CURRENT RESEARCH IN MUSCULAR DYSTROPHY, JAPAN (BIOMEDICAL RESEARCHES)

Vol. 2

The Proceedings of the
Annual Meeting of Muscular
Dystrophy Research Group,
1980, Tokyo

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LATENT MUSCULAR DYSTROPHY IN GENOTYPICALLY DYSTROPHIC-DWARF MICE

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We have proposed a working hypothesis for the development of murine muscular dystrophy, as summarized in Fig. 1. This is based on the following experimental results. In muscular dystrophic mice (dy/dy; strain C57BL/6J-dy), the forelegs seemed to function almost normally even at 3 months of age, whereas the hindlegs showed characteristic signs of the disease at an early age (about 2 weeks), suggesting that comparative studies on the fore- and hindleg muscles might yield clues clarifying the mechanism involved in the development of murine muscular dystrophy. However, the forelegs of dystrophic mice were also found to be affected by the disease at least by 1 month of age. Thus, the endurance of the forelegs was much less than that in normal mice[1]. Moreover, it was found that the foreleg muscles, as well as the hindleg muscles[2,3], were abnormal in collagen content[4], in the level of protease activities[5], and in the histological images (unpublished results from our laboratory). Also, the growth of the fore- and hindleg muscles was arrested at about 1 month of age. From a biochemical study of the hindleg muscles, the drastic increase in the apparent volume per muscle cell which occurs on and after 10 days of age in normal development was not observable in dystrophic mice[6]. Findings thus far showed that there was no remarkable difference to explain the variation in the degree of apparent severity of the fore- and hindleg involvement in any feature of the muscles but rather the difference was in the growth of the bones[7]. The growth (lengthening) of the foreleg bones was much smaller and slower than that of the hindleg bones during the period from 10 to 20 days of age in muscular dystrophic mice as well as in normal mice. It is noteworthy that the onset of the disease observable in the hindlegs (about 14 days of age) occurred in this period.

According to our working hypothesis, the clinical signs of the disease must be alleviated in dystrophic mice in which growth, including bone-lengthening, was arrested. To investigate this, a few effective methods are available at present, such as hypophysectomy and genetic methods. Genes bm (brachymorphic), bp

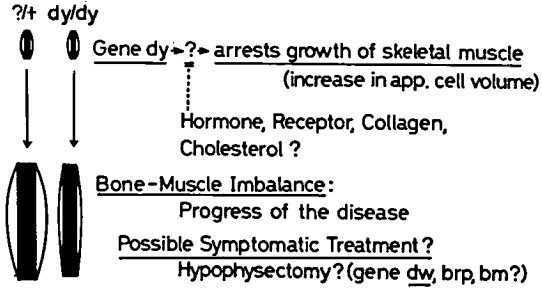

Fig. 1

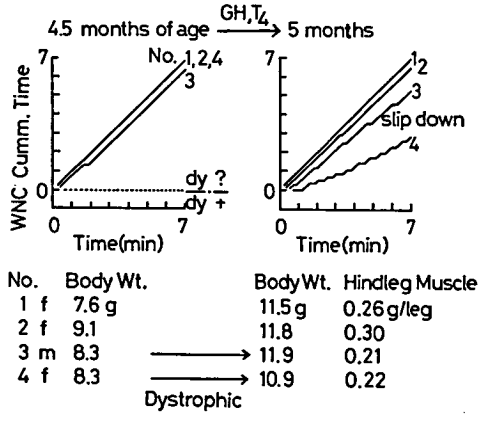

Fig. 2

Fig. 1. A working hypothesis for the development of murine muscular dystrophy.
Fig. 2. Latent muscular dystrophy in genotypically dystrophic-dwarf mice. The genotypically dystrophic-dwarf mice (Nos. 3 and 4) were not dystrophic till they were treated with GH and T_4. f, m: female, male, respectively.

(brachypod), brp (another brachypod), and dw (pituitary dwarf) may be suitable for the purpose. We have already succeeded in producing genotypically dystrophic-brachymorphic (dy/dy•bm/bm), dystrophic-brachypod (dy/dy•brp/brp), and dystrophic-dwarf (dy/dy•dw/dw) mice. In the present study, results for dystrophic-dwarf mice are reported. In order to obtain dystrophic-dwarf mice, crosses were performed between F1 hybrid carriers (dy/+•dw/+), which were produced by crossing C57BL/6J-dystrophic carriers (dy/+•+/+) with DW/J-dwarf[8] carriers (+/+•dw/+; purchased from the Jackson Laboratory). In the F2 hybrid mice, phenotypically normal, dystrophic, and dwarf mice were observed but not the dystrophic-dwarf type. All the mice tested in our wire-net-climbing (WNC) apparatus[9], except for the dystrophic ones which continually dragged their hindlegs after about 3 months of age, showed almost normal locomotive activities even at 4.5 months of age (Fig. 2). Some of the phenotypically dwarf mice, however, began to manifest an abnormal sign in their eyes with age, which is a characteristic of murine muscular dystrophy. From this and the theoretical expectation (*i.e.*, one-sixteenth of the F2 hybrid mice should be genotypically dystrophic-dwarf mice), it was suggested that the dystrophic-dwarf mice were latent in the phenotypically dwarf ones. In fact, this was demonstrated by the following experiments.

F2 hybrid dwarf mice were injected intraperitoneally with bovine growth hormone (GH) and thyroxine (T_4). As the phenotypically dwarf mice grew with the GH-T_4-treatment (100 μg GH and 10 μg T_4/day[10]), every other day for about 15 days), some of them began to exhibit the characteristic clinical signs of muscular dystrophy (sometimes dragging their hindlegs and revealing depressed activity in the WNC apparatus). Thus, genotypically dystrophic-dwarf mice could be identified. These dystrophic-dwarf mice had less muscle in their hindlegs than the nondystrophic dwarf mice which had been raised with GH-T_4-treatment. Five dystrophic-dwarf mice have been observed thus far.

From the present findings, it was concluded that muscular dystrophy was latent in genotypically dystrophic-dwarf (dy/dy•dw/dw) mice in which growth had been arrested by the gene dw. This suggests that hypophysectomy may be an effective symptomatic treatment for murine muscular dystrophy.

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AUTORADIOGRAPHIC STUDIES ON THE REGENERATION OF TRANSPLANTED MUSCLES

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Our studies aim at an analysis of the regeneration of human dystrophied muscles heterotransplanted into nude mice. Previous studies revealed that regenerated muscles of dystrophied muscle origin and control muscle origin are similar under light microscopy in their staining characteristics, their structural features, such as the location of the nucleus, and their histochemical characteristics.

In order to elucidate the mechanism of the regeneration of dystrophied muscles in nude mice, we have been trying to analyze the ability of the satellite cells, which are known to be the origin of regenerated muscles. The present report describes the development of an autoradiographic technique to analyze the ability of the satellite cells during muscle regeneration.

Muscles from normal ddy mice were used. The M. extensor digitorum longus (EDL) from ddy mice were homotransplanted into other ddy mice. Two days and 7 days after transplantation, 5 μCi/g body weight of ^{3}H-thymidine was injected intraperitoneally. Six hours later, the mice were sacrificed and transplanted muscles were subjected to autoradiography.

Two days after transplantation, no uptake of ^{3}H-thymidine was observed. Seven days after transplantation, ample uptake of ^{3}H-thymidine was observed. At this time, the grafted muscles were able to be separated into three zones: the outer, middle, and inner zones.

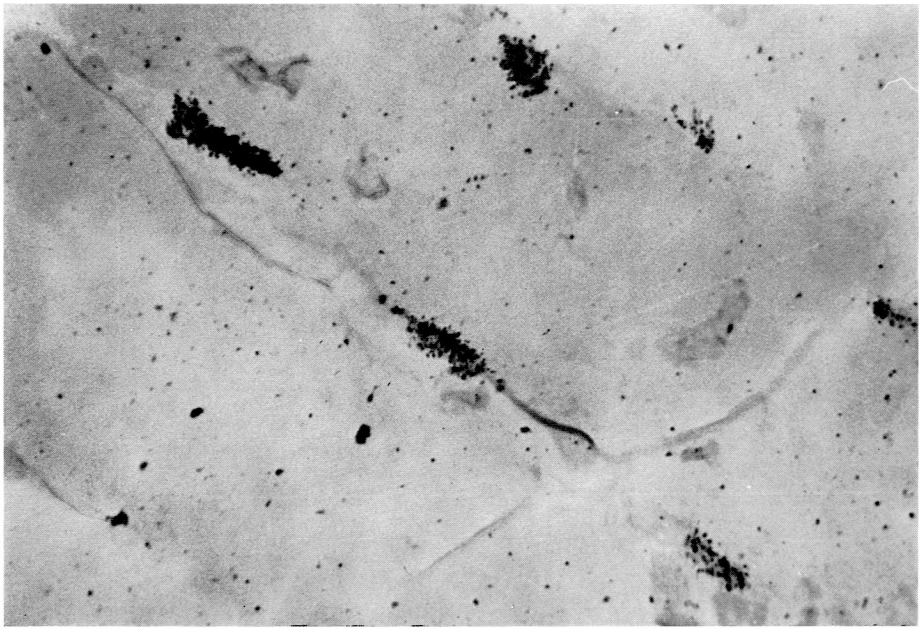

Fig. 1. ^{3}H-thymidine uptake of EDL 7 days after transplantation.

In the outer zone, many nuclei taking up ^{3}H-thymidine were observed. In this zone, many newly regenerated myotubes were also seen. The nuclei of those myotubes, however, did not take up ^{3}H-thymidine, which indicated that those nuclei at this period have no tendency to divide.

The middle zone contained many myofibrils whose nuclei sometimes took up ^{3}H-thymidine. These are myonuclei or satellite cells which are difficult to differentiate under light microscopy.

The inner zone consisted of degenerated muscles which had no ^{3}H-thymidine uptake.

We are planning to analyze the difference in ^{3}H-thymidine uptake in satellite cells between dystrophied and control muscles heterotransplanted into nude mice.

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MUSCLE TRANSPLANTATION IN THE ETIOLOGICAL ELUCIDATION OF AVIAN MUSCULAR DYSTROPHY

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We have previously developed a technique for transplanting an entire muscle without pretreatment from one animal to another[1]. In the present study we applied this technique to avian muscular dystrophy to examine the question of whether dystrophic disorder originates in muscle or nerve tissues, with special reference to neural influence on muscle growth. The extensor carpi radialis longus (ECRL) muscle was chosen. This choice involved a variety of factors which should be noted: This muscle is small so that its reinnervation can be established within several days[1], provided that the rate of axon regeneration has a velocity of about 1 mm/day[2]. The small size of the muscle further made the transplantation of an entire muscle possible without mincing[1], which offers an experimental system that can be analyzed more quantitatively. This surgical procedure gave no regeneration at all when the ECRL muscle was simply removed without being replaced by a transplant (a control for the zero-transplant), although the host muscle "bud" regenerated together with the donor muscle in the minced graft experiment using pectoral muscle[3]. The relatively isolated location of the ECRL muscle facilitated the avoidance of injuries to the transplanted and surrounding muscles during surgery, and consequently the regenerated muscles could be clearly distinguished from the other muscles.

Normal (line 412) and dystrophic (line 413) chickens were used. Two to 3 days *ex ovo*, chickens were anesthetized with ether, and the ECRL muscles were cross-transplanted between two chickens. Four types of transplantation were carried out: normal muscle transplanted into normal or dystrophic chicken (Nn or Dn) and dystrophic muscle transplanted into normal or dystrophic chicken (Nd or Dd). The details of the surgical procedure have been described in a previous report[4]. Animals were kept in an aseptic condition throughout the operation and were not given any antibiotic. The transplanted muscle was allowed to regenerate and was examined 60–65 days after transplantation when tissue reconstitution was virtually complete. Reinnervation of the transplanted muscles was confirmed by observing a muscle contraction induced by nerve stimulation or by histochemical staining of acetylcholine esterase[5].

Table 1. Cross-transplantation of ECRL muscles in normal and dystrophic chickens

Type of transplantation	Number of operation	Survival rate of muscle 60–65 days after operation (%)	Muscle weight for successful transplant (mg)
Nn	19	58	444±99.8
			$n=11$
Nd	25	60	344±72.9
			$n=15$
Dn	25	36	241±81.0
			$n=9$
Dd	19	37	235±62.7
			$n=7$
			Mean±S.E.

N or D: normal host or dystrophic host; n or d: normal muscle or dystrophic muscle.

The survival rates of the transplanted muscles were 58, 60, 36, and 37% for Nn, Nd, Dn, and Dd, respectively (Table 1). The dystrophic hosts had worse conditions than the normal hosts for muscle survival, but the type of muscle, whether normal or dystrophic, did not influence the survival rate.

Since not all of the four types of transplants survived 60–65 days after the operation, the muscle weight was compared among the surviving muscles in the four types of transplantation, and thus contamination by a possible rejecting response was avoided. The results are summarized in Table 1.

Despite there being no statistically significant difference in the muscle weight of non-operated ECRL muscles between normal and dystrophic chickens, 1,040±28.8 mg (mean±S.E., n=21) and 1,104±36.8 mg (n=33), respectively, the normal muscle transplanted into the normal host (Nn) attained a slightly larger muscle weight than the dystrophic muscle transplanted into the dystrophic host (Dd). However, the statistical significance level was $P<0.2$ (Table 1).

The growth in weight of the dystrophic ECRL muscle transplanted into the normal host (Nd) was as good as that of the normal ECRL muscle transplanted into the normal host (Nn). On the other hand, both the normal ECRL muscle transplanted into the dystrophic host (Dn) and the dystrophic ECRL muscle transplanted into the dystrophic host (Dd) were hampered in growth. These results strongly suggest an important role of extramuscular factor(s) in muscle growth, and that the factor(s) may be defective in dystrophic chickens. The present results are consistent with those obtained in murine muscular dystrophy[6], but not with those obtained in avian muscular dystrophy using minced grafts of pectoral muscles[3]. These experiments are different at least in the three following respects: kinds of muscles and nerves used, sizes of the muscles, and pretreatment of the transplanted muscles.

Table 1 includes the results of transplantation of normal muscle into the normal host (Nn) and dystrophic muscle into the dystrophic host (Dd). Therefore, even if some problems in histocompatibility existed, these effects should be annulled by comparing Nd and Dn with Nn and Dd controls. The results showed that the effect of the hosts is more important than the effect of the donors and that dystrophic muscles have an ability to respond to the host-side influence (*cf.* refs. 3, 7).

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THE ECTOPIC DIFFERENTIATION OF STRIATED MUSCLES IN RAT PINEAL BODY CELL CULTURE

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Mammalian pineal bodies are an endocrine organ. Their ancestral origin as a visual organ, however, still seems to be evidenced by the presence of structures suggesting a photoreceptor function during fetal stages. Thus, it seemed interesting to us to examine the possibility of multipotentiality in cell differentiation of pineal cells in cell culture. Most unexpectedly, the differentiation of well-developed myofibers occurred consistently in such cultures[1].

Pineal bodies were taken from newborn rats. Covering tissues were carefully removed under a dissection microscope and very clean fragments consisting of homogeneous pineal cells were dissociated by treatment with trypsin and collagenase. Cells were inoculated onto Falcon plastic culture dishes using Eagle's MEM supplemented with 6% fetal calf serum as a culture medium. Culture plates were incubated at 37°C under 5% CO_2-95% air for about 20 days. The cellular changes occurring within this period were observed daily.

By 2–3 days after inoculation two types of cells were recognized: (1) flattened cells attached directly to the culture substrate and (2) smaller round cells which were not directly attached to the substrate but were superimposed on top of the cells of the first type. Flattened cells were provided with piliform processes, the shape of which changed constantly.

By about 2 weeks, the differentiation of two other distinct cell types took place. One was neuronal cells which were provided with elongated axon-like processes. These cells were often assembled or interconnected with each other by the processes. The neuronal cells possibly originated from round cells. The other type was striated muscles which, according to daily observations of living cultures, seemed to be derived from the cells directly attached to the culture substrate (Fig. A). Muscle fibers were assembled into bundles and contracted, though irregularly, in response to external mechanical stimuli (Fig. B).

This muscle fiber differentiation occurred in all different inocula so far cultured. On each culture plate, several muscle cell foci were formed. Thus, it seems to be very unlikely that muscle differentiation is due to an inadvertent inclusion of muscle cell tissues in the original inocula. We assume that the pineal body contains either myoblasts or multipotential cells which can differentiate into both endocrine and muscle cell directions.

Indeed, there have been several reports indicating muscle fibers in *in situ* pineal bodies in some vertebrate species. Another example of muscle differentiation from unexpected origin is in mammalian thymus cultures[2]. To sum up the situation, the present finding of muscle differentiation in pineal body cultures is not a sporadic, but rather, a consistent event which may occur in the course of normal development of this organ *in situ*. The developmental origin of this differentiation is not yet known. The physiological meaning of the ectopic occurrence of muscle differentiation remains to be solved.

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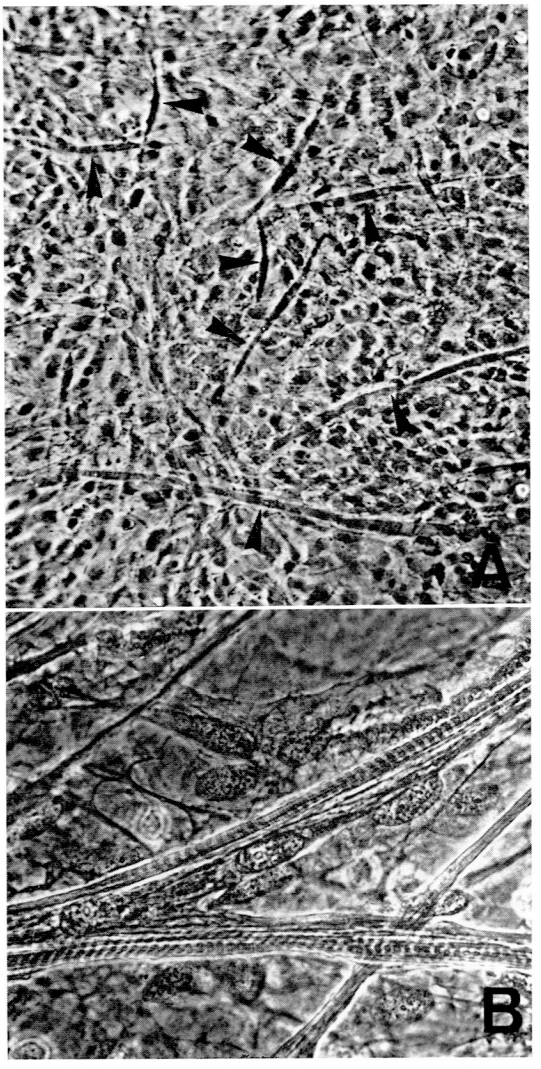

A: an early step in the muscle differentiation in cultures of pineal cells after 2 weeks' cultivation *in vitro*. Arrows indicate early myotubes with several nuclei each. A microphotograph of a living culture with a phase contrast microscope. *ca.* ×250.

B: the differentiation of well-striated muscle fibers in a 4-week culture of pineal cells. A microphotograph of a fixed culture with a phase contrast microscope. *ca.* ×500.

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TISSUE CULTURE STUDIES OF DYSTROPHIC MUSCLES

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For observation of the sequences of development of disease processes, tissue culture is one advantageous and preferable technique. Muscle fibers from Duchenne-type muscular dystrophy, and from a carrier and healthy individuals were introduced into an *in vitro* system and dystrophic alterations were studied. A quantitative analysis of the proliferation activity of myogenic cells was also performed using cell culture techniques.

Experiment I. Development of dystrophic changes *in vitro*.

Materials and Methods: Fragments of muscle tissue obtained from 6 patients with Duchenne dystrophy, 1 carrier, and healthy individuals with matched ages were cultivated on collagen-coated coverslips. One to 3 days after explantation cross-sections of spinal cord from fetal mice or rats, 14 or 15 days *in utero*, were added to the cultures and maintained with Maximow's double-coverslip method. Feeding media were composed of equal parts of Gey's BSS, Eagle's MEM, horse serum, chick embryo extract, and glucose to produce a final concentration of 600 mg%.

Results: When the muscle fibers became attached to the growing motor axon from the spinal cord, rapid deterioration of the muscle occurred. Spindle-shaped myoblasts may grow around the explants. When the muscle fibers were not innervated, the original cross-striations remained unchanged and myoblastic proliferation was markedly retarded. Around 2 weeks or so, outgrowing spindle cells began to fuse with each other. Longitudinal fibrillar structures were often observed. Around 4 weeks some of the fused myotubes developed cross-striation. Throughout the experimental course twitching was not seen in either striated or nonstriated multinucleated cells. In these myotubes, vacuolation was often observed. In Duchenne muscle fibers, as compared to healthy muscle, myotube formation seemed to be disturbed and vacuolar changes were more frequently observed. Some of the fibers developed segmental granular changes in the sarcoplasm which is identical to segmental necrosis. These alterations, vacuolation of the sarcoplasm and segmental granular degeneration, were interpreted as dystrophic changes *in vitro*.

Experiment II. Proliferative activity of dystrophic muscle cells *in vitro*.

Method: Muscle fragments from Duchenne dystrophy and a nonclinical carrier sibling were transferred to plastic flasks and Petri dishes, and maintained in a CO_2 incubator. The feeding medium was composed of 85% L15 and 15% fetal bovine serum. Subcultivation was carried out four times as shown in the following table.

Trypsinization
↓
Wash and centrifuge
↓
Filter through lens paper
↓
Incubation in feeding medium, 40 min.
↓
Collection of floating cells
↓
Cultivation in CO_2 incubator

5×10^5 cells were subcultured in each time.

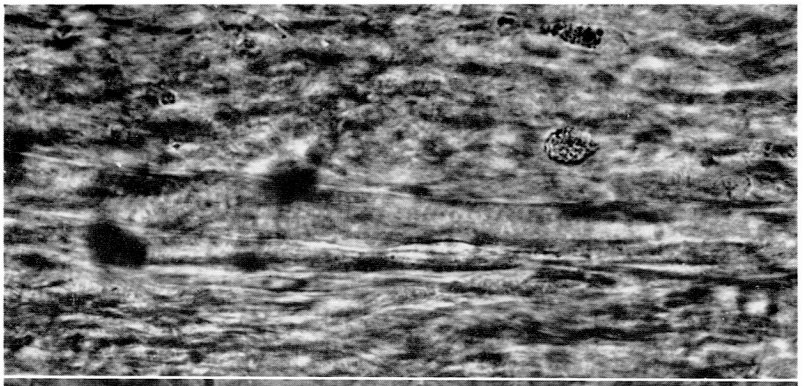

Fig. 1

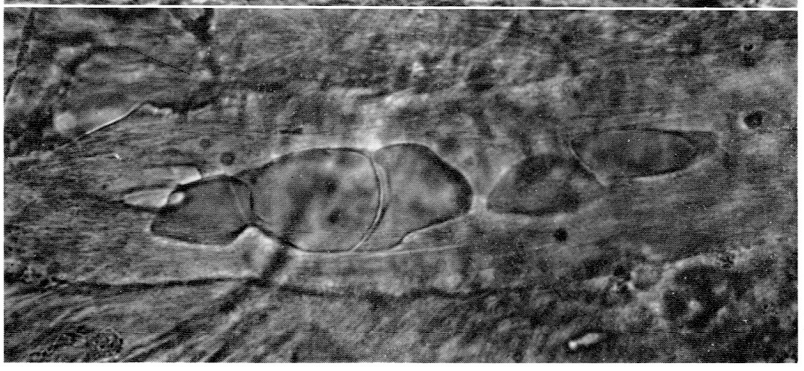

Fig. 2

Fig. 1. Cross-striation of living dystrophic muscle grown *in vitro*.
Fig. 2. Vacuolization of living dystrophic muscle.

Results: In the primary culture, outgrowth was visible on the 4th day and myotubes with more than 10 nuclei were seen after the 17th day in both dystrophic and carrier groups. In the first subcultures myoblastic cells in the carrier group were 2.5 times more numerous than in dystrophic cultures. No difference was seen in the activities of myotube formation between carrier and dystrophic muscle cultures. That is, myotubes with more than 10 nuclei were first observed on the 17th day after explantation in both groups. However, in the following subcultivation, dystrophic muscle produced a lower activity of myoblastic proliferation, and consequently retarded myotube formation. These differences were more emphasized in the succeeding subcultures.

Comments: Though maturation and differentiation of human muscle *in vitro* are at present incomplete, several differences in the developmental processes among Duchenne-type dystrophy, carrier, and control healthy muscle were obtained. First, regenerative activity was less prominent in Duchenne dystrophy. This might be a direct expression of a gene defect in this disease. Morphological features, such as vacuolation of the sarcoplasm and segmental degeneration, observed in dystrophic muscle were closely simulated to the findings in dystrophic chick muscles found in both *in vivo* and *in vitro* systems. By improving the culture techniques for human muscle, more details of alterations can be analyzed.

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

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The mitotic activities of mononucleate cells, cultured *in vitro*, originated from regenerating muscles from either normal or dystrophic mice were studied.

Adult normal (C57Bl/6 +/+) and dystrophics (C57Bl/6J dy/dy) mice were used throughout. The procedures for the preparation of regenerating muscles and the liberation of mononucleate cells from either source were described previously by Kagawa *et al.*[1-3]. In our standard clonal cultures (Experiments 1 and 2)[4], cultures were established by plating 500 cells from either source in 5 ml of the "fresh medium (1)" 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 (1)" 1 day after plating. Mononucleate cells from either origin were successfully cultured under the present culture conditions and they yielded colonies of myogenic cells and of fibroblasts. At 6 days after the initiation of cultures, all cultures were fixed with ethanol and stained with hematoxylin. Mitotic activity was shown by the number of cell divisions which occurred during the culture period. The number of cell divisions (X) was calculated from the number of nuclei of cells (N) forming each colony according to the following formula: $X=\log N/\log 2$.

Experiment 1 and Experiment 2: Ten normal and 11 dystrophic cultures in exp. 1 and 11 normal and 11 dystrophic ones in exp. 2 were carried out (Table 1). The average number of colonies formed per dish and the plating efficiency in each series of cultures are shown in Table 1. In exp. 1, the number of cell divisions (NCD) of normal myogenic cells was 6.93 ± 1.21 and that of dystrophic ones was 5.38 ± 1.13. The difference between them was significant ($P<0.001$) (Table 2). The NCD of normal fibroblasts and that of dystrophic ones were 4.50 ± 0.90 and 4.23 ± 0.84, respectively. The difference between them was much smaller than that observed between normal and dystrophic myogenic cells, but it was also significant ($P<0.02$). In exp. 2, the NCD of normal myogenic cells was 6.14 ± 1.39, that of dystrophic ones was 5.16 ± 1.15, and the difference between them was significant ($P<0.001$) (Table 2). The NCD of normal fibroblasts and that of dystrophic ones were 4.44 ± 1.12 and 4.22 ± 1.05, respectively, and the difference between them was not significant.

Experiment 3: As shown in Table 1, more colonies per dish were formed in normal cultures than in dystrophic ones both in exp. 1 and in exp. 2. These findings suggested that population effect may have enhanced the mitotic activity of myogenic cells in normal cultures, in which more colobies were formed per dish than in dystrophic ones. To test this possibility, mononucleate cells from either normal or dystrophic source were cultured under the same culture conditions at varying cell densities of 500, 250, and 125 per dish and the NCDs of myogenic cells and fibroblasts from either source in cultures at each cell density were obtained. In these cultures in which cell densities were successively altered as described above, the average number of colonies formed per dish was found to be 18.0, 12.1, and 6.7 in normal and 33.8, 16.1, and 10.0 in dystrophic cultures, respectively (Table 1). The number of cultures at each cell density from either source is shown in Table 1. In the cultures at the different cell densities described above, the NCDs of normal myogenic cells were 6.99 ± 1.29, 6.89 ± 1.21, and 7.00 ± 1.53 and those of dystrophic ones were 4.98 ± 1.20, 5.22 ± 1.34, and 4.89 ± 1.19, respectively (Table 2). No significant differences among the NCDs

Table 1. Plating efficiency and number of colonies formed per dish in each series of experiments

Series of exp.	Exp. 1		Exp. 2		Exp. 3					
Source of cells	Norm.	Dystr.	Norm.	Dystr.	Norm.			Dystr.		
Inoculum size	500	500	500	500	500	250	125	500	250	125
No. of cultures	11	10	11	11	10	10	10	12	10	8
Pl. efficiency (%)	10.0	5.1	4.9	3.8	3.6	4.8	5.3	6.8	6.4	8.0
No. of col./pl.	50.2	25.4	24.7	19.1	18.0	12.1	6.7	33.8	16.1	10.0
Myoblast	35.6	13.9	13.2	4.7	12.9	9.1	4.1	12.3	6.3	3.5
Fibroblast	14.6	11.5	11.5	14.4	5.1	3.0	2.6	21.5	9.8	6.5

Table 2. Number of Cell Divisions of Myoblasts

Series of exp.	Inoculum size	Normal ($\bar{X}_n$)	Dystr. ($\bar{X}_d$)	$\bar{X}_n - \bar{X}_d$	d.f	t-stat.
Exp. 1	500	6.93±1.21 (373)	5.38±1.13 (139)	1.55	510	$P<0.001$
Exp. 2	500	6.14±1.39 (124)	5.16±1.15 (51)	0.98	173	$P<0.001$
Exp. 3	500	6.99±1.29 (116)	4.98±1.20 (140)	2.01	254	$P<0.001$
	250	6.89±1.21 (83)	5.22±1.34 (58)	1.67	139	$P<0.001$
	125	7.00±1.53 (27)	4.89±1.19 (26)	2.11	51	$P<0.001$

n: normal; d: dystrophy; $\bar{X}$: average number of cell division; t-stat.: t-statistics; d.f: degree of freedom; ( ): number of colonies used for the count of number of cell divisions.

of myogenic cells in cultures at different cell densities in either normal or dystrophic cultures could be found (Table 2). In addition, the NCDs of normal myogenic cells were always significantly larger than those of dystrophic ones in cultures at any cell density, at least under the present culture conditions (Table 2). The NCDs of fibroblasts cultured at different cell densities were not significantly different from one another in cultures from either source and the NCD of normal fibroblasts was not significantly different from that of dystrophic ones in cultures at any of the cell densities described above. These findings showed that myogenic cells from normal origin showed higher mitotic activity than those from dystrophic origin, regardless of cell density or of the number of colonies of cultures in which they were cultured. The present results suggest the three following questions: (a) Is the decrease in the number of cell divisions of dystrophic myogenic cells due to a prolongation of generation time or to a limited number of cell divisions (cell lineage theory[6])? (b) Is it due to the expression of a dystrophic gene (dy/dy)? (c) Could it be one of the causal factors of various dystrophic changes observed in dystrophic muscles (dy/dy) *in vivo*? An analysis of these problems is now in progress in our laboratory.

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Muscular Dystrophy Group

A FACTOR EXTRACTED FROM THE BRAIN AFFECTING DEVELOPMENT OF TETRODOTOXIN-SENSITIVE SPIKE POTENTIAL IN CULTURED CHICK SKELETAL MUSCLE

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The action potential of adult innervated vertebrate skeletal muscle fibers is generated by tetrodotoxin (TTX)-sensitive sodium channels. The TTX-sensitive sodium channels in the skeletal muscle cell membrane of newborn rats increase in density after birth in relation to the establishment of normal innervation[1]. Conversely, denervation of adult rat skeletal muscle diminishes the density of the TTX-sensitive sodium channels in the muscle cell membrane[2]. The development and maintenance of the TTX-sensitive sodium channels in the muscle cell membrane could then be considered as one of the membrane properties which are under the trophic control of innervation.

Using chick skeletal muscle cell cultures, it was shown that cell-free nerve extracts could duplicate the effect of innervation on the development of the TTX-sensitive sodium channels in noninnervated skeletal muscle cells[3-5]. Thus, the results indicate that trophic control of the TTX-sensitive sodium channels of muscle cells might well be mediated by a soluble substance produced by nervous tissue, as suggested by Harris[6] and Gutmann[7]. In the present study, we report that a protein fraction isolated from chick brain extract has a neurotrophic activity in that it enhances the development of the TTX-sensitive sodium channels in cultured chick skeletal muscle cells.

Muscle cultures were prepared from the breast muscle of 11-day-old White Leghorn chick embryos according to the method described previously[3]. The basic culture medium was Eagle's minimal essential medium supplemented with horse serum (10% by volume), nerve-free chick embryo extract (2–5% by volume), glutamine (1 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). The nerve-free chick embryo extract was made from 10-day-old chick embryos deprived of the brain and spinal cord, by homogenizing the embryo bodies in saline and centrifuging the homogenate at 2,000$\times g$ for 10 min. Muscle cultures were also maintained in culture medium containing brain extract. The brain extract was prepared from whole brains of 19-day-old chick embryos by centrifuging a 1:2 homogenate in saline at 2,000$\times g$ for 10 min.

When muscle cells were grown in the presence of brain extract for 2 weeks, the maximum rate of rise of TTX-sensitive spike potential of the cells was significantly greater than in the control cultures maintained in the basic culture media. This suggests that the brain extract has a trophic activity that enhances the development of the TTX-sensitive spike potential of the cultured muscle cells.

The brain extract was centrifuged again at 20,000$\times g$ for 30 min. Trophic activity could be detected in the supernatant, but not in the resuspended precipitate. The same result was obtained after centrifugation at 100,000$\times g$ for 60 min, *i.e.*, the soluble fraction may be responsible for the activity.

The active component in the 20,000$\times g$ supernatant was isolated by consecutive ammonium sulfate precipitation, and retained after removal of low molecular weight compounds by dialysis against saline. Table 1 summarizes these results.

The protein nature of the active component in the brain extract was further suggested by the results of enzyme degradation. The 100,000$\times g$ supernatant was treated with enzyme for 60 min at 37°C. The trophic activity was abolished by trypsin and protease, but was resistant to DNase and RNase (Table 2). The susceptibility to trypsin and protease suggests that the active component is a protein.

Table 1. Effect of brain extract on the maximum rate of rise of TTX-sensitive spike potential of muscle cells in 2-week-old cultures

Fraction added	Protein (μg/ml)	Maximum rate of rise (V/sec)
Control	—	(59/4) 73.8±3.2
2000×g sup.	300	(56/4) 84.3±3.0*
20 K×g ppt.	40	(31/2) 59.8±2.5
20 K×g sup.	260	(62/4) 96.8±3.4*
Saltrated (Amm. sulf.)		
0-40%	100	(63/4) 96.4±3.1*
40-60%	50	(61/4) 87.5±3.2*
Dialyzed		
Inner sol.	240	(35/2) 91.1±4.2*
Outer sol.	10	(61/4) 65.3±3.0

Mean±S.E. * indicates a statistically significant increase from the control.

Table 2. Effect of enzyme-treated brain extract on the maximum rate of rise of TTX-sensitive spike potential of muscle cells in 2-week-old cultures

Treatment	Maximum rate of rise (V/sec)
Control	(8) 70.9±5.0
100 K×g Sup.	
Non-incubated	(16) 113.8±3.5*
Incubated	(16) 99.8±3.7*
DNase-treated	(16) 93.9±5.4*
RNase-treated	(12) 88.7±2.9*
Trypsin-treated	(16) 68.6±4.3
Protease-treated	(14) 67.3±4.6

Mean±S.E. * indicates a statistically significant increase from the control.

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Muscular Dystrophy Group

CLASS SPECIFICITY OF SERA AND MUSCLE TROPHIC FACTOR FOR MUSCLE CELL GROWTH *IN VITRO*

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Chick myogenic cells do not grow in a medium composed of Eagle's minimum essential medium (MEM) and horse serum, but grow well when a small amount of chick embryo extract (EE) is added to the medium. EE can be replaced by chick or duck serum, and by chick muscle trophic factor (MTF), which is a component of chick serum[1,2]. The cells also grow in a medium composed of MEM and chick serum[3]. Thus it is clear that chick serum is different from horse serum in regard to chick myogenic cell growth, and it is possible that this difference is mainly based on the difference of MTF which could be also contained in mammalian serum. The present work was concerned with whether there is species or class specificity among sera, and whether the above possibility is a valid one.

Chick cells were cultured with a medium composed of 15% horse serum and various concentrations of various sera and MEM. After 4 days of incubation, the cells were harvested for creatine kinase (CK) assay, which was interpreted as showing the growth rate. Figure 1 shows the relationship between the concentration of sera and CK activity. In the cases of chick and duck sera, CK activity began to increase at 0.1 and 0.3%, respectively. Then it increased gradually with the increase in concentration. In the cases of dove and fetal bovine sera, it began to increase at 3%, and increased fairly sharply. In the case of adult bovine serum, it remained at an almost unassayable level, although some myotubes could be seen under a microscope.

In order to compare the effects of various sera on chick myogenic cell growth, the cells were incubated with media composed of 82% MEM, 14% horse serum, and 4% various kinds of sera. The growth rates are shown semiquantitatively in Table 1. Avian sera were more or less effective, but mammalian sera except for fetal bovine serum were not effective. Sera from *Gallus* and *Corturnix* were very effective, but those of *Phasianus, Numida,* and *Meleagaris* were not so effective, although all of them belong to *Galli.* On the other hand, sera from *Anseriformes* were fairly effective.

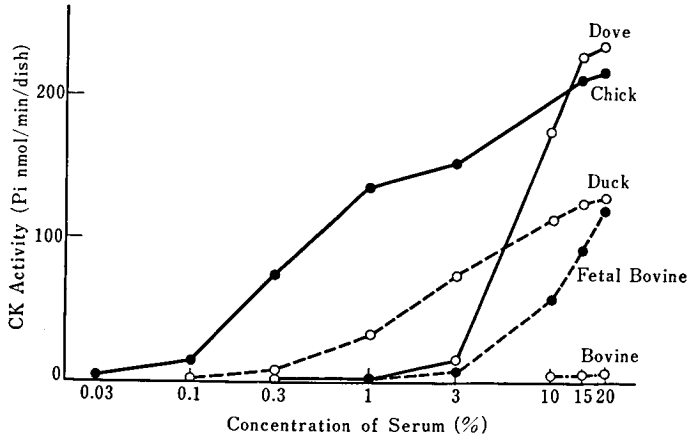

Fig. 1. Effects of various sera on chick myogenic cell growth.

Table 1. Effects of various sera on chick myogenic cell growth *in vitro*

Birds		Mammals	
Chick	(+++)	Fetal bovine	(+)
Cochin-China fowl	(+++)	Calf	(−)
Bantam	(+++)	Bovine	(−)
Japanese quail	(+++)		
Golden pheasant	(+)	Dog	(−)
Korean pheasant	(+)	Rat	(−)
Turkey	(+)	Rabbit	(−)
Guinea fowl	(+)	Swine	(−)
Duck	(++)	Monkey	(−)
Goose	(++)	Human	(−)
Herring gull	(+)		
Dove	(+)		
Kite	(+)		

Table 2. Effects of various sera on rat myogenic cell growth *in vitro*

Birds		Mammals	
Chick embryo extract	(+)	Rat	(++)
Chick	(−)		
		Fetal bovine	(++)
Japanese quail	(−)	Bovine	(++)
Golden pheasant	(−)		
Korean pheasant	(−)	Rabbit	(++)
Turkey	(−)	Dog	(++)
Guinea fowl	(−)	Swine	(++)
Duck	(−)	Horse	(++)
Goose	(−)	Monkey	(++)
Dove	(−)	Human	(++)

MTFs were prepared from chick and fetal and adult bovine sera. Chick MTF was effective at 3 μg/ml, but fetal and adult bovine MTF were only slightly effective at 300 μg/ml.

Newborn rat myogenic cells did not grow in a medium composed of chick serum and MEM, but grew well when a small amount of rat sera was added to the medium. In order to compare various kinds of sera, rat cells were incubated with media composed of 77% MEM, 19% chick serum, and 4% various kinds of sera for 5 days. The growth rates are shown semiquantitatively in Table 2. Mammalian sera were effective but avian sera were not; EE was slightly effective. In this table, the effects of all the mammalian sera were expressed by (++), although they were not necessarily the same.

Rabbit MTF was effective on rat cell growth at 8 μg/ml, but chick MTF was not effective at 240 μg/ml.

Conclusion: There is a class specificity among sera from mammals and aves in regard to myogenic cell growth, although there are some exceptions, and this specificity is mainly based on MTF.

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A FREEZE FRACTURE STUDY OF THE PLASMA MEMBRANE OF DYSTROPHIC CHICK MUSCLES

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To elucidate the role of the plasma membrane in the development of muscle cell degeneration in muscular dystrophy, we conducted a freeze fracture study of the skeletal muscle of dystrophic (line 413) and control (line 412) chickens of various ages starting at day 0. Superficial pectoralis and lateral adductor muscles were chosen for this study simply because the post-natal development of these muscles has been well characterized[1].

Small pieces of muscle tissue were obtained under pentobarbital anesthesia and they were immediately fixed with 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.4, 285 mOsm) for a few hours at 4°C. The fixed 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 $1–2 \times 10^{-7}$ Torr with an Eiko freeze etch device. The fracture surface was immediately shadowed with platinum and carbon. The replicas were retrieved after digestion with commercial bleach solution and washing with several changes of distilled water. Photographs were taken with a Hitachi H-600 electron microscope operating at 100 kV.

The most remarkable finding in the plasma membrane of dystrophic chick muscles was the presence of a large number of caveolae, which had lost the orderly distribution seen in control muscles (Fig. 1). Such a change was evident in both superficial pectoralis and lateral adductor muscles of 5-week-old chickens but it was apparent only in the superficial pectoralis muscle in chickens 2 weeks of age. On day 0, however, the density and distribution pattern of the caveolae in dystrophic chickens were indistinguishable from those in controls. For a further assessment of the density of caveolae, approximately 300 μm^2 of

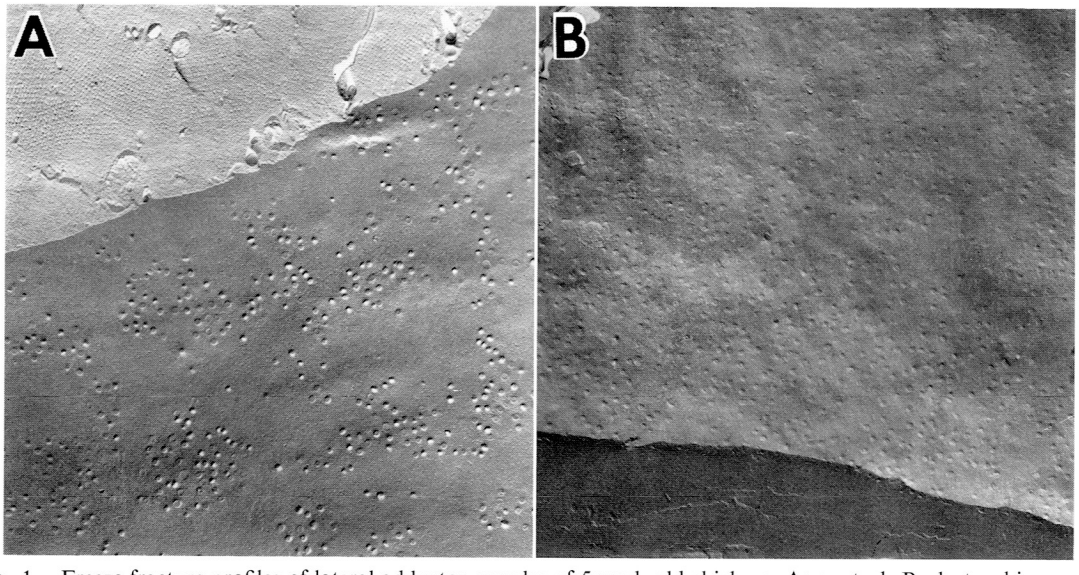

Fig. 1. Freeze fracture profiles of lateral adductor muscles of 5-week-old chickens. A: control; B: dystrophic; magnification: ×10,000.

Table 1. Densities of caveoles (per 10 μm^2)

	day 0	2 weeks	5 weeks
Breast muscle			
Control	108.6±3.6	128.1±14.8	140.1±4.0
Dystrophic	111.5±5.3	199.7±17.3	241.5±5.2
	$P<0.5$	$P<0.01$	$P<0.001$
Leg muscle			
Control	106.4±1.1	140.0±9.2	139.7±8.0
Dystrophic	105.3±2.9	131.1±9.3	261.8±11.7
	$P<0.5$	$P<0.5$	$P<0.001$

the plasma membrane with an apparent continuity with the cytoplasma of muscle cells was chosen for each muscle and the density of caveolae was measured with the aid of a Kontron Image Analyzer. The results of the measurements are summarized in Table 1. The results indicate that an increase in the number of caveolae coincides with a disorderly caveolae distribution.

Although no precise correlation has been established between the freeze fracture profile and the transmission image of caveolae, it is generally assumed that the caveolae on the cell membrane represent pinocytotic vesicles and, in muscle cells, openings of the T-system as well. If this is the case, an increased number of caveolae on the cell membrane can be indicative of an elevated transmembrane transport activity and may be a morphological expression of a "leaky membrane" in muscular dystrophy since a concomitant observation of these muscle tissues with a transmission electron microscope revealed no detectable abnormality in the plasma membrane. A histopathological study of these muscle tissues disclosed grossly normal tissue except for the occasional presence of lipid droplets in the breast muscle of 5-week-old dystrophic chickens. Therefore, it can be assumed that the alteration in the plasma membrane is an early event in the degenerative process of muscle tissues.

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Muscular Dystrophy Group

STRUCTURAL DIVERSITY IN CHICKEN SKELETAL MUSCLES

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The structural details of muscle fibers vary to a great extent in different muscles. Careful analyses of such structural diversity may lead to a better understanding of not only functional correlates but also of local factors for the selectivity of affected muscles in muscular dystrophy. From this viewpoint, we are studying the variations in the cell structures, especially in the membranous system, among different muscles in the same species of animals. In the present study the fine structural variations in the sarcoplasmic reticulum and T-system in various muscles of the chicken were examined under an electron microscope.

Adult chickens (male, white Leghorn), 1–1.8 kg in body weight, were used for this study. Under intraperitoneal anesthesia, various muscles were dissected out and fixed in a stretched state with 2.5% glutaraldehyde-2% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, followed by post-fixation with 1% OsO_4 in the same buffer. Tissues were dehydrated in ethanol and embedded in Epon 812. Thin sections were examined under a Hitachi HU-12 electron microscope.

The following chicken muscles were examined in this study: pectoralis major muscles (superficial layer), anterior and posterior latissimus dorsi muscles, anterior tibialis, extensor digitorum communis, sartorius, ischio-pectineus muscles, and extrinsic (rectus medialis) and intrinsic (ciliaris) eye muscles.

Thin sections of muscle tissues cut longitudinally and transversely were examined to analyze the overall distribution and regional differentiation of the sarcoplasmic reticulum (SR) and T-system. Chicken muscles were classified into two major types according to the distribution levels of the T-system: (1) muscle comprised of muscle fibers in which the T-tubules run at the level of the Z disc (Z-level type); and (2) muscle comprised of muscle fibers in which the T-tubules take their course at the level of the A-I junction (A-I-level type). Of the muscles we examined, only the pectoralis major muscle belongs to the Z-level type (Fig. 1). The rest of the muscles fall under the A-I-level type (Fig. 2). Observations that the pectoralis muscles contain T-tubules at the Z-level have been reported not only in chickens[1,2] but also in pigeons[3]. Since our examination of finch breast muscles adds another example, these two different positionings of the T-tubules seem to be common to many other avian skeletal muscles. This also may be emphasized in relation to the fact that the pectoralis muscle is the major muscle affected in chicken muscular dystrophy.

The overall development and regularity of the T-system vary among different muscles. In the pectoralis, leg, and posterior latissimus dorsi muscles, the T-system was distributed very regularly with respect to the sarcomere pattern, whereas the T-system in the extrinsic and ciliary muscles was much less regular in its arrangement. In anterior latissimus dorsi muscles, the T-system was seen to be distributed in a very irregular pattern. However, high-voltage electron microscopy of thick sections combined with selective staining revealed that transversely running T-tubules were positioned almost exclusively at the level of the A-I junction. Furthermore, triads were formed even along longitudinally running T-tubules, predominantly at the A-I level.

The overall development and regional differentiation of the SR with respect to the sarcomere also varied among different muscles of the chicken. *En face* views of the SR were only seen in longitudinal sections where myofibrils were cut tangentially. Careful comparison between the images of the SR in both longitudinal and transverse sections showed that the regional differentiation of the SR could be imaged and quantitated based on the membrane profiles seen in transverse sections.

The pattern of the SR network can be categorized into two types corresponding to the types in the T-system distribution. In pectoralis muscles, triads were formed at the Z-level. The SR extended from the

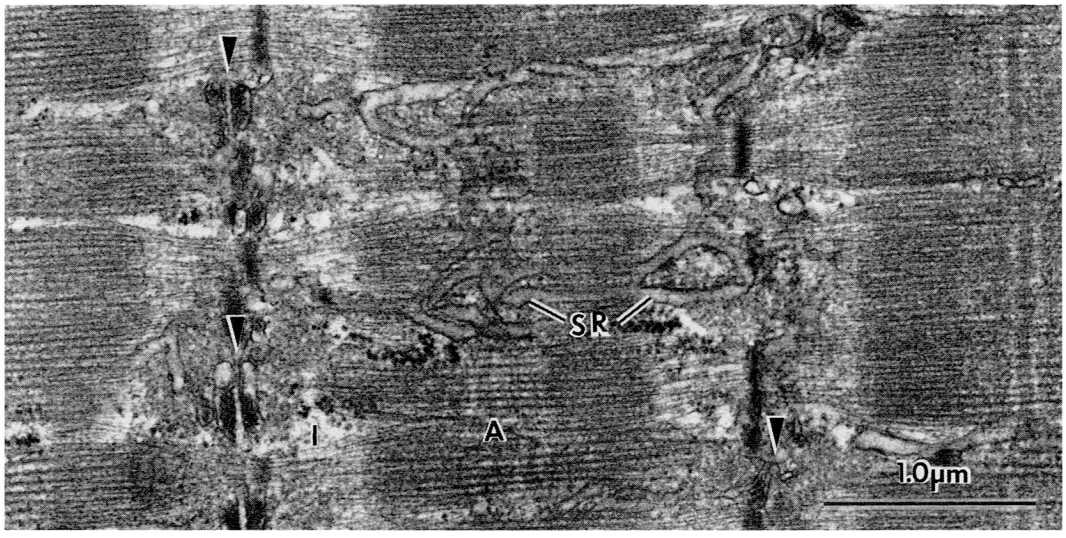

Fig. 1. Electron micrograph of a longitudinally cut muscle fiber from chicken pectoralis major muscle. The T-tubules run transversely forming triads at the level of the Z-band (*arrows*). The sarcoplasmic reticulum (*SR*) extends from the I-band (*I*) to the middle of the A-band (*A*).

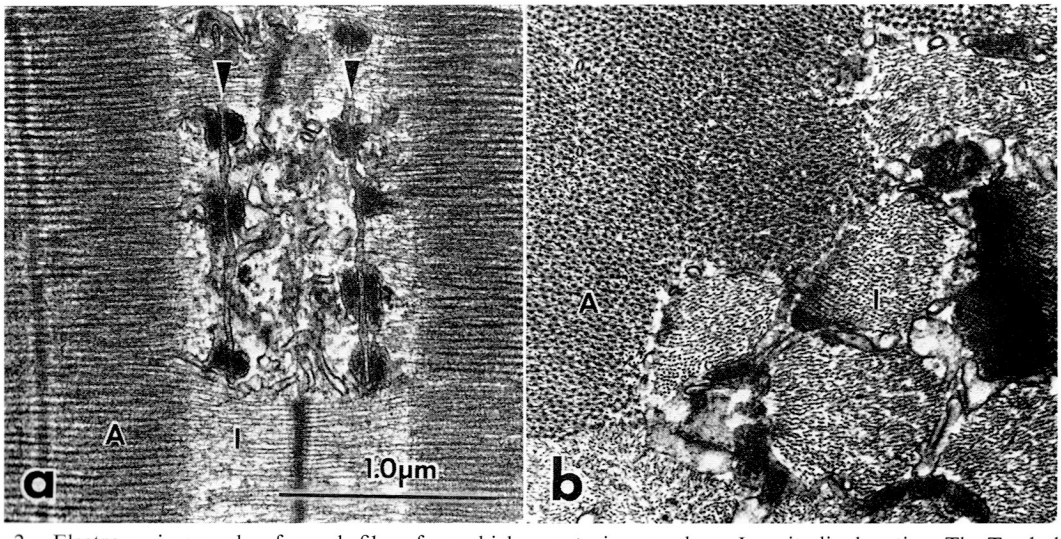

Fig. 2. Electron micrographs of muscle fibers from chicken sartorius muscle. *a*: Longitudinal section. The T-tubules (*arrows*) run at the level of the A-I junction. The sarcoplasmic reticulum (*SR*) is well developed, connecting adjacent triads in the I-band (*I*) but not in the A-band (*A*). *b*: Transverse section. The sarcoplasmic reticulum profiles well reflect the images seen in the longitudinal section.

terminal cisternae at the Z-level toward the middle of the sarcomere, where the SR formed fenestrated collars (Fig. 1). In leg muscles, on the other hand, when the triads were formed at the level of A-I junction, the SR was developed much more in the I-band connecting between adjacent triads than in the A-band (Fig. 2). Such features were well reflected on the membrane profiles in transverse sections. The amount of the SR in the A-band varied among different muscles. In certain leg muscles, the SR networks were poorly formed at the level of the A-band so that the boundary between individual myofibrils might be barely discernible in transverse sections (Fig. 2).

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TROPONIN IN CARDIAC AND SKELETAL MUSCLES IN CHICKEN EMBRYOS STUDIED BY IMMUNOFLUORESCENCE MICROSCOPY

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The differentiation of troponin (TN) in cardiac and skeletal muscles of chicken embryos was studied by indirect immunofluorescence microscopy. Serial sections of embryos were stained with antibodies specific to TN components (TN-T, -I, and -C) from adult chicken cardiac and skeletal muscles. Cardiac muscle began to be stained with antibodies raised against cardiac TN components in embryos after stage 10 of Hamburger and Hamilton[1] (Fig. 1). It reacted also with anti-skeletal TN-I from stage 10 to hatching. Skeletal muscle was stained with antibodies raised against skeletal TN components after stage 14. It also reacted with anti-cardiac TN-T and -C from stage 14 to hatching. Immunoelectron microscopy revealed that the newly synthesized regulatory proteins are assembled at their characteristic position from the initial phases of myofibrillogenesis. The results are summarized in Table 1.

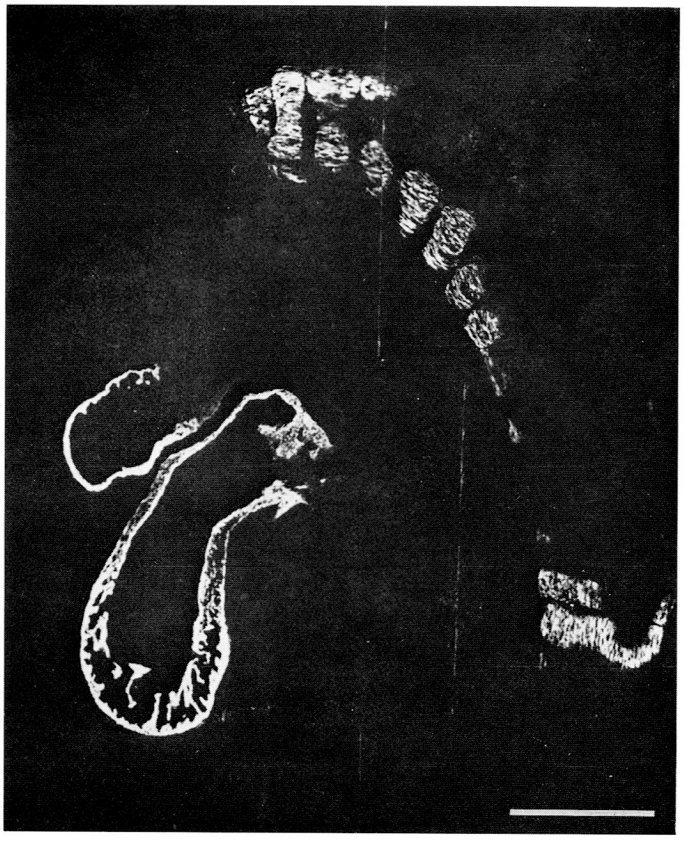

Fig. 1. Sagittal section of a whole chicken embryo at stage 20 stained with antibody against cardiac TN-T. Skeletal and cardiac muscles were stained. ×37. Bar, 0.5 mm.

Table 1. Differentiation of TN in cardiac and skeletal muscles of chicken embryos

Tissue	Antibody against	Stages (Hamburger-Hamilton) of chicken embryos					
		8 (26-29 hr)	10 (33-38 hr)	14 (50-53 hr)	20 (70-72 hr)	45 19-20 days)	Adult
Cardiac muscle	Cardiac TN-T	−	+	+	+	+	+
	Cardiac TN-I	−	+	+	+	+	+
	Cardiac TN-C	−	+	+	+	+	+
	Skeletal TN-T	−	−	−	−	−	−
	Skeletal TN-I	−	+	+	+	−	−
	Skeletal TN-C	−	−	−	−	−	−
Skeletal muscle	Cardiac TN-T		−	+	+	±	−
	Cardiac TN-I		−	−	−	−	−
	Cardiac TN-C		−	+	+	±	−
	Skeletal TN-T		−	+	+	+	+
	Skeletal TN-I		−	+	+	+	+
	Skeletal TN-C		−	+	+	+	+

The present immunofluorescence microscopic study demonstrated that TN components in cardiac muscle begin to appear at about stage 10. It is at this stage in chicken embryos that the heart begins to beat[2] and that myofibrillar structures begin to be observed by electron microscopy of the myocardium[3]. Thus, the first appearance of TN components corresponds well with the time of initiation of functional and structural integrity formation in cardiac muscle. The observation that TN components of cardiac muscle are synthesized much earlier than those of skeletal muscle probably reflects the fact that the heart begins to function at an early stage when the skeletal musculature is still immature.

During embryonic development, cardiac muscle synthesizes TN-T and -C possessing cardiac-type antigenicity, and TN-I with antigenic determinants similar to those present in cardiac as well as in skeletal muscles. Embryonic skeletal muscle synthesizes TN-I possessing antigenicity for skeletal muscle, and TN-T and -C which share antigenicities for both cardiac and skeletal muscles. Thus, in the development of cardiac and skeletal muscle, it is apparent that a process occurs in which the fiber changes its genomic programming: it ceases synthesis of the TN components which are immunologically indistinguishable from each other, and synthesizes only tissue type-specific proteins after hatching. The problem of whether these developmental changes in the gene expression for TN components are programmed endogenously or are due to the influence of an exogenous factor(s) requires further investigation. However, if an exogenous factor(s) does affect the muscles, the role of innervating nerves cannot be ruled out, since it has been known that innervation leads to the differentiation of individual muscle fibers to specific types[4].

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Muscular Dystrophy Group

IMMUNOELECTRONMICROSCOPIC OBSERVATIONS OF THE LOCALIZATION OF TROPONIN ALONG THE THIN FILAMENT OF DYSTROPHIC CHICKEN BREAST MUSCLE

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Previous immunoelectronmicroscopy showed that troponin distributes along the whole length of the thin filament of chicken skeletal muscle at regular intervals of 38 nm. The number of anti-troponin striations formed along the thin filament bundle was always found to be twenty-four.

Recently an immunocytological procedure called the "saponin method" was developed that has made it possible to observe developing or immature myofibrils *in situ* without damaging the overall structure of muscle fibers. Application of this technique to troponin localization in chick embryonic breast muscle revealed that the number of troponin striations along the thin filament was definitely more than that in adult thin filament. This finding strongly suggests that the final maturation of the thin filament consists of a shortening of the longer filaments into a constant length after hatching.

In the present investigation, we undertook to follow the process of thin filament shortening in dystrophic (413) and intact (412) chicken breast muscles after hatching.

The thin bundles were separated from breast muscle 1 to 5 weeks after hatching. These fibers were either used directly or immersed in glycerol solution to make glycerinated muscles. Two methods were used for anti-troponin staining. The first was a saponin technique in which muscle fibers were treated with 10^{-4} g/ml saponin and then with anti-troponin antibody, in a relaxing solution containing 0.1 M KCl, 10 mM phosphate (pH 7.0), 10 mM $MgCl_2$, 5 mM ATP, and 0.5 mM GEDTA. Then, the specimens were fixed and embedded in Epoxy resin for thin sectioning. In the second method, the thin filaments were dissociated from thick filaments by homogenization in the relaxing solution, treated with antibody solution, and stained negatively with uranyl acetate. These specimens were observed under an electron microscope with an acceleration voltage of 100 kV.

Figure 1 shows a longitudinal section of thin filament regions in myofibrils of dystrophic chicken breast muscle 7 days after hatching. Anti-troponin formed twenty-five to twenty-eight striations along the thin filament bundle. No thin filaments with twenty-four anti-troponin striations were observed. These matured thin filaments with twenty-four striations first appeared 4 weeks after hatching, but longer filaments with more than twenty-four striations could be observed even at 3 months after hatching.

Anti-troponin antibody formed twenty-five to thirty-three periodic striations along the thin filaments in intact chicken breast muscle 1 week after hatching, but formed twenty-four striations along all thin filament bundles 3 weeks after hatching so far observed.

The above findings clearly indicate that the process of shortening of thin filaments into a constant length in dystrophic chicken breast muscle is much slower than in intact muscle fibers.

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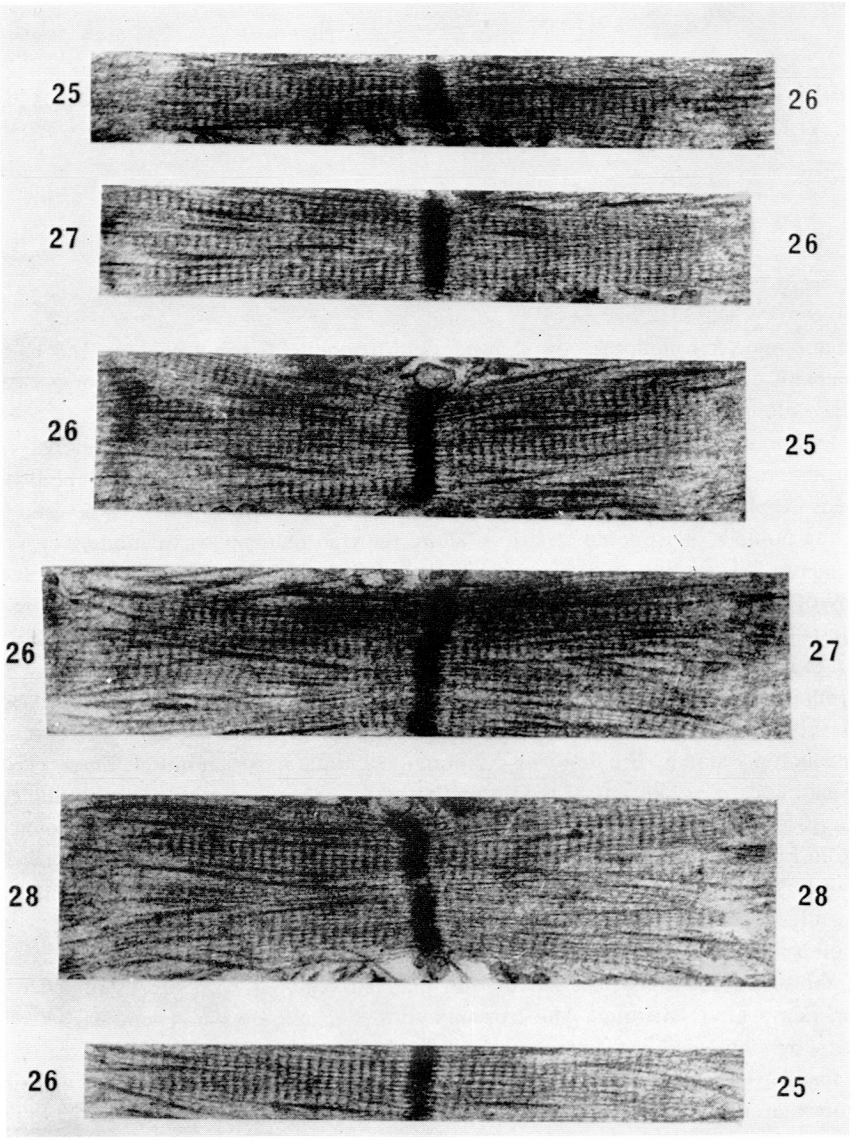

Fig. 1. Anti-troponin staining of thin filaments of dystrophic chicken breast muscle 7 days after hatching. ×40,000. Anti-troponin formed regular transverse striations on the thin filaments which extended in both directions from the Z-line situated at the center of each figure. Numbers on both sides of the figure indicate the number of anti-troponin striations along each thin filament bundle.

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DEVELOPMENTAL STUDIES OF THE MEMBRANE PROPERTIES OF STRIATED MUSCLE FIBERS

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Calcium action potentials have been observed in various excitable membranes, such as in crustacean striated muscles, heart muscles, smooth muscles, neuroendocrine cells, and especially axon terminals where neurotransmitters have been considered to be released[1]. In addition, the egg cell membranes from where cellular differentiation starts show a calcium action potential in many species, such as ascidians, echynoderms, and mammals[2].

On the other hand, intracellular ionized calcium facilitates muscle contraction[3], transmitter release, egg cell fertilization changes[4], and cell cleavage. It has been reported that the calcium ionophore which enhances the permeability of the membrane to calcium has considerable effects upon various kinds of cytokinetic phenomena. It is reasonable to suspect that the Ca-channels, the native calcium ionophores, are essential constituents of muscle cell membrane which has a specialized cytokinetic apparatus. Since especially in the striated muscle cell of ascidian tadpole larva the myofibrils are located just beneath the plasma membrane, the calcium influx through Ca-channels from the surface may play an important role in the control of the contractile apparatus. Thus, the present study aims to analyze the differentiation process of the striated muscle of the ascidian tadpole larva with special regard to the Ca current through Ca-channels in the membrane.

1. Methods: Adult ascidian *Halocynthia roretzi* which had matured eggs and sperm were kept in a cold aquarium at 4°C. The eggs and sperm were obtained from the spawning animals after 24 hr in a warm bath at 12°C. The eggs were fertilized by sperm from heteronymous animals. The developmental time was measured from this fertilization period in an incubation bath at a constant temperature of 10°C. At the 8- or 16-cell stage, the embryos were transferred to another bath containing 1 μg/ml cytochalasin B in order to arrest their cell cleavage and cultured further until the control intact tadpole larvae hatched. After removing the chorion membrane by digestion with 10 mg/ml pronase the cleavage-arrested embryos were kept in a high-proteinous external solution and were penetrated with two microelectrodes. The experiments were carried out with either constant current stimulation or voltage-clamp. The external solution was usually 100 mM $SrCl_2$, 400 mM NaCl, 10 mM KCl, and 5 mM PIPES-Na (pH 7.0). When necessary, sodium was replaced with choline or strontium with calcium. The reason for the use of strontium solution was to increase the current through the Ca-channels.

2. Results:

1) Development of Ca current in cleavage-arrested 16-cell embryos. It has been reported that Na and Ca action potentials develop in cleavage-arrested embryos as in the excitable cells in the intact tadpole larvae[5]. Since the blastomeres in 16-cell embryos were large enough to be penetrated with two microelectrodes, the Ca current was analyzed by the voltage-clamp technique at all stages of development. Until 50 hr after fertilization the intercellular communication between the blastomeres was so tight that the embryo behaved as a cell and the sum of membrane currents was observed on the embryonic surface. The total calcium current in an embryo was initially decreased until 20 hr of development and was enhanced again after 35 hr. After 50 hr most of the intercellular communication disappeared and the membrane currents in individual blastomeres could be analyzed. The presumptive fate of each blastomere in the 16-

cell embryo has been described classically and individual blastomeres have been named. The enhancement of the Ca current appeared in all observed B_{5-1} blastomeres which contained the presumptive muscle region and were located in the vegetal hemisphere. This Ca current was always followed by a delayed K current. In the blastomere a Ca spike which was identical to that in the muscle cells of intact tadpole larvae was evoked by constant current stimulation (muscle type). However, the B_{5-2} blastomere which also contained the presumptive muscle regions did not show any calcium current. Blastomeres A_{5-1} and A_{5-2} did not show any voltage-dependent inward currents (non-excitable type). The group of small blastomeres, b_{5-3} and b_{5-4}, in the animal hemisphere showed a pure Ca current without a following delayed K current. These blastomeres have been assigned to the presumptive epidermal regions and they produced extremely long-lasting action potentials under constant current stimulation (epidermal type). Another group of small blastomeres, a_{5-3} and a_{5-4}, showed in 60% of cases a Na current followed by a delayed K current (neuron type) and they have been known as the presumptive brain region of tadpole larvae. When these blastomeres did not show the Na current, they became the epidermal type.

2) Development of Ca current in cleavage-arrested 8-cell embryos. Even in cleavage-arrested 8-cell embryos, the large blastomere in the posterior portion of the vegetal hemisphere, B_{4-1}, showed a Ca current followed by a delayed K current in 30% of the cases observed. The same blastomere B_{4-1}, was electrically coupled with the group of small blastomeres in the animal hemisphere and differentiated into the epidermal type 40% of the time. Sometimes, the blastomere was isolated as in the case for the muscle type, but did not show any excitability. In a few cases a small blastomere, a_{4-1}, was isolated and showed neuron-type differentiation. As described above, blastomere B_{4-1} showed three types of differentiation: muscle type, epidermal type, and non-excitable type. The acetylcholinesterase was stained by the Karnowsky method and the total surface area of the blastomere was found to increase 5-fold with measurement of the total capacitance only in the case of the muscle type. It was suggested that the differentiation into the muscle type induced infolding or expansion of the surface area of the blastomere. The epidermal-type differentiation also induced an increase in the total capacitance at the penetrated blastomere. However, this was not caused by real expansion of the surface area but by electrical coupling with the group of small blastomeres in the animal hemisphere.

Discussion: The B_{5-1} blastomere in cleavage-arrested 16-cell embryos or B_{4-1} in 8-cell embryos showed the muscle-type differentiation with a Ca spike and acetylcholinesterase activity. In these blastomeres, preliminary observations under an electron microscope showed myofibrils underneath the plasma membrane and an accumulation of the mitochondria, which is characteristic of the muscle cells in intact tadpole larvae. Thus, the entire large blastomere seemed to have only muscle characteristics and did not show any mosaic properties mixed with other cellular types. Since the B_{5-1} blastomere contains the presumptive endodermal region as well, it is concluded that the blastomere selects only one cellular type from more than two possibilities. Durther, in the case of 8-cell embryos, the B_{4-1} blastomere actually chose either one of three cellular types equally and the expression of the differentiation seemed to be always of one type in one blastomere.

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Muscular Dystrophy Group

SHORTENING VELOCITY OF SKINNED MUSCLE FIBERS FROM DYSTROPHIC CHICKEN MUSCLES

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Using anti-troponin antibody, Ohtsuki *et al.*[1] found that thin filaments in breast muscles from dystrophic chickens are longer than those from normal chickens, and in this respect, resemble those from embryonic muscles. On the other hand, we[2] have shown that in the region of sarcomere lengths below the point at which thin filaments from both sides meet each other in the middle of A-bands, only very small tension could be produced by a lower activating range of free Ca-ion concentrations as compared with that at longer sarcomere lengths, in contrast to the fact that the tension produced by a higher or saturating range of free Ca-ion concentrations at such short sarcomere lengths is not much different from that at longer sarcomere lengths. A phenomenon probably related to this is the fact[2] that the maximum shortening velocity under no external load, V_{max}, of muscle fibers activated by a lower activating range of free Ca-ion concentrations, again unlike that by higher free Ca-ion concentrations, becomes very small at such short sarcomere lengths. Although it is not yet certain at all that this mechanical behavior at short sarcomere lengths is really due to meeting and overlap of thin filaments from both sides, it is considered quite possible that fibers from dystrophic muscles with longer thin filaments might start showing such mechanical behavior at sarcomere lengths longer than those at which normal fibers start to show it. Therefore, in this study the V_{max} of normal and dystrophic fibers has been measured at various sarcomere lengths.

Posterior latissimus dorsi muscles were isolated from adult (24 months) dystrophic (line 413) and normal (line 412) chickens and immersed in a relaxing solution containing 100 μg/ml saponin to skin the fibers[3]. Single fibers were isolated from the muscles in the relaxing solution, and about 3-mm long parts of the fibers with uniform sarcomere lengths were used. One end of the parts was connected to a strain-gauge transducer and the other to the lever of a moving-coil microammeter. By feeding a sufficient magnitude of current to the microammeter in a direction to stretch the fiber but preventing the lever from moving with a stop, an isometric condition was obtained. V_{max}, or strictly speaking V_0, was measured in the following way. When the isometric tension reached a steady level after an application of Ca-containing solution, the current fed to the microammeter was suddenly switched to that of the reverse direction, which made the fiber shorten quickly to a prefixed level. Since the movement of the lever of the microammeter was much faster than V_{max}, fibers slackened and myofibrils then started shortening with the speed of V_{max}, and after taking up the slack, started redeveloping tension. The time from release until the start of redevelopment of tension was used as an indicator of V_{max}. Experiments were performed at 20°C.

An activating solution containing 3×10^{-6} M free Ca-ion concentration was applied to skinned fibers from normal and dystrophic muscles, and quick-release experiments were performed as described above. The time for the fiber to take up the slack produced by quick shortening of 0.9 μm per sarcomere was measured at three different sarcomere lengths. The results are shown in Table 1. As shown in the table, the time for shortening of normal fibers became longer at sarcomere ranges from 2.9 to 2.0 μm compared to the time required by longer sarcomeres. In dystrophic fibers, while the time for shortening of sarcomeres from 3.3 to 2.4 μm was not significantly different from that of normal fibers, that from 3.1 to 2.2 μm was definitely longer than that of normal fibers at the same length and that of dystrophic fibers from 3.3 to 2.4 μm. The figures in Table 1 are a collection of data of only the first contraction of each fiber to avoid any influence of deterioration during the course of experiments. However, when the experiments were

Table 1. Time required for 0.9 μm shortening per sarcomere

	Normal (msec)	Dystrophy (msec)
From 3.3 to 2.4 μm	127±15 (n=9)	153±11 (n=7)
From 3.1 to 2.2 μm	128±8 (n=4)	227±12 (n=3)
From 2.9 to 2.0 μm	163±39 (n=3)	343±69 (n=4)

repeated on the same fibers at different sarcomeres, qualitatively similar results were obtained.

The results obtained here are as we expected originally. However, it is now unlikely that the present results are due to longer thin filaments in dystrophic fibers, since Ohtsuki (personal communication) has shown that the frequency of encountering longer-than-normal thin filaments is rather small in adult dystrophic muscles of the age we used here. One possibility is that the Ca sensitivity of dystrophic fibers might be slightly less than that of normal fibers. This and other possibilities, as well as the problem of why V_{max} at lower activating free Ca-ion concentrations sharply decreases at short sarcomeres, are currently under investigation.

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STUDY OF THE ELECTROPHYSIOLOGICAL, MORPHOLOGICAL, AND METABOLIC ASPECTS OF DYSTROPHIC CHICK MUSCLE MEMBRANE IN CULTURE

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We reported previously that the membrane potential of dystrophic muscle cells was significantly lower than that of normal controls[1,2], In this study, the development of specific features of dystrophic muscle was followed by means of tissue culture from the very early stages of genesis.

The material used was the myotomes obtained from chick embryos (N.H. 413) which had been incubated at 37°C for 6 days. The myoblasts attached to the myotomes fused and grew rapidly, forming myotubules within 1 or 2 days after explantation, as shown in Fig. 1. Striations began to appear inside of the myotubules in about 1 week and they were completed as muscle fibers in about 2 weeks' cultivation. However, there was no noticeable difference between dystrophic and normal muscle fibers as far as light microscopic observations were concerned.

1) Membrane potential: Microelectrodes could penetrate into the tubules 2 days after explantation. As shown in Fig. 2, the membrane potential of the cell became deeper and deeper from −20 mV (2 days) to −50 mV (8 days) and further to about −70 mV after 15 days. The cells of the dystrophic strain (413) always exhibited a lower membrane potential: the difference was small but statistically significant from that of controls (412). There was no difference between the control and the Broiler strain (Fig. 2).

2) Ultrastructure: Soon after the myotubules were formed, many filamentous structures appeared inside the cells (Fig. 3). These structures grew into myofibrils as the days in culture proceeded. However, the structural integrity of cultivated cells was weak comparing with that of cells grown *in vivo*. The ultrastructural characteristics of dystrophic muscle which were observed in adult muscle[3] could not be seen.

3) Metabolism of acethylcholine receptor (Ach-R): Breast muscle cells obtained from chick embryos after 4 days in culture were used for this experiment. ^{3}H-labeled Erab-toxin b, a specific blocker of Ach-R, was able to bind more to the normal muscle membrane than to dystrophic muscle membrane. This means the receptor content is less in dystrophic muscle. The metabolic rate of Ach-R measured by the appearance of metabolic products of Erab-toxin b was higher in dystrophic muscle although

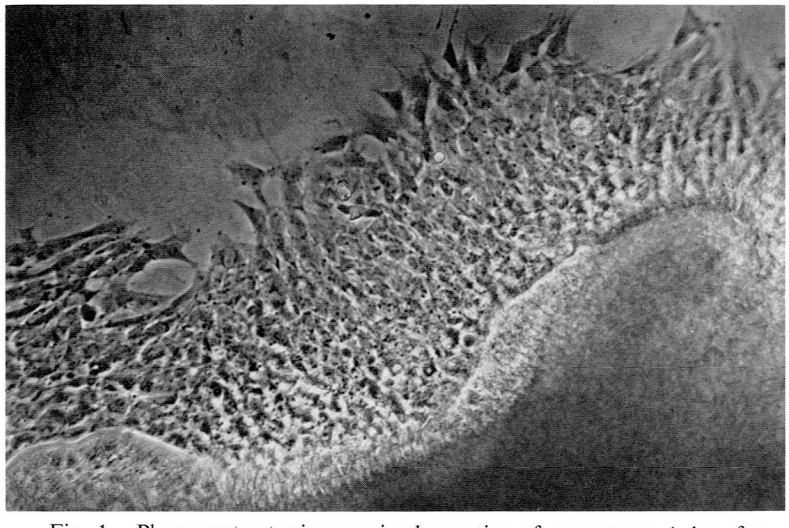

Fig. 1. Phase contrast microscopic observation of a myotome, 1 day after explantation (N.H. 412, ×200).

this result was a preliminary one.

4) Discussion: The results shown here confirmed that the membrane potential of dystrophic muscle cells was lower than that of normal controls from the stage of myotubule formation. The turnover of membrane proteins as reflected by the metabolism of Ach-R, also seems to be different in dystrophic muscle in early stages of development. These facts may indicate that a defect in the membrane system exists in dystrophic muscle from its genesis although morphologically it appears no different.

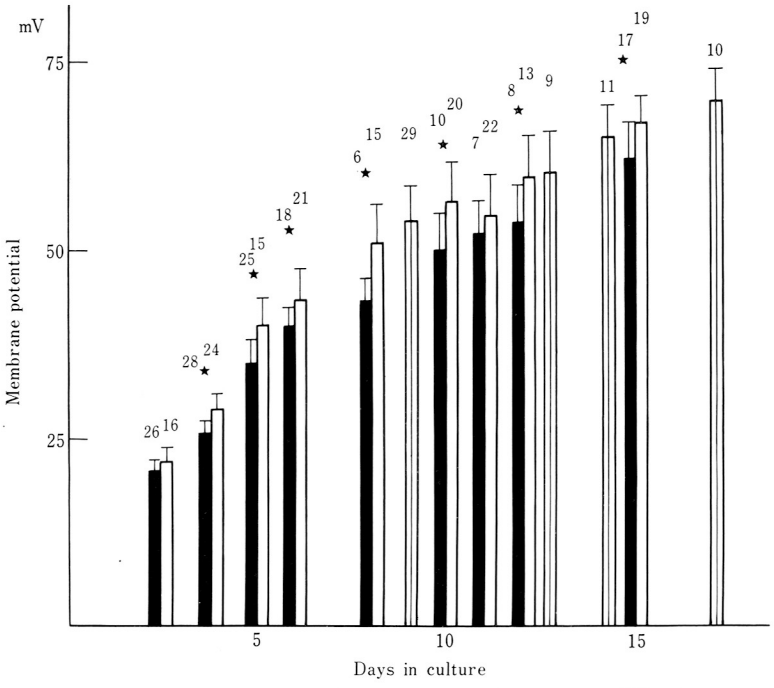

Fig. 2. Development of membrane potential in culture. □: N.H. 412 (control); ■: N.H. 413 (dystrophy); ▤: broiler (control). Numbers indicate the numbers of measurements. $*P<0.05$.

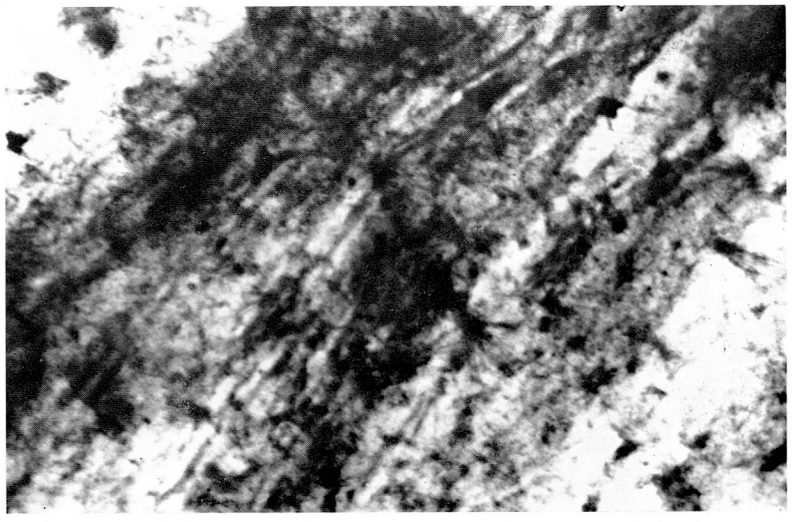

Fig. 3. Electron micrograph of filamentous structures appearing inside myotubules in a 3-day-cultured sample (N.H. 412, ×44,000).

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PROPERTIES OF MUSCLE FIBER AND NEURO-MUSCULAR TRANSMISSION IN ANTHRACENE-9-CARBOXYLIC ACID-INDUCED MYOTONIA

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Controversy exists as to the role of nervous factors in the induction of experimental myotonia. Caccia and associates[1] reported that the initiation of myotonia with 20,25-diazacholesterol did not occur in denervated skeletal muscle; Eyzaguirre *et al.*[2] demonstrated the generation of myotonia by application of 2,4-D to muscle denervated for 10 days; however, Iyer *et al.*[3] and Eberstein and Goodgold[4] claimed that the severity of experimental myotonia is dependent on time after denervation as myotonia could not be induced by 2,4-D 10 days after denervation.

We attempted to clarify whether neural factors influence the generation of myotonia induced by anthracene, using the microelectrode and electromyogram methods.

Young adult Wistar King rats of either sex were given anthracene intraperitoneally (250 mg per day/kg) for 2–3 days.

Myotonia was also induced by adding anthracene to the muscle chamber in concentrations ranging between 10^{-2} to 10^{-6} M. The neuromuscular preparations of the rat hemidiaphragm or extensor digitorum longus (EDL) were used in these *in vitro* studies.

Spontaneous discharges recorded by EMG procedures *in vivo* could be classified into three types, *i.e.*, (i) regular bursts of trains of repetitive discharges alternating with silent periods or discharges at very low frequency; (ii) waxing and waning discharges at high frequency; and (iii) continuous and regular discharge frequencies. The discharge patterns mainly appeared in the present experiments as type (i) or (ii). The amplitude of overshoot and the maximum rate of rise of the action potential declined concomitantly, in proportion to the depolarization of the membrane during the last stage of burst discharges, and abortive action potentials were observed. In the myotonic animals, burst discharges were also induced by direct muscle stimulation. Myotonic activity lasting for few hundred msec was triggered after sciatic nerve stimulation with a brief rectangular pulse during the silent period between the train discharges. This repetitive firing was not observed in the control rats.

To observe the effects of nervous factors on burst discharges, neuromuscular transmission was blocked by surgical sectioning of the nerve or an intravenous injection of curare. Neither of these approaches had any effect on the myotonic burst discharges. However, after curarization, the irregular and intermittent discharges which occurred between the main burst discharges were blocked.

The resting membrane potential remained unaffected after treatment with anthracene in both denervated and curarized preparations. After application of anthracene, spontaneous spike discharges were recorded from both the diaphragm and EDL. Full-amplitude action potentials with a frequency of 10–30 Hz often appeared just after insertion of the microelectrode and these spikes subsequently decreased in frequency and ceased after several sec.

Table 1 shows the various passive membrane parameters measured from control and myotonic diaphragm and EDL muscles. In both muscles, the input resistance, specific membrane resistance, and length and time constants of the membrane were increased during the application of anthracene. However, the passive parameters measured on specific internal resistance or specific membrane capacitance and the fiber radius remained the same in both cases of the diaphragm or EDL in normal and myotonic preparations. This means that the action of anthracene appears mainly on the membrane resistance of muscle

Table 1. Passive membrane parameters measured from the diaphragm and EDL in the presence or absence of anthracene (10^{-4}M)

		n	R_{in} (MΩ)	λ (mm)	ρ (μm)	T (msec)	R_m (Ωcm^2)	R_i (Ωcm)	C_m (μF)
Dia.	C	9	1.24±0.48	0.50±0.07	15.5±4.4	1.7±0.3	1,072±36	318±18	1.6±0.2
Dia.	Anth.	11	1.92±0.70	1.07±0.09	17.2±4.6	5.1±0.8	4,012±220	302±20	1.4±0.2
EDL	C	11	0.65±0.14	0.69±0.06	24.3±3.7	2.3±0.6	1,320±130	334±60	1.7±0.3
EDL	Anth.	11	1.92±0.29	1.97±0.19	23.2±3.2	15.0±1.3	10,820±780	324±50	1.5±0.2

C and Anth: control and anthracene (10^{-4}M), respectively; R_{in}: input resistance of membrane; λ: length constant; ρ: radius of muscle fiber; T: time constant of membrane; R_m: specific membrane resistance; R_i: longitudinal myoplasmic resistance (internal resistance); C_m: specific membrane capacitance.

fibers.

Miniature end-plate potentials (m.e.p.p.s) were recorded before and during treatment with anthracene from the same end-plate in each experiment. With application of anthracene, the mean amplitude of m.e.p.p.s was increased, the duration was prolonged, and the mean amplitude was further increased by the addition of prostigmine. With the addition of curare, the amplitude of m.e.p.p. was markedly reduced. These results indicate that the potentiating action of anthracene on the amplitude of m.e.p.p.s is not due to inhibitorty effects on cholinesterase activity.

After application of anthracene, the bell-shaped distribution of m.e.p.p.s in the amplitude histogram shifted to the right along the horizontal line, and "composite m.e.p.p.s" of the amplitude over 10 mV were recorded. As the threshold depolarization required for the generation of the action potential was decreased after the application of anthracene, the "composite m.e.p.p.s" triggered the action potential of the muscle membrane. The hump observed during the rising phase of the spontaneous action potential was probably generated by the "composite m.e.p.p.s" due to an increase in the amplitude of m.e.p.p.s in the presence of anthracene.

To elucidate the role of the nervous system on myotonic discharges in the presence of anthracene, experiments were performed using denervated muscles. Rats were surgically denervated by excision of a 10-mm segment of the right sciatic nerve, just proximal to the bifurcation of the tibial and common peroneal nerves. The treated animals were separated into three groups, according to the duration of the denervation. One group was given 2 or 3 days intravenous administrations of anthracene 5 to 7 days after denervation, and the second group was given similar injections 20 to 30 days after denervation. The third group served as the controls (5 to 30 days after denervation). All of the denervated animals showed clinical signs of stiffness or a prominent response, to mechanical stimulation of the muscle. After the anthracene treatment, remarkable increases in the frequency of spontaneous electrical activities were observed. The discharges were continuous and regular-type with a high frequency, but dive-bomber patterns with a prepotential were not observed. The microelectrode studies done *in vivo* also demonstrated the generation of action potentials with a regular amplitude and high frequency.

The present results support the concept that chemically-induced myotonia is mainly due to alterations of the electrical properties of the muscle membrane.

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Muscular Dystrophy Group

MECHANISM OF THE INCREASE IN PROTEIN SYNTHETIC ACTIVITY IN CHICKEN DYSTROPHIC MUSCLE

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One of the most striking features of dystrophic muscle is the decay and disappearance of muscle cells after the onset of the disease. This phenomenon is essentially the result of the balance between the rates of synthesis and degradation. Therefore, it is interesting to know which is the more important reason, protein synthesis or degradation, causing this catastrophe to the muscle. To clarify this problem, the protein synthetic activity in chicken dystrophic muscle and that in the normal muscle were compared.

Dystrophic chickens (New Hampshire, line 413) and normal chickens (line 412) were used. Polyribosomes and the 160,000 $\times g$ supernatant were prepared from 13-day chick embryos by the usual method. The results of experiments on the incorporation of animo acids by these factors show that the synthesis of polypeptides by the factors from dystrophic breast muscle increased 1.35±0.10 (average ± S.E., n= 10) times more than that of factors from normal muscle, while that by the cardiac muscle is almost the same. These data clearly show that the protein synthetic activity of dystrophic muscle is activated even in an *in vitro* system. As shown in a previous paper, using the crude elongation factor fraction prepared from the dystrophic and normal 160,000 $\times g$ supernatants, it was shown that polyphenylalanine synthesis from phenylalanyl tRNA was activated by this dystrophic crude elongation factor. This difference between the

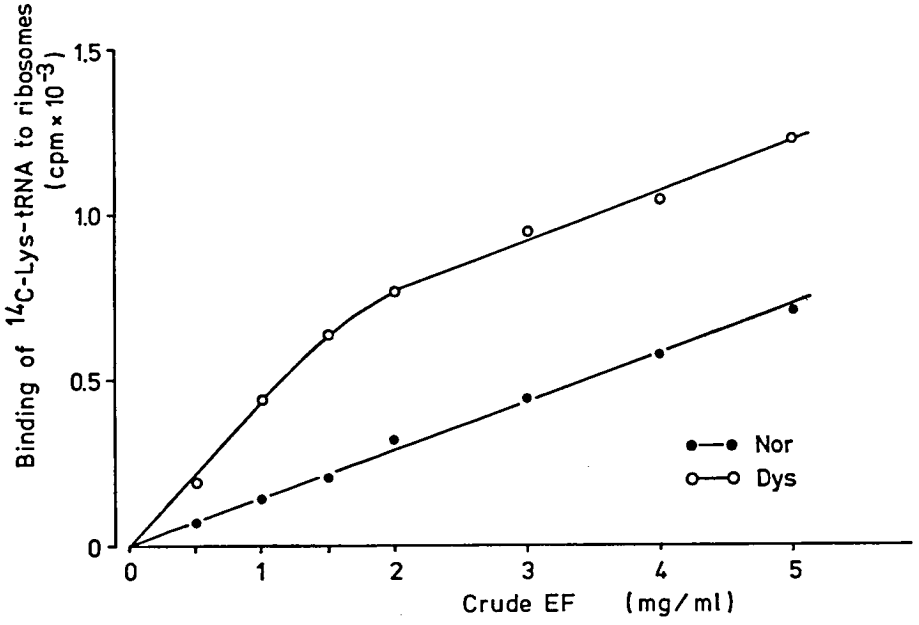

Fig. 1. Binding of [^{14}C]-lysyl-tRNA to puromycin-treated ribosomes in the presence of guanylyl (β,γ-methylene) diphosphonate (GMP-PCP) and crude elongation factors from normal and dystrophic chicken muscles 4 weeks after hatching.

dystrophic and the normal factors may be due to the difference in the amount of the elongation factors contained in both fractions rather than to a qualitative difference between the two factors.

To clarify this difference, the binding of ^{14}C-lysyl-tRNA to puromycin-treated ribosomes was measured in the presence of this factor and guanylyl (β,γ-methylene)-diphosphonate (GMP-PCP). One mole of ^{14}C-lysyl-tRNA binds to 1 mole of ribosome, and protein synthesis does not proceed to further steps under this condition. As shown in Fig. 1, ^{14}C-lysine bound to ribosomes increases as an increment of the elongation factor fraction. Above a 2 mg/ml concentration of the elongation factor fraction, the binding of ^{14}C-lysyl-tRNA to ribosomes increases linearly as an increment of the crude elongation factor fraction. The lines of the dystrophic and the normal factors form parallel lines. Therefore, from this data, the amounts of both the dystrophic and the normal protein which show the same effect on the binding of lysyl tRNA to ribosomes can be compared. Approximately three times more protein of the normal fraction is needed to induce the same effect as that of the dystrophic fraction. A similar experiment can be done using diphtheria toxin. The result obtained by this experiment corresponds very well to the results mentioned above. The ratio of the protein concentration of the 160,000$\times g$ supernatant of the dystrophic muscle to that of normal muscle at various developmental stages were as follows: 1.48 ± 0.39 ($n=4$) for 13-day embryos; 0.95 ± 0.11 ($n=2$) for the 2nd and 3rd days after hatching; and 0.68 ± 0.04 ($n=5$) for the 7–8th week after hatching. mhese data show that dystrophic muscle has a different turnover mechanism from that of normal muscle, and also suggest that the increase in the amount of protein factor necessary for protein synthesis in dystrophic muscle is not due to the apparent increase in muscle atrophy.

These data strongly suggest that the increase in the protein synthesis in chicken dystrophic muscle is due at least partly to the increment in the amount of elongation factor 1 in dystrophic muscle.

Other data also suggest an increase in the soluble protein factors. Kohama *et al.* showed a differential increase in the turnover rate of each myofibrillar protein in dystrophic muscle when compared with that in normal muscle. The present results together with their results suggest that in dystrophic muscle the synthesis of the some soluble proteins were activated. This problem is now under further investigation.

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Muscular Dystrophy Group

THE PROPORTION OF MONOMERIC AND POLYMERIC ACTINS IN EMBRYONIC AND DYSTROPHIC CHICKEN SKELETAL MUSCLES

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In developing muscle cells, contractile and regulatory proteins are synthesized and polymerized into filamentous forms, and then assembled into myofibrillar structures. Many investigations have been done to characterize the myofibrillar proteins in embryonic and dystrophic muscle tissues, and it has been demonstrated that the types of myofibrillar proteins present in embryonic[1,2] and dystrophic[3,4] skeletal muscles are somewhat different from those in normal adult tissues. However, the assembly of myofibrillar proteins in embryonic muscle cells has scarcely been investigated.

In the present investigation, the amount of monomeric and polymeric actins in embryonic and dystrophic chicken skeletal muscles was examined so as to understand the polymerization of nascent actin molecules into the filamentous forms in the developing muscle and the degradation of actin filaments in the dystrophic muscle.

The breast muscle from chicken embryos of various ages or the superficial pectoralis muscle of dystrophic (N.H., line 413) or normal (N.H., line 412) post-hatched chickens was used as materials. The isolated muscle tissues were homogenized in 3 to 5 volumes of phosphate-buffered saline containing 0.9% NaCl, 10 mM phosphate buffer (pH 7.0), 1 mM $MgCl_2$, 0.5 mM ATP, 0.1% Triton X-100, and 0.1 mM PMSF. The homogenates were centrifuged at 100,000×g for 2 hr and the resultant supernatant was used for the determination of the amount of monomeric actin in the tissue. An aliquot of the total homogenate was treated with 0.6 M KI to depolymerize all of the actin filaments into monomeric form and used for the determination of total actin concentration. In the presence of 0.6 M KI, both synthetic F-actin and actin

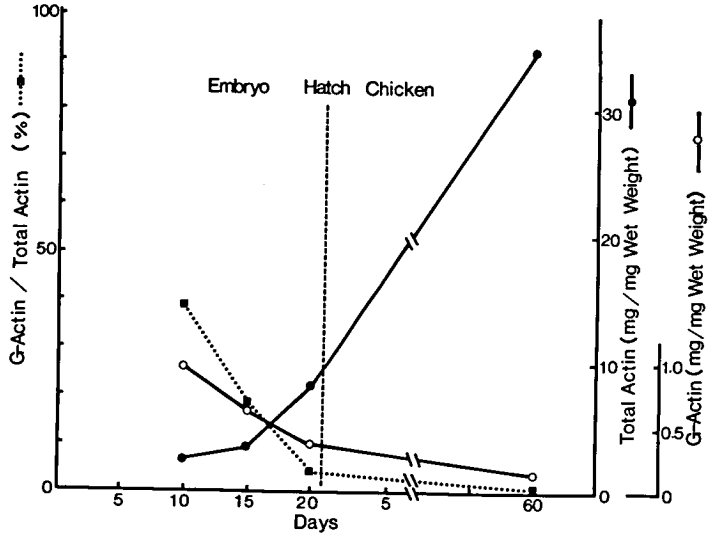

Fig. 1. Changes in G-actin concentration, total actin concentration, and the percent of G-actin in the total actin present in chicken breast muscle druing development.

Table 1. Comparison of G-actin concentration, total actin concentration, and the percent of G-actin in the total actin between dystrophic (D) and normal (N) chickens

	20-Day embryo	2-Month N-chicken	2-Month D-chicken
Total protein (mg/g wet weight)	81.3	222	172
G-actin (mg/g wet weight)	0.36	0.14	0.43
Total actin (mg/g wet weight)	10.4	34.8	24.8
G-actin/total actin (%)	4.3	0.4	1.73
Actin type	$\alpha(\beta,\gamma)$	α	α

Total embryo breast muscle and superficial pectoralis chicken muscle were examined. No difference in actin type was detected between normal and dystrophic chickens, as examined by isoelectric focusing electrophoresis.

filaments in myofibrils at a protein concentration range of up to 2 mg/ml were almost completely depolymerized within a half-hour. Actin was quantified by the DNase I inhibition assay method devised by Blikstad *et al.*[5] with minor modifications.

Figure 1 shows the changes in G-actin concentration, total actin concentration, and percent of G-actin in the total actin in chicken breast muscle during development. The embryonic chicken skeletal muscle at early developmental ages contains a larger amount of G-actin in comparison with well-developed muscle. In the breast muscle of 10-day embryos, about 1 mg/ml of G-actin was present, and about 40% of the total actin was present in the monomeric form. As muscle development progresses, the amount of total actin present in the skeletal muscle markedly increases, while the concentration of G-actin remarkably decreases. At 2 months after hatching, only about 0.14 mg/ml of G-actin, which constitutes about 0.4% of the total actin, was detected in the breast muscle. It is well known that if purified G-actin is put in physiological salt solution it is promptly polymerized to form F-actin, and G-actin coexists only at a level of about 0.05 mg/ml. In comparison with this value, the concentration of G-actin in embryonic muscle seems to be extremely high. When the monomeric actin in the soluble fraction of embryonic muscle was purified by ion-exchange column chromatography and ammonium sulfate fractionation, the actin became polymerizable. These observations suggest that polymerization of actin is suppressed by some factor in young embryonic muscle tissue.

Next, the G-actin concentration and the percent of G-actin in the total actin present in dystrophic chickens 2 months after hatching were compared with those in normal chickens at the same age. As shown in Table 1, the breast muscle of dystrophic chickens contains a higher concentration of G-actin than the breast muscle of normal chickens. It is suggested that the depression of actin-polymerization and/or the stimulation of the degradation of actin filaments may be stimulated.

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Muscular Dystrophy Group

NEONATAL DENERVATION OF RAT SCIATIC NERVE
I. THE EFFECT OF DENERVATION ON MYOSIN DIFFERENTIATION

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Myosin light chains in normal and neonatal denervated rat muscle were studied to examine the neural effect on the differentiation of myosin molecules. Those of fast or slow twitch muscle were identified by single fiber gel electrophoresis.

To study the role of nerves in the determination of fiber type, we investigated the effect of sciatic denervation in newborn rats on the postnatal development of fiber type and the change in myosin molecules. When the left sciatic nerve was severed at birth, the left hindlimb in all rats became paralytic. The diameter of all muscle fibers was decreased except for a few hypertrophied fibers in the extensor digitorum longus (EDL) and we could not detect any evidence of fiber type grouping or normal-sized fibers. Furthermore, when the peripheral nerves in the atrophic limb were examined, all myelinated fibers were small in diameter and poorly myelinated, suggesting a previous complete denervation in the proximal portion of the nerve. Figure 1 shows the protein patterns of denervated rat soleus and EDL muscles. The myofibrillar protein pattern of adult soleus fiber bundles showed that more than 70% of the fibers have a slow-type myosin light chain. Since two-dimensional gel electrophoresis of a single fiber in the adult rat soleus and EDL showed only one type of light chain in one fiber, the heterogeneity of L1 in lanes 1 and 2 resulted from a mixture of the slow and fast fibers in the bundle. On the other hand, the adult rat EDL muscle has only fast-type fibers as far as we observed (Fig. 1, lanes 3, 4). The blurred dissolution of L2 in Fig. 1 was attributed to the difference in the concentration of acrylamide. L1 is thought to be one of the most type-discriminating proteins on the gel over the wide range of acrylamide concentrations (13–16%). We observed an unexpected change in L1 after neonatal denervation. Almost all immature fibers in the neonatal soleus muscle turned into the mature fast-type biochemically (Fig. 1, lane 5) and histochemically (Fig. 2B) as described by Rubinstein *et al.* An embryonic light chain was not found in these muscles upon two-dimensional gel electrophoresis. On the other hand, an unusual thick band which corresponded to slow-type L1 (Fig. 1, lane 6) was observed in the denervated EDL muscle. The myosin ATPase stain revealed that a small number of intermediate fibers in the EDL muscle were hypertrophied, whereas the remaining type-2 fibers were fairly atrophied (Fig. 2D). Therefore the band of slow-type L1 was possibly derived from the hypertrophied and histochemically intermediate fiber. On the basis of these findings, it can be concluded that immature neonatal muscle fibers can become the fast type after denervation whether they are presumptive fast or slow.

Neonatal sciatic denervation caused a developmental arrest in the soleus muscle. Almost all the fibers began to synthesize a fast-type myosin. Since a newly synthesized fast-type myosin has L3 light chain, the denervated soleus muscle might not dedifferentiated, but differentiate into the fast-type. From these results it is concluded that in the soleus muscle neonatal sciatic denervation prevents the synthesis of slow-type myosin light chains without affecting the synthesis of fast-type light chains. The results suggest that the synthesis of slow-type myosin depends on a specific motor innervation at this stage of development.

On the other hand, a few hypertrophied fibers (5% of the total muscle fibers) were observed in EDL muscle after sciatic denervation. These fibers, which may have once been destined to become slow fibers, showed intermediate properties on gel electrophoresis (Fig. 1) and ATPase staining (Fig. 2). We cannot explain the appearance of such fibers only in EDL muscle. We observed that such intermediate fibers atro-

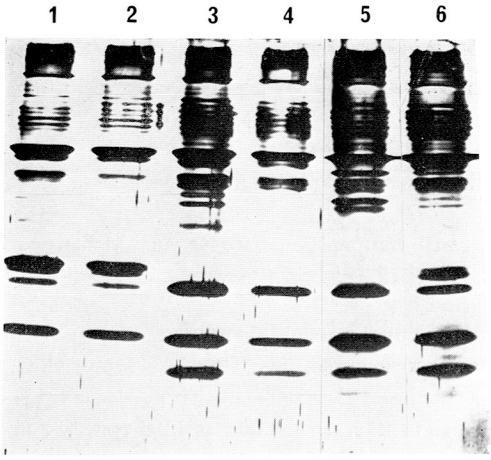

Fig. 1. SDS gel electrophoresis (14%) of denervated rat muscle. Lanes 1, 2: control adult soleus fiber bundle; lanes 3, 4: control adult EDL fiber bundle; lane 5: denervated soleus, single fiber; lane 6: denervated EDL, hypertrophied single fiber.

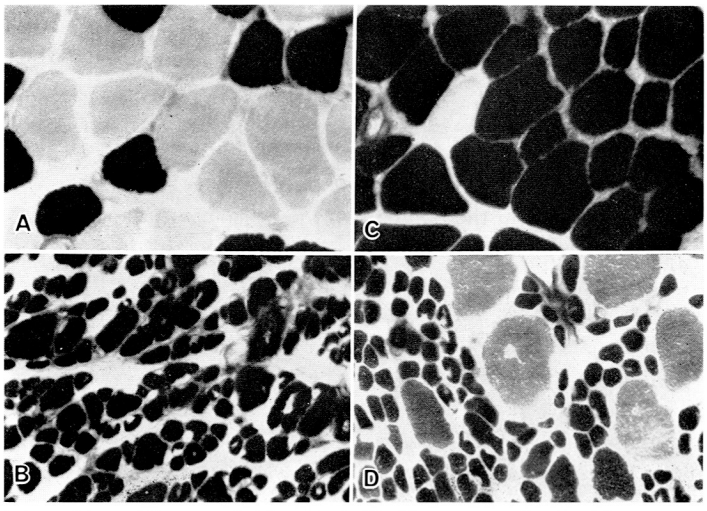

Fig. 2. 39-day-old rat. The type-1 (1) and -2 (2) fibers are mixed with a type-1 fiber predominance in the control soleus (A), whereas all muscle fibers behaved as type 2 with numerous myotubes in the soleus with neonatal denervation (B). In the control EDL (C), approximately 97% of the fibers are type-2 fibers. Note hypertrophic intermediate fibers with atrophic type-2 fibers in the EDL after neonatal denervation (D). A–D: routine ATPase.

phied after 60 days of neonatal denervation.

Whether immature muscle cells are programmed to be fast or slow, they have the potential to synthesize both types of myosin. We have directly demonstrated the coexistence of both types of myosin in denervated EDL muscle fiber using single-fiber gel electrophoresis (Fig. 1, lane 6). The same result was reported by Pette *et al.* with an experimental effect induced by electrical stimulation. There are two hypotheses suggested by Rubinstein *et al.* to explain these events. First, a change in muscle fiber type would involve atrophy of one fiber accompanied by the appearance of the other type. Alternatively, a muscle fiber itself could be changed from one type to the other. We favor the latter possibility on the basis of the results reported here.

Muscular Dystrophy Group

CONNECTIN, AN ELASTIC PROTEIN OF MUSCLE

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There are two kinds of major cytoskeleton structures in vertebrate skeletal muscle. One is the so-called intermediate filament surrounding the Z disc and linking it transversely to the neighboring Z lines[1]. The other is the connectin net longitudinally linking neighboring Z lines[2]. The intermediate filaments consist of desmin, a 55,000-dalton protein, and the connectin nets consist of connectin, very high molecular weight protein (probably more than one million daltons).

The present report is concerned with the localization of connectin within a myofibril, and the effects of muscle proteases.

1) Preparation of connectin: Connectin was purified from chicken breast muscle according to the method of Wang *et al.*[3]. We have already reported that Wang's titin is identical with connectin[4]. Chicken breast muscle was homogenized and well washed with 50 mM KCl containing 5 mM EGTA and 1 mM $NaHCO_3$. In 3% polyacrylamide gel electrophoresis, the connectin band with a doublet was very immovable; it was on the top of the gel, as seen in Fig. 1. When EGTA was omitted in the preparation of myofibrils, the connectin band was somewhat deteriorated (Fig. 1A, 1), and the addition of 1 mM $CaCl_2$ resulted in a decrease in the amount of one of the doublets (Fig. 1A, 2). This was probably due to the action of calcium-activated protease during preparation of myofibrils, as shown below. The connectin band slowly disappeared due to spontaneous breakdown in 1% SDS at room temperature. Thus, after 1 week's storage, it completely disappeared (Fig. 1B). Therefore, connectin should be prepared as quickly as possible, and must be stored at 0°C even in the presence of 0.1% SDS. The myofibrils were dissolved in SDS, and after removal of insoluble collagen, the solution was subjected to gel filtration using a Biogel A 50-m column[3]. Connectin was recovered in the first several fractions immediately following the elution of void volume.

2) Localization of connectin in myofibrils: Antiserum against isolated connectin was raised in a rabbit by injecting it with connectin from chicken breast muscle. The immunoglobins were obtained by affinity chromatography. Connectin was mixed with 10% polyacrylamide and the resultant gel sus-pension was packed onto a column and the adsorbed proteins were eluted by concentrated $MgCl_2$. The indirect immunofluorescence technique was applied to reveal the localization of connectin in myofibrils. Connectin was most concentrated in the A-I junction region, and the middle part of the A band and the whole I band were weakly stained with the antibodies. The M lines and Z lines were not stained at all, contradictory to the results of Wang *et al.*[3]. After removal of myosin, the free end region of the actin filaments was strongly fluorescent. Further extraction of actin with 0.6 M KI resulted in the appearance of fluorescent fibers between the Z lines of the sarcomere, but the Z lines were not stained. It appears that the connectin nets are involved in locating myosin filaments at the center of a sarcomere by attaching to both ends of myosin filaments. This view is in good agreement with Locker's gap filament theory[5]. After myosin and actin are dissolved away, the connectin nets are probably associated with each other to form bundles that can be seen under a microscope.

3) Effect of muscle proteases on connectin in situ: Connectin nets in myofibrils are very sensitive to chymotrypsin or trypsin. However, calcium-activated neutral protease (CANP)[6] did not hydrolyze connectin rapidly. With a weight ratio of 1:100 to myofibrillar proteins, CANP slowly split the upper band of the connectin doublet, and the lower band was broken down even slowly. This is of some interest, since in the breast muscle of dystrophic chicken the connectin content is appreciably decreased and the

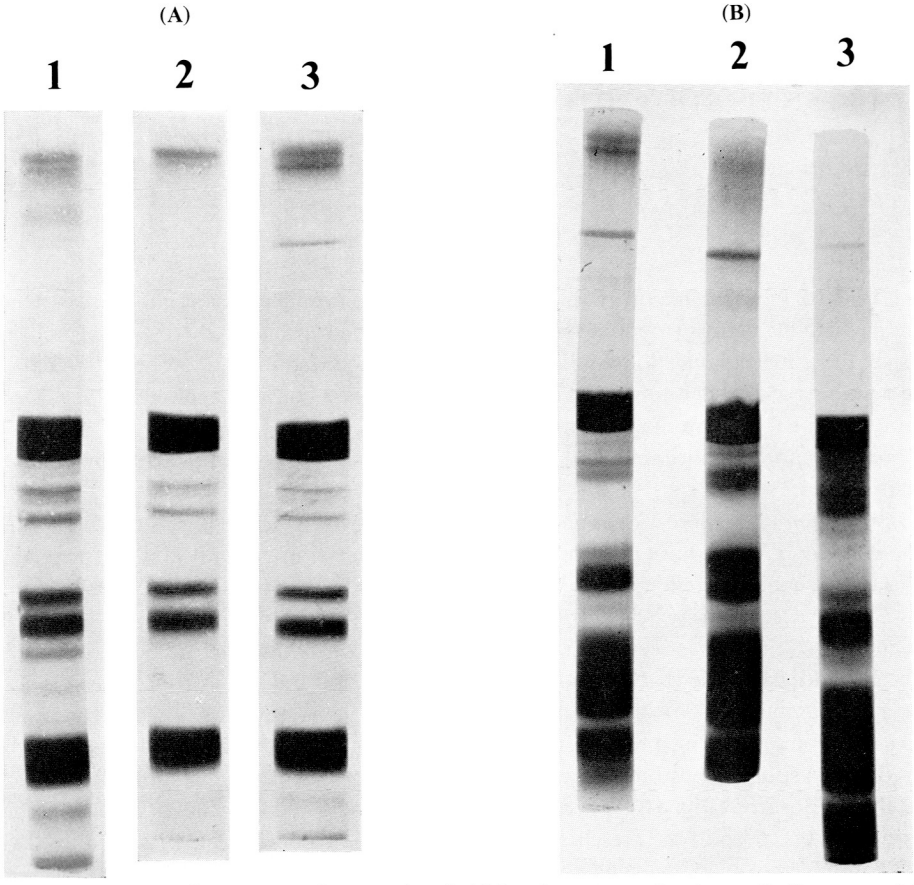

Fig. 1. SDS gel electrophoresis patterns of connectin of chicken breast muscle. A:myofibrils prepared in 50 mM KCl and 1 mM $NaHCO_3$ (1), prepared in the presence of 1 mM $CaCl_2$ (2), and prepared in the presence of 5 mM EGTA (3). B: effect of storage in 1% SDS at room temperature. Freshly prepared (1), stored for 2 days (2), and for 4 days (3).

amount of the upper band protein relative to the lower one is decreased. In dystrophic muscle it has been shown that CANP activity is enhanced. On the other hand, serine protease of mast cell origin[7] very quickly hydrolyzed connectin into small peptides, but they were still larger than the myosin heavy chain.

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MEMBRANE COMPOSITION AND CA-UPTAKE ABILITY OF SARCOPLASMIC RETICULUM FROM DYSTROPHIC CHICKEN

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Isolation of the sarcoplasmic reticulum (SR) from skeletal muscle of dystrophic chicken has been done by several investigators. We also attempted to prepare the fragmented sarcoplasmic reticulum (FSR) from the pectoralis muscles of dystrophic chicken (line 412 and 413) using the modified method of Nishijima *et al.*[1] at 8,000–365,000$\times g$ with Tris-buffer at pH 6.8 (H-fraction). In this preparation, the maximum value of Ca-uptake on FSR from dystrophic muscle was 180–198$\times 10^{-9}$ mol/mg protein/60 sec. This value showed no significant difference with comparing the normal muscle, 190–210$\times 10^{-9}$ mol/mg protein/30 sec. But the Ca-uptake ability decreased a 15% as compared to that of the normal muscles. The results as mentioned above might suggest the Ca-accumulating capacity of the SR from dystrophic muscle is almost similar to that of normal muscle.

It is suggested that the Ca-accumulating capacity of the SR seems to be smaller in dystrophic muscle[2], and also, destruction of the T-SR junction and especially long tubular parts have been observed by electron microscopic study. There exists a possibility that the FSR fraction alone keeping the normal function was prepared from dystrophic muscle in above experiment. Then, we examined a lighter fraction (L-FSR) than 36,500$\times g$ in which a part of long tubular one would be included. In the present experiment, we obtained two facts as follows: 1) the yield of this fraction in dystrophic muscle increased as compared with normal muscle; 2) the Ca-uptake ability of the L-FSR from normal muscles showed 120$\times 10^{-9}$ mol/60 sec, with maximum value of 135$\times 10^{-9}$ mol/120 sec, while in the L-FSR extracted from dystrophic muscle it showed to 65$\times 10^{-9}$ mol/60 sec, with a maximum value of 77$\times 10^{-9}$ mol/120 sec. Namely the Ca-uptake ability of this fraction was markedly inhibited.

In the experimental procedure, we recognized that the dystrophic muscle in buffer solution by Warring blender was more easily homogenized than the normal muscle. This indicates that dystrophic muscle has a weaker physical structure than normal muscle. From these results we considered that the increase in the yield of the FSR was not because of the proliferated membrane as speculated by Scales *et al.*[3] and Gideon *et al.*[4], but rather was due to the weakened membrane of long tubular system.

In next, we tried to fractionate the protein in the light-and heavy-fractions from normal and dystrophic muscles. These composite proteins were prepared with polyacrylamide gel electrophoresis carried out on disc gel using the Tris/Glycine system of Laemmli (1970)[7], as described by Weber and Osborn[8]. The amount of Ca-pump protein[5] of 100,000–110,000 daltons in the H-fraction of the FSR from dystrophic muscle was shown to be almost equal to that of the H-fraction from normal muscle. This protein has been reported by Mark and Baskin[6] to be the (Ca-Mg)ATPase protein, the main protein in the FSR-membrane. In the L-fraction of the FSR from dystrophic muscle, the (Ca-Mg)ATPase protein was markedly decreased in comparison with that from normal muscle.

The gels of the H- and L-fractions of the FSR showed two additional major bands in which one was a Ca-binding protein (mol. wt.$\approx$65,000) and another was protein $\approx$55 (mol. wt.$\approx$55,000). In the H-fraction of FSR from dystrophic muscle, the quantity of both proteins was the same or smaller than that in normal muscle. However, the quantity of both proteins in the L-fraction of the FSR from dystrophic muscle was markedly decreased.

It is well known that activity of the (Ca-Mg)ATPase is obviously related to the lipid environment,

Table 1. Yield of microsome from dystrophic muscle

	Control	Dystrophy
	(mg/g wet wt muscle)	
H-fraction	0.46	0.98
L-fraction	0.23	0.41

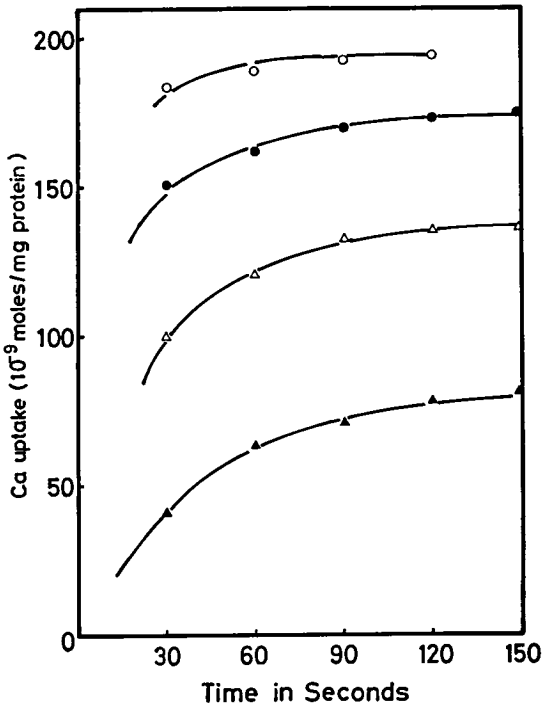

Fig. 1. The Ca-uptake activity of H- and L-FSR. ○△control; ●▲dystrophic muscle; circules: heavy fraction of FSR (H-FSR); triangles: light fraction of FSR (L-FSR).

and particularly to changes in cholesterol levels[9].

We have already reported that the cholesterol and FFA contents in the FSR from dystrophic muscle were increased 2-fold, and the T-G content was increased more than 10-fold. In the L-fraction of the FSR from dystrophic muscle, both contents were markedly increased and especially the T-G content was increased 20-fold. But the protein/lipid ratio in the L-fraction of the FSR from dystrophic muscle was decreased. Therefore, the increase in the yield of the long tubular membrane could be explained by increased buoyancy with the decrease of the protein/lipid ratio and the physically destructible membrane structure. In a tentative summary, we considered that the major factors in change of the biochemical activities have a close relation to the increase of T-G, FFA and cholesterol in the L-FSR of dystrophic muscle.

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STUDIES OF MYELIN-ASSOCIATED ENZYMES IN THE CENTRAL NERVOUS SYSTEM OF MURINE MUSCULAR DYSTROPHY

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Previous biochemical investigations showed a consistent pattern of myelin deficiency in the central nervous system of dystrophic mice. A pronounced reduction in the total amount of myelin recovered from the spinal cord of dystrophic mice at one month of age by differential ultracentrifugation has been demonstrated[1]. A severe decrease in the galactolipid concentration and a considerable increase in the cholesterol concentration, which are the main components of myelin structure, among total lipids have also been shown in the brain and spinal cord of dystrophic mice[2]. The findings of reduced activities of 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP) and cholesterol ester hydrolase (CEH), both enzymes known to be closely associated with the myelin fraction, in the brain and spinal cord of dystrophic mice also provide additional evidence for hypomyelination in the central nervous system of dystrophic mice[3]. In order to elucidate more clearly the disturbances of myelin metabolism in the central nervous system of dystrophic mice and to clarify the relationship between the neural abnormality and the development of muscular dystrophy, we investigated the developmental changes in CNP and CEH activities in the purified myelin fraction of the brain and spinal cord of dystrophic mice.

C57BL/6J-dy strain mice carrying dystrophia muscularis (dy) were bred in our laboratory. Homozygous affected (dy/dy) and unaffected littermate animals (+/?) were studied at several different ages. Mutants were identified by observation of periodic dragging of the rear feet and clasping of the hind limbs when the animal is lifted by the tail beginning 12–14 days after birth. The brain, including the cerebellum, brain stem, and medulla oblongata was removed and weighed. The spinal cord was removed by opening the spinal column. The tissues were homogenized in 10 volumes of ice cold 0.32 M sucrose in a teflon homogenizer. The purified myelin fraction was isolated with a conventional subcellular fractionation procedure, essentially according to Norton and Poduslo[4]. The homogenate was centrifuged at $900\times g$ for 10 min in a refrigerated centrifuge. The precipitate (P_1) was washed two times with 0.32 M sucrose. The supernatant (S_1) and washing solution were combined and layered over 0.85 M sucrose in a transparent tube. The tubes were centrifuged with a swing bucket rotor at $75{,}000\times g$ for 30 min. The layers of crude myelin at the interface of the two sucrose solutions were collected with a Pasteur-pipette and suspended in water by homogenization. After 1-hr dispersion for osmotic shock, this suspension was centrifuged at $75{,}000\times g$ for 15 min. The crude myelin pellet (P_2) was again dispersed in water and centrifuged at $12{,}000\times g$ for 10 min. The resulting supernatant (S_2) was centrifuged two times in the same manner. Each pellet was combined with the original myelin pellet (P_3) and resuspended in 0.32 M sucrose. This suspension was layered over 0.85 M sucrose in a transparent tube and centrifuged with the swing bucket rotor at $75{,}000\times g$ for 30 min. The purified myelin was removed from the interface with a Pasteur-pipette and used for determination of enzyme activity.

For the determination of CNP activity, the method of Olafson *et al.*[5] with 2′,3′-cyclic AMP as substrate and the method of Sogin[6] with 2′,3′-cyclic NADP as substrate were used with the purified myelin pretreated with 0.3 mg/ml Triton X-100 solution used as an enzyme source.

CEH activity in the purified myelin fraction was determined by the colorimetric procedure previously described[7].

The total activity of myelin CNP in both the brain and spinal cord of control mice was extremely low

during the first 8 post-natal days, after which time there was a rapid increase lasting until day 28. A slight and gradual decline in the activity appeared to occur from this period to adulthood. In the spinal cord of dystrophic mouse, the enzyme activity was extremely depressed, showing approximately 60% of the normal level during the maximum period, while the activity showed a similar age response, peaking at around 30 days after birth, in dystrophic as well as in control mice. At 14 days of age immediately after the appearance of symptoms, the activity in spinal cord of dystrophic mice already showed only 70% of the activity of normal littermates.

In the brain of dystrophic mice, the pattern of increase in total myelin CNP activity was identical to that of the controls during the first 28 post-natal days. After this period, a sudden decrease in the activity occurred in the brain of dystrophic mouse, reaching approximately 60% of the control level at around 40 days of age. Expressed on the basis of the protein content, however, the activity of myelin CNP in both the brain and spinal cord of dystrophic mice was not affected. These findings indicate that the reduction in the total activity of myelin CNP in the central nervous system of dystrophic mice correlates with a reduction in the total amount in the myelin sheath rather than with a specific lack in the synthesis of CNP enzyme protein.

A rapid increase in the specific activity of myelin CEH in the developing and maturing spinal cord of control mice occurred during the period from 8–20 days to reach almost a 20-fold increase over the newborn level. After this period, the activity remained constant or possibly declined slightly toward the adult level. The activity in dystrophic mice was extremely depressed, showing approximately 60% of the control level immediately after the appearance of the symptoms, and reached only about half of the normal level at adulthood. The developmental pattern of myelin CEH in the brain was different from that in the spinal cord.

In the brain of control mice, a rapid increase in the activity of myelin CEH occurred during days 8–30. After this period, a gradual but steady increase appeared to continue toward the adult level. In the brain of dystrophic mice, the pattern of increase of specific activity of myelin CEH was identical to that of normal littermates during the first 20 post-natal days, after which time a sudden decrease occurred lasting until 30 days to reach approximately 50% of the control level.

The developmental changes in CNP and CEH activities indicate that these enzymes are exclusively localized in the myelin fraction of the central nervous system. Not only does the sharp increase in activity coincide with the period of active myelination, but the changes also appear to reflect precisely the differences in developmental characteristics of the brain and spinal cord. The results obtained in this experiment clearly point out that the process of myelin formation in the central nervous system of dystrophic mice was affected more severely in the spinal cord than in the brain and that there might be some differences in the causes of myelin deficiency in these two organs. The present findings correlating enzyme activity with the reduced synthesis of myelin suggested that myelin CEH activity plays an important role in the development of hypomyelination in the central nervous system of dystrophic mice. Earlier work by Rabinowitz[8] and Kabara[9] has shown an increased rate of cholesterol synthesis and a decreased concentration of cholesterol-esters in the brain of dystrophic mice. This, in combination with our observations, suggests that the central nervous system of dystrophic mice is unable to bring about the combination of cholesterol with the other myelin constituents to form a mature myelin membrane.

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MODE OF PROTEOLYSIS OF MYOFIBRILLAR STRUCTURAL PROTEINS BY CATHEPSIN-B

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The intracellular concentrations of myofibrillar proteins are regulated by their rates of synthesis and degradation. In atrophic muscle, the contents of myofibrillar structural proteins and the intensity of muscular contractions are markedly decreased. We have reported that a marked increase in cathepsin-B activities was observed in atrophic muscles of DMD patients[1], dystrophic mice (C57BL/6J-dy/dy)[2], and dystrophic chickens (line 413)[3]. It is possible that cathepsin-B may play some role in both the decreases in the contents of structural proteins and in the intensity of muscular contractions. Some myofibrillar proteins are degraded by cathepsin-B. At a molar ratio of cathepsin-B to substrate of 1:100, the order of susceptibilities was myosin$\gg$troponin$>$tropomyosin$\gg$actin. α-Actinin was not degraded. The myosin heavy chain was degraded into several fragments with molecular weights of 175,000, 170,000, 160,000, and 145,000, whereas the light chains were scarcely degraded. Cathepsin-B degraded troponin-T rapidly, and troponin-I more slowly, but did not degrade troponin-C. Troponin-T and troponin-I were degraded into three fragments with molecular weights of 30,000, 18,000, and 12,800. Tropomyosin was degraded slightly and its product had a molecular weight of 32,000. Actin was also degraded only slowly, and no liberated product could be detected. Morphological changes in myofibrils prepared from glycerinated rabbit psoas muscle during incubation with cathepsin-B were observed. Three notable phenomena were observed under phase-contrast microscopy: 1) a disappearance of the Z-band in the early stage of incubation: 2) a decrease in the density of the A-band; and 3) an apparent enlargement of the width of the H-band. Electron microscopic observations and liberated products from myofibrils during incubation with cathepsin-B gave appropriate explanations for these phenomena. α-Actinin, the chief substance constituting the Z-band, was released from the myofibrils by cathepsin-B treatment. The Z-band may be peeled off from the thin filaments through the degradation of some protein contributing to the attachment. The A-band lost its density but the cross-bridge part might be not destroyed during incubation with cathepsin-B. Myosin may be degraded at the edge of the rod portion.

We were surprised to find that the M-line was released from myofibrils into the supernatants of the incubation medium without any change in antigenecity against the antibody to M-protein. No protease has been reported that can strip the M-line from myofibrils. The disappearance of the M-line may result in the enlargement in the breadth of the H-band under phase-contrast microscopy.

We have already reported that cathepsin-B degrades aldolase preferentially and the increase in the activity of cathepsin-B is correlated with the decrease in the activity of aldolase. Therefore it is possible that the contents of myofibrillar structural proteins in dystrophic animals decrease and that their qualitative function changes due to attack by cathepsin-B, and that the anaerobic ATP supply for muscular contraction is also reduced. For these reasons, the whole muscular function may be strongly damaged by cathepsin-B.

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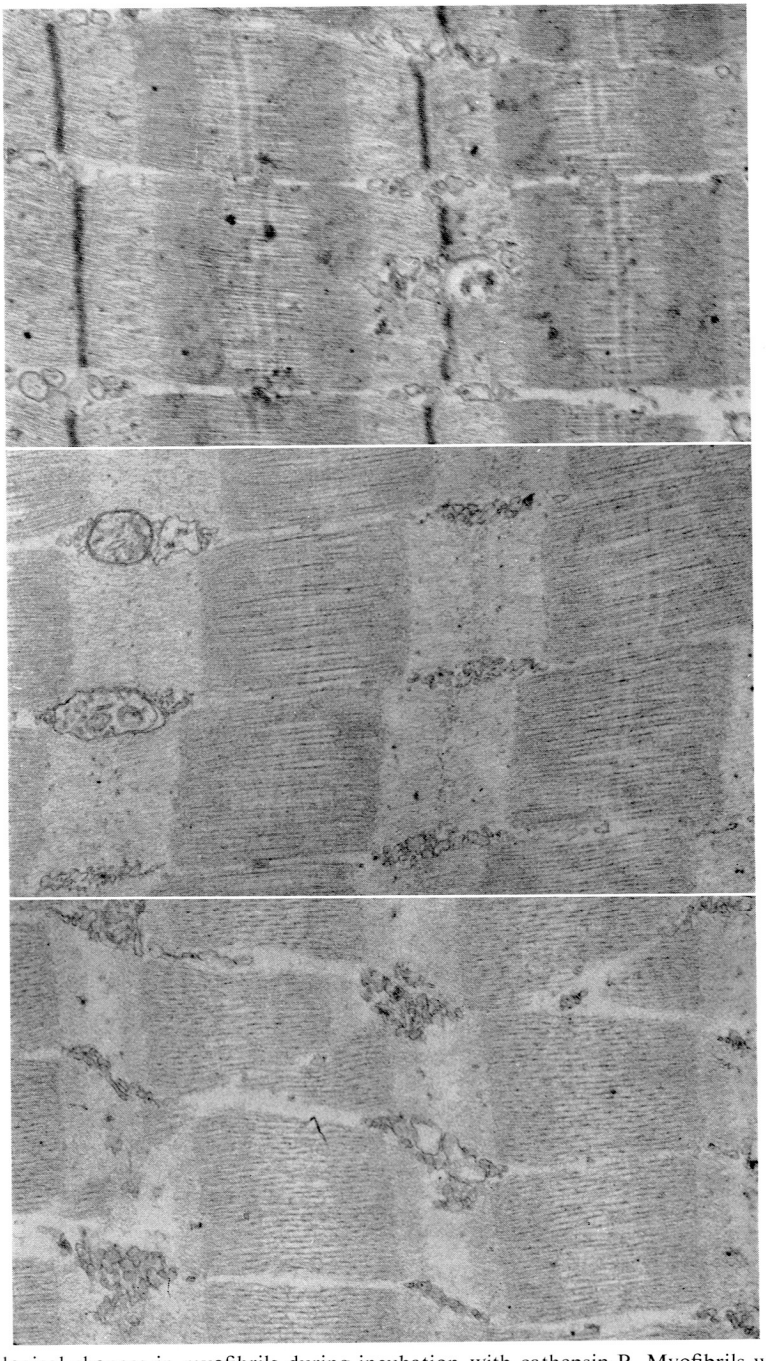

Fig. 1. Morphological changes in myofibrils during incubation with cathepsin-B. Myofibrils were incubated with 50 mM KPB, pH 6.0, 5 mM 2-mercaptoethanol, and 1 mM EDTA with cathepsin-B. Reaction was terminated by adding E-64 and centrifuged at 3,000 rpm for 5 min. The resultant pellet was applied to electron microscopy.

Muscular Dystrophy Group

ENZYMATIC RELATIONS IN DYSTROPHIC MICE AND CHICKENS

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In previous papers we demonstrated that the activities of aminopeptidases, endopeptidases, glycosidases, phosphatase, esterase, and ribonuclease are abnormally elevated in various organs of dystrophic mice and chickens when compared to the controls[1,2]. This suggested the potential usefulness of several enzyme inhibitors found in our laboratories[3,4]. The purpose of the present study was to elucidate the relationship of enzymatic changes in dystrophic mice to those in dystrophic chickens.

Normal and dystrophic mice (C57BL/6N) as well as normal and dystrophic chickens (line 412 and line 413) were used as experimental animals. The animals were killed by cervical dislocation and homogenates of organs and muscles were prepared in 10 times their volume of phosphate-buffered saline (PBS, pH 7.2) by using an Ultraturrax at the maximal speed for 1 min. The homogenate was centrifuged (5,000 rev./min, 20 min), and the supernatant was withdrawn and used for enzyme sources. The methods for enzyme assays have been presented in detail elsewhere[1].

In muscle from dystrophic mice, the most significant difference was seen in activities of glycine aminopeptidase (Gly-AP), Ala-AP, Leu-AP, Met-AP, Phe-AP, Trp-AP, Gly-His-Lys-AP, N-Ac-β-D-glucosaminidase, and free RNase ($P<0.001$). A highly significant difference was seen in aminopeptidase A (AP-A), cathepsin-C, α-D-glucosidase, and total RNase activities ($P<0.01$). A significant difference was seen in Ser-AP, Gly-Pro-Leu-AP, cathepsin-D, β-D-galactosidase, α-D-mannosidase, and esterase ($P<0.05$).

In dystrophic chickens, highly significant increases were seen in the activities of AP-A, AP-B, Gly-AP, Ala-AP, Ser-AP, Pro-AP, Leu-AP, Met-AP, Trp-AP, cathepsin-D, N-Ac-β-D-glucosaminidase, esterase, and total RNase ($P<0.01$). Significant increases were seen in Phe-AP, chymotrypsin-like, elastase-like, and phosphatase activities ($P<0.05$), and possibly significant increases in Gly-His-Lys-AP, N-Ac-

Table 1. Comparisons between controls and dystrophic mice of aminopeptidases in muscle

Enzyme	Activities ± S.D. ($n=4$)		t-Value	Significance of difference
	Control	Dystrophy		
AP-A	0.73±0.08	1.68±0.32	5.76	$P<0.01$
AP-B	5.02±1.11	5.38±1.14	0.45	NS
Gly-AP	0.65±0.17	2.10±0.27	9.07	$P<0.001$
Ala-AP	5.77±0.96	14.13±0.50	15.42	$P<0.001$
Ser-AP	0.51±0.18	0.85±0.17	2.72	$P<0.05$
Pro-AP	1.10±0.50	1.08±0.43	−0.06	NS
Leu-AP	3.59±0.31	7.70±0.20	22.26	$P<0.001$
Met-AP	4.64±0.51	11.80±0.60	18.26	$P<0.001$
fMet-AP	5.32±1.31	4.58±0.34	−1.10	NS
Phe-AP	4.97±0.70	12.40±0.80	13.59	$P<0.001$
Trp-AP	0.96±0.15	3.18±0.05	27.42	$P<0.001$
Gly-Pro-AP	0.52±0.03	0.85±0.13	4.94	$P<0.01$
Gly-Pro-Leu-AP	0.50±0.14	0.80±0.14	3.04	$P<0.05$
Gly-His-Lys-AP	0.09±0.04	0.40±0.00	14.48	$P<0.001$

Enzyme activities are expressed as nmol/min/mg protein.

Table 2. Comparisons between control and dystrophic mice of various enzymes in muscle

Enzyme	Activities* S.D. ± (n=4)		t-Value	Significance of difference
	Control	Dystrophy		
Trypsin-like	17.00±10.70	8.40± 3.74	−1.51	NS
Chy-try-like	12.20± 4.80	18.60± 2.40	2.39	$P<0.1$
Elastase-like	8.12± 8.95	15.60± 6.90	1.33	NS
Cathepsin-C	0.41± 0.09	1.53± 0.36	6.02	$P<0.01$
Cathepsin-D**	0.00± 0.00	0.01± 0.00	3.27	$P<0.05$
α-D-glucosidase	0.20± 0.01	0.43± 0.05	5.20	$P<0.01$
β-D-galactosidase	0.04± 0.05	0.20± 0.08	3.28	$P<0.05$
α-D-mannosidase	0.08± 0.09	0.20± 0.00	2.87	$P>0.05$
N-Ac-β-D-glucosaminidase	0.17± 0.05	0.98± 0.10	14.72	$P<0.001$
Phosphatase	1.25± 0.30	1.78± 0.53	1.72	NS
Esterase	14.10±16.00	80.10±45.40	2.74	$P<0.05$
RNase (free)***	1.08± 0.17	5.53± 0.70	12.30	$P<0.001$

*Enzyme activities are expressed as nmol/min/mg protein.
**Cathepsin-D activity is expressed as absorbance (at 280 nm)/min/mg protein.
***RNase activities are expressed as unit/mg protein. 1 unit is equivalent to the activity of 1 ng standard RNase from bovine pancreas.

β-D-glucosaminidase, and free RNase ($P<0.1$). α-D-glucosidase, β-D-galactosidase, and α-D-mannosidase activities were greatly and significantly decreased in dystrophic chickens ($P<0.01$).

In order to compare these enzymatic changes in dystrophic chickens with those in dystrophic mice, another statistical approach was adopted. The t-values obtained from Student's t-test (Tables 1 and 2) were considered to be useful indices to express the statistical difference between the dystrophic animals and their controls. The t-values for 14 kinds of aminopeptidases showed a significant correlation between the two kinds of model animals ($\gamma=0.55$, $P<0.05$). This may mean that the increases in these enzymes are not independent of each other, but that they collectively perform some sequence of events. In contrast, no significant correlation was seen in the other enzymes.

These results indicate that the intramuscular metabolic abnormalities in dystrophic chickens are generally different from but are partly correlated with those in dystrophic mice.

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STUDIES ON NEUTRAL PROTEASES IN MUSCLE

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Previously we reported the occurrence and some properties of neutral protease(s) firmly bound to the microsomal membranes of rat skeletal muscle[1] as well as rat[2] and monkey livers[3], which could be solubilized only in the presence of certain detergents such as sodium cholate and deoxycholate. As a continuation of these studies, we have investigated in more detail the effects of various protease inhibitors on the caseinolytic activity and coexisting aminopeptidase activity of a partially purified enzyme preparation obtained as described previously[1]. We have also investigated the substrate specificity of the enzyme preparation toward various urea-denatured proteins and aminopeptidase substrates. In addition, we have compared the activities of such neutral proteases in the microsomal and/or supernatant fractions of the breast muscle of normal and dystrophic adult chickens.

As partly reported previously[1], the caseinolytic activity (pH 8.0, 25°C)[2] was inhibited significantly by diisopropylphosphorofluoridate (DFP), phenylmethanesulfonylfluoride (PMSF), N^{α}-tosyl-L-lysine chloromethyl ketone (TLCK), N^{α}-tosyl-L-phenylalanine chloromethyl ketone (TPCK), *p*-chloromercuriphenyl sulfonate (pCMPS), chymostatin, antipain, EDTA, EGTA, and *o*-phenanthroline. Other reagents tested (dithiothreitol, pepstatin, leupeptin, elastatinal, bestatin, soybean trypsin inhibirot, adzuki-bean proteinase inhibitor I, heparin, $CaCl_2$, and $MgCl_2$) were much less effective or without any effect. On the other hand, the aminopeptidase activity (measured with L-leucine *p*-nitroanilide as substrate at pH 7.0 and 37°C)[3] was inhibited by *o*-phenanthroline more strongly than the caseinolytic activity, whereas DFP, PMSF, TLCK, and TPCK were much less inhibitory. These results clearly show that the enzyme preparation is a mixture of neutral endopeptidase(s) and aminopeptidases.

The microsomal membrane-bound enzyme preparation hydrolyzed various urea-denatured proteins and the relative rates of hydrolysis were in the order: histone (793)> protamine (181)> hemoglobin (175) > casein (100)> myosin B (37)> fibrinogen (25)> γ-globulin (21)> bovine serum albumin (19)> ovalbumin (16)> myosin A (3). Since histone and protamine are good substrates, the enzyme(s) appears to be trypsin-like. On the other hand, the specific activities (nmol/hr/mg protein) toward various aminopeptidase substrates were in the order: Gly-Pro-MCA (616)> Ala-2NA (169)> Leu-2NA (108)> Arg-2NA (57)> Gly-Phe-2NA (41)> Cys-di-2NA (13)> Pro-2NA (12) (MCA: methylcoumarine amide; 2NA: 2-naphthylamide). The high activity toward Gly-Pro-MCA may be due mainly to membrane-bound dipeptidylaminopeptidase IV.

The microsomal fraction of chicken breast muscle was also shown to contain membrane-bound neutral proteases including aminopeptidases. The caseinolytic activities both per protein weight and tissue weight were found to be higher in dystrophic chicken muscle than in normal chicken muscle, but the differences were within 2-fold. The differences were much greater in aminopeptidase activities. The specific activities (nmol/hr/mg protein) of the microsomal membrane-bound aminopeptidases of normal chicken muscle toward synthetic substrates were in the order: Leu-2NA (153)> Ala-2NA (125)> Gly-Phe-2NA (83)> Arg-2NA (67)> Cys-di-2NA (25)> Pro-2NA (21)> Gly-Pro-MCA (16). This result is quite different from that obtained for the microsomal aminopeptidases of rat skeletal muscle and appears to reflect a species difference. The dystrophy to normal ratios (D/N) of the specific activities were Arg-2NA (7.9, 11.5), Gly-Pro-MCA (4.1, 6.0), Leu-2NA (3.2, 4.7), Ala-2NA (3.1, 4.6), Pro-2NA (2.0, 3.0), Gly-Phe-2NA (1.4, 2.0), and Cys-di-2NA (1.2, 1.7) (the values in parentheses are the D/N ratios of the specific activities per protein weight and tissue weight, respectively). The elevation of activities toward Arg-2NA and Gly-Pro-MCA

in dystrophic muscle is especially notable.

The aminopeptidase activities in the supernatant fraction of chicken breast muscle had a spectrum different from that of the microsomal membrane-bound aminopeptidase activities. The specific activities (nmol/hr/mg protein) were in the order: Ala-2NA (1220)> Arg-2NA (766)> Leu-2NA (560)> Pro-2NA (186)> Gly-Phe-2NA (93)> Cys-di-2NA (30)> Gly-Pro-MCA (2). These aminopeptidase activities were also found to increase markedly in dystrophic muscle. The D/N ratios were Gly-Pro-MCA (6.5, 2.5), Pro-2NA (4.7, 1.8), Gly-Phe-2NA (4.3, 1.7), Ala-2NA (3.7, 1.4), Arg-2NA (3.6, 1.4), Leu-2NA (3.6, 1.4), and Cys-di-2NA (2.6, 1.0) (the values in parentheses are those per protein weight and tissue weight, respectively).

Increased levels of calcium-activated neutral protease and cathepsin-D activities in the supernatant fraction were also observed for dystrophic chicken muscle. When the supernatant fraction was passed through a column (1.7×150 cm) of Sepharose CL-6B, the aminopeptidase activities toward Gly-Phe-2NA, Arg-2NA, and Leu-*p*-nitroanilide were eluted at a position corresponding to an apparent molecular weight of 63,000, and cathepsin-D at a position corresponding to an apparent molecular weight of 30,000–35,000. No difference in elution position was observed between normal and dystrophic muscle. On the other hand, the elution patterns of the calcium-activated neutral protease were apparently different between normal and dystrophic muscle. In the case of normal muscle, a single protease peak was eluted at a position corresponding to an apparent molecular weight of 47,000, while in the case of dystrophic muscle, the major peak was eluted at a position corresponding to an apparent molecular weight of 100,000, with a shoulder corresponding to an apparent molecular weight of 47,000. The molecular weight of the calcium-activated neutral protease of chicken muscle has been reported to be 80,000[4]. The reason for this apparent discrepancy remains to be elucidated. It may be due to the presence of an inhibitor of this protease in the eluted fractions.

In order to elucidate the roles of the membrane-bound neutral proteases in the metabolism of muscle proteins, it is necessary to further purify them to homogeneity and investigate their properties including their mode of action on muscle proteins. Further studies are also necessary on their localization in muscular tissues. On the other hand, for comparison of protease activities in normal and dystrophic muscle, it seems important to do similar comparative studies with mammalian species such as mice. It will be also necessary to investigate and compare the levels of protease inhibitors in muscle tissues.

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THE EFFECT OF CALCIUM IONS ON CALCIUM-ACTIVATED NEUTRAL PROTEINASE

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We consider that calcium-activated neutral proteinase (CANP) has a very important role in the initial step of myofibrillar protein degradation because (i) CANP specifically removes the Z-band, which is important in maintaining the myofibrillar structure[1]; and (ii) CANP does not locate itself in lysozomes but in the Z-band as well as in the soluble fraction[2]. Our studies on CANP were initiated under the assumption that it plays an important role in the degradation of myofibrillar protein in muscular dystrophy, where serious muscle atrophy is observed.

Recently, we purified CANP to a homogeneous state for the first time from chicken breast muscle[3]. We also purified it from human[4], monkey, and rabbit skeletal muscle. The CANPs thus far purified from various sources resemble each other as far as their enzymatic properties are concerned, though varying results have been reported on the molecular weight and the subunit composition of this proteinase. This enzyme clearly has an essential thiol group at its catalytic site, but also has an absolute requirement for Ca^{2+} for its activity. The role played by the metal ion in the reaction mechanism has not been elucidated, but it is very important. The results obtained concerning this problem will be reported here.

We used purified rabbit skeletal muscle CANP. Judging from disc gel electrophoresis, this proteinase was purified to a homogeneous state by a method similar to that applied for the preparation of human muscle CANP[4]. The proteinase gave two bands upon SDS gel electrophoresis. The molecular weights of these two subunits were estimated to be 80,000 (80 K) and 31,000 (30 K), respectively, and their amount ratio was 1.0: 0.8–1.2 by tracing the gels. The active site was located on the 80 K subunit.

It is very difficult to examine the role of Ca^{2+} on CANP, since the proteinase is easily inactivated by autolysis in the presence of Ca^{2+}. We found, however, that autolysis was fully suppressed when E-64 was present in the enzyme solution. In this condition, the presence and absence of Ca^{2+} made CANP behave differently upon disc gel electrophoresis or on the DEAE cellulose column chromatography. Furthermore, it dissociated into its subunits only in the presence of Ca^{2+}. These phenomena are specific for Ca^{2+}, and are also observed in carboxymethylated CANP (CM-CANP), which was modified with IAA and was completely inactivated. CANP was carboxymethylated as follows. After the addition of IAA (molar ratio: 100/one enzyme), the CANP solution was incubated for 4 hr at 30°C. Caseinolytic activity was lost by this incubation. The mixture was dialyzed against IAA-free buffer. By this procedure, IAA modified about 1.4 cystein residures of the enzyme (the 80 K subunit). We used this modified enzyme in further experiments. Many properties tested so far, except for the catalytic activity of CM-CANP, are indistinguishable from those of native CANP. As stated above it was indicated that the p*I* of the CANP changed from 4.3 to 4.9 when Ca^{2+} (5 mM) was added, as seen in the electrophoresis on celluloseacetate strips. This observation suggested that: (i) Ca^{2+} ions cancelled some of negative charges on the molecule of CANP, thus the apparent p*I* changed; or (ii) Ca^{2+} induced a conformational change in CANP. Although the former possibility cannot be ruled out, evidence which supports the conformational changes was obtained. The CD spectra drastically changed when Ca^{2+} was added to the CANP in the wavelength range from 260 to 340 nm. This wavelength range usually gave us information on the structure in the vicinity of the Trp, Tyr, Phe, and Cys-Cys residues. In this case, these CD spectral changes were attributed to a change in the environment of some Trp and/or Tyr residues. These spectral changes were specific to Ca^{2+} and showed dependency on the Ca^{2+} concentration. Furthermore, this structural change and the appearance of activity

of the native enzyme gave the same Ca^{2+} dependency. Similar results were obtained for the UV difference spectra. On the other hand, we could hardly observe the CD spectral change in the wavelength range (200–250 nm) which reflects the secondary structure. That is, gross structural changes in the polypeptide backbone will not take place through Ca^{2+} binding. As previously stated, CANP dissociated into its subunits when Ca^{2+} was added. One might think that the CD and UV spectral changes may be due to the dissociation of the 30 K from the 80 subunit, but this probability was ruled out since the isolated 80 K subunit itself showed the same structural changes as those of CANP. Thus Ca^{2+} induces rearrangements of some specific amino residures of CANP which are necessary for the appearance of the activity.

These results suggested that CANP could have two forms: an inactivated form and an active form. The role of Ca^{2+} on the enzyme is to change the structure from the inactive form to the active form, and to dissociate the proteinase into its subunits. The metal ion concentration that is required for these structural changes is, however, much higher than that of intracellular Ca^{2+}. A factor that enables CANP to change its structure at the physiological concentration of Ca^{2+} may exist *in vivo*. We are now examining this possibility. Moreover, another type of CANP, which operates under the μM order concentration of Ca^{2+} was discovered by Mellgren[5]. The relationship between our CANP and the new CANP is also now under investigation.

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Muscular Dystrophy Group

EFFECT OF *N*-[*N*-(L-*TRANS*-3-CARBOXYOXIRANE-2-CARBONYL)-L-LEUCYL]-3-METHYL-BUTYLAMINE, A PROTEASE INHIBITOR, ON PROTEIN DEGRADATION IN INTACT MUSCLE CELLS

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The effect of a low concentration of *N*-[*N*-(L-*trans*-3-carboxyoxirane-2-carbonyl)-L-leucyl]-3-methyl-butylamine (E64C) upon protein degradation in isolated or cultured muscles was examined.

1) Binding of E64C to muscle: The amount of E64C retained in adult rat soleus muscle was 6.6 times larger than the amount of inulin (Fig. 1), when it was incubated in Krebs-Ringer solution (KR)[1] containing 12.6 μCi/ml of ^{3}H-E64C (3.4×10^{-5} g/ml) or ^{3}H-inulin. The amount of E64C bound to the muscle was 2.3 μg/g wet weight of muscle.

2) Effect of E64C on degradation of muscle proteins as a whole: The rate of degradation of proteins in muscle as a whole was estimated from the amount of tyrosine released in KR containing cycloheximide (KR-CH) after 2 hrs' incubation[1,2]. Larger amounts of tyrosine indicate higher rates of protein degradation. The amount of tyrosine in the absence (TyrC) or presence (TyrEC) of 2.5×10^{-6} g/ml E64C was 235.8 ± 91.5 ($n=13$) or 196.2 ± 74.8 ($n=13$) (nmol/g wet weight of muscle, $\pm$ S.D.), respectively (Table 1). The differences between TyrC and TyrEC were significant ($P<0.05$).

There was a tendency for the effect of E64C to be more pronounced in muscles for which the TyrC value was higher. If we take the values higher than 250 into consideration, it is highly significant ($P<0.005$).

E64C was effective even at a concentration as low as 2.5×10^{-8} g/ml in KR-CH. E64C was more effective when 10^{-5} g/ml of ionophore A23187 coexisted in KR-CH; the value of [TyrC-TyrEC]/TyrC in the

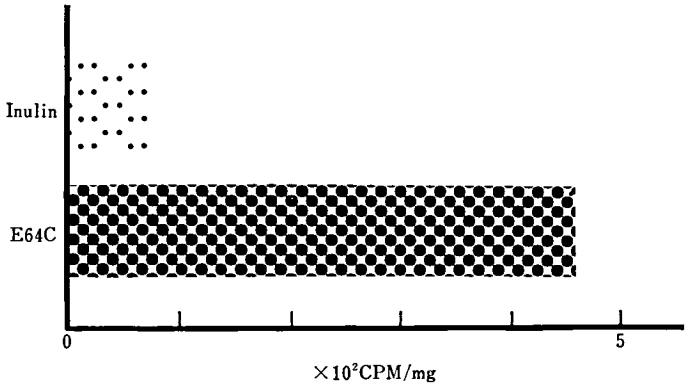

Fig. 1. A couple of adult rat soleus muscles were isolated from tendon to tendon. After weighing, one was incubated for 2 hr in KR containing 12.6 μCi/ml of ^{3}H-inulin and 0.17 μCi/ml of ^{14}C-inulin, and the other in KR containing 12.6 μCi/ml of ^{3}H-E64C (3.4×10^{-5} g/ml) and 0.17 μCi/ml of ^{14}C-inulin. The muscles were taken out of the KR and homogenized in 0.3 M KCl containing 0.2 N NaOH. The radioactivity of the homogenate was counted with a scintillation counter. Solvent space was calculated by the radioactivity of ^{14}C, and ^{3}H radioactivity in the space was subtracted from the total ^{3}H radioactivity. Bars show ^{3}H radioactivity remaining in the muscles.

Table 1. Amounts of tyrosine released in KR-CH (TyrC) or KR-CH containing E64C (TyrEC) (nmol/g wet weight of muscle)

No. of experiment	TyrC	TyrEC
1	252	103
2	388	292
3	195	199
4	203	180
5	188	184
6	447	362
7	230	214
8	221	246
9	283	117
10	209	228
11	168	166
12	112	103
13	170	156
Mean	235.8	196.2

Procedures for isolation, weighing and incubation of the soleus muscle were the same as described in the legend to Fig. 1 except that the muscle was incubated in KR-CH or KR-CH containing 5×10^{-6} g/ml E64C.

absence or presence of the ionophore was 16.8% (Table 1) or 18.6%, respectively (*cf.* ref. 2).

3) Effect of E64C on degradation of myofibrillar proteins as a whole: The same numbers of myoblasts from chick embryonic breast muscle were inoculated on culture plates[3] and cultured for 3 days under the same conditions. The plates were pulsed with ^{3}H-labeled leucine for 24 hr and divided into two groups. One group (G-EC) was chased in the presence of 10^{-6} g/ml E64C and the other (G-C) in its absence. After 2 days' chasing, cultured cells were harvested from each plate and homogenized in 0.1 M KCl. Myofibrillar proteins were recovered as the 0.1 M KCl-insoluble fraction[4]. The radioactivity of this fraction was determined by liquid scintillation counting.

The radioactivity ($\times10^3$ cpm$\pm$S.D.) of myofibrillar proteins per plate in G-C was 814 ± 28 ($n=5$) (Exp. 1), 826 ± 23 ($n=6$) (Exp. 2), and 910 ± 78 ($n=5$) (Exp. 3). That in G-EC was 841 ± 38 ($n=5$) (Exp. 1), 912 ± 37 ($n=6$) (Exp. 2), and 996 ± 43 ($n=3$) (Exp. 3). The difference in radioactivity between G-C and G-EC was significant ($P<0.01$) according to block design analysis.

4) Effect of E64C on degradation of myosin heavy chain: Muscle cells, cultured, pulsed, and chased as described above, were harvested. Myosin-B was extracted from myofibrillar proteins prepared as described above and run on SDS polyacrylamide gel electrophoresis[5]. After staining with Coomassie Brilliant Blue, the band of the myosin heavy chain was cut out of the gel. The radioactivity[4] and the amount of protein[6] in the band were determined. Low specific activities mean higher rates of protein degradation.

The specific activity (cpm per arbitrary unit of protein$\pm$S.D.) of the myosin heavy chain in the presence of 5×10^{-6} g/ml E64C was 26.5 ± 2.9 ($n=6$) and that in its absence 22.0 ± 2.6 ($n=6$). The difference in the specific activities was significant ($P<0.05$).

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