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CONTENTS

Muscular Dystrophy Group, 1981

STRUCTURAL CHANGES IN STRIATED MUSCLES OF DYSTROPHIC HAMSTER

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Since the first introduction of a strain of myopathic hamster by Homburger's group[1] in 1962, attention has been given to the myopathy of this animal, especially its cardiomyopathy. This strain has an autosomal recessively inherited polymyopathy, but has no other defective organs or tissues except for these striated muscles.

The present work aimed at checking the degree of damage in various skeletal muscles at different ages. BIO 15.6 male hamsters were used for the studies. After being sacrificed by a blow on the head and bleeding, the animal was fixed on a board and the various muscles were carefully removed including both terminal tendons. The muscles were fixed *in situ* length on dental wax and 4% glutaraldehyde buffered with cacodylate was poured on the materials. They were then teased into small bundles of fibers by forceps 30 min later and transferred to a small bottle containing a glutaraldehyde fixative. The usual methods were then followed of a second fixation of osmium tetroxide, staining *en bloc*, dehydration with alcohol and acetone, and embedding in epoxy resin. A relatively thick section with toluidine blue staining was prepared for light microscopy and a thin section with lead staining for electron microscopy. The cross section view for light microscopy was mainly used to determine the grade of muscle damage and this was done by the criteria according to Bajusz *et al.*[2], slightly modified as follows: ±, less than 4 cells with central nuclei (CN) among 100 cross sectioned cells; +, less than 8 (CN) cells in 100; ++, more than 8 (CN) cells in 100; +++, abundance of CN cells and other abnormalities, ++++, severe abnormalities with necrotic damage.

As shown in Table 1, 12 striated muscles of different ages were tested with fixed materials. All had some degree of abnormalities, particularly severe ones in the shoulder muscle. When the central nuclei were seen in a longitudinal section, they were arranged in a long row. In this same section an abnormal arrangement of nuclei was recognized in a long row although they were not localized at the center but at the periphery (Fig. 1). Other nucleic abnormalities such as perinuclear cavities or pores were sometimes present. Myofibrils usually looked generally normal even in a grade +++ muscle, except for the nuclear anomaly. When the muscle was observed in detail under high magnification, both free polysomes and membrane-bound ribosomes were distributed around the nucleus and submembraneous region, which could not be seen in a normal adult muscle. In a grade ++++ muscle some vacuoles and disarrangement of myofibrils appeared here and there together with abundant CN.

TABLE 1. The Degree of Muscle Damage

		77 days	97 days	110 days	130 days
Arm muscles	M. extensor digitrum communis			±	
	M. extensor carpi radialis brevis				++
Shoulder muscles	M. omotransversarius		+++*	++++*	
	M. omobrachialis				+++
Back muscle	M. lumbodorsal aponeurosis				+++*
Abdominal muscle	M. external adbominal oblique			+	
Esophagus	M. esophagus		+*	+	
Diaphragma	M. diaphragma		+++	++*	
Leg muscles	M. samitendinosus		++	+	
	M. soleus	++			
	M. extensor digitrum longus	+++			
	M. sartorium	++		++++*	

* Appearance of necrotic cell.

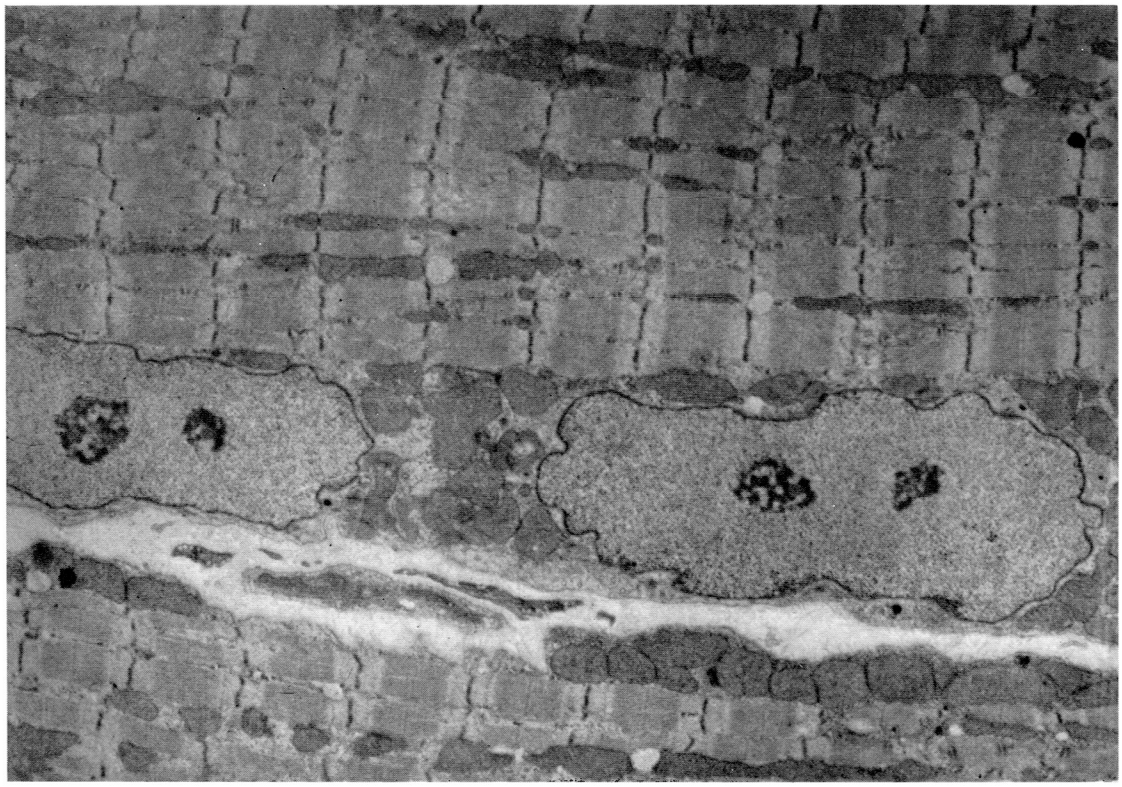

FIG. 1. Electron micrograph of longitudinal section of leg muscle in dystrophic hamster. Nuclei are arranged in a long row at the peripheral region. Nucleoli are clearly observed.

The appearance of degenerative cells marked by asterisk (*) in Table 1 seemed to be induced by at least three different mechanisms. The first type was a so-called fatty degeneration, but this appearance was very rare in dystrophic hamster and only one case was found in the sartorius muscle. The second type was the most common, accompanied by vacuole degeneration and disarrangement of myofibrils. Typically seen in the longitudinal section was a panoramic view which had gone from relatively slight damage with small vacuoles to severe damage with disarrangement of myofibrils and the disappearance of myofilaments. The third type rarely appeared but its image was very impressive; this type was macroscopically recognized as being like refined white wax. The muscles were replaced with fibroblasts or interstitial cells in a certain area and some muscle cells remained lying scattered among the concentrated fibroblasts. While some muscles were normal except for their central nuclei, others were degenerated with vacuoles. These structural changes were not progressive, but such changes were frequently observed in the tissue from 70 to 130 days after birth.

In conclusion, all the striated muscles of a dystrophic hamster including the esophageal muscle show overall structural abnormality, especially the central nuclei, despite the apparent normal arrangement of myofibrils. Furthermore, the process of occurrence of degenerative cells is very like the genesis of human dystrophy. An experimental approach using dystrophic hamsters would make good progress in our studies on this disease.

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Muscular Dystrophy Group, 1981

CELL ADHESION MOLECULE OF CLEAVAGE STAGE MOUSE EMBRYOS—FUNCTIONS IN "COMPACTION"

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"Compaction" is one of the very important events in the preimplantation stage of mammalian embryonic development. In mouse embryos at the 8-cell stage, contact surfaces between each cell (blastomere) become very wide, there is a modification of the cell shape, and thus an embryo becomes a compact mass[1]. It is believed that interactive relationships between individual embryonic cells start to be established only after this stage of "compaction." In this report, we show that a cell surface protein of m.w. of 140K (t-CDS) functions in compaction by means of immunological techniques.

Cell adhesion molecules

Cell-cell contact in vertebrate cells is established by the presence of hypothetical "dual adhesion mechanisms"[2]. We have previously shown that each mechanism is operated by the presence of two distinct cell surface sites. One of them, CDS, a function of which is dependent on Ca^{2+}, is particularly important for cellular recognition between different cell types as well as holding adjoining cells in stable contact[3]. CDS was clearly identified as a protein of m.w. of 150K on the electrophoresis of a total lysate of isotopically labelled cell surface components of a variety of differentiated cells as well as of several established cell lines *in vitro*.

Cells of a particular line of mouse teratocarcinoma, F9, which are characterized by their inability in cell differentiation, contain an unusually high content of CDS on their cell surfaces[4]. However, the molecular weight of F9 cells (t-CDS) is not 150K as found in other cell types, but 140K. Then, CDS in F9 cells (and other teratocarcinoma cells also) is not the same molecule as the CDS present in differentiated cells, though both share the identical function[5].

Specific antibody against cell-adhesion molecule

An antibody specific to t-CDS was raised by injecting F9 cells into a rabbit and by proper absorption to remove non-specific antibodies. This antibody has been successfully utilized as a diagnostic reagent to

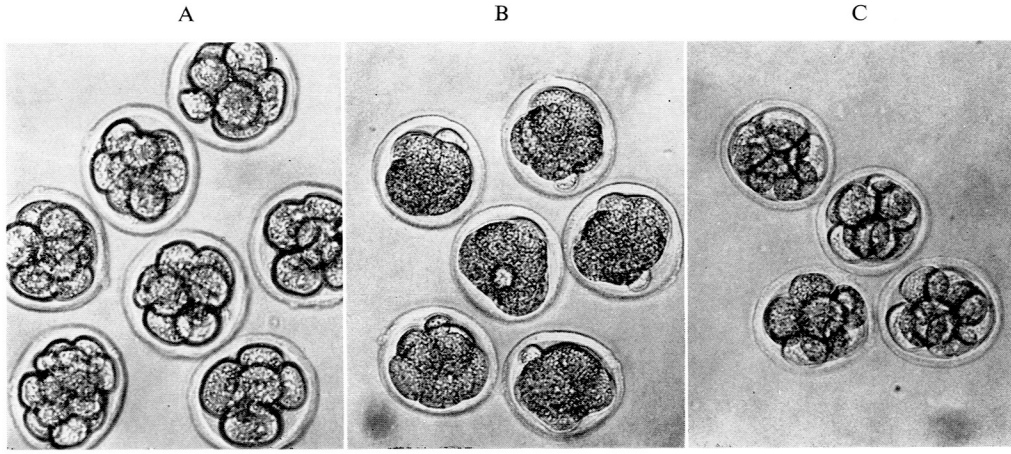

FIG. 1. A: 8-cell stage mouse embryos prior to "compaction." B: Embryos at the stage of "compaction." C: "Decompaction" of once compacted embryos induced by the treatment with the Fab fragment of anti-F9.

see whether t-CDS is present in early mouse embryos before implantation. This was carried out by examining whether the Fab fragment of anti-t-CDS can specifically inhibit the aggregation of embryos placed under a constant gyration. The results clearly demonstrated that mouse embryos ranging from one-cell to 8-cell stages are provided with t-CDS, but not with CDS of m.w. of 150K. Thus, we can conclude that cleavage stage mouse embryos share a common cell adhesion molecule (t-CDS) with teratocarcinoma cells but not with differentiated cells[6].

"Decompaction" of embryos by anti-t-CDS

When mouse embryos at the stage of compaction (the late 8-cell stage) were treated with the Fab fragment of rabbit antibodies against F9 cells, the shape of each cell soon became round and thus the phenomenon called "decompaction" of embryos was induced. Such effect of the Fab of non-absorbed anti-F9 was completely removed by absorbing it with F9 cells, but not with several types of differentiated mouse cells or with several *in vitro* lined cells. It is known that the treatment of F9 cells by protease and EDTA under specified conditions removes only t-CDS, leaving most surface proteins intact[4]. An absorption of the Fab of anti-F9 with such treated F9 cells also abolished its effect to induce "decompaction." Therefore, it becomes clear that "decompaction" is due to a specific effect of the antibody against t-CDS and the latter molecule functions in "compaction" of embryos.

It remains to be investigated why "compaction" occurs at the 8-cell stage, in spite of the continuing presence of t-CDS since the one-cell stage. Perhaps an accumulation of t-CDS at this particular stage becomes high enough to cause "compaction" of embryos.

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PHAGE PARTICLE-MEDIATED GENE TRANSFER INTO CULTURED MAMMALIAN CELLS

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Gene-transfer into cultured cells including cells from human hereditary diseases will become an essential procedure for cell biology. However, several improvements should be made because the efficiency of transformation by genomic DNA has not been high enough to overcome the spontaneous back mutation of the mutant cells, the transformants obtained by DNA transfer have been reported to be unstable, and the DNA sequences introduced exogenously suffer from rearrangement.

Metaphase chromosomes have also been used for the transfer of single-copy genes in eukaryocytes and have at least two advantages as a donor, as compared to genomic DNA: the efficiency of gene transfer is found to be always 10 to 100 times higher for chromosomes than for genomic DNA, and the transformants obtained from chromosome transfer experiments are more stable than those from genomic DNA transfer. These advantages, we think, are partially derived from the organization of chromosome structure where DNA sequences are covered with nuclear proteins, packaged compact into chromatin structure, and protected from DNase attacks. In spite of these advantages, however, metaphase chromosomes are not a choice as donor materials for the isolation of the selectable marker gene because DNA sequences in chromosomes cannot be manipulated before application to the recipient cells.

As a donor of genetic materials, we have selected phage particles in which an exogenous gene is encapsulated for the following reasons: they have a structural organization analogous to that of chromosomes. DNA sequences are covered with phage coat proteins packaged compact into phage particles and protected. From this structural analogy, we expect a high efficiency of gene transfer and good stability of the resultant transformants when phage particles are applied to recipient cells. The method for the construction of a gene library of mammalian DNA in lambda phage vectors has been already established, and sequences for genomic DNA and lambda vectors can be easily manipulated before packaging into phage particles.

In this work phage particles coprecipitated with calcium phosphate were applied to the recipient cells for gene transfer in place of either DNA or chromosomes. Using Charon 4A recombiant phage carrying HSV-1 tk gene and Ltk$^-$ cells, we have established the conditions of phage-mediated gene transfer, attained a high efficiency of gene transfer, and also have found that the resultant transformants were apparently stable. Furthermore, to our surprise, the exogenously introduced DNA sequences in the transformants have been stably conserved with only slight rearrangement.

Abstract of the results

Recombinant phage particles carrying the thimidine kinase gene of herpes simplex type 1 and coprecipitated with calcium phosphate efficiently transform mouse Ltk$^-$ cells to the tk$^+$ phenotype. The conditions necessary to achieve high efficiency transfer of the tk gene by phage particle-mediated gene transfer were investigated. Of the parameters examined, pH of a buffer used for the coprecipitation of phage particles with calcium phosphate, the length of time for the coprecipitation, and the length of adsorption time were found to significantly alter the transfer efficiency. The optimal values for these parameters were as follows: the buffer of pH 6.87 at 25°C; the coprecipitation time of 7 to 20 min; the adsorption time of 18 to 30 hr. Treatment with dimethylsulfoxide, glycerol, or sucrose did not enhance gene transfer. The optimal conditions yielded about one transformant per 10^5 phage particles per 10^6 cells without carrier DNA. Increasing the dosage of phage particles, at least up to 5×10^7 per 100 mm dish, resulted in a linear increase in the number of transformants. Addition of carrier phage up to 10^{10} phage particles

per 100 mm dish, scarcely affected the number of transformants. The transformants were apparently stable and the exogenously introduced DNA sequences in them were stably conserved with only slight rearrangement.

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A STUDY OF DYSTROPHIC MUSCLE MAINTAINED *IN VITRO*

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Our previous work on the alterations of dystrophic muscle maintained *in vitro* (California Davis Line 413) disclosed characteristic degenerative changes, such as abnormal fusing processes of myoblasts, vacuolation of sarcoplasm, fenestration of sarcolemma, dilatation of transverse tubules, hypercontraction of myofibrils, and segmental necrosis of sarcoplasm. Hypercontraction of myofibrils and segmental necrosis were closely related to the dilated T system, which seemed to be continuous to the sarcolemmal openings. To clarify the causative relationships of these alterations and the accumulation of external fluid in the T system, HRP techniques were applied to cultures of dystrophic muscle. Ultrastructural studies were also performed. Fragments of superficial breast muscle from dystrophic chick and cross sections of spinal cord were cultivated on collagen-coated coverslips and maintained with the double coverslip method. Cultures were observed under the ordinary light microscope and Nomarsky optics with high magnification. When needed, cultures were immersed in a balanced salt solution (BSS) containing HRP (2.5 mg/ml) and maintained for one hour. After rinsing with BSS, cultures were fixed with 2.5% glutaraldehyde for 30 min and washed overnight, and then stained with DAB in the presence of 0.01% of H_2O_2.

Fenestrations of muscle surface exhibited with light microscopic observations were found to correspond to the widening of orifices of the transverse tubular system. In addition, endocytotic vesicles increased in number, and were mainly located underneath the sarcolemma. Similar vesicular structures were also seen in the deeper sarcoplasm. HRP reaction seemed to be closely related to these structures, suggesting accumulation of external fluid inside the muscle fibers. Furthermore, with light microscopic observation it was found that HRP positive regions were often associated with either hypercontraction of myofibrils or segmental necrosis of sarcoplasm.

Nerve innervation of muscle fibers maintained *in vitro* may promote better maturation and maintenance than would otherwise occur. Development of dystrophic changes in muscle culture may be enhanced in the innervated muscle fibers, suggesting the alterations were more frequently produced in muscles maturated both biochemically and structurally. Among the alterations described above, vacuolation of the sarcoplasm seemed to be nonspecific, while other types of alteration were characteristic for this disorder. Widening and dilatation of the tubular system are closely related to the necrotic fibers which exhibited HRP deposition. Furthermore, damage of the tubular system seemed to be an initial change leading to the production of necrotic inclusion and hypercontraction as well. Though these necrotic processes differed from the disturbance of fusing processes of myotubes, there may be a possibility of a single causative factor, that is, membrane abnormality which is genetically inherited. If true, it is quite possible that some other type of cell may develop a similar membrane anomaly on a genetic basis.

In the Duchenne type of muscular dystrophy it has been suggested that Ca^{2+} deposition in the tubular system activates the calcium dependent proteinase, resulting in segmental necrosis of muscle fibers and production of opaque fibers. These changes in patients are identical to those observed in chick dystrophic muscles *in vitro*.

Muscular Dystrophy Group, 1981

MITOTIC ACTIVITIES OF MONONUCLEATE CELLS CULTURED *IN VITRO* ORIGINATED FROM REGENERATING MUSCLES OF NORMAL (+/+) AND DYSTROPHIC (dy/dy) MICE (II)

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We have reported that during the same culture period the number of cell divisions (NCDs) of dystrophic myogenic cells (dy/dy) was significantly smaller than that of normal (+/+) ones[1]. To clarify whether the difference in the NCDs was due to the prolongation of generation time of dystrophic myogenic cells or to their earlier deprivation of mitotic activities, we carried out clonal cultures of either normal or dystrophic myogenic cells and obtained their growth curves.

Adult normal (+/+) and dystrophic (dy/dy) mice were used throughout. The procedures for the preparation of regenerating muscles[2,3] and the liberation of mononucleate cells from either source[4] were described previously by Kagawa *et al.* Cultures were established by plating 500 cells from either source in 5 ml of the "fresh medium"[2] onto a 60-mm Falcon plastic petri dish coated with 0.2% gelatin[5]. Incubation was continued at 37°C in a humidified atmosphere gased with 5% CO_2 and 95% air. Each culture was fed with 5 ml of the "growth medium"[2] one day after plating. Three series of cultures of the same type (cultures 1, 2, and 3) were carried out. Each of them consisted of 10 normal and 10 dystrophic cultures. In each series, 30–45 colonies of myogenic cells from either origin were randomly selected and marked with numbers 2 days after plating. The cell number of each was enumerated every day thereafter and growth curves were obtained. Colonies which lost the potency of cell growth were excluded and, in consequence, the number of colonies used for the counting of NCDs decreased with the advancement of the days of cultures.

The average number of cell divisions occurring during the culture period of dystrophic myogenic cells (7 days) was smaller than that of normal in all cultures and the differences were significant in cultures 1 and 2 (Table 1). As shown in Fig. 1, the differences in the NCDs between normal and dystrophic myogenic cells which occurred during the first two culture days were also significant in cultures 1 and 2. To exclude the possibility that the difference in the NCDs was due to the increase of cell number by the fusion of two or more colonies in normal cultures, NCDs occurring after 2 through 7 days of incubation were

TABLE 1. Average Number of Cell Divisions (0–7 days)

	Culture 1	Culture 2	Culture 3
Normal	6.21±2.08	5.02±2.04	5.24±2.18
	(43)	(31)	(23)
Dystrophy	3.80±1.73	2.37±1.11	4.25±1.86
	(29)	(16)	(26)
t-stat. ($P<$)	0.01	0.01	NS

t-stat.: *t*-statistics. ( ): number of colonies used for the count of number of cell divisions. NS: not significant.

TABLE 2. Average Number of Cell Divisions (2–7 days)

	Culture 1	Culture 2	Culture 3
Normal	3.06±1.89	2.99±1.86	2.93±1.63
	(43)	(31)	(23)
Dystrophy	1.45±1.47	1.32±0.92	2.51±1.50
	(29)	(16)	(26)
t-stat. ($P<$)	0.01	0.01	NS

t-stat.: *t*-statistics. ( ): number of colonies used for the count of number of cell divisions. NS: not significant.

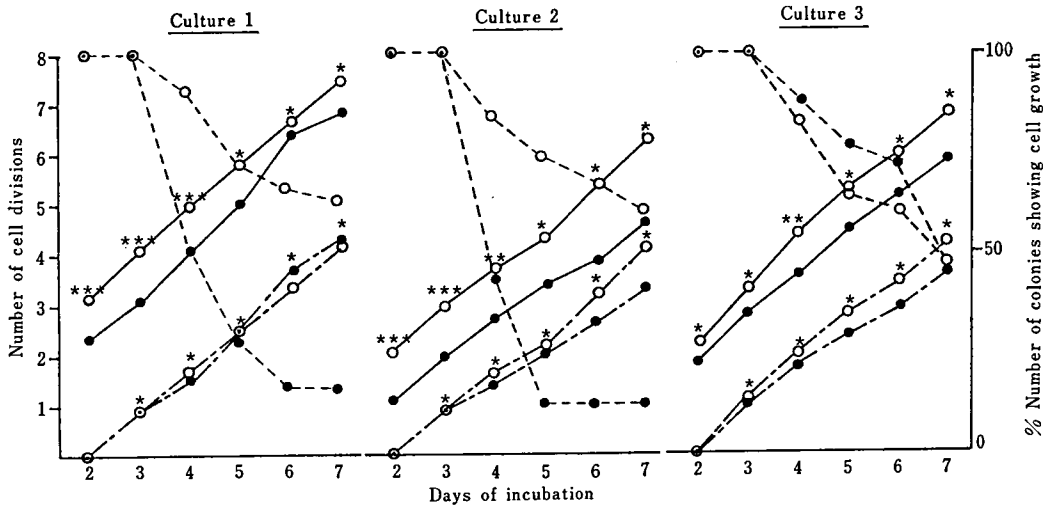

FIG. 1. Growth curves and the curves showing the number of colonies with mitotic activity. —— average number of cell divisions (0–7 days). ——— average number of cell divisions (2–7 days). --- % number of colonies showing cell growth. The *t*-statistics for two means was used to compare the number of cell divisions of normal myogenic cells and that of dystrophic ones: *** $P<0.01$, ** $P<0.05$, *NS.

also obtained. They were also smaller in dystrophic cultures than in normal ones in all series of cultures and the differences were significant in cultures 1 and 2 (Table 2). These findings agreed with the results we previously reported[1].

The grade of each growth curve (Fig. 1) shows the velocity of cell divisions. The grades of the growth curves of dystrophic myogenic cells were approximately parallel to those of normal in all cultures (Fig. 1) indicating that the generation time, which is the reciprocal of the velocity of cell division, of dystrophic myogenic cells was approximately the same as that of normal cells.

Colonies showing cell growth decreased in number much more rapidly in dystrophic cultures than in normal ones in cultures 1 and 2, in both of these the number of cell divisions of dystrophic myogenic cells was significantly smaller than that of normal myogenic cells (Fig. 1).

The present study suggested that the decrease in the number of cell divisions found in dystrophic cultures was not due to the prolongation of generation time of dystrophic myogenic cells but to their earlier deprivation of mitotic activity.

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REQUIREMENT OF TRANSFERRIN FOR MYOGENIC CELL GROWTH *IN VITRO*

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Chick myogenic cells do not grow in a basal culture medium (BCM) composed of 85% Eagle's minimum essential medium and 15% horse serum but grow well when a small amount of muscle trophic factor (MTF) is added to the medium[1]. MTF is a protein prepared from chick adult serum[2] which is salmon pink in color and has a molecular weight of 80K dalton.

In order to determine the nature of MTF, it was compared with chick serum transferrin (sTf) prepared from chick serum, and ovotransferrin (oTf) from chick egg white[3,4].

The molecular weight of sTf was also 80K dalton and that of oTf was a little smaller when determined by SDS acrylamide gel electrophoresis. Proteolytic digests of MTF, sTf, and oTf by α-chymotrypsin and papain showed indistinguishable patterns.

A single and identical precipitin line was formed in a double immunodiffusion precipitation test when anti-sTf and oTf-rabbit sera reacted in agar gel with MTF, sTf, oTf, or chick serum.

These three proteins bound ferric ion. Absorption at 465 nm, which is characteristic of iron-bound transferrin, increased as the amount of ferric ion bound to the apo-type of MTF, sTf, and oTf increased. These two reached a plateau when the molar ratio of 80K dalton protein against iron was approximately 1 : 2. This stoichiometric ratio is also characteristic of transferrin, indicating that the main proteins belong to the transferrin family.

Iron-free proteins showed no myotrophic activity. Myotrophic activity appeared when the protein bound iron. It increased almost proportionally with the amount of the bound iron, and reached a plateau at

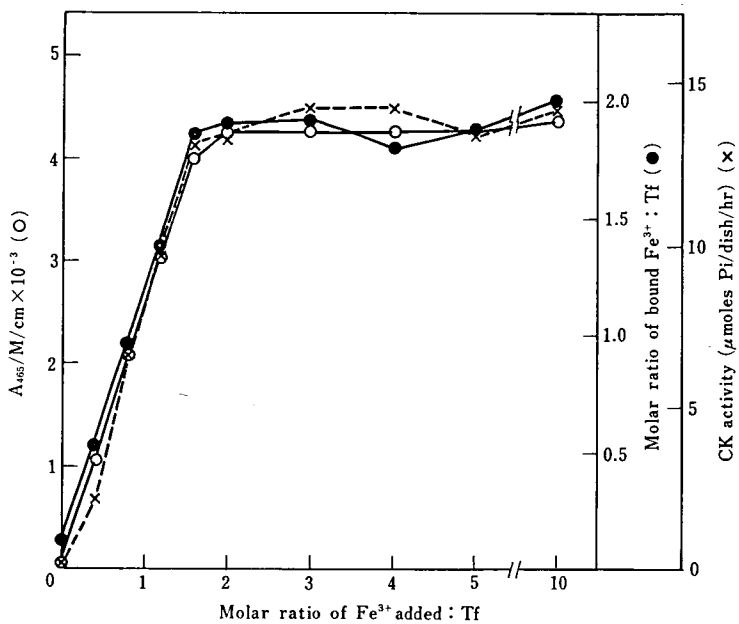

FIG. 1. Relation of molar ratio of Fe^{3+} *vs.* Tf to molar ratio of bound Fe^{3+} *vs.* Tf, optical absorption at 465 mμm, and creatine kinase (CK) activity as an index of myotrophic activity.

the same molar ratio (Fig. 1). Complexes of transferrin with metals other than iron so far examined were without activity; therefore, the activity can be solely ascribed to iron-bound transferrin. All of these data also indicate that MTF is identical to transferrin.

Ferric or ferrous ion, not bound to transferrin, was also effective in promoting myogenic cell growth, although more than 200 times the amount of iron as a complex with transferrin was required. Other metals, such as Mn, Cr, Cu, Co, Ni, Cd, and Zn, were not effective.

We have also prepared transferrin from chick embryo extract (EE). The preparation method was similar to that used for MTF preparation, with some modifications which will be detailed elsewhere[5]. This transferrin (EE Tf) and sTf were indistinguishable from each other physicochemically, immunologically and in myotrophic activity so far examined, except that EE Tf was mainly composed of transferrin species with 0 or 1 sialic acid residue, whereas sTf had mainly 2 or 1 sialic acid residues. Myotrophic activity did not depend on the number of sialic acid residues.

When transferrin was removed from EE by immunoprecipitation or iron bound to transferrin was removed from EE by dialysis at pH 4.5, myotrophic activity was lost but was restored by the addition of transferrin or iron to both kinds of inactivated EE. This indicates that transferrin is a very important component of EE for myogenesis. Further, one of the most important role of EE is to give iron to the cells.

Horse transferrin present in BCM did not show myotrophic activity on *chick* cells. This can be explained on the basis of the class-specific nature of transferrin; mammalian transferrin, except bovine transferrin, was not effective but avian transferrin was effective in promoting chick myogenic cell growth.

Chick transferrin, irrespective of the sources or methods of preparation, promoted chick myogenic cell growth so far as it bound ferric ion. It is deduced that iron is absolutely required either for multiplication of myoblast or growth of myotubes. Transferrin serves as an efficient iron-donner to cells. Without it, grown myotubes degenerate and are destroyed[6].

Some of the results on this subject have been previously published[7-10].

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EFFECTS OF INNERVATION ON MUSCLE REGENERATION

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In transplanted muscles myotubes are formed by satellite cells followed by striate formation, and subsequently develop into new mature muscle fibers. A major difference in the muscle structure before and after transplantation is that the diameter of the regenerated muscle fiber is smaller.

Major factors contributing to the increase in the diameter of these regenerated muscles are the recovery of blood supply and muscle tension as well as the reinnervation to the muscles, which includes the regeneration of nerves from the host body and their connection to the regenerated muscle.

The present studies were undertaken in order to elucidate the influence of reinnervation on the regeneration of transplanted muscles.

Under nembutal anesthesia, orthotopical autotransplantation of both sides of the extensor digitorum longus muscle was performed in rats. Ishiadic nerve of one side was denervated and the intact side served as a control.

The transplanted muscles were extirpated successively after the transplantation and were weighed and sectioned with a cryostatt. The histological differences were analyzed between the control and denervated muscles both by ordinary stainings such as Hematoxylin-Eosin and Trichrome, and histochemical stainings such as ATPase, DPNH, and SDH.

Four days to one week following the muscle transplantations, numbers of fibroblasts, myoblasts, and macrophages appeared in the peripheral regions of the transplanted muscles followed by the development

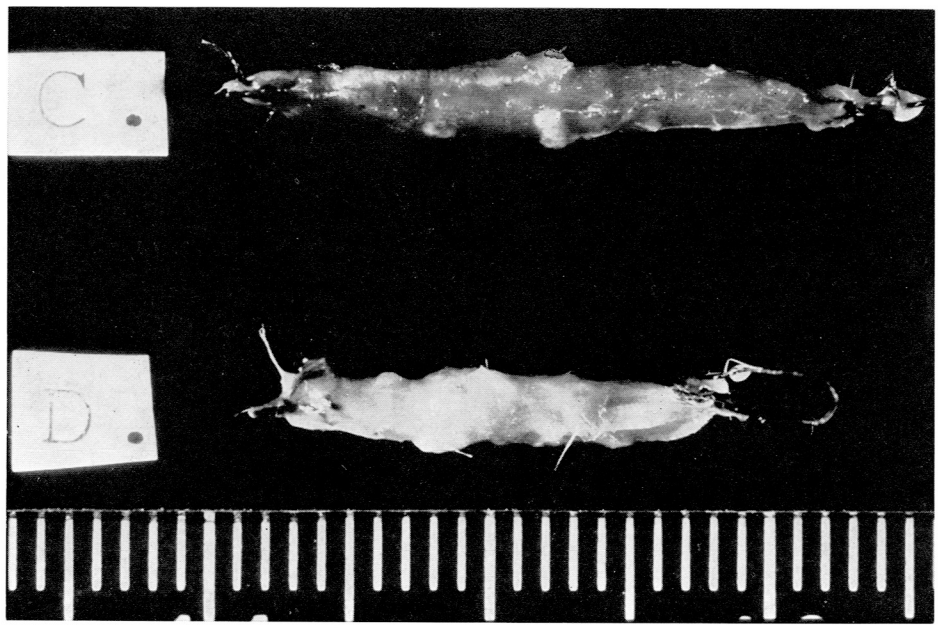

FIG. 1. Regenerated muscles three weeks after transplantation. c: control side, d: denervated side.

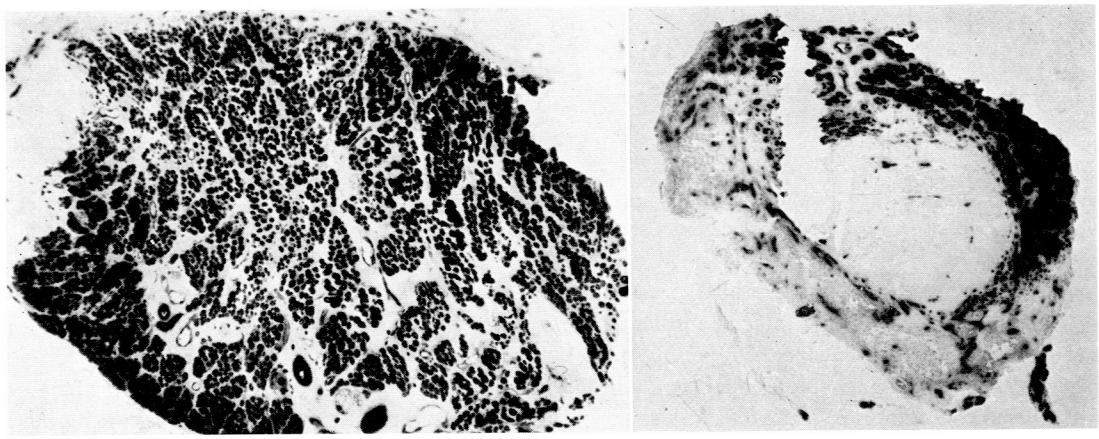

FIG. 2. Histology of the regenerated muscles three weeks after transplantation. ATPase staining. left: denervated side, right: control side.

of new regenerated muscles. After two weeks, the regenerated muscles grew to occupy the whole area of the grafts. An analysis of the size of the regenerated muscle fibers revealed little difference between the denervated and the intact muscles, although the diameters of denervated muscle fibers tended to be smaller than those of the control.

Thereafter, the differences became clearer. Figure 1 shows regenerated muscles of the denervated and the control sides three weeks after transplantation. As shown, the size of the regenerated muscle from the denervated side is apparently smaller than that of the central. The difference increased gradually and successively. At four weeks after transplantation the average weights of the regenerated muscles from the control side were approximately 50 mg, whereas those of the denervated muscles were usually less than half that of control.

Figure 2 shows the histology of the regenerated muscles of the denervated and control sides three weeks following transplantation. Here the denervated muscle reveals poorer regeneration with plenty of connective tissues, which lead to the fibrosis frequently observed in the central portion. The number of regenerated muscle fibers decreased in the denervated side. Further, the controls were apparently uniform in size, whereas the denervated muscles were variable and tended to be smaller.

The above results indicate that the formation of the myoblast and the myotube during muscle regeneration was not influenced by the innervation. However, the development of the regenerated muscles was progressively impaired with time. Further studies are needed to analyze these phenomena.

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Muscular Dystrophy Group, 1981

POSTNATAL ALTERATIONS IN THE NUMBER OF MUSCLE FIBERS IN NORMAL AND DYSTROPHIC CHICKENS

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Previous results obtained from cross-transplantation experiments on extensor carpi radialis longus (ECRL) muscles suggested that some extramuscular factor(s) plays an important role in muscle growth in normal and dystrophic chickens[1]. On the other hand, abnormal discharges originating from the central nervous system have been demonstrated in the muscles of the dystrophic chicken[2].

The normal development of skeletal muscle involves a series of interactions between motor neurons and muscle fibers. For instance, muscle fibers are innervated polyneuronally in neonatal animals and some of these synaptic inputs are eliminated during subsequent development resulting in a single innervation by 2–3 weeks of age[3–7]. There are also several reports of an increase in the number of muscle fibers after birth[6,8–11].

It is, therefore, interesting to examine whether or not there exist any disorders in muscle growth and maturation in the dystrophic chicken. In the present study, we have explored the postnatal changes in the number of muscle fibers in normal and dystrophic chickens. It was found that the postnatal muscle proliferation was greatly suppressed in the dystrophic chicken.

Normal (line 412) or dystrophic (line 413) chickens were anesthetized with ether and the ECRL muscle was exposed. The excised muscle was fixed in a phosphate-buffered formalin solution, and transverse sections were taken from the widest portion of the muscle belly. The total number of muscle fibers was counted on a preparation stained with haematoxylin and eosin.

The results are summarized in Table 1. The ECRL muscles in normal and dystrophic chickens at 2–3 days *ex ovo* contained about the same number of muscle fibers. From 2–3 to 12–14 days postpartum there was a 77% increase in the number of muscle fibers in the normal chicken, whereas only a 15% increase was observed in the dystrophic chicken. The number of muscle fibers then gradually decreased in both the dystrophic and the normal muscles, reaching a steady-state in about 60 days; however, the decrease was much more pronounced in the dystrophic chicken than in the normal chicken. A kinetic analysis of the decreasing phase using the "steady-state theory of mutation rates"[12] gave the rate constants of 0.128/day and 0.134/day in normal and dystrophic chickens, respectively, suggesting that there is no essential difference in the rate of degradation between the two.

The present study showed that a postnatal increase and a decrease in the number of muscle fibers occur in the ECRL muscle of both normal and dystrophic chickens. With the light microscope, there were some uncertainties in identifying individual muscle fibers, especially in young animals. Although the electron

TABLE 1. Number of Fibers in Normal and Dystrophic ECRL Muscles of the Chickens

Age	2–3 days	12–14 days	25–26 days	42–43 days	60–65 days
Normal	14,375 ±1,699 $n=4$	25,382 ±1,777 $n=5$	20,664 ±1,472 $n=3$	19,893 $n=1$	18,077 ±2,028 $n=5$
Dystrophic	14,912 ±1,311 $n=4$	17,173 ±869 $n=4$	13,316 ±660 $n=4$	10,106 ±196 $n=2$	7,462 ±1,013 $n=4$
Significance*	no	$P<0.01$			$P<0.01$

Mean±S.E. * t-test for the difference between normal and dystrophic.

microscopic count gave higher values by 20% than those obtained by light microscopic count in rat lumbrical muscle[6], this would give similar biases to the values in both normal and dystrophic chickens. Therefore, it is likely that no significant abnormalities in muscle proliferation existed at the embryonic stage, but this process was greatly affected in dystrophic muscles in the early postnatal period of 2–14 days. In terms of the nerve-muscle interactions, it is conceivable that the dystrophic nerves have some defect in their ability to promote postnatal proliferation of the muscle fibers. Alternatively, the dystrophic muscle fibers themselves may be unable to respond properly to the neural influence. Further study is needed to elucidate the mechanisms of the suppressed proliferation during the early postnatal stage in the dystrophic chicken.

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Muscular Dystrophy Group, 1981

ARRESTED GROWTH OF MUSCLE FIBERS IN MUSCULAR DYSTROPHIC MICE

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We have proposed a bone-muscle imbalance hypothesis for the pathogenesis of murine muscular dystrophy and possible symptomatic treatments (Fig. 1). Our recent finding that a major dystrophic symptom in dystrophic mice could be masked by the introduction of gene dw strongly supports this hypothesis[1]. Recently a similar human case has been reported by Zatz *et al.*[2], and an improved righting ability (flip number) has been reported in dystrophic chickens treated with an anti-thyroid drug (propylthiouracil) by King *et al.*[3].

In the hindlimbs of the dystrophic mouse, impaired growth of skeletal muscles is due to maturational defects of muscle fibers[4]. Thus, in the present study three types of experiments were carried out in order to ascertain the maturational defects of muscle fibers in both the fore- and hindlimbs of the dystrophic mouse (C57BL/6J dy/dy).

Muscular evoked potentials

Evoked potentials of gastrocnemius muscles of normal and dystrophic mice were recorded by a sciatic nerve stimulation (0.05 msec pulse). They displayed a biphasic wave with high amplitudes (Fig. 2A, upper half). When the frequency of stimulation was raised from 0.5 to 5 Hz, amplitudes of the muscular potentials in normal adult mice were rapidly and severely reduced, whereas those in normal young and dystrophic (both young and adult) mice changed only slightly (Fig. 2A, lower half and Fig. 2B). Thus, muscles of dystrophic mice appear to be fatigue-resistant compared with those of normal adult mice, a finding similar to that of Sandow and Brust (tension analysis)[5]. Muscles of dystrophic mice can therefore be said to be very similar to those of normal young mice.

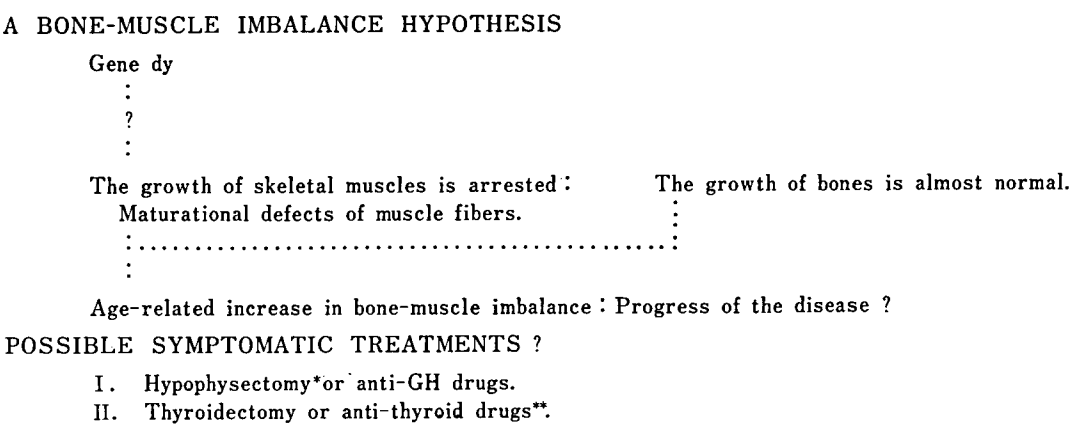
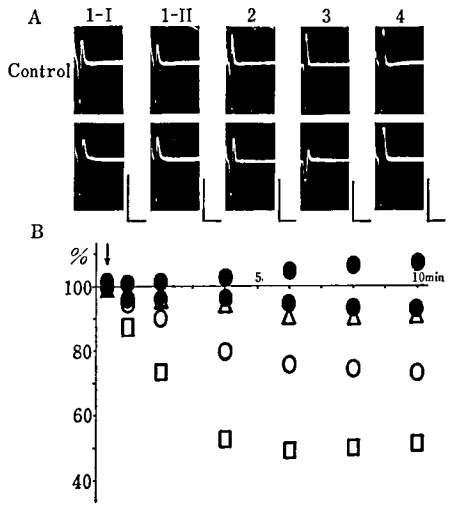


FIG. 1. A bone-muscle imbalance hypothesis for the pathogenesis of murine muscular dystrophy and possible symptomatic treatments. *Latent dystrophic symptom in genotypically dystrophic-dwarf mice[1], and a similar human case[2]. **Improved righting ability of propylthiouracil-treated dystrophic chickens[3].



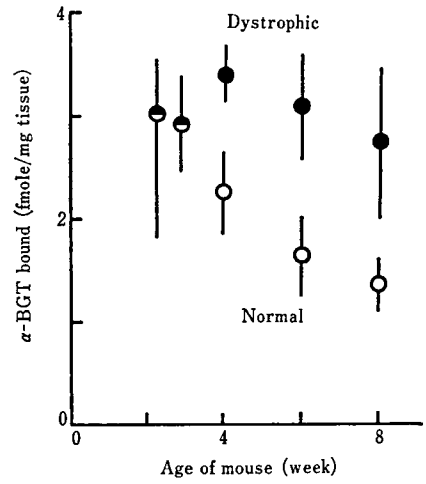


FIG. 2 (left). Evoked potentials of m. gastrocnemius by sciatic nerve stimulation in normal and dystrophic mice. A) Representative patterns by 0.5 (upper) and 5 Hz-stimulation (lower). 1-I and 1-II: 2-month-old dystrophic mice. 2, 3, and 4: 2-month, 7-month, and 16-day-old normal mice. Calibration: 40 mV and 4 msec. B) Time-courses of changes in muscular potentials during 5 Hz-stimulation. Mean amplitudes (% of the initial level) are plotted *versus* time. Arrow indicates values obtained immediately after the start of 5 Hz-stimulation. ●: 2-month-old dystrophic mice (n=7 and 7). △, ○, and □: 16-day (n=8), 2-month (n=13), and 7-month-old (n=7) normal mice.
FIG. 3 (right). Age-related changes in [^{125}I] α-BGT binding by forelimb triceps of dystrophic and normal mice.

Muscle fiber diameters

Histological observations were performed on muscle specimens (7 μm-thick cross sections of paraffin-embedded forelimb triceps and hindlimb biceps). Increase in fiber-splitting, fiber necrosis, central and clustered nucleation, atrophic fiber, connective tissue, and fiber-diameter variation was pronounced throughout the fore- and hindlimb muscles of dystrophic adult mice[6]. In the present study, the diameters of muscle fibers were measured. Those in hindlimbs of normal young and dystrophic (both young and adult) mice averaged only about 25 μm, whereas those of normal adult mice were very large (around 40 μm). Forelimb muscle fibers of adult mice had a large diameter (37 μm in average) in normal mice and a small one (23 μm in average) in dystrophic mice. These results suggest an age-related increase in the diameter of muscle fibers in forelimbs as well as in hindlimbs in normal mice, but not in dystrophic mice.

α-Bungarotoxin binding

The forelimb triceps was quickly removed, weighed and homogenized in a buffered saline with Polytron PT-10. After centrifugation at 28.000×g for 30 min, the pellet was suspended in the same saline, sonicated and used for [^{125}I]α-bungarotoxin (α-BGT) binding experiments. Muscles from normal young and dystrophic (both young and adult) mice were found to bind similar amounts of α-BGT on a wet weight basis (Fig. 3). An age-related change (decrease) in α-BGT binding was observed only in normal mice. This may be at least partly attributable to an age-related decrease in the surface area-volume ratio of muscle fibers resulting from the maturation (enlargement) of these fibers.

In conclusion, the present findings demonstrate that maturational processes of muscle fibers in the fore- and hindlimbs may be severely inhibited in muscular dystrophic mice.

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Muscular Dystrophy Group, 1981

COMPARISON OF THE ALTERATION PATTERNS OF SEVERAL BIOCHEMICAL MARKERS IN MICE WITH SOME NEUROMUSCULAR MUTATIONS

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The purpose of this work was to compare abnormal changes of certain enzyme activities in several tissues and of creatine concentrations in urine from mice with three different mutations of muscular dystrophy, such as Dystrophia muscularis (dy), Myodystrophy (myd), and newly discovered mutation (mut), and to elucidate the differences of intrinsic or genetically controlled changes in the development of disease among these mutations.

The experiments were carried out with 6–12 week old affected homozygotes and unaffected littermate animals from strains of C57BL/6J-dy, MYD/J-myd, and SM/J-mut.

Hind leg muscles and brain were removed from the mice immediately after cervical dislocation and were homogenized with 10 volumes of ice cold 0.32 M sucrose in a teflon homogenizer. The muscle homogenates were then fractionated by the Schneider method and the cytosol fractions were used as enzyme sources. The purified myelin fraction was isolated from the brain homogenate with a conventional subcellular fractionation procedure, essentially according to Norton and Poduslo[1]. The blood sample obtained from the carotid artery was allowed to coagulate and the serum was separated in the centrifuge and then used for enzyme assay. Urine samples collected from five mice of each experimental trial were appropriately diluted and filtered to remove impurities.

For the determination of creatine kinase (CPK) activity in muscle and serum, the spectrophotometric method by Hess *et al.*[2] was used. Pyruvate kinase (PK) and lactate dehydrogenase (LDH) activities in muscle and serum were also measured by the spectrophotometric methods of Tanaka *et al.*[3] and of Wroblewski and LaDue[4], respectively. 2′,3′-Cyclic nucleotide 3′-phosphohydrolase (CNP) and cholesterol-ester hydrolase (CEH) activities in the purified myelin fraction of brain were determined by the methods previously described[5]. Estimation of creatine and creatinine in urine was performed by the method of Jaffe reaction according to Taussky[6].

In Table 1 are shown the calculated results of the alteration of the enzyme activities in serum, muscle, and brain, and of creatine concentrations in urine of the affected mice. Each value in the table shows the percent of change of affected mice to that of unaffected control mice. It is noted that in myd mice, serum levels of all those enzymes reported to be high in human muscular dystrophy are also significantly elevated.

TABLE 1. Alterations of Enzyme Activities in Several Tissues and of Creatine Contents in Urine from Affected Mice Mediated by Three Different Neuromuscular Mutations.

		Dystrophy	Myodystrophy	New mutant
Serum	CPK	128.1**	257.0*	194.3*
	PK	329.3*	643.2*	89.0
	LDH	107.4	247.2*	69.5**
Muscle	CPK	54.9*	17.4*	41.0*
	PK	25.6*	61.9*	50.2*
	LDH	72.9*	58.3*	99.6
Brain	CNP	66.7*	72.3*	105.8
	CEH	52.3*	125.4	40.8*
Urine	Creatine	235.5*	211.1*	175.2*
	Creatinine	88.5	90.0	81.0
Creatine/Creatinine ratio		276.5*	237.7*	220.6*

*$P<0.01$, **$P<0.05$.

The activity levels for corresponding muscle enzymes show significant decrease which is analogous to the result obtained in dy mice. LDH activities in serum of dystrophic mice and in muscle of new mutant mice are normal. All serum enzyme levels except CPK level in new mutant mice tend to decrease. There is significant decrease of myelin CNP activities in the brain of both dystrophic and myodystrophic mice, but this is unchanged in the brain of new mutant mice. However, significant decrease of myelin CEH is observed in the brain of dystrophic and new mutant mice. On the other hand, myelin CEH activity in brain of myodystrophic mice shows a slight but not significant increase.

Significant increase of creatine concentrations and slight but not significant decrease of creatinine concentrations are observed in all affected mice as compared with those of unaffected littermates. Therefore, the creatine/creatinine ratio in urine of dystrophic, myodystrophic, and new mutant mice is much higher than that of their normal littermates.

The results obtained in this experiment indicate that many similarities in the alteration patterns of some biochemical markers of muscle disease could link new mutant mice with dystrophic mice. It is of interest that the decrease of myelin CEH activity which involves in cholesterol metabolism of the membrane structure in the nerve tissue is also observed in the brain of both dystrophic mice and new mutant mice. The alteration pattern of some biochemical markers in this experiment resulted in myodystrophic mice comparable to those in a pseudohypertrophic type of human muscular dystrophy.

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EFFECT OF BRAIN EXTRACT ON DEVELOPMENT OF TETRODOTOXIN-SENSITIVE SODIUM CHANNEL IN L6 MYOTUBES

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In adult innervated vertebrate skeletal muscle the action potential is generally blocked by tetrodotoxin (TTX 10^{-6} M), indicating that the action potential is operated by a TTX-sensitive sodium channel. Immediately after birth, however, rat skeletal muscle fibers are capable of generating an action potential that is not blocked by TTX, and by 20 days of age the action potential become TTX-sensitive in relation to the establishment of normal innervation[1]. Conversely, after denervation of adult skeletal muscle the action potential loses its normal sensitivity to TTX, but is still sodium-dependent[2]. These observations indicate that there are two different sodium channels: one is sensitive to TTX whereas the other is not. Mainly TTX-resistant sodium channels are present in rat skeletal muscle at birth and, in contrast, the sodium channels in adult rat innervated skeletal muscle are TTX-sensitive but revert back to TTX-resistant sodium channels following denervation. The development and maintenance of TTX-sensitive sodium channels in the skeletal muscle cell membrane could be considered, then, as one of the membrane properties regulated by innervation.

Using skeletal muscle cultures, it was shown that cell-free nerve extracts can duplicate at least a part of the effect of innervation on the development and maintenance of the TTX-sensitive sodium channels in uninnervated skeletal muscle cells[3,4]. Thus, the results indicate that the trophic control of the sodium channels of muscle cells might well be mediated by a trophic substance produced by nerve tissues.

The L6 myotubes[5] share many characteristics of embryonic and denervated skeletal muscle cells, including resistivity of the action potential to TTX[6,7]. This may be correlated with a lack of the neurotrophic effect. The present study deals with the trophic effect of brain extract on the sodium channels in L6 myotubes. We have found that brain extract is capable of inducing the development of TTX-sensitive sodium channels in uninnervated L6 myotubes.

Stock cultures of L6 myoblasts (gift of Dr. T. Amano, Mitsubishi-Kasei Institute of Life Sciences) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Experimental dishes were prepared by plating 3×10^6 cells per 60 mm tissue culture dish containing 3 ml of culture medium, to which horse serum was added in place of the fetal calf serum in order to accelerate myotube formation. Brain extract was prepared from brain of 20-day old rat fetuses by centrifuging a 1 : 2 homogenate in Tyrode's solution at $20{,}000 \times g$ for 30 min. Cultures were maintained in the absence or the presence of brain extract for 18 or 19 days.

A myotube in 18- or 19-day old cultures was penetrated with closely adjacent conventional recording and current-passing microelectrodes. To minimize the effect of variation in the resting membrane potential and to ensure maximal action potential generation, the membrane potential was preset in each case at −80 mV by passing a steady hyperpolarizing current, and an action potential was evoked by a superimposed depolarizing current pulse.

The action potential was composed of a spike operated by sodium channels, followed by a plateau

TABLE 1. Maximum Rate of Rise of Sodium Spikes (V/sec) in Cultured L6 Myotubes

	Normal saline	TTX containing saline
Control	132.50±10.72 (14)	122.23±15.54 (13)
Brain extract	153.34±10.23 (16)	78.75± 9.04 (16)*

Values are mean±S.E. Numbers in parentheses are myotubes examined. *Indicates a significant decrease.

operated by calcium channels, as reported by others[6,7]. The maximum rate of rise of the spike was measured with a differentiating circuit and used as an index of the density of the sodium channels.

As shown in Table 1, the mean value of the maximum rate of rise of the sodium spikes in cultures grown in the presence of brain extract does not significantly differ from that in control cultures grown in the absence of brain extract. However, the value in cultures with brain extract is reduced on the average by about 50% by TTX (10^{-6} M), although the value in control cultures is not affected by TTX.

These results indicate that there is a loss of TTX-resistant sodium channels and a gain of TTX-sensitive sodium channels in cultures with brain extract, *i.e.*, the L6 myotubes can differentiate with respect to the sodium channels when grown in the presence of brain extract, even in the absence of innervation.

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DEVELOPMENTAL STUDIES OF THE MEMBRANE PROPERTIES OF STRIATED MUSCLE FIBERS—CHANGES IN THE PROPERTIES OF Ca CHANNELS DURING DIFFERENTIATION OF EXCITABLE CELLS

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The mechanism that induces the Ca influx from outside by depolarization of the membrane is one of the essential components in the excitable membrane and is called the Ca channel. This Ca channel shows wide distribution among excitable cells of various adult animals[1]. The existence of Ca channels in the heart muscle membrane and the smooth muscle membrane of the vertebrates has been well established. Although the striated muscle fibers in the vertebrates have been considered to evoke only Na spikes and induce no Ca current, the recent precise analysis of the membrane current with TTX and TEA presence reveals Ca-dependent inward current which is clearly distinguished from Na current by the time course under voltage-clamp. Further during ontogenesis, the Ca channels are characteristic components of differentiating excitable membranes in the early stages of development. For example, the presence of a Ca-dependent plateau following after a fast Na-dependent spike has been reported in the membrane of the myotubes in vertebrate embryos[2]. Therefore, changes in the quality and quantity of Ca channels can usually be expected during the differentiation of excitable membranes.

The Ca channel which was first described in the crustacean striated muscle fiber and then found in the neurons of the molluscan ganglia has been known to show characteristic high permeability to Ba ions and a long-lasting inward current with much less inactivation than the Na channel. Further in the Ca channel the Ca current is more inactivated than the Sr or Ba current, indicating that the inactivation is dependent on the ion species carrying the current. This is assumed to be due to Ca-induced inhibition of Ca channels by the accumulation of Ca ions carried by the current itself. On the other hand, recent studies in our laboratory have revealed an other type of Ca channel in various oocytes of the mouse, the tunicate and sea urchin[3]. This Ca channel shows a potential-dependent inactivation which is very similar to the Hodgkin and Huxley type found in the Na channel. The time course and the inactivation are not dependent upon the ion species carrying the current. Further, the Ca channel is the most permeable to Sr ions. Thus, the egg Ca channel is clearly different from the Ca channel found in other excitable membranes. Since the egg cell membrane is actually at the starting point of the differentiation of the membrane function, the Ca channel may change its properties from egg cell type to excitable cell type in association with the differentiation of the embryonic membrane.

In the tunicate embryo of the protochordate it has been reported that the blastomeres in the cleavage-arrested 8- or 16-cell embryo can show differentiation of the excitable membrane by inducing Na or Ca action potentials[4]. Even in large blastomeres of the cleavage-arrested 1-, 2-, and 4-cell embryo differentiation of the membrane also occurs. In those cases, a single blastomere may be expected to have mosaic properties of various types of excitable cells, because those blastomeres are not uniquely determined. However, in reality, the differentiated unicellular embryo shows the characteristic long-lasting action potentials which are identified as the epidermal cell type. The membrane current in the differentiated unicellular embryo is shown to be pure Ca-channel current. Thus, using a unicellular embryo, analysis of the changes in the properties of Ca channels are attempted during the differentiation of the membrane from that of the original egg cell.

Methods

Adult ascidian, *Halocynthia roretzi* or *aurantium*, which had matured eggs and sperm, were kept in a

cold aquarium of 4°C. The eggs and sperm were obtained from spawning animals after 24 hr in a warm bath of 12°C. The eggs were fertilized by the sperm from heteronymous animals. Developmental time was measured from this fertilization in the incubation bath at a constant temperature of 10°C. Before first cleavage the embryos were transferred to another bath containing cytochalasin B 1 μg/ml in order to arrest their cell-cleavage and were further cultured until the control intact tadpole larvae hatched. After removing the chorion membrane by digestion with pronase 10 mg/ml, the cleavage-arrested embryo was kept in the high proteinous external solution and was penetrated by two microelectrodes. The experiments were done by either constant current stimulation or voltage-clamp, as described previously[5]. The standard external solution was 100 mM $SrCl_2$, 400 mM NaCl, 10 mM KCl, 5 mM PIPES-Na (pH 7.0), 10 mg/ml BSA, and 2 μg/ml cytochalasin B. When it was necessary, sodium was replaced by equimolar choline or strontium with equimolar calcium or barium. In order to analyze the inactivation process of the Ca channel, the tail current of the Ca current at various periods from the voltage-step was measured and the time course of the inward Ca current was decided without contamination of the outward current.

Results

1) After culturing in seawater containing cytochalasin B until the control intact larvae hatched, the resting potential of the differentiated unicellular embryo was about −70 mV, being at K equilibrium potential in the standard external solution. The action potential was evoked by depolarization above −30 mV and its duration was more than two seconds. The action potential was abolished by eliminating Sr ions, but not changed by the replacement of Na with choline. The overshoot was about 50 mV. No evidence of the developed delayed K rectification was not obtained. This type of action potential has been reported as the epidermal type.

2) Under a voltage-clamp condition, the less inactivating inward current was observed with depolarization above −30 mV and the current showed the maximum value at 15 mV in standard external solution. In Mn external solution the inward current was eliminated but no outward current was observed except the leakage current without time-dependent kinetics. Thus, the membrane current in the diffrentiated unicellular embryo was identified as almost pure Ca-channel current. The time course of the decay phase in standard solution was much slower than that of the egg Ca-channel current.

3) In Ca external solution the membrane current showed the outward component, but this was abolished by injecting EGTA in the unicellular embryo. Thus, the outward current was considered to be Ca-dependent K current and not delayed K rectification.

4) The ion selectivity of the Ca channel in the differentiated unicellular embryo was Ca < Sr < Ba, being different from that of the egg Ca channel, Ca≒Ba < Sr. The inactivation of the Ca-channel current was only found in Ca external solution in the case of the differentiated unicellular embryo, while the inactivation of the egg Ca-channel current was potential-dependent and observed in either Ca or Sr or Ba external solution.

Discussion

All the above results indicate that the Ca channel in the differentiated unicellular embryo was exactly identical with those found in other differentiated excitable cells and definitely different from those found in various oocytes. Thus, it is suggested that the difference of the properties in Ca channels is partly due to the changes of channel structure during the differentiation of excitable membranes.

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Muscular Dystrophy Group, 1981

SENSITIVITY TO Ca^{2+}, Sr^{2+}, AND Ba^{2+} IONS OF THE CONTRACTILE SYSTEM OF SLOW AND FAST FIBERS OF AMPHIBIAN MUSCLES

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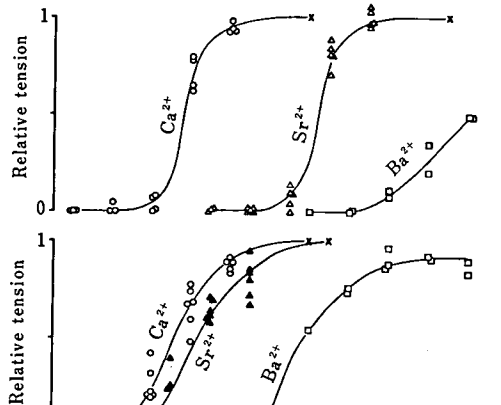
It is well known that there are two types of muscle fibers, slow and fast, in amphibian skeletal muscles. The slow fibers of amphibian muscles are different from slow twitch fibers of mammalian muscles at least in that action potentials cannot be evoked in the former but can be in the latter. It has been reported that in mammalian muscles, whereas Ca^{2+}-sensitivities of slow and fast twitch fibers are rather similar, Sr^{2+}-sensitivities are quite different, the Sr^{2+}-sensitivity of slow twitch fibers being significantly higher than that of fast twitch fibers[1,2]. It is interesting, therefore, to examine whether or not a similar difference exists between fast and slow fibers of amphibian muscles. We have examined sensitivities to Ca^{2+} and Sr^{2+} ions as well as to Ba^{2+} ions of the contractile system of fast and slow fibers of amphibian muscles as the first step of our studies on differentiation and development of muscle tissues.

Iliofibularis muscles were isolated from the adult African clawed toad, *Xenopus laevis*, and small bundles were dissected out of them in a frog Ringer solution. The bundles were immersed in a relaxing solution and single fibers were isolated and then skinned either by tearing them longitudinally with forceps or by immersing them in a detergent (0.5 w/v% Brij 58)-containing relaxing solution for 30 min. Isometric tension was recorded with a strain-gauge transducer (UC-2 or -20, Shinkoh) and displayed on a pen-recorder. Maximum shortening velocity was measured by the slack test technique as reported previously[3] and denoted as V_0. Experiments were performed in the presence of 1 mM free Mg^{2+}, 4 mM $MgATP^{2-}$, 10 mM total EGTA, and 20 mM PIPSE (pH 7.0) at an ionic strength of 0.15 M and at 2°C. Activating solutions contained Ca^{2+}, Sr^{2+}, or Ba^{2+} and the free divalent cation concentrations in these solutions were calculated by assuming apparent association constants for Ca-, Sr-, and Ba-EGTA to be $10^{6.20}$, $10^{3.71}$, and $10^{3.62}$, respectively[4,5].



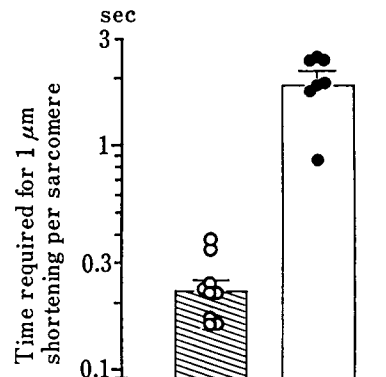


FIG. 1 (left). The relation between free divaldnt cation concentrations and tension develpoed by fast (upper) and slow (lower) fibers of amphibian skeletal muscles.
FIG. 2 (right). Correlation between shortening velocity and Sr^{2+}-sensitivity in two types of fibers.

Histochemically, the amphibian skeletal muscle fibers have been divided into five types which were named from their appearances as large pale, large dark, small dark, small pale, and small clear[6]. The first three types are considered to be the fast fibers, the last the slow fibers, and the fourth the intermediate fibers[6–9]. Fibers of the large pale type were dissected as a typical fast fiber and their divalent cation-sensitivities were determined. As shown in the upper part of Fig. 1, Sr^{2+}- sensitivity of these fibers was some 30 times lower than their Ca^{2+}-sensitivity. With Ba^{2+} ions, the sensitivity was still lower and maximal tension could not be reached even at the concentration of 1 mM. Skinned fibers from the large dark and probably the small dark fiber types showed similar divalent cation-sensitivities to those of the large pale fibers. However, skinned fibers from fibers of the probably small clear type exhibited quite different properties. As shown in the lower part of Fig. 1, while their Ca^{2+}-sensitivity was not much different from that of the large pale fibers, their Sr^{2+}-sensitivity was so much higher that the Sr^{2+} requirement of the contractile system of this fiber type was almost the same as its Ca^{2+} requirement. Ba^{2+}-sensitivity of this fiber type was also much higher than that of the large pale fibers and almost maximal tension could be obtained with 0.1 mM of the Ba^{2+} ions. We could not recognize three fiber types among the small fibers and, therefore, small pale fibers described in the literature might be included either in our small dark fibers or our small clear fibers.

The results described above clearly show that there are two distinct types of fibers in amphibian muscles showing either high or low sensitivities to Sr^{2+} and Ba^{2+} ions. From fiber appearance and previous reports, fibers showing the lower sensitivities may be fast fibers and those showing the higher sensitivities slow fibers. Indeed, measurements of V_0 of these fibers indicated that this is the case. As shown in Fig. 2, all the highly Sr^{2+}-sensitive fibers shortened very slowly and required a period of time several times longer for shortening of the 1 μm distance per sarcomere than did fibers of lower Sr^{2+}-sensitivity.

It was thus shown that Sr^{2+}-sensitivity of fast and slow fibers of the amphibian skeletal muscles is similar to those of fast and slow twitch fibers of mammalian muscles. The difference between Sr^{2+}- and Ba^{2+}-sensitivities of these fiber types might reflect the difference between the affinities of troponin to these divalent cations. In fact, in mammalian and avian muscles it has been reported that troponin from red or slow muscles has higher affinity to Sr^{2+} ions than that from white or fast muscles[1,10]. If this is the case, troponin of slow and fast fibers of amphibian skeletal muscles are different from each other. Whether this difference in troponin structures has a physiological significance in performing contractions with different speeds, and how the differentiation of troponin structures is developed are among the problems to be investigated next.

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ANTIBODIES TO ACETYLCHOLINE RECEPTOR IN OCULAR MYASTHENIA GRAVIS

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Anti-acetylcholine receptor (ACh-R)-antibodies are detected in the sera of most patients with myasthenia gravis[1,2]. However, the sera of patients with ocular myasthenia often showed a negative titre or a significantly lower titre than those observed in cases of generalized myasthenia gravis[1,3]. To improve the sensitivity of the radioimmunoassay method for anti-ACh-R-antibody, large amounts of sera from patients with myasthenia gravis, and higher concentrations of antigens and rabbit anti-human-IgG-antiserum, were used. These procedures enabled measurement of the titre value of over 0.04 pmol/ml serum and this value revealed a sensitivity about 10 times higher than that predicted using the previous method.

Using the more sensitive radioimmunoassay method, we compared the titre values of the antibody to ACh-R in the sera of ocular and generalized myasthenia gravis in relation to the clinical status of these patients.

Sera were obtained from 17 Japanese patients with myasthenia gravis restricted to the eyelids and ocular muscles, 37 patients with generalized myasthenia gravis, and 10 normal controls. All the patients (10 men and 24 women aged 21–63 and with definite generalized myasthenia gravis) had classic histories, signs and symptoms, and the diagnosis was supported by clinical improvement following anticholinesterase medication.

As patients with definite ocular myasthenia, 11 males and 6 females (aged 9–65 years) were selected. These individuals had been affected for two months to 18 years. All patients had been on anticholinesterase medication but had not undergone thymectomy nor been given steroids.

Radioimmunoassay of anti-ACh-R-antibody was fundamentally the same as that described elsewhere for human antigens[2].

The amount of anti-ACh-R-antibody was measured by incubating increasing amounts of human serum with a fixed quantity of ^{125}I-α-BuTx-ACh-R and subsequently precipitating the ^{125}I-α-BuTx-ACh-R complexed to anti-ACh-R-antibodies by rabbit anti-human-IgG serum. In the routine assay, the test serum was diluted 10-fold with phosphate-buffered saline-0.5% Triton X-100, and 10, 20, and 50 μl aliquots of the diluted serum were incubated with a constant volume (500 μl) of ^{125}I-α-BuTx-ACh-R (0.4 pmol/ml), at 4°C. After 10 hr of incubation, 450 μl of rabbit antiserum (which contained sufficient anti-human-IgG antibodies to precipitate all the IgG in the test serum) was added and the mixture was further incubated at 4°C for 16 hr. The final volume was 1 ml following addition of PBS. The insoluble immune complex formed was precipitated by centrifugation at 2,500 rpm for 5 min at 2°C, washed three times with ice cold PBS, and the radioactivity in the precipitate was measured by a gamma scintillation counter.

When the sera showed less than 1 pmol/ml titre values (less than 800 cpm/5 μl serum), these sera were reassayed using the more sensitive procedures. When an increased amount of diluted sera (250–500 μl) was used, and in order to obtain the same final tube volume as in the routine assay (1 ml), concentrated ^{125}I-α-BuTx-ACh-R (2.0 pmol/ml) and rabbit anti-human-IgG were used to precipitate the anti-ACh-R-antibodies. Namely, the labelled ACh-R and rabbit anti-human-IgG were concentrated about 5- or 10-fold using a macromolecular concentrator (Amicon, PM 30) or 33% Na_2SO_4, respectively.

Thirty-five out of 37 patients with generalized myasthenia gravis showed measurable amounts of the antibody titres in routine radioimmunoassays. The two patients with a negative titre value were in a remission. The antibody concentrations in these patients ranged from 0.44 pmol/ml to 252 pmol/ml (the mean value was 25.42±43.30 (±S.D.) pmol/ml). The range of normal control was 0 pmol to 0.4 pmol with a

mean value of 0.18±0.10 pmol/ml (±S.D.). On the other hand, in the ocular group, anti-ACh-R-antibodies were detected in only 5 of 17 patients and the titre values of the ocular patients ranged from 0 pmol to 11.24 pmol.

Thus the incidence of anti-ACh-R-antibody in the ocular group (30%) was lower than in the generalized myasthenia gravis (90%), as determined using a routine assay. However, if patients with ocular myasthenia gravis have antibodies of less than 0.4 pmol/ml, the incidence of anti-ACh-R-antibody in the ocular group increase.

With the sensitive radioimmunoassay method, anti-ACh-R-antibodies were detected in 8 of 12 ocular patients with a negative titre, as measured by routine assay. The antibody concentrations of these patients ranged from 0.042 to 0.16 pmol. In the ocular group, therefore, 13 of 17 patients had measurable titre values. These values ranged between 0.042 and 11.2 pmol/ml, and the mean titre value (1.79±3.18 (±S.D.)) was significantly lower than the values obtained in the generalized group ($P<0.01$).

With this improved assay procedure (10-fold increase in the sensitivity), the incidence of antibody against ACh-R in cases of ocular myasthenia was elucidated in about 70% and that of generalized myasthenia gravis in about 90% of the patients. The incidence of anti-ACh-R-antibody in the two groups did not differ, yet absolute titre values were significantly lower in the ocular group than in the generalized group. Furthermore, anti-ACh-R-antibody was not detectable in four patients with ocular symptoms of several months duration. The sensitivity of the present assay may not be sufficient to detect minute amounts of antibodies in the sera of these patients, and/or the antibodies against ACh-R may not even be present in their sera. Another possibility is that there is some different antigenic determinant between ACh-R of the ocular muscles and that of the pectoral muscles.

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NEURONAL FACTOR STABILIZING ACETYLCHOLINE RECEPTORS ON CULTURED MUSCLE CELLS

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The distribution and metabolic stability of the acetylcholine receptors (ACh-R) on the skeletal muscle of vertebrates are influenced by the innervating nerve. The ACh-R of adult muscle fibers are localized at neuromuscular junctions and degraded slowly, whereas the ACh-R of embryonic muscle fibers are distributed diffusely and degraded rapidly. The ACh-R on cultured myotubes are metabolized as rapidly as those on embryonic muscle fibers.

The neurotrophic mechanism by which the ACh-R on the muscle membrane are stabilized metabolically during development is unclear. Here we report that a factorprodu ced and released by neuronal cells regulates the turnover of the ACh-R on cultured myotubes.

Primary cultures of embryonic skeletal muscle cells were prepared from hindlimbs of rat embryos as described by Christian *et al.*[1]. Four or five days after plating cultures were treated for one day with 10^{-5} M fluorodeoxyuridine and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum and 0.5 μg/ml tetrodotoxin. On the day before treatment with neuronal conditioned medium, horse serum was replaced by 2 mg/ml bovine serum albumin. Neuronal conditioned medium (NCM) was prepared using the neuroblastoma×glioma hybrid cell line NG108-15[2]. NG108-15 cells were grown in DMEM containing hypoxanthine, thymidine, and 10% fetal calf serum. Serum free medium was exposed to confluent cultures of NG108-15 cells for one day, concentrated by ultrafiltration, dialyzed against deionized water, and stored at −20°C. The lyophilized material was redissolved at a concentration of 0.4 mg/ml in DMEM containing bovine serum albumin and tetrodotoxin and added to myotube cultures. Medium conditioned by the glioma cell line C6BU-1 (GCM) was also prepared by the same procedure.

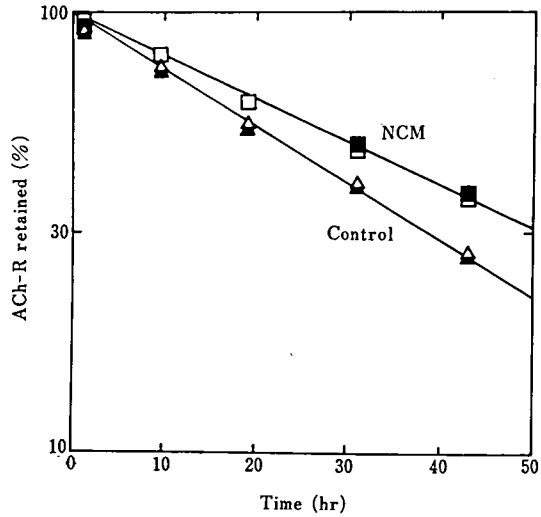

FIG. 1. Rate of degradation of ACh-R. Two nine day old cultures were pre-treated for 17 hr with NCM or control medium. Cultures were then labelled with ^{125}I-ABT and the rate of degradation of ^{125}I-ABT-ACh-R complex was measured.

The rates of degradation of the ACh-R on the surface membrane of myotubes were determined according to the procedure of Devreotes and Fambrough[3]. Seven to ten day cultured myotubes were treated with 0.4 mg/ml NCM or GCM for 16 to 45 hr and incubated with 10 nM ^{125}I-α-bungarotoxin (ABT) for 60 min. Unbound ^{125}I-ABT was removed. Then the loss of ^{125}I-ABT-ACh-R complex on myotubes was monitored in NCM, GCM, or control medium over 40 hr by measuring radioactivity released by myotubes into the medium.

The size of the intracellular pool of internalized ACh-R and its degradation products was calculated by subtracting the amount of label bound to surface ACh-R during 1-hr incubation with ^{125}I-ABT from the amount of label bound to surface ACh-R and internalized ACh-R during 20–24 hr incubation with ^{125}I-ABT.

The degradation curves of the ACh-R on myotubes followed a single exponential process over 40 hr (Fig. 1). The half-time (T 1/2) of the degradation of ^{125}I-ABT-ACh-R complex on myotubes in control cultures was calculated to be 22.6$\pm$0.9 hr (mean$\pm$S.E.). T 1/2 of the degradation of ^{125}I-ABT-ACh-R on myotubes in NCM-treated cultures was 132% of that in control cultures. Since T 1/2 of the ACh-R degradation in GCM-treated cultures was similar to that in control cultures, the effect of conditional medium on stabilizing ACh-R on cultured myotubes is specific to neuronal cells.

The decrease in the rate of degradation of surface ACh-R that was observed by measuring the degradation products is caused by slowing one of the following processes: 1) internalization of the surface ACh-R, 2) intracellular degradation, or 3) release of degradation products into the extracellular medium. If the treatment of myotubes with NCM slows the second or the third process, the increased accumulation of internalized ^{125}I-ACh-R is expected. Therefore, the effect of NCM on the intracellular accumulation of ^{125}I-ABT-ACh-R complex and its degraded products was examined. The amount of intracellularly accumulated ^{125}I-ABT-ACh-R and its degraded products in NCM-treated myotubes was similar to that in control cultures. In myotubes treated with 10 μM chloroquine instead of NCM, the amount of internalized ^{125}I-ABT-ACh-R complex increased to three times that of control cultures and T 1/2 of the ACh-R degradation increased to eight times that of control.

In our experiments both NCM and chloroquine decreased the rate of degradation of ACh-R. Whereas chloroquine inhibited the release of internalized ^{125}I into the extracellular medium[4], NCM had no effect on this release. NCM, therefore, acts by decreasing the rate of ACh-R internalization. Our results suggest that a diffusible factor which is produced and released by neuronal cells is involved in the neurotrophic mechanism for the stabilization of ACh-R on the surface membrane of muscle cells.

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SYNTHESIS AND DIFFERENTIATION OF ACETYLCHOLINE RECEPTORS IN CULTURED SKELETAL MUSCLE CELLS

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Two types of acetylcholine receptors (AChRs) have been found in the membrane of vertebrate skeletal muscle, one localized at a high density at the adult neuromuscular junctions and the other distributed at a lower density over the extrajunctional regions of denervated muscles. These two types of receptors, junctional and extrajunctional, differ in a number of properties in addition to their location and densities. Biochemically, the junctional receptor has a slightly lower isoelectric point than does the extrajunctional receptor. Embryonic muscles have the extrajunctional type of receptor, and the junctional type of receptor appears, at least *in situ*, only after motor innervation of the muscles occurs. Although the differentiation of AChRs is considered to be under the influence of the innervating nerves, the underlying mechanism remains largely unknown.

In the present study, we examined the biochemical properties of the receptors on the surface membranes and intracellular α-bungarotoxin (αBT) binding components of cultured muscle cells from newborn rats, chick embryos, and mouse clonal muscle cells G8-1 prepared as described previously[1]. The surface receptors or intracellular αBT-binding components were differentially labeled with ^{125}I- or ^{3}H-labeled αBT[2], extracted with 2% Triton X-100, and analyzed by gel filtration, sucrose density gradient centrifugation, or isoelectric focusing. The detailed experimental procedures have been described previously[3,4].

The results showed that in the absence of neuronal influences cultured muscle cells of the rat and the mouse can produce two forms of receptors on the cell surface membranes, as demonstrated by isoelectric focusing, with pI values indistinguishable from those of junctional and extrajunctional receptors. In contrast, only a single major component with a pI value close to that of the extrajunctional receptors was found on the surface membranes of cultured chick muscle cells (Table 1).

The cultured cells had two classes of intracellular αBT-binding components: one had the same sedimentation coefficient as that of surface receptors (9S), and the other had much smaller apparent molecular

TABLE 1. Isoelectric Points of AChRs of Different Forms from Various Sources Complexed with Radiolabeled αBTs

Preparation source	pI values of AChRs of various types complexed with radiolabeled αBTs[a]		
	Acidic	Basic	Degraded
Chicken embryo muscle cells			
Surface	—[b]	5.39±0.09 (7)	6.02±0.22 (10)
Inside	—[b]	5.60 (1)	n.d.[c]
Mouse			
Diaphragm	5.15±0.10 (2)	n.d.[c]	5.72±0.11 (2)
G8-1 cell surface	5.09±0.11 (9)	5.45±0.08 (9)	6.01±0.16 (8)
Rat			
Diaphragm	5.18 (1)	5.41±0.02 (2)	5.75±0.12 (2)
Newborn rat muscle cells			
Surface	5.14±0.08 (8)	5.44±0.10 (8)	5.94±0.19 (7)
Inside	—[b]	5.56±0.10 (9)	5.88±0.12 (5)

[a] Values determined using ^{3}H-αBT (mono[^{3}H]propionyl derivative)[1] and ^{125}I-αBT (mono- and di[^{125}I]iodo derivatives) were pooled without discrimination, and expressed in pH unit; mean±standard deviation (number of determinations).
[b] Not detectable under our conditions.
[c] Not determined.

weights. Only a single major component was detected by isoelectric focusing analysis of the 9S intracellular αBT-binding component, with a pI value close to that of the extrajunctional receptor (Table 1, "Inside").

These results suggest: 1) that the junctional and extrajunctional types of AChRs of rat or mouse muscles may be synthesized through a common precursor and differentiated into the two forms with different pIs by some post-translational modifications, and 2) that in the case of the chick muscles, the junctional and extrajunctional AChRs may have an identical pI value.

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Muscular Dystrophy Group, 1981

STUDIES ON ACETYLCHOLINE RECEPTOR OF DYSTROPHIC CHICK MUSCLE IN CULTURE

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We reported previously that the electrical properties of chick dystrophic muscle cells were significantly different from normal controls even in very early stages of genesis[1]. In this study the turnover of acetylcholine receptor (ACh-R) of dystrophic muscle cells was investigated to learn if there were any differences from normal muscle using the cell culture technique.

The starting material was chick embryo (N.H. 412, 413) which had been incubated at 37°C for 12 days. The breast muscle was dissected and explanted on collagen coated petri dishes containing the medium (MEM, 10% HS, 3% FCS, and 2% CEE). Cultivation was performed under 5% CO_2-air atmosphere at 37°C for 5 to 11 days. The myoblasts in culture grew rapidly and fused with each other forming myotubules, as shown in Fig. 1 (6 days). Striations appeared approximately 8 days after the explantation. There was no recognizable difference between dystrophic muscles and normal ones by phase contrast microscopic observations up to 11 days.

^{3}H-labelled erabutoxin (30 nM) or α-bungarotoxin (2 nM), which binds ACh-R specifically, was applied on the sample in culture. After washing excess toxin in the medium, the sample was incubated in the new medium. The appearance of toxin and/or its degradated products into the medium was followed for 30 hr.

Typical examples of toxin binding and its degradation are shown in Fig. 2 (erabutoxin) and Fig. 3 (α-bungarotoxin). The initial binding of the toxin to dystrophic muscle was only 50–80% of that to normal muscle in both erabutoxin and α-bungarotoxin (Fig. 2a, 3a). This probably indicates the number of the binding sites (ACh-R) is less in dystrophic muscles than in normal ones. The half-life time of the receptor (τ) was obtained by plotting the logarithm of remaining toxin *versus* incubation time (Fig. 2b, 3b). The value obtained from erabutoxin binding was considerably shorter than that from α-bungarotoxin binding; apparently, dystrophic muscle always exhibited faster turnover of ACh-R. The relative toxin binding of dystrophic to normal muscles (D/N) and the apparent half-life time (τ) of ACh-R obtained from different samples are listed in Table I. τ obtained from α-bungarotoxin binding is in agreement with that of rat skeletal muscle[2].

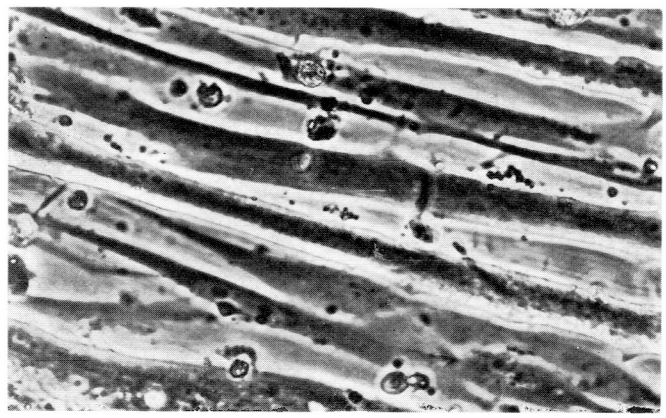

FIG. 1. Phase contrast microscopic observation of cultured dystrophic chick myotubules (6-day old in culture) (×160).

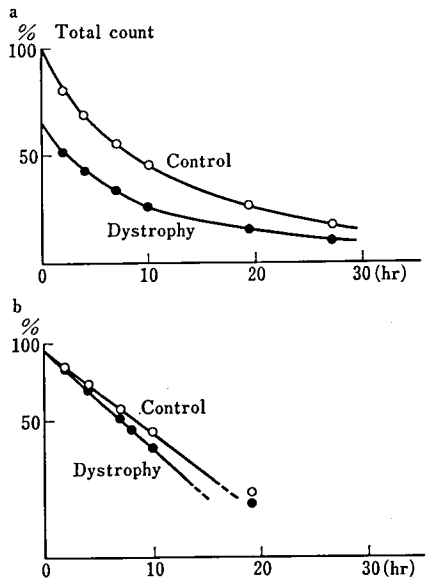

FIG. 2. Erabutoxin binding to ACh-R and its degradation. ○ normal, ● dystrophy. a: relative binding, initial value of normal muscle is taken as 100%. b: log of relative binding *versus* time, each initial value is taken as 100%.

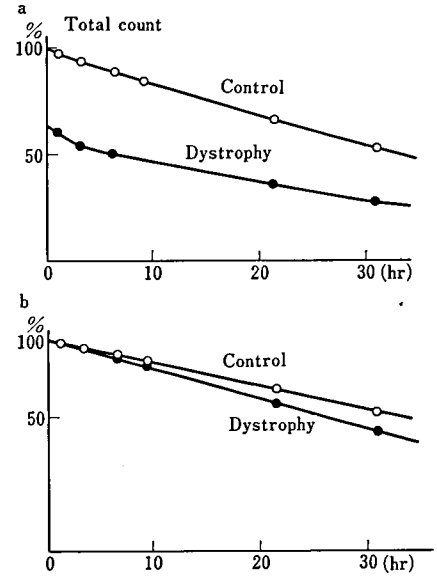

FIG. 3. α-Bungarotoxin binding to ACh-R. Notations and symbols are same as Fig. 2.

TABLE 1. Numbers of Binding Sites and Half Life Time (N, normal muscle; D, dystrophic muscle; D/N, ratio of toxin binding (100%, initial value of normal muscle); τ, half-life time (hr)).

Erabutoxin B

Exp. No.		8	9	11	12	17	18
Total D/N		0.65	0.40	0.40	0.85	0.72	0.74
τ (hr)	N	9.3	9	7.7	7.7	7.7	7.7
	D	8	8.3	6.1	6.7	6.5	5.7
	D/N	0.86	0.92	0.78	0.87	0.84	0.74

N: 8.3±0.8. D: 7.1±1.0.

α-Bungarotoxin

Exp. No.		21	22	24	27	28	29
Total D/N		0.62	0.61	0.36	1.0	0.78	0.68
τ (hr)	N	39.2	34.4	31.8	41.5	31.0	34.2
	D	39.2	34.4	31.8	40.0	26.4	26.7
	D/N	1	1	1	0.96	0.84	0.80

N: 35.5±4.0. D: 33.2±5.7.

The fast turnover of ACh-R measured by erabutoxin binding may be due to the weak binding of the toxin to the receptor sites. Some of the bound erabutoxin could be released directly into the medium without degradation. Nevertheless, there seems to be significant difference in these turnover rates between dystrophic and normal muscles.

The results obtained in these experiments suggest that the ACh-R in a developing dystrophic muscle has distinct properties compared with that in a normal control: 1) density of the ACh-R on the membrane is less, 2) it has weak affinity to erabutoxin, and 3) it exhibits faster turnover. These findings are consistent with the previously known fact that the turnover of muscle proteins is, in general, enhanced in dystrophic muscle.

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Muscular Dystrophy Group, 1981

FREEZE-FRACTURE ANALYSIS OF THE CAVEOLAE IN CHICK SKELETAL MUSCLES

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Last year we reported that increase in number and loss of orderly alignment of caveolae on the plasma membrane might precede the development of fiber degeneration in dystrophic chick skeletal muscles. For the further understanding of the significance of these caveolar changes in dystrophic muscles, we analyzed the size distribution of caveolae in skeletal muscles of developing normal control chickens and compared the results with those obtained from age-matched dystrophic chickens. As reported previously, superficial pectoralis and adductor lateral muscles of newly hatched, 2 and 5 week old chickens of lines 412 (control) and 413 (dystrophic) were obtained under pentobarbital anesthesia. Small pieces of muscle tissue were immediately immersed in 2.5% glutaraldehyde in phosphate buffered saline (pH 7.4, 285 mOsm). After fixation in glutaraldehyde for a few hours at 4°C, the tissues were glycerinated in 25% glycerin in PBS and quenched in liquid nitrogen cooled Freon 12. The tissue was fractured at −115°C under a vacuum of $1-2\times10^{-7}$ Torr with an Eiko freeze etch device. The fracture surface was immediately coated with platinum and carbon. The replicas were retrieved after digestion with a commercial bleach solution and washed with several changes of distilled water. Photographs were taken with a Hitachi H-600 electron microscope at a magnification of 40,000. The size of caveolae was determined by measuring the smallest diameter and enlarging it to a final magnification of 120,000 with the aid of a Kontron Image Analyzer.

Based on their diameters the caveolae of chick skeletal muscles seemed to be divided into two groups; those with a diameter of less than 50 nm and others more than 50 nm in diameter. The size distribution of those in each muscle group is summarized in Table 1. Except for the superficial pectoralis muscle of newly hatched chickens, the caveolae are apparently heterogeneous in size; small and large are mixed in various ratios. The most striking finding in dystrophic muscles is the increased density of caveolae in superficial pectoralis muscles of 2 and 5 week old chickens; in the lateral adductor muscle of 5 week old chickens this is largely due to the increased number of small caveolae less than 50 nm in diameter. This observation agrees with the report on human muscular dystrophy by Bonilla, Fischbeck, and Schotland[1]. Even though the underlying mechanisms of muscle degeneration may differ in human and chick muscular dystrophy, it is of interest that the increased number of small caveolae developed as an early change of muscle degeneration in both. We are still uncertain about the physiological role of caveolae in skeletal muscles. The present observation may suggest that a certain population of small caveolae can be associated with abnormal transmembrane transports only manifested under pathological conditions, if such exist. It is also interesting that the plasma membrane of the superficial pectoralis muscle of newly hatched chickens was dominated by small caveolae even in control chickens. Since it is known that these muscles are very immature at the time of hatching with a small fiber diameter and poorly developed T-system, it can be assumed

TABLE 1. Size Distribution of Caveolae

Time	Muscle	<25 nm	25–49 nm	50–74 nm	75 nm<
Day 0	Superficial pectoralis	0* (4°)	97 (102)	15 (2)	0 (0)
	Lateral adductor	0 (0)	4 (15)	62 (85)	39 (6)
2 weeks	Superficial pectoralis	0 (0)	138 (37)	60 (71)	2 (19)
	Lateral adductor	0 (0)	34 (81)	86 (56)	10 (2)
5 weeks	Superficial pectoralis	2 (0)	206 (87)	39 (55)	0 (3)
	Lateral adductor	26 (0)	155 (11)	102 (108)	3 (22)

* Dystrophic chickens, ° control chickens, per 10 μm^2.

these small caveolae represent increased pinocytotic activity to compensate for the function of this immature T-system.

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Muscular Dystrophy Group, 1981

FREEZE-ETCH REPLICA STUDY OF THE CYTOSKELETON IN SKELETAL MUSCLE FIBERS

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The cytoskeleton may be defined as a cytoplasmic structure which mechanically supports the cell and is mainly comprised of microtubules, intermediate filaments and microfilaments. In muscle cells the cytoskeleton seems to be involved in the contractile activities and overall organization of myofibrils and membranous systems. We have been studying electron microscopically the roles of the cytoskeletal components in the differentiation and development of skeletal muscle cells in culture. There is a transient increase of microtubules and intermediate filaments during the early stage of cell elongation and myofibril formation. In mature skeletal muscles microtubules and intermediate filaments are small in number, but often show more or less regular distribution with respect to the sarcomere pattern of myofibrils.

Morphological studies on the cytoskeleton have been carried out mostly by thin-section electron microscopy. Recent progress in a freeze-etch replica method has prompted us to use this method to ultrastructurally analyze the cytoskeletal components in muscle cells with special reference to myogenesis. In particular, the deep-etch replica method combined with rapid freezing is most pertinent to three-dimensionally observe the ultrastructures of unfixed, fresh tissues. However, one serious problem in this method arises from the fact that only the superficial layer of tissue is morphologically well-preserved and free of any detectable ice crystals, even by rapid freezing. It is difficult to expose the interior of tissue within this superficial layer for freeze-etch replication in commercially available freeze-etch devices. To overcome this difficulty we have made some technical improvements, the major point of which is to apply cryomicrotomy to the freeze-etch replica method[1].

A small piece of tissue is mounted on a specimen holder (Fig. 1a) and rapidly frozen by bringing the tissue sample into contact with a copper block cooled by liquid helium[2]. The sample thus frozen is brought into a cryokit (FTC/LTS-2) fitted to a Sorvall MT-2 ultramicrotome and cut with a glass knife with a setting of 1 μm thickness at -110°C to -120°C to remove the outermost, metal-contacted surface of the sample (Fig. 1b-1). The preferable depth of removal is usually 3 to 15 μm for samples frozen at the temperature of iiquid helium. Samples with an exposed surface are transferred to a conventional freeze-etch device (Eiko FD-2S) in which the exposed surface is deeply etched (Fig. 1b-2) and then rotary-shadowed with platinum and carbon (Fig. 1b-3). Replicas are obtained by cleaning in household bleach, are picked up on formvar-filmed copper grids and then examined under a Hitachi HU-12 electron microscope.

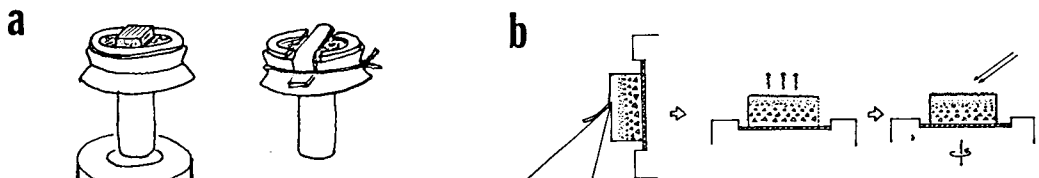

FIG. 1. Improved rapid-freeze, deep-etch replica method. **a**: unfixed, fresh tissue samples on specimen holders. **b**: (1) cutting a sample in a cryokit to expose the tissue within the superficial layer, (2) deeply etching in a freeze-etch replica device, and (3) rotary-shadowing with platinum and carbon for replication.

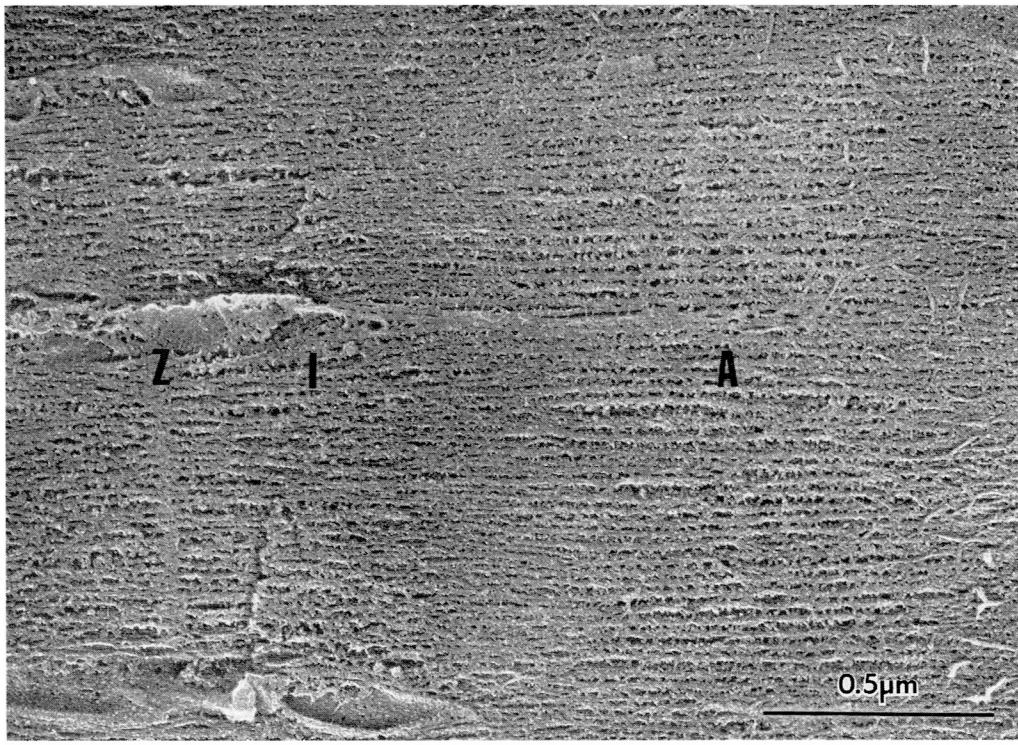

FIG. 2. Freeze-etch replica feature of unfixed, fresh skeletal muscle. Thick and thin myofilaments are clearly visible within the myofibrils. **A**: A band, **I**: I band, **Z**: Z disc. Frog sartorius muscle.

For preparing freeze-etch replicas of skeletal muscles, small strips of muscle tissues were freshly dissected and fastened in an extended state onto specimen holders (Fig. 1a) and, without chemical fixation, were frozen rapidly at the temperature of liquid helium (4°K). The ultrastructure of myofibrils as seen in replicas was basically the same as that in thin sections (Fig. 2). The thick and thin filaments within myofibrils were clearly visualized in three-dimension and at high resolution[3].

In summary, we improved the rapid-freeze, deep-etch replica method by combining cryomicrotomy and showed the feasibility of using this improved method for an ultrastructural examination of the cytoskeleton.

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Muscular Dystrophy Group, 1981

DIFFERENT RATE OF CHANGE IN ISOFORMS OF MYOSIN HEAVY AND LIGHT CHAINS DURING DEVELOPMENT OF SKELETAL MUSCLE

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It is well known that several contractile proteins, such as myosin, actin, tropomyosin, troponin, and α-actinin, have multiple isoforms. Immunochemical and electrophoretic studies have shown that the different forms of each contractile protein are usually located in different cell types, respectively[1-6]. Thus, a specific 'kit' of proteins characteristic for each of the cellular types is shown in the differentiated cell. The hypothesis proposed for the mechanism of this specific expression is that the genes of each 'kit' are under coordinate control during differentiation of the cells[6-8].

In the present work we have attempted to study what type of control is operating in the synthesis of myosin molecules in the developing muscle. One mole of myosin molecule is composed of six moles of subunits which are classified into three types of two moles each: heavy chains (HC), phosphorylatable light chain (PLC), and unphosphorylatable light chains (UPLC). So far, six isoforms of HC, three of PLC and four of UPLC, which would be coded in different structural genes, have been detected in the same animal. Although about 270 types of myosin are expected from the random assembly of isoforms subunit, differentiated cells contain only a few types of myosin[2-5]. As myosin is composed of three types of subunits, the presence of one specific form of myosin requires the regulation of three structural genes to be expressed from the gene pools.

We used anterior latissimus dorsi (ALD) muscle of chicken, because this muscle in an adult animal is predominantly composed of one cellular type, the slow twitch muscle fibers, and contains only one isoform of HC, PLC, and UPLC[5].

Total cellular proteins extracted from embryonic muscle at a variety of stages are fractionated by two dimensional (IEF/SDS) polyacrylamide gel electrophoresis (2D-PAGE). As reported previously, seven isoforms of the LC subunits have different molecular weights and/or different isoelectric points[5] and hence each of them is mapped at different positions on 2D-PAGE. The accumulated LC's in embryonic muscle were identified by three criteria: 1) to be comigrated with authentic LC markers, 2) to have LC-antigenicity, 3) to show identical peptide patterns with LC markers by limited proteolysis. Results indicated that in embryonic ALD muscle, the LC isoforms corresponding to the fast muscle form (fPLC and fUPLC) were expressed in addition to the slow muscle forms (sPLC and sUPLC) which are specific for the differentiated ALD muscle. At a very early stage of development (10 days embryonic stage), fPLC and sUPLC could be faintly seen in combination with predominantly fUPLC and sPLC. This combination of the LC patterns of myosin was also seen in embryonic ALD muscle. The spot of fPLC first became fainter and then was no longer detectable in muscle of the 17-day embryo. The sUPLC/fUPLC ratio gradually increased with development, and fUPLC was still detectable at a few days post-hatching. Thus, fPLC and fUPLC disappear at different rates from the ALD muscle as it develops. These results suggest that at some stage of ALD muscle development, a hybrid myosin molecule might exist which is composed of fast form LC in combination with slow form LC. To confirm this further, we examined the assembly of HC and LC by non-denaturant pyrophosphate gel electrophoresis (PPi-PAGE)[9]. Results indicated that myosin in adult ALD muscle migrated as one band, whereas embryonic myosin of ALD muscle showed additional bands

(emb.M), besides the adult type (ad.M). The emb.M/ad.M ratio was gradually decreased with development, but emb.M was still detectable in ALD muscle at 16 weeks post-hatching. As the LCs expressed at this stage were only slow muscle form, the different mobility of emb.M from that of ad.M might be due to the difference of HC. This is likely because the limited proteolytic peptide maps of HC from emb.M and ad.M are distinctly different. The HC peptide maps of ad.M in embryonic muscle by cleaning with proteolytic enzyme was identical with ad.M in adult muscle. The ad.M of embryonic muscle, however, contains a fast form of LC. It is likely that in embryonic ALD muscle, hybrid molecules might exist. In addition, the change in HC expression from the embryonic to the adult type takes a longer time course than the case of LC subunits. These results clearly indicate the different rates of expression among the three subunits of myosin molecules during development. The accumulation of each subunit can be controlled at a number of steps such as replication, splicing, translation, post-translational modification, assembly, and filament formation. The results presented here suggest that the genes of each subunit are not under simple coordinate control; some steps before assembly may determine the rate of accumulation of the subunits.

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NUMBER OF ANTITROPONIN I STRIATIONS ALONG THE THIN FILAMENT OF ADULT AND FETAL SKELETAL MUSCLES OF RABBIT

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Immunoelectron microscopic study on the chicken skeletal muscle showed that troponin distributes along the whole length of the thin filament at regular intervals of 38 nm. The number of antibody striations along each bundle of thin filaments was always twenty-four. It was also shown that, in the developing myofibrils, antitroponin forms more than twenty-four striations. This finding indicates that the final maturation of thin filament in chicken skeletal muscle consists of a shortening of the longer filaments into a constant length after hatching. This shortening of thin filament occurred much later in the dystrophic muscle of chicken than in intact muscle.

The present study was undertaken to investigate whether the shortening of thin filament is also found during the development of rabbit skeletal muscle. Glycerinated muscle fibrers from the iliopsoas muscle of an adult and a 29-day fetus were used in the experiments. Antitroponin I antiserum from goat was purified by the method of ethanol fractionation. Two methods were used for antibody staining. The first was a saponin technique in which muscle fiber was treated with 10^{-4} g/ml saponin and then with antitroponin I in a relaxing solution containing 0.1 M KCl, 10 mM $MgCl_2$, 10 mM K-phosphate (pH 7.0), 3 mM ATP, and 0.4 mM EGTA. The muscle fiber was fixed and embedded in epoxy resin for thin sectioning. In the second method, thin filaments were dissociated from thick filaments by homogenizing in the relaxing solution, treated with antibody, and stained with uranyl acetate. These specimens were observed by an electron microscope (Hitachi HS-8).

Antitroponin I antibody always formed twenty-six striations along the thin filament of adult muscle at intervals of 38 nm. On the other hand, the antibody formed more than twenty-six striations along the thin filament of the 29-day fetus as shown in Fig. 1. Up to thirty-four striations could be counted along the bundle of the filaments. The length of the thin filaments in each bundle seemed to be variable but was longer than the filament of the adult muscle. These findings clearly indicate that the shortening of the filament also occurs during the development of skeletal muscle in rabbit.

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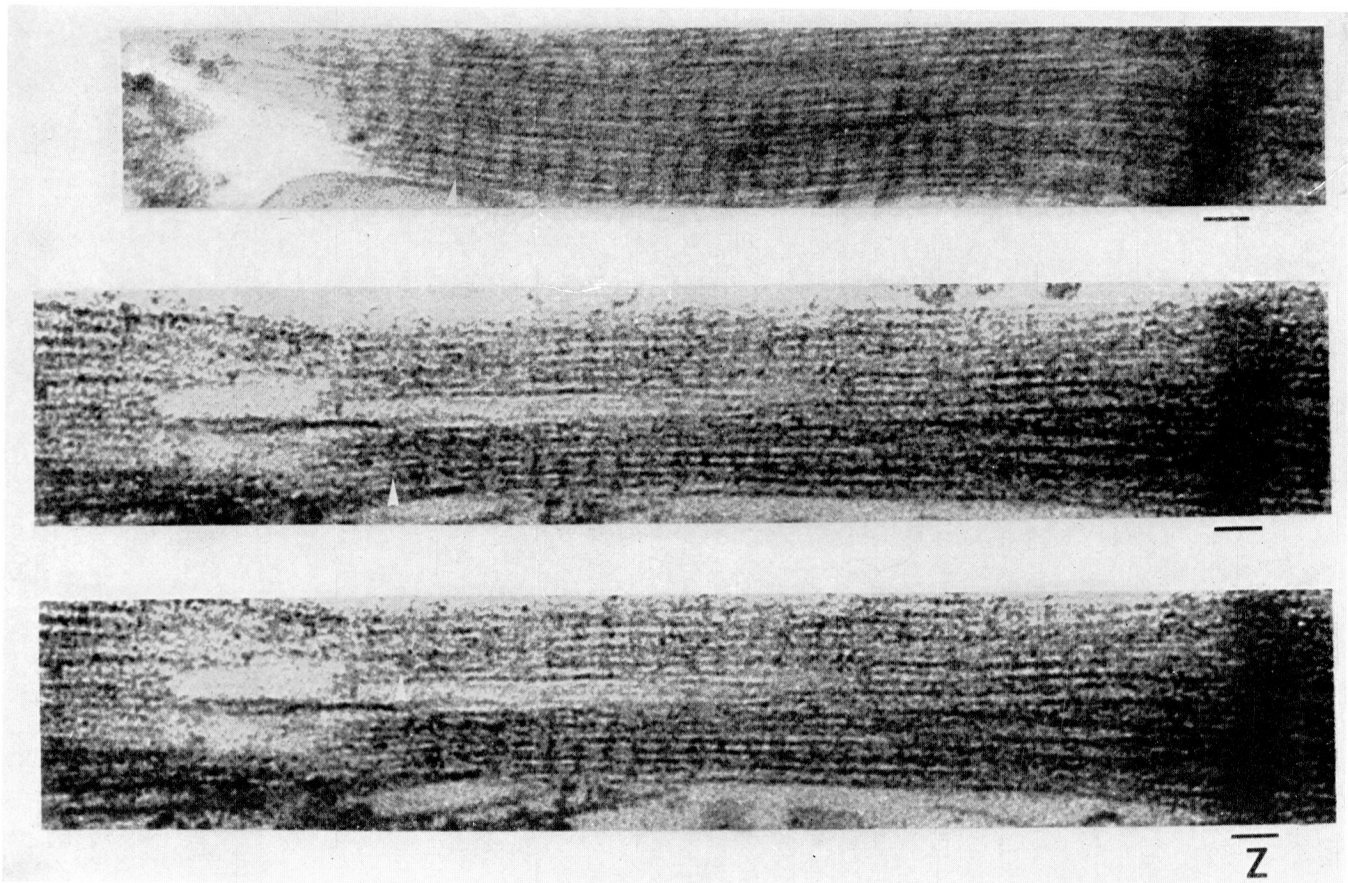

FIG. 1. Electron micrograph of skeletal muscle from a 29-day fetus rabbit stained with antitroponin I. White triangle (△) indicates the position of 26th striation counted from Z-line (×120,000).

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ISOFORM VARIANTS OF MYOFIBRILLAR PROTEINS DURING MUSCLE REGENERATION AFTER COLD INJURY

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Skeletal muscle fibers of adult chickens were focally injured by a liquid-nitrogen cooled brass rod, and the regeneration of the frozen zones were examined by immunofluorescence microscopy and two-dimensional electrophoresis. Transverse sections of injured fibers were stained with antibodies specific for troponin (TN) components (T, I, and C) from adult chicken breast and cardiac muscles, and with monoclonal antibodies MF-21 and E101 D6 raised against adult and embryonic breast C-protein, respectively.

Anterior latissimus dorsi (ALD) muscle of normal animals bound only anti-cardiac TN-C, while the regenerating muscle stained positively for breast TN-T, -I, and -C and cardiac TN-T and -C. Normal posterior latissimus dorsi (PLD) muscle stained positively with antibodies to all breast muscle TN subunits, while the regenerating muscle reacted strongly with antibodies to all breast TN subunits and to cardiac TN-T and -C (Fig. 1). We have reported previously that all embryonic skeletal muscles are stained with antibodies against breast TN components as well as cardiac TN-T and -C[1]. Thus, our present results showed that reactivities of regenerating myotubes in ALD and PLD muscles to antibodies against TN components are the same as those seen in embryonic skeletal muscles.

Monoclonal antibody MF-21 did not stain control ALD muscle, yet reacted strongly with regenerating ALD myotubes. E101 D6 only stained regenerating ALD and PLD; uninjured fibers were unreactive for this antibody.

Two-dimensional electrophoresis confirmed the presence of isoform variants of TN and myosin during regeneration of each muscle. In addition to proteins of each type, in regenerating ALD cardiac type TN-T and -C and PLD type myosin light chains were found, and in regenerating PLD cardiac type TN-C was detected. The results are summarized in Table 1.

From the results obtained in the present study, it is concluded that embryonic-like TN, myosin, and C-protein isoforms are synthesized within adult muscle during the regeneration from cold injury.

TABLE 1. Regeneration of Myofibrillar Proteins

Myofibrillar proteins	Methods		ALD			PLD		
			Normal	1 week	4 weeks	Normal	1 week	4 weeks
Troponin	Immuno-fluorescence and two-dimensional electrophoresis	Breast TN-T	−	+	−	+	+*	+*
		Breast TN-I	−	+	−	+	+*	+*
		Breast TN-C	−	+	−	+	+	+
		Cardiac TN-T	−	+	−	−	+*	−*
		Cardiac TN-I	−	−	−	−	−*	−*
		Cardiac TN-C	+	+	+	−	+	−
C-protein	Immuno-fluorescence	MF-21 (Adult breast)	−	+		+	+	
		E101 D6 (Embryo breast)	−	+		−	+	
Myosin light chain	Two-dimensional electrophoresis	PLD LC_1	−	+		+	+	
		PLD LC_2	−	+		+	+	
		PLD LC_3	−	+		+	+	
		ALD-Cardiac LC_1	+	+				
		ALD-Cardiac LC_2	+	+				

* Not confirmed by two-dimensional electrophoresis.

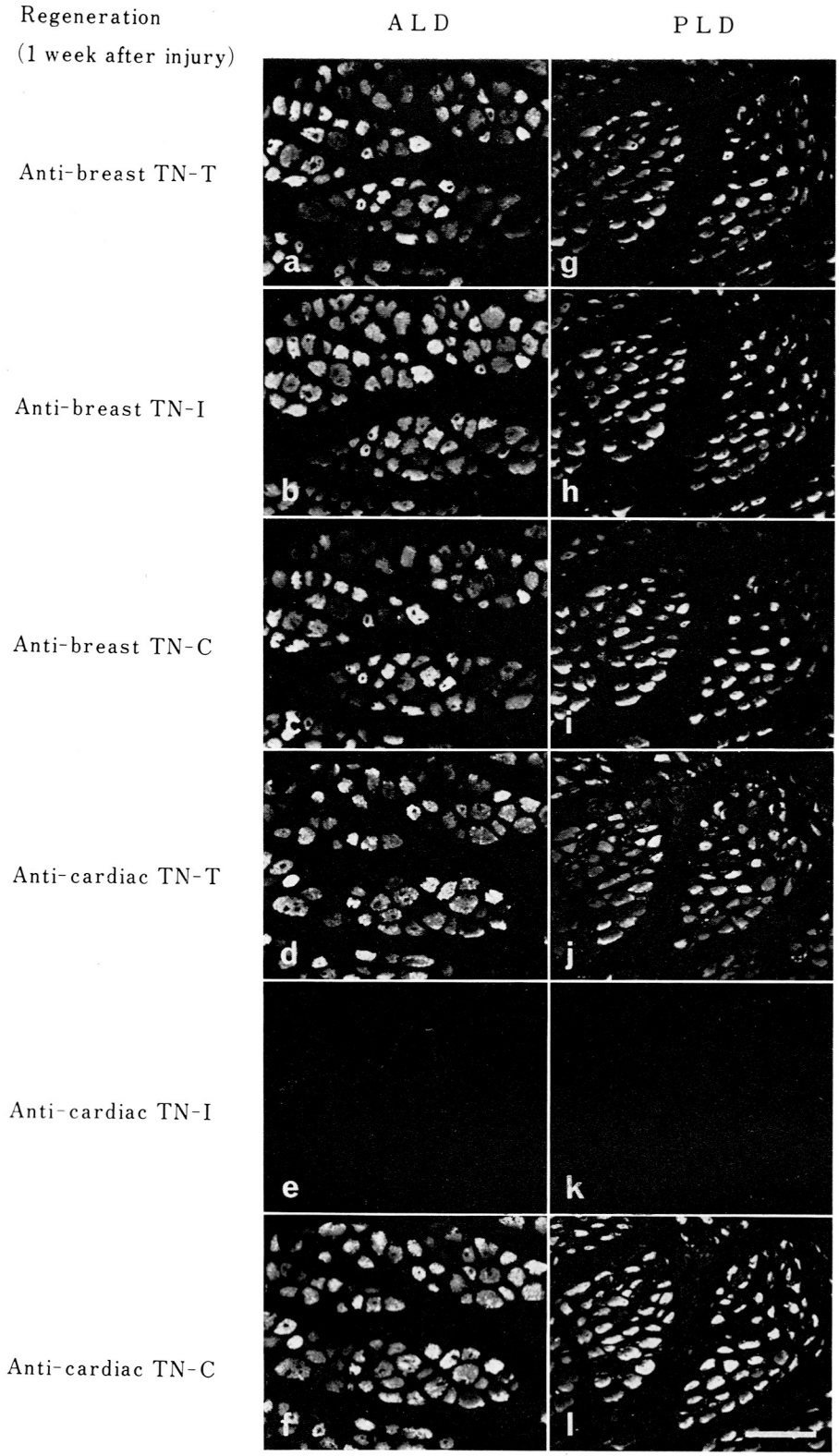

FIG. 1. Regenerating ALD and PLD, 7 days after cold injury. ALD (a–f) and PLD (g–l) were stained with antibodies to all skeletal TN components (a–c and g–i) and to cardiac TN-T and C (d, f, j, and l) (Bar, 10 μm).

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Muscular Dystrophy Group, 1981

MYOSIN ISOZYMES IN EMBRYONIC AND ADULT CHICKEN SKELETAL MUSCLE

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Myofibrillar proteins are known to exist in several different isoforms, and many recent studies have demonstrated that the proteins in embryonic muscle are different from those in the normal adult tissue. Changes in myosin isoforms during muscle development have particularly been a matter of wide interest and an increasing body of literature now exists which strongly indicates that the type of myosin present in embryonic skeletal muscle differs from that in homologous mature muscles[1-5]. However, the precise chemical nature of the embryonic variations remains controversial. In the present investigation, we observed that myosin isozymes from chicken embryonic and adult pectoralis muscle are distinguishable from each other by pyrophosphate (PPi)-acrylamide gel electrophoresis, and obtained additional evidences which suggests that the light chain composition and peptide fragments of heavy chains differs in embryonic and adult myosin of the chicken[6].

Myosin was prepared from pectoralis muscle of various developmental ages of chicken embryo or post-hatched chicken as described earlier[2], and embryonic and adult myosin isozyme in a monomeric form was examined by PPi-acrylamide gel electrophoresis according to Hoh[6]. As shown in Fig. 1, three fast myosin isozymes, FM_1, FM_2, and FM_3, were detected in adult chicken pectoralis muscle as previously demonstrated[7] (Fig. 1e). In contrast, when the myosin from the pectoralis muscle of 10- to 13-day old chick embryo was examined, only two bands, one major (EM_3) and one trace (EM_2), were observed (Fig. 1a, 1b). After 15 days of incubation another myosin isozyme (EM_1) appeared in the pectoralis muscle in addition to EM_3 and EM_2 (Fig. 1c). EM_3 was clearly distinguished from the adult myosin isozymes by the difference in electrophoretic mobility on PPi-acrylamide gel; it migrated more slowly than the adult three isozymes and therefore when it was mixed with adult myosin isozymes, four bands were obtained on the PPi acrylamide gel (Fig. 1g). The electrophoretic mobilities of the other embryonic myosin isozymes, EM_2 and EM_1, were close to those of adult myosin isozymes FM_3 and FM_2, respectively, but not exactly identical. The mobility of EM_2 was also very close to that of cardiac myosin isozyme (CM). EM_2 could be an ectopic cardiac-type myosin[1] which appeared in the embryonic pectoralis muscle. Slow myosin isozymes from the anterior latissimus dorsi (ALD) did not comigrate with embryonic pectoralis myosin isozymes. The myosin from the muscle of a 10-day old post-hatched chicken contained three myosin isozymes (Fig. 1d) and the mobilities of these isozymes on PPi-acrylamide gel were not distinguishable from those of the three adult myosin isozymes (Fig. 1f). It has been pointed out that myosin from embryonic tissue is occasionally

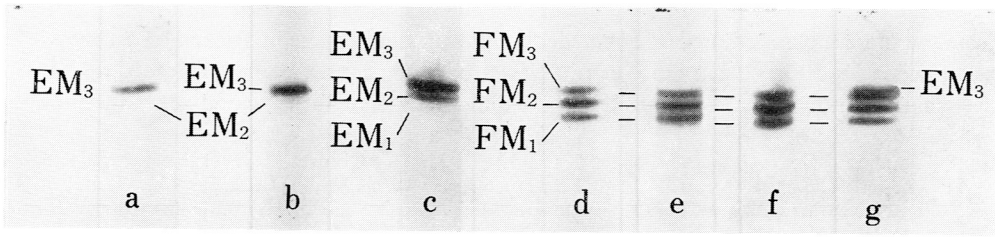

FIG. 1. PPi-acrylamide gel electrophoresis of myosin from the breast muscle of 10-day embryo (a), 13-day embryo (b), 15-day embryo (c), 10-day post-hatched chicken (d), and adult chicken (e). (f) Mixture of 10-day embryo and adult myosin. (g) Mixture of 10-day chicken and adult chicken myosin. Other symbols: see text.

TABLE 1. Subunits in Chicken Embryo and Adult Chicken Breast Myosin

	Myosin isozymes	Heavy chains	Light chains
Embryo	EM_3	EMHC	L_{f1}, L_{f2}, L_{s1}, L_{s2} (L_{c1}, L_{c2})
	EM_2	EMHC	L_{f1}, L_{f2}, L_{s1}, L_{s2} (L_{c1}, L_{c2})
	EM_1	EMHC	L_{f2}, L_{f3}
Adult	FM_3	FMHC	L_{f1}, L_{f2}
	FM_2	FMHC	L_{f1}, L_{f2}, L_{f3}
	FM_1	FMHC	L_{f2}, L_{f3}

EMHC, embryonic myosin heavy chain; FMHC, adult fast-type myosin heavy chain. Other symbols: see text.

contaminated with RNA[8], but the difference in electrophoretic mobility between embryonic and adult myosin isozymes was not due to the binding of RNA to embryonic myosin because the mobilities of embryonic myosin isozymes were not affected by the treatment of embryonic myosin with RNase. We cannot exclude the possibility that some myosin-binding proteins are associated with embryonic myosin and therefore the mobilities of embryonic myosin isozymes differed form those of adult myosin isozymes. However, C-protein, one of the typical myosin-binding proteins, could not be detected on the myosin band on PPi-acrylamide gel. We assumed that the difference in the mobility of embryonic and adult myosin on PPi-acrylamide gel was mainly due to the structural difference in myosin subunits, and we examined both heavy and light chain subunits of embryonic and adult myosin.

In order to analyze the type of light chains present in each myosin isozyme, PPi acrylamide gels were stained faintly with Coomassie brilliant blue and each band of myosin isozyme was cut out. Each gel slice was then applied for SDS-polyacrylamide gel electrophoresis after treatment with an SDS-solution containing 2% SDS, 2% 2-mercaptoethanol[5]. The pattern of light chains was visualized by means of a highly sensitive silver according to Oakley *et al.*[9]. Confirming the previous report[7], the myosin isozymes from adult chicken pectoralis muscle contained only fast light chains (Lf_1, Lf_2, and Lf_3) but each isozyme exhibited a specific combination of light chains (see Table 1). The light chain combination in embryonic myosin isozymes, EM_3 and EM_2, was quite different from those of adult myosin isozymes: both embryonic myosin isozymes contained two slow (Ls_1, Ls_2) and/or two cardiac light chains (Lc_1, Lc_2) in addition to two fast light chains (Lf_1, Lf_2) (Table 1). Coexistence of fast and slow (or cardiac) light chains in EM_3 was further confirmed by an immunological method.

In order to examine the structural differences in the heavy chains of pectoralis myosin in embryonic and adult chickens, myosin was partially digested with α-chymotrypsin, and then the resultant peptide fragments were analyzed by means of two-dimensional gel electrophoresis[6]. The pattern of the cleavage product of embryonic myosin was different from that of adult myosin. Most of the peptide fragments from embryonic myosin corresponded to those from adult myosin, but embryo-specific peptides also existed. Some of the peptide fragments from adult myosin were missing in the cleavage products of embryonic myosin. The pattern of the peptide fragments from embryonic pectoralis myosin was quite different from those of slow skeletal or cardiac myosin. These results indicate that the peptide structure of embryonic myosin heavy chain resembles that of adult breast (fast) myosin. In the preliminary experiment, we did not find any remarkable difference in heavy chain structure between embryonic myosin isozymes.

From the present observations, we conclude that the myosin isozyme in chicken breast muscle contains heavy chain which is distinct from those in adult tissue as in the case of mammalian fetal myosin[5], but we further conclude that light chain compositions also differ between embryonic and adult myosin isozymes.

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Muscular Dystrophy Group, 1981

NATIVE HIGH MOLECULAR WEIGHT PROTEIN FROM CHICKEN BREAST MUSCLE

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We have shown that an elastic protein designated "connectin" is responsible for elasticity and mechanical continuity of skeletal muscle, and that connection consists of protein very high in molecular weight (one million) and forming very thin filaments (2 nm in diameter)[1]. Recently Wang and his associates reported that the high molecular weight protein that they called titin can be isolated by gel filtration from a direct SDS extract of intact muscle fibers[2]. We confirmed Wnag's work, but reached the conclusion that their titin is identical with the connectin we previously described[3]. Previously connectin was extracted with an SDS solution from thoroughly extracted muscle residues[1]. In any case, connectin was isolated as a denatured form.

In the present study we have tried to isolate connection as a native form without use of a denaturing agent.

The indirect immunofluorescent technique revealed that connectin is located in the A–I junction area and in I-bands but not in Z-lines[1]. Observations that some connectin is coextracted with myosin[3] and that both ends of isolated A segments are covered with connectin filaments[4] suggest that some of these filaments play a role in locating the A-band in the center of a sarcomere. Based upon this view, we partially succeeded in releasing connectin filaments into a salt solution by suddenly contracting swollen myofibrils with the addition of salt.

Chicken breast muscle was freshly cut out, and the myofibrils were prepared by washing them more than ten times with 50 mM KCl containing 1 mM $NaHCO_3$. They were then washed exhaustively with 5–10 mM $NaHCO_3$ to remove water-extractable proteins such as alpha-actinin. Following this, the myofibrils were washed with pure water to reduce the ionic strength to approximately 0.001, and they became swollen. NaCl was rapidly added to a final concentration of 0.2 M, while being vigorously mixed. After one hour extraction, the suspension was filtered through a sheet of filter paper. The filtrate contained the high molecular weight protein (HMW protein) together with some myosin and actin.

TABLE 1. Amino Acid Composition

		Connectin from			
	Actin[3]	HMW	Whole[3]	Residue[3]	HS ext[3]
Asp	97	102	95	93	94
Thr	72	71	75	66	77
Ser	61	68	69	67	60
Glu	116	122	116	118	108
Pro	*43*	*56*	74	67	73
Gly	81	82	71	76	74
Ala	82	71	62	75	64
Cys/2	4	7	11	6	12
Val	*53*	*78*	85	78	89
Met	*47*	*21*	10	16	11
Ile	*76*	*59*	59	56	60
Leu	75	68	67	76	66
Tyr	*41*	*29*	30	30	29
Phe	31	28	27	29	28
Lys	*53*	*77*	82	79	85
His	18	14	15	18	15
Arg	50	47	55	50	55

The way of extracting exclusively HMW protein has not yet been established, but occasionally more than 90% purity has been obtained using SDS gel electrophoresis criteria. Using this sample, some preliminary characterization has been performed.

From the mobility in 3% acrylamide gel electrophoresis, the apparent molecular weight of HMW protein was more than one million, which is in good agreement with the values of connectin preparations[1,3]. UV absorption spectra were typical of protein nature with a maximum at 280 nm. The amino acid composition was almost identical with that of isolated connectin in a denatured state (Table 1). Although the amino acid composition of the native connectin was similar to that of actin, several amino acid components were distinctly different (Table 1). The viscosity of native HMW protein was very small when measured by the usual Ostwald type of viscometer: less than 1.1 cp for 0.2 mg/ml at a velocity gradient of 1,000 sec^{-1}. However, at a very low velocity gradient, *e.g.* 0.0008 sec^{-1}, measured in a Low-Shear Rheometer, it was as large as 30,000 cp. This suggests that three dimensional nets are formed in solution and the structure is easily broken down by a weak external force. This is not the case with denatured connectin solution in 1% SDS. In fact, the frozen replica method revealed that the thread formed by concentration consisted of very thin filaments. Furthermore, negatively stained HMW protein solution exhibited a filamentous image under an electron microscope. More work is in progress to elucidate the structure and function of native high molecular protein as well as its possible interaction with myosin filaments.

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Muscular Dystrophy Group, 1981

A PROTEIN IN CHICKEN BREAST MUSCLE WHICH INHIBITS THE ADP-RIBOSYLATION OF EF2 BY DIPHTHERIA TOXIN *IN VITRO*

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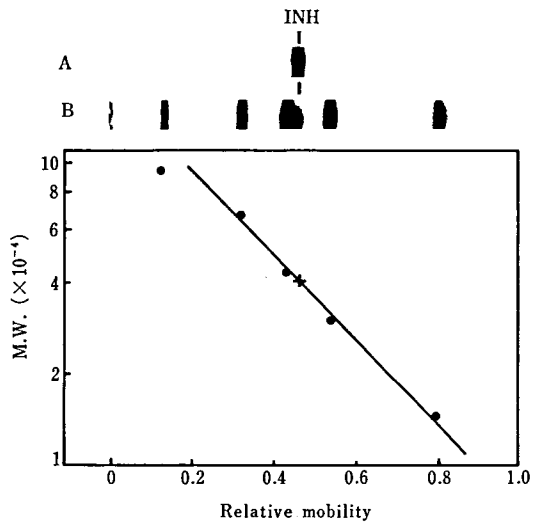
We reported on the activation of protein synthesis directed by poly(U) in the presence of protein factors in the 100,000×*g* supernatant of dystrophic chicken muscle in a previous paper[1,2]. To clarify the mechanism of activation of protein synthesis in dystrophic chicken muscle, we measured the amount of elongation factor 2 (EF2) in the 100,000×*g* supernatant. The EF2 content can be conveniently determined by measuring the amount of [^{14}C]ADP-ribosyl EF2 formed by diphtheria toxin catalysis because EF2 is the only protein which is known to be ribosylated by diphtheria toxin[3,4].

In some preparation we found that EF2 was not always ribosylated by diphtheria toxin and that in these samples a protein which inhibited the ADP-ribosylation of EF2 by this toxin (INH) was contained. To further identify this protein, purification and characterization of INH was performed. So far as we know, INH is not a substance which has previously been known.

We purified INH as follows: chicken breast muscle was homogenized with 2 volumes of buffer A (0.1 M KCl, 10 mM $Mg(OAc)_2$, 20 mM Tris-HCl (pH 7.4), 6 mM β-mercaptoethanol, and 0.25 M sucrose) in a Waring blender. The homogenate was centrifuged at 100,000×*g* for 15 min. The supernatant was centrifuged at 40,000 rpm for 4 hr in a Beckman 60Ti rotor. The supernatant (100,000×*g* sup) was fractionated by ammonium sulfate precipitation and the fraction between 50–75% saturation was collected. The pre-



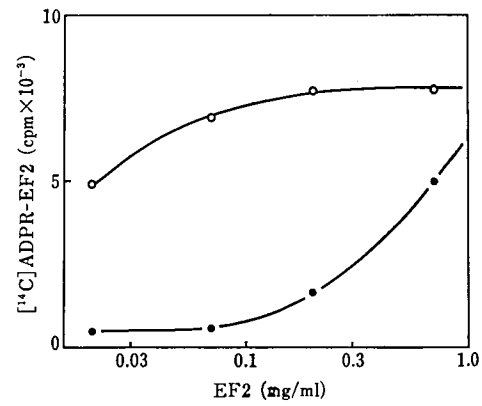


FIG. 1 (left). Molecular weight of INH determined by SDS-polyacrylamide gel (10%) electrophoresis by the method of Weber and Osborn[5]. A column: INH. B column: phosphorylase B; 94,000, BSA; 67,000, ovalbumin; 43,000, carbonic anhydrase; 30,000, lactoalbumin; 14,400, and INH.
FIG. 2 (right). Inhibition of formation of [^{14}C]ADP-ribosyl EF2 by INH. The reaction mixture in 0.1 ml contained 4 μmol DTT, 5 μmol Tris-HCl (pH 7.4), 0.1 mg BSA, 2 μmol histamine, 56 pmol [^{14}C]NAD (534 mCi/mmol), 3 μg diphtheria toxin, 40 μg INH, and various amounts of pig liver EF2. The activity of INH is decreased with the increasing amount of EF2.

cipitate was dissolved in buffer B (0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 6 mM β-mercaptoethanol, 1 mM EDTA, and 0.25 M sucrose) and applied on a Sephacryl S-200 column (2.6×95 cm). The peak which showed inhibiting activity was fractionated again by ammonium sulfate and the fraction between 66.5–75% saturation was collected. The precipitate was dissolved and dialyzed against buffer B and applied on a hydroxyapatite column (2×10 cm). The eluate between 0.1 and 0.2 M phosphate buffer was collected, to which ammonium sulfate was added to 60% saturation. INH was eluted with 10 mM Tris-HCl (pH 7.4).

To measure EF2 content, the reaction mixture contained 4 μmol DTT, 5 μmol Tris-HCl (pH 7.4), 0.1 mg BSA, 2 μmol histamine, 56 pmol [^{14}C]NAD (534 mCi/mmol), 3 μg diphtheria toxin, 21 μg pig liver EF2, and various amounts of INH or 100,000×g sup in 0.1 ml. Incubation was carried out at 37°C for 15 min. An aliquot of the reaction mixture was spotted on a Whatman 3MM filter paper disk and washed 3 times with cold 5% TCA and once with 99% ethanol. After drying the paper disk, [^{14}C]ADP-ribosyl EF2 was measured by a liquid scintillation counter.

The molecular weight of INH is 40,000 daltons as determined by SDS-polyacrylamide gel electrophoresis by the method of Weber and Osborn[5] (Fig. 1). INH is also present in liver as well as in muscle, however the amount seems to be less than that in muscle. SDS-gel electrophoresis shows that chicken embryonic breast muscle contains only a small amount of INH and the amount increases with the stage of development. This is confirmed by the fact that the rate of ADP-ribosylation of EF2 by diphtheria toxin in the 100,000×g sup from embryonic chicken is higher than in that from adult chicken. It is also probable that the content of EF2 in a chicken breast muscle at a younger stage of development might be larger than that of adult chickens because the inhibiting activity of INH is blocked by increasing amounts of EF2 (Fig. 2) and not by an increasing amount of diphtheria toxin or [^{14}C]NAD. INH has no ADP-ribosyl EF2 hydrolase activity. This suggests that the mechanism of inhibition by INH may involve binding at the specific site of EF2 where diphtheria toxin binds and INH shows competitive inhibition. In normal chicken breast muscle, the inhibiting activity of INH appears at 2 or 3 weeks after hatching, while in dystrophic chicken breast muscle it appears at 4 or 5 weeks after hatching. Ribosylation of EF2 in the 3-week old dystrophic 100,000×g sup is inhibited by addition of 3-week old normal 100,000×g sup which shows INH activity.

In future we must clarify the question of whether or not this phenomenon is due only to developmental delay of dystrophic muscle as shown by Nonaka *et al.*, Takeda *et al.*, and Obinata *et al.*[6–8].

With the exception of mono-ADP ribosylation, many reports about poly-ADP ribosylation have been published. Though the physiological significance of poly-ADP ribosylation is still unclear, some reports have suggested that poly-ADP ribosylation of histones is related to the differentiation of cells[9].

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Muscular Dystrophy Group, 1981

A TROPHIC INTERACTION IN DENERVATED MUSCLES FROM THE STANDPOINT OF RECOVERY OF Ca-UPTAKE ABILITY OF FSR

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In previous papers[1,2], we have reported a characteristics of Ca-uptake ability of fragmented sarcoplasmic reticulum (FSR) in M. pectoralis, fast posterior latismus (PLD) and anterior latismus dorsi (ALD) of dystrophic chicken and also have described a modification of the preparation method.

It is known that the M. soleus and M. extensor digitorum longues (EDL) of rat leg muscle are equivalent to the slow and fast muscle, respectively, which exactly correspond to ALD and PLD in chicken. Therefore, in the present experiment, we measured the Ca-uptake ability of fragmented sarcoplasmic reticulum from EDL and M.soleus in atrophy induced by denervation or in recovery by reinnervation. From the results obtained it was considered whether or not the changes of Ca-uptake ability of FSR from dystrophic muscle have an analogous connection with those from denervated muscle. Further, a procedure of denervation and reinnervation on nerves innervated to the EDL and soleus muscle were done at sites different distances from the muscle, *i.e.*, at the terminal branch to each muscle, (EDL-N. tibialis, M.soleus-N.fibralis) and at the N. ichiadic in which several sensory and motor nerves are involved. Using our modified method, the difference in Ca-uptake ability of FSR from denervated and reinnervated muscles was compared[3,4].

In the separation of FSR from the denervated muscle in the lower leg of rats, in contrast to that of dystrophic muscles no changes of pH in the process of homogenization or of normal muscle were done. The yield of H-fraction (8,500–36,500 $\times g$) and L-fraction (above 36,500 $\times g$) of FSR from muscle under denervation for 4 weeks was much the same as that from normal muscle. Therefore, we conjectured that in the SR of denervated muscle there were no membrane distractions which could be seen such as those in dystrophic muscle.

In the present experiments the maximum amount of Ca-uptake on FSR from the soleus muscle was about 180×10^{-9} mol/45 sec; from EDL, it was 240×10^{-9} mol/mg protein/30 sec. The amount of Ca-uptake on FSR from the soleus muscle was, therefore, lower than that from EDL. This lower value of Ca-uptake on

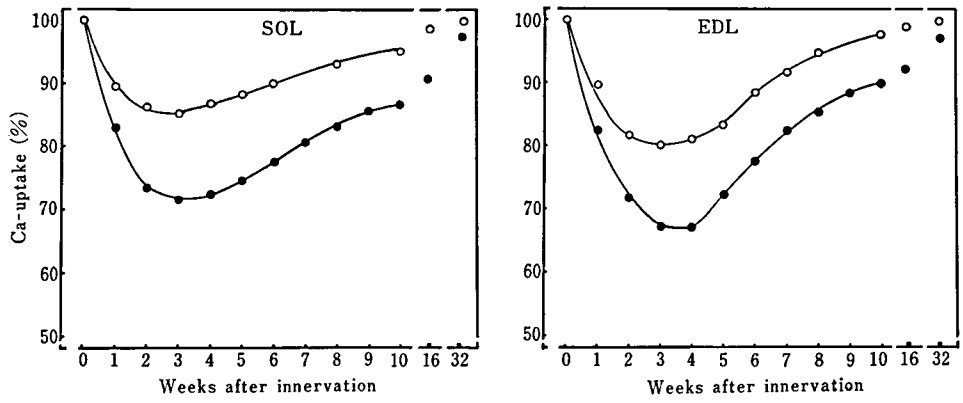

FIG. 1. Changes of Ca-uptake ability of FSR from EDL or soleus muscles by denervation of N.tibialis or N.fibralis (—○—) and N.ichiadics (—●—).

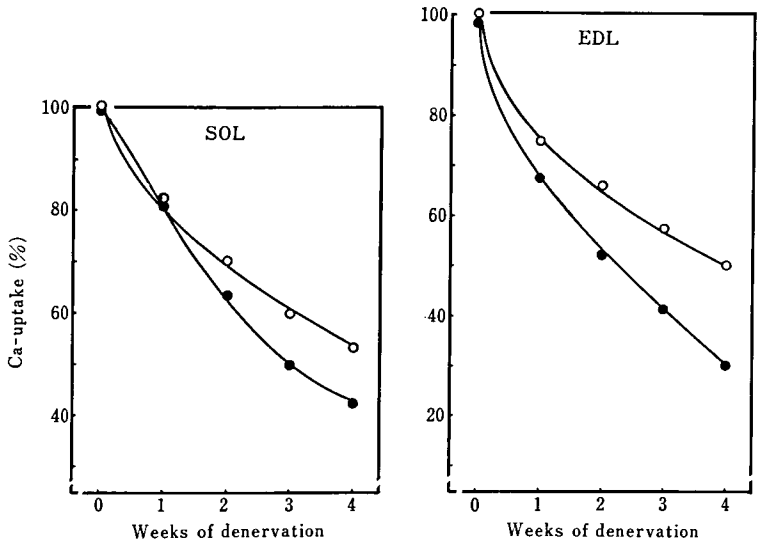

FIG. 2. Changes of Ca-uptake ability on FSR from EDL or soleus muscles by reinnervation of N.tibialis or N.fibralis (—○—) and N.ichiadics (—●—).

FSR from the soleus muscle could also be seen on FSR from ALD of the dystrophic chicken.

The degree of change in Ca-uptake ability in the denervation process was the same in FSR from EDL and from the soleus muscle, when only the terminal branch to each muscle was cut (Fig. 1). But when the ichiadic nerve was cut, the Ca-uptake ability of FSR from EDL decreased more markedly than that of FSR from the soleus muscle. Also, the Ca-uptake rate in the H-fraction and that in the L-fraction of FSR from PLD and M.pectoralis of dystrophic chicken was reduced more than in ALD. From these results, it was assumed that the function of Ca-uptake on FSR from fast type muscle was inhibited more than that from slow type muscles.

In order to observe a reinnervation effect, the changes of Ca-uptake ability of FSR from the reinnervated muscle were studied. Even if the terminal branch of the nerve to the soleus and EDL muscles or N. ichiadic was reinnervated soon after denervation, the Ca-uptake ability of FSR from both muscles was suppressed and was minimal 3 weeks after reinnervation (Fig. 2).

When the N.ichiadics were cut, suppression of Ca-uptake ability of FSR from both muscles, EDL and M.soleus, was more remarkable than that of the former experimental result mentioned. But the suppressed values of Ca-uptake ability on FSR as shown in Fig.2 increased slowly up ot that of normal muscles after showing the minimum at 3 weeks of reinnervation.

These results seem to suggest that the Ca-uptake ability of FSR recovered without regard to distance between cutting point of the nerve and muscles and the number of funicules in the nerve. In summary, we would suggest that trophic interaction plays a prominent role between the muscle and nerve on those muscles which were experimentally tested for procedure of denervation and innervation.

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CHANGES IN PROTEASE ACTIVITIES DURING DEVELOPMENT OF CHICKEN MUSCLES

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There have been few reports so far on the developmental changes in the levels of protease activities in muscular tissues. In order to gain some knowledge about such changes, we investigated the changes in the levels of certain protease activities during the early stage of development of chicken muscles.

Muscles were obtained from 14-day and 19-day (*i.e.*, 1-day before hatching) embryos and 7-day chickens of White Leghorn. Each muscle was homogenized in 5 volumes of 5 mM Hepes buffer, pH 7.5, containing 0.25 M sucrose and 10 mM 2-mercaptoethanol with a Potter-Elvehjem type glass-Teflon homogenizer. The homogenate was centrifuged at $10,000 \times g$ for 10 min and the supernatant was further centrifuged at $78,000 \times g$ for 60 min. The supernatant fraction thus obtained was used for protease assay. The precipitate (microsomal fraction) was washed with 1 M KCl to obtain 1 M KCl-insoluble microsomal fraction which was thought to contain firmly membrane-bound proteases, and these fractions were also used for protease assay. Cathepsin D activity was measured at pH 3.0 with hemoglobin as a substrate. Dipeptidylaminopeptidase IV activity was determined at pH 7.5 with glycyl-L-proline-4-methyl-coumaryl-7-amide as a substrate. Aminopeptidase activities were determined at pH 7.0 with 2-naphthylamides of L-alanine, L-leucine, and L-arginine as substrates.

The specific activities (activity/protein weight) of cathepsin D of the supernatant fractions of leg and breast muscles did not change significantly during the period examined, whereas those of heart and gizzard muscles showed a marked increase: those of the 7-day chickens were more than five times higher than the 14-day embryos. Similar but less marked increases in the activity (about 1.5–2-fold) were also observed for the microsome fractions of heart and gizzard muscles, and with the 1 M KCl-washed microsome fraction of gizzard muscle. The specific activity of the 1 M KCl-washed microsome fraction of heart muscle, however, decreased somewhat toward hatching and then maintained a constant level. On the other hand, the specific activities of the microsome and 1 M KCl-washed microsome fractions of leg and breast muscles showed a gradual decrease during development.

The specific activities of dipeptidylaminopeptidase IV of most muscle fractions showed a general tendency to increase toward hatching, and after hatching to keep a constant level or to gradually decrease. A notable increase (about 4-fold) in specific activity toward hatching was observed for the 1 M KCl-washed microsome fraction of gizzard muscle. This may reflect the fact that dipeptidylaminopeptidase IV is thought to be one of the microsomal membrane-bound enzymes.

The changes in the level of alanine aminopeptidase activity were roughly similar to those of dipeptidylaminopeptidase IV. In most cases the specific activity of the aminopeptidase increased toward hatching and after hatching it was almost constant or decreased gradually. Similar patterns of activity changes during development were also observed for leucine aminopeptidase and arginine aminopeptidase.

So far as examined, the specific activities of proteases in leg and breast muscles were very close and changed almost in parallel with each other, whereas those of heart and gizzard muscles were different and changed separately both from each other and from leg and breast muscles. This may reflect the difference in the type of muscles and suggests that there may be significant difference in protein catabolism in the early stage of development among these muscles. In order to obtain more conclusive results, however, further experimental data is necessary, including those on several other proteases, especially endopeptidases such as cathepsin B, calcium-activated neutral proteinase, alkaline proteinase, and membrane-bound neutral proteinase[1]. Changes in the level of protease activities in the later stage of development must also be

investigated. In the previous studies[2,3] the activities of various proteases were found to be markedly elevated in the breast muscle of dystrophic chickens. Therefore it will be also important to investigate the developmental changes in protease activities in the muscles of dystrophic chickens in comparison with normal ones.

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Muscular Dystrophy Group, 1981

ENDOGENOUS THIOL PROTEINASE INHIBITOR AND ITS CHANGES OF ACTIVITY IN SKELETAL MUSCLE OF DYSTROPHIC HAMSTERS

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Endogenous thiol proteinase inhibitor is found in various organs of many animals[1]. We have recently purified the inhibitor from the cytosol fraction of rat liver using affinity chromatography on papain-bound Sepharose[2]. Purified inhibitor is monomeric protein and has a molecular weight of 12,500. It inhibits various thiol proteinases (cathepsin B, H, L, C and papain) but not serine proteinases or carboxyl proteinase. Calcium dependent proteinase is a thiol proteinase but is not inhibited by this inhibitor. An immunodouble diffusion test using antibody against thiol proteinase inhibitor from rat liver showed that liver and muscle inhibitor are indistinguishable immunologically.

Activities of lysosomal thiol proteinases (cathepsin B and H) and of their endogenous inhibitor were assayed with thigh muscle of normal (F_1B) and dystrophic (BIO 14.6) hamsters. Muscle homogenate from 15 week old hamsters was centrifuged at 12,000×g for 20 min and the centrifuged precipitates were used for assay of the inhibitor. As shown in Fig. 1, a marked increase of cathepsin B (Z-Arg-Arg-βNA hydrolase) and cathepsin H+B (BZ-Arg-βNA hydrolase) were observed. But another lysosomal marker, acid phosphate, was not increased, suggesting specific increase of proteases in muscle lysosomes of dystrophic hamsters. Thiol proteinase inhibitor is also increased in muscle of dystrophic animals, but other cytosolic enzymes, aldolase and lactic dehydrogenase are rather decreased in dystrophic animals. It is unknown at present whether the increase of thiol protease inhibitor in muscles of dystrophic hamsters has any biological significance. No significant increase in thiol proteinase inhibitor in livers of dystrophic animals suggests that increase of the inhibitor is limited to target organs of dystrophy.

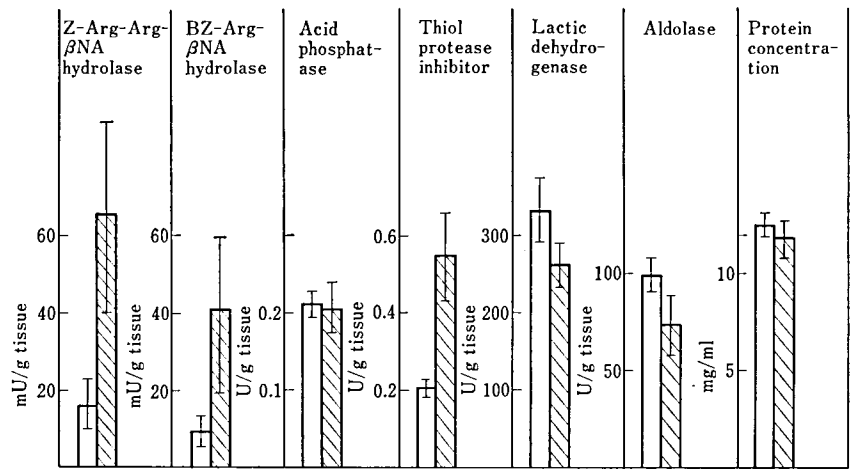

FIG. 1. Various enzyme activities in skeletal muscle of normal and dystrophic hamsters. Values are expressed as means±standard deviation of 10 hamsters. ☐; normal, ▨; dystrophic.

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Muscular Dystrophy Group, 1981

THE EFFECTS OF LOW MOLECULAR ENZYME INHIBITORS ON ENZYME NETWORKS IN VARIOUS ORGANS OF DYSTROPHIC MICE

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In previous papers we suggested the possibility that not only abnormalities in endopeptidases but also abnormalities in exopeptidases might play a role in pathogenesis of muscular dystrophy[1,2]. This prompted the trial of bestatin for treatment of mouse muscular dystrophy. It actually showed an effect in suppressing the occurrence of dystrophy[3]. We doubted whether or not this effect was due to the inhibition of elevated aminopeptidases in dystrophic muscle. It is also possible that bestatin which binds to cells induces a change in protease patterns in dystrophic muscle and has the effect of suppressing the progress of dystrophy. Thus we decided to compare the changes of enzyme activities in various organs of dystrophic mice after the administration of leupeptin[4], bestatin[5], and forphenicinol[6], which have different spectra of enzyme inhibiting actions *in vitro*[7].

Three-week-old dystrophic mice (C57BL/6J dy/dy) were obtained from the Central Institute for Experimental Animals, Japan. Mice were killed by cervical dislocation 3 hr after the final injection, and organ homogenates were prepared in phosphate-buffered saline (PBS) using an Ultra-turrax at maximum speed for 1 min. The homogenate was centrifuged (5,000 rpm for 20 min) and the supernatant fluid was withdrawn to measure enzyme activities. The inhibitors were dissolved in 0.2 ml of saline, and were given to experimental animals daily intraperitoneally for 8 days in the following doses: leupeptin 500 μg, bestatin 200 μg, and forphenicinol 500 μg. Control animals were given 0.2 ml saline daily for 8 days. The supernatants of organ homogenates were dispensed into test tubes (1.5×10 cm) with PBS containing the respective substrates. The test tubes were incubated for 1 hr at 37°C. Assays were done at pH 7.2[1,2].

Table 1 shows the enzymatic changes in the forelimb muscle of dystrophic mice induced by the low molecular inhibitors. As can be seen, leupeptin tended to induce the decrease of all aminopeptidase activities except fMet-AP. Bestatin and forphenicinol tended to decrease not only aminopeptidase activities but

TABLE 1. Enzymatic Activity Changes in Forelimb Muscle of Dystrophic Mice Induced by Low Molecular Inhibitors

Enzyme	Specific activity* ± SD			
	None	Leupeptin	Bestatin	Forphenicinol
AP-A	1.29± 0.22	0.95± 0.60	1.26± 0.22	1.28± 0.06
AP-B	10.28± 1.61	8.39± 2.29	9.22± 1.14	9.08± 1.42
Pro-AP	2.97± 1.75	2.42± 0.49	1.45± 0.43	2.54± 0.85
Leu-AP	8.13± 0.90	5.68± 3.45	7.67± 0.74	7.58± 0.12
fMet-AP	5.33± 0.40	6.34± 1.41	5.17± 0.67	4.60± 0.07
Phe-AP	13.17± 0.91	10.87± 5.32	10.93± 2.31	13.59± 0.58
Gly-Pro-Leu-AP	1.00± 0.02	0.94± 0.48	0.97± 0.18	1.03± 0.15
Trypsin-like	86.62± 5.20	102.07±47.93	54.42±21.40	55.11±13.43
Chy-try-like	100.97±17.47	134.26±25.32	63.53±23.28	90.06± 5.96
Elastase-like	56.35± 7.78	48.74±18.50	45.76±18.82	54.11± 7.22
Cathepsin C	1.19± 1.01	2.37± 0.49	0.99± 0.19	1.38± 0.11
α-D-Glucosidase	1.04± 0.42	0.74± 0.16	0.40± 0.04	0.43± 0.00
α-D-Mannosidase	0.16± 0.28	0.31± 0.28	0.14± 0.03	0.19± 0.05
Glc-NH_2ase**	2.67± 0.91	2.22± 1.07	1.22± 0.25	1.13± 0.14
CPK	22.51± 4.76	19.75±10.43	16.05± 2.74	19.27± 3.48
Phosphatase	6.27± 1.58	1.67± 1.23	1.46± 0.20	1.42± 0.19
Esterase	206.20±79.30	67.62±16.80	62.57± 2.85	57.31± 4.81

*n mol/min/mg protein. **N-acetyl-β-D-glucosaminidase.

TABLE 2. Correlations Among Enzymatic Changes Induced by Low Molecular Weight Inhibitors in Various Organs

Organs	x / y	Leupeptin		Bestatin
		Bestatin	Forphenicinol	Forphenicinol
Muscle				
Forelimb		0.12	0.05	0.69**
Hindlimb		0.69**	0.55*	0.31
Heart		0.66**	0.22	0.43(*)
Spleen		0.71**	0.81***	0.75***
Liver		−0.04	0.49(*)	0.13
Kidney		0.37	0.59*	0.21

(*): $P<0.1$, *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$.

Spearman's rank correlations (r_s values) were calculated by the equations below.

$$r_s=1-\frac{6\Sigma(Tix-Tiy)^2}{n^3-n} \quad n=16,\ i=1,2,3.....16$$

also the activities of endopeptidases and glycosidases. Similar studies were done on 5 other kinds of organs such as hindlimb muscle, heart, spleen, liver, and kidney. The degree of enzymatic changes in 6 organs are expressed as Student's *t*-values. As judged from these *t*-values, the inhibitors significantly affected many enzyme activities in all 6 organs tested. However, there were differences among the response patterns of the various organs. In order to quantitatively compare the enzymatic response patterns among the 3 inhibitors and the 6 organs, Spearman's rank correlations (r_s values) were adopted. Each of the *t*-values for the 16 enzymatic activities was given a rank according to its size. The series of ranks were evaluated for the degree of correlation among various mouse groups which were given the inhibitors. The obtained r_s values are shown in Table 2. There were high correlations among the effects of the 3 inhibitors in several organs.

The *in vivo* effects of enzyme inhibitors revealed in the present study were hardly imaginable from the viewpoint of *in vitro* actions of enzyme inhibitors. First, the movements of the various enzyme activities were seemingly independent of the purely biochemical actions of the inhibitors. Second, the responses of the various organs were not uniform; even between the muscular tissues of forelimb and hindlimb there were discrepancies in enzymatic responses. Third, there were frequent correlations among the response patterns of organs to different enzyme inhibitors. Significant changes of enzymatic activity in muscle and various organs induced by leupeptin, bestatin, and forphenicinol suggest that the therapeutic effect of leupeptin and bestatin on mouse muscular dystrophy may not be due to the inhibition of enzymes sensitive to these inhibitors but due to their indirect effects[3,8,9]. It moreover suggests that forphenicinol which binds to cell surfaces may be a compound worthy of further study against muscle dystrophy.

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Muscular Dystrophy Group, 1981

THE ROLE OF PROTEASE IN THE DEVELOPMENT AND DIFFERENTIATION OF MUSCLES

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Some proteases are thought to be involved in the development and differentiation of muscles, especially in the turnover of muscle proteins and the activation of protein kinases. As a candidate of such a protease we selected calcium activated neutral protease (CANP) and purified it[1]. However, since this CANP required an unphysiologically high concentration of Ca^{2+} it may not operate under physiological conditions. Recently another CANP which required micro-molar Ca^{2+} (μ-CANP) was found[2] besides the one which necessitates milli-molar Ca^{2+} (m-CANP). Thus we have undertaken work to clarify the relation between these two CANPs and have learned that μ-CANP is derived from m-CANP by its autolysis. This conversion may trigger some important step in the differentiation of muscle cells. Another important regulatory system of CANP activity would be the endogenous inhibitor. Thus we purified and characterized it in detail. This report will deal with the conversion of CANP and the characterization of the inhibitor.

Results

The conversion of m-CANP to μ-CANP was found by chance[3]. We tried to purify CANP by affinity chromatography. Partially purified m-CANP of chicken muscle[1] was applied to a column of casein-Sepharose in the presence of 20 mM Ca^{2+}. After washing out the unabsorbed material CANP was eluted with a solution containing 10 mM EGTA. The eluted CANP was highly sensitive to Ca^{2+} with Kd 30 μM while the original CANP was far less sensitive to Ca^{2+} with Kd 0.8 mM. Thus we called the former and the latter μ-CANP and m-CANP, respectively. The molecular weights of m- and μ-CANPs were identified as 82K and 79K, respectively, as judged by SDS-gel electrophoresis. This suggested that μ-CANP was derived by the autolysis of m-CANP in the column where enough Ca^{2+} existed to activate m-CANP.

In order to prove this suggestion we conducted the following experiments[4]. Purified m-CANP was incubated with 10 mM Ca^{2+} at 0°C. At intervals an ample amount of the incubation mixture was withdrawn and assayed in the presence of 50 μM Ca^{2+}. In parallel, a part of the sample was analyzed on SDS-gel electrophoresis. The activity of μ-CANP appeared very rapidly, reached the maximum at 3 min and then gradually decreased. In the sample taken at 3 min, the 82K band was replaced by the 79K band as judged by SDS-gel electrophoresis. Afterwards the 60K band also appeared and they were finally reduced to the 30–33K band. We have separated components of 79K, 60K, and 30K from the digestion mixture. The component with the molecular weight of 79K gave the same Ca^{2+} sensitivity as that of μ-CANP obtained by affinity chromatography. The component of 60K had properties similar to the 79K component, while the 30K component was totally inactive. These results indicated that μ-CANP is derived from m-CANP through autolysis of the latter.

It is interesting that the autolysis m-CANP stops at the stage of 79K and no further degradation takes place when it is carried out in affinity chromatography. This may suggest that in the presence of the substrate, as in that cellular cytoplasm, the conversion to μ-CANP will proceed in a proper way.

In order to clarify another regulatory mechanism of CANP activity we purified its endogenous inhibitor[5]. From the extracts of the muscle cell CANP was precipitated at its isoelectric pH. Through several purification steps, including DEAE-cellulose, QAE-Sephadex chromatography, isoelectric focusing, and phenyl Sepharose chromatography we succeeded in purification of the endogenous inhibitor in homogenity. The molecular weight was estimated as 70K from gel filtration but 35K from SDS-gel electrophoresis. Thus

the inhibitor was identified as a dimeric protein, judged by its susceptibility to several hydrolases. When CANP activity was assayed in the presence of several amount of the inhibitor it was clarified that each subunit of the inhibitor inhibits one molecule of CANP by forming a tight complex. The inhibitor was effective against both m- and μ-CANPs but the complex formation took place only when CANPs were activated by Ca^{2+}. The inhibitor was ineffective against any other proteases so far tested.

Although it has not yet been clarified how CANP is involved in the differentiation of muscle cell, the present results do provide a breakthrough for the problem. Under the physiological condition m-CANP would behave as a precursor of μ-CANP. When it is necessary transient or local influx of Ca^{2+} ions will take place and the conversion of m-CANP to μ-CANP will be triggered. The resulting μ-CANP would fulfill its physiological role but its unfavorable action may be prevented by the endogenous inhibitor. On the other hand, when an uncontrolled influx of Ca^{2+} takes place m-CANP will digest several muscle proteins and result in a disease such as muscular dystrophy.

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THE EFFECT OF PROTEASE INHIBITOR ON THE CALCIUM-INDUCED DEGENERATION OF MYOFILAMENT *IN VITRO* AND *IN VIVO*

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A non-lysosomal calcium-activated neutral protease (CANP) is suggested to be involved in muscle protein turnover, but its intracellular role is still obscure. To clarify the role of CANP *in vivo*, we employed a new technique of muscle incubation with calcium *in vitro*. Calcium ions were introduced into intact rat soleus muscle by A23187 and the effect of the protease inhibitor was studied morphologically and biochemically.

Intact rat muscle was removed with tendons and incubated in a solution containing 4 ml of Krebs-Ringer buffer, pH 7.4, and cycloheximide. When calcium ions were involved, Z-line loss and concomitant release of α-actinin into the medium were observed. The released α-actinin was determined by SDS-gel electrophoresis. A thiol protease inhibitor, E-64-c, added exogenously into the incubation mixture, suppressed the calcium-induced release of α-actinin by 45% (Table 1).

TABLE 1. The Release of α-Actinin from Intact Rat Soleus Muscle

Experiments	Additions	Released α-actinin (g/3 hr)
Control	EGTA	9.1 ± 2.0 ($n=5$)
	$CaCl_2$	55.3 ± 5.2 ($n=5$)
	E-64-c, $CaCl_2$	33.1 ± 7.4 ($n=5$)
E-64-c injected	EGTA	12.1 ± 3.2 ($n=5$)
	$CaCl_2$	56.1 ± 5.5 ($n=5$)

Normal male rats (100 g) were sacrificed by pentobarbital overdose administration. The paired soleus muscles were carefully removed with tendons intact and weighed. Muscles were immediately incubated in Krebs-Ringer buffer (4 ml) containing 25 μg/ml cycloheximide under 95% O_2-5% CO_2 at 37°C. A23187 was dissolved in DMSO (final concentration, 0.5%). The control was treated as mentioned above except that Krebs-Ringer buffer was prepared without calcium but contained 0.1 mM EGTA. After 3 hr incubation, aliquots of the medium were subjected to SDS-gel electrophoresis. The released α-actinin, identified by immunoreplica, was determined spectrophotometrically. In separate groups of rats, E-64-c was injected subcutaneously (10 mg/kg) in a volume of 0.2 ml of saline. Animals were sacrificed after 24 hr and the soleus muscle was rapidly removed as described above. Calcium-induced release of α-actinin was determined in the same manner. Intramuscular distribution of [^{3}H] E-64-c was determined by preparing three fractions, myofilaments, mitochondrial, and lysosomal fractions (ML) and supernatant. Almost 70% of the injected E-64-c was localized in the cytosol, not in the lysosome.

However, subcutaneous injection of E-64-c (10 mg/kg, 24 hr later) had no effect on the calcium-induced Z-line loss (see Table 1). The intracellular concentration of injected [^{3}H] E-64-c was also studied. The radioactivity in the postmitochondrial supernatant indicated that approximately 0.23 μg of E-64-c was taken up into 1 g of muscle. Therefore, we concluded that CANP was not inhibited by E-64-c in injected muscle because of the low content of E-64-c in the muscle cell.

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