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Ca^{2+} Binding, ATP-dependent Ca^{2+} Transport, and Total Tissue Ca^{2+} in Embryonic and Adult Avian Dystrophic Pectoralis

Earl M. Ettienne and Robert H. Singer

Departments of Physiology and Anatomy, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01605

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Summary. Avian muscular dystrophy is an autosomal recessive genetic disease characterized by early hypertrophy and loss of function of the pectoralis major. The disease is progressive, ultimately resulting in atrophy and heavy lipid deposition.

Previous investigators have noted a decrease in the ability of the dystrophic sarcoplasmic reticulum to concentrate Ca^{2+}. More recently, other investigators have shown an abnormal calcium uptake in avian dystrophic sarcoplasmic reticulum. They indicated, using freeze-fracture techniques, that a 90 Å particle of the vesicle membrane exhibited a decreased population and suggested that they might be the ATPase involved in calcium transport.

Our studies confirm the earlier observations of a decreased rate of Ca^{2+} uptake and Ca^{2+} binding capacity of dystrophic fragmented sarcoplasmic reticulum vesicles which are isolated from both embryonic and adult pectoralis. These observations correlate in turn with a 75% drop in the Ca:ATP transport efficiency of the dystrophic sarcoplasmic reticulum determined by measuring the rate of $^{32}P_i$ liberation from γ-ATP^{32} during active calcium transport by the isolated sarcoplasmic reticulum SR.

In addition, we have found a quantitative deficiency in a 65,000 dalton component of the dystrophic fragmented SR at the time of myoblast fusion by measuring ^{35}S-Methionine incorporation into the SR, coupled to high resolution polyacrylamide gel electrophoresis and radioautography. Analysis of total tissue calcium by atomic absorption spectroscopy revealed a decrease in the total calcium content of dystrophic muscle.

Duchenne muscular dystrophy is a human genetic lesion which is characterized clinically by progressive loss of proteins in affected muscles. The loss of proteins is a degenerative process eventually resulting in loss of normal muscle functions such as normal tension development and relaxation [3–5, 7]. An avian model system which mimics this disease is available and has been used in the present study [1].

Previously, investigators have noted a decrease in Ca^{2+} concentrating ability of fragmented sarcoplasmic reticulum in avian, mouse, and human dystrophic muscle [8, 11, 13–15]. It has been reported that the

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apparent affinity (K_m) for Ca^{2+} transport by the membrane associated ATPase remains close to control values. However, the V_{max} of transport is decreased. Recent freeze-fracture electron microscopial techniques by Sabbadini *et al.* [10] showed that a 90 Å component was reduced in density and distribution in dystrophic sarcoplasmic reticulum. They suggested that the particles might be involved in Ca^{2+} transport. Sabbadini *et al.* [8] have also investigated the protein composition of sarcoplasmic reticulum from normal and dystrophic muscle by gel electrophoresis but reported that no differences were immediately evident. More recently, W.K. Engel (*personal communication*) has shown an increase in cytosolic Ca^{2+} of human dystrophic muscle histochemically utilizing potassium pyroantimonate as *in situ* Ca^{2+} precipitating agent.

The inability of the sarcoplasmic reticulum from dystrophic muscle to sequester and store Ca^{2+} as rapidly as that from normal muscle could result in a prolongation in the Ca^{2+}-dependent ionotrophic state of the sarcoplasm following activation. Many of the symptoms of this particular muscle pathology could be attributable to an elevation in cytosolic Ca^{2+} in the resting muscle since Ca^{2+} at cytosolic concentrations which are just subthreshold for contraction could still be sufficiently high to affect cellular metabolic reaction rates [2]. Preliminary studies in this laboratory confirm the findings of a decreased initial rate of Ca^{2+} uptake as well as Ca^{2+} binding capacity of fragmented sarcoplasmic reticular vesicles from dystrophic embryonic and adult chicken pectoralis muscles. We have also found quantitative differences in the incorporation of ^{35}S-methionine in some of the protein components of dystrophic sarcoplasmic reticulum isolated from fusion-ready myoblast in 14 day old embryos by using high resolution polyacrylamide gel electrophoresis of the isolated vesicles. One of these proteins correspond in molecular weight to the high affinity Ca^{2+}-binding proteins of MacLennan.

The analysis of total tissue calcium by atomic absorption spectroscopy revealed a decrease in the calcium content of dystrophic muscle compared to normal controls.

Materials and Methods

White Leghorn fertilized dystrophic eggs and adult dystrophic roosters were obtained from Dr. Pierro at the Department of Animal Genetics, University of Connecticut at Storrs, Connecticut. Normal eggs and adult animals were obtained from SPAFAS, Inc. Eggs were incubated in a Lehy Manufacturing Co. (Higginsville, Missouri) incubator at 38 °C and a relative humidity of 70%. Adult animals were over 120 days old and

maintained on Ralston Purina Feed. The pectoralis and deep pectoralis muscles were excised from 14–20 day old dystrophic and normal chick embryos. The samples were washed in saline, cleaned of fat and connective tissue and minced. Adult animals were decapitated and subjected to the same procedure.

The minced tissue was homogenized for 40–90 sec in a Tekmar homogenizer using 4 volumes (wt/vol) of 0.3 sucrose and 10 mM imidazole, pH 7.0. The homogenate was centrifuged in an SS-34 Sorvall rotor at 17,000 × *g* for 20 min to pellet tissue fragments and large organelles. Mitochondrial contaminants of the supernatant were further removed by straining through four layers of cheesecloth. After spinning at 34,000 × *g* for 20 min, the pellet was resuspended in 0.6 M KCl and 10 mM imidazole, pH 7.0, for 15 min to remove contaminating contractile proteins. A final spin at 198,000 × *g* for 20 min effectively pellets the microsomal fraction representing the sarcoplasmic reticulum. The pellet was resuspended in 0.3 M sucrose to a final concentration of 1 to 5 mg per ml and used for either further purification or for the various assays.

Sucrose Density Gradient Fractionation

Microsomal suspension (4 ml containing 5 mg/ml) was layered on the surface of a sucrose gradient (20–40% wt/vol) in 10 mM imidazole, pH 7.0. Centrifugation was performed in Beckman (L5–65) SW 27 rotor (12 ml) at 50,000 × *g* for 2 hr. In a second set of experiments, microsomes (final concentration 100 μg/ml) were incubated in 10 mM oxalate, 0.1 M KCl, 1.6 mM EGTA, 0.6 mM $CaCl_2$, 0.5 μCi/ml $^{45}CaCl_2$, 5 mM $MgCl_2$, 5 mM ATP, 40 mM creatine phosphate and 18 mM imidazole, pH 7.0 for 1 hr. After incubation, 5-ml samples of the preparation were layered onto a 20–40% sucrose gradient and centrifuged for 1 hr at 51,000 × *g* in an SW 27 Beckman rotor.

The gradients obtained from both oxalate loaded and unloaded vesicles were photographed, then scanned at 280 nm on a Gilford continuous flow, recording spectrophotometer and collected in 1.5-ml fractions on a fraction collector. Pellets were diluted to 50 μg concentrations and assayed for protein composition on SDS-PAGE 10% to 20% gradient slabs. Samples were also taken from the sucrose gradient for transmission electron microscopy.

Electron Microscopy

The sarcoplasmic reticular microsomal vesicles were fixed in 2% glutaraldehyde, post-fixed in 1% osmium, sectioned and observed with a Phillips EM 300.

^{35}S-Methionine Incorporation and Acrylamide Gel Analysis

Fourteen day old embryos *in ovo* were given 20 μl of 6 mCi/ml of ^{35}S-methionine for a 6 hr pulse. Subsequently, the sarcoplasmic reticulum (SR) was isolated. Samples of the isolated SR microsomes representing 50 μg of protein were reduced in 2.5% 2-mercaptoethanol at 100 °C for 1 min and layered in wells of a 2% acrylamide-agarose spacer of a 10%–20% gradient acrylamide slab gel. The samples were then electrophoresed in standard Laemmli buffer until the tracking dye reached the bottom of the gel (approximately 8 hr at 100 V, 60 mA). The gels were then stained in 0.1% Coomassie brilliant blue for 30 min and destained overnight in 5% methanol, 7% acetic acid. After destaining, the gels were dried and exposed to an X-ray plate for autoradiography and photographed. The appropriate columns were scanned with a Gilford Recording Spectrophotometer at 550 nm. The normal and dystrophic samples were treated equivalently at all points in the

procedure. This procedure allowed for the resolution of as little as 1 ng protein within a gel band as well as to determine differences in the relative rate of incorporation of the labeled methionine.

Measurement of Fast Uptake of Calcium

The rates of calcium uptake were measured potentiometrically with a Ca^{2+} electrode in 2.5 ml of reaction medium containing 5 mM $MgCl_2$, 0.2 mM ATP, 50 mM KCl, 5 mM Tris, pH 7.0, 0.2 mM $CaCl_2$ and 0.2 mM EGTA, and microsomal protein to 0.04 mg/ml.

The potentiometric set-up was according to Madeira [9]. A radiometer 2117 Ca^{2+} electrode was powered with the radiometer, model PHM64, pH meter and monitored through a Grass model 713 polygraph. Calibration of the system was achieved by successive additions of 10-µl aliquots of 50 nmol of $CaCl_2$ at the end of the experiment.

Measurement of the calcium storage capacity of the microsomes in the presence of oxalate was achieved by monitoring $^{45}CaCl_2$ levels in a medium of 100 mM KCl, 18.5 mM Imidazole (pH 7.0), 10 mM K-oxalate, 4.0 mM creatine phosphate, 5 mM ATP, 5 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 1.6 mM EGTA to $\simeq 10^{-5}$ M free calcium, 0.1 µCi/ml $^{45}CaCl_2$ and 0.04 mg/ml of microsomes. Samples were taken for millipore filtration at 1 min and at 5 min intervals for 20 min using a millipore apparatus and 0.45 µm filters. The amount of Ca^{2+} removed by the microsomes was determined as the product of the total calcium and the fraction of tracer $^{45}CaCl_2$ removed from the medium by the microsomes.

Assay for Ca^{2+} Stimulated ATPase Activity

Calcium stimulated ATPase activity was determined by monitoring at 10-sec intervals ^{32}P liberated from γ-ATP^{32} in a reaction medium of 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 10 mM K-oxalate, 10 mM imidazole, pH 7.0, 0.2 mM EGTA and 10 µM $CaCl_2$/mg SR protein in 0.01 µCi/ml ATP^{32}. The reaction was started by addition of SR vesicles to the reaction mixture to yield a concentration of 500–700 nmol of ATP per mg of SR microsomal protein. Samples taken at 10-sec intervals are stopped in 0.5 ml of 5% Trichloroacetic acid with 50 mg/ml Norit A and spun at 2,000 rpm for 10 min. Ten µl of the deactivated sample were counted in a Packard scintillation counter. The procedure was repeated on normal and dystrophic pectoralis SR microsomes.

Atomic Absorption Spectroscopy: Determination of Total Tissue Calcium

As a final measure of the total calcium storage capacity of the sarcoplasmic reticulum of normal and dystrophic adult and embryonic pectoralis, total tissue Calcium was measured on an Instrumentation Laboratories, Inc., atomic-absorption flame spectrophotometer. Excised muscle was cleaned of fat and connective tissue and dissolved in a tared crucible containing 0.5 ml nitric acid at 110 °C for 12–14 hr. The tissue was then ashed in a 700 °C furnace for 24–48 hr. The tissue ash was then suspended in 1.0 ml of 0.5 N HCl and an aliquot (100–300 µl) was transferred to 5 ml of 1 mg/ml $LaCl_3$ in 0.5 N HCl. Samples were aspirated into the flame and determined for Ca^{2+} against standards measured at 422.7 nm wavelength. Several readings were made of each sample. Total molar tissue Calcium was normalized against wet wt using the equation:

$$\frac{\text{Total Calcium (M)}}{[(\text{wet wt})(\text{mg ml})] \times \text{aliquot added to 5 ml } LaCl_3 \div \text{Dilution Factor}} = \text{M } Ca^{2+}/\text{mg wet wt.}$$

Extracellular calcium was replaced by prewashing tissue in 10 mM $LaCl_3$ for 15 min. La^{3+} a nonpermeant cation, irreversibly binds to Ca^{2+}-binding sites in the extracellular space eliminating the contribution of external calcium to total Ca^{2+} measurements.

Results

Ultrastructural Characteristics of Microsomes Isolated by Differential Centrifugation

The centrifugation and filtration procedures employed in the purification of SR vesicles from the pectoralis, is designed to minimize contamination by membranous contributions from other cellular organelles. An easily determined measure of contamination is the presence of fragmented mitochondrial cristae as determined by electron microscope (EM) observations. Microsomes obtained from all preparations after the final step of differential centrifugation, showed only smooth, clear vesicles when thin sections were viewed through the EM. Their morphology was consistent with that which has been described for purified sarcoplasmic reticular fragments [12] (Fig. 1). There was no visible mitochondrial contamination.

Sucrose Density Gradient Fractionation of Purified Vesicles from Dystrophic and Normal Muscle

Microsomes isolated from adult dystrophic and normal pectoralis, run on isopycnic sucrose gradients showed major differences in sedimentation patterns. There is a dramatic change in the content and resolution of the resolved fractions. The normal pectoralis microsome leaves a narrow, highly concentrated band which comigrates with the leading front of the dystrophic pectoralis microsomes (Fig. 2). The dystrophic microsome forms a nearly continuous band of membrane vesicles which are most concentrated near the top of the gradient, between 22 and 24% sucrose.

Unlike the normal microsomes, the gradient containing the dystrophic microsomes did not yield a pellet following Ca loading and one hour spin at $5{,}000 \times g$.

The increased density and pelleting of the vesicles in the normal pectoralis microsomes could be attributable to the accumulation of

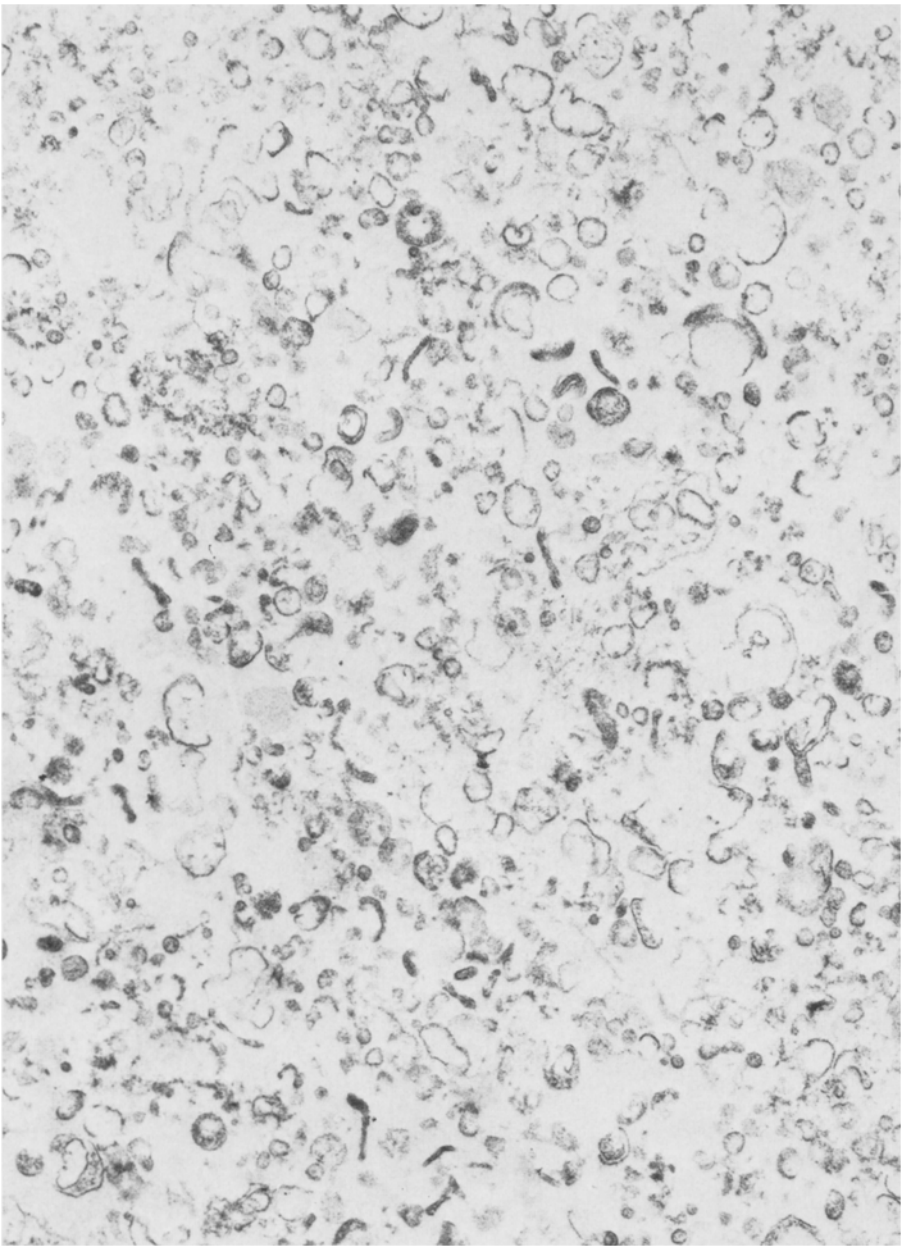

Fig. 1. Skeletal muscle microsomes. The membranous vesicles were first isolated as described in *Methods* and subjected to further purification through passage on a sucrose gradient (Fig. 2) (20–40% wt/vol). The purified vesicles were then sedimented and the pellet fixed in 2% glutaraldehyde for 30 min followed by post fixation in 1% osmium for 1 hr at 4 °C. The fixed pellet was dehydrated, embedded in epon 812, and sectioned. Staining was with lead citrate. Sections were observed and photographed with a Phillips EM 300, magnification 30,000 ×

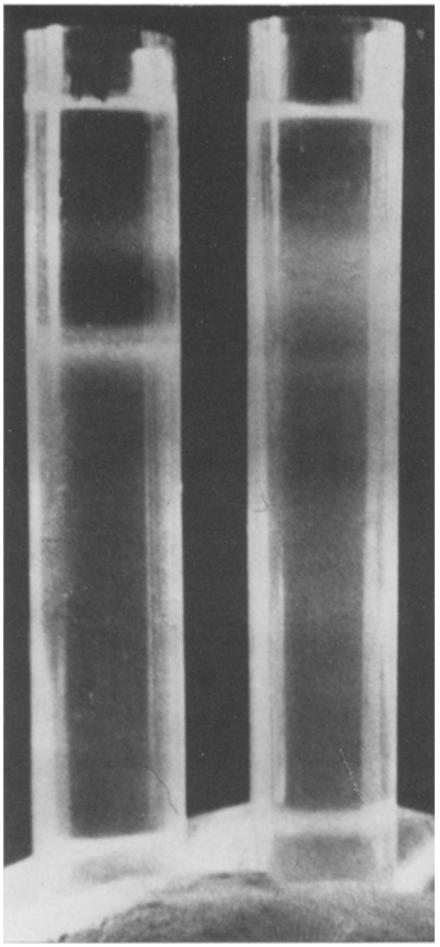

Fig. 2. Sucrose density gradient. The fractionation of the isolated normal muscle microsomes on an isopycnic sucrose gradient (20–40% wt/vol) resulted in the formation of two distinct bands at 26% sucrose and a faint band at 24%. A similar treatment of the dystrophic microsome revealed a broad band of membranes between 24 and 26%, with none of the sharp resolution characteristics of the normal. The gradient was obtained by layering 4 ml of 5 mg/ml microsomal suspension onto 30 ml of 20–40% sucrose in 10 mM imidazole, pH 7.0, and centrifuging for 1 hr at 51,000 × *g* in a Beckman SW27 rotor

calcium oxalate precipitates within them. Over the same incubation interval, the dystrophic microsomes failed to accumulate enough calcium oxalate to alter their sedimentation characteristics. Such differences could likely result from lower calcium storage characteristics and/or differences in the rates of uptake in dystrophic and normal sarcoplasmic reticular

vesicles. We designed the following experiments to determine if such differences did exist and whether they were detectable as primary biochemical lesions in the differentiating embryonic tissue.

Measurement of Calcium Uptake in Microsomal Fractions (SR) from Normal and Dystrophic Muscle

The initial rates of Ca^{2+} uptake in isolated SR represents the maximum rate of uptake at the calcium concentration in the incubation medium. At the start of each experiment, our protein concentration was consistently adjusted to total calcium in the incubation medium to yield 5 μmol Ca^{2+} per mg protein. With the potentiometric technique, we were able to determine the differences in transport rates between the normal and dystrophic sarcoplasmic reticulum from adult and embryonic tissues. We found that the embryonic dystrophic sarcoplasmic reticulum initial uptake was consistently greater than normal by as much as 30% (Fig. 3*b*). Adult dystrophic sarcoplasmic reticulum exhibited an initial rate which was 65% of the normal (Fig. 3*a*). These observations are summarized in Table 1 and Fig. 3*a* and *b*.

Furthermore, a measure of the ratio of the *oxalate-potentiated* calcium storage capacity of homogenates to SR from the embryonic system, demonstrated that the normal homogenate has only 26% of the storage capacity of the isolated SR. The embryonic dystrophic homogenate shows a storage capacity which is 32% of the isolated SR. In the adult, calcium oxalate storage functions are more completely relegated to the SR. In the normal adult muscle, the SR is responsible for 69% of the calcium storage functions, whereas the dystrophic SR is responsible for storing up to 94% of the cell calcium in the presence of oxalate. It is

Table 1

Source	Initial velocity Ca^{2+}	Initial ATPase velocity	Efficiency $Ca/^{32}P$	Capacity at 10 min
	μmol/mg/min			μmol Ca/mg
Embryonic				
Normal SR	14.12	18.0	0.77	0.76
Dystrophic SR	20.4	8.98	2.27	0.65
Adult				
Normal SR	0.68	0.40	1.76	0.96
Dystrophic SR	0.44	1.56	0.38	0.79

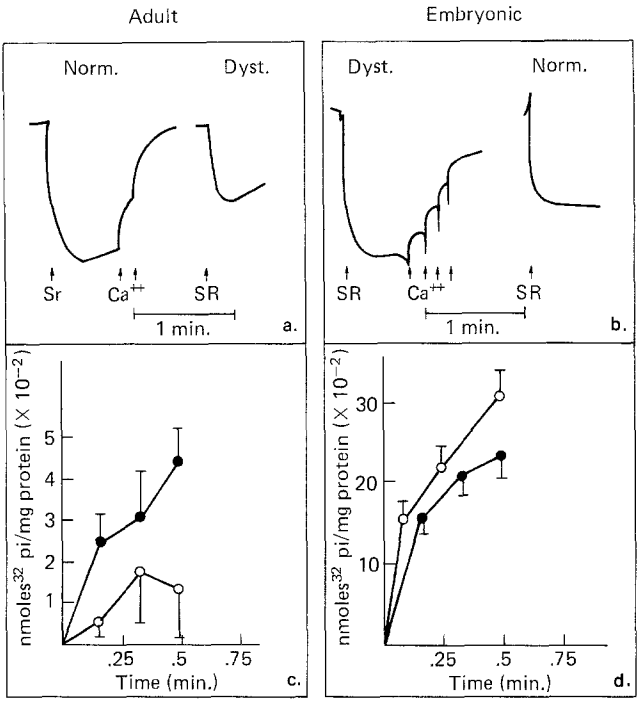

Fig. 3. (*a*): Adult normal and dystrophic calcium uptake. Purified sarcoplasmic reticulum (SR arrow) microsomes were added in an initial concentration of 0.1 mg in 2.5 ml of volume of uptake medium as defined under methods. Ca^{2+} uptake was monitored with a calcium Selectrode (2117) (Radiometer) coupled to a Radiometer pH meter (PHM 64). Initial rate of uptake was measured as the rate of deviation from baseline. Calcium concentration changes were determined by calibrating the electrode following uptake through the addition of 10 µl of 3.4 nmol of CaC_2 ($Ca^{++}\uparrow\uparrow$). The initial velocity for the normal adult SR was measured as 685 nmol/mg/min. Similar determinations for the dystrophic adult SR was 442.5 nmol/mg/min. Addition of H_2O, NaCl, KCl, ATP and Mg did not result in any significant change in electrode output. (*b*): Embryonic normal and dystrophic calcium uptake. Uptake for the embryonic sarcoplasmic reticulum was determined in the same manner as with the adult. Final calibrations were achieved by adding 50 µl of 50 nmol of $CaCl_2$ ($\uparrow\uparrow$). The reactions were initiated by adding 100 µl of SR in a concentration of 0.1 mg in a final volume of reaction medium of 2.5 ml for each measurement. The embryonic normal SR showed an initial uptake rate of 14.20 µmol/mg/min, whereas the dystrophic embryonic SR had an uptake rate of 20.40 µmol/mg/min. (*c*): ATPase activity associated with adult SR calcium transport. ATPase activity was measured according to procedures described in *Methods* by monitoring P^{32} liberated from γ-ATP^{32}. Adult dystrophic SR (●) shows an initial ATPase rate which is close to 4 times the rate for the normal (–○–) (Table 1) under identical conditions of incubation and using the same concentrations of SR and Ca as in the uptake measurements. When the transport of Ca by dystrophic SR is compared to the corresponding P^{32} release, the measured efficiency becomes 0.30 $Ca:P^{32}$. The normal adult exhibits an efficiency of 1.76 $Ca:P^{32}$. (*d*): Shows ATPase activity associated with embryonic SR calcium transport. ATPase activity in embryonic SR was measured as in *c*. The normal ATPase rates were overall 20 times higher than in the adult, and 5–6 times higher in the embryonic dystrophic compared to the adult dystrophic, (Table 1). Embryonic normal SR exhibited an initial rate of 18 µmol P^{32}/mg/mg which was more than twice the dystrophic which was measured to be 8.98 µmol/mg/min. The normal SR showed an efficiency of 0.77 $Ca:P^{32}$ which compares unfavorably to the 2.27 $Ca:P^{32}$ efficiency of the dystrophic SR

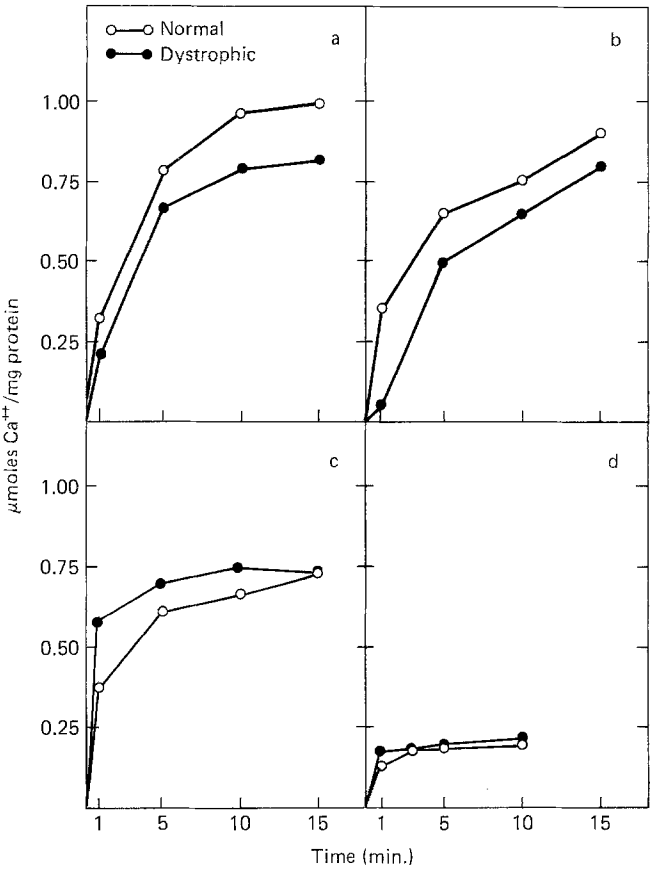

Fig. 4. (*a*) and (*b*): oxalate potentiated calcium storage capacities from adult and embryonic sarcoplasmic reticulum, respectively. Conditions of incubation were augmented by phosphocreatine and oxalate. (*c*) and (*d*): calcium storage values for adult and embryonic whole muscle homogenates (*see* Table 1 for values)

important to note, however, that both embryonic and adult isolated dystrophic SR operate at 80–87% of the storage capacity of their normal counterparts (Fig. 4*a–d*).

ATP^{32} Hydrolysis and Transport Efficiency

Measurements of ^{32}P liberated from γ-ATP^{32} during transport, showed significant differences between the adult and embryonic rates and between the values obtained for the adult SR. Figure 2*c* and *d* show that over the same interval the rate reached by the adult normal was 401 nm

P_i/mg/min and in the adult dystrophic, 1556 nm P_i/mg/min, 2% and 17%, respectively, of their embryonic values at equal concentrations of SR protein. We determined the stoichiometry of the various systems during initial uptake by comparing the rates of calcium transported to $^{32}P_i$ liberated at 10 sec. The values summarized in Table 1 indicate that embryonic dystrophic SR show 3 times greater initial efficiency than the embryonic normal, translocating ~2 Ca ions for every ATP hydrolyzed. In the adult, the dystrophic SR operates at 17% of the efficiency of the normal, translocating ~one calcium for every three ATP molecules hydrolyzed.

These observations further suggest that, during normal development, the efficiency of Ca transport improves by a factor of 2.3 in the adult over embryonic values. This relationship is markedly reversed in the development of dystrophic SR, where the efficiency of the adult dystrophic SR is 13% of the embryonic.

Measurement of Total Tissue Calcium

The accumulated experimental data suggest that in dystrophic muscle the sarcoplasmic reticulum not only sequesters calcium at a reduced rate, but is also incapable of storing Ca in amounts comparable to normal controls. It was anticipated that these measurable differences in storage and uptake rates might be reflected in an overall diminution of whole tissue Ca. Furthermore, if the dystrophic cell Ca bears the same relationship to the normal controls in both the adult and embryonic tissues, then the evidence would be compelling that the genetic disorder was manifested as a primary lesion in the Ca transport and storage functions of the SR. We first measured total bound Ca simply by excising the pectoralis and washing in phosphate-buffered saline. Total Ca was then measured in small aliquots with an atomic absorption spectrometer. In a second set of experiments, extracellular Ca was replaced by preincubation of the excised tissue in lanthanum chloride. When data from these two sets of experiments were compared, it was apparent that for both the adult and embryonic tissue, 80% of the cell calcium was found in the extracellular space. We consistently found higher amounts of total cellular calcium in the embryonic normal tissues than in their adult counterparts.

When values for the intracellular tissue Ca from the normal embryos were compared to values for dystrophic embryonic tissue, they were

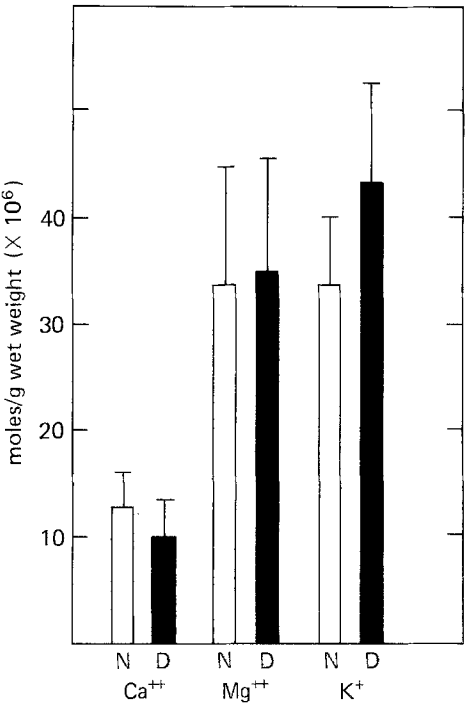

Fig. 5. Atomic absorption measurement of total tissue calcium, potassium and magnesium. The tissue in each case was washed in phosphate-buffered saline, weighed in a tared crucible, and hydrolized in HCl at 700 °C and reweighed. Total Ca, Mg and K were determined against measured standards in small aliquots by atomic absorption spectroscopy. Extracellular Ca was displaced by washing the tissue in 10^{-3} M lanthanum chloride prior to hydrolysis during calcium determination. Measurements indicated that as much as 80% of tissue Ca is bound extracellularly. Values for cellular Ca in normal muscle was 12.82 ($\pm$3.27) mM *vs.* 9.77 ($\pm$3.70) ($P<0.05$) for dystrophic. Values for K and Mg from dystrophic tissues were not plastically significantly different from normal ($P<0.10$)

found to exceed the dystrophics by a factor of 1.3:1 ($P<0.05$) (Fig. 5). The ratio was consistent whether the tissues were pretreated with lanthanum to remove extracellular Ca or left untreated.

^{35}S-*Methionine Incorporation*

Dystrophic and normal chick pectoralis were excised from 14 day old embryos which had been exposed to ^{35}S-methionine following 6 hr incubation. The muscles were washed with extraction solution and the sarcoplasmic reticulum isolated as indicated in *Methods*. 50-μg samples of each of the SR were loaded onto 10–20% gradient polyacrylamide slab

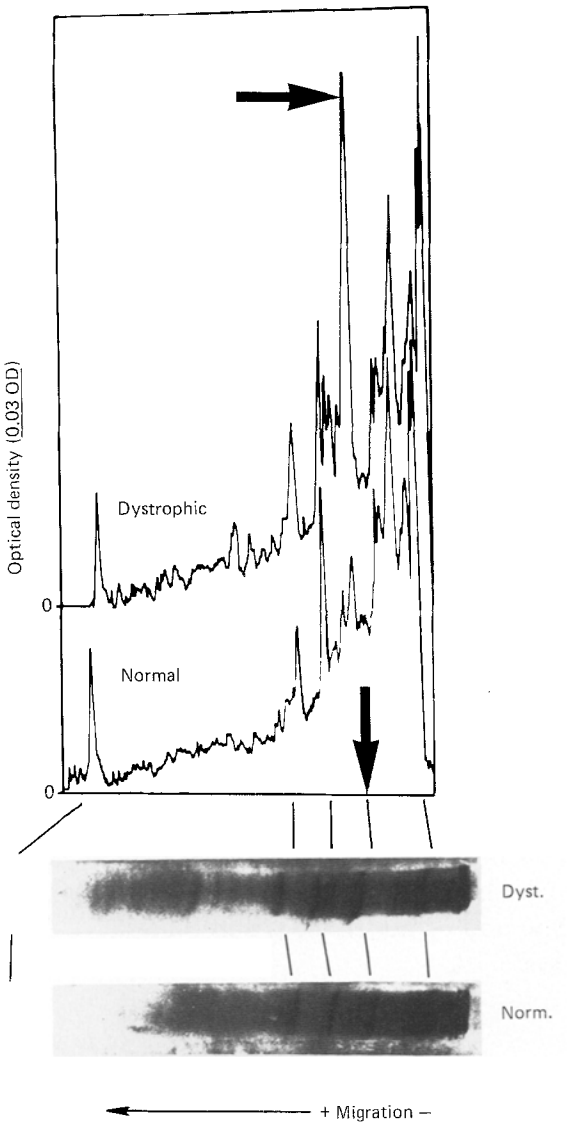

Fig. 6. ^{35}S-Methionine autoradiograph and densitometric plan of labeled muscle microsomes gel electrophoretic pattern. Embryos were labeled *in vivo* with ^{35}S-methionine as described under *Methods*. The embryos were pulsed with the label for 6 hr during which fusion of myoblast and the differentiation of muscle membranes was in process. The tissue was excised, washed in saline, and the labeled microsomes were extracted. 50-μg samples were loaded onto a 7.5–15% gradient polyacrylamide slab gel. The gels were dehydrated and exposed to a Kodak XRP-1 X-ray photographic plate. The component running at approximately 65,000 daltons (arrows) in the dystrophic lane has incorporated label at a greater rate than the normal tissue over the 6-hr interval

gels. The gels were subsequently dried and placed in direct contact with an unexposed X-ray plate for 24 hr. The positive print of the plate is shown in Fig. 6. It is evident that a component migrating at 65,000 daltons has incorporated a greater amount of the label in the dystrophic tissue. However, the same dystrophic protein band stains much less intensely with Coomassie blue. The amount incorporation of the label in this protein is, however, more than twice that of normal controls. The high rate of incorporation of the label in the dystrophic band is a feature which has been suggested to be a characteristic of abnormal proteins exhibiting high turnover rates. It is important to note that none of the other SR proteins on the gels, in both dystrophic and control samples, show any significant differences in incorporation.

Discussion

The active maintenance of electrochemical gradients by exclusion or sequestration of certain cations is essential for the regulation of normal cell function. Calcium is a cation which is an essential cofactor in motility and important in the regulation of fundamental aspects of the physiological and biochemical enzymatic mechanisms of cells; therefore, all cells and tissues must be able to regulate intracellular Ca ion concentration gradients in order to maintain a cytosolic concentration of 1 μM or lower in the cytoplasm. In muscle, cells must remove cytosolic calcium through the expenditure of energy by use of a Ca−Mg dependent ATPase and/or buffer the Ca in a nonionic form with calcium-binding proteins.

In muscle cells, the role of Ca-binding proteins is essential to both the regulation of contraction and the maintenance of low cytoplasmic Ca^{2+} concentrations. Skeletal muscle sarcoplasmic reticulum amplifies the Ca-regulating ability of the sarcomere, in association with a system of Ca-binding proteins so that the bulk of the cell calcium is found in the SR. Calcium binding and release by the SR can affect cytosolic calcium concentrations over 4 logarithmic units within milliseconds. This allows for controlled contraction and relaxation of the muscle fibers [16].

These results indicate that dystrophic muscle in embryonic chicken pectoralis have dysfunctional sarcoplasmic reticular fractions. Calcium uptake and the capacity to bind Ca within sarcoplasmic reticular vesicles is significantly depressed in the diseased muscle (20% of normal).

The observation that embryonic dystrophic muscle SR is defective in calcium metabolism indicates that the lesion is manifested early in the course of the disease, implying that there may be a primary lesion in the

synthesis of Ca-binding SR proteins. This deficiency in Ca-binding proteins would result in an impaired capacity to bind Ca by the SR. The decreased ability to sequester Ca would result in a prolonged Ca transient in the sarcoplasm following activation. The lower efficiency of the Ca binding by the proteins of the SR in dystrophic muscle would also result in a decreased rate of Ca loading. This would, in turn, account for the slow relaxation time of dystrophic muscle after contraction, a phenomenon observed by Roe *et al.* [9].

The higher cytoplasmic calcium, though subthreshold for contraction, may be sufficient to initiate other cellular calcium-dependent enzymatic systems, giving rise to fundamental deviations from normal cellular metabolism which would cascade into the many defects seen in the clinical manifestations of this disease. It is entirely possible that this defect may also be manifested in cells other than striated muscle. Preliminary results indicate that isolated heart and brain membrane vesicles from dystrophic chicken embryos are also exhibiting abnormal Ca metabolism (*unpublished*).

Possibly this defect is manifested not in the *de novo* synthesis of the calcium-binding proteins, but rather in their assembly and/or incorporation into the membranes of the reticulum. We have assayed for this possibility incident to the preparation of SR for the relative distribution of these protein bands. Normal and dystrophic chick pectoralis, which have been incubated in a ^{35}S-methionine enriched medium for 6 hr incorporation of the label and encompassing the fusion of cells to form myotubes, show that a component running at 65,000 daltons incorporates a greater amount of the label in the dystrophic cultures. However, the much less intensity of Coomassie blue staining in the dystrophic SR samples leads us to the tentative conclusion that the diminished amounts of protein in the dystrophic is possibly due to increased turnover of this protein. Experiments are in progress in which turnover rates for all SR proteins are being further investigated. Hopefully, these possibilities will enable us to extend these results to human dystrophic muscle cell culture.

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