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A SPECIFIC DECREASE OF THE FLUORESCENCE DEPOLARIZATION OF PERYLENE IN MUSCLE MEMBRANES FROM MICE WITH MUSCULAR DYSTROPHY

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SUMMARY

The microviscosity of erythrocyte membranes and muscle microsomes from age matched 6-week old control mice REJ 129 Dy/Dy, and mice with muscular dystrophy REJ 129 dy/dy has been estimated by measuring the fluorescence depolarization of perylene. There was no difference between the erythrocyte membranes. The muscle microsomes from dystrophic animals had about 20 % lower values than the controls. The temperature dependence indicated that a transition occurs in both sets of muscle microsomes, but the transition temperature was lower in the dystrophic microsomes. Cholesterol, phospholipid and triglyceride analyses of the membranes showed no difference between the erythrocyte membranes. The largest difference in the muscle microsomes was a two-fold increase in cholesterol level found in the dystrophic microsomes. No simple correlation could be made between the lipid analysis and the microviscosity measurements. Since the change in microviscosity is found in membranes isolated from the tissue primarily affected by the dy gene, we suggest that the change in microviscosity may be important in the development of the disease.

INTRODUCTION

Recently several reports have appeared which show that there are abnormalities in the erythrocyte membranes from human beings, as well as other animals, suffering from muscular dystrophy [1, 2]. Particularly important for our work are the reports by Butterfield et al. [3, 4] that indicate there is an increase in the fluidity of erythrocyte membranes isolated from humans with myotonic muscular dystrophy as well as a decrease in the polarizability in the environment of the probe. These author's conclusions were based on studies using an electron spin resonance probe. Such studies allow qualitative comparisons between different types of membranes but they do not provide a quantitative measure of viscosity. For our studies we have chosen to use the fluorescent dye perylene as a probe of membrane viscosity. It has been proposed that

perylene can be used to estimate the microviscosity of membranes [5–10], although this suggestion has yet to be proven satisfactory for biological membranes [11, 12]. Multiple domains of lipids may exist in biological membranes and the role of perylene binding to membrane proteins has not been completely assessed.

In our studies we have used the 129 ReJ dy/dy strain of mice from Bar Harbor, Maine [13–15]. These animals inherit muscular dystrophy as an autosomal recessive trait. This dystrophy is a progressive disease that seems to be highly selective for skeletal muscle, i.e., in contrast to human myotonic muscular dystrophy [4] no other musculature seems to be affected [14, 16, 17]. There are few if any abnormalities in these mice at birth, but muscular weakness develops slowly and the animals ultimately die from the disease or from its secondary consequences. A number of characteristic biochemical abnormalities has been described in these animals, which, in many instances are similar to those seen in human forms of muscular dystrophy [14].

Although the primary event responsible for the degeneration of muscle is not known, several authors have suggested that the genetic effect is on cellular membranes [18], and several studies have shown biochemical changes in the skeletal muscle membranes of these animals [16, 17].

Since our studies were undertaken, a report has appeared on the measurement of microviscosities of cellular membranes isolated from normal and dystrophic chickens [19]. Our results on the mouse are not in agreement with this report.

METHODS

Mice (129 ReJ/dydy and 129 ReJ/DyDy) shipped from the Jackson Laboratories in Bar Harbor, were sacrificed immediately on their arrival in the laboratory. We used female mice that were six-weeks old. At this time the effects of dystrophy are discernible as a weakness in the hind limbs, but the animals are roughly the same size as the control animals. Despite the presence of considerable histological change in the muscles of animals of this age, most of the tissue is identifiable histologically as muscle, and there is little or no fatty infiltration [20]. The control animals were homozygous normal DyDy.

At first we attempted to use well-defined muscle groups, since the dy gene affects the white or fast muscle preferentially [14]. Efforts were made to compare soleus muscle (red) with extensor digitorum longus (white). Even pooling muscles from several animals, however, did not provide us with enough material for study. Our results, therefore, were obtained on two broad classes of muscle from the hind limbs of the mice: (1) the upper hind limb muscles, the "thigh" muscles and (2) the lower limb muscles, the "calf" muscles, including the gastrocnemius, soleus and extensor digitorum longus. By pooling these muscles from 2 to 5 animals we were able to obtain sufficient crude microsomal membranes.

Immediately after the animals were bled, the muscles were removed, dissected free from fat and connective tissue, and minced. All manipulations after the removal of the muscle were done at 4 °C. The membranes were prepared by homogenizing minced muscle in 0.25 M sucrose, 0.2 mM EDTA, 10 mM Tris · HCl pH 7.4, 10 μg/ml 2,6 di-*t*-butyl-4-hydroxymethyl phenyl alcohol (DTA) with a power driven Teflon pestle. When the material was homogeneous, it was centrifuged at about $1000 \times g$. The pellet was resuspended and homogenized and centrifuged again under the same

conditions as before. The supernatant fractions were pooled. Phase contrast microscopy showed that the homogenate was free of nuclei, but there was an occasional myofiber present. This material was centrifuged at about $10\,000 \times g$ for 20 min in a Sorval RC 2B centrifuge. The supernatant fraction of this centrifugation was free of myofibers and large particulate matter. This supernatant fraction was centrifuged at $100\,000 \times g$ in a Beckman L2-75B centrifuge for 90 min. The pellet was resuspended in 139 mM NaCl/10 mM sodum phosphate buffer pH 7.0/10 μg/ml DTA alcohol with a hand homogenizer and then sonicated for 15 s. These membranes were used without further purification.

Erythrocyte ghosts were prepared according to the method of Steck et al. [21]. The animals were bled from the retroorbital sinus with a heparin-treated glass Pasteur pipette. The yield in mg protein of ghosts and microsomes per animal was about the same for normal and dystrophic animals.

All experiments were carried out in 139 mM NaCl, 1.5 mM NaH_2PO_4 at pH 7. The membranes were labeled at 1, 0.5 and 0.1 μM perylene. Perylene was purchased from Aldrich Chemical Company. A 10^{-6} M dispersion of perylene in the buffer described above was prepared by blowing 25 μl of a 1 mM solution of the hydrocarbon in acetone into 25 ml of vigorously vortexed buffer. Vortexing was continued for 15 min. This dispersion was diluted with buffer to give 0.5 and 0.1 μM perylene dispersions. Labeling of the membranes was begun by blowing a concentrated membrane suspension into the perylene dispersions to give a final membrane concentration of 0.04 mg protein/ml. The solutions were vortexed for about 15 s and then incubated at 0 °C for 2 h. After this, measurements were made immediately. Generally, polarization measurements were made on the same day the microsomes were isolated, but we found the membranes could be stored frozen at −20 °C for up to two days without altering the polarization values significantly.

All steady-state fluorescence measurements were carried out with a Perkin Elmer fluorescence spectrometer MPF 3, operated in the ratio mode and equipped with a 018-0054 polarization accessory and a temperature bath. The samples were excited at 436 nm, emission was recorded at 470 nm. Excitation and emission slits were adjusted to 10 nm. Unless stated otherwise, the measurements were made at 18 °C. The samples were allowed to equilibrate at the indicated temperature in the cuvette for 20 min. Before taking the readings, the suspensions were carefully mixed by bubbling N_2 through the samples.

For the calculation of the anisotrophy values, the emission intensities obtained for a labeled membrane suspension at the 4 polariser settings were corrected by subtracting the value obtained for an unlabeled sample at the respective polariser setting. This correction was usualy between 1 and 7 % for membranes labeled at 0.5 μM perylene and about 25 % for membranes labeled at 0.1 μM perylene. The emission intensities of the perylene dispersion used for labeling were negligible at the setting of the instrument used for measuring the fluorescence of the labeled membranes. Anisotropy values were calculated according to Eqn. 1

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \tag{1}$$

where I_{VV} is the emission intensity obtained when the both excitation as well as the emission polariser are in the vertical position, I_{VH} is the emission intensity, when the

excitation polariser is in the vertical position and the emission polariser in the horizontal position. G is defined by Eqn. 2 and corrects for polarization of light by the instrument.

$$G = \frac{I_{HV}}{I_{HH}} \tag{2}$$

where I_{HH} is the emission intensity, when both excitation and emission polariser are in the horizontal position and I_{HV} is the emission intensity, when the excitation polariser is in the horizontal position and the emission polariser is in the vertical position. For each sample, r was determined 3 times and the values obtained were averaged.

The α values were calculated using the equation:

$$\alpha = \left(\frac{r_0}{r} - 1\right)^{-1} \tag{3}$$

$r_0 = 0.37$ (r_0 = limiting anisotropy value [9])

The α value is related to the microviscosity by the following equation:

$$\eta = C(r)T\tau \left(\frac{r_0}{r} - 1\right)^{-1} \tag{4}$$

η is the microviscosity, T is degrees K, τ is the lifetime of fluorescence, and $C(r)$ is a constant [6, 7]. Calibration curves for the evaluation of $C(r)$ are available [9], but we have chosen not to calculate microviscosities because of difficulties in evaluating the published calibration graphs which are difficult to read accurately and show a slight curvature in the range of r values of our measurements. Since our primary goal is to compare different sets of membranes, it is not necessary for us to calculate η, and we will use α which is directly proportional to η at constant τ and T.

Fluorescence lifetime was measured with an Ortec nanosecond fluorometer. The excitation wavelength was 400 nm. The spectra were corrected for scattering of light by the membranes by subtracting the counts obtained from an unlabeled sample. The precision of these measurements was about 1 %.

Lipid analyses were carried out on chloroform/methanol extracts of freshly isolated muscle microsomes or red blood cell ghosts. The extracts were prepared by the method of Folch et al. [22], with special modifications [23] of this procedure. Two extractions of the microsomes or ghosts with redistilled chloroform/methanol (2 : 1, v/v) were followed by extraction in chloroform/methanol (1 : 1, v/v) and then chloroform/methanol (1 : 2, v/v). The combined extracts were washed with Folch upper phase containing 0.017 % $MgCl_2$.

After evaporation of the less polar phase under dry nitrogen to near dryness, neutral lipids were separated by thin-layer chromatography according to the method of Mangold and Malins as described by Skipski and Barclay [24]. Preparative thin-layer plates (Kontes) 200 × 200 mm were spread with layers of silica gel H (Sigma) 0.250 mm in thickness. After development of the plates in glass chambers containing equilibrated petroleum/ether-diethyl/ether-acetic acid, (90 : 10 : 1, v/v/v), triglycerides were visualized with I_2 vapor and were scraped from the plates. After evaporation of the iodine, the triglycerides were eluted with chloroform/ethanol/H_2O/acetic acid, (97 : 97 : 4 : 2, v/v/v/v), according to the procedure of Olivercrona [25].

Triglyceride content of the eluted spots was determined by the method of Sardesai and Manning [26]. Triolein (obtained from Sigma) and ^{3}H-triolein (New England Nuclear) were also separated on the same plates and radioactivity and triglyceride content determined after elution of the spots containing these compounds. Blank areas of the silica gel plates served as controls for both assays. Colour development was determined with a Gilford model 2400 spectrophotometer.

Cholesterol was measured spectrophotometrically by the procedure of Glick et al. [27]. Cholesterol standards were obtained from Steraloids, Inc., Pawling, N.Y. and recrystallized from absolute ethanol prior to use.

Total lipid phosphorous was determined in total lipid extracts by the method of Chen et al. [28]. Ascorbic acid was obtained from Calbiochem and Sulfuric acid (reagent grade) from Dupont. A qualitative determination of phospholipid classes was accomplished by the methods of Skipski et al. [29] on "basic" thin-layer chromographic plates of silica gel H. The plates were pre-run prior to development in the separating solvent mixture. Spots were visualized with I_2 vapor. Phospholipid standards were obtained from Sigma or Supelco. Phosphatidylethanolamine was the gift of Dr. D. Nelson.

Acetylcholinesterase activity was assayed spectrophotometrically by the method of Ellman et al. [30]. The specific inhibitor of acetylcholinesterase, BW284-C51 (Wellcome Reagents Ltd, Beckenham, U.K.) was used to inhibit acetylcholinesterase activity at a concentration of $3 \cdot 10^{-5}$ M. Iso-OMPA (Kock-Light Ltd., Colnbrook, U.K.) was used to inhibit butyrylcholinesterase activity. Dithiobisnitrobenzoic acid was obtained from K & K Fine Chemicals and acetyl- and butytylcholinesterase were obtained from Sigma. Color development was monitored in a Gilford model 2400 spectrophotometer.

RESULTS

Fig. 1 shows the emission spectra of perylene in acetone and benzene as well as in the muscle microsomes from normal and distrophic mice. The spectra in benzene and in the two types of membranes are nearly identical, while in acetone the two emission peaks are shifted to shorter wavelengths. This suggests that perylene exists in a highly nonpolar environment in the membranes, presumably the hydrocarbon interior of the lipid phase. The dystrophic microsomes are found to give consistently higher perylene emission intensities per mg protein compared with the normal microsomes. Since, as will be shown later, the microsomes have a higher cholesterol and triglyceride content, the reason for the higher intensity might be that more perylene is taken up. The emission intensity of perylene in membranes was about 100-times weaker than that of the organic solutions containing the same amount of perylene as was used in the aqueous dispersions for labeling. The lifetime of the excited state of perylene at 18 °C is 4.3 ns for a 0.5 μM solution in benzene and 6.5 ns for both types of muscle membranes, labeled with 0.5 μM perylene dispersion. These values are nearly identical to those obtained by Rudy and Gitler [8] on erythrocyte membranes. The lower emission intensity in the membranes and the fact that the lifetime in the membranes is longer rather than shorter, most likely means that not all the perylene in the dispersion is taken up into the membranes [7]. If all perylene was taken up in the membrane an apparent quenching could be due to a reduced absorption

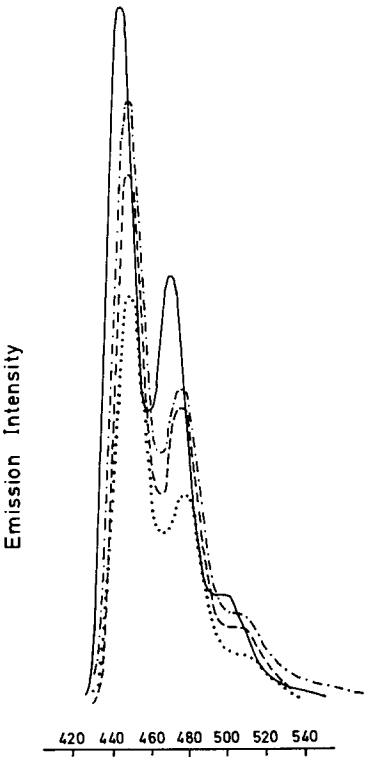

Fig. 1. Emission spectra of perylene for excitation at 400 nm (uncorrected). The membrane preparations were labeled with a 0.5 μM perylene dispersion at a concentration of 0.04 mg protein/ml. The perylene concentration in the organic solvents is 0.5 μM. The spectra of the organic solutions were recorded at a setting of the fluorimeter that was 100 times less sensitive than that used to measure the emission of perylene dissolved in the membranes. (—), acetone; (– – –), benzene; (–.–), dystrophic muscle microsomes; (. . .) normal muscle microsomes.

of light for perylene inside the membrane or due to some of the perylene being in clusters, as in aqueous solution, and not being evenly dispersed. The finding that normal and dystrophic membranes show the same perylene lifetime, however, allows us to use the "α" values (Eqn. 3) for comparing measurements made on these membranes, since in this case, α is directly proportional to the microviscosity (Eqn. 4).

In order to determine the optimal conditions for labeling the membranes and to evaluate the possibility of energy transfer between the perylene molecules, we studied the fluorescence polarization at various concentrations of perylene (1 μM to 0.1 μM). The α value increases slightly with decreasing perylene concentration, but when a difference between membranes was found, the percent change was independent of the concentration of perylene used. The percent change in α with decreasing perylene concentration was variable and ranged between 0 and 30 K. We decided to carry out all experiments at 0.5 μM perylene in order to avoid the high scattering corrections that must be made for labeling at 0.1 μM (see Methods) and that might introduce artifacts. It should be emphasized, however, that the differences

TABLE I

THE VALUES FOR α DETERMINED FOR NORMAL AND DYSTROPHIC ERYTHROCYTE GHOSTS

For the erythrocyte ghosts of 1A and 1B, the blood of two animals was pooled for each preparation. The muscles from four animals were pooled for the muscle microsome preparation.

Membrane	Preparation number	α
Normal	1A	0.355± .028
Normal	1B	0.365± .030
Dystrophic	1	0.340± .020
Normal	2	0.397±0.031
Dystrophic	2	0.369±0.026
Normal	4	*0.405±0.041
Dystrophic	4	*0.385±0.038

* Sensitivity of the instrument was set at 30 for these measurements. All other measurements were done at a sensitivity 10. The difference between the normal and dystrophic ghosts is not significant as tested with the Student's *t*-test.

TABLE II

THE VALUES OF α DETERMINED FOR NORMAL AND DYSTROPHIC MUSCLE MICROSOMES

Membrane	Preparation number	α
Normal Thigh	1	0.334±0.011
Dystrophic Thigh	1	0.271±0.028
Normal Calf	1	0.328±0.023
Dystrophic Calf	1	0.307±0.027
Normal Thigh	2	*0.275±0.017
Dystrophic Thigh	2	*0.245±0.012
Normal Thigh	3	*0.308±0.026
Dystrophic Thigh	3	*0.268±0.023
Normal Thigh	4	*0.329±0.016
Dystrophic Thigh	4	*0.281±0.032

* Sensitivity of the instrument was set on 10 for these measurements. All other measurements were done at a sensitivity of 3. The difference between normal and dystrophic muscle was significant at the 0.5 % level except for the calf muscle in which the difference is significant only at the 2.5 % level.

between normal and dystrophic muscle membranes were found with all concentrations of perylene used.

Table I shows the calculated α values for the normal and dystrophic erythrocyte ghosts. There is a small difference between the two types of membranes; the tendency is for the α value of dystrophic ghosts to be between 5 and 10 % lower than those of the normal ghosts. However, since this difference is of the same magnitude as the standard deviations, more data would be needed to be certain that this difference is significant.

TABLE III

LIPID ANALYSIS OF MEMBRANES ISOLATED FROM NORMAL DYSTROPHIC MOUSE MUSCLE MICROSOMES AND ERYTHROCYTE GHOSTS

Membrane	μmol cholesterol / μg atom phosphorous	μg triglyceride / μg protein	μmol cholesterol / μg protein	μg atom phosphorous / μg protein
Normal ghosts	0.223	0.117	$8.0 \cdot 10^{-6}$	$3.6 \cdot 10^{-5}$
Dystrophic ghosts	0.257	0.104	$7.7 \cdot 10^{-6}$	$3.0 \cdot 10^{-5}$
Normal muscle microsomes	0.179	0.144	$1.1 \cdot 10^{-5}$	$6.2 \cdot 10^{-5}$
Dystrophic muscle microsomes	0.338	0.161	$2.6 \cdot 10^{-5}$	$7.7 \cdot 10^{-5}$

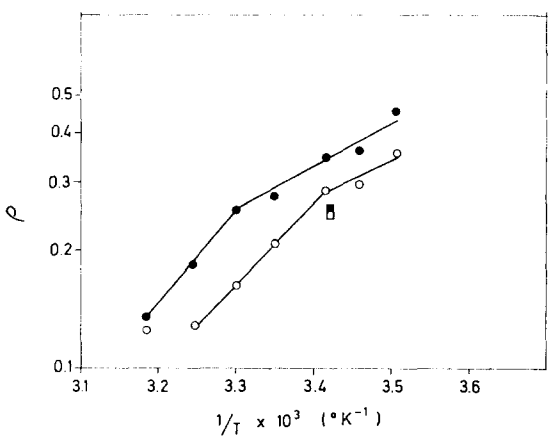

Fig. 2. The effect of temperature on the values of α calculated using muscle microsomes from normal (●) and dystrophic (○) animals. These experiments were done by cooling the membranes to the lowest indicated temperature and then heating them slowly (at each temperature the membranes were allowed to equilibrate for 30 min). After the membranes were heated to the highest temperature used, a few measurements were made at lower temperatures (■) normal, (□) dystrophic. The calculated slope $\pm$ standard deviation for best fit straight lines, using the method of least squares are: normal, 2.34 ± 0.11, $r = 1.00$, and 1.02 ± 0.13, $r = 0.98$; dystrophic, 2.08 ± 0.04, $r = 1.0$ and 1.01 ± 0.29, $r = 0.97$. (The 41 °C point for the dystrophic microsomes was not included in this calculation.) The transition temperatures are about 30 °C for the normal microsomes and 21 °C for the dystrophic ones.

Table II summarizes our results on the muscle microsomes. The α values of the muscle membranes tends to be lower than the erythrocytes. There is, however, a striking difference between the membranes of the dystrophic muscle and normal muscle. The α value of the dystrophic membrane is significantly less than normal. This difference was observed in both sets of muscles studied. There is a possibility, however, that the difference is greatest in the thigh muscle, since in comparisons of muscles from the same animal the thigh muscle showed the largest difference. For this reason we used thigh muscle in all subsequent experiments.

TABLE IV

CHOLINESTERASE ACTIVITY ON MEMBRANES ISOLATED FROM NORMAL AND DYSTROPHIC MICE

Units for each of these assays are mol acetylthiocholine hydrolyzed/min/per g protein.

Membrane	Supernatant	Pellet	Total
Normal ghosts	$1.7 \cdot 10^{-5}$	$9.0 \cdot 10^{-4}$	$2.6 \cdot 10^{-5}$
Dystrophic ghosts	$8.8 \cdot 10^{-4}$	$1.1 \cdot 10^{-5}$	$2.0 \cdot 10^{-5}$
Normal muscle microsomes	$7.1 \cdot 10^{-5}$	$9.5 \cdot 10^{-5}$	$1.6 \cdot 10^{-4}$
Dystrophic muscle microsomes	$2.5 \cdot 10^{-5}$	$6.9 \cdot 10^{-5}$	$9.4 \cdot 10^{-5}$

In order to evaluate the possible cause of the low microviscosity in the dystrophic muscle membranes we have determined the cholesterol, triglyceride and phospholipid content of the membranes used for the α values (Table III). This table shows no difference between normal and dystrophic ghosts whereas, there are differences between the dystrophic and normal muscle microsomes. The largest difference is in the cholesterol level which is about 2-times higher in the dystrophic microsomes compared to normal. There is not a simple relationship, however, between the cholesterol content and microviscosity for the four sets of membranes. The triglyceride values, on the other hand, vary in an inverse fashion with the microviscosity measurements, i.e. the higher the triglyceride value the lower the microviscosity. Our qualitative studies on the major classes of phospholipids, i.e. lysolecithin, sphingomyelin, lecithin, phosphatidylserine and phosphatidylethanolamine, showed no differences between the muscle microsomes.

The temperature dependence of the fluorescence polarization is shown in Fig. 2. It is clear from these data that the α values are different in the two sets of microsomes at all temperatures below 35 °C. The lines drawn in this figure were calculated using the method of least squares with the assumption that there is a break indicating a transition at 30 °C for the normal microsomes and 21 °C for the dystrophic microsomes. The data shown in this figure cannot be rigorously interpreted because a sufficiently large number of temperatures was not studied. Corroboration of a transition temperature was obtained using light scattering and a difference in this temperature was found for the two sets of membranes. The transition temperature measured by light scattering, however, did not agree with the values obtained with perylene. Values of about 32 °C for the normal microsomes were obtained and about 29 °C for the dystrophic ones. Additional work, therefore, is required before a definitive statement can be made about the molecular nature of these transitions. From the studies reported here it is clear, however, that transitions do occur in the microsomes and that there are differences between normal and dystrophic microsomes.

In an effort to determine if the changes in α values could be correlated with the biochemical changes that are known to occur in the dystrophic muscle, we have measured the acetylcholinesterase activities in the four sets of membranes (Table IV). As has been reported by other authors [31, 32] the acetylcholinesterase activity in both the ghosts and muscle microsomes from dystrophic animals is lower than that in the normal membranes. There is not a simple linear relationship, however, between the acetylcholinesterase activities and the "α" values shown in Tables II and III.

DISCUSSION

Our results clearly show that there is a difference between the α-values of the microsomes isolated from normal and dystrophic muscle. In contrast, the erythrocytes from normal and diseased animals have nearly identical values. Since α-changes are only observed in the tissue that is primarily affected by the dy gene this quantity seems to be, in fact, an important parameter linked to the functional state of muscle. In this context, we might mention that we did one experiment using mice that were 10-weeks old, in which the disease has proceeded much further than in the animals used in the study reported in this paper. In this case, the erythrocyte ghosts also had about 20 % lower microviscosity values than the controls. Since animals of 10 weeks of age are in very poor physical condition, it might be that membrane effects develop in tissues other than the muscle at the later stage of the disease. Because we were interested in identifying the primary cause of the disease, we chose to use young animals in an effort to avoid complicationsdue to secondary changes in the health of the older animals.

There are several reports in which the microviscosity of various biological membranes has been estimated by using fluorescence depolarization of hydrocarbon embedded in the membranes. [7, 8, 19]. Unfortunately, however, it is difficult to make comparisons between these reports since material from different animal species was used, as well as, different fluorescent probes. In addition, in those studies using perylene [8, 19], these authors have not reported the values of $C(r)$, see Methods, they have used to calculate microviscosity. It is impossible therefore to determine whether the discrepancy in the value for the erythrocyte membranes between the two reports is due to different $C(r)$ values. For the same reason we can compare our data only with the study by Rudy and Gitler who reported the polarization values for perylene in human erythrocytes. These polarization values agree with our measurements on mice erythrocytes to within 30 %.

The results from our lipid analysis (Table III) agree with previous reports in that there is a large increase in the cholesterol levels of the microsomes from the muscle from *dy/dy* mice [16, 17, 33]. Furthermore, the finding that there is no change in the lipid composition of the erythrocyte membranes from dystrophic mice is in line with results by Shull and Alfin-Slater [16], that an increase in cholesterol is only found in skeletal muscle, but not in brain, liver, kidney, lung or heart.

A straightforward interpretation of our fluidity data on the basis of the changed cholesterol content, however, is not possible. When comparing cholesterol content and microviscosity, we find a decreased microviscosity at an increased cholesterol level, in contrast to observations by Shinitzky and Inbar [7]. On the other hand, our observation that the transition temperature in the dystrophic microsomes is lowered compared with the normal ones may be due to the increased cholesterol content [34].

Quantitative studies on the lipid composition of muscle microsomes from normal and dystrophic mice [17] have shown changes in the phospholipid content, e.g. sphingomyelin, phosphatidylserine and phosphatidylethanolamine increased and lecithin plus phosphatidylcholine decreased, as well as small changes in the fatty acid acyl groups of the phospholipids. These authors, however, did not find that dystrophic membranes had lipids of longer chain length or a higher degree of unsaturation; two properties known to decrease membrane viscosity [35, 36]. The only trend in our

lipid analysis which qualitatively changes in the same direction as the microviscosity of the four groups of membranes studied is the triglyceride content.

More knowledge about the complex lipid/protein mixtures which occur in biological membranes will be necessary before a molecular interpretation of our data will be possible. Membrane proteins are not generally thought to play a significant role in controlling the fluidity of the membrane lipids [37]. On the other hand, Shinitzky and Inbar [7] found that the microviscosity of protein free liposomes prepared from lymphocytes was about one-half the value obtained for intact membranes. A general problem with microviscosity measurements using fluorescent hydrocarbons is that these probes may not be located in a homogeneous lipid phase of the membrane [11, 12]. On the basis of fluorescence intensity Papahadjopoulos et al. [10] have suggested that perylene may have two binding sites, one at the lipid interior and the other at the aqueous-membrane interface. As far as our measurements are concerned, the fact that the perylene lifetime is the same in both normal and dystrophic muscle microsomes suggests that there is no dramatic change in the binding sites of the probe between the two sets of muscle microsomes. We suggest, therefore, that the differences in α values between normal and dystrophic muscle microsomes do, in fact, reflect a difference in microviscosity. It is of course possible that multiple lipid domains exist in the membranes [11], and a phase separation may be present in the dystrophic microsomes which results in the perylene binding preferentially in the more fluid phase. We cannot conclude that the total microviscosity of the membrane has decreased. Similarly, since a change in temperature may change the location of perylene*, we hesitate to define the temperature induced transition we have observed as being due exclusively to a change in microviscosity of the lipid phase.

While our investigations were in progress, a study on the microviscosity of membranes from normal and dystrophic chickens was published by Sha'afi et al. [19]. In striking contrast to our results, these authors found a large difference in microviscosity between all tissues studied (muscle, liver and erythrocyte ghosts) and an increase of microviscosity rather than a decrease in the dystrophic membranes. Apart from the fact that the direction of the microviscosity changes is opposite from our results, in the mouse the only microviscosity or lipid changes observed were in muscle and not in erythrocytes (this study) or several other organs [16]. We have no explanation other than that this disagreement may be due to the different animal species and possibly to a different stage of the disease at which the studies were carried out. Insofar as dystrophic muscle resembles denervated muscle, chicken and rat muscle do behave differently upon denervation [38, 39] so that there is some reason to

* Since we have not determined the temperature dependence of the fluorescence lifetime, we cannot be sure that the observed changes in the mobility of the probe directly reflect changes in microviscosity. We did not feel confident to derive the temperature dependence of the lifetime from the temperature dependence of the steady-state fluorescence [5] since we could not rule out that the observed increase in fluorescence with increasing temperature is due to more perylene being taken up into the membranes. (Attempts to separate prylene dispersions from the membranes were unsuccessful, since ultrafiltration or ultracentrifugation methods which allow the separation of membranes from the solution also removed most of the perylene from control dispersions.) The increase of perylene fluorescence with increasing temperature resembled that observed by Papahadjopoulos et al. [10] in synthetic membranes except that the transition occurred over a much broader temperature range (20 °C) and that the limiting values at high and low temperatures were not defined well enough to derive the transition temperature from the midpoint.

believe that the neuromuscular systems of these species may react differently to the disease. The direction of microviscosity change observed by us is, however, in line with the studies reported by Butterfield et al. [3, 4], who, by the use of an ESR technique found a decrease of microviscosity in erythrocyte ghosts from humans suffeing from myotonic dystrophy.

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