addition, the parallel axis of the electron-nuclear hyperfine tensor is approximately parallel to the long axis of the spin label.^{17a}

A typical spectrum of 5-NS in intact erythrocyte membranes is given in Figure 2. 5-NS in erythrocyte

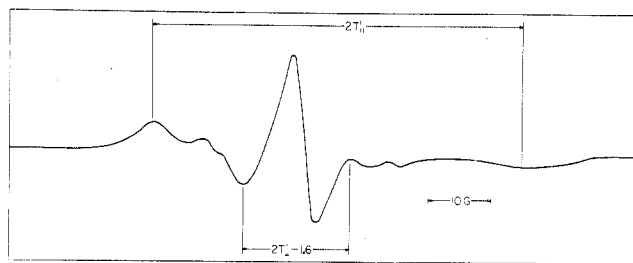


Figure 2. A typical ESR spectrum of 5-NS incorporated into intact erythrocyte membranes. The T -tensor measurements are indicated.

membranes undergoes rapid anisotropic motion about the long axis of the probe. This rotational motion of the spin label gives rise to the new effective T -tensor values, T_{\parallel}' and T_{\perp}' , indicated in Figure 2. The order parameter, S , is calculated^{17b} from these averaged T -tensor values from

$$S = \frac{T_{\parallel}' - T_{\perp}'}{(T_{\parallel}' - T_{\perp}')_{XL}} \frac{(a_N)_{XL}}{a_N'}$$

where

$$a_N' = (T_{\parallel}' + 2T_{\perp}')/3$$

is the nitrogen nuclear isotropic hyperfine coupling constant and XL refers to values obtained from nitroxide radicals incorporated into appropriate host single crystals. The polarity correction factor used by Hubbell and McConnell^{17b} is included in the calculation of S in order to normalize the environments seen by nitroxides in the membrane and the reference single crystal since the T -tensor values are polarity dependent.¹⁶

Regardless of whether the random-walk model of Jost et al.^{18a} (which relates S to the degree of wobble of the hydrocarbon chain axis containing the nitroxide group about a probable mean of 0°) or the model of Mason et al.^{18b} (which involves an inverse relationship between the rate of rotation of the symmetry axis and the apparent value of S) is used to explain spectra like that in Figure 2, the interpretation of S as a measure of local fluidity is valid. The smaller the value of S , the more fluid is the local environment seen by the paramagnetic center of the spin label.

In contrast to the noncovalently bound lipid-specific spin-labels, MAL-6 is covalently bound to membrane protein sulfhydryl (SH) groups, although a small amount of amino group binding may also occur. MAL-6 may competitively inhibit the binding of tritiated N -ethylmaleimide ($^3\text{H-NEM}$) to erythrocyte membrane proteins, suggesting that the binding sites of $^3\text{H-NEM}$ may also be those of MAL-6, although a definitive statement cannot be made at this time.¹⁹ Erythrocyte membrane proteins predominately labeled by $^3\text{H-NEM}$ (and presumably MAL-6) include spectrin, the higher molecular weight proteins present in smaller amounts, and band III.¹⁹

A typical ESR spectrum of erythrocyte ghosts labeled by MAL-6 is shown in Figure 3. Spectra similar to that in Figure 3 have been described previously^{19,20} as having

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(19) D. A. Butterfield, A. D. Roses, S. H. Appel, and D. B. Chesnut, *Arch. Biochem. Biophys.*, **117**, 226 (1976).

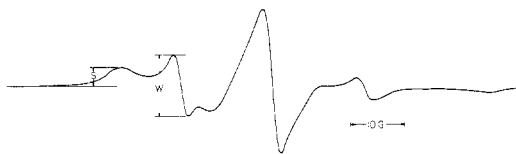


Figure 3. A typical ESR spectrum of MAL-6 incorporated into erythrocyte ghost membranes. The amplitudes of the ($M_1 = +1$) low-field strongly and weakly immobilized signals are given by S and W, respectively.

ESR parameters reflecting at least two different classes of SH group binding sites in the erythrocyte membrane. One type of site is indicated by the powder-like spectrum whose amplitude of the low-field line is indicated by S (for strongly immobilized). Sulfhydryl groups in the S sites are essentially completely immobilized, as evidenced by comparison of one-half the splitting of the outer hyperfine extrema to the T_{zz} principal values of nitroxides doped in appropriate host single crystals.^{18a,19,21} The second SH group environment reported by MAL-6 is one in which the spin probe is only weakly immobilized; the low-field peak-to-peak amplitude of this weakly immobilized spectrum is indicated by W (for weakly immobilized). Since the low- and high-field weakly immobilized signals are resolved from their strongly immobilized counterparts, a measure of a_N , the nitrogen isotropic hyperfine coupling constant, can be made.

In all spectra recorded employing MAL-6 as a probe of erythrocyte membrane protein conformation, a value of 16.6 G was obtained for a_N compared to 16.7 and 13.8 G for that of MAL-6 in aqueous buffer and dodecane, respectively.¹⁹ This result indicates that, in all systems described below, the W sites are highly polar in nature; therefore, they are not likely to be buried in the lipid milieu of the membrane but rather exposed to the aqueous medium, a finding also supported by ascorbate decay studies.¹⁹ The amplitudes of the high-field lines of spectra like that of Figure 3 are quite small and the central lines overlap; consequently, analyses of MAL-6 ESR spectra were confined to the low-field lines. The ratio of the ESR spectral amplitude of MAL-6 attached to weakly immobilized SH groups (W) to that of MAL-6 attached to strongly immobilized SH groups (S) is a monitor of conformational changes in membrane proteins.^{19,20} 5-NS and MAL-6 were used with other spin-labels to study erythrocyte membranes from patients with myotonic and Duchenne muscular dystrophy and congenital myotonia. The changes in the physical state of the membranes in each case and their possible role in the molecular mechanisms of myotonia and dystrophy are the principal subjects of this Account.

Results and Discussion

Myotonic Muscular Dystrophy. The three methyl stearate spin-labels 5-NMS, 12-NMS, and 16-NMS

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were used to probe various levels of the lipid environment of intact erythrocytes in MyD.^{22,23} Low concentrations of the probes were employed in order to avoid spin exchange and morphological effects.²⁴ The T_{\parallel} and T_{\perp} components of the motionally averaged nitrogen hyperfine tensor were measured; from these the order parameter S was calculated. S is significantly smaller for 5-NMS in MyD membranes compared to normal controls, suggesting a greater membrane fluidity near the membrane surface.^{22,23} No apparent difference in membrane fluidity as reported by 12-NMS and 16-NMS could be demonstrated.²³ The increased surface fluidity in MyD is not due to any measurable constitutive differences of proteins, lipids (phospholipids, gangliosides, cholesterol, fatty acids), and carbohydrates (sialic acid and inositol).^{25a}

Possibly, very subtle differences in chemical composition are responsible for these ESR differences. For example, an altered amount of highly unsaturated boundary lipid might materially affect enzymes like protein kinase (see below) and also result in an increased membrane fluidity. If these lipids constituted only a small percentage of the total lipid composition, they would not have been detected by the thin-layer or gas-liquid chromatographic techniques previously employed.^{25a} Small changes in charge of the membrane perhaps as a result of an altered state of endogenous membrane phosphorylation might give rise to an altered physical state of the lipid phase. This increased membrane fluidity in MyD could be completely abolished by incubation of intact erythrocytes with diphenylhydantoin (DPH),²⁶ a drug which clinically relieves myotonia in MyD and CM while having no effect on normal controls.²⁷

The increased membrane fluidity in MyD may be a secondary consequence of an altered protein-lipid organization in the membrane, a question potentially amenable to experiment by use of a protein-specific spin-label. Accordingly, normal and MyD erythrocyte ghost membranes were spin-labeled with MAL-6.¹⁹ The W/S amplitude ratios (described above) were calculated from spectra similar to that in Figure 3 and the results compared for normal and MyD erythrocyte membranes. The data indicated that W/S is significantly increased in MyD ghosts compared to those in normal controls.¹⁹ The individual S and W amplitudes corrected for spectrometer gain and membrane protein content were also compared. MyD erythrocyte membrane proteins appeared to take up less MAL-6 in S sites than did those of controls, while the incorporation of MAL-6 into W sites in MyD is apparently not different from that of control ghosts.¹⁹

Changes in the W/S ratio in erythrocyte ghosts have been used to monitor red cell membrane organizational

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alterations caused by various procedures and agents.²⁰ The increased value of W/S in the ESR spectra of MAL-6 labeled proteins in MyD erythrocyte ghosts¹⁹ therefore suggests membrane organizational or protein conformational changes to be present. The increased surface membrane fluidity in MyD may be the cause or the effect of the protein spin-label changes. It is possible that changes in the membrane protein conformation which result in a decreased binding by MAL-6 to S sites may be manifested in lipid changes near the membrane surface. Likewise changes in charge on the surface of the membrane or an alteration in the lipid packing may make otherwise reactive SH groups inaccessible to MAL-6. Interrelationships of membrane proteins and lipids are probably involved, and alterations in these protein-lipid interactions may help explain the differences in ouabain-sensitive sodium efflux, protein phosphorylation, and response to fixation which have been observed in MyD erythrocytes.²⁵ The changes in the physical state of erythrocyte membranes in MyD detected by the spin-labeling method support the concept that MyD is associated with a basic membrane abnormality.

Duchenne Muscular Dystrophy and Congenital Myotonia. The specificity of the membrane alterations in MyD was studied by electron spin resonance.^{28,29} In addition to erythrocytes from patients with MyD (which has both myotonia and dystrophy), erythrocyte membranes from patients with Duchenne muscular dystrophy who demonstrate dystrophy with no myotonia and congenital myotonia who present myotonia with no dystrophy were utilized. The difference between S_{control} and S_{MyD} or S_{CM} was found to be significantly greater than zero, suggesting more fluid environments near the membrane surface in these myotonic conditions.²⁸ In contrast, there was no significant mean difference of S_{control} and S_{DMD} and, accordingly, no difference in surface membrane fluidity in this nonmyotonic disease.²⁸ The difference between $(W/S)_{\text{control}}$ and $(W/S)_{\text{MyD}}$ or $(W/S)_{\text{DMD}}$ was significantly less than zero, suggesting alterations in the membrane protein conformation and/or organization in these dystrophic conditions.²⁹ As opposed to these findings, no significant difference in the W/S ratio of control and the nondystrophic disease state CM could be demonstrated.²⁹ These results suggest that increased erythrocyte membrane fluidity may be correlated with the presence of myotonia (discussed further below) while alterations in the physical state of membrane proteins may be correlated with dystrophy. This result may allow study of the separate symptoms of myotonia and dystrophy.

Although membrane protein alterations are present in each of the dystrophic states studied, the biophysical and biochemical defects peculiar to each disease which account for these ESR changes may be different. Spin-label studies indicated MyD erythrocytes were more fluid than controls near the membrane surface,^{22,23} while no difference in membrane fluidity was observed in DMD.²⁸ Increased phosphorylation of "spectrin" in RBC membrane has been observed in DMD,^{25e} while decreased phosphorylation of component a is reported

for MyD.^{25c} Similarly, while increased fluidity is detected in both myotonic conditions, the specific biochemical alterations responsible for the change in these diseases may be quite different. Phosphorylation of component a is normal in CM RBC ghosts in contrast to MyD membranes.³⁰ Physiological data also support different mechanisms of myotonia. Muscle membranes from CM demonstrate increased resistance and decreased chloride conductance, while these parameters are not apparently altered in MyD.³ Membrane fluidity is a function of many variables, including the nature of membrane lipids (type of phospholipid, fatty acid chain length and degree of unsaturation, head group, and cholesterol content), temperature, water content, presence of ions (especially divalent cations), and the sum total of the protein-lipid interactions.³¹ Activity of membrane-associated transport enzymes in bacterial^{32a} and animal^{32b,c} systems can be altered by affecting membrane fluidity. Increased membrane fluidity in MyD and CM may affect expression of a transport or enzyme system, resulting in the clinical sign of myotonia in muscle.

Notwithstanding the uncertainty of the specific mechanisms responsible for these phenomena, increased membrane fluidity may be the common factor in the myotonia process. Each of the previously discussed ESR results is consistent with this suggestion. Erythrocyte membranes from the myotonic diseases MyD^{22,23} and CM²⁸ are more fluid near the membrane surface than those of controls, while RBC membranes from the nonmyotonic disease state DMD do not possess an altered membrane fluidity with respect to normal controls.²⁸ Moreover, diphenylhydantoin, which clinically relieves myotonia, also caused MyD erythrocyte membranes to become as rigid as those of controls.²⁸ Support for this suggestion of increased membrane fluidity in myotonia also is found in ESR and biochemical studies of a cogent animal model of human congenital myotonia.^{33,34}

Possible Relationships of the ESR Results to Biochemical and Clinical Abnormalities. Each of the three diseases examined by the spin-labeling method has a distinct clinical and genetic expression and probably involves a separate inborn error. Nonetheless, the ESR results suggest that increased membrane fluidity may be correlated with myotonia,^{22,23,28} while membrane protein alterations may be correlated with dystrophy.²⁹ The observed alterations in the physical state of these membranes which are outside the nervous and neuromuscular systems strongly suggests that MyD, DMD, and CM are diseases of widespread membrane involvement. These results are, therefore, not consistent with the neurogenic^{35a,b} and vascular^{35c,d} theories of the pathogenesis of muscular dystrophy.

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Table I
Summary of ESR and Some Biochemical Alterations in Erythrocyte Membranes in MyD, DMD, and CM

	MyD	DMD	CM
ESR—increased membrane fluidity ^a	Yes	Normal	Yes
ESR—membrane protein alterations ^b	Yes	Yes	Normal
Protein kinase activity ^c	↓, III	↑II, III	Normal
Na ⁺ -K ⁺ pump exchange ratio ^d	2:2	Normal	Normal
Scanning electron microscopy—increased stomatocytes ^e	Yes	Yes	Yes

^a References 22, 23, and 28. ^b References 19 and 29. ^c References 25c, 25e, and 30. ^d References 25b. ^e Reference 25d.

Comparison of results of several biochemical studies in MyD, DMD, and CM erythrocyte membranes have been made. Phosphorylation of band III is decreased in MyD erythrocyte membranes, increased in bands II and III in DMD ghosts, and unaltered in CM membranes.^{25c,e,30} A 2:2 Na⁺:K⁺ active exchange by the sodium pump was observed in MyD erythrocytes compared to the 3:2 value found in control cells.^{25b} No such alteration is apparently detected in DMD or CM.^{25b} A nonspecific increased number of stomatocytes were observed in unwashed erythrocytes in all three disease states.^{25d} This latter observation was probably the result of an intrinsic biophysical membrane difference in each case that led to an abnormal response to fixation. These results are summarized in Table I.

Consider the results for CM. Of the various procedures examined, only increased membrane fluidity²⁸ and an abnormal response to fixation^{25d} are noted in this disease. The apparent absence of changes in the physical state of erythrocyte membrane proteins in CM as determined by ESR²⁹ and the concomitant absence of any alterations in enzymatic activities studied in the three diseases may suggest that the abnormality which causes the increased surface fluidity is probably not located in the "boundary" lipids. Differential assemblage of these latter lipids which are in intimate contact with integral membrane proteins might be expected to cause protein structural and enzymatic alterations. The increased fluidity of membrane lipids may in some as yet undetermined manner give rise to the decreased chloride conductance and consequent increased membrane resistance with the resulting myotonia.

Interpretation of the results for MyD is more difficult. The chloride conductance and membrane resistance are normal.³ The alteration in the stoichiometry of the sodium pump^{25b} may explain the resulting membrane potential of -62 to -68 mV in MyD^{36a} compared to a normal value of -90 mV.^{36b} This former potential requires less stimulation than normal for membrane depolarization and may explain myotonia in MyD. There is much evidence to support the suggestion that the sodium pump and the (Na⁺ + K⁺)-ATPase are the same moieties.³⁷ The isolated (Na⁺ + K⁺)-ATPase reportedly requires the negatively charged lipid phosphatidylserine for activation.³⁸ The abnormality found in the sodium pump in MyD could conceivably result from a slight diminution of the amount of phosphatidylserine in its boundary lipid layer. Such

a change might result in a decreased binding of Ca²⁺ to the membrane, thereby affecting the conformation of the enzyme and causing an increase in lipid fluidity near the membrane surface. Ca²⁺ has been shown to selectively cause a phase separation in mixed-lipid systems containing phosphatidylserine and result in an increase in rigidity of the lipid phase.³⁹ A decreased Ca²⁺ binding to sarcoplasmic reticulum has been observed in MyD.⁴⁰

A second alternative explanation for the relationship between changes in the physical state of MyD membranes and the presence of myotonia and dystrophy may involve the state of phosphorylation of membrane proteins and lipids. As before, if there is less phosphate on the membrane, decreased Ca²⁺ binding might occur and the increased membrane fluidity and altered protein conformation might result. Several membrane proteins in erythrocytes are labeled by MAL-6,¹⁹ the protein spin probe used in the investigations reported here. Preliminary experiments⁴¹ which may provide insight to the myotonic and dystrophic processes in MyD indicate the (Na⁺ + K⁺)-ATPase can be selectively spin-labeled under certain conditions. Other possible interpretations than those enumerated above are also conceivable.

In DMD, only evidence for an altered protein conformation and/or organization²⁹ and increased phosphorylation of membrane proteins^{25e} is observed (Table I). However, the Ca²⁺ binding in DMD is reported to be normal,⁴⁰ suggesting the speculation above relating Ca²⁺ binding to phosphorylation levels may not be applicable here. The two alterations in DMD listed in Table I may be related and give rise to dystrophy.

The understanding of the molecular mechanisms by which myotonia and dystrophy arise is now a difficult problem. Illumination of the basic alterations in the physical state of membranes in myotonic and Duchenne muscular dystrophy and congenital myotonia and acceptance of the concept that these are general membrane diseases are being aided by the spin-labeling technique.

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