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Tohoku Journal of Agricultural Research
Vol. 32, No. 1, July 1981
Printed in Japan

Genetic Heterozygous Carriers in Hereditary Muscular Dystrophy of Chickens

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(Received Dec. 1, 1980)

Summary

The inheritance pattern of *am* gene was analyzed in chickens with hereditary muscular dystrophy. During the course of analysis, comparative histochemical observations on mitochondrial oxidative enzymes (SDH, NADH-TR) and acetylcholinesterase (AchE) in pectoralis muscles were attempted among normal, heterozygous carrier, and homozygous dystrophic chickens. The data obtained indicated that a single incompletely dominant gene was responsible for the difference between normal and dystrophic patterns.

In the early stages of symptom, hypertrophic αR fibers in heterozygous pectoralis muscle exhibited high oxidative enzyme activity. The αW fibers showed relatively normal characteristics in SDH and NADH-TR activity until later stages, and did not show the heavy hypertrophy as is typical of the αW fibers in homozygous dystrophic muscle. However, intense AchE activity diffused in sarcoplasm of αW fibers were often observed even when they showed normal oxidative enzyme activity. The necrotic destruction, vacuolization, and splitting of fibers were rarely found in heterozygous muscles.

Serum pyruvate kinase (PK) activity in the homoyzgous dystrophic genotype was approximately 30-fold higher than in normal one, while only a 7-fold elevation was observed in serum creatine phosphokinase (CPK) activity. The PK activity was a more sensitive detector of the heterozygous carrier state than was CPK activity and was useful for screening of heterozygous chickens from other genotypes.

The *am* mutant of hereditary muscular dystrophy in chickens was first described by Asmundson and Julian (1956) (1). This mutant is inherited as an autosomal recessive, designated *am/am*. Depending on muscles such as the pectoralis, biceps and posterior latissimus dorsi to reach a standing position, dystrophic chickens lose their ability to rise to their feet after being placed in supine position due to the effects of dystrophy on these muscles. The flip test, which counts as the number

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of successful attempts to right themsevles, has been one of the specific performance tests for muscular disability and a useful marker to trace the inheritance pattern of *am* recessive gene (1, 2). Classification as normal (+/+) or abnormal (*am*/*am*) has routinely been based on this test.

However, it has been generally accepted that the heterozygotes (+/*am*) did not display appreciable functional disability and, therefore, were not easy to separate from normal genotypes by the flip test alone (1, 2, 3). To identify heterozygotes from the group of other genotypic chickens, other criteria such as the measurement of breast width (1, 4), serum aldolase (3), serum glutamic-oxaloacetictransaminase (GOT) (3), and serum creatine phosphokinase (CPK) (5) were used by other investigators.

The most reliable method for screening heterozygotes from other genotypes is the microscopic observations of skeletal muscles. Results have suggested that the histopathological changes in skeletal muscles, especially in pectoralis muscle, were more sensitive to classify three genotypes than the changes of several blood enzyme activities. Heterozygous carriers readily identified in animal models of muscular dystrophy offer additional opportunities for the study of primary gene effects on pathogenesis with limited complication by secondary changes, and also contribute to the study of more sensitive methods for the detection of human carriers.

It has been reported that blood pyruvate kinase (PK; ATP: pyruvate phosphotransferase, EC 2.7.1.40) was greatly increased in Duchenne muscular dystrophy and other myopathies (6), and that PK was a more sensitive marker enzyme of muscle pathology and the heterozygous carrier state than other blood enzymes (7). However, the findings are still somewhat controversial (8, 9).

We investigated modes of inheritance of *am* gene by focussing on muscle pathology as a phenotypic expression and its relationship to the blood PK and CPK activity as detectors of heterozygous carrier chickens from other genotypes at the several stages of development.

Materials and Methods

One of several lines of dystrophic New Hampshire chickens, line 413, was developed in the Department of Avian Science, University of California, Davis, U.S.A. and were introduced with normal line 412 to Japan in 1976.

Normal White Leghorn (line IWAYA E) males were crossed with dystrophic New Hampshire (line 413) females to produce F_1 progeny. Males of normal chickens were again backcrossed with these F_1 females to produce N_1 progeny. Homozygous dystrophic chickens were extracted by crossing heterozygous N_1 pairs. Normal chickens were produced by crossing normal N_1 pairs. In such genetic processes, homozygous dystrophic chickens were easy to classify from other genotypes by the flip test and with measurement of serum CPK activity for

confirmation. Classification of heterozygous chickens was made by means of histopathological evaluations in microscopic preparations of pectoralis muscles which were removed surgically under ether anesthesia at 4 months age. These chickens were used again to produce offspring of the next generation for the test of segregation pattern of *am* gene.

Progeny were sacrificed at 37, 51, 70, 86, 98, 112, 126, 140, and 475 days after hatching. The number of chickens used for the present experiments was 274. The blood was collected from a wing vein, 2.5–5.0 ml, before sacrifice, clotted at room temperature for about 1 hr, and centrifuged for 20 min at 760 ×g at room temperature. Serum was removed by pipet. Pectoralis muscles (M. pectoralis superficialis) were sliced into small pieces and fixed in Bouin's solution to make paraffin sections, or placed in dry-ice/acetone to make cryostat sections for histochemical procedures. Frozen sections were stained with hematoxylin/eosin (H-E) and serial sections were incubated for the demonstration of succinic dehydrogenase (SDH) (10), nicotinamide adenine dinucleotide-reduced tetrazolium reductase (NADH-TR) (11), and acetylcholinesterase (AchE) (12). Possible non-specific staining of AchE was examined by incubating successive sections in the presence of 10^{-4} M Tetraisopropyl-pyrophosphoramide (iso-OMPA) which is an inhibitor of Butyrylcholinesterase (BchE) (13).

PK activity was determined by the procedure of Gutman and Bernt (1974) (14) with some modifications. The assay mixture (0.5 ml) contained 0.44 ml 0.1 M Triethanolamine-KOH (pH 7.6), 0.025 ml 10.5 mM Phosphoenol pyruvate, 50 mM $MgSO_4$, 0.2 M KCl, 0.025 ml 94 mM NADP (pH 7.0), 0.008 ml 12 mM NADH and 0.001 ml 10 mg/ml LDH. Activity was measured at 25°C using a Gilford model 250 spectrophotometer. CPK activity was measured at 25°C, using NAD^+ method (15). The reaction was measured by recording the absorbance decrease at 340 nm using the same spectrophotometer as in PK assay. Enzyme activities of both CPK and PK were expressed in International milliunit (mU) per milliliter serum under these standard conditions.

Results

I. Genetics

Modes of inheritance for the histopathological abnormality in pectoralis muscles were determined in a series of crosses, whose parental genotypes were NH 413 (*am*/*am*) × WL (+/+), F_1 (+/*am*) × WL (+/+), N_1 (+/*am*) × N_1 (+/*am*) and N_1 (+/+) × N_1 (+/+). All F_1 chickens had moderate symptoms in pectoralis muscle. 14% of F_1 chickens gained less than 6.0, but more than 1.0 in flip score when they were measured at 4 months age. F_1 females were then backcrossed with normal White Leghorn males to produce N_1 chickens. The transmission of dystrophic trait was analyzed for goodness-of fit to a 1:1 Mendelian ratio in N_1 progeny. 54% of all N_1 chickens were classified as heterozygotes, 11% of which

showed abnormal performance in flip test as was seen in F_1 progeny. The other 46% of N_1 progeny were classified as normal. These results are summarized in Table 1. The abnormal performance observed in some of the heterozygotes in these crosses was that they could not gain normal flip scores and were slower in motion to right themsevles from the supine position than normal genotypes, even when they obtained more than 6.0 in flip score. Chickens which could rise 5 times were assigned a socre of 6.0 and consider normal (2).

TABLE 1. *Segregations from Crosses between Dystrophic NH*[a] *(am/am) and Normal WL*[b] *(+/+) Chicken*

Cross	Progeny phenotypes			χ^2 [d]	Ratio
	Normal	Moderate dystrophy	Dystrophy		
NH (*am*/*am*) × WL (+/+)		All (all)[c]		0.00	All
F_1 (+/*am*) × WL (+/+)	43 (40)	37 (40)		0.450	1:1
N_1 (+/*am*) × N_1 (+/*am*)	18 (16.75)	33 (33.50)	16 (16.75)	0.134	1:2:1
N_1 (+/+) × N_1 (+/+)	All (all)			0.00	All

a) NH means New Hampshire chicken (line 413).
b) WL means White Leghorn chicken (line IWAYA E).
c) Expected numbers in parentheses.
d) Chi-square test for homogeneity of the ratio

This data indicated that a single incompletely dominant gene was responsible for the differences between normal and dystrophic patterns. In order to confirm further the transmission modes of *am* gene, the pairs of heterozygote in N_1 generation were crossed between themsevles. At time of sacrifice chickens ranged from 79–140 days of age. As was summarized in table 1, the number obtained are in close agreement with the Mendelian proportions of 1/4 normal homozygotes, 1/2 heterozygotes, and 1/4 dystrophic homozygotes.

II. Histopathological observations

Comparative histochemical observations on the pectoralis muscles were attempted among these different genotypes, focussing especially on the heterozygous chickens. In the pectoralis muscles of heterozygous chickens, numerous αR fibers were found within primary fascicles and they showed heavy hypertrophy. The αW fibers were smaller in size and surrounded αR fibers. The αW fiber showed normal characteristics in SDH and NADH-TR activity, while αR fibers tended to have higher oxidative enzyme activity until relatively later stages. In normal chickens, almost all of the fibers in pectoralis muscle are equal in size and are αW fibers which have weak reaction of SDH and NADH-TR by about 2 weeks of age. The transformation of αR to αW fibers in normal pectoralis is accomplished by a decrease in size and the number of diformazan granules. In heterozygotes, such transformation does not occur and the number of diformazan granules remains high.

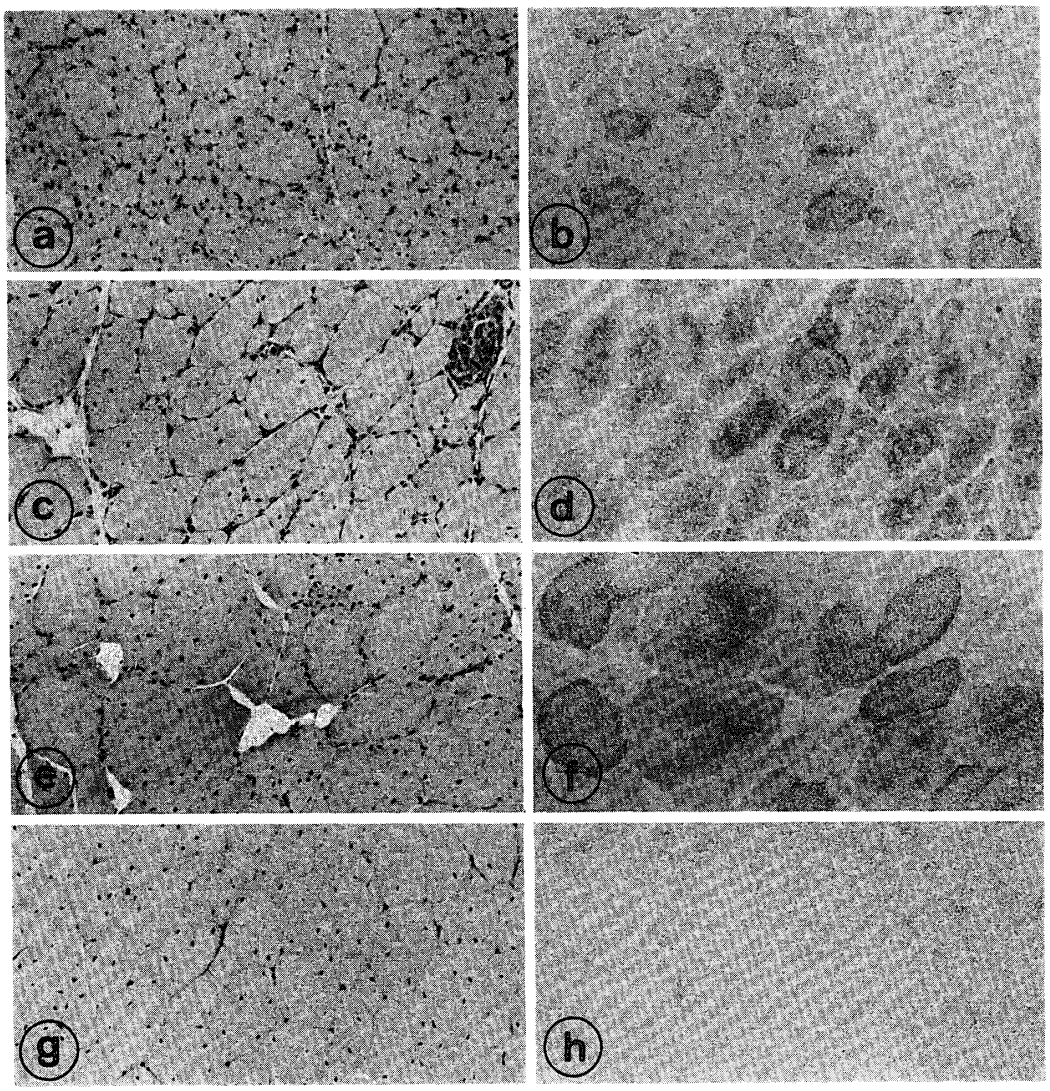

FIG. 1. Serial transverse sections of heterozygous (a, b) and dystrophic (c, d) pectoralis muscle at 51 days after hatching, and heterozygous (e, f) and normal (g, h) pectoralis muscle at 126 days after hatching. hematoxylin-eosin; a, c, e and g. SDH: b, d, f and h. × 150.

At 51 days of age, many large fibers with very intense SDH activity were revealed within primary muscle fascicles in pectoralis muscle of heterozygotes. They were surrounded by smaller fibers which have low SDH activity. The former were classified as αR fibers by their intense SDH activity and peripheral location of nuclei; the latter were classified as αW fibers which compose almost all of fibers in normal pectoralis muscle (Figs. 1a and b).

In contrast to this, the majority of fibers in homozygous dystrophic pectoralis muscles exhibited intense SDH reaction showing increased variability in their sizes by this time (Fig. 1d). Active destruction of muscle fibers were often observed in dystrophic muscles. Some of the cells present in such foci were infiltrated by undifferentiated cells (Fig. 1c). Normal muscle fibers generally have

low SDH activity and have both internal and peripheral nuclei (Fig. 1g).

At 126 days of age, αR fibers in muscles of heterozygotes still tend to show high oxidative activity and hypertrophy, except that some fibers show an abnormal distribution of diformazan granules, like moth-eaten fibers, coupled with an increase of internal nuclei within the fiber (Figs. 1e and f).

Typical myopathic changes were observed in pectoralis muscles of dystrophic chickens at 1 year of age. There was a marked variation in fiber size with numerous hypertrophic fibers, necrotic fibers with active phagocytosis, ring fibers, and splitting fibers. The proliferation of internal nuclei was not as marked at this age (Fig. 2a). NADH-TR activity still remains high in some, but slight in other fibers (Fig. 2b). In heterozygotes, heavy proliferation of internal nuclei in hypertrophied fibers were often observed and identical phenomena were also observed in smaller fibers in some individuals. Fiber destruction, vacuolization, splitting, and ringed fibers were rarely found (Fig. 2c). Stronger NADH-TR reaction than in normal muscles, but lower than in homozygous dystrophic muscles, was observed in heterozygotes irrespective of fiber types (Fig. 2d).

Normal pectoralis muscle fibers exhibited AchE activity only at the motor end-plates (Fig. 3a). However, AchE reaction in muscle fibers of heterozygous pectoralis muscle exhibited diffuse activity in sarcoplasm itself. Intracellular AchE activity in αW fibers was higher than in hypertrophied αR fibers (Figs. 3b and d). These findings were in marked contrast to the AchE reaction seen in dystrophic pectoralis muscle. Muscle fibers were round in shape and had a wider gap among themselves and varied in their sizes. In addition to AchE activity at the motor end-plates, they also exhibited diffuse and high enzyme activity in the sarcoplasm itself (Fig. 3c).

III. Blood serum PK and CPK activity

Serum PK activities of dystrophic chickens were significantly higher than normal chickens at 37 days of age ($p<0.01$). An approximately thirty-fold difference in activity became evident between dystrophic and normal genotypes at this age, although PK activity of both genotypes decreased at 475 days of age. Serum PK values of dystrophic chickens showed wide bird-to-bird variation so that standard deviations were larger in dystrophic genotype than in the normal one. Some CPK activities at the same intervals as in PK measurements are also summerized in Table 2 and showed as approximately seven fold increase which paralleled the relative increase of PK activity. Serum CPK decreased their values with increasing age. On the other hand, serum PK and CPK activities of heterozygous chickens, which were readily identified by means of histopathological observations, do not clearly separate from the normal range by 37 days of age. Conspicuous differences between the two genotypes appeared first in PK activity at 70–86 days of age ($p<0.005$), while there were still no significant differences in CPK values.

TABLE 2. *Blood Serum Pyruvate Kinase (PK) and Creatine Phosphokinase (CPK) Activities in Normal (WL)*[a], *Dystrophic (WL)*[a] *and Heterozygous Carrier Chicks*

Enzyme	Age (days)	Normal (WL)[b] (mU/ml)	Dystrophy (WL)[b] (mU/ml)	Heterozygotes[b] (mU/ml)
PK	37	401±111 (8)	12,430±6,269 (4)[c]	630±209 (5)
	70-86	405± 73 (4)	10,773±6,800 (7)[c]	1,213±552 (10)[d]
	475	229± 33 (7)	8,940±4,032 (5)[c]	516±138 (7)[d]
CPK	37	141± 55 (8)	1,071± 812 (4)[c]	180± 31 (5)
	70-86	164± 41 (4)	1,146± 599 (7)[c]	183± 43 (10)
	475	30± 9 (7)	986± 586 (5)[c]	46± 10 (7)[e]

a WL means White Leghorn.
b Mean enzyme activity±SD. Number of animals tested in parentheses. Statistical analyses were performed using paired t-tests. Comparison with normal chicks,
c $p<0.01$ d $p<0.005$ e $p<0.05$

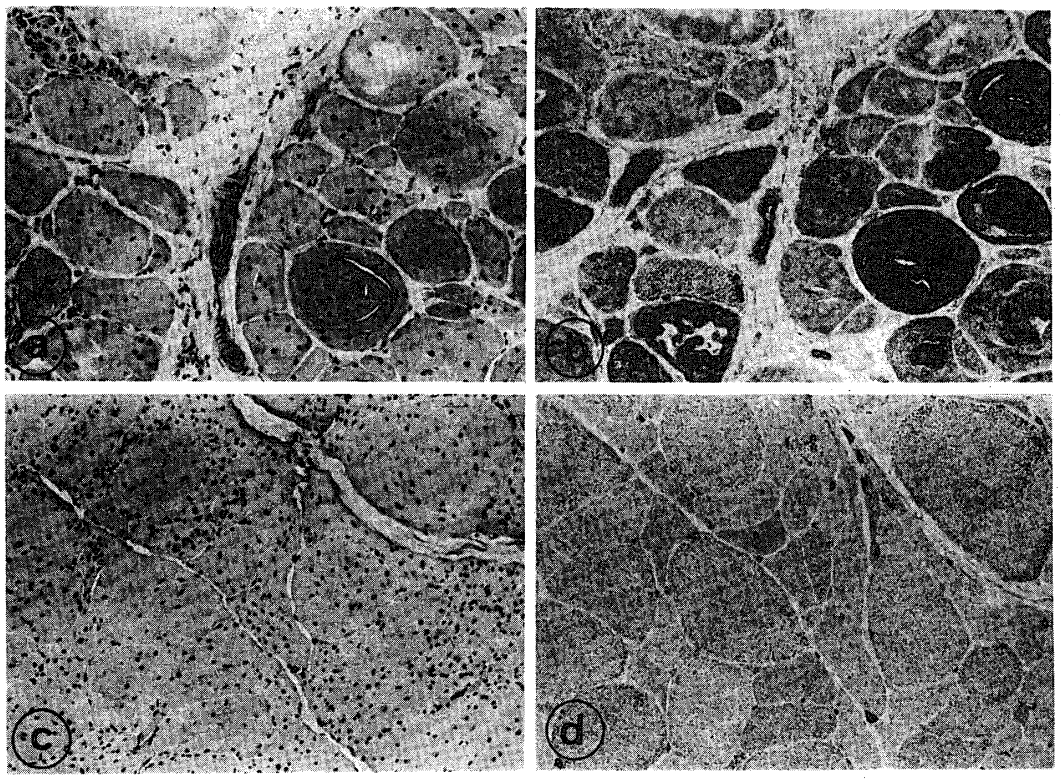

FIG. 2. Serial transverse sections of dystrophic (a, b) and heterozygous (c, d) pectoralis muscle from 1 year old chick. hematoxylin-eosin; a and c. NADH-TR; b and d. × 150.

Significant differences with respect to the CPK activity began to be clear at 475 days of age ($p<0.05$).

A time course of the PK activity of both normal and heterozygous carrier chickens (Fig. 4) demonstrates that the PK values of heterozygotes increased and separated from the normal range in older birds. The lower values in heterozygotes

often overlapped with the higher values in normal range throughout this period. It was reasonable to assume that chickens having PK values more than 1000 mU/ml could be classified as heterozygous chickens.

Dicussion

The muscular dystrophy trait has previously been reported by Asmundson and Julian (1956) to be inherited as an autosomal recessive (1). They analyzed the inheritance pattern of *am* gene using flip test measured at 8 weeks of age to identify homozygous dystrophic chickens from other genotypes, and observed in F_2 data, 10 dystrophic chickens out of 90 rather than the expected 22.5. A reduced number of the dystrophic genotype also occurred in F_2 progeny, when immunodeficiency was used as a parameter of the phenotypic trait (16).

They mated dystrophic males to normal females to produce F_1 progeny which were crossed with each other in next generation to obtain F_2 progeny. Asmundson and Julian (1956) suggested that since the age at which the chickens become abnormal varies and the degree of abnormality may also vary, these variables could well account for the apparent reduction in number of homozygous dystrophic chickens in the data (1). However, such reduced number of dystrophic chickens was not observed in our data when crosses were made between N_1 heterozygotes in which abnormal *am* gene was originally introduced to normal genotype from homozygous dystrophic female chickens and the flip test was made at 4 months of age. The heterozygous chickens were identified in several crosses using histopathological classifications in pectoralis muscles. Their numbers obtained were in close agreement with the Mendelian proportions of 1/2 heterozygotes out of all offspring in crossing between N_1 heterozygotes, and of 1/2 heterozygotes out of all offsprings in crossing between normal male and F_1 female chickens.

It has been pointed out previously that the *am* gene is a simple recessive to the normal gene in young chickens, but in the older chicken the genes act as codominants (2, 17), and furthermore, that the breast width and the activity of some of enzymes tested do not permit accurate classification into three genotypes (2). Such screening methods for genotypes might be too inaccurate to obtain clear ratios of segregating *am* gene. The microscopic histochemical test of inheritance mode of the dystrophic trait seems to be more sensitive than previous method described above.

To the present time, histochemical studies on skeletal muscle of heterozygous chickens have not been reported, except quantitative histopathological observations in F_1 progeny (18). Hereditary musclar dystrophy in chickens is characterized by selective destruction of a specific fiber type (αW fibers) in skeletal muscles. The pectoralis muscles are typically and severely involved since it is composed almost exclusively of αW fibers (19).

The stem-line theory has been advanced that, in embryonic chicken muscle,

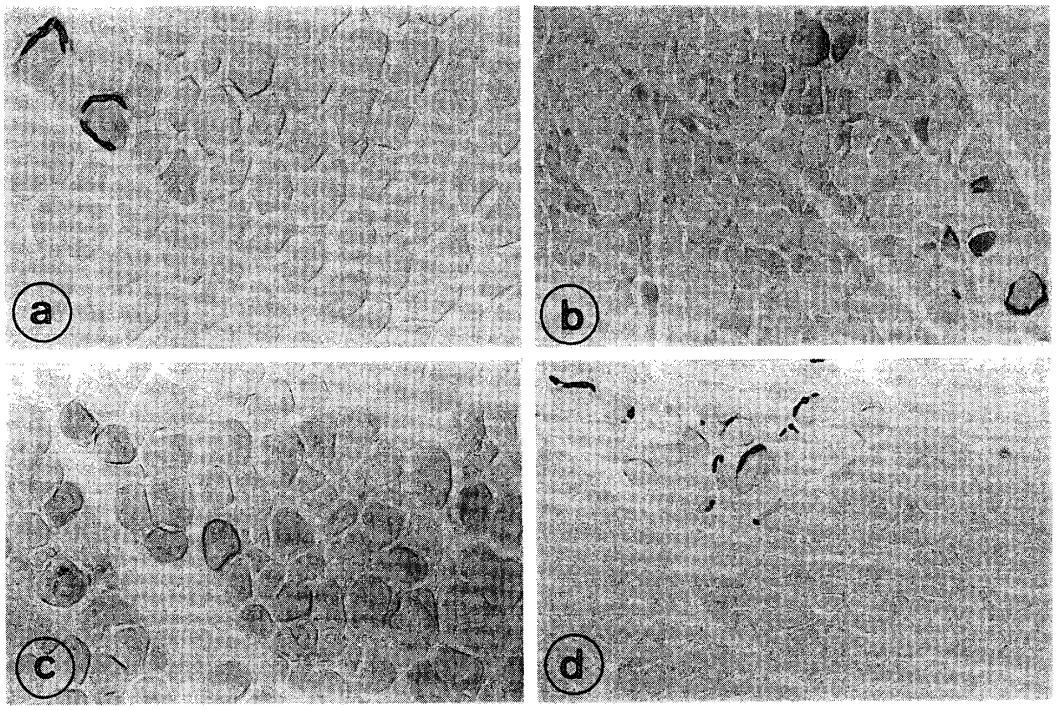

FIG. 3. Transverse sections stained for AchE activity of normal (a) pectoralis muscle at 93 days after hatching, heterozygous (b) pectoralis muscle at 79 days after hatching. AchE activity of dystrophic (c) and heterozygous (d) pectoralis muscle from 51 days old chick. × 100.

primary myotubes are first formed and serve as a structural framework upon which myoblast fusion and formation of secondary myotubes take place, and that the secondary myotubes are under the influence of the primary myotube innervation until such time as they are removed from its surface (20, 21, 22). In contrast, prenatal and postnatal histochemical differentiation of muscle fibers is not determined by prenatal structural differentiation and is neurally regulated (23). In pectoralis muscle from 1 day old normal chickens, two populations of fibers are clearly visible when mitochondria are localized via SDH activity (19). The majority of fibers differentiated from secondary myotubes have many large diformazan granules which are typical of αR fibers. The second population of fibers derived from primary myotubes are usually larger in size, but contain fewer and smaller granules, and become mitochondria-rich fibers with large granules, smaller in size, located in the interior of the fasciculi by 2 weeks of age. The αR fibers derived from secondary myotubes transform to αW fibers soon after hatching. Consequently, the pectoralis muscle exhibits 90–100% αW fibers, with the remaining fibers αR.

With exception of the heavy hypertrophy and high oxidative enzyme activity, the distribution pattern of diformazan granules in αR fibers of heterozygous pectoralis muscle remains relative normal. The αW fibers, however, do not show hypertrophy and intense oxidative enzyme activity as αW fibers in homozygous

dystrophic muscle do. They are equal or smaller in size than normal αW fibers and, with increasing age, become angular shaped fibers as in early stages of neuropathy (24). Mitochondrial SDH reaction is controlled by nuclear genes through a trophic influence of nerve and exhibits abnormal elevation in dystrophic muscle fibers (25). Abnormal appearance of extrajunctional AchE activity is another typical changes seen in diseased fibers (26), and was observed in immature embyronic and cultured muscle cells (27, 28, 29), denervated muscle fibers (26), and regenerating muscle fibers (30). The fact that αW fibers in herterozygous pectoralis muscle exhibited intense activity diffused in the sarcoplasm itself is evidence to suggest that they are in a regenerating or embryonic stage and are not under the normal control of nerve. However, unlike αW fibers in homozygous dystrophic muscle, they are capable of maintaining relatively normal activity of mitochondrial enzymes and fiber size regardless of the abnormal AchE reaction. Although more complicated interpretations are possible, the results of this study suggest that abnormal AchE reaction observed in dystrophic αW fibers may be primary to the secondary changes of mitochondrial enzyme reaction and hypertrophy. In contrast, αR fibers in heterozygotes were associated with a marked elevation of the oxidative enzyme activity and a hypertrophy response suggesting that they hypertrophied compensatory to disordered abnormal circumstances in diseased muscle.

The proliferation of nuclei in fibers is of equal interest. Total number of nuclei per cross section of a fibers was increased in heterozygous peoctralis muscle (18). αR fibers, however, tend to keep nuclear positions beneath the sarcolemma and characteristics of αR fibers in normal muscle. The dislocation of nuclei from a peripheral to an internal portion and marked increase of nuclear number occurred

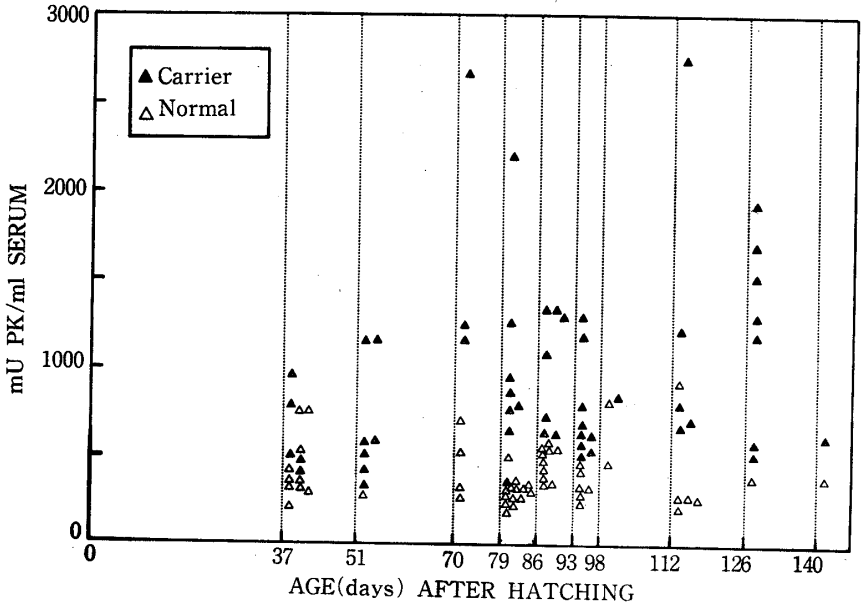

FIG. 4. Comparison between serum pyruvate kinase (PK) activity and age (days) after hatching in normal (△) and heterozygous carrier (▲) chickens.

at later stages coupled with abnormal distribution of diformazan granules of mitochondrial oxidative enzyme. These results support an idea that αR fibers were able to resist the degenerating process in dystrophic muscle (19).

The measurement of blood CPK has been first reported by Ebashi et al. (1959) as a useful marker enzyme of choice for clinical diagnosis of the progressive muscular dystrophy (31) and by other as a useful mean for detection of carriers of Duchenne muscular dystrophy (32). Measurement of CPK activity showed consistently higher serum activity for the dystrophic chickens and an increase in activity to one year of age. The CPK activity in heterozygous carrier chickens showed a significant elevation in the adult, but could not be used to identify the carriers when measurements were made in younger chickens. The results with chickens are in this respect more like those in golden hamsters than those in man (33).

The PK activities in homozygous dystrophic genotypes were approximately 30 fold higher than in normal ones, while only a 7 fold elevation was observed in CPK activity. These data are consistent with the results of Barnard and Barnard (1979) suggesting that the PK appeared in blood rather faster and greater than the CPK (34). Although the precise mechanism of enzyme leaking from dystrophic muscles to blood circulation remains obscure, the PK activity was thought to be more sensitive to identify heterozygous carrier state than the CPK activity. We found that this enzyme was useful for screening of heterozygous chickens from other genotypes if measurements were made after 70 days of age.

The PK activity of higher animals exists in at least 3 different isoenzyme forms (35, 36). The L-type of PK is found as the major component in liver and as a minor component in kidney. Skeletal muscles, heart, and brain contain the M_1 type. The PK of most other tissues is of the M_2 type. The abnormal PK accumulation in blood circulation of dystrophic chickens was entirely due to isoenzyme M_1 type leaking from skeletal muscle and not due to red cell (R type) and other types of isoenzymes (37).

Reinacher et al. (1979) has recently reported that there is considerable difference in the amount of PK in different muscle types, and that the red muscles, such as gastrocnemius and anterior latissimus dorsi, contain less PK activity than white muscles, such as pectoralis and posterior latissimus dorsi (38). The white muscles are typical in flight muscles of chickens and are affected severely by muscular dystrophy as mentioned above. The reasons why the elevated blood PK and CPK activity in dystrophic chickens must be closely related to membrane abnormality of diseased muscle fibers was reviewed by Rowland (1980) (39). The fact that the fiber destruction was rarely observed in heterozygous pectoralis muscle may be coupled with the relatively lower values of CPK and PK activities than those in homozygous dystrophic chickens.

Acknowledgement

This study was supported by Grant No. 80-10 from National Center for Nervous, Mental and Muscular Disorders (NCNMMD) of the Ministry of Health and Welfare, Japan. We are indebted to Dr. H. Sugita, NCNMMD, for giving us an opportunity to analyze serum enzymes. The skilled assistance of Dr. S. Ohwada, Mr. T. Inamoto, Mr. S. Hoshi and Miss. K. Tsuchiya is acknowledged with thanks.

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