DIFFUSIBLE MAGNESIUM IN FROG SKELETAL MUSCLE CELLS

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ABSTRACT Total diffusible magnesium concentration in frog skeletal muscle is 5.2 mM as determined by electron probe microanalysis of 0.2 nl liquid samples. The calculated free Mg concentration, 0.2 mM, is at the lower end of the range of values reported by others as calculated by methods using nuclear magnetic resonance, Mg-selective microelectrodes, and metallochromic indicator dyes. Magnesium is but one of many elements of physiological importance in muscle that can be analyzed using this novel liquid-sampling and x-ray spectroscopic method.

INTRODUCTION

The molecular events of muscle contraction depend on the concentration of both the free magnesium ion (Mg^{2+}) and the substrate magnesium adenosine triphosphate ($MgATP^{2-}$). Thus, a direct measurement of the in vivo concentration of total diffusible Mg concentration (from which both Mg^{2+} and $MgATP^{2-}$ concentrations can be determined) is useful in clarifying the conditions under which muscle contraction normally occurs. I report here a new method of sampling and analyzing intracellular fluid, from which the concentration of total diffusible Mg can be determined. Samples were obtained from segments of relaxed muscle cells by equilibrating 0.2 nl liquid droplets with the cellular contents of fibers skinned under water-saturated mineral oil. The elemental concentration of Mg in all its diffusible forms was found to be 5.2 mM, as determined by electron probe microanalysis of the liquid samples. Of this total, I estimate that the cytoplasmic (bulk fluid) $MgATP^{2-}$ concentration is ~4.2 mM, the Mg^{2+} concentration is ~0.2 mM, with the remaining 0.8 mM Mg bound to diffusible parvalbumin and creatine phosphate.

MATERIALS AND METHODS

Semitendinosus or tibialis anterior muscles were dissected out of the frog *Rana pipiens* or *Rana temporaria* (Hazen Frog Farm, Alburg, VT; Charles Sullivan Co., Nashville, TN). The whole muscles were gently blotted and transferred directly to a glass bottom dish filled with water-saturated mineral oil. A bundle of fibers 1–2 cm long was then cut from the dorsal head of the muscle and a single fiber segment disengaged from the rest. In some cases, muscles were first placed in Ringer's solution containing 2.5 mM KCl, 1.8 mM $CaCl_2$, 109 mM NaCl, 10 mM Tris HCl, and 1 μM tetrodotoxin (pH 7.0). Single fibers were isolated from tendon to tendon in the Ringer's solution, and transferred to the oil-filled dish in a Ringer's solution–filled "transfer boat." Nearly all of the Ringer's solution adhering to a single fiber was removed by gently pulling the fiber out of the solution-filled transfer boat directly into the oil. Following each of these preparatory procedures, the relaxed single fibers or fiber segments were mechanically skinned under the oil by peeling back the sarcolemma with a sharp needle (1). The temperature of the oil and fiber were maintained at 20°C with a thermoregulating unit (Cambion, Cambridge, MA).

Each fiber segment, straightened and trimmed to 3–5 mm, was photographed using an inverted compound microscope (Olympus Corp., New Hyde Park, NY); striation spacings and segment widths were determined from photomicrographs. The striation spacing, width, and general appearance of the fiber segments remained unchanged during the sampling period, indicating that the fibers were relaxed and stable. Loss of fiber water to the oil phase (with possible accompanying changes in intracellular Mg concentration) was insignificant insofar as the volume (width) of the segment did not appear to diminish during the sampling period.

Sampling pipettes were constructed from borosilicate glass capillary tubing that was heated and drawn to form a segment used to store single or sequential (serial) liquid samples. Near the tip of the pipette, a constriction was produced with a deFonbrune microforge, forming a 0.2 nl elongated cavity used as the sampling chamber. The nontapered end of the glass pipette was linked via polyethylene tubing to a syringe used to draw and expel the fluid.

De-ionized water, to which 0.25 M sucrose and 5 μM EGTA was added, was drawn into the sampling chamber of the pipette from a drop placed in the oil-filled dish holding the fiber segment (with sucrose, the solution was isosmotic with the fiber fluid; with EGTA, any residual calcium that would have otherwise activated the fiber upon contact was chelated). The pipette was then placed on the surface of the skinned fiber and allowed to equilibrate for 0–10 min with the fiber fluid (see schematic inset to Fig. 1 *a*). Subsequently, the pipette was removed from the muscle surface and the sample drawn up into the shank of the pipette. Samples, stored singly or sequentially in the pipette, were isolated by segments of mineral oil to avoid cross-contamination and evaporation. Upon completion of each experiment, the pipettes were frozen and stored at −70°C.

Pipettes with their frozen samples were shipped with dry ice by air to the National Laboratory of Biotechnology Resource in Electron Probe Microanalysis at Harvard Medical School. There, the samples were thawed, expelled under oil from the sampling pipettes, and three drops from each sample aspirated with a specially constructed and calibrated 20 or 30 pl volumetric pipette. These drops were then expelled under saline-saturated paraffin oil onto a beryllium block along with five droplets containing various known concentrations of Mg (and other elements analyzed concurrently). The oil on the block covering the droplets was removed by washing with *m*-xylene, and the samples were

frozen and freeze-dried. Each crystal patch resulting from the freeze-drying was exposed to the beam of a Cameca MS 46 microprobe (Cameca, Courbevoie, Paris), and the number of counts from the four Cameca x-ray spectrometers recorded for 10 s. The counts from individual residues of a given sample or calibrating solution were averaged to yield an estimate of the count rate. Linear regression lines relating each count rate to concentration in Mg in the calibrating solutions were computed and used to estimate the Mg concentration of the samples. Phosphorus and cobalt (used as an extracellular marker, as discussed later) were analyzed similarly. Details of the electron probe microanalysis technique are presented elsewhere (2, 3).

RESULTS

Fig. 1 *a* illustrates results from 24 freshly skinned frog muscle fibers. The concentration of Mg in the pipette samples is plotted as a function of the time that the pipettes remained on the fibers (mean values ± 1 SEM from 13 *R. pipiens* and 11 *R. temporaria*). Mg uptake into the pipettes appears to be complete within 5–10 min, leveling off at a concentration of 5.2 mM. The Mg levels in samples from *R. pipiens* tended to be greater than those from *R. temporaria* (e.g., for 10 min samples, 5.7 ± 1.6 mM vs. 4.9 ± 1.8 mM), but the differences were not significant ($P < 0.05$; results of similar sampling periods compared). Also, no significant differences in Mg levels were noted ($P < 0.05$) between samples from five whole fibers that had been first isolated tendon to tendon and samples from six fiber segments that had simply been cut out of the intact muscle (muscles from *R. temporaria* were compared).

To test for direct contamination of Mg from extracellular fluid, cobalt (a benign element found only in trace amounts inside muscle) was used as an extracellular marker in some experiments. Six fibers were examined from muscles that had been allowed to soak for up to 2 h in Ringer's solution containing 10 mM $CoCl_2$. The results from these fibers were then compared with those from four muscles that had been dissected in Co-free Ringer's solution. In both cases, no detectable signal from Co (over background counts) was found in the fluid samples taken from either group of muscle fiber segments, indicating that direct extracellular contamination is negligible (cf. Discussion).

To characterize the overall accuracy and reproducibility of the sampling technique and electron probe microanalysis, a control experiment was carried out in which 24 samples of a test solution were aspirated directly into separate pipettes that had been used to sample the fiber fluid. The test solution contained 4.75 mM $MgCl_2$, among other salts added to mimic the intracellular fluid (see legend to Fig. 1 for details). The electron probe microanalysis of the samples yielded a Mg concentration of 4.6 ± 0.5 mM (mean ± SEM; $n = 24$), close to the expected value of 4.75 mM.

In another control experiment (one that more closely approximated the actual test conditions), a 0.2 nl droplet of sucrose/EGTA solution in the pipette tip was placed in contact with a 10 μl droplet of the test solution under oil (see schematic inset to Fig. 1 *b*). Uptake of the Mg into the pipette solution was complete within 5 min (Fig. 1 *b*), leveling off to a concentration (4.9 mM) close to the expected 4.75 mM.

A parallel analysis of the phosphorus content in the samples from *R. pipiens* yielded a diffusable concentration of 52.3 mM (52 ± 6 mM; mean ± 1 SEM; 13 fibers; 10 min samples).

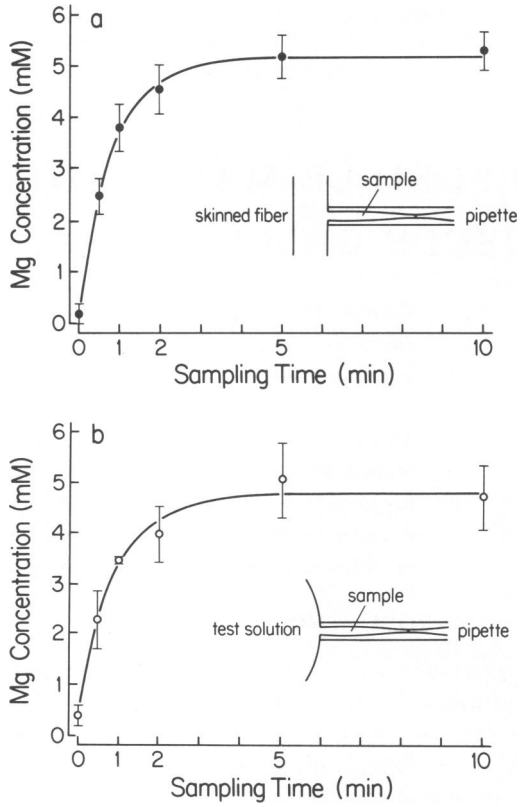

FIGURE 1 Magnesium analysis of liquid samples from frog muscle fibers (*a*) and test solution (*b*). Element concentration determined from electron-probe microanalysis of pipette fluid residues calibrated against residues from standard solutions using the same volumetric pipette (see text). The time axis indicates the duration that the pipette remained on the skinned muscle fiber segment (*a*) or the test solution drop (*b*). The zero-minute sample is that of the water/sucrose/EGTA solution alone. Mean values (± SEM) pooled from 24 experiments (*a*), or 8 experiments (*b*). The experiments of (*a*) sampled fluid from relaxed fibers of 13 *R. pipiens* and 11 *R. temporaria*; those of (*b*) sampled a test solution. The test solution contained 4.75 mM $MgCl_2$, 60 mM KCl, 15 mM Na_2 creatine phosphate, 5 mM $CaSO_4$, 5 mM EGTA, 3.2 mM Na_2ATP, and 20 mM imidazole (pH 7.0). Atomic absorption spectrophotometric check of 1 ml samples of text solution yielded 4.7 ± 0.3 mM (mean ± SEM; $n = 4$) Mg. Fiber striation spacing, 2.20 ± 0.10 μm; width of fiber segments from *R. pipiens*, 73.1 ± 14.7 μm, from *R. temporaria*, 101.7 ± 30.5 μm. Average time under oil, ~30 min from start of experiment. Curves were obtained by a nonlinear least-squares exponential fit to the data points (see text), with time constants of 0.82 s (*a*) and 0.97 (*b*). Sampling chamber length, 0.32 ± 0.05 mm; volume, 0.191 ± 0.063 nl. The mean volume of the skinned fibers examined was 24 nl (over 10^2 times the volume of the sampling chamber, so dilution effects are probably negligible).

DISCUSSION

Equilibrium Time Course of Magnesium

To quantitate the diffusion process underlying the equilibration time courses of Fig. 1, the data points were fit with single exponential curves of the form $c = c_f - c_i \exp(-t/\tau)$, where c is the Mg concentration at time t, c_f the final equilibrium concentration, c_i the initial concentration, and τ is the exponential time constant. As a first-order approximation, the time course of diffusion of Mg into the pipette chamber was described by a simple complementary error function that can itself be expressed as an infinite series of exponential functions (4). Equating the first exponential term of the series to the single exponential curve fitted to the data points, the diffusion constant (which is related to the exponential time constant through the exponents) works out to be 8.5×10^{-6} cm^2/s for Fig. 1 *a* and 7.4×10^{-6} cm^2/s for Fig. 1 *b*.[1] The diffusion constants are, of course, the mean diffusion constant for all Mg moieties present, applicable only to the solution into which Mg is diffusing.

Contamination and Ionic Redistribution

Direct contamination from extracellular Mg is unlikely insofar as the sarcolemma and residual extracellular fluid attached to it are removed during the skinning process. Still, the transverse tubular system (deep invaginations of the surface membrane) may contain some extracellular fluid (even in fibers initially bathed in Mg-free Ringer's solution if the tubules are inadequately washed out). However, the low concentration of Mg in plasma (1.2 mM; reference 5) and the relatively low volume of the transverse tubular system in frog muscle (only 0.3% of the muscle volume; reference 6) suggests that the contamination is negligible. This conclusion is supported by the experiments in which extracellular marker cobalt was not detected in intracellular samples.

It is also possible, but unlikely, that appreciable loss of intracellular Mg occurs during the dissection. The problem of ionic redistribution arises when the fiber segments are initially cut from the bundle or when the fiber itself is skinned. These procedures rapidly depolarize the membrane and produce an increased ionic permeability. For Mg^{2+}, however, the resting permeability is small (equivalent to an inward flux of ~0.6 mM/h [7]). The increase in permeability and inward flux that occurs during prolonged depolarization is also comparatively small and transient (8). Therefore, the loss of intracellular Mg during dissection is probably negligible.

A general systematic dilution or concentration of the Mg in the fluid of the fiber or sample is unlikely, in light of several observations mentioned earlier: First, little or no loss of fiber water to the oil phase was observed, indicating that (accompanying) changes in the Mg concentration in the fiber during the sampling period were probably insignificant. Second, a control solution, sampled and analyzed under conditions that closely approximated the actual test conditions, yielded Mg values within 4% of those obtained by atomic absorption analysis. Thus changes in Mg concentration in the sample following the sampling period were probably small (<4%) and can be neglected. Third, a parallel analysis of phosphorus content in the samples from *R. pipiens* indicated that the mean diffusible P concentration was 52.3 mM. This value is in reasonable accord with the 56.8 mM phosphorus given by Conway (5) and the results of Dawson et al. (9), which indicate that, of the primary moieties of P, creatine phosphate contributes ~39 mM P and ATP ~15 mM P to the diffusible total in relaxed frog muscle (see later section). These correspondences of P values lends credence to the present method and results. Because the cell membrane is relatively impermeable to the P moieties, appreciable redistribution during dissection is unlikely (as is the case for Mg).

The Intracellular Distribution of Magnesium

Frog muscle contains ~9 mM total magnesium; dividing 6.2 mmol Mg/kg whole muscle (7) by 0.69 kg (0.69 liter) cell water/kg whole muscle (10) yields 9 mM Mg, close to the value of 9.6 mM Mg that Conway (5) gives as a median value from 11 references. The present results indicate that 5.2 mM Mg represents a fraction of the total Mg that is readily diffusible, that is, readily exchangeable with the fluid in the sampling pipette.[2] The remaining Mg represents a fraction that is associated with (or incorporated into) fixed structures within the muscle cell.

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[1] Assume, for simplicity, that diffusion occurs from a stirred solution of large volume (the muscle fiber) to an unstirred solution of small volume (the elongated droplet in the pipette); i.e., assume, as a first approximation, that the concentration of Mg in the fiber remains essentially constant during the diffusion process. Expressing the total concentration of Mg in the pipette sample $c(t)$ (where t is the sampling duration) as a fraction of the final equilibrium concentration c_f, the solution to the diffusion equation $\nabla c_i = \nabla^2 c$ in one dimension (4) yields $c(t)/c_f = 1 - \Sigma_{n=0}^{\infty} 8\,(2n+1)^{-2}\pi^{-2} \exp[-D(n+1/2)^2\pi^2 L^{-2} t]$, where L is the length of the droplet and D is the mean diffusion constant of Mg in the droplet. For time constants (τ) of the order of those observed (1 min) the series on the right-hand side of the above equation converges so rapidly that all terms following the first can be neglected with an error <0.1%. Thus, $\exp(-t/\tau) \simeq \exp(-0.25D\pi^2 L^{-2} t)$ so that $D \simeq 4 L^2 \pi^{-2} \tau^{-1}$. Taking L = 0.032 cm (Fig. 1) and τ = 49.2 s (Fig. 1 *a*) yields $D = 4(0.032\text{ cm})^2 \pi^{-2} (49.2\text{ s})^{-1} = 8.5 \times 10^{-6}$ cm^2 s^{-1}. For τ = 58.2 s (Fig. 1 *b*), $D = 7.4 \times 10^{-6}$ cm^2 s^{-1}.

[2] Both the free ions and ion complexes can be considered "freely diffusible" if the energy of the molecule within the aqueous phase differs by less than kT (4×10^{-21} J at 20°C) from its mean energy (11). However, if binding to fixed structures occurs with energies exceeding kT, motion is restricted. Binding will influence measureable parameters such as the diffusion constant, mobility, mean activity coefficient, and nuclear magnetic resonance relaxation time of the ion or ion complexes (11).

The 5.2-mM diffusible Mg concentration is probably indicative of the average Mg concentration in most of the bulk water phase between the filaments. The distance between the negatively charged filaments is much larger than the effective reach of the heavily screened electrostatic field associated with the charged filaments (the surface-to-surface distance being an order of magnitude greater than the 1 nm Debye length [12]). It is thus likely that at equilibrium the microdroplet in the pipette and most of the fluid within the muscle lattice are nearly equipotential since both points are relatively far (many Debye lengths) from the charged filament surfaces. Consequently, in the absence of a significant electrochemical gradient, the concentrations should be similar.

Of the Mg that is not readily diffusible, the amount bound to (or incorporated into) myosin and actin (plus tropomyosin and troponin) is comparatively small, even though these contractile and regulatory proteins occupy most of the nonfluid volume of muscle. Frog muscle contains ~1 mM myosin (monomer) with ~20 ionic binding sites per monomer (13), of which only ~2% are occupied by Mg^{2+} (13, 14). Thus, myosin probably binds less than 0.4 mM Mg (14), or 4% of the total. The actin (monomer) concentration in frog muscle is 4–5 times less than that of myosin (13), but there are two classes of binding sites on "actin" (probably actin and tropomyosin), one with 1 mol sites/mol monomer and an apparent stability constant of $\sim 10^5\ M^{-1}$, and another with 11 mol sites/mol monomer and an apparent stability constant of $2\text{–}4 \times 10^3\ M^{-1}$ (15). Thus both sites on actin bind together ~0.3 mM Mg (or 3% of the total). Mitochondria, occupying only 1–2% of the volume of frog muscle, probably bind <0.2 mM Mg (or <2% of the total), assuming 23 mmol Mg/kg dry wt and a mitochondrial water content of 65% (16). The sarcoplasmic reticulum (SR), on the other hand, may bind as much as 2 mM Mg (or 22% of the total), assuming similar water content (72%) among the two primary components of the sarcoplasmic reticulum (longitudinal SR and terminal cisternae) and a Mg content of up to 59 mmol/kg dry wt (16). It is not yet known whether "backbone" proteins such as connectin (17) bind appreciable amounts of Mg. Thus, roughly a third of the total Mg in frog muscle (2.9 mM, or greater if backbone proteins bind Mg) is probably attached to or incorporated into fixed intracellular proteins and organelles, and therefore not freely diffusible. The remaining half to two-thirds of the total Mg (~5.2 mM) appears to be freely diffusible.

The Moieties of Freely Diffusable Magnesium

In living relaxed frog muscle, the primary moieties of freely diffusible magnesium are $MgATP^{2-}$, free Mg^{2+}, Mg parvalbumin, and Mg creatine phosphate. At near neutral intracellular pH, 0.15 M ionic strength, and 20°C, the apparent stability constant for $MgATP^{2-}$ is likely to be near $2.8 \times 10^4\ M^{-1}$ (18), while that for Mg parvalbumin is $\sim 1.1 \times 10^4\ M^{-1}$ (19), and that for Mg creatine phosphate is $\sim 40\ M^{-1}$ (reference 20, note added in proof). Assuming that the diffusible ligand concentrations (in mmol/liter cell water) are 4.9 mM ATP (converting from 3.4 mM/kg muscle [10, 21]), 39 mM creatine phosphate (converting from 27 mM/kg muscle [9, 10]) and 0.4 mM parvalbumin (0.8 mM Mg^{2+} sites) converting from 0.25–0.30 mM/liter fiber [22]), one can then solve the appropriate set of reaction equations (23) using the total value of Mg of 5.2 mM (Fig. 1 *a*) to yield 0.2-mM free Mg^{2+} concentration, with 4.2 mM $MgATP^{2-}$, 0.5 mM Mg parvalbumin, and 0.3 mM Mg creatine phosphate. If one neglects the binding of Mg to parvalbumin, the free Mg^{2+} concentration estimate increases from 0.2 to 0.3 mM, with slight increases of $MgATP^{2-}$ and Mg creatine phosphate. The effect of doubling or halving the magnitude of the apparent stability constant of $MgATP^{2-}$ (which may be in error by as much as twofold [18]) is to decrease the estimate of Mg^{2+} concentration slightly, in the present case to ~0.1 mM, or increase it slightly, to ~0.3 mM. The effect of increasing or decreasing the concentration of ATP by 1 mM is to decrease Mg^{2+} concentration to 0.1 mM, or increase it to 0.4 mM. Thus, it is likely that, given the above assumptions, the free Mg^{2+} concentration in the bulk fluid of frog muscle is between 0.1 and 0.4 mM, with 0.2 mM the most probable value. However, in light of the number of assumptions involved in the calculations, this estimate must be considered preliminary.

The present results do nevertheless strongly suggest that in muscle a relatively low fraction of the total Mg (0.2 mM/9 mM = 0.02 or 2%) is free Mg^{2+}. Similar results can be found in other cells. For example, the Mg content of pig and mouse lymphocytes was recently measured by Rink et al. (24). They used a variety of methods (null-point ionophore, Mg-sensitive electrode, and ^{31}P NMR) to produce an estimate of free Mg^{2+} that was also a small fraction (4–11%) of the total (12 mM).

Free Mg^{2+} concentration in intact frog muscle has been estimated recently by other methods. Gupta and Moore (14) used a ^{31}P NMR technique to produce a weighted average of the ATP resonances, from which free Mg^{2+} was estimated; their results yielded a free Mg^{2+} concentration of 0.2–0.6 mM (14, 25), i.e., values similar to the present estimate. Cohen and Burt (20), on the other hand, report a value of 3-mM free Mg^{2+}, using a NMR method in which the ^{31}P transverse relaxation time of creatine phosphate was used to obtain parameters from which free Mg^{2+} was derived. Hess and Weingart (26), using a Mg^{2+}-selective electrode, also produce an estimate of free Mg^{2+} concentration in the millimolar range (3.3 mM). Baylor et al. (27) recently estimated free Mg^{2+} using three metallochromic indicator dyes, but found that a different estimate was produced with each dye used: Arsenazo I yielded values of

3–6 mM; Arsenazo III, 0.5–1.2 mM, and Dichlorophosphonazo III, 0.2–0.3 mM (pH range 7.1–6.9, respectively). It is not yet clear what factors are responsible for the wide variation in reported results (including that from the present work). Baylor et al. (27) proposed that the most likely explanation for the variability of the dye results is that (at least two) indicator dyes behave differently inside muscle fibers than in calibrating solutions. The same may be true of NMR probes and microelectrodes.

Extension of Method

Magnesium is but one of many elements of physiological importance in muscle that can be analyzed using these liquid sampling and x-ray spectroscopic methods. In fact, one can measure the diffusible concentration (above 0.1 mM or so) of any chemical element after boron in atomic weight in the cytoplasmic samples (2, 3). One can also measure the concentrations repetitively, in the same preparation, so that reliable correlative studies can be performed on changes in elemental composition which may occur under different physiological or pharmacological conditions (3). Moreover, by applying microprecipitation (3), ultrafiltration (3), and ion-selective electrode techniques to the liquid samples, it may be possible to extend the method to enable one to distinguish the concentration of an ionized free element from that of the element complexed to another mobile molecule.

This work was accomplished with help from the National Biotechnology Resource in Electron Probe Microanalysis, Harvard Medical School, Claude Lechene, Director. I am especially grateful to Dr. Lechene for providing the resources of his laboratory and to Mr. Christopher Recchia whose careful experiments provided the basis of this report. I also thank Ms. Kathy Edgerly, Dr. George Webb, Dr. Jan Koniarek, and Ms. Cathy Ward for assistance, and Drs. Michael Berman, Joseph Patlak, Robert Godt, Avril Somylo, and especially Graham Mainwood for suggestions and inspiration. I also appreciate the helpful comments of the reviewers.

This was work was supported by the Muscular Dystrophy Association of America, US PHS (5P41 RR00679), the American Heart Association (79 165), and the Vermont Heart Association. This work was done during the tenure of an Established Investigatorship of the American Heart Association.

Received for publication 22 June 1982 and in final form 22 December 1982.

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