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ORDER MEASUREMENTS IN PLASMA MEMBRANES FROM DUCHENNE DYSTROPHY FIBROBLASTS

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Plasma membranes have been isolated using different methods from Duchenne dystrophy and control human skin fibroblasts. Fluorescence techniques were utilized to resolve the rotational properties and the degree of hindered rotation of the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene in the membranes. Under specific conditions of fibroblast processing and membrane fractionation, plasma membranes from Duchenne fibroblasts showed significantly less order ($0.0125 > P < 0.0025$) and less hindrance to probe rotation than membranes from control fibroblasts. The order differences did not seem to be the result of heterogeneity in the membrane environment sampled by the probe. The frequency dependence of the fluorescence lifetime for diphenylhexatriene indicated no measurable contribution by a short lifetime component. Analysis of diphenylhexatriene rotation in the plasma membranes using the 'wobbling-in-cone' theory suggested that both the angle of probe rotation (θ_c) and the rotational rate (D_w) were important parameters in understanding the variations between Duchenne and control membranes at 16, 22 and 30°C. Electron spin resonance studies with 5′-doxylstearic acid at 25°C confirmed our fluorescence results. The segmental motion exhibited by the spin label revealed less order in the Duchenne membranes.

Introduction

The molecular defect in the pathogenesis of muscular dystrophy has been suggested to be a genetic alteration in a structural protein or enzyme associated with membrane function [1,2]. In support of this hypothesis, a significant number of membrane-related differences have been documented in myotonic and Duchenne dystrophy cells and tissues, including erythrocytes [3,4], cultured fibroblasts [5,6], sarcoplasmic reticulum [7], lymphocytes [8], muscle fibers [9] and myoblasts [10].

Electron spin resonance (ESR) was one of the first physical techniques utilized for examining human dystrophy tissue. Butterfield et al. [11], using a nitroxide-labeled fatty acyl methyl ester in their ESR measurements of myotonic erythrocytes, found a less ordered and less polar region in the myotonic samples relative to the controls. The differences reported by Butterfield et al. [11] have been difficult to reproduce in the laboratories of Gaffney et al. [12] and Chalikian and Barchi [13]. Recently, Butterfield [14] reported that increased fluidity in myotonic erythrocytes does not occur in fresh samples of erythrocytes but only after a 2-day aging period.

Electron spin resonance studies have also been conducted using erythrocytes from Duchenne dystrophy patients. Significant differences in the de-

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Abbreviations: SDS, sodium dodecyl sulfate; F-12, Ham's F-12 medium; 5′-nitroxide stearic acid, 5-doxylstearate.

gree of order relative to controls have been observed when the nitroxide spin label was located close to the terminal methyl group of the fatty acid [15]. Wilkerson et al. [16] have used saturation transfer ESR with a fatty acid spin label and observed differences in probe partitioning or diffusion in the lipid-protein environment between Duchenne and control erythrocytes. Finally, Dellantonio et al. [17] using stearic acid and maleimide derivative spin labels reported the Duchenne erythrocyte membrane to be more 'fluid' with the more dramatic differences being seen with the maleimide spin label.

Few investigations have been reported on the degree of order in membranes of cells cultured from biopsies of Duchenne patients. Prendergast [18], in preliminary fluorescence studies, has observed no differences in measurable order between intact cultured myotubes from Duchenne and control patients. Likewise, we have observed no consistent differences in membrane order with the fluorescent probe, diphenylhexatriene, when examining homogenized fibroblasts or intact lymphocytes from Duchenne and control patients (Shaw, J.M., Goldsmith, B.M. and Gruemer, H.D., unpublished data). Aware of the difficulties in working with whole cell preparations, we have examined plasma membranes from human skin fibroblasts and confined the order measurements to less heterogeneous populations of cellular membranes. Previous investigations of different cells and tissues have suggested that the effect in Duchenne dystrophy may be associated with the plasma membrane [8,9,19–21]. Furthermore, only few studies are available in the literature concerning possible defects in skin fibroblasts from Duchenne dystrophy patients [22,5,6,23,24]. Therefore, in the present paper, we have performed polarization, lifetime and polarized lifetime fluorescence measurements with the fluorescent probe, diphenylhexatriene, using plasma membranes isolated under defined conditions from Duchenne and control skin fibroblasts. Our results have been extensively analyzed for the degree of order and suggest a less ordered environment in the hydrophobic domain of Duchenne plasma membranes.

A preliminary account of this work has been presented [65].

Materials and Methods

Superficial skin tissue containing the dermis was obtained from either an immediate site on the upper or lower leg, or by needle biopsy from the forearm or shoulder. The skin biopsies were taken from 15 Duchenne dystrophy patients between the ages of 2 and 18, and 15 control individuals representing similar ages. Two samples, one Duchenne and one control, were cultured from skin tissue taken from abortuses at about 20 weeks gestation. Unless specified, a control individual is one who possesses no known neuromuscular disease. Ham's F-12 medium (F-12) with L-glutamine but without sodium bicarbonate was obtained in powdered form from Flow Laboratories and additional components added according to Konigsberg et al. [25].

The fluorescence compound, 1,6-diphenyl-1,3,5-hexatriene was purchased from either Aldrich Chemical Co. or Fluka Chemicals and used without further purification. Excitation and emission spectra of diphenylhexatriene in cyclohexane was virtually identical to zone-refined diphenylhexatriene (a gift from Dr. Y. Barenholz). Spin-labeled derivatives of stearic acid, 5-doxylstearate and 12-doxylstearate were obtained from Syva Associates, Palo Alto, CA. Tetrahydrofuran and acetone were of spectral grade and in some cases the tetrahydrofuran contained an antioxidant. Horse serum was purchases from Gibco and chick embryo extract prepared as described by Konigsberg et al. [25].

Growth of fibroblasts and preparation of membranes

The Duchenne and control skin fibroblast cells were cultured to confluency in gelatin-coated Falcon tissue culture plates (100 mm) under identical conditions utilizing Ham's F-12 medium containing 2.5% chick embryo extract and 15% horse serum. For each experiment, 20 plates containing cells between the 2nd and 10th passage were (after removal of the medium) rinsed three times with cold, modified phosphate-buffered saline, pH 7.4. Modified phosphate-buffered saline consists of 0.9 mM $CaCl_2$/1.5 mM KH_2PO_4/2.7 mM KCl/0.5 mM $MgCl_2$/0.14 M NaCl/0.85 mM Na_2HPO_4/1 mM EDTA, pH 7.4, prepared using deionized water with no precautions taken to remove or

exclude air during preparation. Cells were scraped from dishes with a rubber policeman and lysed in modified phosphate-buffered saline using a Parr nitrogen bomb then centrifuged at 153000 × *g* for 1 h in a SW-41 rotor. The pellet was taken up in 10% (w/v) sucrose in modified phosphate-buffered saline and overlayed on top of a discontinous density gradient consisting of 3 ml each of 10, 30, 48 and 60% (w/v) sucrose in modified phosphate-buffered saline. Centrifugation was performed at 106000 × *g* for 2 h in a SW-41 rotor [26]. Membranes banding at the 10/30 interface (plasma-membrane enriched), 30/48 interface (microsomal-enriched) and 48/60 interface (nuclear-enriched) were placed in 0.25 inch dialysis tubing and dialyzed against modified phosphate-buffered saline, pH 7.4, for periods from 3 to 12 h at 4 °C. In place of dialysis, membrane fractions could also be diluted with appropriate buffer and recentrifuged at 153000 × *g* for 2 h. Membrane pellets were dispersed by using 3–5 strokes with a teflon pestle.

Several additional procedures were also tested for the fractionation of plasma membranes from skin fibroblasts. These included the borate-EDTA lysis technique of Thom et al. [27] and a modified procedure of Thom et al. [27] which involves lysing cells by nitrogen cavitation, centrifugation at 153000 × *g* for 2 h followed by recentrifugation of the resuspended pellet on a 35% sucrose solution in modified phosphate-buffered saline at 106000 × *g* for 2 h. In some cases, the two-phase polymer method of Brunette and Till [28] was utilized for preparing plasma-membrane-enriched membrane fractions.

Marker enzyme assays

5′-Nucleotidase, a plasma membrane marker, was assayed using [^{3}H]adenosine-5′-monophosphate according to Avruch and Wallach [29]. The 5′-nucleotidase inhibitor, α,β-methyleneadenosine 5′-diphosphate, was utilized in the nucleotidase assays. Cathepsin B, a marker endopeptidase for lysosomes was fluorimetrically assayed with a methylcoumarylamide peptide substrate [30]. The mitochondrial membrane marker, succinate dehydrogenase was assayed using succinate, phenazine methosulfate and 2,6-dichlorophenolindophenol [31]. The microsomal enzyme, NADPH cytochrome *P*-450 reductase, was measured using cytochrome *c* as oxidizing agent [32]. The extinction coefficients were 19.1 $mM^{-1} \cdot cm^{-1}$ at 600 nm for dichlorophenolindophenol and 21 $mM^{-1} \cdot cm^{-1}$ for cytochrome *c*.

Preparation of membranes for fluorescence and ESR measurements

Plasma membranes (75–200 μg protein) were placed in a cuvette at a total volume of 2–2.3 ml. Diphenylhexatriene dissolved in tetrahydrofuran or acetone was either injected into the membrane suspension, or the membrane diluted with buffer containing tetrahydrofuran or acetone plus diphenylhexatriene. The percentage tetrahydrofuran never exceeded 0.075% in buffer solutions. The diphenylhexatriene/total membrane lipid molar ratio was maintained between 1/300 to 1/600. Partition of the probe into membranes was allowed to occur at room temperature and in some cases on ice or at 37°C protected from light for 0.5–1 h. During examination in the fluorimeter a small teflon-coated stirring bar or occassional swirling was performed although we experienced no problems with plasma membrane aggregation or settling in the cuvette.

Fluorescence measurements were performed at the lower temperature of 16°C then increased to higher temperatures with the reverse procedure performed in some cases. No evidence of a 'phase transition' was observed in any of the preparations between 14 and 37°C. A concentrated and diluted sample of the plasma membrane was examined. The concentrated sample was essential for lifetime (τ) and polarized lifetime measurements, whereas the diluted sample was critical for the polarization or anisotropy value used in further calculations. Light scattering depolarization was not found to be a critical problem in the examination of the fibroblast plasma-enriched membranes. Light scattering depolarization, however, can be especially severe in certain lipid-enriched membranes (Ref. 33; Shaw, unpublished data).

Fluorescence measurements

The majority of fluorescence measurements were conducted on an SLM subnanosecond fluorimeter (4800 series). Electronic data output was entered directly into a 9815 Hewlett-Packard calculator for

programming. Steady-state polarization measurements (P) were made after placement of a polarizer fixed parallel in the exciting beam and two polarizers oriented parallel ($\|$) and perpendicular ($\perp$) at right angles to the sample in T-format design. The excitation monochromator was set at 355 nm from a xenon lamp source when examining diphenylhexatriene and fluorescence emission intensity (I) above 410 nm examined by use of 3-72 or 3-73 Corning filters. The ratio output ($\|/\perp$) from the photomultiplier tubes was recorded on a ratio digital voltmeter after rotation of the exciting polarizer from 90° back to 0° for polarization values corrected for instrumental fixed optics.

$$P = \frac{I_{\|} - I_{\perp}}{I_{\|} + I_{\perp}} \tag{1}$$

Polarization was converted to anisotropy values (r) by the equation $r = 2P/3 - P$. Sample temperature was monitored by a YSI Telethermometer, and thermocouple immersed in an additional cuvette. Temperature variation of the turret and samples was accomplished by a Lauda bath at the rate of 1°C/1–2 min.

Fluorescence lifetimes were measured by the phase modulation technique with the exciting light modulated in most cases at 18 MHz [34]. 6 MHz and 30 MHz frequencies were used in addition for examining the frequency dependence of the lifetime. After excitation by the sinusoidally-modulated light the fluorescence emission intensity was phase shifted and demodulated relative to the exciting light. This process was dependent upon the lifetime (τ) of the fluorophore. The phase and modulation values were measured relative to a glycogen scattering solution. The phase lifetime (τ_p) was calculated as $1/\omega \tan \psi$ where 'ω' is the circular modulation frequency of the exciting light ($1.131 \cdot 10^8$ radians/s for 18 MHz). ψ represents the difference in the phasemeter reading in degrees between diphenylhexatriene and glycogen. The modulation lifetime (τ_m) was calculated as $(1/\omega)(1/D^2 - 1)^{1/2}$ where 'D' is the ratio of diphenylhexatriene modulation to glycogen modulation. Polarization effects on the lifetime were corrected by orienting the emission polarizer at about 55° as described by Lakowitcz et al. [35]. The lifetime data represent actual data, uncorrected for 'color delay' which results from variations in the kinetic energy of photoelectrons with shift in the excitation wavelength. Severe 'color delay' will normally lead to an unusually high phase lifetime relative to the modulation lifetime (see Table IV and Ref. 36). 'Color delay' was not readily apparent in the lifetime values for diphenylhexatriene in the fibroblast membranes. The rotational relaxation time (ρ) was calculated from the Perrin equation $1/P - 1/3 = (1/P_0 - 1/3)(1 + 3\tau/\rho)$. The value used for P_0, the polarization at low temperature, was 0.492 [37].

Differential phase measurements were made as described in detail by Lakowicz et al. [35] in which differences in phase angles between the polarized ($\|$) and ($\perp$) components were determined. Phase angle differences were printed out directly by the calculator when both polarizers were oriented ($\|$), ($\|$); then ($\|$), ($\perp$). Subtraction of the two phase angle difference readings gives the instrumental corrected phase angle difference (Δ) which is used to determine the differences in lifetime between ($\|$) and ($\perp$) components (τ_d). Tan Δ is calculated from τ_d by the equation $\tan \Delta = \omega \tau_d$. After measurement of '$P$' (and therefore '$r$'), '$\tau$', and tan Δ, the rotational rate, R, in radians/s can be determined. In solving the quadratic equation for 'R' (see detailed equations in Ref. 35), the positive root was utilized. With 'R', and 'r' determined, the limiting anisotropy (r_∞), can be calculated by the equation:

$$r_\infty = r + \frac{r - r_0}{6R\tau} \tag{2}$$

The anisotropy value in the absence of depolarizing rotations, r_0, was 0.392 [36]. 'r_∞', the limiting anisotropy at times long relative to the lifetime of diphenylhexatriene is a function of the parameter for angular distribution (θ) of the probe.

$$\frac{r_\infty}{r_0} = \frac{(3\cos^2\theta - 1)}{2} \tag{3}$$

Values of 'r_∞' close to 0.0 suggest little hindrance to probe rotation and give an angular distribution approaching 54.47° (at $r_\infty = 0$). Higher values of 'r_∞' suggest restricted and hindered rotation of diphenylhexatriene.

'Wobbling-in-cone' theory [38,39] was utilized

for determining two parameters, D_w, the wobbling diffusion constant and θ_c, the cone angle of diphenylhexatriene rotation. The limiting anisotropy, r_∞, determined by steady-state and differential phase measurements was utilized to solve for θ_c by the following equation:

$$\frac{r_\infty}{r_0} = \left[\tfrac{1}{2}\cos\theta_c\right)(1+\cos\theta_c]^2 \quad (4)$$

The relaxation time, ϕ, determined by differential phase measurements as $\phi = 1/6R$ [79], θ_c, r_∞ and r_0 were applied to the following equation for solving D_w (ns^{-1}) where $x = \cos\theta_c$ [39,40].

$$\frac{D_w\phi(r_o - r_\infty)}{r_o} = -x^2(1+x)^2\frac{\{\ln[(1+x)/2]+(1-x)/2\}}{[2(1-x)]} + \frac{(1-x)(6+8x-x^2-12x^3-7x^4)}{24} \quad (5)$$

Electron spin resonance measurements of plasma membrane fractions

Electron spin resonance has been performed at 9.5 GHz on a Varian E-9 spectrometer with variable temperature control. A spin-labeled derivative of stearic acid, 5-doxylstearate (5′-nitroxide stearic acid) was tested. The mobility and differential anisotropic rotation about the long axis of the fatty acid spin label were estimated in two ways. The outer one-half line width at one-half height of the low field peak (Δl) was determined as described by Mason et al. [41]. Greater Δl values indicate a greater degree of isotropic motion. The maximum outer hyperfine extrema could be resolved for 5-doxylstearate and $T_{\|}$ and $T_{\perp}$ measurements were made and an order parameter (S) calculated as described by Gaffney [42].

Miscellaneous methods

Protein was determined using bovine serum albumin as a standard by the method of Lowry et al. [43] modified by the inclusion of Triton X-100 for membrane solubilization. Lipid phosphorus was estimated by the Bartlett method [44] and cholesterol measured using the enzyme, cholesterol oxidase [45].

Total lipids from membranes were extracted with chloroform/methanol by the procedure of Bligh and Dyer [46]. The total lipid fraction was subjected to trans-esterification with methanolic sodium methoxide overnight at room temperature. Methyl esters were extracted with petroleum ether and analyzed on a Varian Model 3700 gas chromatograph using a 10% SP-2340 Supelco column. Polyenoic fatty acids were identified primarily by their retention times relative to available standards.

Electron microscopy was performed on the membranes as described by Kartner et al. [26] with uranyl acetate and lead citrate staining of the thin sections as described by Stempak and Ward [47] and Venable and Coggeshall [48]. Electron micrographs were taken on a Hitachi 11-E electron microscope.

Significance testing of the fluorescence data was accomplished by the two-tailed t-test between Duchenne and control sample means with population variation not known [49]. The Duchenne and control fibroblasts were generally cultured and analysed as a pair under identical conditions.

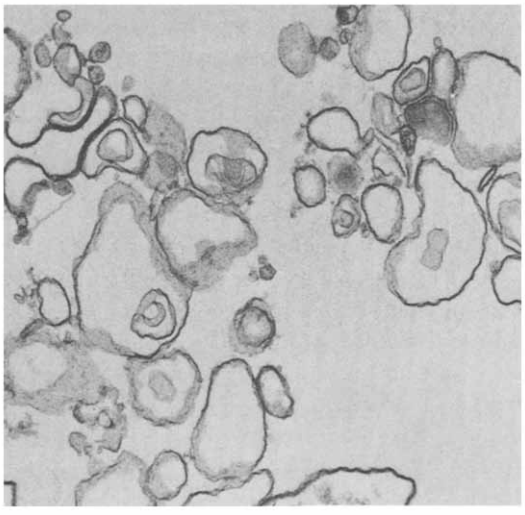
Results

Effect of membrane fractionation technique on the fluorescence polarization of diphenylhexatriene

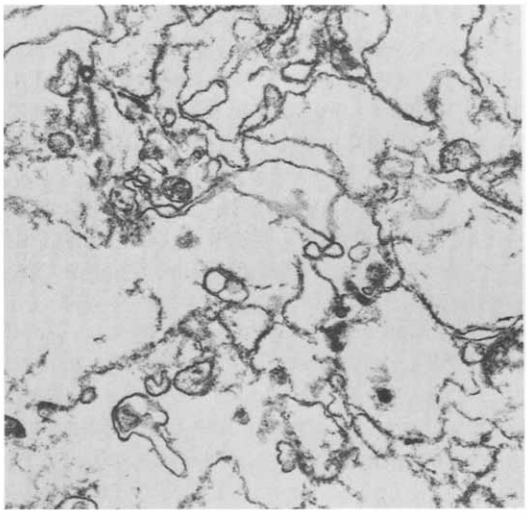
Our first objective was to find a rapid and reproducible procedure for preparing plasma membranes from the cultured fibroblasts. Three different techniques for obtaining plasma membranes have been examined, namely the two-phase polymer of Brunette and Till [28], the sucrose cushion of Thom et al. [27] and the sucrose-step gradient of Kartner et al. [26].

The two-phase polymer method gave the most contaminated plasma membrane preparation as evidenced by electron microscopy of thin sections which revealed mitochondrial and microsomal fragments. Succinate dehydrogenase and NADPH cytochrome c reductase activities in plasma-enriched membranes were slightly elevated above the total cell homogenate. [^{125}I]Lactoperoxidase surface labeling of the fibroblasts failed to show more than a 2-fold enrichment of ^{125}I above the total cell homogenate. 5′-Nucleotidase was apparently inactivated by the Zn^{2+} used in the procedure.

The sucrose cushion technique of Thom et al. [27] employs borate-EDTA lysis of fibroblasts fol-



SUCROSE STEP GRADIENT



SUCROSE CUSHION

Fig. 1. Electron microscopy of plasma membrane derived from cultured fibroblasts. Plasma membranes were prepared from fibroblasts by two procedures. The sucrose-step gradient technique was used with cells being disrupted by nitrogen cavitation and the sucrose cushion technique with fibroblasts having been disrupted by borate-EDTA. Membranes were fixed, stained and sectioned by standard procedures and electron micrographs taken. The micrographs represent a magnification of 44200× for the sucrose-step gradient membranes and 19550 × for the sucrose cushion membranes.

lowed by differential centrifugation then recentrifugation of total membranes on a 35% sucrose cushion. In some cases, the borate-EDTA lysis step has been replaced with nitrogen cavitation for cell disruption. Electron microscopy of the plasma membranes showed a mixture of large fragments and small membrane vesicles with some contamination from intracellular organelles (Fig. 1). 5′-Nucleotidase activities were elevated about 11-fold above the homogenate. We have not used the sucrose cushion technique as the method of choice in our studies for two main reasons. First, contamination of the plasma membranes with intracellular membrane fragments is apparent in Fig. 1. Second, in using a 35% sucrose cushion only plasma membranes can be collected, with other cellular membranes being pelleted during centrifugation.

The remainder of our studies have been conducted using a sucrose-step gradient technique originally developed by Kartner et al. [26] for use with human skin fibroblasts. In addition to plasma membranes, intracellular membrane fractions can also be collected from the gradient. Plasma membranes isolated by the sucrose-step gradient technique have been analyzed for morphology and marker enzymes. Transmission electron microscopy revealed vesicle, sac-like structured bilayer membranes relatively free of microsomal or mitochondrial fragments (Fig. 1). Membrane or organelle marker enzymes were assayed for four Duchenne and four control sample preparations (Table I). 5′-Nucleotidase activities for plasma

TABLE I

MARKER ENZYME ACTIVITIES FOR MEMBRANES ISOLATED BY THE SUCROSE-STEP GRADIENT TECHNIQUE

The assays represent the average of four Duchenne and four control sample preparations. The activity for each respective enzyme is nmol [^{3}H]5′-AMP hydrolyzed/min per mg protein (5′-nucleotidase), nmol cytochrome *c* reduced/min per mg protein (NADPH cytochrome *c* reductase), nmol dichlorophenolindophenol reduced/min per mg protein (succinate dehydrogenase) and nmol 7 amino-4-methylcoumarin cleaved/min per mg protein (cathepsin B).

	Homogenate	Plasma membrane
5′-Nucleotidase	480.0 ± 80.0	4200.0 ± 50.0
NADPH cytochrome *c* reductase	1.90 ± 0.7	0.8 ± 0.7
Succinate dehydrogenase	8.70 ± 2.3	2.7 ± 1.9
Cathepsin B	4.90 ± 1.2	2.2 ± 1.1

TABLE II

FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE IN PLASMA MEMBRANES PREPARED BY DIFFERENT TECHNIQUES

Fibroblasts were collected, disrupted and membranes fractionated using modified phosphate buffered saline. The technique for fibroblast disruption was either nitrogen cavitation or osmotic lysis. Fluorescent polarization experiments were initiated at 36°C. Polarization values for each technique represented a separate Duchenne and control pair examined twice for the two-phase polymer, twice for the sucrose-step gradient and three times for the sucrose cushion technique. A total of four Duchenne fibroblasts from patients ages 2–14 years were examined and four controls ages 0.2–18 years. Fibroblasts were all <10 passages and Ham's F-12 medium was utilized. The level of significance is indicated by the probability, 'P' value or n.s. (not significant) as determined by a two tailed t-test.

	Method of membrane preparation		
	Two-phase polymer	Sucrose cushion	Sucrose-step gradient
16°C Control	0.272 ± 0.025	0.295 ± 0.024	0.338 ± 0.018
Duchenne	0.252 ± 0.038	0.255 ± 0.017	0.302 ± 0.012
	n.s.	$P < 0.025$	$P < 0.05$
21°C Control	0.252 ± 0.021	0.273 ± 0.017	0.317 ± 0.013
Duchenne	0.233 ± 0.036	0.245 ± 0.019	0.289 ± 0.010
	n.s.	$P < 0.05$	$P < 0.025$
27°C Control	0.222 ± 0.027	0.249 ± 0.020	0.284 ± 0.016
Duchenne	0.209 ± 0.037	0.213 ± 0.022	0.254 ± 0.008
	n.s.	$P < 0.025$	$P < 0.025$
36°C Control	0.190 ± 0.022	0.213 ± 0.011	0.268 ± 0.017
Duchenne	0.175 ± 0.027	0.154 ± 0.014	0.230 ± 0.014
	n.s.	$P < 0.0005$	$P < 0.025$

membranes were about 8-fold above homogenate values with the remaining three enzyme markers showing reduced activities in the plasma membranes relative to homogenates. Plasma membranes isolated from the cultured fibroblasts showed a fatty acid composition consisting of 37% saturated, 23% monoenoic, 19% dienoic and 22% polyenoic fatty acids. Cholesterol levels in the membranes revealed values of approx. 190 μg cholesterol/mg membrane protein.

Fluorescence polarization results of plasma membranes isolated by the two-phase polymer method are shown in Table II. Although Duchenne membranes showed lower polarization values than control membranes the differences were not significant. The lack of significance was due mainly to the high variability in the polarization values illustrated by the standard deviations in Table II. Fluorescence polarization results with membranes prepared by the sucrose-cushion technique are presented in Table II. The results represent our first observations of significant differences ($P < 0.05$) between Duchenne and control plasma membranes at temperatures from 16 to 36°C. The Duchenne plasma membranes revealed a less ordered lipid-protein domain since polarization values were decreased relative to control membranes. Polarization differences for membranes isolated by the sucrose-step gradient technique are significant ($0.05 > P < 0.025$) at all temperatures with the Duchenne plasma membrane again revealing a less ordered lipid domain relative to control samples. In summary, the results shown in Table II illustrate that two different techniques for lysing fibroblasts and preparing plasma membranes (sucrose cushion and sucrose-step gradient) both reveal statistically significant polarization differences between Duchenne and control samples. Furthermore, each cell fractionation-membrane isolation procedure gives different polarization values with the highest values being associated with membranes prepared by the sucrose-step gradient technique. Lastly, the error between sample measurements was smallest with the membranes prepared by the sucrose step-gradient technique.

Rotational properties of diphenylhexatriene in plasma membranes

Having chosen the sucrose-step gradient as our method of choice, we next examined a larger number of samples. Furthermore, polarization (P), fluorescence lifetimes (τ), and differential polarized phase measurements were performed on all samples. With these measurements we could apply relationships developed by Weber [50] and Lakowicz et al. [35] to determine the rotational properties of diphenylhexatriene in the hydrophobic domains of the plasma membranes. In addition, we have applied an earlier approach for determining fluorescent probe rotation, the Perrin rotational relaxation time [51], as well as the recent wobbling-in-cone analysis of diphenylhexatriene rotation modeled by Kinosita et al. [38,39]. It is important to present our results analyzed by a

TABLE III

ROTATIONAL PROPERTIES OF DIPHENYLHEXATRIENE IN PLASMA MEMBRANES

Fibroblasts were scraped from dishes and disrupted by nitrogen cavitation in the presence of phosphate-buffered saline (Ca^{2+}/Mg^{2+}/EDTA) buffer. The total membranes were fractionated on a sucrose-step gradient and the plasma-enriched membranes collected. Fluorescence measurements were initiated at 16°C. Nine Duchenne samples were examined from fibroblasts representing passages 4–9 with two at passage 17. The ages of the Duchenne patients were between 2 and 18 years with one Duchenne sample representing an abortus at 20 weeks gestation and confirmed for Duchenne dystrophy by muscle biopsy and elevated creatine kinase levels. The nine control samples were examined from fibroblasts at passages 3–10 with one at passage 20. The ages of the controls included seven between 4 and 14 years, one at 0.2 years and one abortus at approx. 20 weeks gestation. Ham's F-12 containing 2.5% chick embryo extract and 15% horse serum represented the culture medium in all cases. 'n.s.' indicates not significant at 'P' values greater than 0.05.

Fluoresence parameter	16°C		22°C		30°C	
	Control	Duchenne	Control	Duchenne	Control	Duchenne
Polarization (P)	0.328 ± 0.015	0.309 ± 0.008 $P < 0.0025$	0.306 ± 0.016	0.289 ± 0.008 $P < 0.0050$	0.263 ± 0.016	0.248 ± 0.014 $P < 0.0125$
Hindered anisotropy (r_∞)	0.230 ± 0.011	0.212 ± 0.008 $P < 0.0005$	0.210 ± 0.012	0.195 ± 0.010 $P < 0.0050$	0.172 ± 0.008	0.164 ± 0.014 $P < 0.05$
Average angle of distribution (θ) degrees	31.9 ± 1.1	33.6 ± 0.8 $P < 0.0025$	33.8 ± 1.3	35.4 ± 1.0 $P < 0.0025$	37.7 ± 0.77	38.5 ± 1.4 $P < 0.05$
Fluorescence lifetime (τ) ns	9.74 ± 0.95	10.10 ± 0.80 n.s.	9.71 ± 1.09	9.99 ± 0.86 n.s.	9.07 ± 1.24	9.53 ± 0.85 n.s.
Rotational relaxation time (ρ) ns	49.1 ± 6.2	42.8 ± 5.1 $P < 0.0125$	40.4 ± 5.1	35.1 ± 5.7 $P < 0.025$	26.4 ± 2.1	24.7 ± 4.3 n.s.

number of different approaches for comparative purposes by other investigators using different instrumentation.

Table III gives the values of the various fluorescence parameters (P, r_∞, θ, τ and ρ) which were used to determine the rotational properties of diphenylhexatriene in the hydrophobic domains of plasma membranes. The lower polarization values for Duchenne membranes relative to the control membranes indicate that the hydrophobic domains probed by diphenylhexatriene in dystrophy samples are less ordered (Table III). These results are consistent with the polarization values for Duchenne plasma membranes in Table II. Polarization values, however, vary inversely with the fluorescence lifetime of the probe. Therefore, it follows that a shift in the fluorescence lifetime of the probe could be responsible for changes in polarization rather than differences in microdomain order. As illustrated in Table III, however, there were no significant differences in lifetimes between Duchenne and control membranes. The steady-state polarization and lifetime values of Table III were used to determine the rotational relaxation time (ρ) defined by the Perrin equation. The average rotational relaxation times in ns for the nine Duchenne plasma membranes at 16 and 22°C were significantly shorter ($0.025 > P < 0.0125$) when compared to the nine control samples. The values for the dystrophy and control membranes converge at the 30°C temperature and are not significant (Fig. 2).

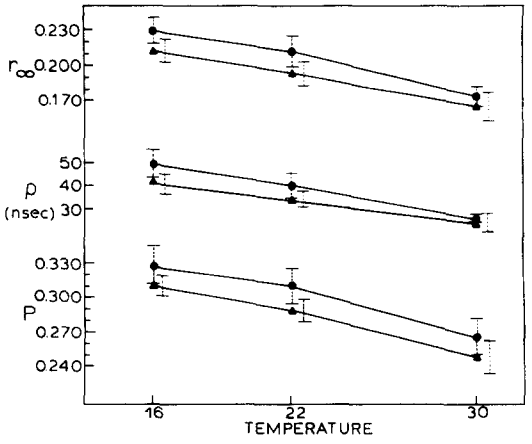


Fig. 2. Temperature dependence of the rotational parameters for diphenylhexatriene in plasma membranes. The values and the standard deviations are taken from Table III for control (●) and Duchenne (▲) membranes.

The rotational rates (R) for diphenylhexatriene

in the plasma membranes were determined as described in Experimental procedures. Next, the rotational rates (R), fluorescence lifetimes (τ) and steady-state anisotropy values (r) were recombined as described by Lakowicz et al. [35] to determine the degree to which diphenylhexatriene was hindered (r_∞) in the plasma membranes. At 16, 22 and 30°C, r_∞ values for Duchenne membranes suggested less hindrance to probe rotation than in control membranes (Table III). From R_∞ values, the average angular distribution (θ) of diphenylhexatriene at times greater than the 8–11 ns fluorescence lifetime can be determined. The θ values of 31.9°–38.5° reported in Table II demonstrate that diphenylhexatriene in lipid domains of plasma membranes experiences significant hindrance to rotation. The Duchenne membranes revealed greater θ values than controls which again were significant ($P < 0.0025$) at the two lower temperatures of 16 and 22°C.

Wobbling-in-cone analysis of diphenylhexatriene rotation in plasma membranes

Rotational properties of diphenylhexatriene in plasma membranes have also been determined from the data in Table III after modeling the rod-shaped probe as wobbling in the membranes in a cone of semiangle (θ_c) with a diffusion constant or rotational rate in the cone equal to D_w [38,39]. Numerical values for D_w, θ_c and ϕ (relaxation time from differential phase measurements) for control and Duchenne membranes are presented in Fig. 3 with a plot of D_w vs. θ_c illustrated in Fig. 3. The curve for Duchenne membrane data is displaced to the right-hand side of Fig. 3 relative to the control membrane curve. Consquently, the cone angle (θ_c) or range for rotation of diphenylhexatriene is greater at all temperatures in the Duchenne membranes. The θ_c data of Fig. 3 and the hindered-anisotropy (r_∞) measurements of Table III are both in good agreement in describing the lesser constraint on diphenylhexatriene rotation in Duchenne membranes. A determination of D_w provides a measurement of the rate of rotational diffusion within the cone of semiangle, θ_c. In addition to θ_c, r_∞ and r_0, D_w is also dependent upon ϕ, the relaxation time of the fluorescent molecule. At 30°C, D_w for Duchenne and 'control' membranes differs markedly ($D_w = 0.189$ and

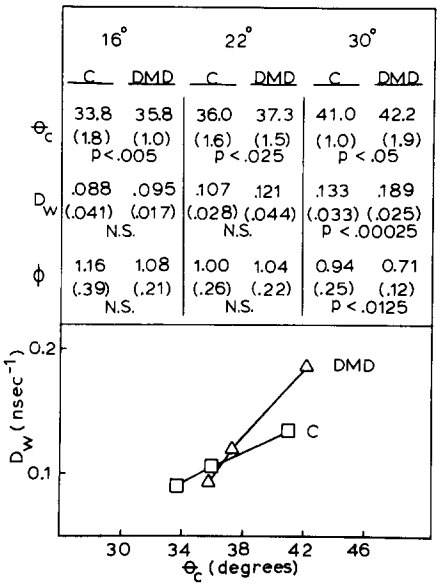


Fig. 3. Wobbling-in-cone analysis of diphenylhexatriene rotation in plasma membranes. The plasma membranes represent the control and Duchenne samples from Table III. 'θ_c' (degrees) is the semiangle for probe rotation with a rotational diffusion rate equal to 'D_w' (ns^{-1}). 'ϕ' (ns) is the relaxation time of the probe determined from differential phase measurements. Control (C) and Duchenne (DMD) values are numerically illustrated with the standard deviation shown in parentheses and the level of significance (P or n.s.). A plot of D_w vs. θ_c is illustrated.

0.133, Fig. 3). The D_w variations are also reflected in the relaxation times determined at 30°C from differential polarized fluorescence measurements ($\phi = 0.94$ and 0.71 ns, Fig. 3). The rotational relaxation times calculated by the Perrin equation (ρ, Fig. 2) from steady-state and fluorescent lifetime measurements, however, do not follow similar patterns as D_w or ϕ at 30°C.

Electron spin resonance studies of plasma membranes

Fibroblasts from four control and four Duchenne patients were cultured, then prepared as described in Table III for enrichment of plasma membranes by the sucrose-step gradient technique. The membranes were examined by electron spin resonance techniques using the fatty acid spin label, 5'-doxylstearic acid. The spin-label studies were performed at 25°C and two approaches [41,42] were taken in analyzing spectra for determining the degree of segmental motion ex-

TABLE IV

FREQUENCY DEPENDENCE OF THE FLUORESCENCE LIFETIME IN PLASMA MEMBRANES

Three control and four Duchenne fibroblast samples were processed and plasma membranes collected. The frequency dependence of the fluorescence lifetime for diphenylhexatriene at three temperatures was recorded. The phase and modulation values represent averages of Duchenne or control samples. The difference between the averaged phase and modulation values is indicated in the Table. Averages of the lifetimes (ns) and the standard deviations for the three frequencies are given below each indicated line. The ages of individuals were 2–14 years and passages for the fibroblasts were between 5–12.

	Frequency	Phase	Modulation	Difference
Control 16°C	6 MHz	10.53 ± 0.39	12.12 ± 2.00	1.59
	18 MHz	9.18 ± 0.31	10.30 ± 0.40	1.12
	30 MHz	10.01 ± 0.59	10.85 ± 0.73	0.84
		9.91 ± 0.43	11.09 ± 1.04	1.18
22°C	6 MHz	9.98 ± 0.36	11.38 ± 1.43	1.40
	18 MHz	10.43 ± 0.57	10.14 ± 0.66	−0.29
	30 MHz	9.00 ± 0.69	9.48 ± 0.91	0.48
		9.80 ± 0.54	10.33 ± 1.00	0.53
30°C	6 MHz	9.55 ± 0.23	10.64 ± 1.76	1.09
	18 MHz	9.32 ± 1.16	9.41 ± 0.70	0.09
	30 MHz	9.12 ± 1.24	9.55 ± 0.81	0.43
		9.33 ± 0.88	9.87 ± 1.09	0.54
Duchenne 16°C	6 MHz	10.28 ± 0.49	11.39 ± 2.14	1.11
	18 MHz	10.05 ± 0.51	10.91 ± 1.14	0.86
	30 MHz	9.86 ± 0.69	10.46 ± 0.42	0.60
		10.06 ± 0.56	10.92 ± 1.23	0.86
22°C	6 MHz	10.15 ± 0.71	10.18 ± 2.06	0.03
	18 MHz	9.81 ± 0.38	10.44 ± 0.95	0.63
	30 MHz	9.97 ± 0.64	9.91 ± 0.58	−0.06
		9.98 ± 0.58	10.18 ± 1.20	0.20
30°C	6 MHz	10.17 ± 0.37	12.14 ± 1.97	1.97
	18 MHz	9.55 ± 0.28	10.59 ± 0.97	1.04
	30 MHz	9.76 ± 0.35	9.93 ± 0.45	0.17
		9.83 ± 0.33	10.89 ± 1.13	1.06

hibited by the nitroxide spin label. The order parameter, S, for control plasma membranes was 0.630 ± 0.009, whereas Duchenne membranes revealed 0.606 ± 0.015. The outer half line width at half height of the low field peak (Δl) [37] for Duchenne membranes was 3.88 ± 0.20, whereas for control membranes Δl was 3.15 ± 0.34. Both sets of measurements indicate a greater degree of freedom of segmental motion of the spin label in Duchenne membranes relative to controls.

Lifetime heterogeneity in plasma membranes

Table IV gives phase and modulation fluorescence lifetimes for light modulated at three frequencies; 6, 18 and 30 MHz for Duchenne and control plasma membranes. Normally, lifetime heterogeneity is present if significant differences occur between phase and modulation lifetimes at a fixed frequency. As can be seen in Table IV, the differences between phase and modulation lifetimes for either Duchenne or control membranes were usually within the standard deviation for at least one of the lifetime measurements. The modulation lifetimes at 6 MHz showed a high degree of fluctuation (approx. 2.00 ns) between samples, presumably due to a low degree of light modulation that we invariably obtained at this frequency. Heterogeneity is also apparent if either the phase

or modulation values vary significantly as a function of the modulation frequency (i.e., 6, 18 or 30 MHz). At all temperatures, the phase or modulation lifetimes at different frequencies in most cases were within standard deviations for the lifetime measurements.

Discussion

Plasma membranes were prepared from Duchenne and control fibroblasts by several techniques for cell disruption and membrane fractionation. The plasma membranes prepared by each technique varied in the percentage of cross-contamination from intracellular membranes. Consequently, each plasma membrane preparation revealed a characteristic composition and had distinct fluorescence polarization values for diphenylhexatriene. The highest polarization values and the smallest levels of cross-contamination were found in plasma membranes prepared by the sucrose-step gradient technique. Using the sucrose-step gradient technique, nine Duchenne and nine control fibroblasts were processed and fractionated using a buffer system consisting of phosphate-buffered saline containing small amounts of Ca^{2+}/Mg^{2+}/ EDTA.

The plasma membranes were examined at three temperatures using diphenylhexatriene and the rotational properties of the fluorescent probe in the membrane domains analyzed. The rotational parameters, θ and θ_c are angle measurements. The hindered anisotropy (r_∞) is also an angle measurement due to its dependence upon the average angular distribution (θ). In contrast, D_w, ϕ and ρ are rate and time measurements. We observed that values for D_w and ϕ in the Duchenne and control membranes seemed to be closely dependent at all temperatures. The D_w and ρ values, however, between Duchenne and control membranes were not in close agreement at 30°C (Figs. 2 and 3). This finding most likely relates to the theoretical formalism involved in defining ρ and D_w. The Perrin equation which derives ρ, restricts rotation of the fluorophore in a sphere [51,52] and clearly does not apply to diphenylhexatriene rotation in lipid bilayers [53,35,40,54,55]. In contrast, D_w, the rate of diffusion, gives the rate dynamics of rotation for the rod-shaped diphenylhexatriene as being restricted in a volume such as a cone. Rotational properties for diphenylhexatriene in a restricted volume would also appear to apply in plasma membranes from cultured cells.

Table III, Fig. 2 and Fig. 3 illustrate the different analyses applied to diphenylhexatriene rotation in the plasma membranes. The r_∞, θ and θ_c measurements suggest that, in Duchenne plasma membranes, the rotational properties of the probe are less hindered and less constrained and exhibit a larger angle of rotation than in control membranes. The differences measured at 16 and 22°C were significant ($0.005 > P < 0.0025$). At the higher temperature of 30°C, the degree of significance declined ($0.05 > P < 0.0125$). Several compositional related changes are known to alter r_∞, θ and θ_c. Reduced levels of cholesterol in membranes increase the angle, θ_c, with a corresponding decrease in r_∞ [53,35,56]. Decreased protein to lipid ratios also increase θ_c and decrease r_∞ resulting in a less ordered membrane [39,54]. In addition, the degree of unsaturation in phospholipid acyl chains [57] and the extent of lipid peroxidation in phospholipid acyl chains have been shown to affect r_∞, θ and θ_c [58]. The rate of diffusion, D_w, showed no significant differences at 16 and 22°C between Duchenne and control membranes. At 30°C, however, there was a marked increase in D_w for Duchenne membranes but not in the angle or r_∞ measurements. The elevation in D_w for Duchenne membranes at 30°C seems to correlate with the significant differences in relaxation time, ϕ, between Duchenne and control membranes at 30°C. The possibility may exist that the Duchenne membranes are more sensitive to elevated temperatures than the control membranes. If subtle denaturation or disassembly of the membrane occurred it might be possible to observe shifts in rotational rates or angle measurements. Kinosita et al. [39] has recently observed a marked increase in D_w but only a small change in θ_c for diphenylhexatriene when the temperature was raised from 10 to 35°C in purple membranes.

The composition of the plasma membranes from Duchenne and control fibroblasts is undoubtedly complex, consisting of 15–20 major proteins, four phospholipid classes and cholesterol (Shaw, unpublished data). Consequently, diphenylhexatriene probes ill-defined hydrophobic lipid-protein re-

gions in biological membranes with the signal representing an average of multiple domains. Diphenylhexatriene residing in multiple membrane domains might be expected to have different fluorescence lifetimes. Heterogeneous lifetimes can be analyzed numerically with appropriate phase shift and relative modulation fluorimetry measurements of diphenylhexatriene [59]. The phase and modulation lifetimes reported in Table IV showed no consistent variations as a function of frequency at the three temperature measurements. Consequently, our results support a single lifetime for the domains being probed in the Duchenne and control plasma membranes. Kinosita et al. [39] recently observed a single lifetime component for diphenylhexatriene in erythrocyte ghost and sacroplasmic reticulum membranes. Lifetime hetrogeneity for diphenylhexatriene has been described, however, in lymphocyte membranes [60]. Although multiple lifetimes may exist in fibroblast plasma membranes, short lifetime components cannot be accurately assessed from the phase-modulation data reported here. Likewise, if a multiplicity of domains occur in the plasma membranes, the fluorescence lifetime of diphenylhexatriene in each domain must be very similar.

We have utilized two methods for analyzing the ESR spectral line shapes and hence the degree of motion of the fatty acid spin label in the plasma membranes. The conventional order parameter, S, predicts decreases or increases in the amplitude of segmental motion relative to the long axis of the fatty acid by measuring inner and outer hyperfine extrema ($T_{\parallel}$ and $T_{\perp}$) [61]. In contrast to S formalism, Mason and Freed [62] have proposed that chemical exchange theory demands shifts in the outer half-widths of the low (Δl) or high field (Δh) extrema peaks. Furthermore, Δl or Δh measurements are suggested to be more sensitive indicators of motion and at longer rotational correlation times than S measurements. The Δl and Δh measurements unlike S are independent of polarity effects [62]. Decreases in S and increases in Δl indicate greater motion (less order) of the fatty acid spin label in membranes [41]. Our results confirm those of Mason et al. [41] in that a less ordered membrane showed a marked increase in Δl (approx. 19%) and a smaller percentage decrease in S (approx. 4%). Corresponding shifts in both S and Δl are important in ruling out aggregation of the fatty acid spin label in the plasma membranes leading to broad line widths as a result of spin-spin exchange.

The ESR Δl and S measurements performed on the control and Duchenne plasma membranes support our fluorescence measurements. Both S and Δl spectral measurements were significantly different and suggested the Duchenne fibroblast membrane to be less ordered when using the 5′-doxylstearic acid. The segmental motion exhibited by the 5′-doxylstearic acid diffuses in a cone-like motion [63]. Consequently, the order parameter, S from ESR measurements, θ_c from the wobbling-in-cone theory calculations, and r_∞ from differential phase and steady-state fluorescence measurements should be in close agreement. The limiting behavior of r_∞ has recently been described as a measure of the order parameter of diphenylhexatriene by the relationship $S = \sqrt{\frac{r_\infty}{r_0}}$ and in principal is analogous to ESR or NMR order parameters [40,64].

The Duchenne and control fibroblasts used in our studies from closely age-matched individuals at similar cell passage were indistinguishable in their morphological and growth characteristics. However, we have consistently observed order differences between Duchenne and control plasma membrane isolated by two different techniques. We suggest that the order differences reported here are the result of a distinct compositional alteration between Duchenne and control membranes. The compositional alteration is either present during cell culture or occurs during fractionation of the fibroblasts and isolation of the plasma membranes. The protein/lipid ratio in biological membranes is an important compositional variable since it has been observed to alter θ_c and r_∞ [39,54]. We are first searching for differences in the protein profiles of silver-stained polyacrylamide gels of SDS-solubilized plasma membranes from Duchenne and control fibroblasts. Secondly, cholesterol and phospholipid levels in the membranes are examined using the sensitive cholesterol oxidase method and two-dimensional thin layer chromatography, respectively. In addition, the protein and lipid analyses are correlated with order

measurements from membranes prepared with and without $Ca^{2+}/Mg^{2+}/EDTA$ in the buffers used for processing the fibroblasts. By this approach the membrane order differences we observe can be related to specific membrane components. Clearly, any membrane defect reported in cells or tissues from Duchenne dystrophy patients must be completely characterized and a relationship to the onset and progression of the muscle specific disease established.

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References

1 Howland, J. (1974) Nature 251, 734
2 Roses, A. and Appel, S. (1974) Nature 250, 245
3 Roses, A., Hartwig, G., Mabry, M., Nagano, Y. and Miller, S. (1980) Muscle and Nerve 3, 36–54
4 Howland, J. and Iyer, S. (1977) Science 198, 309–310
5 Wyatt, P. and Cox, D. (1977) Lancet 1, 172–174
6 Gelman, B., Davis, M., Morris, R. and Gruenstein, E. (1981) J. Cell Biol. 88, 329–337
7 Takagi, A., Schotland, D. and Rowland, L. (1973) Arch. Neurol. 28, 380–384
8 Verrill, H., Pickard, N. and Gruemer, H. (1977) Clin. Chem. 23, 2341–2343
9 Schotland, D., Bonilla, E. and Van Meter, M. (1977) Science 196, 1005–1007
10 Thompson, E. (1980) Br. Med. Bull. 36, 181–185
11 Butterfield, D., Chesnut, D., Roses, A. and Appel, S. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 909–913
12 Gaffney, B., Drachman, D. and Lin, D. (1980) Neurology 30, 272–276
13 Chalikian, D. and Barchi, R. (1980) Neurology 30, 277–285
14 Butterfield, D.A. (1981) J. Neurol. Sci. 52, 61–67
15 Sato, B., Nishikida, K., Samuels, L. and Tyler, F. (1978) J. Clin. Invest. 61, 251–259
16 Wilkerson, L., Perkins, R., Roelofs, R., Swift, L., Dalton, L. and Park, J. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 838–841
17 Dellantonio, R., Angeleri, F., Capriotti, M., Lenaz, G., Curatola, G., Mazzanti, L. and Bertoli, E. (1980) Int. J. Biochem. 29, 121–128
18 Prendergast, F.G. (1982) in Disorders of the Motor Unit (Schotland, D.L., ed.), pp. 321–347, John Wiley and Sons, New York
19 Mawatari, S., Schonberg, M. and Olarte, M. (1976) Arch. Neurol. 33, 489–493
20 Roses, A.D., Herbstreith, M.H. and Appel, S.H. (1975) Nature 254, 350–351
21 DePirro, R., Lavro, R., Testa, I., Ferretti, G., DeMartinis, C. and Dellantonio, R. (1982) Science 216, 311–313
22 Jones, G. and Witkowski, J. (1979) J. Neurol. Sci. 43, 465–470
23 Gelman, B.B., Papa, L., David, M.H. and Gruenstein, E.I. (1980) J. Clin. Invest. 65, 1398–1406
24 Wrogemann, K., Rosenmann, E., Dobbs, M., Adachi, A., Kreis, C. and Hamerton, J. (1982) Fifth International Congress on Neuromuscular Diseases, Abst. 10.1
25 Konigsberg, I.R. (1979) Methods Enzymol. 58, 511–527
26 Kartner, N., Alon, N., Swift, M., Buchwald, M. and Riordan, J. (1977) J. Membrane Biol. 36, 191–211
27 Thom, D., Powell, A., Lloyd, C. and Rees, D. (1977) Biochem. J. 168, 187–194
28 Brunette, D.M. and Till, J.E. (1971) J. Membrane Biol. 5, 215–224
29 Avruch, J. and Wallach, D. (1971) Biochim. Biophys. Acta 233, 334–347
30 Barrett, A. (1980) Biochem. J. 187, 909–912
31 Ackrell, B., Kearney, E. and Singer, T. (1978) Methods Enzymol. L111, 466–475
32 Svingen, B., Buege, J., O'Neal, F. and Aust, S. (1979) J. Biol. Chem. 254, 5892–5899
33 Lentz, B.R., Moore, B.M. and Barrow, D.A. (1979) Biophys. J. 25, 489–494
34 Spencer, R.D. and Weber, G. (1969) Ann. N.Y. Acad. Sci. 158, 361–376
35 Lakowicz, J., Prendergast, F. and Hogan, D. (1979) Biochemistry 18, 508–519
36 Jameson, D.M. and Weber, G. (1981) J. Phys. Chem. 85, 953–958
37 Lakowicz, J. and Prendergast, F. (1978) Science 200, 1399–1401
38 Kinosita, K., Kawato, S. and Ikegami, A. (1977) Biophys. J. 20, 289–305
39 Kinosita, K., Kataoka, R., Kimura, Y., Gotoh, O. and Ikegami, A. (1981) Biochemistry 20, 4270–4277

40 Lipari, G. and Szabo, A. (1981) Biophys. J. 30, 489–506
41 Mason, R., Giavedoni, E. and Dalmasso, A. (1977) Biochemistry 16, 1196–1201
42 Gaffney, B. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 664–668
43 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
44 Bartlett, G. (1959) J. Biol. Chem. 234, 466–468
45 Moore, N., Patzer, E., Barenholz, Y. and Wagner, R. (1977) Biochemistry 16, 4708–4715
46 Bligh, E. and Dyer, W. (1959) Can. J. Biochem. Physiol. 37, 911–917
47 Stempak, J.G. and Ward, R.T. (1964) J. Cell Biol. 22, 697–701
48 Venable, J.H. and Coggeshall, R. (1965) J. Cell. Biol. 25, 407–408
49 Bahn, A. (1972) Basic Medical Statistics, Grune and Stratton, New York, 136–143
50 Weber, G. (1977) J. Chem. Phys. 66, 4081–4091
51 Perrin, F. (1926) J. Phys. Radium 7, 390–401
52 Weber, G. (1953) Adv. Protein Chem. 8, 415–459
53 Kawato, S., Kinosita, K. and Ikegami, A. (1977) Biochemistry 16, 2319–2324
54 Heyn, M.P., Cherry, R.J. and Dencher, N.A. (1981) Biochemistry 20, 840–849
55 Engel, L.W. and Prendergast, F.G. (1981) Biochemistry 20, 7338–7345
56 Hildenbrand, K. and Nicolau, C. (1979) Biochim. Biophys. Acta 553, 365–377
57 Stubbs, C.P., Kouyama, T., Kinosita, K. and Ikegami, A. (1981) Biochemistry 20, 4257–4262
58 Shaw, J., Shaw, K., Bembeneck, M., Litman, B. and Konigsberg, I. (1980) Fed. Proc. 39, 2098, Abst. 2604
59 Weber, G. (1981) J. Phys. Chem. 85, 949–953
60 Klausner, R.D., Kleinfeld, A.M., Hoover, R.L. and Karnovsky, M.J. (1980) J. Biol. Chem. 255, 1286–1295
61 Gaffney, B. and McConnell, H. (1974) J. Magn. Res. 16, 1–28
62 Mason, R. and Freed, J. (1974) J. Phys. Chem. 78, 1321–1323
63 Israelachvili, J., Sjosten, J., Eriksson, L.E., Ehrstrom, M., Graslund, A. and Ehrenberg, A. (1975) Biochim. Biophys. Acta 382, 125–141
64 Prendergast, F.G. (1981) Period. Biol. 83, 69–79
65 Shaw, J., Schlag, E. and Konigsberg, I. (1979) Biophys. J. 25, 168a