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Increased Turnover of Proteins from the Sarcoplasmic Reticulum of Dystrophic Chicken Muscle Cells in Tissue Culture*

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Chicken myoblasts were cultured from the pectoralis muscles of dystrophic and normal 11-day-old embryos. Cells were allowed to grow to fusion (differentiation) and exposed to [^{35}S]methionine for a short period. Subsequently, the decay of labeled proteins in the presence of cycloheximide was measured for various cellular fractions as well as individual proteins isolated from the sarcoplasmic reticulum and separated by gel electrophoresis. Some dystrophic material showed an increased decay when compared to normal material. The most significant ($p < 0.005$) difference was found in a M_r = 65,000 component of the sarcoplasmic reticulum. This same component accumulates label at an accelerated rate in the presence of the protease inhibitor leupeptin. Increased turnover of this protein, possibly calsequestrin, may be a manifestation of the genetic disease.

Avian muscular dystrophy is a genetic disease resulting in the progressive degeneration of skeletal muscle tissue (1), with visible swelling of mitochondria and the sarcoplasmic reticulum. Similar ultrastructural changes are also seen in cloned muscle from dystrophic human muscle biopsies (2). Physiological changes related to the ultrastructural events include the partial loss of normal tension development and delayed relaxation (2-5). However, the most significant change is characterized by progressive atrophy of the muscle (4), which reflects an accelerated degradation of the muscle fiber proteins (6-8).

It is possible that, by the time the pathological aspects are apparent, the molecular pathology of the primary defect is obscured by other secondary manifestations of the disease. We have shown that embryonic dystrophic sarcoplasmic reticulum vesicles showed an increased calcium transport over normal SR[1] vesicles (9). However, there was a decreased transport in the adult dystrophic SR vesicles. This observation emphasized the importance of an investigation into the early events in the development of the muscle pathology. This work extends these observations to the identification of differences in the protein composition of the sarcoplasmic reticulum from normal and dystrophic chicken muscle.

Evidence that proteins resulting from genetic errors may be selectively degraded at high rates (10, 11) led us to investigate the possibility that abnormally functioning proteins contained in the sarcoplasmic reticulum may turn over more rapidly. We proposed to investigate the process of development which involves myoblast fusion in cell cultures for evidence of abnormal protein synthesis and degradation during the differentiation of the sarcoplasmic reticulum, before the onset of the pathology. This approach enabled us to examine early events in molecular differentiation for evidence of protein abnormalities which may relate to the latter onset of the disease. A number of separate investigations have reported various changes in the collective rates of synthesis of dystrophic muscle proteins *in vivo* (12-14). Other studies suggest higher average rates of degradation (6-8). We focus here on the unusual kinetics of synthesis and decay of a specific protein component of the sarcoplasmic reticulum which may result from a genetic alteration.

MATERIALS AND METHODS

Chemicals—[^{35}S]methionine (500-780 Ci/mmol) was obtained from New England Nuclear. Soybean trypsin inhibitor and marker proteins for electrophoresis standard were obtained from Sigma. Ampholines for electrophoresis were obtained from Bio-Rad. Cell culture media and supplements were obtained from Microbiological Associates.

Animals—White Leghorn fertilized eggs were obtained from SPAFAS, Inc. Dystrophic eggs were supplied from the Department of Animal Genetics, University of Connecticut, Storrs, CN. Eggs were incubated in a Leahy Manufacturing Co. incubator (Higginsville, MO) at 38 °C and a relative humidity of 70%.

Cell Cultures—Muscle cell cultures were prepared from the pectoralis muscles of 10- to 12-day-old chicken embryos according to the procedures of Paterson and Strohman (15). Plating density was usually 2.5×10^6 cells/100-mm plate. Cells were grown for the first 24 h in minimal essential medium supplemented with 10% horse serum and 2% embryo extract. All culture plates were precoated with a 0.1-mg/ml of gelatin solution. Following a change of medium, myoblasts were allowed to grow to confluence. Fusion was observed to be initiated within 48 h after plating. Cell in minimal essential medium deficient in methionine were rountinely labeled with [^{35}S]methionine (20 μCi/ml) final concentration after 70% of nuclei were in multinucleated fibers. For procedures involving suppression of protein synthesis and determination of protein half-lives, 0.5 μg/ml, final concentration, of cycloheximide was added to each plate following an appropriate pulse with the labeled amino acid.

Isolation of Sarcoplasmic Reticulum—Harvesting of labeled cells was accomplished by washing four times with Earle's balanced salt solution, scraped free of the plate with a rubber policeman, and suspended in 1 ml of 0.3 M sucrose and 10 mM imidazole, pH 7.0, at 4 °C, with 50 μg/ml of soybean trypsin inhibitor. The cell suspension was homogenized with a dounce homogenizer. Samples were taken of the cell homogenate for counting and for SDS-polyacrylamide gel electrophoresis. Membranes were isolated from the homogenate according to procedures published elsewhere (9). One-μl samples of the cell homogenates and isolated membrane fractions were spotted onto glass fiber filters, and proteins were precipitated by boiling in 10%

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; SDS, sodium dodecyl sulfate.

trichroloacetic acid. Subsequent washes in water and acetone removed any unincorporated label. The activity on the filters was then counted.

SDS-Gel Electrophoresis—Samples were also solubilized in 1% SDS and 10 mM dithiothreitol, 12.5 mM iodoacetamide, and glycerol/bromphenol blue for 1 min at 100 °C. They were subsequently layered onto a 7.5 to 15% gradient slab gel and subjected to electrophoresis according to the method of Laemmli (16). Gels were vacuum heat dried and exposed to Kodak XR-5 x-ray film for autoradiographic analysis. The exposed film was developed in a Kodak X-omatic processor. Processed XR-5 negatives were scanned with an Ortec (4310) microdensitometer. The molecular weights of the resolved protein bands were determined with [^{35}S]methionine labeled vesicular stomatitis virus coat proteins of known molecular weight.

The amount of label in each band is directly proportional to the optical density of the band and decreases exponentially following a cold chase in unlabeled methionine. The values of optical density at hourly intervals for each protein band in the XR-5 negatives were used to determine the half-life of the protein following washout of the label. Data points were correlated by linear regression analysis, and graphical print-outs on a digital PDP/1104 computer were obtained.

Two-dimensional Gel Electrophoresis—The method of O'Farrell (17) was used. Pelleted sarcoplasmic reticulum microsomes from [^{35}S]methionine-labeled myotubes were suspended in lysis buffer and applied to tube gels for isoelectric focusing. Electrophoresis conditions were 600 V for 12 h, then 2000 V for 2 h. The isoelectric focusing gels were removed from the tubes and the pH gradient was determined by a contact electrode (LKB) as a function of gel length. They were then equilibrated for 30 min in a solution containing SDS and subsequently overlayed on a 12% polyacrylamide slab gel. Electrophoresis was for 4 h at 150 V according to Laemmli (16). The slab gels were subsequently fixed, dried, and used to expose to Kodak XR-5 film at −80 °C.

RESULTS

Incorporation into Muscle in Ovo—Dystrophic and normal pectoralis muscle removed from 14-day-old embryos which had been exposed to [^{35}S]methionine for 6 h showed significant incorporation of the label into several prominent protein bands of sarcoplasmic reticulum microsomes, with M_r = 100,000, 65,000, 54,000, 46,000, and 36,000 (Fig. 1). The M_r = 100,000 component had the mobility reported for the (Ca^{2+} + Mg^{2+})-dependent ATPase (18). Components with M_r = 65,000 and 54,000 migrated on gels with the same apparent mobility as the calcium binding proteins calsequestrin and the high affinity calcium binding protein, respectively (18). The other component at M_r = 12,000 may be a hydrophobic lipoprotein (19) and at M_r = 36,000, a glycoprotein (19). These latter two proteins have unknown functions in the sarcoplasmic reticulum (19). Fig. 1 shows that the protein which M_r = 65,000 from dystrophic cells incorporated three times the label than the equivalent band in the normal controls, suggesting that "calsequestrin" was synthesized at a higher rate in dystrophic tissue than control values. Increased labeling was also apparent in other lower molecular weight components of dystrophic SR.

Incorporation of Methionine into Total Protein and Sarcoplasmic Reticulum of Muscle Cell Cultures—In order to assess whether a putative biochemical defect in dystrophic muscle may be associated with an increased turnover of the proteins of the sarcoplasmic reticulum, the incorporation of [^{35}S]methionine into pectoralis myotubes in culture was observed as well as the subsequent decay of label after replacement of the medium with unlabeled methionine. The isolated sarcoplasmic reticulum was compared with total homogenate in both normal and dystrophic cells with respect to kinetics of labeling and decay. Cells were harvested at 3-h intervals and homogenized. Samples of the total homogenates were counted and also used to extract the sarcoplasmic reticulum microsomes. The uptake of the label into the cell homogenates as well as its decay appeared nearly identical for both dystrophic and control cultures (Fig. 2*A*). However, as shown in Fig. 2*B*, at the end of the incorporation, the specific activity of the label was 3.5 times greater in the dystrophic SR than in the dystrophic homogenate. The amount of label in the normal SR was 2.7 times greater than in the corresponding homogenate. The loss of label from dystrophic and normal SR appeared to be biphasic. Unlike the homogenate, the rate of initial labeling as well as the subsequent loss of label from dystrophic SR is increased over the normal SR.

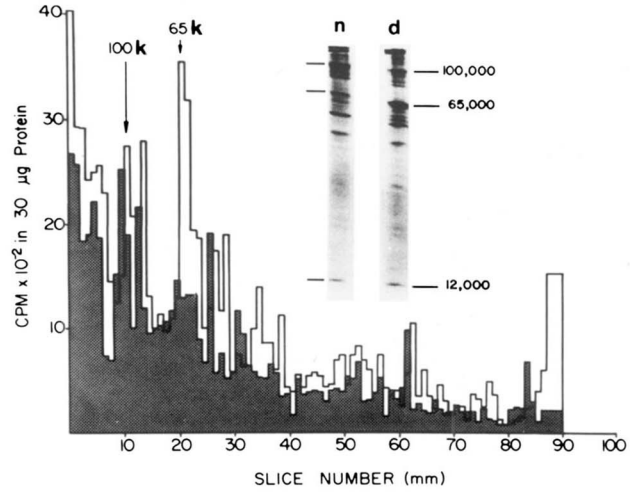

FIG. 1. **Electrophoresis and autoradiography of labeled muscle microsomes.** Chicken embryos after 14 days of incubation were labeled *in ovo* with 250 µCi of [^{35}S]methionine for 6 h during which fusion of myoblast and the differentiation of muscle membranes was in process. The tissue was excised and washed in saline, and the labeled microsomes were extracted. Fifty-µg samples were loaded onto a 7.5 to 15% gradient polyacrylamide slab gel. The gels were dehydrated and exposed to a Kodak XR-5 x-ray film. 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-h interval. The *speckled region* on the graph shows label incorporated into 1-mm slices of the lane carrying the normal SR and is shifted 1 mm to the left with respect to the dystrophic slices (open faced). The gels which were scanned are shown in the *inset* (*n* = normal, *d* = dystrophic); the *lines on either side of the lanes* indicate the position of the molecular weight markers.

Cycloheximide-treated Cultures—Cycloheximide (0.5 µg/ml) was added to each cell culture after a labeling period of 6 h. This concentration effectively inhibits any further incorporation of label; hence, the results reflect only the decay of the proteins. As was seen in the case of Fig. 2, biphasic decay rates were obtained for both dystrophic and normal controls (Fig. 3).

The half-lives of loss of radioactivity in the homogenate fractions were shown to be 19 h for the dystrophic and 32 h for the normal (data given in Table I).

Unlike the homogenates, the 5-h decay profile of sarcoplasmic reticulum from cultures treated with cycloheximide was biphasic in both dystrophic and normal controls. The computer-fit half-lives for the fast decaying component of the biphasic curve was 1 h for normals and 0.5 h for dystrophic and a proportional increase of 20 times longer for the slow decaying components. Thus, some proteins in the dystrophic SR are being collectively degraded at nearly twice the rate of the normal SR.

Decay of Specific Proteins from the Sarcoplasmic Reticulum—Isolated, labeled sarcoplasmic reticulum described above was analyzed on 7.5 to 15% polyacrylamide gradient slab gels. Equivalent amounts of four samples, taken at hourly intervals after chasing, were loaded into each lane of the gel, and electrophoresis and autoradiography were performed as

described. Fig. 4 shows the resultant profile for decay in the radioactivity of specific proteins over a 4-h period. Since the cultures were treated with cycloheximide, the gel profiles represent only degradation of individual protein constitutents of the SR. The film in Fig. 4 was then cut into strips corresponding to the lanes, and the optical density for each protein (not saturating the film) was obtained. These values were then plotted on a semilog scale *versus* time to arrive at an estimate of their decay profiles and determination of half-lives as shown in Fig. 5. Table II shows values obtained for half-lives of each major component of the SR in normal and dystrophic cell cultures. In all cases except that of the M_r = 100,000 component, the half-lives of the dystrophic proteins are faster than those for normal controls. The most significant difference in decay rate occurs in the M_r = 65,000 component ($P < 0.005$). All half-lives can be represented as monophasic components, some of which fit the short lived kinetics observed in Fig. 5 for the total decay profile of the SR and some of which fit the long lived kinetics. Components migrating at M_r = 140,000, 100,000, 68,000, and 54,000 in both dystrophic and normal controls show half-lives which correlate well with the half-lives displayed by the shorter lived populations in both normal and dystrophic SR and are not significantly different from each other (Table II). Other minor components present in the gel may represent either precursor forms of these membrane proteins or specific degradation intermediates of any of the larger protein components.

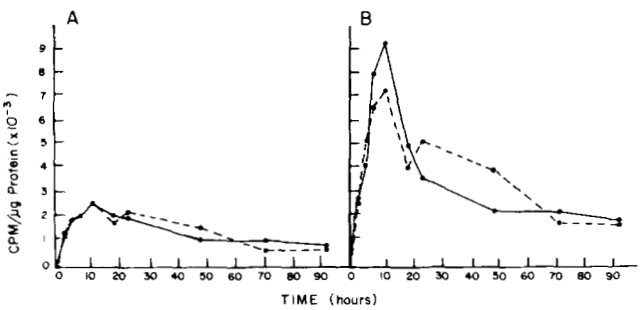

FIG. 2. **Kinetics of methionine incorporation in total homogenate and isolated microsomes from normal and dystrophic chick embryo muscle cultures.** Pectoralis tissue from 12-day-old chicks was removed and plated at 10^6 cells/plate. After 3 days of culture the medium was replaced with medium lacking methionine and the cultures were labeled for 10 h with [^{35}S]methionine (250 μCi/plate). After 10 h, the labeling medium was replaced with fresh medium and the incubation of plates of cells continued for an additional 80 h. The cells were harvested at specific intervals during labeling and chasing by washing twice with 0.3 M sucrose, pH 7.0, 10 mM imidazole and then scraping cells into a total volume of 2 ml. The homogenate was assayed on filter paper and washed with hot trichloroacetic acid (*a*) after cellular disruption by dounce homogenization. The remaining homogenate was centrifuged according to the procedures previously described (9). The SR pellet was suspended in extraction solution and aliquots were counted (*B*). In all cases protein concentrations were measured colorimetrically, and data were expressed as counts per min/μg of protein. - - -, normal cells; ——, dystrophic cells.

Two-dimensional Gel Electrophoresis of Leupeptin-treated Cultures—Normal and dystrophic cultures were labeled with [^{35}S]methionine for 6 h and lysosomal protease activity in the differentiating myotubes was blocked by treatment with leupeptin (20–22). The sarcoplasmic reticulum was subsequently isolated from each culture and equal amounts subjected to two-dimensional gel electrophoresis. The resulting pattern from exposure of the gel on film is shown in Fig. 6. A major feature of the gel is the dramatic amount of label incorporated into the M_r = 68,000 component in dystrophic cells, as well as a much diminished M_r = 65,000 protein. Normal sarcoplasmic reticulum showed both M_r = 68,000 and 65,000 proteins with approximately equal amounts of incor-

TABLE I

Half-lives ($t_{1/2}$) of decay for cellular homogenates and sarcoplasmic reticulum components

	$t_{1/2}$ ± RMS[a]
	h
Homogenate	
Normal	19.11 ± 0.01[b]
Dystrophic	31.75 ± 0.02
Sarcoplasmic reticulum (short lived)	
Normal	1.12 ± 0.01
Dystrophic	0.57 ± 0.02
Sarcoplasmic reticulum (long lived)	
Normal	22.45 ± 0.01
Dystrophic	13.8 ± 0.01

[a] Root mean square residual.
[b] n = 4 for each mean value.

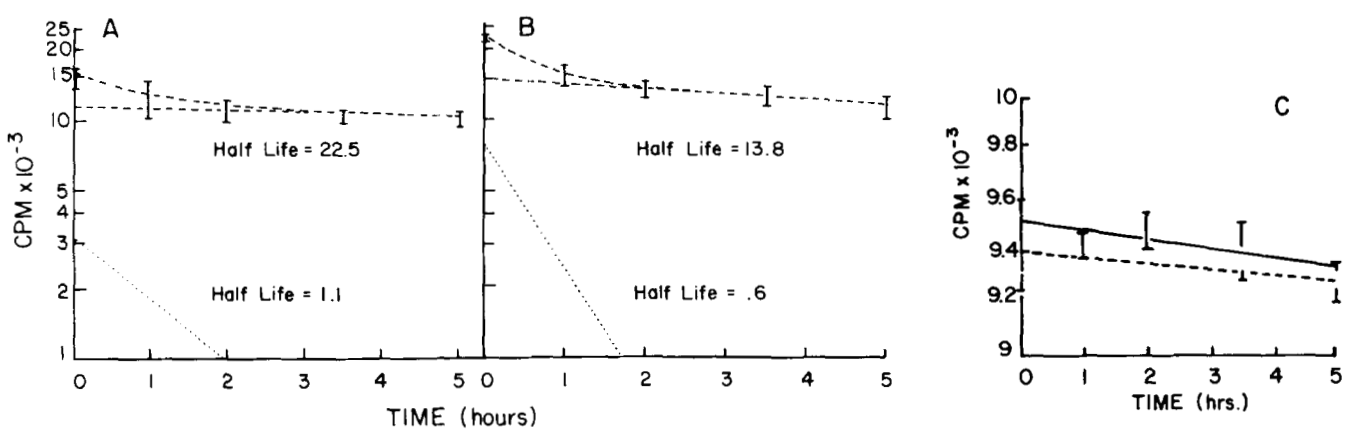

FIG. 3. **Decay of methionine-labeled proteins in the presence of cycloheximide.** For the suppression of protein synthesis and determination of protein half-lives, 0.5 μg/ml, final concentration, of cycloheximide was added to each plate following a 6-h exposure to [^{35}S]methionine. Labeled cells were harvested at the indicated intervals subsequent to the addition of the drug by washing four times with Earle's balanced salt solution, scraped with a rubber policeman, and suspended in 1 ml of 0.3 M sucrose and 10 mM imidazole, pH 7.0, at 4 °C, with 50 μg/ml of soybean trypsin inhibitor. Aliquots of the cell homogenates and isolated membrane fractions were then counted. Loss of label is plotted as a function of time after drug addition and normalized per μg of protein. Computer plots for each biphasic component of the projected half-lives (in hours) are included for the normal (*A*) and dystrophic (*B*) SR, but not for the homogenate (*C*) where the difference between the normal (- - -) and dystrophic (——) was not significant.

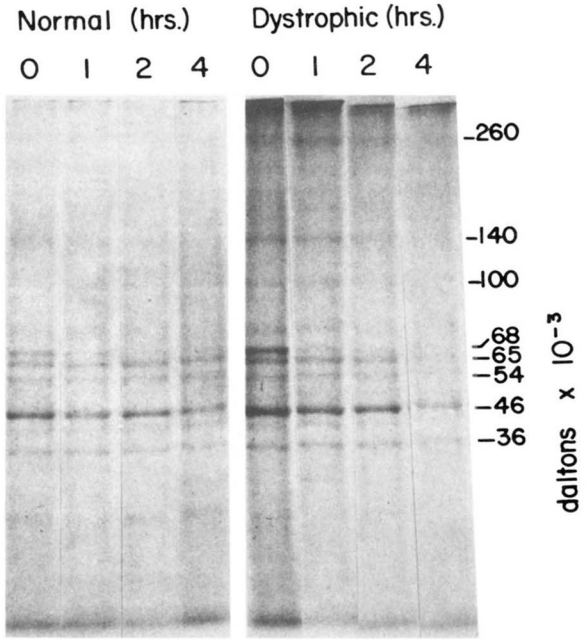

FIG. 4. **Electrophoresis separation of labeled proteins from isolated sarcoplasmic reticulum of normal and dystrophic cells after cycloheximide treatment.** At the times noted, equal amounts of samples from Fig. 3 were removed for further analysis by SDS-acrylamide gradient slab gel electrophoresis for the purpose of identifiying the decay rates of particular proteins. The dried gel was used to expose the x-ray film presented here.

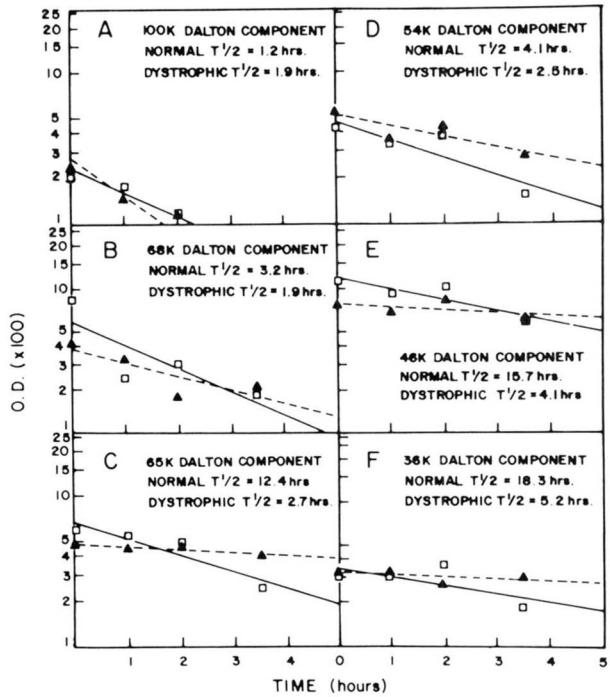

FIG. 5. **Computed degradation half-lives for particular proteins.** The exposed bands on the x-ray film from Fig. 4 were scanned for optical density as described and the densities of each band in arbitrary absorbance units was plotted as a function of time in cycloheximide. Each molecular weight species is plotted in a separate panel as noted. Normal points are represented by *triangles;* dystrophic points are represented by *squares.*

poration. However, the amount of label incorporated into each of these components was dramatically lower than that incorporated into the M_r = 65,000 component of the dystrophic tissues. Other significant increases in labeling were evident in specific proteins of the dystrophic gel patterns, particularly at higher molecular weights and more acid pH, possibly indicating additional precursor forms.

TABLE II

Half-lives of decay for individual SR proteins

M_r	$t_{1/2}$	S.D.	p
	h	*h*	
140,000			
Normal	2.3	0.59	10%
Dystrophic	2.1	0.27	
100,000			
Normal	1.2	0.35	0.02
Dystrophic	1.9	0.25	
68,000			
Normal	3.2	0.86	0.07
Dystrophic	1.9	0.87	
65,000			
Normal	12.4	0.43	0.0005
Dystrophic	2.7	0.48	
54,000			
Normal	4.1	0.71	0.025
Dystrophic	2.5	0.65	
46,000			
Normal	15.7	2.16	0.0015
Dystrophic	4.1	0.67	
36,000			
Normal	18.3	1.76	0.001
Dystrophic	5.2	1.45	

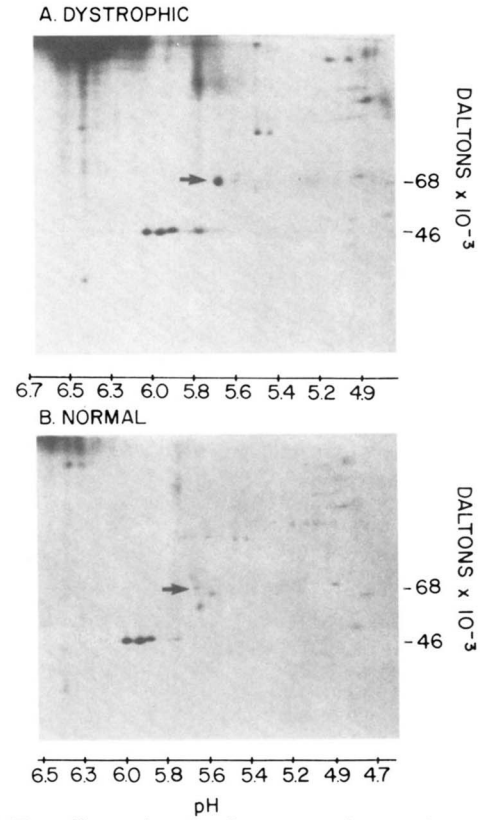

FIG. 6. **Two dimensional gel pattern of sarcoplasmic reticulum proteins from normal and dystrophic cultures.** Isolated microsomes labeled with [^{35}S]methionine for 6 h from dystrophic (*A*) and normal (*B*) cell cultures were subjected to two-dimensional gel electrophoresis. Cultures were treated with 8 μg/ml of the protease inhibitor leupeptin during this time. The *arrow* in *A* points to a 68,000-dalton component of the dystrophic which is virtually absent in the normal SR.

DISCUSSION

We have shown in Fig. 1 that, during the culturing of cells from dystrophic pectoralis of embryonic chicken, the sarco-

plasmic reticulum in general, and a M_r = 65,000 component thereof in particular, exhibited an increased rate of incorporation of [^{35}S]methionine compared to normal cells. Additionally, this event occurs in cells undergoing differentiation and, hence, early in the ontogeny of the muscle tissue. This observation led us to a more detailed kinetic study in muscle cell cultures, with particular emphasis on the sarcoplasmic reticulum, over a time interval which included the fusion of myoblasts into myotubes and the subsequent synthesis of muscle proteins.

Our earlier observations that embryonic dystrophic sarcoplasmic reticulum was physiologically and biochemically different from either embryonic normal or adult dystrophic SR (9) contributed to our exploration of the SR proteins for evidence of degradation of abnormal proteins. The labeling of the dystrophic SR proteins showed a higher specific activity than normal SR despite the fact that the total cellular homogenates in either case were similar. Consistent with the observation of increased labeling is the fact that dystrophic sarcoplasmic reticulum shows a higher degradation rate for constituent proteins than either cellular homogenates or normal SR.

When we examined the comparative degradation of individual protein components of the dystrophic and normal sarcoplasmic reticulum, after separation by gel electrophoresis, we observed that the greatest stabilities were exhibited by the M_r = 140,000 and 100,000 components. These proteins provided an internal standard of control such that any significant comparative variations in the degradation of other proteins would suggest a possible candidate for a primary product of the genetic dysfunction based on evidence for selective degradation of abnormal proteins (10, 11). Confirmation of increased degradation of the M_r = 65,000 component ($p < 0.0005$) compared to the other components or to normal SR proteins is consistent with the observed increased incorporation and, hence, an increased turnover. The lower molecular weight components with increased turnover may be either degradation products of the same defect or additional proteins from nearby genes affected by the genetic defect. Spradling *et al.* (23) have found in *Drosophila* that the genetic lesion *ocelliless* affects the transcription of nearby genes. Alternatively, however, the elevated degradation may not be a primary defect but could be attributable to errors in post-translational modifications such as protein processing, membrane insertion, or glycosylation. Finally, these proteins may all be components of a supramolecular complex involved in Ca^{2+} transport as is the putative calsequestrin (65,000) component. If such a complex functioned "abnormally" in the SR membrane, it could conceivably be "wholly" susceptible to enzymatic degradation.

Increased incorporation into the M_r = 68,000 component in the presence of leupeptin (Fig. 6) at the expense of the 65,000 component suggests that the former may be a precursor of the latter. The observation that the protein accumulates in the presence of leupeptin also supports the concept that it is highly susceptible to protease activity. It is possible that the larger protein contains a "signal" sequence (24) for insertion into the SR membrane which may not be functioning properly in the dystrophic cells. Both of these proteins are present in normal cells, perhaps representing normal processing activity.

It is worth noting that any abnormality in the biochemical function of the sarcoplasmic reticulum would result in abnormal Ca^{2+} metabolism. Since calcium is a key cofactor in cellular energy metabolism and motility, it is logical that cellular catabolism and motility would be adversely affected in a manner consistent with that observed in the pathology of dystrophic muscle as described in the Introduction.

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