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EXPERIMENTAL MANIPULATION WHICH INTERFERES WITH THE PHENOTYPIC EXPRESSION OF THE DYSTROPHIC PROCESS: CROSS REINNERVATION OF A FAST TWITCH MUSCLE BY THE NERVE OF A SLOW TONIC MUSCLE IN CHICKENS WITH HEREDITARY MUSCULAR DYSTROPHY

By

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A Thesis

Submitted to the School of Graduate Studies

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for the Degree

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December 1980

CROSS REINNERVATION OF AVIAN DYSTROPHIC MUSCLE

לאשתי אדיבה
בן אדיב ובת נועה
באהבה.

To my wife Aviva

and my children Irad and Noa

DOCTOR OF PHILOSOPHY (1980)
(Medical Sciences)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Experimental Manipulation which interferes with the Phenotypic Expression of the Dystrophic Process: Cross Reinnervation of a Fast Twitch Muscle by the Nerve of a Slow Tonic Muscle in Chickens with Hereditary Muscular Dystrophy

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ABSTRACT

Hereditary muscular dystrophy of the chicken is a primary defect of muscle which is expressed specifically in the fast twitch, focally innervated glycolytic muscle, while slow tonic, multiply innervated oxidative muscles are spared disease characteristics (for review, Cosmos et al., 1979b). Since motor nerves influence the characteristics of skeletal muscles, and since slow tonic muscles in the chicken do not express dystrophic phenotypes, it was decided to replace the motor innervation of the fast twitch posterior latissimus dorsi (PLD) muscle with the motor innervation of the slow tonic anterior latissimus dorsi (ALD) muscle within a dystrophic chicken, in order to alter the fate of the dystrophic fast twitch muscle.

Selected mechanical, histochemical and structural properties of the ALD and PLD muscles of normal (White Leghorn) and dystrophic (Storrs line) chickens 15-800 days ex ovo were compared to determine which of these properties were altered as a result of the disease, and to provide baseline data for the analysis of the cross reinnervation experiments. ALD muscles of dystrophic chickens exhibited normal phenotypes, i.e. slow tonic isometric contraction in response to nerve stimulation in vivo, acid and alkaline stable myosin ATPase activity, "en grappe" innervation, weak phosphorylase (Pase), strong succinic dehydrogenase (SDH) enzymic activities and peripheral location of nuclei. PLD muscles of dystrophic genotype demonstrated structural and histochemical

alterations but retained contraction and relaxation times characteristic of fast twitch muscle of normal genotype. Further, they exhibited focal "en plaque" innervation and alkaline stable myosin ATPase activity similar to that of normal PLD muscles. The abnormalities identified in the dystrophic PLD included the following: lower muscle weight, abnormal size and shape of fibres, increased number of internal nuclei, abnormal Pase and SDH enzymic activities and lower twitch and tetanic tensions.

The surgical cross reinnervation between the ALD nerve and the PLD muscle was performed at hatching. The muscles were examined at various time intervals postoperatively using the criteria established in the study of the unoperated muscles. Self reinnervation of PLD muscles by their own nerves, and cross reinnervation within normal chickens served as control experiments.

Fibres of both normal and dystrophic PLD muscles which had been successfully cross reinnervated acquired mechanical, structural and histochemical properties characteristic of the ALD muscle. Thus, muscles of both genotype responded similarly to the ALD nerve. These results established that the PLD muscle of dystrophic chicken is able to accept the new innervation, and is as capable of responding to the influence of the ALD nerve as is the normal PLD. Furthermore, the successfully cross reinnervated fibres are spared disease characteristics.

The present cross reinnervation experiment demonstrates, for the first time, an experimental manipulation which interferes with the expression of phenotypic characteristics of dystrophy during development ex ovo. The results support the rationale underlying the present study:

the PLD muscle of dystrophic genotype is unable to respond appropriately to the demand from its own motor nerve to complete successfully the transition from embryo to adult metabolism, thus expressing the dystrophic phenotypes. However, when this request is removed by cross reinnervating the PLD muscle with the ALD nerve, the dystrophic phenotype is not expressed. These findings strongly suggest that regardless of the time during development when slow tonic characteristics are achieved, i.e. either during development in ovo or by surgical manipulation ex ovo, slow tonic fibres are spared dystrophic phenotypes.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS
(alphabetical order)

Ach	- Acetylcholine
AchE	- Acetylcholinesterase
ALD	- Anterior latissimus dorsi
ATPase	- Adenosinetriphosphatase
cm	- centimetre
df	- degree of freedom
DNA	- deoxyribonucleic acid
DLD	- dorsocutaneous latissimus dorsi
e.g.	- for example
gm	- gram
hr	- hour
Hz	- Hertz (cycle per second)
ID	- identification
i.e.	- that is
log	- logarithm
m	- metre
µm	- micrometre
mg	- milligram
ml	- millilitre (cc)
MLD	- metapatagial latissimus dorsi
mm	- millimetre
mos	- months
pase	- phosphorylase
PLD	- psoterior latissimus dorsi
RNA	- ribonucleic acid
SDH	- succinic dehydrogenase
sec	- second
S-RI	- self reinnervation
v	- volt
X-RI	- cross reinnervation

INTRODUCTION

It has been established that at least two main types of muscle fibres exist in vertebrates: focally innervated twitch (fast and slow) muscles and multiply innervated tonic (slow) muscles (for review see Hess, 1970; Close, 1972).

ANTERIOR AND POSTERIOR LATISSIMUS DORSI MUSCLES

In the chicken, the anterior latissimus dorsi (ALD) and posterior latissimus dorsi (PLD) muscles consist almost entirely of slow tonic and fast twitch fibres respectively. This circumstance provides an ideal opportunity to study, in the same animal, the different charateristics of the two main fibre types. What follows is a summary of the known features of these two muscles.

Structural Properties

The terms Fibrillenstruktur and Felderstruktur have been used to differentiate between fast twitch and slow tonic muscle respectively in histological cross sections (Krüger, 1950). The PLD fibrillenstruktur fibres show regular punctuate fibrils evenly distributed throughout the sarcoplasm, while the ALD felderstruktur fibres show large irregularly shaped fibrils which are clumped together (Hess, 1961; Bock and Hikida, 1968). These histological differences reflect underlying ultrastructural differences between the PLD and ALD muscles (Hess, 1961, 1967; Page and

Slater, 1965; Page, 1969; Shear and Goldspink, 1972). The fibrils of the PLD are equal in size and regularly separated from each other; the sarcoplasmic reticulum has a well developed tubular system with triads regularly distributed on both sides of the Z-line near the A-1 junction; The Z-line is straight and the M-line is present. In contrast, the fibrils of the ALD are irregular in size and distribution; the sarcoplasmic reticulum has a less developed tubular system; the diads or triads are rare and irregularly distributed; the Z-line is thicker with a zig-zag shape; and the M-line is also present (although the M-line is usually not seen in other slow tonic fibres). The difference in internal organization between ALD and PLD muscles may account for the major differences in their mechanical responses. Three other major structural differences have also been noted between ALD and PLD muscles: fibre diameter is larger in the ALD even during in ovo development (Shear and Goldspink, 1972; Gordon et al., 1977a); nuclei in the ALD are at the periphery while they are scattered in the PLD (Cosmos et al. 1979b), and the mitochondria are greater in number but smaller in size than in the PLD (Kiessling, 1976).

Innervation Characteristics

Histochemical localization of acetylcholinesterase (AChE) activity reveals that ALD fibres have multiple "en grappe" nerve endings scattered along each fibre, while PLD fibres are characterized by single "en plaque" motor nerve endings (Ginsborg and MacKay, 1960, 1961; Hess, 1961; Silver, 1963; Koenig, 1970; Bennett et al., 1973). The distance between endplates in the ALD muscle fibres appears to be directly

proportional to the length of the muscle fibre which suggests that the number of endplates on an ALD fibre remain constant during growth (Ginsborg and MacKay, 1961). At the ultrastructural level, no post-junctional sarcolemmal infoldings occur under the nerve terminals of ALD fibres but they are conspicuous under PLD nerve terminals (Hess, 1967; Zelená and Sobotková, 1973).

Results with conventional electrophysiological techniques, e.g. recordings of intracellular potentials and iontophoretic application of chemical substances, supported the histochemical observation cited above. Spontaneous miniature endplate potentials and evoked potentials recorded from individual ALD fibres indicate that: the ALD is multiply innervated; the amount of transmitter released at the neuromuscular junctions is very low; and that axons of different lengths and/or excitation threshold are innervating individual muscle fibres (Ginsborg, 1960a, b; Bennett et al., 1973). Moreover, multiple sensitivity peaks of iontophoretically applied acetylcholine (ACh) are distributed along ALD fibres, which correlates with the previous findings that the ALD fibres are multiply innervated (Fedde, 1969; Bennett et al., 1973). Similar electrophysiological studies with the PLD muscle reveals the presence of focal innervation with single axons releasing relatively high amounts of transmitter (Ginsborg, 1960a,b; Fedde, 1969; Vyskočil et al., 1971; Bennett et al., 1973).

Kuffler and Vaughan Williams (1953) have shown that slow tonic muscle fibres of the frog are innervated by nerve axons of small diameter which conduct an action potentials at 2-8 m/sec while fast twitch muscle fibres are innervated by nerve axons of larger diameter which conduct action potentials at 8-40 m/sec. Close and Hoh (1968) have shown that in

the toad the nerve axons innervating slow tonic muscle fibres have a high threshold for electrical stimulation, whereas the nerve axons innervating fast twitch muscle fibres have a lower threshold for electrical stimulation. Hess (1961), reported that preliminary studies of the nerves to the ALD and PLD muscles of the chicken indicated that the ALD nerve contains many myelinated axons with smaller diameter than those in the PLD nerve. Although Hess (1961) suggested that additional analyses of the nerves of the ALD and PLD muscles are needed, his preliminary findings might suggest that there are differences in properties such as conduction velocity and threshold for electrical stimulation.

Electrical Properties

Fedde (1969) reported that the passive electrical properties of the ALD and PLD muscles in 3-6 month old chickens are clearly different. In the ALD muscle, the membrane resistance and the time and length constants are much greater than in the PLD muscle. Gordon et al. (1977a) showed that the passive electrical properties of ALD and PLD muscles are similar in early embryonic development (14-16 days) but differ during later development. Whereas the length constant of PLD fibres remains the same throughout development, that of ALD fibres increases. The alteration noted in the length constant measurements can be explained by the type of innervation characterizing the two types of fibres. Since PLD muscle fibres are focally innervated, a well developed action potential mechanism activates the whole muscle fibre (Vyskocil et al., 1971). The ALD muscle fibres are multiply innervated and small non-propagated endplate potentials are evoked at each synaptic site (Bennett et al., 1973). Since the

distance between adjacent endplates increases as the muscle grows (Ginsborg and Mackay, 1961), the increased length constant permits the spread of the endplate potentials over a greater distance.

Mechanical Properties

Ginsborg (1960b) has demonstrated that ALD muscle fibres are able to conduct an action potential which leads to a twitch contraction in response to single maximal nerve stimulation in vitro. The ability of the ALD fibres of the chicken to produce an action potential in vitro may be related either to the fact that the endplates along the ALD fibre are distributed regularly, unlike any other slow tonic multiply innervated muscle (Hess, 1970), and/or to some ionic changes occurring in vitro, since no twitch contraction can be evoked in response to single nerve stimulation in vivo (Hník et al., 1967, 1977; Jirmanová and Zelená, 1973). Whatever the reason may be, the twitch response evoked either as a result of maximal nerve stimulation in vitro (Ginsborg, 1960b) or as a result of post tetanic potentiation in vivo (Hník et al., 1967) is a slow twitch with contraction and relaxation times 5 to 8 times longer than the twitch response of the fast PLD muscle. The range of twitch contraction and half relaxation times reported for the PLD muscle is 20 to 40 msec and 25 to 60 msec respectively (Ginsborg, 1960b; Hník et al., 1967; Gutmann and Syrový, 1967; Hoekman, 1968; Page, 1969; Canfield, 1971; Shear and Goldspink, 1972; Melichna et al., 1974).

A most striking characteristic of the ALD muscle is its unique ability to sustain a contraction in response to either repetitive nerve stimulation (tetanic stimulation) or to depolarizing agents lasting for

minutes. This is not observed with the PLD muscle (Ginsborg, 1960b; Page, 1969; Hoekman, 1968; Canfield, 1971; Melichna et al., 1974).

Other mechanical properties which differ between the ALD and PLD relate to their respective length tension characteristics, heat production during contraction, fusion frequency and tension development (Hoekman, 1968; Canfield, 1971).

The contractile properties of the ALD and PLD muscles have also been studied developmentally between 13 to 21 days in ovo (Gordon and Vrbová, 1975; Gordon et al., 1977a, b). It was reported by Gordon and Vrbová (1975), using direct stimulation, that both muscles at 13 to 16 days in ovo contract slowly. However, it is possible that the slow response recorded for the PLD muscle at this stage is due to the response of the slow tonic dorsocutaneous and metapatagial latissimus dorsi muscles which lie over the PLD. These muscles resemble the ALD and at the times studied they are far more advanced in their development than the PLD (Grim, 1971; Butler, Personal Communication). Gordon and Vrbová (1975) and Gordon et al. (1977a,b) have also reported that by 17 or 18 days in ovo the contractile response of the PLD is faster than that of the ALD. At hatching, both muscles exhibit their specific contractile characteristics, i.e., fast twitch and slow tonic respectively (Gutmann and Syrový, 1967; Melichna et al., 1974; Gordon et al, 1977a). In contrast, a study by Shear and Goldspink (1972) describes both muscles as slow three days prior to hatching and "equally fast" two days after hatching; thereafter, the rate of contraction of the ALD decreases, while that of the PLD remains rather constant. The ALD achieves a completely slow tonic response by 29 days ex ovo. The differences noted in this study may be

due to the use of different chicken lines; Rhode Island Red were used by Shear and Goldspink and White Leghorn were used by the others. An alternative explanation may be attributed to the difference in recording conditions. Shear and Goldspink recorded muscle contraction in vivo while the others used an in vitro condition. The findings that the rate of contraction of the PLD muscle after hatching and that the rate of contraction of the ALD muscle, following 29 days ex ovo development, remain rather constant (Shear and Goldspink, 1972) agree with the findings of Melichna et al. (1974) and Gordon et al. (1977a) that the contraction properties of the ALD and PLD muscles show only slight changes during development ex ovo.

Enzymic Properties

Histochemical and biochemical studies have demonstrated that the slow tonic ALD muscle has an aerobic metabolism with low glycolytic and high oxidative enzymic activity while the fast twitch PLD muscle has primarily an anaerobic metabolism with high glycolytic and low oxidative enzymic activity (Nene and Chinoy, 1965; Gutmann and Syrovy, 1967; Bass et al., 1970; Kiessling, 1976; Butler et al., 1978; Cosmos et al., 1979b). However, based on histochemical localization of succinic dehydrogenase (SDH), phosphorylase (Pase), fat and glycogen, Nene and Chinoy (1965) demonstrated the occurrence of different fibre types in both these muscles in the adult "domestic fowl". The PLD has three distinguishable fibre types, two of the fibre types exhibit anaerobic metabolism characterized by high and intermediate Pase activity. The third fibre type resembles the aerobic enzymic profile of the ALD fibres which is characterized by

low Pase activity. The existence of ALD type fibres in the PLD muscle was reported by Hess (1961) on the basis of nerve ending morphology. The third fibre type was also confirmed on the basis of myosin ATPase activity in histochemical preparations by Koenig and Fardeau (1973); Toutant et al., (1979) and Cosmos et al. (1979b). Toutant et al. (1979) state that ALD type fibres comprise 23% of the fibres in the PLD muscle at days 14 and 15 in ovo and decrease steadily to a very few or none at hatchings. This phenomenon is also confirmed by our laboratory (unpublished observations). The proportion of ALD type fibres in PLD muscles of adult chickens, although variable, is found to be very low: 3% according to Koenig and Fardeau (1973); less than 3.5% according to Jirmanová and Zelená (1973); and less than 2% according to Cosmós et al. (1979b).

The ALD muscle consists of two types of fibres. Both fibre types exhibit multiple endplates and aerobic metabolism, but differ in size and in the activity of their enzymes as noted histochemically. Fibres with smaller diameters exhibit higher Pase and SDH activity than do fibres with larger diameters (Nene and Chinoy, 1965; Asmussen et al., 1969; Asiedu and Shafiq, 1972; Koenig and Fardeau, 1973; Ovalle, 1978; Cosmos et al., 1979b).

Ontogenetic analyses of the biochemical and histochemical characteristics of these muscles have shown that while ALD fibres achieve adult metabolic characteristics during embryonic development, PLD fibres differentiate adult metabolic profile only after hatching (Gutmann and Syrový, 1967; Bass et al., 1970; Melichna et al., 1974; Butler et al., 1978; Cosmos et al., 1979b). At hatching, biochemical analyses of PLD muscles demonstrate metabolic enzymic activities which do not differ

significantly from those of ALD muscles; both demonstrate predominantly aerobic metabolism (Bass et al., 1970). However, the PLD muscle alters its metabolism during further development ex ovo and within 3-4 weeks is predominantly anaerobic. As has been reported by Cosmos (1966; 1970) and Cosmos and Butler (1967), this timetable of metabolic differentiation characterizes other fast twitch (pectoralis) and slow tonic (soleus) muscles of the chicken.

It was also demonstrated by Syrový et al. (1965) and Gutmann and Syrový (1967) that the ALD has a higher rate of incorporation of amino acids into proteins, a higher RNA content and a higher activity of proteolytic enzymes. All these differences suggest that a higher turnover of proteins occurs in the slow tonic oxidative fibres of the ALD muscle. It has been postulated that these characteristics may very well be related to the ability of the ALD muscle to maintain tensions for longer time periods than the fast twitch PLD muscle.

Myosin ATPase activity which is usually correlated with the rate of contraction of muscle (Bárány, 1967), also correlates well with the overall contractile properties of the ALD and PLD muscles. Syrový (1973) demonstrated that in adult chickens the calcium ATPase activity of myosin of the ALD is 5 times lower than that of PLD muscles. Using histochemical analyses of myosin ATPase activity, it was observed that these two muscles also exhibit differences in their myosin ATPase activity following preincubation at pH 4.35 and 10.4 (Asiedu and Shafiq, 1972). The fast twitch fibres exhibit myosin ATPase activity following alkaline (pH = 10.0) preincubation only, while the slow tonic fibres of the ALD exhibit activity following both acid (pH = 4.35) and alkaline (pH = 10.0) pre-

incubation (Butler et al., 1978; Cosmos et al., 1979b). Moreover, in mature chickens the PLD muscle contains three molecular weight classes of myosin light chains while the ALD has only two types (Lowey and Risby, 1971; Syrový, 1973; Hoh, 1978).

Histochemically, the myosin ATPase activity of the ALD and PLD, after both acid and alkaline preincubation, show no alteration during in ovo and ex ovo development (Butler et al., 1978; Butler and Cosmos, 1979a, b; Cosmos et al., 1979b). However, while the PLD in ovo contains only myosin light chains characteristic of fast twitch muscles, the ALD in ovo contains myosin light chains characteristic of both slow and fast muscle; the latter, i.e. fast myosin, disappears during ex ovo development (Rubinstein et al., 1977; Hoh, 1978; Pette et al., 1979). The relation between myosin light chains and myosin ATPase activity following acid and alkaline preincubation is not yet understood. These results may indicate that the speed of muscle contractions during embryonic development is not necessarily related to myosin. The PLD muscle of 14 to 16 day embryos contracts slowly (Gordon et al. 1977a) yet its myosin light chains as well as its myosin ATPase pH specific activity are both characteristic of a fast twitch muscle (Rubinstein et al., 1977; Hoh, 1978; Pette, et al., 1979). Thus, the slow contraction of the PLD muscle during this time of development may well be dependent upon other internal structures and/or upon the metabolic differentiation of the PLD muscle which is still not fully developed at this stage (Gutmann et al., 1969; Butler et al., 1978; Cosmos et al., 1979b; Pette, 1979).

Effects of Denervation and Hereditary Muscular Dystrophy

Differences between the multiply innervated, oxidative, slow tonic ALD muscle and the focally innervated, glycolytic, fast twitch PLD muscle are apparent in normal intact chickens. These differences also appear in muscles subjected to experimental alterations or undergoing diseases processes.

Experimental alterations: Feng et al. (1963) demonstrated that following denervation the PLD atrophies while the ALD hypertrophies. Since the wing droops as a result of denervation and subsequent stretching of the ALD, these workers concluded that hypertrophy of the ALD fibres during the first two weeks of denervation may be largely in response to stretch. However, the hypertrophy which persists during the later weeks cannot be so explained, as after the first two weeks no significant drooping of the wing occurs. Jirmanová and Zelená (1970) and Sola et al. (1973) confirmed the results of Feng et al. (1963). In addition, Jirmanová and Zelená (1970) showed that as a result of both denervation and tenotomy of the ALD muscle, the muscle fibres atrophied. Sola et al. (1973) reported that hypertrophy of the ALD muscle fibres was obtained by stretching the chicken wing whether the ALD muscle was innervated or not. As a result of these findings Sola et al. (1973) suggested that stretch may be the only reason for the denervation hypertrophy of the ALD muscle. However, the differential reaction to denervation of the slow tonic and fast twitch fibres in the biventer cervicis muscle which is not subjected to stretch (Feng et al., 1963) supports the conclusion that hypertrophy of the ALD and atrophy of the PLD represent (at least in part) a typical reaction to denervation.

Natural Disease Processes: Hereditary Muscular Dystrophy of the chicken demonstrates a differential response of these two muscles to a naturally occurring genetic disorder. In the chicken with Hereditary Muscular Dystrophy, both muscles are genotypically identical; however, only the PLD expresses the dystrophic phenotype, the ALD remains unaffected. (Cosmos, 1979b).

NERVE AND MUSCLE

The previous literature clearly shows that although the ALD and PLD are synergistic muscles, they have different characteristics. The ALD is adapted for long, sustained contraction, as a holding muscle, while the PLD is adapted for phasic motion. The functional properties of these two muscles probably depend on the differences in myosin ATPase activity, internal structure, membrane properties and metabolism.

The above observations direct one to investigate factors which influence the development of these muscle properties. Clearly, one of the main extramyogenic factors is the nerve, as shown by the dependence of a muscle on its nerve during development and its degeneration following denervation (Gútmann, 1976).

Cross Innervation in Mammals

In 1960, Buller, Eccles and Eccles investigated the possible relationships between nerve and muscle in determining the speed of muscle contraction "in an attempt to determine whether the appropriate matching of motoneurons and muscle were brought about by motoneurons influencing muscle differentiation or vice versa by muscle influencing motoneurons" (Buller et al., 1960a, p.399). They showed that in the newborn kitten

all muscles contract slowly and that only after several weeks of postnatal development does the contraction time of the fast muscle decrease to its adult value. During this period, the contraction time of the slow muscle also decreased but it again became prolonged to achieve its adult value. In experiments where nerves to muscles were cut and allowed to self reinnervate, there was no change in the speed of contraction of the slow twitch and the fast twitch muscles. However, when nerves which innervate slow muscles were crossed to fast muscles and vice versa, within one hind-limb of 16-22 day old kittens, the contraction time of the fast muscle increased while that of the slow muscle decreased. Similar results were obtained with muscles of adult cats (Buller et al., 1960a,b).

The demonstration by Buller et al. (1960a,b) that the isometric contraction time of muscles in cats can be regulated by the motor nerve initiated many more studies in an effort to correlate physiological, biochemical and histochemical properties with innervation. It was demonstrated that cross reinnervation of the fast and slow twitch muscles in mammals at any time during postnatal development results in a conversion of many of the physiological, biochemical and histochemical properties that are specific to these muscles. However, it was generally found that cross reinnervated muscles are not completely transformed in that some fibres within the cross reinnervated muscle retain their original properties (Romanul and Van der Meulen, 1966, 1967; Dubowitz, 1967; Prewitt and Salafsky, 1967, 1970; Yellin, 1967; Guth and Watson, 1967; Guth et al., 1968; Robbins et al., 1969; Close, 1969; 1972; Bárány and Close, 1971). Nevertheless, cross reinnervation experiments have clearly demonstrated the ability of the motor nerve to influence muscle properties. Furthermore,

based on qualitative differences noted between myosins of fast and slow twitch muscles, Samaha et al. (1970) suggested that nerves can influence gene expression of muscle fibres.

The cross reinnervation experiments raise questions on the mechanisms by which the nerve exerts its influence upon muscle fibres and the specificity of this neuronal influence. Buller et al. (1960b) suggested that a trophic mediator from nerve to muscle may be responsible for the changes. However, muscle activity alone has been shown to be effective (Salmons and Vrbová, 1969; Salmons and Sréter, 1976). Although the mechanism is still not known, it seems unlikely that only one factor is involved and multiple mechanisms may be responsible (see review in Guth, 1969; Gutmann, 1976).

Cross Reinnervation in Chickens

Results of cross reinnervation experiments with mammalian muscles prompted similar studies in other vertebrates such as the frog (Miledi and Orkand, 1966), the toad (Close and Hoh, 1968) and the chicken (Feng et al., 1965). The ALD and PLD muscles in the chicken have been used as a target for the cross reinnervation studies. Since Feng et al. (1965) demonstrated that when given an equal opportunity, the denervated PLD and ALD muscles of adult chickens are reinnervated selectively by their own nerve, they questioned the absolute ability of the ALD nerve to re-innervate the PLD muscle, i.e. when the PLD nerve was effectively excluded. These workers indicated that the degree of success of the cross reinnervation of the PLD by the ALD nerve was "very poor" and that no more than one-tenth of full reinnervation occurs. Based on visual observation, they

reported that the contraction of the cross reinnervated PLD muscle was as fast as the control PLD muscle. However, in their experiments, a high frequency nerve stimulation was needed in order to record maximum electrical responses from the cross reinnervated PLD, whereas, control PLD muscles required a single nerve stimulation only. This difference in response to nerve stimulation was attributed to differences at the neuromuscular junctions.

Zelená et al. (1967) and Hník et al. (1967) also cross reinnervated the PLD muscle with the ALD nerve in adult chickens. These investigators tested whether the type of motor innervation would be changed according to the new nerve supply and whether the structural electrical and mechanical properties of the cross reinnervated muscles would be transformed. Using AChE staining, they found that all the motor endplates examined in the cross reinnervated PLD were of the "en grappe" type with low AChE activity characteristic of the normal ALD. However, the number of endplates along the cross reinnervated fibres was smaller, i.e. one or two endplates within a segment where eight to ten junctions normally occur in the control ALD. Only endplate potentials were recorded extracellularly in response to indirect stimulation whereas propagated action potentials were evoked only as a result of post tetanic potentiation. The contraction time of the twitch, evoked during post tetanic potentiation, as well as the ultrastructure of the cross reinnervated muscle fibres did not change. Hník et al. (1967) suggested that the low evoked potential recorded in the cross reinnervated PLD is due to the properties of the ALD nerve terminal rather than to changes in the membrane properties of the cross reinnervated PLD. They assumed that the small quantity of transmitter released by the ALD

nerve terminals is not enough to generate a propagated action potential in response to single nerve stimulation. Vyskočil et al. (1971) gave direct evidence for this assumption when they recorded both miniature endplate potentials and evoked potentials from PLD muscles cross reinnervated by the ALD nerve. They showed that the low synaptic response at the neuromuscular junctions of the cross reinnervated PLD can be explained by the decrease in the number of quanta released by single pulses. However, no changes in the input resistance, threshold for action potential or the size of individual quanta could be detected.

Koenig (1970), Koenig and Fardeau (1973) and Bennett et al. (1973) have also cross reinnervated the PLD muscle by the ALD nerve in adult chickens and obtained similar results. Koenig (1970) studied the motor endplate of the cross reinnervated PLD muscle using AChE and silver staining. Koenig and Fardeau (1973) extended the initial study (Koenig, 1970) by using histochemical tests such as myosin ATPase activity following alkaline (pH = 9.6) and acid (pH = 4.35; 4.63) preincubation, oxidative and glycolytic enzymic activity. Bennett et al. (1973) tested the distribution of AChE sensitivity along individual fibres, the evoked potential, and AChE activity of cross reinnervated PLD muscle fibres.

The above studies demonstrate that in adult chickens PLD muscles cross reinnervated by the ALD nerve have motor endplates which resemble the "en grappe" type of the ALD in gross morphology, in AChE activity (Zelená et al., 1967; Hník et al., 1967; Koenig, 1970; Bennett et al., 1973). and in quantal content (Vyskočil et al., 1971; Bennett et al., 1973). Because the quantal content is low, single nerve stimulation produces low evoked potential, no propagated action potential and no twitch

contraction (Feng et al., 1965; Zelená et al., 1967; Hník et al., 1967; Bennett et al., 1973). However, the number of endplates per fibre, as assessed by either AChE activity or ACh sensitivity along the fibres, is smaller than is usually observed in control ALD muscles (Zelená et al., 1967; Hník et al., 1967; Koenig, 1970; Bennett, 1973). Furthermore, the contraction time (Zelená et al., 1967; Hnik et al., 1967), the histochemical properties (Koenig and Fardeau, 1973) and the ultrastructural characteristic (Hník et al., 1967) of the cross reinnervated PLD muscles were not transformed to those of a slow tonic muscle.

The failure of the ALD nerve to transform PLD muscles of adult chickens led Jirmanová and Zelená (1973, 1974) to perform the cross reinnervation experiments in newly hatched chickens. They predicted that younger fibres would be more responsive to changes influenced by the nerve. In order to avoid self reinnervation, they did not cross the ALD nerve to the intact PLD muscle as had been performed in adult chickens; instead they transposed the insertion of the PLD muscle from the right side of the animal to the left side by cutting the tendon, the nerve and the blood supply and leaving the muscle origin intact. They then removed the left ALD muscle and allowed the ALD nerve to reinnervate the transposed PLD muscle. Following this procedure, they observed that at a very early stage postoperatively, many fibres had degenerated due to ischemia, and significant regeneration followed. This process resulted in the existence of many ultrastructurally recognizable "young fibres" at a relatively late stage of development. From 5 to 18 months postoperatively these muscles revealed much the same results as those noted in the cross reinnervated PLD in adult chickens, i.e. the motor endplates, stained for AChE, were of the

slow type, their number per fibre remained small and single nerve stimulation did not elicit contraction of the cross reinnervated PLD muscle except during post tetanic potentiation. However, there were some differences. In the muscle cross reinnervated in newly hatched chickens, about 30% of the fibres had ultrastructural properties closely resembling the slow ALD fibres, indicating a transformation of PLD fibres to ALD fibres. The remaining fibres were a heterogeneous population which showed a range in the degree of ultrastructural transformation from a partial change to none.

The studies by Vyskočil and Vyklický (1974), Bennett and Pettigrew (1974) and Hník et al. (1977), using the same experimental technique described by Jirmanova and Zelená (1973), support the conclusion that the transformation to slow type fibres occurs only when cross reinnervation of the PLD muscle is performed in young chickens. Vyskočil and Vyklický (1974) performed the cross reinnervation in young (7 days old) as well as adult chickens, and demonstrated that only in young chickens were multiple areas of increasing ACh sensitivity formed along cross reinnervated PLD muscle fibres. (However, they tested a very small number of fibres.)

Hník et al (1977) examined the contractile response and the myosin ATPase activity of cross reinnervated PLD muscles and found that at 2 and 6.5 months postoperatively both the contractile times of the cross reinnervated PLD muscle were significantly longer and that the myosin ATPase activity was lower than that of the unoperated PLD. Neither of these two properties, however, equalled the results obtained with unaltered ALD muscles which indicated again that not all fibres had been transformed.

Bennett and Pettigrew (1974) reported that in two successfully cross

reinnervated muscles, two groups of fibres were identified. "The large group of fibres had localized ChE deposits of high intensity and of 'en plaque' configuration..." while, "the other, smaller group of fibres had localized ChE deposits of low intensity and of 'en grappe' configuration." (p.566) Using the silver impregnation technique they showed that both types of endplates are associated with nerves. Recordings of miniature endplate potentials also demonstrated the existence of two kinds of fibres. Jirmanová and Zelená (1973) claimed that "all fibres" in the cross reinnervated PLD muscle have "en grappe" innervation as suggested by Hník et al. (1967) in the cross reinnervated experiment performed with adult chickens. Although Jirmanová and Zelená (1973) and Bennett and Pettigrew (1974) demonstrated that only some of the fibres in the cross reinnervated PLD muscle show characteristics of slow tonic fibres, Jirmanová and Zelená (1973) claimed that this occurs despite the fact that all fibres received "en grappe" types of innervation. However, Bennett and Pettigrew (1974) showed that both "en plaque" and "en grappe" types of innervation exist in the cross reinnervated PLD muscle.

The results of these studies indicate that the degree of transformation of the fast twitch fibres, cross reinnervated by the ALD nerve at hatching, differs from fibre to fibre and that many fibres remain unchanged. It is unknown yet which factors are responsible for this heterogeneity. Jirmanová and Zelená (1973) suggested that the fully transformed fibres are the fibres which have been cross reinnervated during regeneration. Hník et al. (1977) on the other hand, have suggested that the degree of transformation depends on the density of the nerve terminals along a cross reinnervated fibre.

The mechanism(s) involved in the neuronal influence are also still unresolved. Since the amount of transmitter released by the ALD nerve innervating the PLD muscle is low (Vyskočil et al., 1971), Vyskočil and Vyklický (1974) suggested that it is unlikely that any muscle action potential activity could be generated in vivo in the cross reinnervated PLD muscle and thus, muscle action potential activity cannot be considered as a factor involved in the transformation of cross reinnervated PLD muscles as has been suggested with mammalian muscle (Salmons and Sréter, 1976).

Cross reinnervation experiments on both mammalian and avian muscles have demonstrated the ability of the motor nerve to influence the expression of muscle properties. Mammalian fast twitch muscle can be transformed in both adult and young animals. However, the transformation of the fast twitch fibres in chickens can only occur when cross reinnervation is performed in newly hatched chickens. Furthermore, not all muscle fibres innervated by the foreign nerve in the cross reinnervated muscle have been transformed. The failure to achieve full transformation may indicate that other internal and external factors besides the motor nerve may also be involved in the expression of muscle properties.

HEREDITARY MUSCULAR DYSTROPHY OF THE CHICKEN

Characteristics of the dystrophic chicken

Hereditary muscular dystorphy in the chicken was first reported by Asmundson and Julian (1956). The abnormal chickens were discovered in a commercial New Hampshire flock which had been developed for heavy breast muscles. The clinical criterion for the abnormality is the inability of

the chick to right itself after falling or when placed on its back on a flat surface. The data from breeding studies done by these authors indicated that the abnormality is the consequence of an autosomal recessive gene abnormal muscling (am). Preliminary studies reported by Asmundson and Julian (1956) indicated that most of the major muscles of the chicken are affected, although some of the muscles (e.g. pectoralis major) are more extensively involved than others. Each muscle also shows a different time of onset and rate of progression of the disease which is grossly characterized by early hypertrophy followed (at different times) by atrophy and degeneration of muscle fibres, increase in fat deposition and increase in the number of muscle fibre nuclei. Extensive studies have been carried on in an effort to explore and to characterize all the differences between normal chickens and those with hereditary muscular dystrophy. As a result of these earlier studies, several lines of dystrophic chickens were developed by selectively mating chickens on the basis of special characteristics, e.g. high fat content, early or late onset of the disease and early atrophy of muscles. The major characteristics of the various lines of dystrophic chickens (developed and maintained at the University of California at Davis) have been reviewed by Julian and Asmundson (1963); Asmundson et al. (1966) and Wilson et al. (1979).

It was noted that unless a selection for rapid growth was performed in either normal or dystrophic chickens, the growth and body weight of chickens with hereditary muscular dystrophy did not differ from those of normal chickens (Asmundson et al., 1966). However, in contrast to body weight, muscles of the different lines of dystrophic chickens have many characteristics that distinguish them from normal muscles (Cardinet et al.,

1972; McMurty et al., 1972; Randall and Wilson, 1980). All dystrophic chickens of the California lines exhibit early muscle hypertrophy with the exception of line 307 which was selected for high fat, and line 433 which was an outcross between dystorphic New Hampshire and White Leghorn breeds. These two lines show early muscle atrophy (Randall and Wilson, 1980). Furthermore, the dystrophic muscle demonstrates abnormal electromyographic activity which suggests that these fibres are myotonic (Holliday et al., 1965). High creatine phosphokinase activity in the plasma of dystrophic chickens has been found to increase progressively with age and is probably related to the progressive damage of dystrophic muscle fibres (Holliday et al., 1965). Histological analyses have revealed structural abnormalities such as high variation in the size of muscle fibres (McMurty et al., 1972), increased number of nuclei, extensive destruction of muscle fibres and abnormal levels of fat deposition (Julian and Asmundson, 1963). Abnormal metabolism in the dystrophic muscles is suggested by high levels of oxidative enzymes, and by low levels of glycolytic enzymes (Cardinet et al., 1972). High levels of proteolytic enzymes (e.g. cathepsins) (Peterson et al., 1972) and changes in the levels and molecular forms of lactic dehydrogenase and AChE have also been reported (Kaplan and Cahn, 1962; Wilson et al., 1970).

Chung et al. (1960) produced two isogenic chicken lines which differ only in the presence or absence of the dystrophic gene. New Hampshire dystrophic hens derived from the original line (N3) reported by Asmundson and Julian (1956) were crossed with White Leghorn roosters. Also the White Leghorn roosters were crossed with local New Hampshire hens yielding F1, F2 and backcross generations. The progeny of this dystrophic line (known as

the "Storrs Line") is currently maintained in the University of Connecticut at Storrs (Cosmos et al., 1980). The Storrs line was used in the present study. As with all the other dystrophic lines, the Storrs line is homozygous for the hereditary muscular dystrophy mutation (am). Chickens of this line fail the righting test by 8 weeks of age. Unlike most dystrophic lines, the Storrs line is characterized by lower weight of the pectoralis muscle very early ex ovo instead of hypertrophy followed by atrophy. Many characteristics noted in the other dystrophic lines are expressed also in the muscles of the Storrs bird. These include variation in size and shape of fibres, increased number of nuclei, destruction, high fat content, changes in the level of metabolic enzyme (Cosmos, 1966, 1970; Cosmos and Butler, 1967; Cosmos et al., 1979b; Cosmos et al., 1980) and increased level of proteolytic enzymes (Iodice et al., 1972).

Although the degree of variation of a specific property may not be the same in every dystrophic line studied, one feature appears to be common to all: the dystrophic phenotype is specifically expressed in the fast twitch, focally innervated glycolytic fibres while the slow tonic, multiply innervated oxidative fibres are spared (Julian and Asmundson, 1963; Cosmos, 1966, 1970; Cosmos and Butler, 1967; Linkhart and Wilson, 1975).

ONTOGENETIC ANALYSIS OF GENETICALLY DYSTROPHIC MUSCLES

The metabolic differences noted between muscles of normal and dystrophic adult chickens could result from a regression from the normal pattern or equally well from an arrest of differentiation early in development.

Studies of adult dystrophic muscles at various ages led Julian and Asmundson (1963) to suggest that hereditary muscular dystrophy in the chicken is a progressive degenerative disease. However, differences between the pectoral muscles of normal and dystrophic chicks were noted at 14 days in ovo (Cosmos, 1964) as alterations in the intracellular distribution of calcium ions. The importance, therefore, of examining the dystrophic process during development of the chicken was stressed.

Cosmos (1966, 1970) and Cosmos and Butler (1967) peformed comparative biochemical and histochemical analyses of muscles of normal and dystrophic (Storrs line) chickens from early embryos up to sexual maturity. The activities of the glycolytic enzyme Pase and the odixative enzyme SDH were selected as markers for the metabolic state of the muscle during development. The results of such comparative studies showed that the slow tonic soleus muscles of both genotypes achieved chemical maturation early in ovo and that their enzymic profile remained unaltered during development ex ovo. In contrast, the fast twitch pectoral muscle of normal genotype alters its enzymic profile from aerobic in ovo to anaerobic ex ovo. Fast twitch pectoral muscles of dystrophic genotype did not successfully complete these metabolic changes with the result that they demonstrate abnormal levels of both oxidative and glycolytic enzymes.

Such results indicate that hereditary muscular dystrophy in the chicken is expressed in the fast twitch muscles as an arrest of metabolic differentiation early in development (for a review, see Cosmos et al., 1979a; 1980).

ETIOLOGY OF THE DISEASE - NERVE OR MUSCLE?

As has been described earlier, the ability of motor nerves to influence muscle fibre properties has been demonstrated in both mammals and birds. Since hereditary muscular dystrophy of the chicken is expressed specifically in fast twitch glycolytic fibres, it seemed reasonable to implicate a neuronal factor in the etiology of the disease. Cosmos and Butler (1972) performed heterotransplantation of pectoral muscles between newly hatched normal and dystrophic chickens in order to test the involvement of extramyogenic factors, as well as the motor nerve, in the phenotypic expression of the disease. Using a variety of histochemical and structural analyses they studied these transplants between 2 to 37 weeks postoperatively. Their results showed that both normal and dystrophic transplants differentiated their specific characteristics regardless of the genotype of the host. In subsequent studies, Cosmos (1973) and Cosmos et al. (1979a) performed heterotransplant experiments whereby pectoral muscles from 12 day old normal or dystrophic embryos were transplanted to normal or dystrophic one-day-old chickens. These studies confirmed their earlier results and indicated that neither the motor nerves nor the other extramyogenic factors determined the properties of the dystrophic pectoral muscles. Furthermore, these results demonstrate that genetically dystrophic nerves innervating the dystrophic pectoral muscle are competent since these nerves support the proper growth and differentiation of transplanted genetically normal muscle. However, fast twitch muscles of dystrophic genotypes seem unable to respond to the appropriate neuronal demands to properly differentiate the mature characteristics of glycolytic muscles.

Other investigators (Linkhart et al., 1975, 1976; Wilson et al., 1979; Rathbone et al., 1975; Rathbone and Stewart, 1979) have attempted to determine whether an extramyogenic factor such as the motor innervation can influence the expression of dystrophy earlier than 12 days in ovo. Linkhart et al. (1975) transplanted limb buds between dystrophic (413 California line) and normal (White Leghorn) embryos at 3.5 days in ovo. Muscles were analyzed at 25 weeks postoperatively. The analyses focused mainly on the biceps but pectoral and extensor carpi radialis muscles were also examined. Using electromyographic recording, structural changes and enzymic activity (AChE, LDH, SDH) as criteria, they concluded that genetically dystrophic muscle exhibits the phenotype of dystrophy regardless of the genotype of the host or the motor innervation.

Rathbone et al. (1975) have explored the possibility that the neural tube induces the phenotype of dystrophy earlier than 3.5 days in ovo. They transplanted a neural tube between normal and dystrophic (Storrs line) embryos at 2 days in ovo and measured the activity of thymidine kinase at 18 days in ovo as a criterion for dystrophy (Weinstock and Dju, 1967). In subsequent works, Rathbone and Stewart (1979) measured the cholesterol concentration of pectoralis muscles at 11 days in ovo as another criterion. In both these studies the neural tube transplant affected the level of thymidine kinase and cholesterol irrespective of the host genotype. These investigators hypothesized that the neural tube has an early inductive influence on muscle precursor cells and that as these cells develop they retain the induced characteristics even when placed in different environments. This is a reasonable conclusion, if indeed induction occurs. However, reasons exist to doubt

these conclusions. The phenotypic expression of many muscle characteristics can be influenced by surgical manipulation of its motor nerve supply (Gutmann, 1976). Rathbone et al. (1975, 1979) have not shown that the muscle characteristics which they study, i.e. thymidine kinase activity and cholesterol level, can or cannot be altered by surgical manipulation, (e.g. cross reinnervation). Evidence demonstrating that the transplanted neural tube of either genotype is viable in the foreign environment and that the pectoralis muscle at the stages examined is innervated fully by the appropriate transplanted motor nerve is insufficient. And finally, Rathbone and Stewart (1979) indicated that two important limitations exist in the technique used in their study.

In general, the evidence to date favours the conclusion that hereditary muscular dystrophy of the chicken arises from a defect in the muscle rather than in the motor nerve. Additional support for such conclusions is derived from the studies of Sansone and Lebeda (1976) who observed that spinal cords of normal and dystrophic chickens are remarkably similar except for the large amount of glycogen found in the ventral horn neurons of dystrophic chickens. In addition, Di Giamberardino et al. (1979) demonstrated that the transport of AChE molecular forms along the peripheral nerves (sciatic and superior brachialis nerves) is similar between normal and dystrophic chickens.

PLD Muscle of Dystrophic Chickens

The PLD muscle of dystrophic chickens is a fast twitch glycolytic muscle. Like the pectoralis, it demonstrates abnormalities related to the dystrophic gene, i.e. abnormal muscle weight (Hoekman, 1976), increased

number of muscle fibre nuclei, abnormal size and shape of fibres, destruction of fibres and high fat content (Cosmos et al., 1979b); metabolic changes (Mazliah et al., 1976; Cosmos et al., 1979b) and changes in AChE activity and molecular forms (Wilson et al., 1973; Lyles et al., 1979). Unlike the pectoralis, the size and position of the PLD muscle in the chicken makes it more suitable for electrophysiological and mechanical analyses.

Albuquerque and Warnick (1971) studied the passive electrical properties of PLD muscle of normal and dystrophic chickens from both the 304 California line and the Storrs line using intracellular recording from several surface muscle fibres under in vitro conditions at 23°C. No differences were noted in the membrane potential between PLD fibres of normal and dystrophic muscles. However, in the dystrophic muscles, membrane resistance, membrane capacitance and duration of miniature end-plate potentials were increased, while the rate of rise of the action potential is decreased and with repetitive indirect stimulation (10-20 Hz), the action potential decreased in size and disappeared.

Subsequent studies (Warnick and Albuquerque, 1978; Warnick et al., 1979) using similar methods were performed on the newer dystrophic 413 California line. These studies failed to show differences between the action potential and other membrane properties of normal and dystrophic PLD fibres. Thus, the differences noted in the membrane cable properties (Albuquerque and Warnick, 1971) may not be related to the primary genetic defect. However, all the dystrophic lines used in these electrophysiological studies show the decremental response of the muscle action potential to repetitive indirect stimulation. This decremental response

is attributed to depression of neurotransmitter release during tetanic stimulation (Warnick et al., 1979). These observations have led Warnick and Albuquerque (1978) and Warnick et al. (1979) to suggest that the various signs of atrophy and weakness noted in dystrophic chickens could be the result of altered calcium metabolism influencing evoked transmitter release.

Hoekman (1976) studied the in vivo contractile properties of PLD muscles in normal and dystrophic (304 California line) chickens 8 to 16 weeks of age. His results show no significant difference between the contraction time or time to half-relaxation of normal and dystrophic PLD muscles. The PLD muscle of dystrophic chickens demonstrated tetanic tension comparable to normal following indirect stimulation at the rate of 10 to 300 Hz. However, the twitch tension and the maximum rate of rise of tension during both twitch and tetanus are lower in the dystrophic PLD. Furthermore, the fusion of the tetanus response is higher in the dystrophic PLD, and higher stimulation frequencies were needed to obtain the same tetanic tension in dystrophic compared to normal muscles.

The demonstration that PLD muscles of dystrophic chickens can evoke tetanic tension comparable to normal in response to 10-300 Hz nerve stimulation (Hoekman, 1976) contradicts the findings of Albuquerque and Warnick (1971) that the muscle action potential disappears with a 10-20 Hz nerve stimulation. It is possible that Albuquerque and Warnick (1971) based their data on recordings from a relatively small number of fibres and were actually examining hypertrophied fibres as suggested by Lebeda and Albuquerque (1975). These hypertrophied fibres might have undergone

abnormal changes at the neuromuscular junctions, or, the *in vitro* conditions employed may not be suitable for dystrophic muscle since small changes in ionic concentration or differences in temperatures may cause the decremental response of the dystrophic muscle action potential.

Hoekman (1976) suggested that the changes in the contractile response noted in the dystrophic PLD represented a shift towards slow muscle characteristics. In subsequent work, Hoekman (1977) studied the relative fatigability of the dystrophic and normal PLD muscles during twitch and tetanus response. His results demonstrate a fatique resistance in the dystrophic muscle which probably indicates a change in overall muscle metabolism. Hoekman (1977) also demonstrated that the twitch contraction elicited directly is larger than the twitch contraction elicited indirectly in the dystrophic PLD. No difference between direct to indirect twitch tension ratio was noted in normal PLD muscle. Since potassium chloride infusion resulted in reduction of the direct to indirect twitch tension ratio toward unity, he concluded that the low contractile response of the dystrophic PLD is due to synaptic failure (as suggested by Albuquerque and Warnick, 1971) and that potassium chloride infusion resulted in recovery of neuromuscular transmission.

Although these results may imply an abnormal relation between nerve and muscle in the dystrophic PLD muscle, it does not necessarily mean a nerve defect since Cosmos (1974) has suggested that a retrograde influence of disease muscle fibres on the innervating neurons may lead to an abnormal neuromuscular relationship. The results of Albuquerque and Warnick (1971) and Hoekman (1977) may indicate that non-functional muscle fibres exist in the dystrophic PLD (Warnick *et al.*, 1979). Furthermore,

DeSantis et al. (1977) demonstrated that retrograde transport of horseradish peroxidase in PLD nerves of both normal and dystrophic genotypes is similar. No differences in the number of the location of horseradish peroxidase labelled motor neurons could be observed between normal and dystrophic chickens.

The studies on the PLD muscle and nerves support the conclusion (Cosmos and Butler, 1972) that hereditary muscular dystrophy in the chicken arises from an intrinsic defect in the fast twitch glycolytic muscle, possibly affecting its ability to respond appropriately to normal neuronal demands.

THE RESEARCH PROBLEM

SPECIFIC BACKGROUND

It has been well documented that a close relationship exists between the type of innervation, the speed of contraction and the enzymic metabolism of a given vertebrate skeletal muscle (Buller et al., 1960a,b; Close, 1972). There remain, however, many questions regarding neuronal control of muscles, e.g. how specific is this control and through what mechanism does it operate. Furthermore, the involvement of nerves in the expression of pathological changes of the muscle in many neuromuscular disorders is not fully understood. Hereditary muscular dystrophy of the chicken is a good example of this dilemma.

Hereditary muscular dystrophy of the chicken is expressed specifically in fast twitch, focally innervated glycolytic muscles while slow tonic, multiply innervated oxidative muscles are spared disease characteristics (Cosmos, 1966; Cosmos and Butler, 1967; Wilson et al., 1973; Mazliah et al., 1976; Mazliah and Cosmos, 1979a,b; Cosmos et al., 1979b). Ontogenetic analyses of the two muscle types reveal that slow tonic muscles of both normal and dystrophic chickens achieve chemical maturation early in ovo and that their enzymic profiles are not altered during subsequent development ex ovo. In contrast, fast twitch muscles of normal chickens show an alteration in their metabolic profile from aerobic in ovo to anaerobic during development ex ovo. Fast twitch muscles of dystrophic chickens during a similar time period do not success-

fully complete this change; instead, they maintain abnormal levels of both oxidative and glycolytic enzymes (Cosmos, 1966; Cosmos and Butler, 1967). Since only fast twitch muscles of chickens are affected by the disease, a neurogenic etiology was investigated. However, the transplantation studies of Cosmos and Butler (1972) and Cosmos (1973) in which the pectoral muscles from newly hatched normal and dystrophic chickens were exchanged, and the work of Linkhart et al. (1976) which involved limb bud transplantation between 3.5 day old normal and dystrophic embryos, give strong evidence that hereditary muscular dystrophy of chickens is a primary muscle defect and not a primary defect expressed extramuscularly. The studies of Cosmos et al. (1972, 1973, 1979a) indicate that motor nerves of dystrophic genotype are competent since they are able to support the differentiation of fast twitch muscles of normal genotype. Fast twitch muscles of dystrophic genotype, however, even when innervated by nerves of either normal or dystrophic genotype, cannot respond to controls governing proper differentiation of glycolytic muscles.

THE HYPOTHESIS

The results cited above suggest that the fast twitch muscle of dystrophic genotype is incompatible with its environment in that it is unable to respond to factors of the environment to alter completely its metabolism from aerobic in the embryo to anaerobic during development ex ovo. Thus it seemed reasonable to postulate that if the fast twitch muscle of dystrophic genotype is not asked to alter its embryonic metabolism, it will not express dystrophic phenotypes.

RATIONALE

Since motor nerves influence the metabolic characteristics of skeletal muscle, and since slow tonic muscles do not alter their embryonic metabolism and do not express dystrophic phenotypes, it was decided to replace the motor innervation of a fast twitch muscle with the motor innervation of a slow tonic muscle, within a dystrophic chicken, in order to alter the metabolic fate of the dystrophic fast twitch muscle.

EXPERIMENTAL STRATEGY

The ALD and PLD muscles in the dystrophic chicken were selected to test the above hypothesis. These two synergistic muscles are positioned side by side, and while both are genotypically dystrophic, only the fast twitch focally innervated PLD muscle shows the disease phenotype. The slow, tonic, multiply innervated ALD muscle is spared all known disease characteristics. The anatomical position of these muscles permitted a surgical cross innervation of the PLD muscle with the nerve of the ALD muscle. The surgical cross innervation was performed at hatching, since, at this time fast twitch muscles are metabolically undifferentiated and are expected to be more responsive to nerve influence than mature muscles (Cosmos, 1966, 1970; Cosmos and Butler, 1967, 1972).

To test the effect of the ALD nerve on the ex ovo development of the PLD muscle, it was decided to examine the cross reinnervated PLD muscle at various times postoperatively, since both hereditary muscular dystrophy in the chicken and cross reinnervation are time dependent. Certain structural, mechanical and histochemical properties of muscles

were selected for analyses.

CONTROL ASPECTS

The progression of dystrophy in chickens of the Storrs line has been studied extensively in the pectoralis and soleus muscles (Cosmos, 1964, 1966, 1970; Cosmos and Butler, 1967; Cosmos et al., 1979b, 1980). At the time the present study was initiated, similar studies examining the progression of dystrophy in the ALD and PLD muscles during ex ovo development were non-existent. Furthermore, the present study is the first attempt to cross reinnervate the PLD muscle with the ALD nerve in the dystrophic chicken. Thus, in order to properly assess the cross reinnervated PLD muscles it became necessary to perform the following control studies:

1. In order to reveal the progression of dystrophy in these muscles and to establish base line data for the analyses of the cross reinnervation PLD muscles, the properties of ALD and PLD muscles in dystrophic chickens of the Storrs line were analyzed and compared with normal chickens using the same criteria selected for the analyses of the cross reinnervated PLD muscles.
2. In order to have a proper normal control for the cross reinnervated experiments in the dystrophic chickens, PLD muscles of normal chickens were cross reinnervated with ALD nerves.
3. To identify changes due to the type of innervation and to check on the operative trauma, PLD muscles were self reinnervated by their own PLD nerves in both normal and dystrophic chickens, as an operative control.

CONCLUSION

The present study contains two major parts -

Part I - Developmental study; Includes results and discussion of the properties of the ALD and PLD muscles in both normal and dystrophic chickens during development ex ovo.

Part II - Experimental study: Includes results and discussion of the self reinnervation and cross reinnervation experiments in both normal and dystrophic chickens.

METHODS

ANIMALS

Fertilized eggs from normal White Leghorn chickens were obtained from a local Ontario hatchery (Martindale). Fertilized eggs homozygous for dystrophy (*am*) were obtained from the University of Connecticut, Storrs, Connecticut, U.S.A.

Eggs were incubated until hatching in a Petersime incubator and raised in the animal quarters at McMaster University Health Sciences Centre. Before hatching, day 19 *in ovo*, normal and dystrophic eggs were separated in covered cages placed at the bottom of the incubator. Newly hatched chickens were numbered with leg bands and marked on their heads before being placed in separate brooder cages. At two weeks of age, a wing band was placed permanently on the left wing and the leg band was removed. At 6 weeks of age, the chickens were moved from the brooder cages to adult cages (2 chickens per cage).

The chickens were kept in well ventilated rooms maintained at $18^{o}C$. Food and water were provided *ad lib*. The chickens were checked daily and weighed either weekly or biweekly, depending on age.

SURGICAL PROCEDURES

Anesthesia

All operations were performed on newly hatched normal and dystrophic chickens (body weight 30-40g). Initially, sodium pento-

barbitol (Somnotol® MTC) was used as the anesthetic. However, since difficulties were experienced in determining an effective dose per body weight with pentobarbitol, as has also been experienced by Shear and Goldspink (1972), Combuthal (Diamond Lab.) was used as suggested by the late Dr. D.Y.E. Perey (Personal Communication).

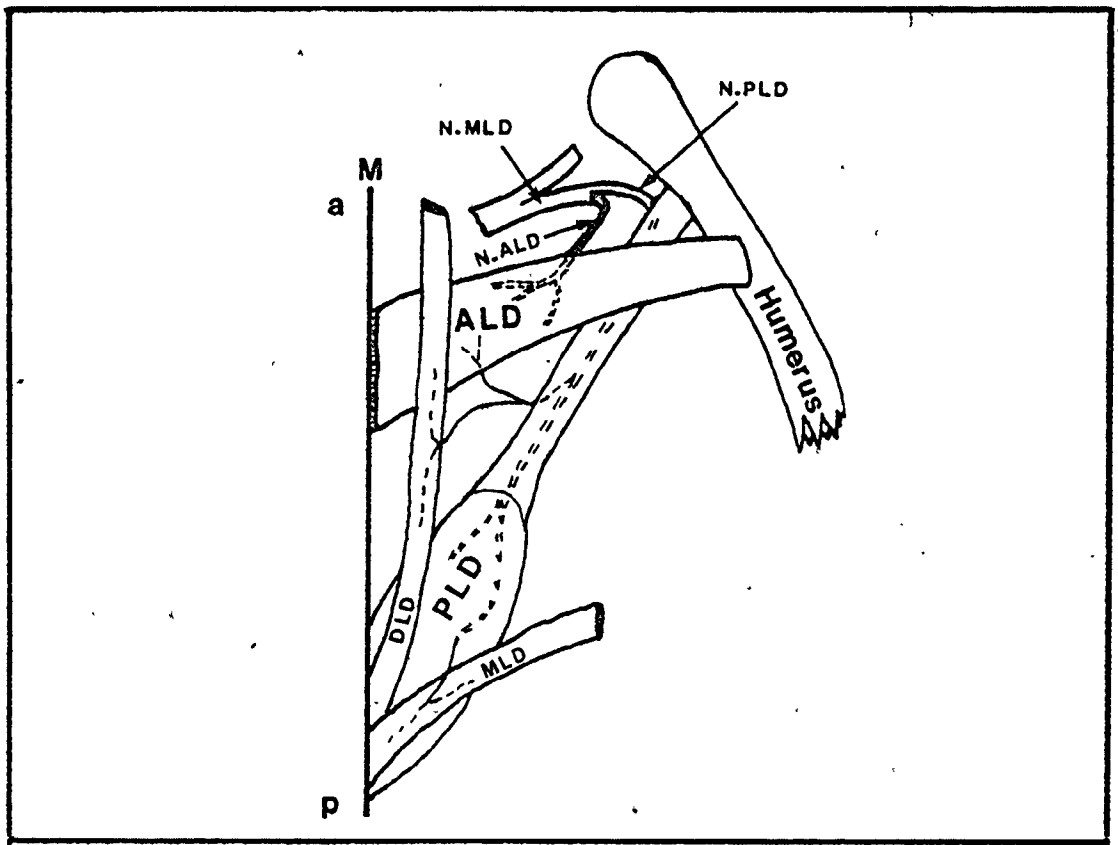
In 1978, Combuthal became unavailable from the Diamond Lab., and a similar anesthetic was prepared by dissolving 1g of pentothal sodium (Abbot Lab.) in 5.2 cc of sodium pentobarbital (65 mg per cc) diluted with 30 to 50 cc of sterile water. This anesthetic was injected intramuscularly. Although the dosage required to induce deep anesthesia varied even among chickens of the same age, the average dose found to produce satisfactory anesthesia was 0.04-0.5 cc per gram body weight (concentration 20 mg/ml).

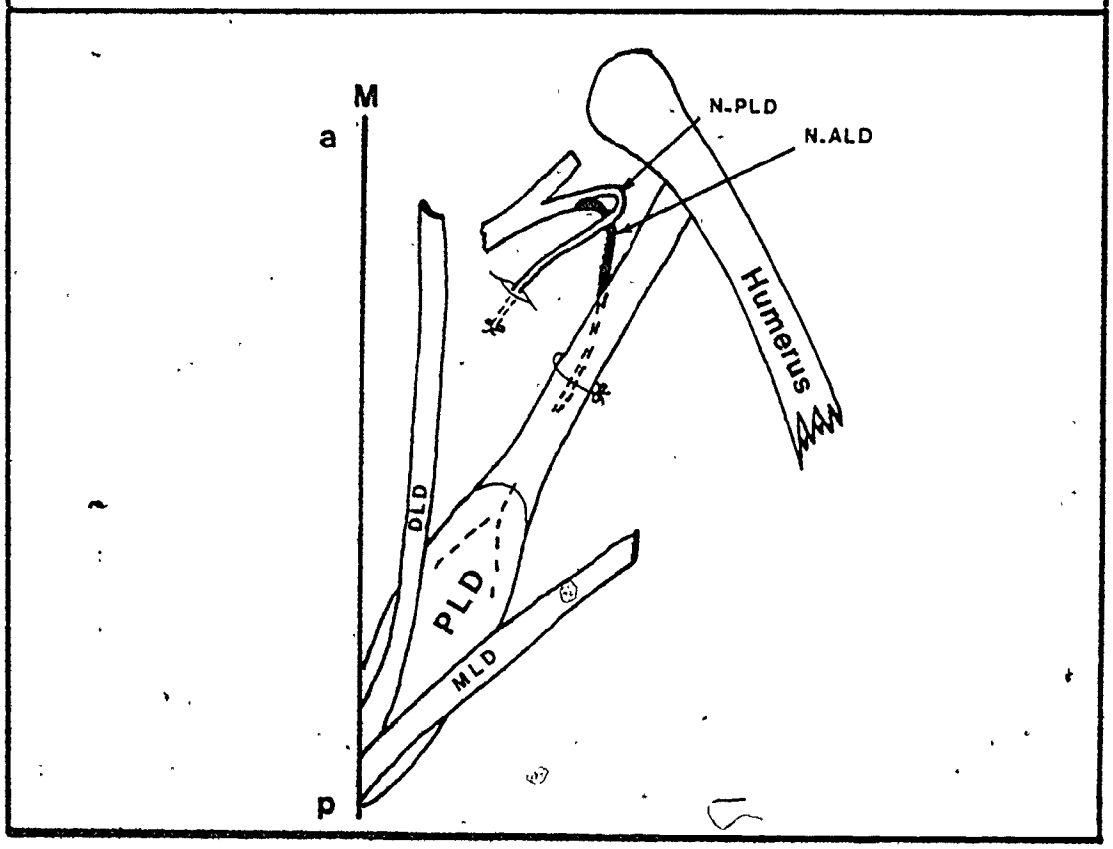
Intact Muscle Position and Innervation

The latissimus dorsi muscles in the chicken (Fig. 1) form the most superficial muscle layer in the back. They lie on either side of the dorsal mid-line. The ALD has its origin on the neural spines of the caudal cervical vertebrae. The PLD has its origin posterior to the ALD on the spines of the lateral thoracic vertebrae. The ALD enters the wing musculature by passing under the triceps brachii muscles and inserts on the humerus. The PLD has a long, narrow tendon which passes under the ALD and inserts on the fascia of the triceps brachii muscle. Both ALD and PLD muscles are innervated via the brachial plexus through the common brachiales dorsales nerve. This divides into four branches, one of which is the musculi latissimi dorsi which subsequently divides into the ALD

Figure 1: A schematic drawing of the relative position and innervation of the latissimus dorsi muscles on the back of the chicken (right side only). M - represents the dorsal midline; ALD - anterior latissimus dorsi; PLD - posterior latissimus dorsi; DLD - dorsocutaneous latissimus dorsi; MLD - metapatagial latissimus dorsi; NMLD - nerve musculi latissimus dorsi; NALD - ALD nerve; NPLD - PLD nerve.

Figure 2: A schematic drawing showing the relative position of the latissimus dorsi, muscles and nerves of the right side following the surgical manipulation at hatching, to produce cross innervation of the PLD muscle by the ALD nerve (NALD). The PLD nerve (NPLD) was cut, tied and inserted into the underlying muscle. The ALD nerve was cut and loosely tied to the PLD tendon; the ALD muscle was then removed.


N.PLD
N.MLD
M
a
N.ALD
ALD
Humerus
DLD
PLD
MLD
p


M
a
N.PLD
N.ALD
Humerus
DLD
PLD
MLD
p

and PLD nerves (Koch, 1973). The PLD nerve runs under and along the PLD tendon before it enters the muscle, the PLD nerve branches inside the muscle. The ALD nerve branches prior to its entry into the muscle and each branch further subdivides within the muscle itself. One small branch which also innervates the ALD, separates from the PLD nerve distal to the main division of the musculi latissimi dorsi nerve and proximal to the entry of the PLD nerve into its muscle. According to George and Berger (1966) and Grim (1971), the latissimus dorsi muscles consist of two more dermal muscles: the dorsocutaneous and the metapatagial latissimus dorsi, both of which are long and narrow. These two muscles have the same origin as the PLD muscle and cover the caudal end part of the PLD muscle as they run forward. The dorsocutaneous and metapatagial latissimus dorsi muscles insert in the skin at different points. Both muscles resemble the ALD muscle in their characteristics and both are innervated by branches of the PLD nerve (Grim, 1971). The PLD nerve thus contains a mixed population of axons, supplying focally innervated fibres of the PLD and multiply innervated fibres of the ALD and the two dermal muscles.

Surgical Manipulation

After the feathers of the dorsal skin were plucked, the anesthetized chick was placed on a dissecting board with a groove which fitted the ventral side and allowed for flat positioning of the dorsal side. Visualizing the area with a dissecting microscope, an incision was made along the dorsal midline between the shoulder down to the pelvic region, exposing the ALD and PLD muscles on the right side. Care was taken not to damage the dorsocutaneous and metapatagial latissimus dorsi

muscles. The connective tissues around the ALD and PLD muscles were dissected clear and the nerves exposed.

One of two surgical manipulations was then performed on the right side leading to either cross reinnervation of the PLD muscle by the ALD nerve or to self reinnervation of PLD muscle. Upon completion of the surgical manipulations, the cut dorsal skin of the chick was closed using either surgical thread or skin closures (Steri-strip® x 3M).

The operated chicks were placed under observation until the anesthesia wore off. They were kept in the laboratory area for 2 to 3 days to facilitate postoperative care and were then returned to the animal quarters.

1. Cross Reinnervation of the PLD muscle by the ALD Nerve: To cross reinnervate the PLD muscle by the ALD nerve a modification of the technique of Hnik et al. (1967) was used (Fig. 2). The ALD nerve was cut as close as possible to the muscle. The ALD muscle was then completely removed to prevent the ALD nerve from reinnervating its natural target. The PLD nerve was cut more centrally, tied off with a surgical thread, deflected back and inserted deep into an underlying muscle. The area of insertion was then covered with gelfoam (UpJohn) to hold the PLD nerve in its new position and to prevent the denervated PLD from becoming self reinnervated. The cut ALD nerve was directed to the tendon of the PLD muscle and loosely tied with a surgical thread to the PLD tendon to keep it in place and to facilitate cross reinnervation of the denervated PLD muscle. Two types of cross reinnervation experiments were performed. In one, the PLD muscle was injured along its exposed dorsal surface with iridectomy scissors to further encourage reinnervation by the foreign ALD

nerve (Studitsky 1974); care was taken to avoid injury to the two dermal muscles which cover part of the PLD. In the other, the PLD was left uninjured.

In all cross reinnervation experiments, ALD and PLD muscles of the contralateral side were used as unoperated control muscles to identify changes due solely to experimental manipulation.

2. Self Reinnervation Experiments: To self reinnervate the PLD muscle, the PLD nerve was cut and allowed to self reinnervate the denervated PLD muscle. Since the cross reinnervation of the PLD muscle by the ALD nerve was accompanied by the removal of the ALD muscle, self reinnervation experiments were also accompanied by removal of the ALD muscle. However, this procedure might have allowed for unwanted innervation of the PLD muscle by the cut ALD nerve. Therefore, in a few self reinnervation experiments the ALD muscle was left intact. In experiments where the ALD muscle was removed, the ALD nerve was tied and inserted into underlying muscles. The area of insertion was then covered with gelfoam to hold the ALD nerve in its new position and prevent it from innervating the denervated PLD. As described in the cross reinnervation experiments, the PLD was either left intact or injured along its dorsal surface in an attempt to facilitate reinnervation, and the contralateral side was used as a control.

METHODS FOR ANALYSIS OF MUSCLE PROPERTIES

Unoperated ALD and PLD muscles of both normal and dystrophic chickens were analyzed in the same way as the cross reinnervated and self reinnervated PLD muscle of both genotypes. All muscles were analyzed for

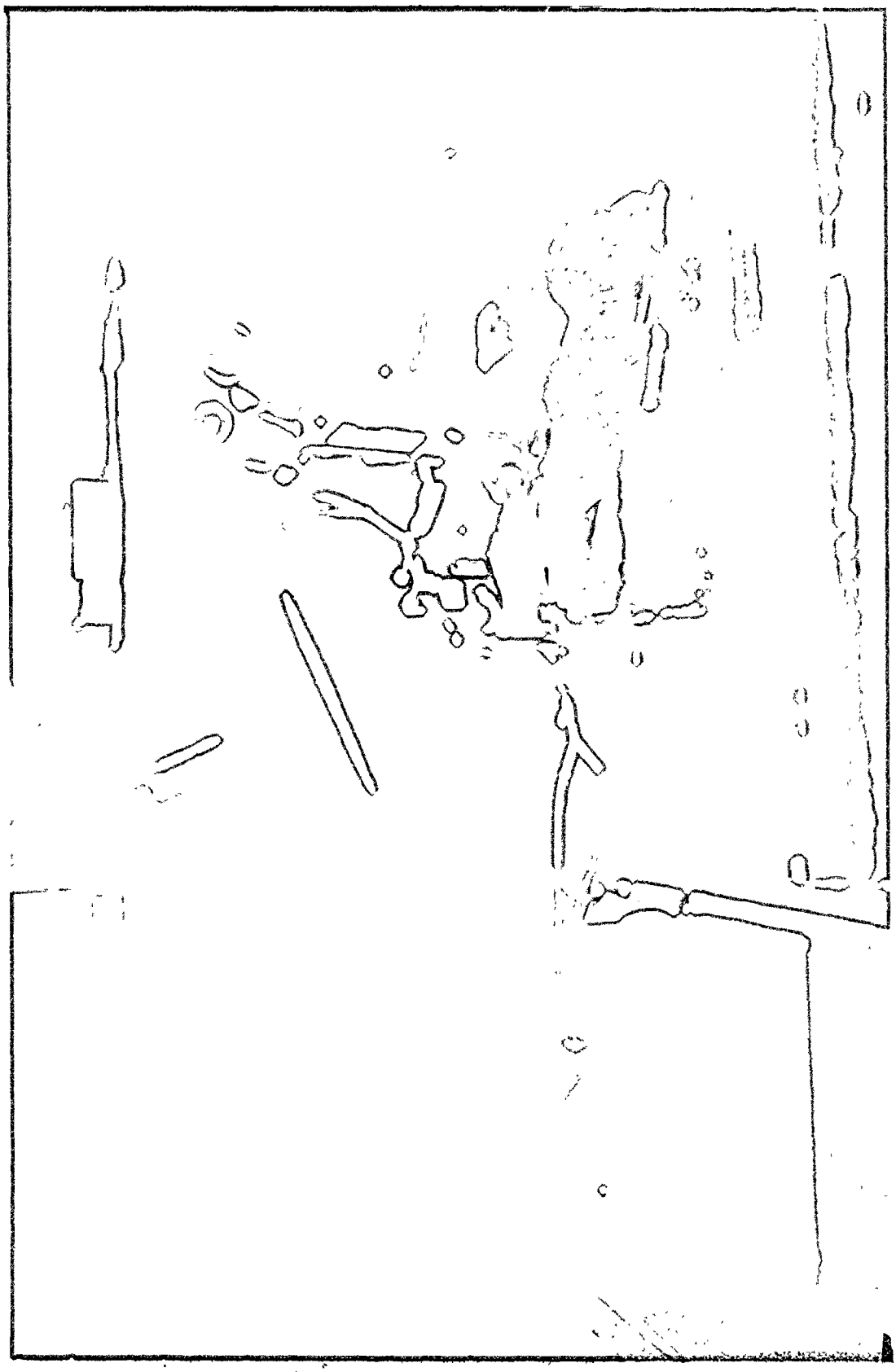
mechanical, histochemical and structural properties according to the procedures described below. These analyses were carried out on muscles from chickens 15 to 800 days ex ovo. The number of chickens of each experimental and control group are given in the appropriate section of the RESULTS.

The apparent discrepancies between the number of chickens operated on and the number for which data were obtained, result from the fact that not all measurements were obtained for all muscles. In some cases chickens died before completing the mechanical measurements.

Mechanical Analyses

The chickens were anesthetized with Combutal in a concentration of 20 mg/cc at a dosage level of 0.4-0.5 cc per 100 g body weight, administered into the leg muscles. Under anesthesia the feathers which cover the back and the proximal dorsal side of the wing were plucked. The chicken was held on a custom made base which positioned its back horizontally (Fig. 3). The base itself was fixed with button magnets to a heavy iron plate. During the experiment, the chicken was maintained on a conventional small animal respirator (Harvard 681) using a tracheal cannula through the pharynx. The flow rate was adjusted to maintain a normal respiratory rate; the minute ventilation rate was 300-500 cc (Hoekman, 1976; Duke, 1977). An incision was made along the dorsal midline between the shoulders along the thoracic vertebrae. A second incision was made laterally in the pelvic region. The skin was deflected laterally to expose the superficial muscles of the back. Paraffin oil saturated with Hanks' balanced salt solution (Hanks and Wallace, 1949) heated to 40°C (chicken body temperature)

Figure 3: A photograph showing an experimental chicken in position, immediately prior to measurement of muscle contraction. (For details, see text p. 44).



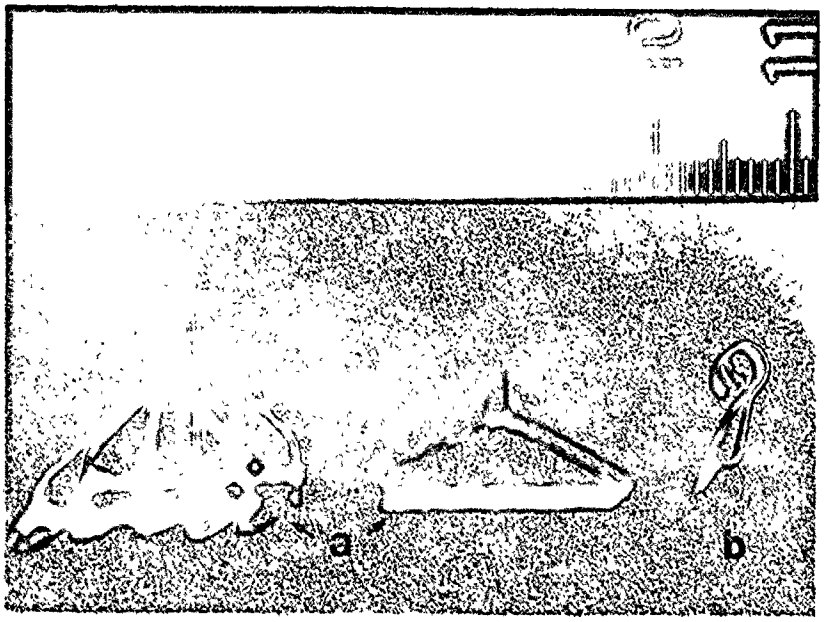
was dropped continually on the muscles to keep them wet and at the proper temperature. A heating lamp was placed beside the chicken to keep it warm. The body temperature of the chicken was checked at the beginning and periodically during the experiment with an anal thermometer. Under these conditions, a drop in body temperature of only 1-2^{o}C was observed during the course of the recordings.

Because of the anatomical differences between the positioning of the ALD and PLD muscles, it was necessary to use a different method to support each muscle for mechanical recording. In general, the technique followed was that described by Hnik et al. (1967) and Shear and Goldspink (1972), i.e. one end of the muscle was freed and tied to a mechanical transducer while the other end was maintained with its normal anatomical connection. However, since these investigators gave insufficient details of their techniques, the following method of preparing the PLD and ALD muscles was developed for in vivo measurements of their isometric contractions.

PLD Preparation: To prepare the PLD muscle for the in vivo measurement of its isometric contraction, two clamps were attached to the vertebral column, one anterior and the second one posterior to the origin of the PLD. Both clamps were fixed rigidly to the iron plate by magnetic holders placed on both sides of the chicken base. This effectively fixed the position of the origin of the PLD muscle.

The PLD muscle was freed as much as possible from the surrounding connective tissue without damaging the large blood vessels which supply the muscle at its posterior end. The long tendon was cut from its attachment and a small stainless steel hook tied to it (Fig. 4b). The PLD

Figure 4: Photograph of the stainless steel hooks used to connect the muscles to the isometric strain gauge (a) used with the ALD muscles, and (b) used with the PLD muscles; for the ALD, the particular hook used depended on the size of the muscle.

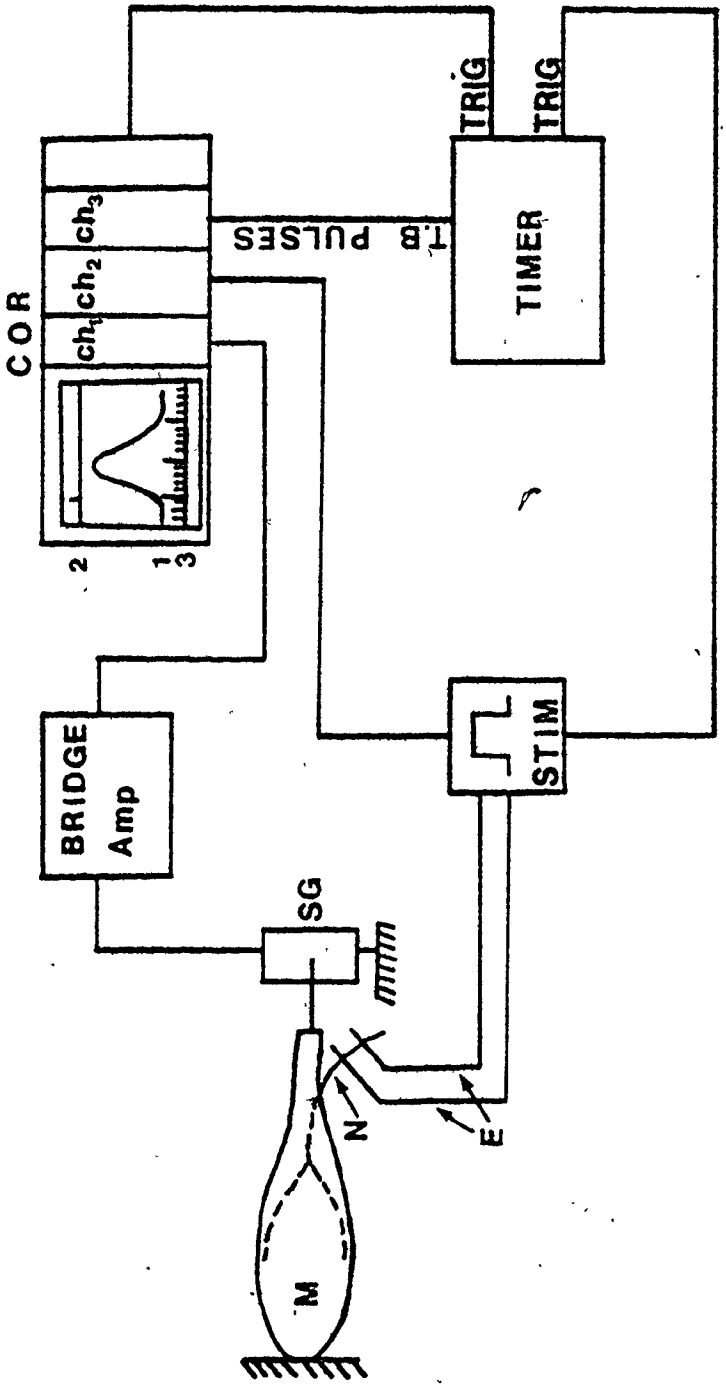

a
b

nerve was cut as close as possible to its separation from the ALD nerve. The muscle was then ready for attachment to the test equipment.

ALD Preparation: In initial experiments, the technique described by Hnik et al. (1967) and Shear and Goldspink (1972) was used. However, exposure of the insertion of the ALD muscle on the humerus and the need to cut small pieces of bone attached to the muscle, caused great damage to the surrounding wing muscles and their blood supply. Thus, an alternative technique was necessary. Mechanical tension from the ALD muscle was recorded by freeing it at its origin and attaching a special hook (Fig. 4a) to its short tendon. The muscle was then freed from the surrounding connective tissues along its length down to its insertion on the humerus. The intact wing was held rigidly by a clamp fixed to the iron plate with a magnetic holder. This minimized damage to the wing muscles. The ALD nerve was also freed from its surrounding connective tissues to allow bipolar stimulating electrodes to be placed on it without damaging the blood supply of the ALD muscle.

Mechanical Recording: The small hook tied to the muscle was attached to an isometric strain gauge (BLH SR-4 semi-conductor) and the strain gauge connected to a bridge amplifier (operational amplifier LI44J5 siliconix) with an adjustable calibration (Appendix A). The output of the bridge amplifier was displayed on a taktronix 5113 dual beam cathode ray storage oscilloscope. The nerve was stimulated with a pair of platinum electrodes with supramaximal rectangular pulses (0.02-0.1 msec, 1-50v) supplied from a stimulator (Digitimer LTD, Model DS2). A Digitimer D4030 controlled the timing and triggering of selected stimulus pulses. This system (Fig. 5) permitted a single stimulus or a train of stimuli from 10/Hz - 1000/Hz

Figure 5: A drawing of the basic circuit diagram for the measurement of the isometric contraction of the muscles. The isometric strain gauge (SG) was connected to the muscle (M) and to the bridge amplifier. The output of the bridge amplifier was displayed on a cathode ray oscilloscope (CRO). The nerve (N) was stimulated through a pair of platinum electrodes (E) by rectangular pulses supplied from the stimulator (STIM). A digitimer (TIMER) controlled the timing (T.B. PULSES) and triggering (TRIG) of the stimulator and the oscilloscope. Channel one (ch_1) of the oscilloscope displayed the muscle response (1); channel two (ch_2) monitored the electrical stimulation (2); channel 3 (ch_3) displayed the time base (3).


TRIG
TRIG
T.B PULSES
TIMER
COR
ch3
ch2
ch1
2
1
3
BRIDGE
Amp
STIM
SG
N
E
M

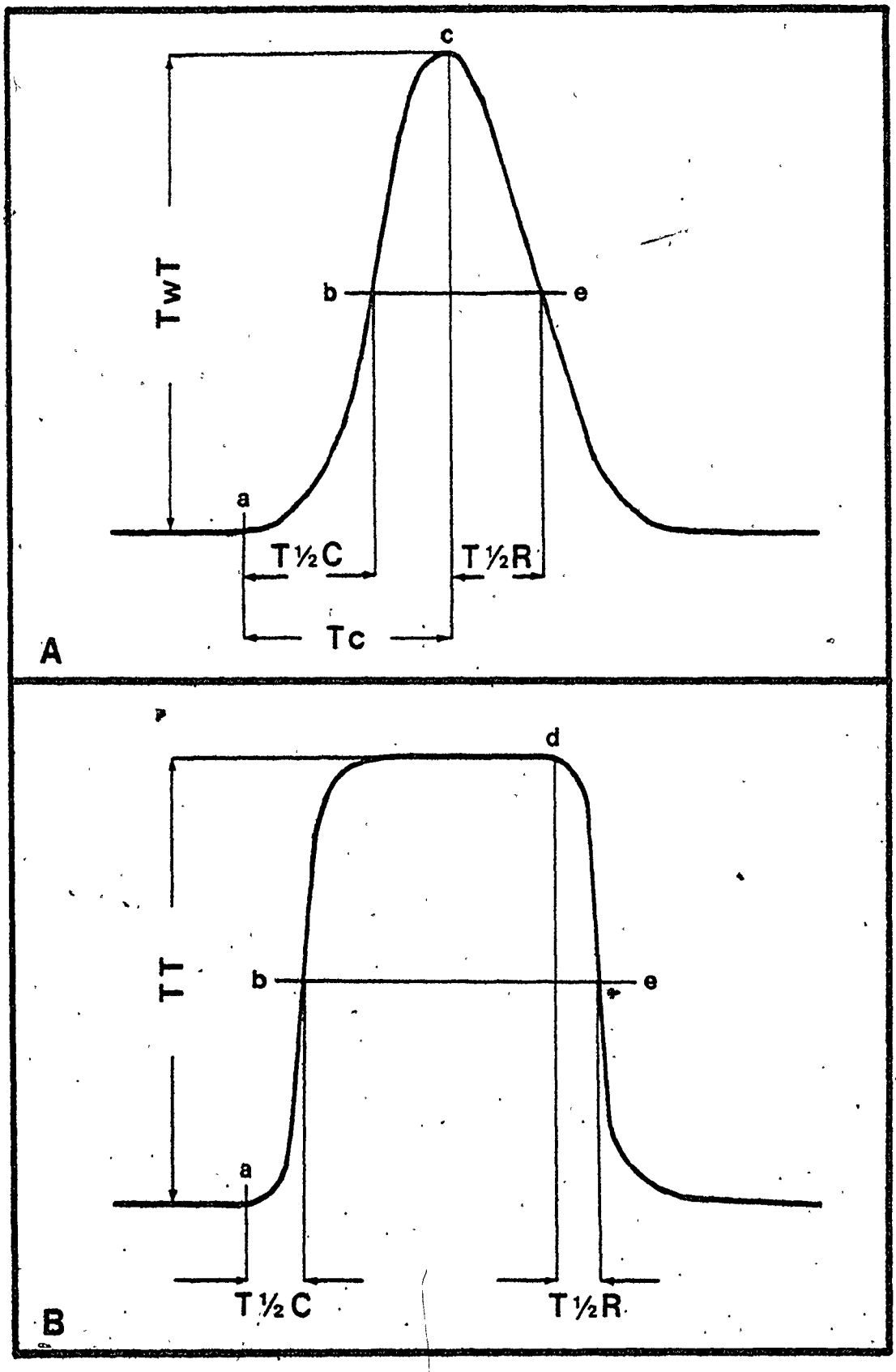
or any combination of the two to be delivered; a range of different durations and delays were used.

When the preparation was completed, resting tension of the muscle was adjusted to produce a maximal contraction. This resting tension was maintained throughout the mechanical recordings. Each muscle was tested for its response to a single stimulus or trains of stimuli at frequencies ranging from 10 Hz to 200 Hz. Preliminary studies of the contraction responses of ALD and PLD muscle of both genotypes, demonstrated that 80-100% of the muscle maximum tetanus tension was obtained at 50, 70 and 100 Hz nerve stimulation. The tetanus response of the muscles at these frequencies was chosen for the measurement of tetanic contraction properties of the muscles. Since the ALD and the successfully reinnervated PLD muscles do not respond to single stimuli, these muscles were also tested to determine their twitch response following post tetanic stimulation, i.e. indirect stimuli of 100 Hz to 300 Hz were used for 500 msec to 7 sec followed by a single stimulus.

These procedures lasted no more than 1-1.5 hours, a time interval which caused a minimal alteration to the metabolic enzymes of the muscle judged by histochemical examination.

The response of the muscle was displayed on a storage cathode ray oscilloscope and was also stored on a magnetic tape recorder (Hewlett-Packard 3964A) for future analyses. Photographs of the muscle responses were taken during the experiments using an oscilloscope polaroid camera (Tektronix C-5A). These photographs were then used to measure parameters of the responses (Fig. 6): Twitch tension (TwT), tetanic tension (TT), time to half contraction ($T_{\frac{1}{2}}C$), contraction time (Tc) and time to half

Figure 6: Illustration of the kind of measurements made from the isometric twitch (A) and tetanic (B) responses of muscles, to determine their isometric contraction properties. Twitch tension (TwT) and tetanic tension (TT) were measured as the maximum amplitude obtained for each response. The beginning of the contraction was defined as that moment (a) when the "contraction curve" first deviated from base line. The time to half contraction (T ½ C) was measured from point (a) to the moment (b) when the muscle exerts 50% of the full force developed by it during that contraction. The full-contraction time (Tc) was measured, for twitch response only, from point (a) to point (c) when the muscle attains its full contraction. The time to half relaxation (T ½ R) was measured from point (c) for the twitch response, or from the moment (d) when the muscle response starts its relaxation phase during tetanus, to the point (e) when the muscle tension has dropped to 50% of its value.


c
TwT
b
e
a
T½C
T½R
Tc
A
d
TT
b
e
a
T½C
T½R
B

relaxation ($T_{\frac{1}{2}}R$). At the end of each mechanical analysis, the muscle was prepared for histochemical analysis.

Histochemical Analysis

Established techniques for the histochemical analysis of muscle were followed (Cosmos, 1966, 1970; Cosmos and Butler, 1967; Cosmos et al., 1979b). The muscle was excised, weighed and cut transversely into several blocks. Each block of muscle was then mounted on a small round previously labelled cork, either alone or with supporting tissue. The supporting medium was either chicken liver or mounting media (Tissue-Tek II), depending upon the block size. In many instances two or three blocks from both operated and unoperated control muscles were mounted together on the same cork to permit a simultaneous exposure to the same histochemical procedure. The blocks were then deep-frozen in isopentane (Practical grade, J.T. Baker) precooled to -150°C to -160°C by liquid nitrogen. Frozen blocks were then stored in a Revco deep freeze at -70°C for at least one day (a block can be stored with no adverse effects for a few years). For cryostat sectioning, a block of tissue was removed from the freezer and mounted on chucks with the aid of mounting media. The entire procedure was performed in a dewar flask partly filled with dry ice. The mounted tissue was then removed and placed in a cryostat maintained at -27°C. Serial sections were cut at a thickness of 14 μm, picked up on microslides, and air dried at room temperature. Minimum drying time was ½ hr for sections of dystrophic muscle and 1 hr for sections of normal muscle. This procedure minimized the muscle contraction which often occurs in these preparations during histochemical reactions. The following

histochemical reactions were done on consecutive serial sections:

1. Phosphorylase (Pase) and succinic dehydrogenase (SDH) as the representatives of the glycolytic and oxidative enzymes, respectively; changes in the activities of these enzymes would indicate changes in the muscle fibres' metabolism.

2. Methyl green-pyronin y stain (RNA-DNA reaction) Barka and Anderson (1963), to identify regenerating muscle fibres, fibre nuclear number and localization.

3. Myosin ATPase after acid (pH = 4.35) and alkali (pH = 10.0 pre-incubation using the technique of Guth and Samaha (1970) as modified by J. Butler (Butler and Cosmos, 1979). This modified technique (Appendix B) provided a simple and direct method for demonstrating the difference between the slow tonic and fast twitch fibres of the ALD and PLD muscles, since slow tonic fibres show both acid and alkali stable myosin ATPase while myosin ATPase activity in fast twitch fibres is only alkali stable.

4. Acetylcholinesterase (AChE) reaction to identify muscle fibre endplates (Silver, 1963) in cross and longitudinal sections (14-20μm). For teased fibre preparations, a fresh block of muscle was stained by AChE by using a modification of the method of Tsuji (1974) (see Appendix C). At the end of the staining procedure the muscle was placed in 20% glycerol and stored at 4^{0}C for at least 24 hours. Individual fibres were teased apart at room temperature with the aid of fine steel needles and watch-makers' forceps under a dissecting microscope.

5. The Oil Red O reaction was used to demonstrate extra- and intra-cellular accumulation of neutral lipids (Barka and Anderson, 1963).

Histochemical cross sections of the muscles were examined and

photographed with a Zeiss Photomicroscope. 35mm colour slides (Kodachrome 64) and black and white (Kodak Panatomic-X) photographs were taken by Dr. E. Cosmos.

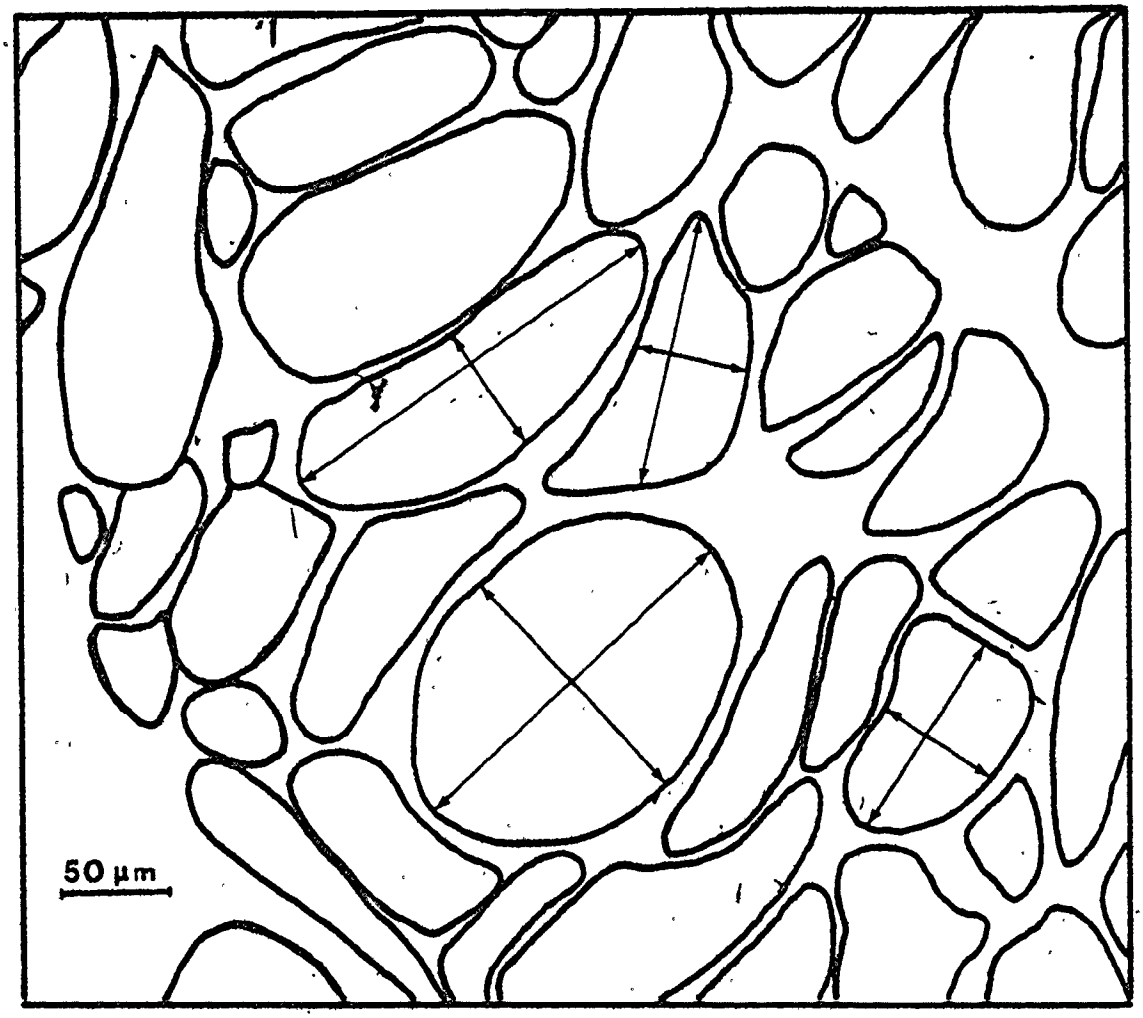
Structural Analysis

Fibre diameters of unoperated ALD and PLD muscles of both genotypes were measured from cross sections of muscles which had been stained for Pase activity. However, the diameters of PLD fibres successfully cross reinnervated by the ALD nerve were measured from preparations stained for acid stable myosin ATPase activity since they could be identified more readily with this reaction than with the Pase reaction (see RESULTS). Both enzymic reactions are acidic and serial muscle sections tested for Pase and pre-acid myosin ATPase activity showed no difference in the distribution of fibre diameters.

Cross sections of muscle were projected on drawing paper with a Leitz projection microscope (Neo Promar). The image of the muscle section was enlarged 300-500 fold and each fibre was individually traced. For every fibre in the projected field, the largest diameter and its perpendicular bisector (the smaller axis) was measured (Fig. 7). The mean fibre diameter was calculated (Jirmanová and Zelená, 1970) and histograms were made to show the percentage distribution of fibre diameters in μm.

This type of histogram does not, however, indicate differences between shapes of fibres of the different muscles. Even though photographs of cross sections of muscle fibres provide clear visual demonstrations of the shape of fibres, in order to compare the shapes of fibres of

Figure 7: Tracing of cross section of fibres (x 300) from a dystrophic muscle to show the measurement made for calculating mean fibre diameter. For every fibre the largest diameter was determined; the smaller diameter was measured at the midpoint of the largest diameter and perpendicular to it. The mean fibre diameter was then calculated in μm by adding the small to the large diameter and dividing it by two.


50 µm

different muscles quantitatively it was found necessary to calculate the ratio (large/small) of the measured fibre diameters. With this calculation, if the cross section of a fibre is a circle, the ratio is one and any deviation from a circle will be expressed as a number larger than 1. Histograms of percent distribution of the ratio of fibre diameters (large/small) were constructed.

Statistical Analyses

Statistical tests were applied in order to answer the following questions:

1. Is there any significant difference between ALD or PLD muscles of normal and dystrophic genotypes during development ex ovo with regard to muscle weight, contraction and relaxation times of their mechanical response, and muscle fibre size?
2. Is there any significant difference in the contraction characteristics and fibre size between cross reinnervated PLD muscles of normal and dystrophic genotypes or between either and its control?

Data from individual chickens were keypunched and stored on discs in a HP 3000 computer (see Appendix D for data record format).

The mean ± the standard deviation (SD) or standard error of the mean (SE) were calculated for each measurement (see Appendix E). Statistical techniques such as multiple regression analyses, Student t-test, and chi square (x^2) were used according to the specific question being asked. For each statistical test, an appropriate appendix has been included to explain the procedure used. Throughout the analyses, a 1% level of significance has been used to protect against the appearance of spurious findings through multiple testing of the data.

PART I: DEVELOPMENTAL STUDY

RESULTS

INTRODUCTION

The aim of this study was to examine selected structural, mechanical and histochemical properties of ALD and PLD muscles of normal chickens and to determine which of these properties were altered in the ALD and PLD muscles of dystrophic chickens (Storrs line) during development ex ovo. The data established in this section were used as baseline data for the analyses of PLD muscles cross reinnervated by ALD nerves (Part II).

The muscles analyzed for Part I include:

1. ALD and PLD muscles from unoperated normal and dystrophic chickens which served as control chickens; and
2. ALD and PLD muscles from the unoperated contralateral side of operated normal and dystrophic chickens.

ALD and PLD muscles of both genotypes were analyzed at selected times ranging from 1 day to 800 days ex ovo.

The following figures and tables are constructed from the raw data presented in the Appendices.

WEIGHT ANALYSIS

Body Weight of Normal and Dystrophic Chickens

During the course of this study, the body weight of each chicken

was taken every 12 weeks. The growth curves of the roosters and hens of normal and dystrophic genotypes are presented in Figures 8 and 9 (Appendices F and G).

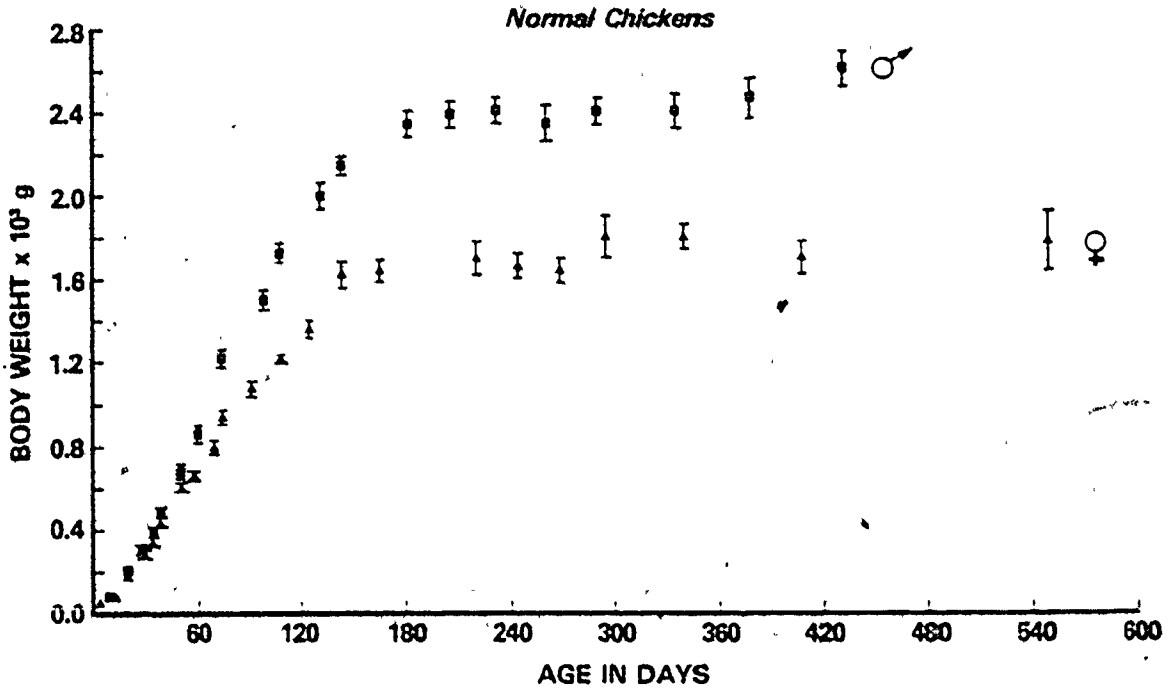
Up to approximately 50 days ex ovo no difference in the rate of growth is observed between the two sexes with either normal or dystrophic chickens. After 50 days, roosters grow faster than hens. Both sexes achieve a steady state level with no further weight gain by 160 to 180 days, which correlates with their sexual maturity (Cosmos, 1970). Regression analysis (Appendix H) was used in order to compare the rate of growth between the four groups of chickens presented in Figures 8 and 9. The regression lines which best fitted the data for chickens from 1 to about 160 days of age were calculated. Table 1(a) presents the slopes which reflect the rate of growth of the chickens and the intercepts of the regression lines. Table 1(b) presents results of the statistical t-test for the difference between the slopes of the lines. No significant difference was obtained between the rate of growth of normal and dystrophic hens or roosters. Highly significant differences ($P < 0.001$). have been calculated between the rate of growth of the two sexes in both normal or dystrophic chickens.

Muscle Weights

It has been shown by Feng et al. (1963) that there are no significant differences between the muscle wet weight of left and right PLD and ALD muscles in normal chickens. Thus, for analyses of muscle weights, the wet weights of the ALD and PLD muscles from unoperated chickens were measured without preference for one side or the other. In

Figure 8: A growth curve of roosters (♂) and hens (♀) of normal White Leghorn chickens, between 1 to 600 days ex ovo. Each point represents the mean ± S.E. of 4 to 24 chickens. The exact numbers are listed in Appendix F.

Figure 9: A growth curve of roosters (♂) and hens (♀) of dystrophic Storrs line chickens, between 1 to 600 days ex ovo. Each point represents the mean ± S.E. of 4 to 24 chickens. The exact numbers are listed in Appendix G.


Normal Chickens
BODY WEIGHT x 10³ g
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
60
120
180
240
300
360
420
480
540
600
AGE IN DAYS

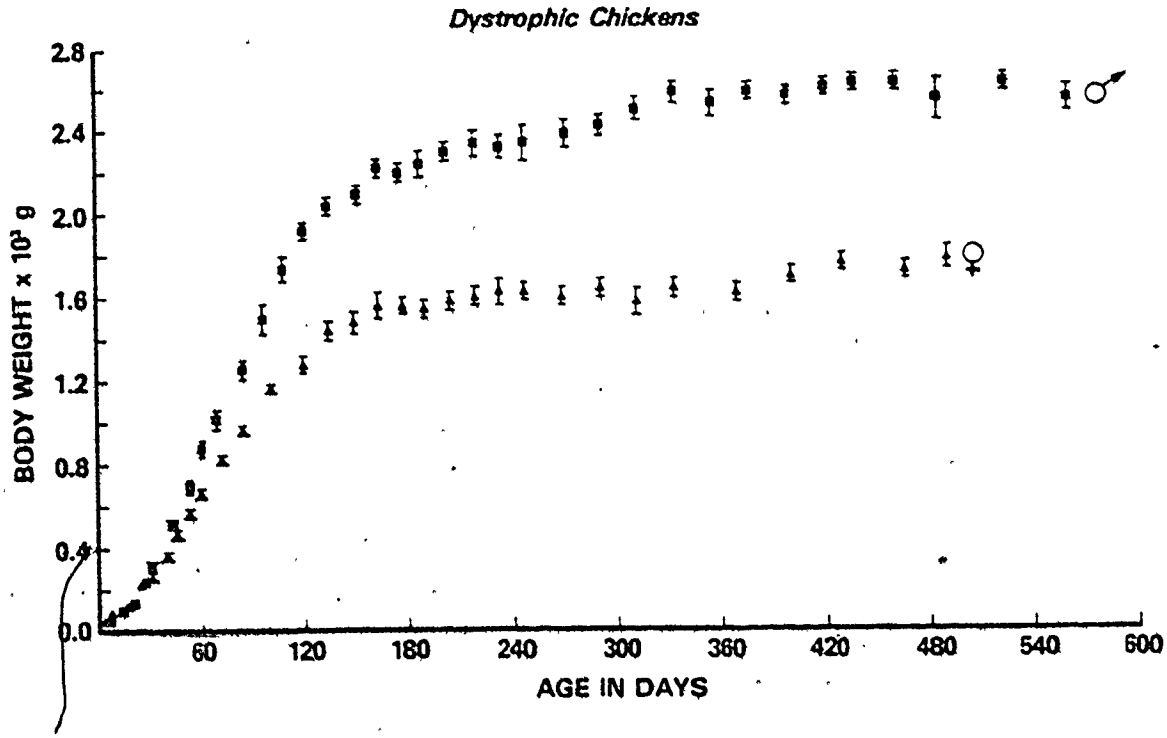

Dystrophic Chickens
BODY WEIGHT x 10³ g
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
60
120
180
240
300
360
420
480
540
600
AGE IN DAYS

TABLE 1 RATE OF GROWTH OF NORMAL AND DYSTROPHIC CHICKENS BETWEEN 1 TO 160 DAYS OF AGE

a The regression lines ($y = a x + b$)

Group	Slope	Intercept
N -♀	11.48	- 9.61
N -♂	15.89	-84.07
D -♀	11.27	-37.83
D -♂	15.27	-70.20

b Test for difference between slopes

Groups	t	df	p*
N -♀ vs D -♀	0.52	24	N.S.
N -♂ vs D -♂	0.87	26	N.S.
N -♀ vs N -♂	9.20	23	<0.001
D -♀ vs D -♂	5.63	27	<0.001

♀ = hens; ♂ = roosters; a = slope; b = intercept; df = degrees of freedom.

See Appendices F and G for individual measurements

* two tail probability

addition, wet weights of the unoperated left ALD and PLD muscles used in the reinnervation experiments were measured. Since no significant differences were found between the two population groups, the data from the two groups were combined to obtain a larger number of results for the muscle weight analysis.

Because differences in body weight between hens and roosters of either normal or dystrophic chickens were observed (Figs. 8 and 9), ALD and PLD muscle weights were related to body weight rather than to age.

ALD Muscle Weight: Figure 10 (Appendices I and J) shows ALD muscle weights plotted against body weights for normal (n = 70) and dystrophic (n = 49) chickens from 1 to 180 days of age; 180 days is about the time at which the body weight of the chickens has reached its steady state level. Appendices K and L present the data for normal and dystrophic chickens older than 180 days.

Regression analyses of the graphs in Figure 10 indicate that:

1. the best fitting straight lines through the data start at the same point, i.e. no significant difference exists between the intercepts of normal and dystrophic regression lines;
2. a positive correlation exists between ALD muscle weight and body weight in both genotypes; and
3. the slopes of the regression lines are significantly different ($P < 0.001$).

Although the general impression is of similarity between the ALD muscle weight of both genotypes, the data show an overall 12% differences in muscle weight in favour of the dystrophic chickens. This difference did

Figure 10: Correlation between ALD muscles wet weight and body weight for normal and dystrophic chickens from 1 to 180 days ex ovo. The two regression lines were compared by statistical test for the differences between the intercepts and between the slopes of the lines. The values of the regression lines are:

Normal ALD $a_1 = -0.0158$ $b_1 = 0.00038$ $R_1 = 0.99$ $n_1 = 70$

Dystrophic ALD $a_2 = -0.0150$ $b_2 = 0.00033$ $R_2 = 0.96$ $n_2 = 49$

Test of intercepts differences:

$a_1 - a_2 = 0.0008$ SE = 0.020 t = 0.04 N.S.

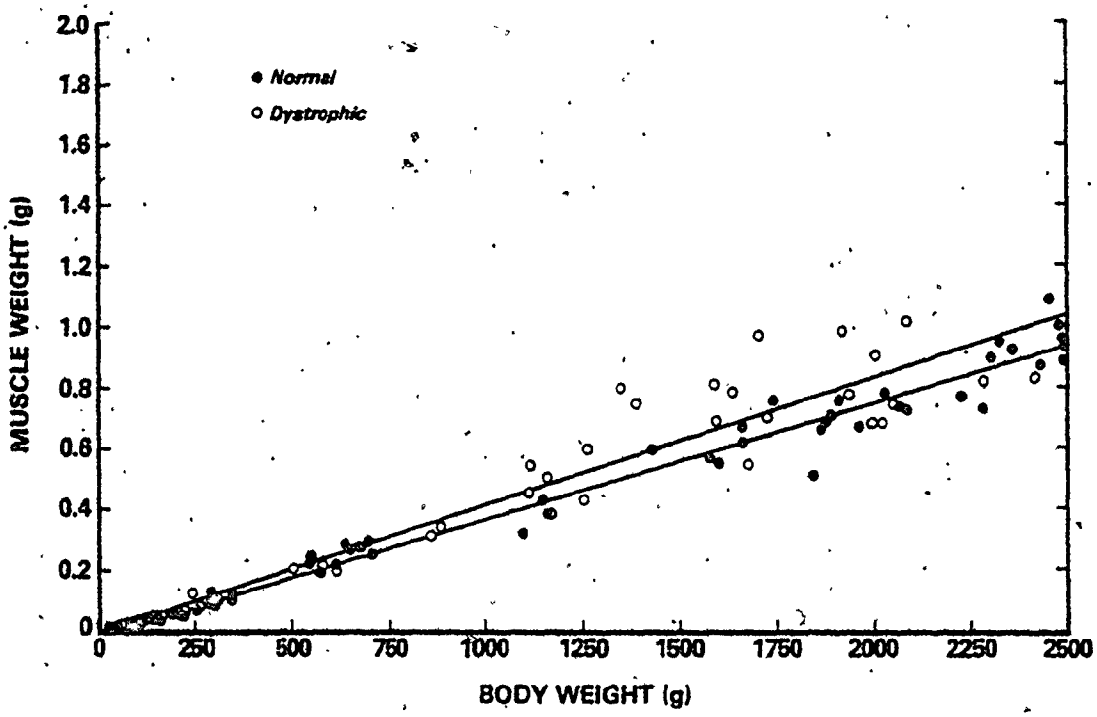
Test of slope differences:

$b_1 - b_2 = 0.00005$ SE = 0.0000152 t = 3.27 P <0.01

Note that the intercepts are identical but the slopes of the lines differ significantly.

a = intercept; b = slope; R = correlation coefficient; n = number of muscles; SE = standard error of the mean; t = t test; N.S. = not significant; p = two tail probability.

AVIAN ANTERIOR LATISSIMUS DORSI



not appear until the chickens were 90 days old, but was statistically significant thereafter.

PLD Muscle Weight: PLD muscle weights of normal (n = 70) and dystrophic (n = 49) chickens aged 1 - 180 days are plotted against body weights in Figure 11 (Appendices I and J). Appendices K and L give the data obtaine from 180 days onward.

The regression lines indicate a positive correlation between PLD muscle weight and body weight during this period for both normal and dystrophic chickens. When tested statistically for the difference between the two regression lines using multiple regression techniques, no significant difference was detected between the interception of the lines. However, a highly significant difference ($P < 0.001$) between the slopes of the two regression lines was obtained, where the slope of the dystrophic PLD was lower than that of the normal one. This result indicates that PLD muscles of both normal and dystrophic chickens have the same weight at hatching, but during further growth the dystrophic PLD muscles have a lower weight than that of the normal PLD for any given body weight.

Since there are no overall differences between the body weight of normal and dystrophic chickens (Figs. 8 and 9), the differences noted between the two regression lines (Fig. 11) can only be attributed to the different weights of the PLD muscles of both genotypes.

The Ratio Between the Weight of ALD and PLD Muscles: To eliminate any possible strain differences, the weights of ALD and PLD muscles were compared by calculating the ratio between ALD muscle weights to PLD

Figure 11: Correlation between PLD muscles wet weight and body weight for normal and dystrophic chickens from 1 to 180 days ex ovo. The two regression lines were compared by statistical test for the differences between the intercepts and between the slopes of the lines. The values of the two regression lines are:

Normal PLD $a_1 = -0.0318$ $b_1 = 0.00060$ $R_1 = 0.99$ $n_1 = 70$

Dystrophic PLD $a_2 = -0.0040$ $b_2 = 0.00037$ $R_2 = 0.96$ $n_2 = 49$

Tests of intercepts differences:

$a_1 - a_2 = 0.0278$ SE = 0.0241 t = 1.15 N.S.

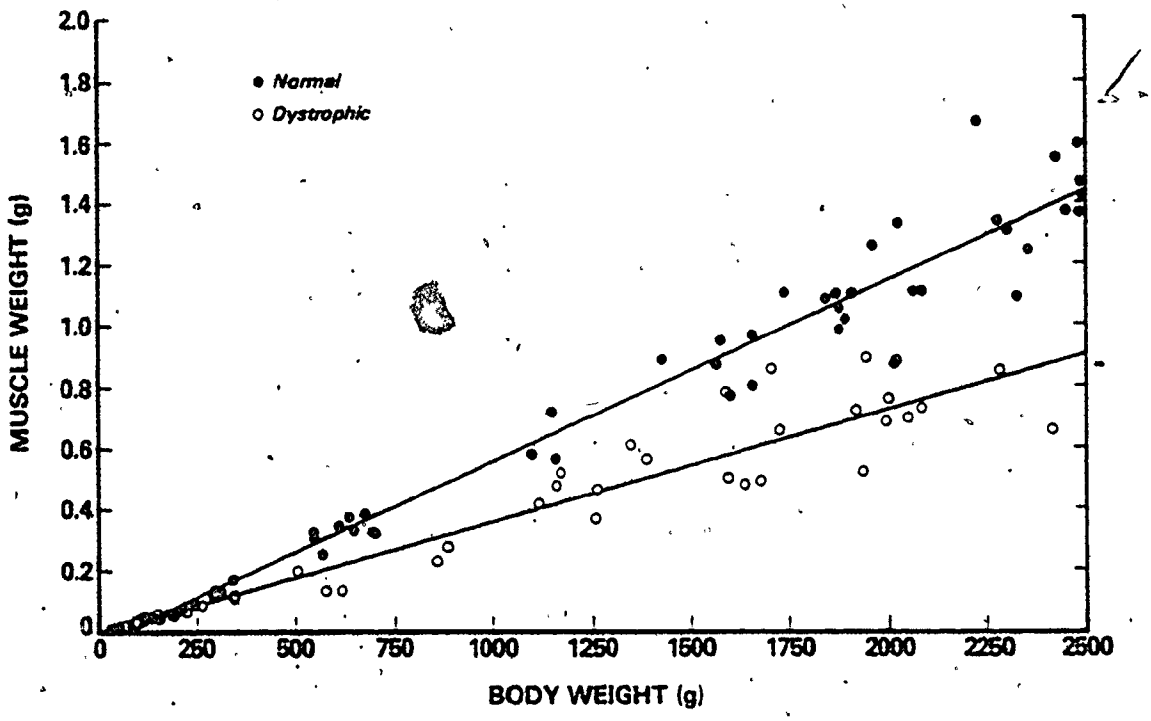
Test of slope differences:

$b_1 - b_2 = 0.00023$ SE = 0.0000193 t = 11.9 P <0.001

Note that the intercepts are identical but the slopes of the line differ significantly.

For glossary see Figure 10.

AVIAN POSTERIOR LATISSIMUS DORSI



muscle weights with data obtained from chickens of the same strain. Tables 2 and 3 summarize the data for the ratio of ALD and PLD muscle weights in normal and dystrophic chickens from 1 to 180 days (Appendices I and J). Tables 2 and 3 demonstrate the change in ratio between the weight of ALD and PLD muscles of either normal or dystrophic chickens compared with their body weight.

In normal chickens from hatching up to about 9 days old, or 60 g body weight, ALD muscles weigh more than PLD muscles. Beyond a 60 g body weight the growth rate of the PLD muscle is faster; at the steady state level (i.e. no more significant change in the ratio of the muscle weight) the ALD muscle weight is about 60% that of the PLD (see Table 2 and Appendices I and K). In dystrophic chickens at hatching, as in normal chickens, the ALD is heavier than the PLD. However, in dystrophic chickens, no steady state level is reached between the weight ratios of these two muscles; in most cases, the ALD remains heavier than the PLD (Table 3 and Appendices J and L).

In Appendix L some unusually heavy ALD and PLD muscles of dystrophic chickens are marked with asterisks. These high muscle weights were observed occasionally in some roosters over 2000 g body weight.

STRUCTURAL ANALYSES

Mean fibre diameters of ALD and PLD muscles (see Methods p 58) were calculated from measurements obtained from projections of histochemically stained cross sections. Figure 12(a-d) is a composite drawing of such projections from normal and dystrophic ALD and PLD muscles. These drawings are presented to emphasize the similarities in size and shape of

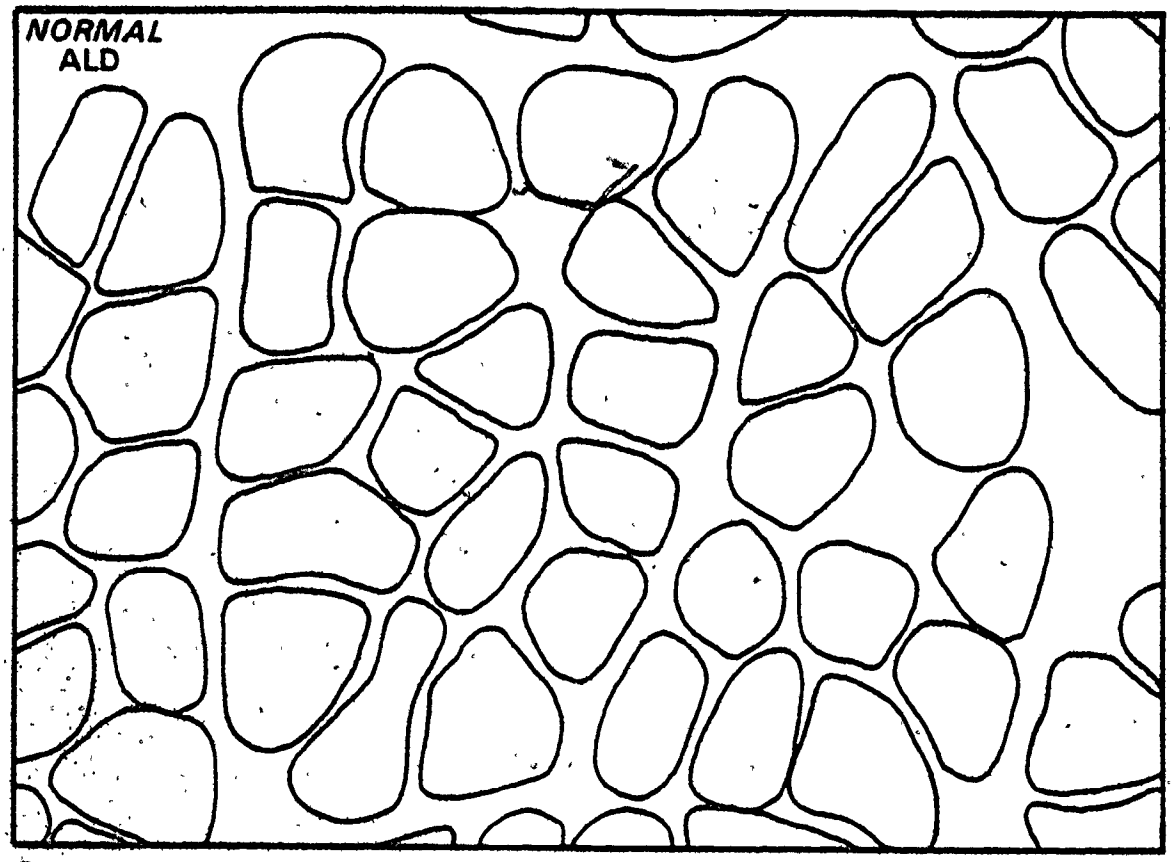
TABLE 2 NORMAL CHICKENS
CHANGE IN THE MUSCLE WEIGHT RATIO OF THE ALD AND PLD CORRELATED TO BODY WEIGHT

n	Body wt (g) (mean ± S.E.)	ALD/PLD (mean ± S.E.)
8	42 ± 1	1.426 ± 0.06
8	79 ± 4	0.887 ± 0.03
5	125 ± 5	0.809 ± 0.02
5	191 ± 8	0.801 ± 0.06
7	298 ± 10	0.764 ± 0.04
9	628 ± 20	0.769 ± 0.03
9	1454 ± 83	0.667 ± 0.03
9	1950 ± 29	0.642 ± 0.02
10	2408 ± 27	0.680 ± 0.03

TABLE 3 DYSTROPHIC CHICKENS
CHANGE IN THE MUSCLE WEIGHT RATIO OF THE
ALD AND PLD CORRELATED TO BODY WEIGHT

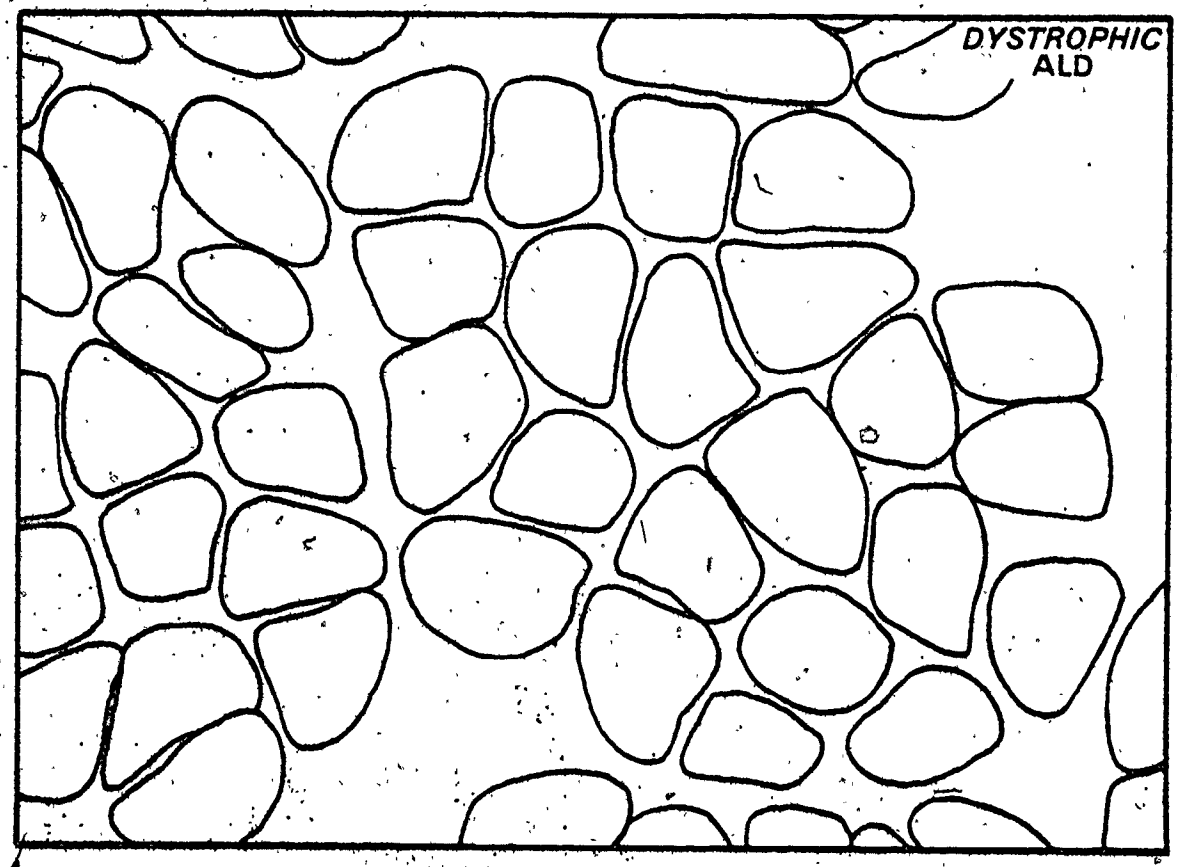
n	Body wt (g) (mean ± S.E.)	ALD/PLD (mean ± S.E.)
5	33 ± 2	1.480 ± 0.12
8	113 ± 23	0.928 ± 0.65
6	288 ± 22	1.030 ± 0.79
5	689 ± 77	1.345 ± 0.10
8	1228 ± 37	1.159 ± 0.07
6	1655 ± 23	1.227 ± 0.10
11	2052 ± 48	1.183 ± 0.09

Figure 12: Drawings of projected microscopic cross sections from representative areas of ALD and PLD muscles. These drawings emphasize the similarity between the shape and size of normal ALD (a) and dystrophic ALD (b) muscle fibres, and the differences in shape and size between normal PLD (c) and dystrophic PLD (d) muscle fibres. Note the magnification in all these drawings is the same.

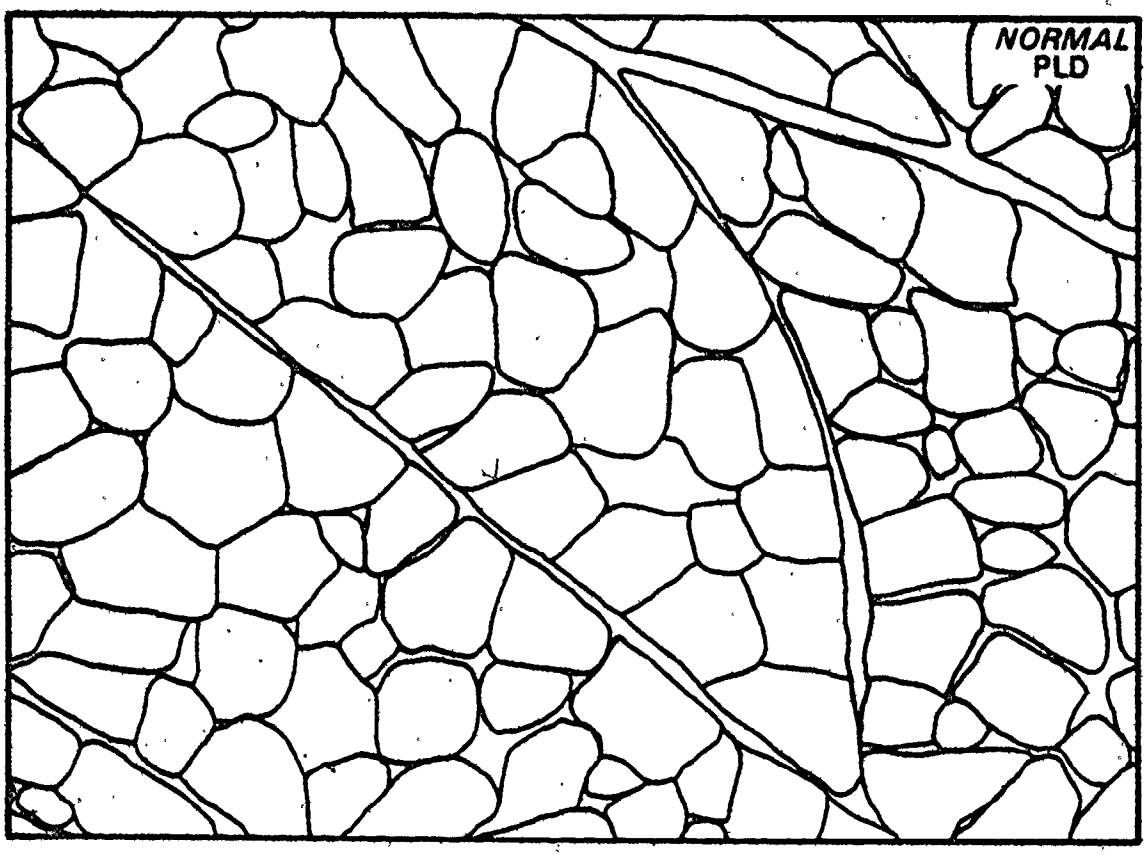


a

50μ



b



c

50μ

d

fibres of normal (Fig. 12a) and dystrophic (Fig. 12b) ALD muscles, and the differences in size and shape of fibres of normal (Fig. 12c) and dystrophic (Fig. 12d) PLD muscles.

ALD Muscle

As can be seen in Figure 12(a,b) in a cross section, both fascicles and fibres of ALD muscle of both genotypes have rounded shapes with large extracellular spaces between fascicles and fibres. Similar projections of muscle cross sections were also used to count the number of fibres within the fascicles. A mean and standard deviation was calculated by counting the fibres in 10 fascicles in each of 6 ALD muscles from 6 normal and 6 dystrophic adult chickens. The fascicles of normal and dystrophic ALD muscle contain 21.4 ± 8.1 and 19.6 ± 4.3 fibres respectively. The Student's t-test (Appendix M) indicates no significant difference between the mean number of fibres within the fascicles of ALD muscles of both genotypes.

PLD Muscle

In cross section, the fascicles of PLD muscle of normal chickens (Fig. 12c) are elongated and the fibres have a polygonal shape. Both fibres and fascicles are compact as very little extracellular space exists between either the fibres or the fascicles. This structure contrasts with the variability of shape and size of both fascicles and fibres noted within the dystrophic PLD muscle (Fig. 12d).

In PLD muscles of mature normal chickens, the fascicles contain a mean of 49.0 ± SE 2.4 fibres. While in PLD muscles of mature dystrophic chickens a mean of 37.1 ± SE 2.4 was obtained. The mean was calculated by counting the fibres in 10 fascicles in each of 6 PLD muscles. The ALD and PLD muscles used for the calculation of mean were removed from the same normal and dystrophic chickens. Analysis by the Student's t-test indicated a highly significant differences between the mean number of fibres within the fascicles of normal and dystrophic PLD muscles.

Fibre Diameter

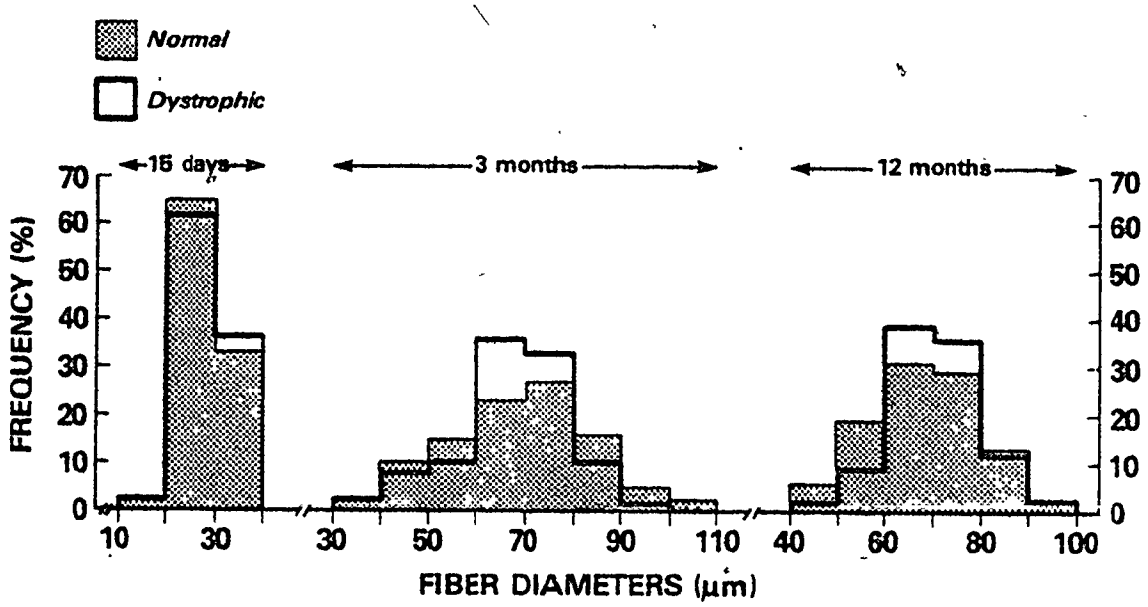
As indicated previously, the large and small diameters of each fibre were measured from the drawings of projected cross sections of muscle (Methods, Fig. 7).

The mean diameter of each fibre was calculated for histogram analyses. Figures 13 and 14 are histograms of the frequency distribution of fibre diameter for ALD and PLD muscles of both genotypes. The histogram for each time period (0.5, 3 and 12 months) includes 3 muscles for which 350-1000 fibres were measured (the exact number of fibres measured for each group is listed in Tables 4 and 5). The histogram obtained for the ALD muscle (Fig. 13) emphasizes the similarity between normal and dystrophic ALD muscle fibres. Both have the same range of diameters and the same major peaks for each of the three time periods studies. Using appropriate techniques for grouped data (Appendix E), the mean and standard deviation of fibre diameter were calculated (Table 4, I). Although very small differences exist between the histogram distributions

Figure 13: Frequency distribution of fibre diameter (μm) of ALD muscles from normal and dystrophic chickens at 0.5, 3 and 12 months ex ovo. The histogram for each time period includes 3 muscles for which a total of 350 to 1000 fibres were measured. The exact number of fibres measured for each group, the calculated means and the statistical differences between normal and dystrophic are listed in Table 4. This histogram emphasizes the similarity between the diameter of normal and dystrophic ALD muscle fibres.

Figure 14: Frequency distribution of fibre diameter (μm) of PLD muscles from normal and dystrophic chickens at 0.5, 3 and 12 months ex ovo. The histogram for each time period includes 3 muscles for which a total of 500 to 1000 fibres were measured. The exact number of fibres measured for each group, the calculated means, and the statistical differences between normal and dystrophic are listed in Table 5. This histogram emphasizes the variability in fibre size within the PLD muscle of dystrophic genotype.

AVIAN ANTERIOR LATISSIMUS DORSI



AVIAN POSTERIOR LATISSIMUS DORSI

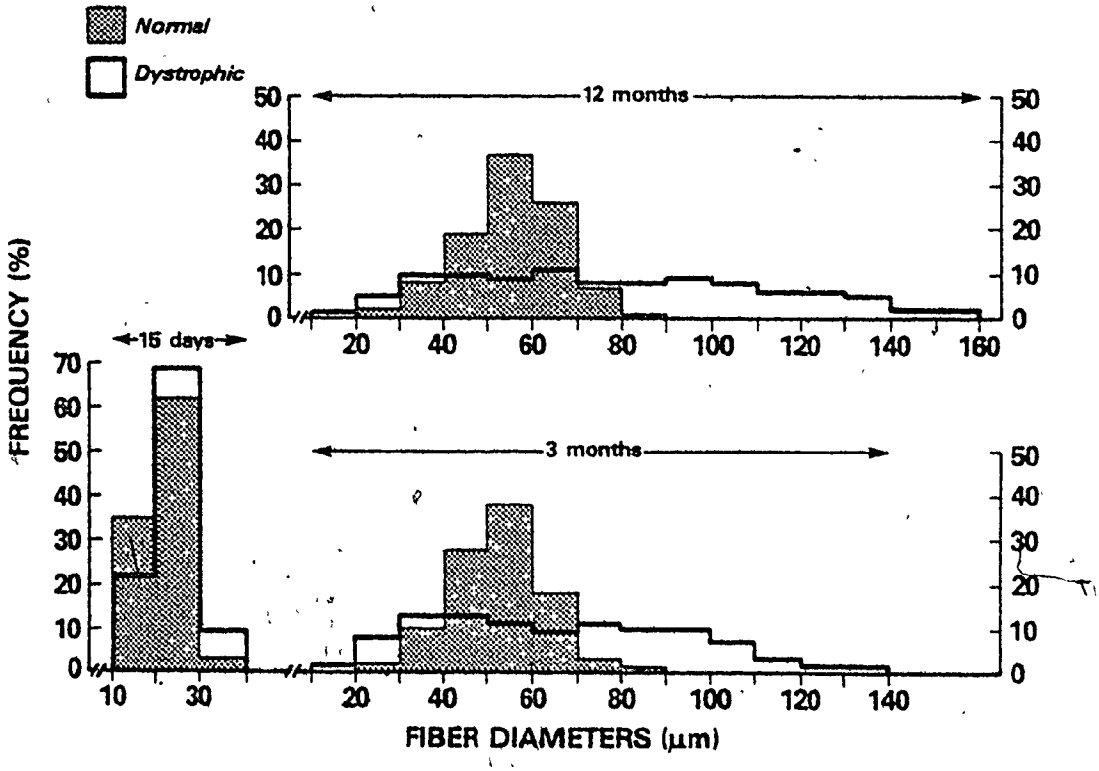


TABLE 4 FIBER DIAMETERS OF THE ANTERIOR LATISSIMUS DORSI OF NORMAL AND DYSTROPHIC CHICKENS

I - Frequency Distribution

Range of Fiber diameter	Number of Fibers					
	Normal ALD			Dystrophic ALD		
	0.5 (mos)	3 (mos)	12 (mos)	0.5 (mos)	3 (mos)	12 (mos)
0- 9	--	--	--	--	--	--
10- 19	12	--	--	20	--	--
20- 29	415	--	--	740	1	--
30- 39	209	7	2	438	12	3
40- 49	--	34	38	--	53	56
50- 59	--	53	138	--	66	63
60- 69	--	84	226	--	250	271
70- 79	--	97	213	--	224	247
80- 89	--	59	96	--	67	83
90- 99	--	18	15	--	11	13
100-109	--	6	--	--	1	2
110-119	--	1	--	--	1	--
fi	636	359	728	1198	686	688
$\bar{X}$	28.1	69.3	68.2	28.5	67.7	70.5
S.D.	5.0	14.9	11.5	5.1	11.9	9.6

II - Comparative analysis (Normal vs Dystrophic)

Age (mos)	Mean Diameter ($\bar{X}$)		Frequency Distribution		
	t	P*	χ^2	df**	P*
0.5	1.60	N.S.	2.52	2	N.S.
3	1.88	N.S.	46.66	6	<0.001
12	4.06	<0.001	54.04	5	<0.001

* two-tail probaility

** ranges combined where appropriate

TABLE 5 FIBER DIAMETERS OF THE POSTERIOR LATISSIMUS DORSI OF NORMAL AND DYSTROPHIC CHICKENS

I - Frequency Distribution

Range of Fiber Diameter	Number of Fibers					
	Normal RLD			Dystrophic PLD		
	0.5 (mos)	3 (mos)	12 (mos)	0.5 (mos)	3 (mos)	12 (mos)
0- 9	--	--	--	--	--	--
10- 19	242	8	--	216	6	5
20- 29	435	51	16	694	53	27
30- 39	19	144	68	90	84	53
40- 49	--	196	170	--	84	57
50- 59	--	92	335	--	72	49
60- 69	--	17	239	--	56	60
70- 79	--	3	68	--	71	43
80- 89	--	--	11	--	69	43
90- 99	--	--	--	--	68	49
100-109	--	--	--	--	45	43
110-119	--	--	--	--	22	32
120-129	--	--	--	--	15	33
130-139	--	--	--	--	8	26
140-149	--	--	--	--	2	9
150-159	--	--	--	--	1	11
160-169	--	--	--	--	--	2
170-179	--	--	--	--	--	2
180-189	--	--	--	--	--	1
fi	696	511	907	1000	656	545
$\bar{X}$	21.8	52.4	55.6	23.7	67.1	78.9
S.D.	5.2	10.6	11.7	5.4	28.9	35.7

II - Comparative analysis (Normal vs Dystrophic)

Age (mos)	Mean Diameter ($\bar{X}$)		Frequency Distribution		
	t	P*	χ^2	df**	P*
0.5	7.20	<0.001	54.40	2	<0.001
3	12.00	<0.001	362.91	6	<0.001
12	14.80	<0.001	554.18	6	<0.001

* two-tail probability
** ranges combined where appropriate

of the fibre diameter and between the mean fibre diameter of normal and dystrophic ALD muscles, this small difference is statistically significant for 3 and 12 month old chickens (Table 4 & II).

The histogram for the PLD muscles (Fig. 14) clearly reveals the variability in fibre size within the PLD muscle of dystrophic genotype. While fibre diameters of the normal PLD muscle at 3 and 12 months range from 20 to 90 μm with most fibres having diameters between 40-70 μm, the dystrophic PLD shows a much wider spread of fibre diameters of 10-160 μm with a flat pattern of distribution. The mean and standard deviation of fibre diameter in μm for normal and dystrophic PLD muscles are listed in Table 5, I. The statistical analyses (Table 5, II) revealed significant differences between the two genotypes, at all ages examined (0.5, 3 and 12 months). Since in some measurements the standard deviation of the results from dystrophic chickens is larger than that of the normal, it requires the Welch form of the Student's t-test for unequal sample variances (Appendix M).

MECHANICAL ANALYSES

Isometric Contraction of ALD Muscle of Normal and Dystrophic Genotypes

Contraction Characteristics: An *in vivo* analysis of the isometric contraction of ALD muscles in response to nerve stimulation was performed on 20 normal and 26 dystrophic chickens ranging from 15 to 800 days of age. Because ALD muscles of normal genotype do not respond *in vivo* to a single stimulation (see Introduction, p 5), the post tetanic potentiation technique had to be used to assess their twitch contraction properties. This distinctive physiological feature also characterized

the ALD muscles of dystrophic genotype. Examples of post tetanic twitch and tetanic responses are shown in Figure 15. The twitch response was evoked after 7 sec of 100 Hz nerve stimulation. The tetanic contractions were evoked in response to 10, 20, 30, 50, 70, 100 Hz nerve stimulation for the normal ALD and 10, 20, 30, 40, 50, 60, 70, 100 Hz for the dystrophic ALD. These recordings demonstrate the slow tonic response of normal and dystrophic ALD muscles and the similarities between their contraction properties.

Time Parameters of Tetanic Contraction: The contraction properties of the ALD muscle in response to nerve stimulation at frequencies of 50, 70 and 100 Hz were measured (see Methods, p 53). Since no significant changes in the time parameters of the tetanic response of normal or dystrophic ALD muscles were measured at various ages, the data obtained from the normal group (n = 19) and the dystrophic group (n = 26) of chickens were combined for each group (Table 6). The times to half contraction and half relaxation of the tetanus response of normal and dystrophic ALD muscles were tested for significant differences between means for independent samples using t-test. No significant differences were observed between ALD muscles of both genotypes for the time parameters of the tetanic response.

Tetanic Tension: Tables 7 and 8 summarize the data for the tetanic tensions obtained from ALD muscles in response to 50, 70, 100 Hz (Appendices N and O). Tension increases are noted with an increase in rate of stimulation and with increased muscle weight in both normal and dystrophic ALD muscles. The relationship between tetanic tension and muscle weight is also expressed in graphic form for 70 Hz nerve

Figure 15: Examples of isometric post tetanic twitch (A) and tetanic (B) responses of ALD muscles of normal and dystrophic genotypes. The twitches were evoked after 7 sec of 100 Hz nerve stimulation followed by single stimuli. The tetanic contractions were evoked in response to 10, 20, 30, 50, 70 and 100 Hz nerve stimulation for the normal ALD and 10, 20, 30, 40, 50, 60, 70 and 100 Hz for the dystrophic ALD. The responses of both normal and dystrophic ALD muscles are similar and typical of slow tonic muscles. The following are the contraction properties measured from the twitch and tetanic (50 Hz) and responses of these recordings:

		Post tetanic twitch			Tetanic (50 Hz)		
	Muscle wt.	TWT	TC	T½R	TT	T½C	T½R
Normal ALD	0.853	8	108	120	147	360	281
Dystrophic ALD	1.335	12	108	108	276	480	300

TWT = twitch tension in grams; Tc = contraction time in msec; T½R = time to half relaxation in msec; TT = tetanic tension in grams; T½C = time to half contraction in msec. Note the difference between the muscle weight in grams which explains the higher tension obtained for the dystrophic ALD. Both chickens were 11 months old.

ISOMETRIC CONTRACTION - AVIAN ALD

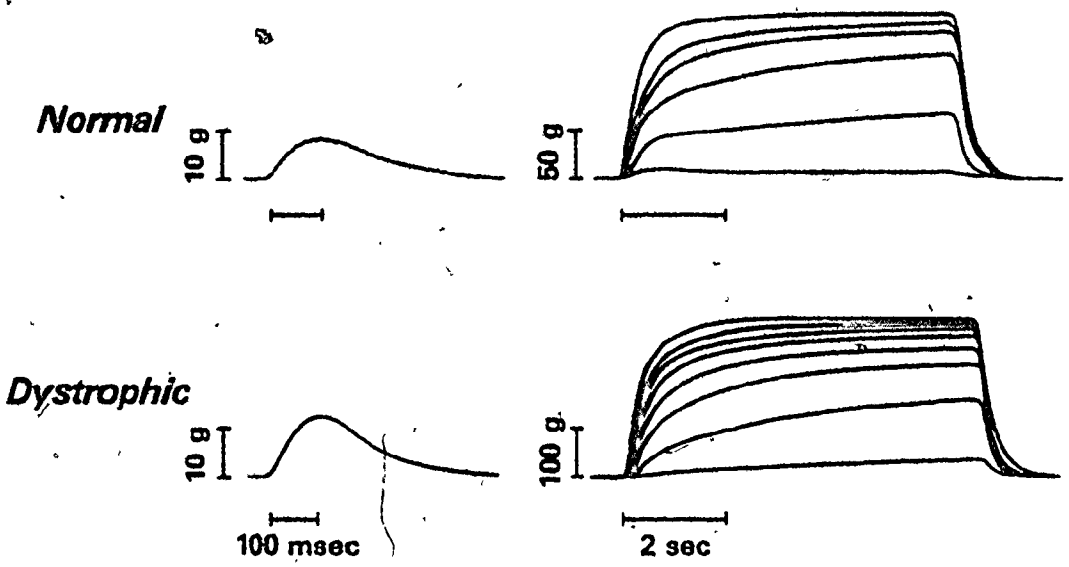


TABLE 6 MECHANICAL PROPERTIES OF ANTERIOR LATISSIMUS DORSI OF NORMAL AND DYSTROPHIC CHICKENS

RESPONSE		NORMAL (msec)	DYSTROPHIC (msec)	*P
TETANUS (50 Hz)	$T_{\frac{1}{2}}C$	365.4 ± 26.0	363.9 ± 21.9	N.S.
	$T_{\frac{1}{2}}R$	189.6 ± 12.5	200.8 ± 10.9	N.S.
TETANUS (70 Hz)	$T_{\frac{1}{2}}C$	294.9 ± 21.5	302.5 ± 16.9	N.S.
	$T_{\frac{1}{2}}R$	181.8 ± 11.7	204.5 ± 11.6	N.S.
TETANUS (100 Hz)	$T_{\frac{1}{2}}C$	238.4 ± 16.6	246.5 ± 11.2	N.S.
	$T_{\frac{1}{2}}R$	187.7 ± 13.1	201.3 ± 11.9	N.S.

$T_{\frac{1}{2}}C$ = time to half contraction; $T_{\frac{1}{2}}R$ = time to half relaxation

Normal, n = 20; Dystrophic, n = 26

Values are mean ± SE

* two-tail probability

TABLE 7 NORMAL CHICKENS
TETANIC TENSION OF THE ANTERIOR LATISSIMUS DORSI
DURING DEVELOPMENT EX OVO

n	Muscle wet wt (g)	Tetanic Tension (g) 50 Hz	70 Hz	100 Hz
4	0.206 (0.027)	36.8 (7.7)	39.8 (7.9)	44.5 (7.8)
5	0.287 (0.013)	50.6 (6.4)	76.6 (7.2)	59.8 (7.3)
7	0.489 (0.011)	86.0 (3.1)	88.4 (4.3)	111.2 (14.1)
3	0.827 (0.149)	164.7 (17.7)	175.3 (18.3)	182.3 (14.0)

The values are mean (± SE). See Appendix N for the individual observations

TABLE 8 DYSTROPHIC CHICKENS
TETANIC TENSION OF THE ANTERIOR LATISSIMUS DORSI
DURING DEVELOPMENT EX OVO

n	Muscle wet wt (g)	Tetanic Tension (g) 50 Hz	70 Hz	100 Hz
3	0.205 (0.006)	48.7 (4.3)	50.7 (4.8)	55.7 (3.5)
6	0.411 (0.016)	88.5 (11.9)	96.2 (12.6)	101.8 (12.9)
7	0.569 (0.022)	123.9 (16.2)	131.4 (14.9)	138.0 (15.4)
5	0.856 (0.049)	133.2 (12.6)	135.2 (6.6)	159.2 (14.7)
5	1.254 (0.055)	296.8 (35.9)	325.6 (36.8)	341.4 (41.1)

The values are mean (± SE). See Appendix O for individual observations

stimulation (Fig. 16). Although it was not possible to match exactly the weights of normal and dystrophic ALD muscles, the histogram shows the similarities between the tetanic tension response of normal and dystrophic ALD muscles. It does not include the data obtained from the last group of dystrophic roosters listed in Table 8 since the ALD muscles of these mature chickens were much heavier than all ALD muscles removed from normal chickens.

Isometric Contraction of the PLD Muscles of Normal and Dystrophic Genotype

Contraction Characteristics: The PLD muscles of 39 normal and 66 dystrophic chickens 15 to 800 days of age were analyzed. In vivo isometric twitch and tetanic contractions were measured in response to nerve stimulation; representative recordings from chickens 500 days old are shown in Figure 17. The isometric fast twitch contractions (Fig. 17a) were obtained in response to single stimulations, while tetanus was obtained in response to 10, 20, 30, 40 Hz (Fig. 17b) and 50, 70, 100 Hz (Fig. 17c) nerve stimulation. Whereas, contractions of both normal and dystrophic PLD muscles are typical of fast twitch muscles, the tension developed by dystrophic PLD is lower than that of the normal ones. This observation was consistent in the PLD muscles of dystrophic genotype at all ages examined (15 to 800 days) (Fig. 18). Thus, the dystrophic PLD retains fast twitch properties regardless of the extent of the dystrophic process, even though the twitch tension is considerably lower than that of the normal PLD muscle.

Time Parameters of Contraction: The time parameters of the twitch response and the tetanic response at 50, 70 and 100 Hz nerve stimulation were

Figure 16: The relationship between the tetanic tension evoked in response to 70 Hz nerve stimulation and muscle weight for ALD of normal and dystrophic genotypes. The number of muscles, the value of mean tension and standard error of the mean are listed in Tables 7 and 8. The histogram shows the similarities between the tetanic tension response of normal and dystrophic ALD muscles.

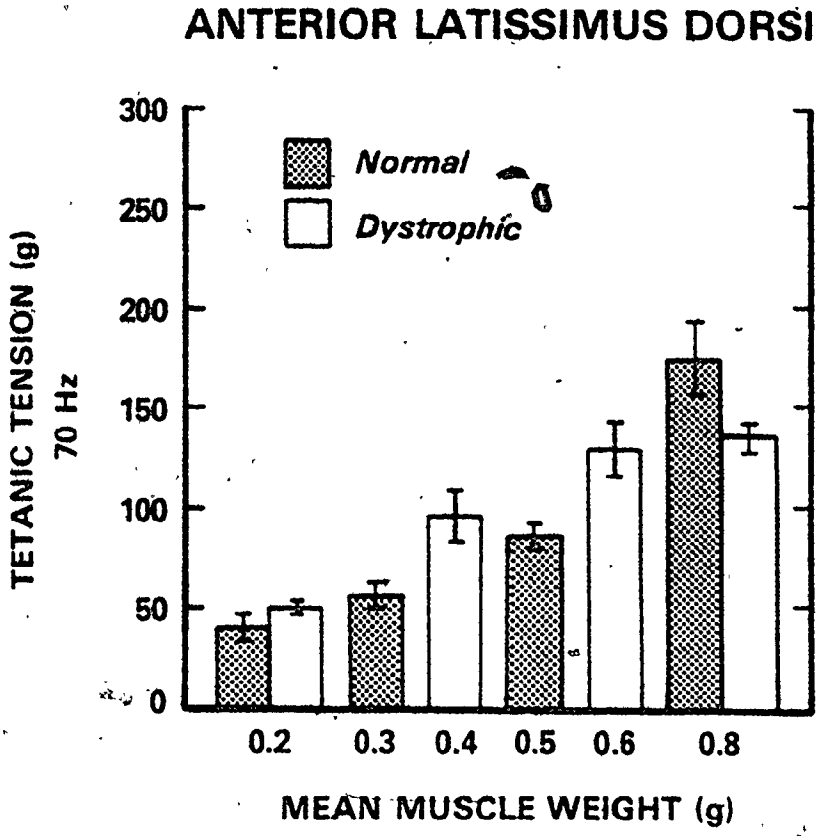

ANTERIOR LATISSIMUS DORSI
TETANIC TENSION (g)
70 Hz
300
250
200
150
100
50
0
Normal
Dystrophic
0.2
0.3
0.4
0.5
0.6
0.8
MEAN MUSCLE WEIGHT (g)

Figure 17: Examples of isometric contraction of PLD muscles of normal and dystrophic genotypes. The twitch contractions (a) were evoked in response to single nerve stimuli. The tetanic contractions were evoked in response to 10, 20, 30, 40 Hz (b) and 50, 70, 100 Hz (c) nerve stimulations. Whereas the time properties of the contractions of both these muscles are similar and typical of fast twitch muscles, the tension developed by the dystrophic PLD muscle is lower than the normal muscle. The following are the contraction properties measured from the twitch and tetanic (70 Hz) responses of these recordings:

		Twitch			Tetanic (70 Hz)		
	Muscle wt.	TWT	Tc	T½R	TT	T½C	T½R
Normal PLD	0.836	228	30	18	648	36	24
Dystrophic PLD	0.829	120	33	27	444	40	24

For glossary see Figure 15. Note difference in calibration of vertical scale between normal and dystrophic tetanus recordings.

ISOMETRIC CONTRACTION - AVIAN PLD

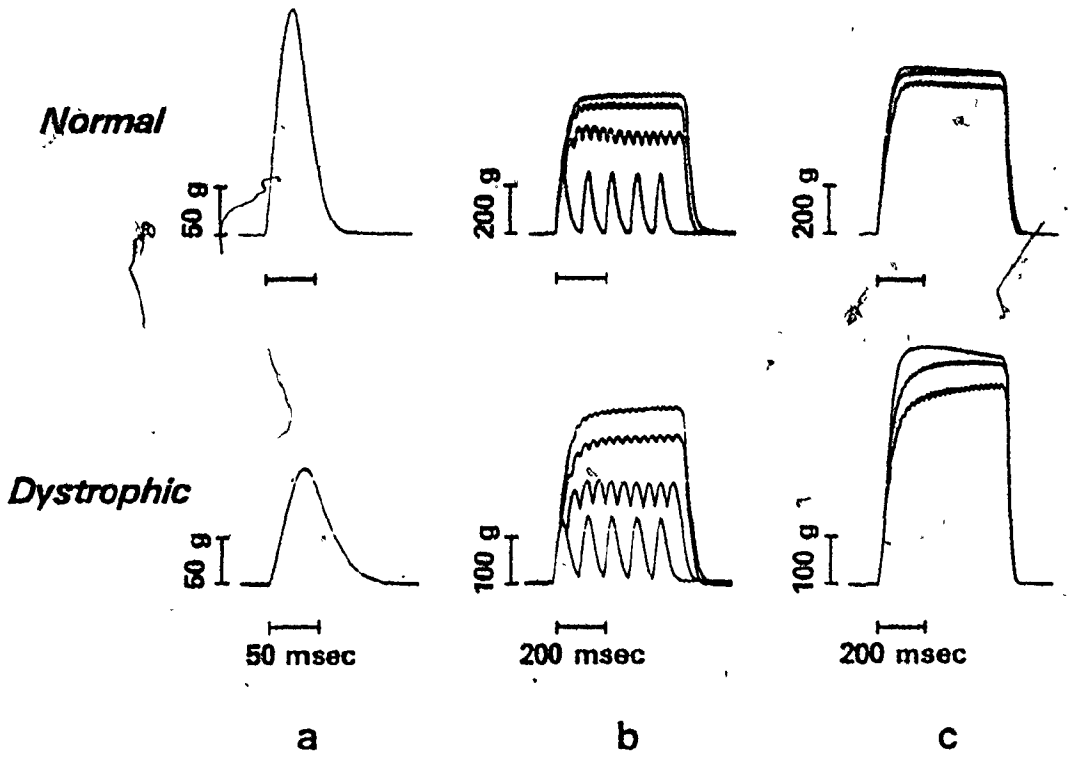
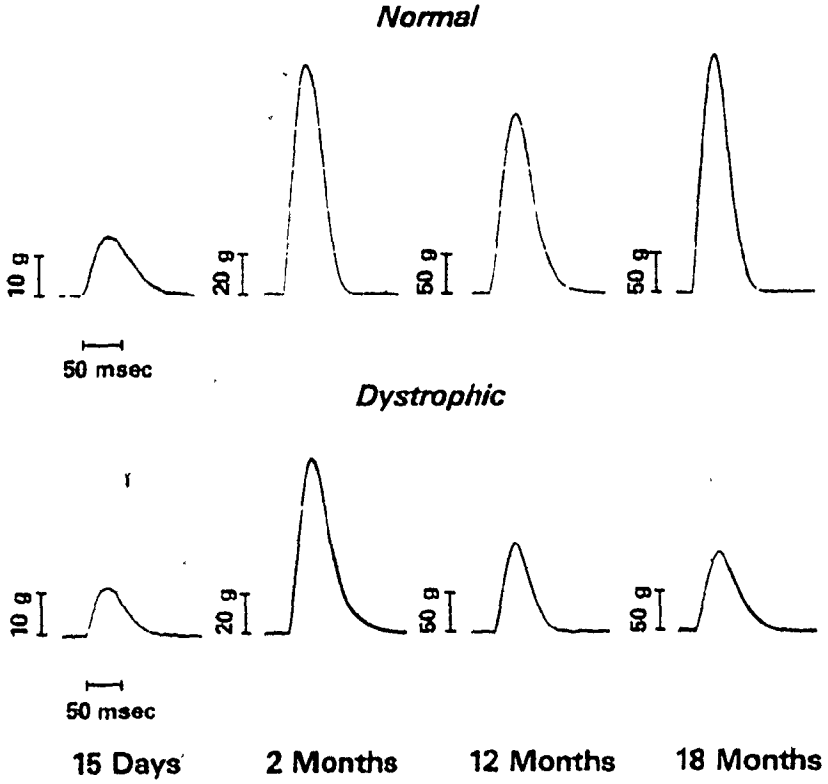


Figure 18: Actual recordings of indirectly evoked, isometric twitch responses from PLD muscles of normal and dystrophic chickens during ex ovo development. These recordings show that both normal and dystrophic PLD muscles have fast twitch characteristics but the twitch tension developed by dystrophic PLD muscles is lower than normal. The weights of the muscles are:

	15 days	2 months	12 months	18 months
Normal	0.060	0.372	0.815	0.845
Dystrophic	0.046	0.416	0.567	0.834

ISOMETRIC TWITCH RESPONSE
POSTERIOR LATISSIMUS DORSI



measured as described in the Methods (p 53). Since no significant changes in time parameters of the isometric twitch or tetanic response of normal and dystrophic PLD were measured at various ages, the data obtained from the normal group (n = 35) and the dystrophic group (n = 61) of chickens were combined for each group as shown in Table 9. The time to half contraction, the contraction time and time to half relaxation of normal and dystrophic PLD muscles were tested for significant difference between means for independent samples (Appendix M). Although the dystrophic PLD reveals fast twitch properties, a difference of 7-18 msec in time to half contraction of the tetanus response was observed.

Twitch and Tetanic Tension: Figures 19 and 20 (Appendices P and Q) reveal the inability of dystrophic PLD muscles to develop both twitch and tetanic tensions equivalent to those obtained by the normal PLD muscles. The histogram in Figure 19 shows the changes in mean twitch tension attained by normal and dystrophic muscles during development ex ovo. The histogram shows that the mean twitch tension of PLD muscles of both genotypes increases as muscle weight increases. But, the rate at which the tension of the dystrophic PLD muscle increases is slower than that of the normal PLD. At any given muscle weight, the twitch tension developed by the dystrophic PLD is lower than that of the normal PLD.

The ratio between maximum twitch and tetanic tension during growth ex ovo is plotted against muscle weight in the histogram in Figure 20. This ratio gives an indirect indication of the properties of the mechanical component of muscle (e.g. duration of active state, series elastic component).

The histogram shows that the twitch to tetanus ratio calculated

TABLE 9 MECHANICAL PROPERTIES OF POSTERIOR LATISSIMUS DORSI OF NORMAL AND DYSTROPHIC CHICKENS

Response		Normal (msec)	Dystrophic (msec)	P*
TWITCH	T½C	14.2 ± 0.4	13.4 ± 0.3	N.S.
	Tc	33.3 ± 0.8	30.3 ± 0.5	0.005
	T½R	22.5 ± 0.8	25.5 ± 0.7	0.01
TETANUS (50 Hz)	T½C	33.1 ± 1.2	42.1 ± 1.1	0.001
	T½R	40.3 ± 1.7	40.0 ± 1.4	N.S.
TETANUS (70 Hz)	T½C	32.8 ± 1.3	39.8 ± 1.1	0.001
	T½R	41.8 ± 1.6	42.0 ± 1.7	N.S.
TETANUS (100 Hz)	T½C	29.8 ± 1.1	47.9 ± 1.3	0.001
	T½R	42.4 ± 1.7	43.3 ± 1.8	N.S.

T½C = time to half contraction; Tc = contraction time;

T½R = time to half relaxation

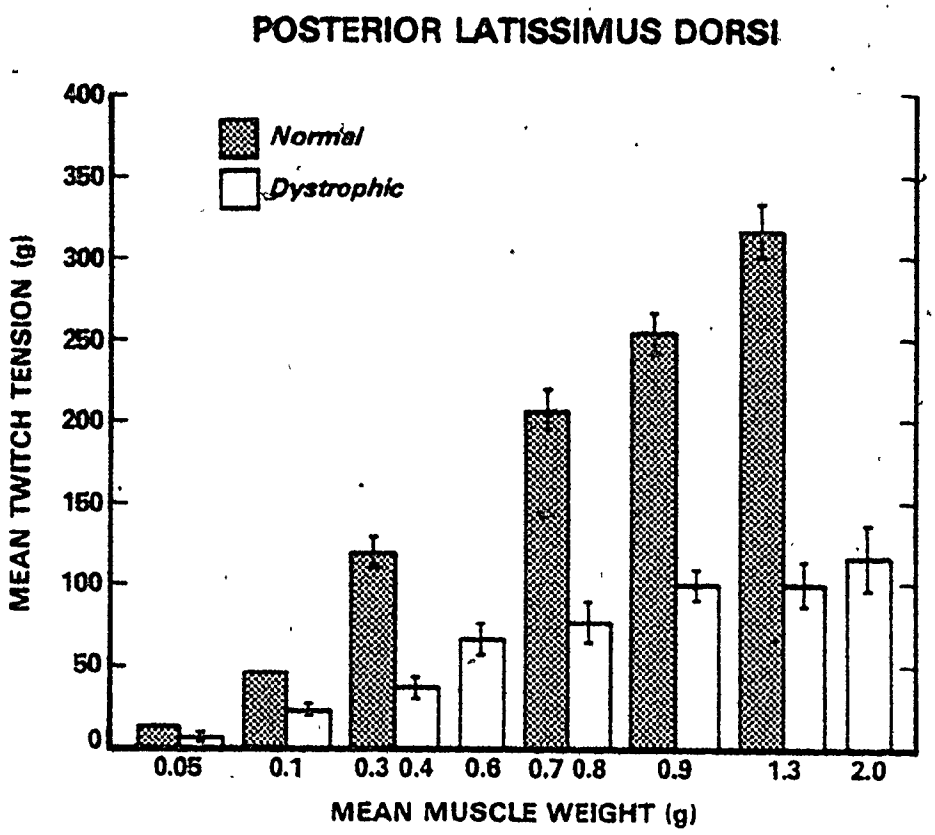
Normal, n = 35; Dystrophic, n = 63

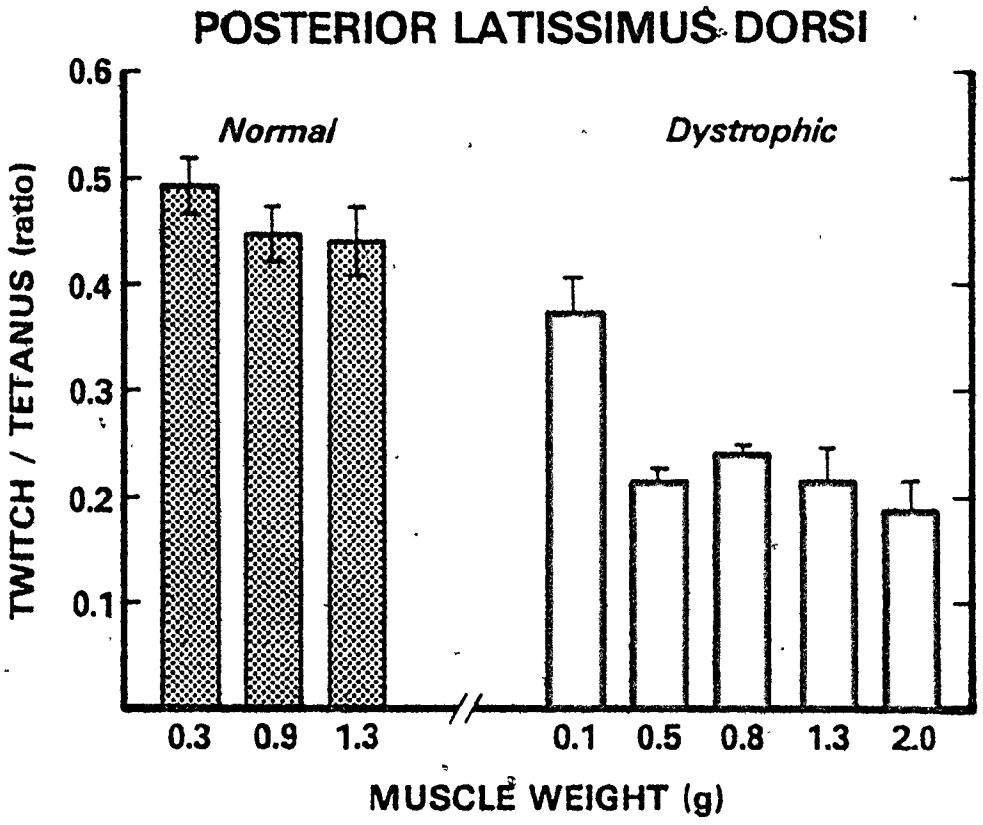
Values are mean ± SE

* two-tail probability

Figure 19: The change in mean twitch tension attained by PLD muscles of normal and dystrophic chickens during growth ex ovo. The mean twitch tension increases as muscle weight increases but the rate at which the tension of the dystrophic PLD muscle increases is slower than that of the normal PLD. The values of the mean twitch tension and the standard error of the mean are listed in Appendices P and Q. The number of columns in the histogram is smaller than the number of divisions in the Appendix as groups of muscles with similar muscle weight were pooled in the histogram for simplicity. Each of the first two columns of the normal PLD represents one muscle only.

Figure 20: The change in the maximum twitch to tetanus ratio of PLD muscles of normal and dystrophic chickens during growth. Whereas the ratio of normal PLD falls within the 0.4 - 0.5 range at all muscle weights, the ratio of the dystrophic PLD muscle decreases to the 0.2 - 0.25 range. The values of the mean and standard error of the mean are listed in Appendices P and Q. The number of columns in the histogram is smaller than the number of divisions in the Appendix, as groups of muscles with similar weights were pooled in the histogram for simplicity.


POSTERIOR LATISSIMUS DORSI
Normal
Dystrophic
MEAN TWITCH TENSION (g)
400
350
300
250
200
150
100
50
0
0.05
0.1
0.3
0.4
0.6
0.7
0.8
0.9
1.3
2.0
MEAN MUSCLE WEIGHT (g)


POSTERIOR LATISSIMUS DORSI
Normal
Dystrophic
TWITCH / TETANUS (ratio)
0.6
0.5
0.4
0.3
0.2
0.1
0.3
0.9
1.3
0.1
0.5
0.8
1.3
2.0
MUSCLE WEIGHT (g)

for the normal PLD muscles falls within the 0.4 to 0.5 range at any given muscle weight. The PLD muscles of dystrophic chickens, 15 to 30 days posthatching, shows a mean muscle weight of 0.1 g and a twitch to tetanus ratio of 0.4. At this period the twitch to tetanus ratio is similar to that of the PLD muscle of normal chickens. However, with subsequent development the twitch to tetanus ratio of the dystrophic muscles decreases to the 0.2 to 0.25 range.

HISTOCHEMICAL ANALYSES

The ALD and PLD muscles of both normal and dystrophic chickens were examined during development ex ovo. Histochemical analyses of Pase, SDH, myosin ATPase following both acid and alkali preincubation, and AChE enzymic activities were assessed. These enzymic activities permitted an analyses of the metabolic differentiation of the muscle, fibre type distribution, myosin ATPase specificity, AChE activity and the distribution of endplates. Furthermore, in order to determine the nuclear number and location and the fat content and distribution, the DNA-RNA and the Oil Red O reactions were also analyzed. A detailed biochemical and histochemical analyses of the development of fast twitch (pectoral) and slow tonic (soleus) muscles in normal and dystrophic (Storrs line) chickens has been reported (Cosmos, 1966, 1970; Cosmos and Butler, 1967; Cosmos et al., 1979b). In the present study, the ALD and PLD muscles were found to follow the same developmental changes described by Cosmos and coworkers.

Representative histochemical analyses at different developmental stages of the ALD and PLD muscles are presented in the following text.

ALD Muscles of Normal and Dystrophic Chickens

Histochemical analyses of the ALD muscle in normal and dystrophic chickens at various ages ex ovo indicated that this muscle has already attained its mature characteristics at hatching. No differences were noted between normal and dystrophic ALD muscles in all subsequent ages examined. Thus, the histochemical phenotype of the ALD muscle presented in the following results equally described muscles of normal or dystrophic genotype throughout ex ovo development.

Phosphorylase and SDH Activities: The response of ALD muscle sections to histochemical tests for Pase and SDH indicates that the ALD muscle has low Pase and high SDH activity as is characteristic of aerobic metabolism. Two fibre types are noted. One type (about 76% of all fibres) exhibits a red-iodine colour indicative of short chain polysaccharide. The second fibre type exhibits a lavender to purple iodine colour indicating the presence of intermediate length chain polysaccharide (Cosmos, 1966). The latter type also exhibits a relatively stronger SDH reaction. Figure 21c demonstrates the distribution pattern of these two fibre types in ALD muscle following Pase reaction.

Myosin ATPase: The myosin ATPase activity of ALD fibres was tested following acid (pH = 4.35) and alkaline (pH = 10.0) preincubations. Examination of cross sections of ALD muscles shows that all fibres demonstrate myosin ATPase activity after both acid and alkaline preincubation, indicating that the myosin ATPase of ALD muscles is both acid and alkali stable. The two fibre types distinguished by their reaction to the metabolic enzymes Pase and SDH are also identified with the ATPase reaction. The fibres exhibiting intermediate Pase and strong SDH activity

Figure 21: Photomicrographs of frozen cross sections of muscles following Pase reaction. These photomicrographs demonstrate the homogeneous strong (blue) response of normal PLD muscle (A x25), the cellular variability and the wasting characteristic of dystrophic PLD muscle (B x25), and the weak (red) Pase activity of the ALD muscle (C x100). The darker fibres in C demonstrate an intermediate (purple) Pase response.

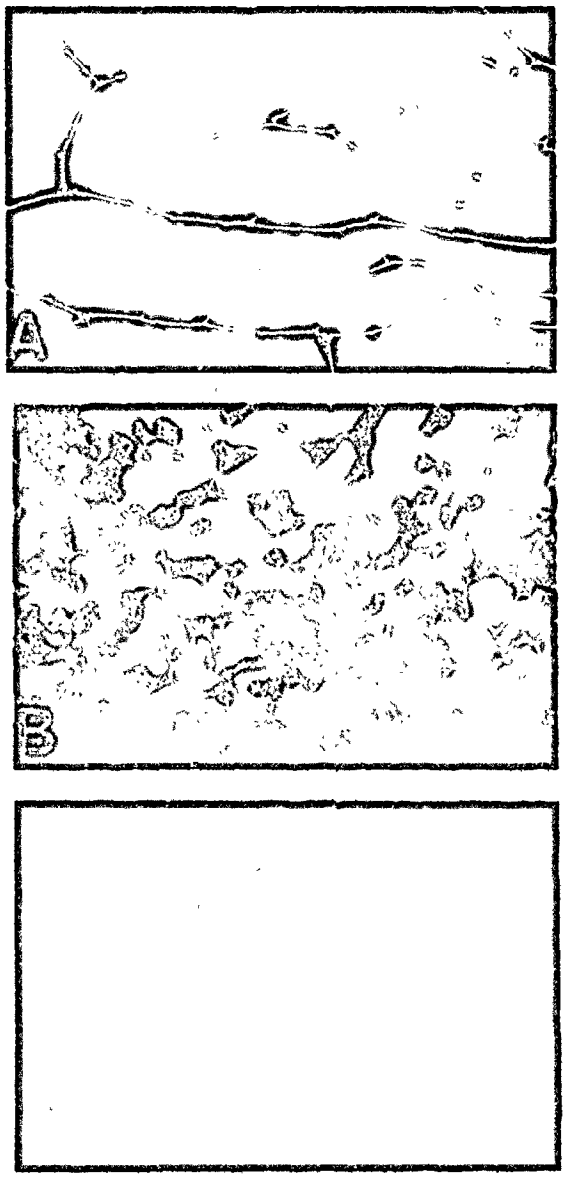
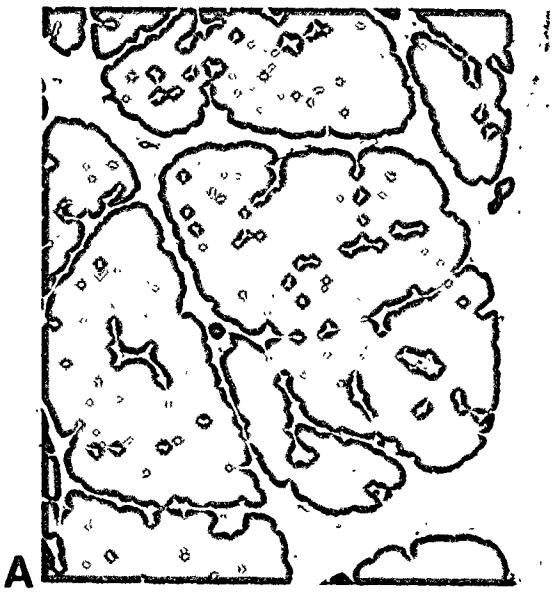

A
B

Figure 22: Photomicrographs of frozen sections of muscles subjected to myosin ATPase reaction after acid (pH = 4.35) and alkaline (ph = 10.0) preincubation (x10). In dual preparation of ALD and PLD muscles, no reaction is noted in the PLD muscle while all fibres of the ALD muscle display a strong reaction following acid preincubation (A). Following alkaline preincubation both ALD and PLD muscles show myosin ATPase activity (B). Muscles from both normal and dystrophic chickens responded the same following myosin ATPase reaction.

MYOSIN ATPase Pre H^+



A

ALD PLD

N or D

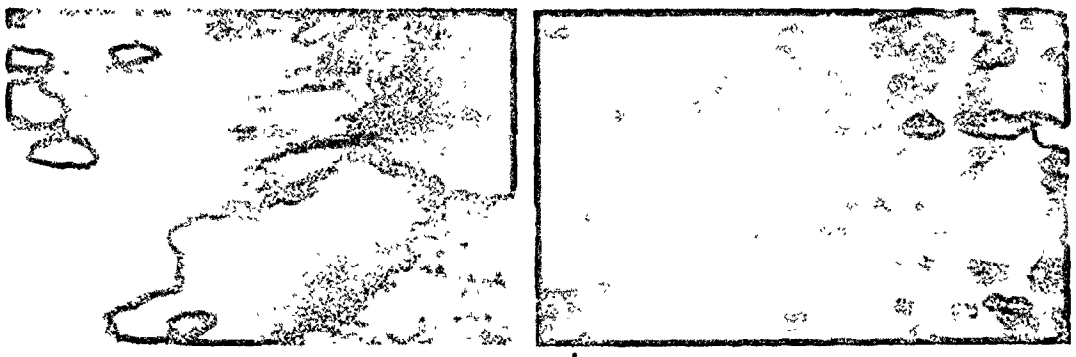
MYOSIN ATPase Pre OH^-

B

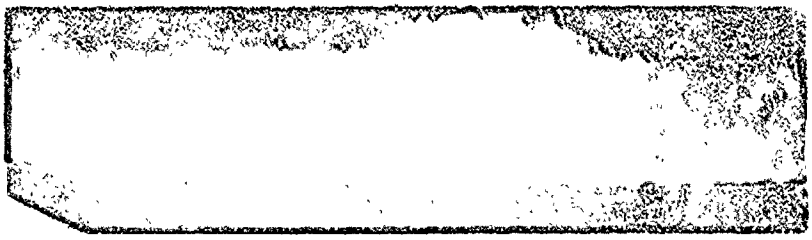
ALD PLD

N or D

Figure 23: Photomicrographs of PLD and ALD muscle fibres following AChE reaction. These photomicrographs demonstrate the focal "en plaque" (a, c, e) and the multiple "en grappe" (b, d, f) endplates as they appear following staining of a block of muscle (a, b); teased fibre preparations (c, d) and frozen cross sections (e, f) of PLD and ALD muscles of both genotypes.



a PLD b ALD



PLD

c

ALD

d

e PLD f ALD

also demonstrate stronger (dark brown colour) myosin ATPase activity after both acid and alkali preincubation. Figure 22 shows the myosin ATPase activity of ALD muscles from chickens of either normal or dystrophic genotype following acid (A) and alkali (B) preincubation.

Acetylcholinesterase: AChE reactions were performed both on frozen cross sections and on whole blocks of unfrozen ALD muscles (Fig. 23). Examination of a block of muscle shows a multitude of small brown dots scattered over the entire surface of the muscle. Examination of single fibres which had been teased apart from the AChE stained block of muscle revealed multiple endplates at short intervals along the fibre. These endings demonstrate the "en grappe" structure, i.e. they appear as fine groups of droplets of AChE stained material. Examination of cross sections of ALD muscles revealed fibres with AChE activity at single motor endplates, each of which appeared as one or more discrete droplets of AChE stained material.

Distribution of Nuclei: Examination of muscle sections following DNA-RNA reaction showed that the nuclei of ALD muscle fibres are located at the periphery of each fibre (Fig. 33). However, in a few fibres (<0.5%) taken from muscles of normal or dystrophic chickens greater than 12 months of age, internal nuclei are occasionally observed. Those fibres showing internal nuclei also reveal other abnormalities, e.g. heterogeneity of enzymic reaction inside the fibres and irregular shape and internal structure. These abnormalities may be related to aging of the fibres.

Oil Red O: The Oil Red O reaction was used to examine the neutral fat distribution in cross sections of muscles. Lipids are observed both

extracellularly around blood vessels and nerves and intracellularly as discrete droplets, perhaps associated with mitochondria (Cosmos, 1970).

PLD Muscles of Normal and Dystrophic Chickens

Phosphorylase and SDH: At hatching, the histochemical tests for Pase and SDH show that the PLD muscle of both genotypes reveal a "mixed" response with fibres showing weak (red), intermediate (purple) and strong (blue) Pase activity, and overall strong but variable SDH activity.

By 1 month, normal PLD fibres exhibit a more homogeneous reaction to Pase and SDH, with the majority of fibres showing strong Pase and weak SDH enzymic activities. This enzymic profile is maintained throughout subsequent development. Among 1941 fibres, counted within 60 fascicles from 6 PLD muscles of 6 normal chickens over a year old, 7% show intermediate Pase and strong SDH, while 93% demonstrate homogeneous strong Pase and weak SDH activity. The percent distribution of these fibres varies between muscles, and a few normal PLD muscles are composed of fibres exhibiting a completely homogeneous activity for both enzymes (Fig. 21). In contrast to the normal PLD muscles, the mixed enzymic response which characterizes the dystrophic PLD muscle from 1 day to 1 month of age remains throughout further development. The mixed fibre population of the dystrophic PLD shows no constant pattern; the typical correlation of Pase and SDH activities as seen in normal PLD muscle cannot be detected. Furthermore, following the SDH reaction, unequal intensity of the colour within many individual fibres were observed, indicating differences in the level of enzymic activity, or an abnormal distribution of mitochondria within different parts of individual fibres.

Some PLD muscles of both genotypes also demonstrate fibres (<2%) which exhibit metabolic characteristics of ALD fibres.

Myosin ATPase Activity: When PLD muscles of both normal and dystrophic genotypes were tested for myosin ATPase activity, no differences were noted in any age group examined. In chickens from 1 day to 2 years of age, PLD muscles of both genotypes show myosin ATPase activity after alkaline preincubation only. The muscle as a whole shows no activity after acid preincubation (Fig. 22) which indicates that the ATPase activity of the PLD muscle of both genotypes is acid labile and alkali stable. However, as noted with Pase and SDH reactions, a few PLD muscles of both genotypes do contain a population of fibres with characteristics similar to the ALD fibres, i.e. they demonstrate activity after acid and alkali preincubation. Analyses of those muscles which contained such fibres revealed that the "ALD type" fibres comprised less than 2% of the total number.

Acetylcholinesterase: Being focally innervated, PLD fibres of both normal and dystrophic genotypes examined at any age ex ovo exhibit only one endplate along their entire length. This can be demonstrated with teased fibre prepartions (Fig. 23). Only a few fibres in any one cross section show endplates. The endplates of the PLD fibres are not as structurally fine as the ALD endplates and they demonstrate strong AChE activity. These focal "en plaque" endplates consist of a number of branches joined to form a unified structure which resembles an oval plate under low magnification. Histochemically, AChE activity of the endplates in dystrophic PLD do not differ from those of the normal PLD. However, many dystrophic PLD fibres demonstrate AChE activity dispersed both within

the cytoplasm and on the extrajunctional membrane.

Distribution of Nuclei: Analysis of nuclei number and localization, using the DNA-RNA reaction on frozen cross sections of muscles, reveal that the nuclei within the normal PLD fibres of newly hatched chickens are located at the periphery of fibres. By 1 to 2 months of age, PLD muscles reveal some fibres with 1 or 2 internal nuclei. During further development, the number of fibres demonstrating internal nuclei increases. The number of the internal nuclei varies between 1 to 5. The mean number of internal nuclei was calculated from 500 fibres of 5 PLD muscles of 5 normal chickens 3 to 12 months old and found to be 1.6 ± 1.4. In contrast, a great difference is noted in the number of internal nuclei of the dystrophic PLD muscle. In the PLD muscle of newly hatched dystrophic chickens, the location of nuclei is similar to that of the normal PLD; however, at 1 to 2 months fibres with 1 to 5 internal nuclei are already apparent. At later stages of development, although peripheral and internal nuclei are observed, as in normal muscle, the number of internal nuclei increases significantly (Fig. 33). The number of internal nuclei per fibre in the mature dystrophic PLD muscle is highly variable and as many as 45 nuclei in the cross sectional area of one fibre have been counted. The mean number of internal nuclei calculated from observations on the same number of fibres and chickens as with the normal PLD is 5.8 ± 6.2.

Oil Red O: Unlike the ALD muscle of both genotypes, the normal PLD from a mature normal chicken gives a weak lipid response following the Oil Red O reaction. Lipid is observed extracellularly around blood vessels and nerves but only in trace amounts intracellularly. In contrast PLD

muscles of mature dystrophic chickens show a high accumulation of lipid not only around blood vessels and nerves but also between fibres and fascicles. Furthermore, variability in the intracellular lipid response is apparent and many fibres exhibit high intracellular lipid concentration while others are totally replaced by fat. The replacement of individual fibres by fat can even be seen in PLD muscles of 1 month old dystrophic chickens. It was especially characteristic of PLD muscle from mature dystrophic hens. Some of the PLD muscle from mature dystrophic roosters exhibited no replacement of fibres by fat at all.

DISCUSSION

INTRODUCTION

Although the structural, mechanical and biochemical properties of the ALD and PLD muscles of normal genotype have been described by many investigators (see Introduction), the ALD and PLD muscles of dystrophic genotype have not been studied to the same extent. The aim of this part of the research was to analyze selected structural, mechanical and histochemical properties of the ALD and PLD muscles of dystrophic genotype during development ex ovo, primarily to provide control data for analyses of PLD muscles which have been cross reinnervated by the ALD nerve in the dystrophic chicken.

The study was performed on normal White Leghorn chickens and on chickens with hereditary muscular dystrophy (Storrs line). The results obtained with the ALD and PLD muscles of dystrophic genotype will also be compared with related studies done on the pectoralis and soleus muscles of the same dystrophic line and on ALD nad PLD muscles of other dystrophic lines developed at Davis, California.

Altered properties in righting ability, metabolic and proteolytic enzymic activity, nuclear number, lipid quantity and distribution are observed in more than one dystrophic line, which indicates that these characteristics are linked with the primary genetic defect. However, the degree of alteration of a specific property is not the same in every line (Asmundson et al., 1966: Wilson et al., 1979; Randall and Wilson,

1980). On the other hand, the observation that early in development ex ovo one dystrophic line shows atrophy while others show hypertrophy of the fast twitch fibres may point to significant differences in genetic background and therefore comparisons between different dystrophic lines should be made with caution.

The present study confirms that fast twitch glycolytic fibres are affected by the disease while slow tonic, oxidative fibres are spared (Cosmos, 1966, 1970; Cosmos and Butler, 1967; Linkhart and Wilson, 1975). Although the PLD muscle of dystrophic genotype shows structural and histochemical abnormalities when compared to the PLD muscle of normal genotype, it retains the fast twitch properties and myosin ATPase specificity of normal muscles. However, the ALD muscle of dystrophic genotype demonstrates a normal phenotype, i.e. its structural, histochemical and mechanical properties resemble those of the ALD of normal chickens.

WEIGHT PROPERTIES

Body Weight

No difference in body weight between normal and dystrophic chickens from 1 day to 500 days ex ovo could be demonstrated. Asmundson et al. (1966) indicated that, unless a selection for rapid growth or large size had been made, the growth and size of normal and dystrophic chickens need not be different. Chou et al (1975) using the Storrs line, also found no difference in body weight between normal and dystrophic chickens up to 105 days of age. However, Chou et al. reported that differences do exist between the body weights of hens and roosters in both normal and dystrophic chickens and that result was confirmed in the

present study. To allow for this difference, ALD and PLD muscle weights analyzed in the present study were related to body weight rather than to age.

Muscle Weights

Normal Chickens: Although studies of the development of the ALD and PLD muscles in ovo showed a markedly faster development of the ALD muscle (Gutmannet al., 1969; Butler et al., 1978), comparison made between the ALD and PLD muscle weights in normal chickens at various ages ex ovo indicate that the PLD muscle is heavier than the ALD muscle (Feng et al 1963; Jirmanova and Zelena, 1970; Shear and Goldspink, 1972). The present study reveals that the ALD muscle is heavier than the PLD muscle at hatching but that the rate of growth of the PLD muscle ex ovo is faster than that of the ALD. By approximately 9 days ex ovo (60 grams body weight) both muscles show equal weights. At 160 to 180 days, the age range when chickens achieve sexual maturity and a steady level of growth (Cosmos, 1970), the weight of the ALD muscle is about 60% that of the PLD muscle.

Dystrophic Chickens: The growth pattern of the ALD muscle of the dystrophic chicken is similar to that of the ALD muscle of the normal chicken. Both develop their mature characteristics in ovo (Gutmannet al., 1969; Butler et al., 1978; Cosmos et al., 1979b), have similar weights at hatching, and grow at identical rates up to 90 days ex ovo. After 90 days, however, the ALD muscle of dystrophic chickens increases in weight compared to the ALD muscle of normal chickens (Fig. 10). This difference may not represent an intrinsic difference but rather some

compensatory mechanism (Gutmann et al., 1970; Sola et al., 1973) within the ALD muscle of dystrophic chickens stimulated by the weakness and wasting of its synergistic PLD muscle.

The growth pattern of the dystrophic PLD muscle does not follow that of the normal PLD muscle. At hatching both muscles show similar weight, but as development proceeds, the growth rate of the dystrophic muscle is greatly decelerated. By approximately 60 days (750 grams body weight) the PLD of the dystrophic chicken weighs only 70% of its normal counterpart, and this difference is maintained throughout further growth. Thus, in both newly hatched normal and dystrophic chickens, the weight relationships of the ALD and PLD muscle are the same; the ALD muscle being larger than the PLD muscle. However, while these ratios are reversed in mature normal chickens, the immature weight relationships are maintained with the ALD and PLD muscle of dystrophic genotypes (Tables 2 and 3). This abnormal relationship likely reflects both the pathological changes characterizing the fast twitch muscle of dystrophic genotype (Cosmos, 1970) and the hypertrophy of the ALD muscle in the dystrophic chicken. The PLD in dystrophic birds of the 304 line also weighs approximately 70% of the normal PLD muscle between 8-16 weeks of age (Hoekman, 1976).

STRUCTURAL PROPERTIES

Morphological Characteristics

ALD muscles of both genotypes are characterized by the presence of small fascicles which contain an average of 20 individual muscle fibres. Both fibres and fascicles have a rounded shape. The large

extracellular spaces between the fascicles give an overall impression of loosely packed muscle. This characteristic appearance of the ALD muscle in cross section is already apparent early in ovo (Gordon et al., 1977b; Butler and Cosmos, unpublished observations).

In contrast, the PLD muscle of normal genotype is characterized by large elongated fascicles which contain an average of 49 individual muscle fibres of polygonal shape. Since very little extracellular space exists between the fibres and fascicles, the appearance of the PLD muscle in cross section is that of a very compact muscle.

Contrary to the appearance of the normal PLD, the dystrophic PLD is characterized by fibres of variable shape and size. This variability of both muscle fibres and fascicles is apparent as early as 15 days ex ovo but it becomes more marked as the disease progresses. Furthermore, the extracellular spacing between both fibres and fascicles increases with age as a result of wasting, and the number of individual muscle fibres within the fascicles decreases to an average of 37 fibres. These abnormal structural characteristics of the dystrophic PLD probably reflect multiple processes such as degeneration, regeneration, and replacement of degenerating fibres by lipids (Cosmos, 1970). The typical appearance of ALD and PLD muscles of both genotypes in cross section is illustrated in Figure 12, and can also be seen in the photomicrographs illustrating the histochemical analyses. In dystrophic chickens of the Storrs line, the soleus and pectoralis muscle have morphological appearances similar to the ALD and PLD respectively (Cosmos and Butler, 1967; Cosmos, 1970).

Fibre Diameters

The mean fibre diameter of the ALD muscle in normal chickens is larger than that of the PLD, and can be seen as early as 14 days in ovo (Gordon et al., 1977a). The results of the present study demonstrate that during ex ovo development the fibres of the two muscles in normal chickens reach their maximum diameter by about 3 months. At this time, the ALD fibres have a mean diameter of 69.3 ± 14.9 μm and the PLD fibres, 52.4 ± 10.6 μm. These values are similar to those reported by Shear and Goldspink (1972).

The two fibre types distinguished in the ALD by histochemistry (Fig. 21) differ in size,with the mean fibre diameter of the small fibres (24% of total) being about 10 μm less than that of the larger fibres. The histogram presented in Figure 13 includes measurements of both fibre types and the two maxima, one at 60-70 μm and the second at 70-80 μm, identify the two fibre populations.

Differences in size between the two fibre types of the normal ALD muscle have been reported by Koenig and Fardeau (1973) and Ovalle (1978). Koenig and Fardeau (1973) reported that the larger fibres, 60% of the total population, have a mean diameter of 150 μm and the smaller, 80 μm. Ovalle (1978) reported that 84% of the fibres have a mean diameter of 63.0 μm while that of the smaller is 45.5 μm. Although the results of the present study more closely resemble the results of Ovalle (1978), the reason for the differences between the results of the three studies is not clear.

The proportion of the two fibre types and the total range of fibre diameters in the ALD muscle of dystrophic genotype are identical

to those of the normal ALD muscle (Fig. 13). Differences exist, however, in the frequency distribution of fibre diameter and the mean fibre diameter (Table 4), consistent with compensatory hypertrophy in the dystrophic ALD suggested as a possible mechanism leading to increased muscle weight of the ALD after three months (90 days).

The PLD muscle of dystrophic genotype has a larger range of fibre diameters than does the normal PLD muscle (Fig. 14). Although the standard deviation of the mean (Table 5) reflects this wide range of fibre diameters, the "mean fibre diameter" with its standard deviation can be a misleading term without reference to the histogram showing the distribution (Fig. 14). The large variability of fibre diameters in the dystrophic PLD muscle may reflect different processes at different times, i.e. different rates of growth, degeneration and regeneration, atrophy, hypertrophy and splitting of fibres; all of these phenomena have been observed in dystrophic fast twitch muscles (Julian and Asmundson, 1963; Cosmos, 1966, 1970; Cosmos and Butler, 1967). The variability in fibre size noted in the PLD of the dystrophic Storrs line has also been reported to characterize the fast twitch pectoralis muscle of the same line (Cosmos, 1966) and of the other dystrophic lines (McMurty et al., 1972).

Two other points arise from the structural analyses:

1. The mean fibre diameter of the ALD muscle is greater than that of the PLD muscle of normal chickens, but the muscle weight of the ALD is lower than that of the PLD, suggesting that the ALD contains fewer fibres than the PLD. A direct count of the total number of fibres of one ALD and one PLD muscle of a normal chicken (16 weeks ex ovo) yielded only

4000 fibres for the ALD and 9000 fibres for the PLD. For the PLD muscle of normal chickens, a range of 4000-6000 fibres has been reported by Jirmanová and Zelená (1973).

2. The fact that the mean fibre diameter of ALD and PLD muscles of normal chickens reaches a constant value by 3 months, while the total muscle weight continues to increase, may indicate that a longitudinal growth of fibres is occurring as suggested by Shear and Goldspink (1972).

MECHANICAL PROPERTIES

Normal Chickens

The first reported study on the mechanical properties of the ALD and PLD muscles of normal chickens was the *in vitro* analyses of Ginsborg (1960a). He showed that the PLD is a fast twitch muscle, while the ALD is a slow tonic one capable of producing twitches in response to single nerve stimuli. These results were confirmed by the *in vitro* studies of Gutmann and Syrový (1967), Hoekman (1968), Page (1969) and Canfield (1971). Yet, when the response of the ALD muscle was tested *in vivo*, twitches could not be elicited by single nerve stimuli and in order to assess the twitch contraction properties of the ALD, post tetanic potentiation techniques were used (Hník *et al.*, 1967, 1977; Jirmanová and Zelená, 1973). With this technique, a single stimulus that follows tetanic stimuli can evoke a twitch response from ALD muscles *in vivo*. The reason for the ability of the ALD muscle to produce a twitch in response to a single nerve stimuli under *in vitro* conditions, but not *in vivo* is not known; it may reflect a change in the ionic distribution resulting from the experimental conditions.

In the present in vivo study no twitches could be evoked from the ALD muscle in response to a single nerve stimulus. Using the post tetanic potentiation technique, the time parameters, i.e. contraction time and time to half relaxation, of the twitch response agreed well with the results reported by Hnik et al. (1967, 1977).

The scientists cited above studied the ALD and PLD muscles of mature chickens. There are, however, three studies in which the mechanical properties of the muscles during development in ovo and/or ex ovo were analyzed (Shear and Goldspink, 1972; Melichna et al., 1974; Gordon et al., 1977a). Although these results are not in complete agreement (Shear and Goldspink, 1972) as to the specific age when the two muscles exhibit their characteristic contractile properties, they do agree that the contractile properties of ALD and PLD muscles show only slight changes during development ex ovo (see Introduction, p 6).

The present study has confirmed that in normal chickens the ALD muscle has slow tonic characteristics and the PLD muscle has fast twitch properties. It has also been shown that from 15 to 800 days ex ovo, the ALD and PLD muscles undergo no appreciable alteration in their mechanical properties. This result agrees with the observations of Shear and Goldspink (1972), Melichna et al. (1974) and Gordon et al. (1977a). The maximum tension produced by each muscle increased as the muscle weight increased. However, the maximum tension produced by the PLD muscle was always greater than that of the ALD muscle when corrected for muscle weight. These findings agree with the developmental studies of Shear and Goldspink (1972), although the maximum tension obtained from the PLD in the present study was higher than that reported by these investigators.

The average twitch to tetanus ratio calculated for the PLD muscle was 0.45. Hoekman (1976), who studied the *in vivo* mechanical response of the PLD of the New Hampshire chickens at 8-14 weeks of age reported a twitch to tetanus ratio of 0.313.

Chickens with Hereditary Muscular Dystrophy

The only reported study of the mechanical properties of the PLD muscle of dystrophic chickens is that of Hoekman (1976) who studied the PLD *in vivo*, of 8-14 week old normal and dystrophic chickens of the California lines (200 and 304 respectively). The present study, however, is the only one to date in which the *in vivo* isometric contraction properties of both the ALD and PLD muscles of dystrophic chickens (Storrs line) have been studied during *ex ovo* development (15-800 days).

Analyses of the mechanical responses of the ALD muscle of dystrophic genotype during development *ex ovo* showed that there are no significant differences between the mechanical properties of ALD muscles of normal and dystrophic chickens. Thus, the mechanical properties characterizing the ALD muscles support the structural and histochemical findings which indicate that the slow tonic muscle of dystrophic genotype expresses a normal phenotype (Cosmos, 1966, 1970; Cosmos and Butler, 1967; Cosmos *et al.*, 1979b).

Analyses of the mechanical responses of the dystrophic PLD muscle during *ex ovo* development showed that at 15 days *ex ovo* the dystrophic PLD muscle is a fast twitch muscle and its fast twitch characteristics are maintained during further development (up to 800 days), despite the structural and histochemical abnormalities incurred during this period.

The contraction and relaxation times of its twitch responses are the same as those of a normal PLD muscle; however, during the tetanic response an increase of 7-18 msec in the time to half contraction ($T_{\frac{1}{2}}C$) is observed with no change in the relaxation time (Table 9). Bárány et al. (1966) showed that myosin from the pectoralis muscles of both mature-dystrophic (Storrs line) and normal chickens has identical ATPase enzymic activity and amino acid composition. In our laboratory, a histochemical analysis of the myosin ATPase activity of the fast twitch PLD (and pectoralis) of both genotypes confirmed the biochemical analysis, i.e. no alterations in this enzyme are noted as a result of the disease process. Thus, the increased time to half contraction may be related to other factors, e.g. calcium concentration or distribution, known to be altered in the dystrophic pectoralis muscle (Cosmos, 1964), or to an increase in the series elastic compliances of the muscle (Hoekman, 1976). The absence of alterations in the relaxation time of the dystrophic PLD indicates that the ability of the intact sarcoplasmic reticulum to reabsorb calcium is not affected, as has been reported with fragmented sarcoplasmic reticulum of dystrophic pectoral muscles (Sylvester and Baskin, 1973).

The main difference noted between normal and dystrophic PLD muscles was the inability of the latter to develop twitch and tetanic tensions equal to those developed by the normal PLD muscle. Since this was observed early in development ex ovo it could not be accounted for by the loss of muscle fibres only. It has been proposed (Warnick et al., 1979) that this lowered tension is due to a loss of functional muscle fibres resulting from a failure of the synaptic mechamism of certain motor units. The twitch to tetanus ratio was also significantly reduced

in the dystrophic PLD. These alterations may reflect an increase in the series elastic compliance of the dystrophic PLD muscle or a change in the time course of the active state (Close, 1972; Hoekman, 1976). More comprehensive studies of the mechanical properties of the PLD muscle are needed to evaluate the differences noted. At present, it can only be concluded that the dystrophic PLD muscle remains a fast twitch muscle and that the changes noted do not indicate a shift towards a slow tonic muscle.

HISTOCHEMICAL PROPERTIES

ALD and PLD Muscles of Normal Genotypes

In the present study, histochemical analyses of the ALD and PLD muscles of normal genotype showed that the ALD muscle possesses its mature characteristics at the time of hatching. These include low Pase and high SDH enzymic activity, acid and alkaline stable myosin ATPase activity, multiple endplates with low AChE activity, high RNA content and peripheral localization of nuclei. In contrast, the PLD muscle at hatching demonstrates a heterogeneous histochemical response to metabolic enzymes which gradually changes to the more homogeneous reactions characterizing fast twitch glycolytic muscles; i.e. weak SDH and strong Pase activity. The localization of nuclei alters slightly during development ex ovo, but other characteristics such as alkaline stable (and acid labile) myosin ATPase activity and focal endplates with high AChE activity show no change.

The studies of Bass et al. (1970), Gutmann and Syrový (1967) and Melichna et al. (1974), on the metabolic differentiation of the ALD and PLD muscles during development ex ovo and the studies of Cosmos (1966, 1970) and Cosmos and Butler (1967) on the pectoralis and soleus muscles of the

normal chickens have demonstrated both biochemically and histochemically that whereas the slow tonic muscle maintains the metabolic characteristics it has at hatching, fast twitch muscles change their metabolism from aerobic at hatching to anaerobic during later growth.

Histochemical analyses showed that a mature PLD muscle still possesses a small population (approximately 7%) of fibres with intermediate Pase and high or intermediate SDH enzymic activity. Similar results were reported by Nene and Chinoy (1965), Melichna et al. (1974), Sansoneand Lebeda (1975) and Beringer (1978). The ALD muscle was also shown to contain two populations of fibres, as first reported by Asmussen et al. (1969) and later confirmed by Asiedu and Shafiq (1972), Koenig and Fardeau (1973), Sola et al. (1973), Ashmore and Doerr (1976) Ovalle (1978), and Cosmos et al. (1979b). Other slow tonic muscles of the chicken such as the soleus (Cosmos and Butler, 1967; Cosmos, 1970) also contain two fibre populations. In the present study these two fibre types were found to differ in their mean fibre diameter and in the intensity of their enzymic reactions. The majority of the fibres (76%) are larger and exhibit weaker Pase, SDH and myosin ATPase activity than the remaining fibres. However, both fibre types are slow tonic, multiply innervated fibres exhibiting both alkali and acid stable myosin ATPase activity.

The studies of Syrový (1973) and Melichna et al (1974) showed that the myosin ATPase activity of the PLD muscle is higher than that of the ALD muscle, which corresponds well with the known contractile properties of these two muscles. Although it was pointed out by Guth (1973) that the intensity of histochemically demonstrated myosin ATPase activity is not necessarily indicative of the level of activity of the enzyme, the histo-

chemical reactions of this enzyme at different pH's provides a reliable method for fibre typing. The slow tonic ALD fibres show positive reactions for myosin ATPase activity following both acid and alkaline preincubation (Asiedu and Shafiq, 1972). These results were confirmed in the present study, using the technique developed by Guth and Samaha (1970) and modified in our laboratory by J. Butler. It was also shown that ALD fibres have both acid and alkaline stable ATPase both at hatching and during later development ex ovo, while PLD fibres express only alkaline stable ATPase. Ongoing work in our laboratory indicates that this pH specificity is characteristic of both muscles during development in ovo and this circumstance provides an enzymic marker capable of distinguishing between fast twitch and slow tonic fibres during avian embryogenesis (Butler and Cosmos, 1979a,b).

The innervation patterns of the ALD and PLD muscles have been demonstrated with teased fibre preparations by Ginsborg and MacKay (1961), Hess (1961) and Silver (1963) and their results were confirmed in this study. The difference in the innervation pattern between the fibres of the two muscles is obvious. Each ALD fibre has many endplates occurring regularly along its length and separated by a distance of about 1000 μm in adult chickens (Hess, 1961), while each PLD fibre exhibits a single endplate. It was also shown that the endplates of the ALD have "en grappe" structures with low AChE activity while the PLD endplates have "en plaque" structures with high AChE activity. The histochemical differences between the endplates of these muscles could also be detected in fibre cross section preparations (Linkhart and Wilson, 1975; Wilson et al., 1973). In the present study, fibre cross sections as well as teased

fibre preparations were used to study the innervation patterns of the ALD and PLD muscles. The histochemical observations of the AChE activity and distribution confirm the results described above. No change in the patterns of innervation was found during development ex ovo. The study of Ginsborg and MacKay (1961) suggested that the number of neuromuscular junctions on the ALD fibres remained constant during growth, but the distance between the endplates increased with the increase in muscle length.

The distribution of nuclei in the ALD and PLD fibres was different (Cosmos et al, 1979b). At hatching, histochemical analyses revealed that the nuclei of the PLD fibres were peripherally located, but, during further development this pattern changes. Cross sections of the mature PLD muscle of normal chickens showed fibres with nuclei at the periphery as well as internally. A maximum of 5 internal nuclei per fibre cross section was found. By contrast, only peripheral nuclei were found in cross sections of healthy ALD fibres during development ex ovo (Fig. 33). This distribution of nuclei characterizes all fast twitch and slow tonic fibres of the chickens which have been examined. It is not yet known what determines nuclei position or distribution, especially since the mammalian fast and slow twitch fibres have peripheral nuclei.

The distribution of lipid in PLD and ALD muscles resembles that of the pectoralis and soleus muscles respectively (Cosmos, 1970). Both fast muscles contain smaller amounts of lipid, mainly around blood vessels and nerves, than do the slow tonic muscles. In the latter, lipid is located extracellularly around blood vessels and nerves and intracellularly as discrete droplets.

ALD and PLD of Dystrophic Genotype

It has been demonstrated that slow tonic muscles (soleus, ALD) of dystrophic chickens of either the Storrs or California lines undergo no alterations in metabolic enzymic activity, nuclei or lipid distribution (Cosmos and Butler, 1967; Cosmos, 1970), or in AChE activity, distribution and molecular form (Wilson et al., 1973; Linkhart and Wilson, 1975), as a result of the disease process. The present study reinforces these observations by demonstrating that the ALD muscle of dystrophic genotype expresses a normal phenotype during development ex ovo, i.e. it has low Pase and high SDH enzymic activity, myosin ATPase activity following acid and alkali preincubation, multiple endplates with low AChE activity, peripheral nuclei, high lipid and two populations of fibres, all of which characterize the normal ALD. These characteristics combined with the structural and mechanical properties discussed above suggest that the ALD muscle of dystrophic genotype expresses a completely normal phenotype.

In contrast, the synergistic fast twitch PLD muscle of dystrophic genotype shows many abnormalities which have already been observed in the fast twitch pectoralis muscle of the same dystrophic line (Cosmos, 1966, 1970; Cosmos and Butler, 1967). Similar to the normal muscle, the dystrophic PLD demonstrates a mixed fibre population during the immediate post hatching period. Later in development, however, when the normal PLD demonstrates a homogeneous population of fibres, the dystrophic PLD still maintains the immature mixed fibre pattern. The abnormal pattern of metabolic enzymes noted with the dystrophic muscle may indicate a fault in the regulatory mechanisms of these enzymes rather than a shift towards a slow fibre type as suggested by Hoekman (1976).

Unlike the alterations noted with the metabolic enzymes, no changes were found in the myosin ATPase activity of the pectoralis muscle (Bárány et al., 1966). In the present study, histochemical analyses of dystrophic PLD muscles reveal that from the time of hatching through maturity, this muscle demonstrates high myosin ATPase activity following alkaline preincubation only. These findings and the mechanical properties characterize a fast twitch muscle.

The focal pattern of innervation of the dystrophic PLD muscle also remained unchanged during development ex ovo. Changes in the molecular forms and AChE activity have been reported to take place at the endplates of fast twitch muscles of dystrophic genotype (Wilson et al., 1973; Lyles et al., 1979). In the present study, these properties were not examined since specific cholinesterase inhibitors were not used. However, an abnormal localization of AChE activity in the cytoplasm and at extrajunctional membranes of the PLD fibres of dystrophic genotype was demonstrated. Similar observations have been reported with PLD fibres of other dystrophic lines (Linkhart and Wilson, 1975; Sansoneand Lebeda, 1975). Other alterations observed with histochemical preparations of the dystrophic PLD were an increase in the number of internally located nuclei and an accumulation of lipid, not only around blood vessels and nerves, but also between and within fibres. These findings agree with the results obtained with other fast twitch muscles of dystrophic chickens of all lines (Julian and Asmundson, 1963; Cosmos, 1966, 1970). Even though the significance of an increased number of nuclei per fibre is not understood, they may originate from satellite cells attempting to regenerate destroyed fibres. Satellite cells are significantly more

numerous in dystrophic pectoral muscle (Nichols and Shafiq, 1979) and are a source of myoblasts during regeneration.

Table 10 summarizes the phenotypic characteristics of the ALD and PLD muscles of normal and dystrophic genotype demonstrated in this study.

Conclusion

A major aim of this part of the research was to establish appropriate criteria for the analysis of PLD muscles successfully cross reinnervated by ALD nerves in either normal or dystrophic chickens and to determine whether converted fibres of dystrophic genotype are spared disease phenotypes. Analysis of the results of the developmental study indicated that the following properties should be analyzed to determine if the successfully cross reinnervated PLD muscles demonstrated phenotypic characteristics of slow tonic ALD fibres:

1. Mechanical properties: Measurement of isometric tension evoked by nerve stimulation would indicate that the muscle is reinnervated and responsive. PLD muscles of both genotypes are fast twitch muscles; therefore, the absence of a response to a single nerve stimulus, but a slow tonic contraction in response to a train of stimuli (characteristics of slow tonic ALD muscles) would indicate that mechanical changes have followed the reinnervation.

2. Myosin ATPase activity: This enzyme analysis tests the specific response of the muscle fibres to myosin ATPase following acid (pH = 4.35) and alkaline (ph = 10.0) preincubation. Using this technique, it was shown that a response following both acid and alkali preincubation is

TABLE 10 PROPERTIES OF THE POSTERIOR AND ANTERIOR LATISSIMUS DORSI MUSCLES OF ADULT NORMAL AND DYSTROPHIC CHICKENS

Muscle Properties	PLD		ALD
	Normal	Dystrophic	Normal and Dystrophic
Speed of contraction	Fast twitch	Fast Twitch	Slow Tonic
Type of innervation	Focal	Focal	Multiple
Myosin ATP activity:			
Alkali stable	Positive	Positive	Positive
Acid stable	Negative	Negative	Positive
Metabolic Enzymic Activity:			
Phosphorylase	Strong	Variable (weak to strong)	Weak
Succinic dehydrogenase	Weak	Variable (weak to strong)	Strong
Position of nuclei (No. of internal nuclei per cross section)	Scattered (n = 1-5)	Scattered (n = 1-45)	Peripheral (none)
Fibre structure	Polygonal	Variable in shape	Rounded
Fibre diameter			
Range (μ)	20-90	10-160	40-100
Major peaks (μ)	50-70	No major peaks	60-80
Destruction	Absent	Present	Absent

restricted to the slow tonic fibres; fast twitch fibres of both genotypes demonstrate activity following alkaline preincubation only. Therefore, the successfully cross reinnervated fibres of both genotypes should have both alkali and acid stable myosin ATPase activity.

3. Endplates distribution: Since fast twitch PLD fibres of both genotypes are focally innervated and slow tonic ALD fibres of both genotypes are multiply innervated, successfully cross innervated PLD fibres should demonstrate multiple endplates.

Since this developmental study demonstrated that the above properties show no alterations as a result of the dystrophic process, they cannot be used to identify the dystrophic phenotype, but, instead to indicate only that conversion of fibres in the cross reinnervated PLD muscles of both genotypes has been achieved. An additional set of properties (4-6 below) were selected not only to further assess the success of cross reinnervation but also to identify the dystrophic phenotype.

4. Pase and SDH activity: Since slow tonic fibres of both genotypes exhibit high SDH and low Pase activity, successfully cross reinnervated PLD fibres of both genotypes will exhibit this pattern of enzymic activity. Although PLD muscles of both genotypes demonstrate glycolytic metabolism, the dystrophic PLD is characterized by a more variable enzymic response and therefore fibres expressing the disease should have abnormal levels of activity. This test should therefore indicate whether the cross reinnervated PLD muscle of dystrophic genotype demonstrates disease characteristics.

5. Distribution of nuclei: Since slow tonic fibres of both geno-

types are characterized by peripherally located nuclei, examination of the successfully cross reinnervated PLD fibres of both genotypes should demonstrate a similar distribution. However, since the dystrophic PLD fibres are characterized by an increased number of internal nuclei, this characteristic identifies cross reinnervated PLD fibres of dystrophic genotype which still express this abnormal phenotype.

6. Structural analysis: Since the mean fibre diameters, distribution and shape of ALD fibres of both genotypes differ from those of PLD fibres, examination of the cross reinnervated PLD muscles will show which morphological pattern they exhibit.

To determine whether converted PLD fibres of dystrophic genotype express a dystrophic phenotype they should be compared with fibres of normal genotype similarly converted. Thus, any abnormalities appearing in cross reinnervated PLD fibres of both normal and dystrophic chickens cannot be attributed to the disease process.

PART II: EXPERIMENTAL STUDY

RESULTS

INTRODUCTION

A primary objective of this study was to investigate the response of a fast twitch muscle of dystrophic genotype to a foreign innervation. The questions that were asked involved

1. the ability of the fast twitch PLD muscle to accept the nerve which usually innervates the slow tonic ALD muscle;
2. Once innervation was successful, could the fast twitch dystrophic muscle respond to the influence exerted by the "slow tonic" nerve; and
3. would such a response include an expression of dystrophic phenotype?

The surgical cross reinnervation between the ALD nerve and the PLD muscle was performed within the same chicken at hatching. The muscles were examined at various time intervals postoperatively using the criteria established in the preceding part of the present study. To identify alterations resulting from the operative procedures and from reinnervation, the cross reinnervated muscles were compared to PLD muscles which were self reinnervated by their own PLD nerve. Comparable experiments performed with normal chickens served as normal controls.

The results will be presented in two sections: Those obtained from PLD muscles self reinnervated by the PLD nerve; and those obtained from PLD muscles cross reinnervated by the ALD nerve.

SECTION 1: SELF REINNERVATION OF PLD MUSCLE

Number of Experiments

Self reinnervation experiments were performed on newly hatched normal and dystrophic chickens. In these experiments the nerve to the PLD muscle was severed *in situ* and allowed to reinnervate its own muscle. In addition, the PLD muscle was either injured or left intact and the ALD muscle removed to control for the same procedure done in the cross reinnervated experiments (see Methods p 42). Nine experiments were done on normal chickens and thirteen on dystrophic chickens with the following combinations:

Type of Preparation	Normal	Dystrophic
ALD intact, PLD injured	5	3
ALD removed, PLD injured	2	5
ALD removed, PLD uninjured	2	5
Total	9	13

The self reinnervation experiments were analyzed at various intervals 4 to 18 months postoperatively. Since some chickens died from oversensitivity to the anesthesia, not all of the experiments were analyzed for contraction characteristics. However, all muscles were analyzed histochemically.

Gross Examination

The PLD muscles were exposed for mechanical testing and were examined for the success of self reinnervation. All injured muscles showed excessive connective tissue around the muscle when compared to

the muscle on the unoperated contralateral side. This was more evident in operations with dystrophic chickens. Self reinnervation by the PLD nerve usually occurred at the point of nerve entry noted with the unoperated PLD muscles. In normal chickens, the ALD nerve deflected to underlying muscles could be seen clearly and when stimulated, elicited a response from the underlying musculature. However, the ALD nerve was more difficult to find in the dystrophic chicken due to the increased connective tissue and the presence of wing stiffness characteristic of the dystrophic chickens. In one normal chicken, the deflected ALD nerve sent a branch toward the PLD muscle. Unfortunately, it was not possible to test whether a mechanical response could be elicited from the self reinnervated PLD muscle by stimulating this small nerve branch.

In all experiments where the ALD muscle had been removed, it was noted that the metapatagial latissimus dorsi (MLD) muscle (which runs along the PLD muscle and inserts onto the skin overlying the wing musculature - Fig. 1) formed an indentation on the PLD muscle as illustrated in the sketch below. When compared to the contralateral side, the MLD muscle in the operated side appeared hypertrophied (see Discussion).

contralateral side operated side

Mechanical Analyses

Contraction characteristics: An in vivo analysis of the isometric contractions of the self reinnervated PLD muscles in response to nerve stimulation was performed on 9 normal and 10 dystrophic muscles. All

self reinnervated muscles of both genotypes responded to a single stimulus as well as to a train of stimuli (10-100 Hz) with the twitch and tetanus responses characteristic of a fast twitch PLD muscle. Figure 24 shows representative recordings of the contraction responses of self reinnervated PLD muscles of normal and dystrophic chickens. These recordings indicate that the mechanical response of the self reinnervated muscles are similar to those of the unoperated PLD (Fig. 17).

Time parameters of contraction: The contraction and relaxation times of the twitches and the tetanic (50, 70, 100 Hz) responses were measured as described in the Methods (Fig.7). The data obtained from the self reinnervated PLD muscles of both genotypes (Appendix R) is summarised in Table 11 and compared to the data obtained from the unoperated PLD muscles. No significant differences were observed between the two sets of observations (Student's t-test).

Twitch and tetanic tensions: The maximum twitch and tetanic responses obtained from the self reinnervated PLD muscles of both genotypes is presented in Table 12. For each chicken the age, body weight and muscle weight are given to allow direct correlation with the various responses obtained. The data show that the twitch tension developed by the self reinnervated PLD muscles of dystrophic chickens is lower than that of the self reinnervated muscles of normal chickens. The ratio of the maximum twitch to tetanic tension (Pt/Po) of the self reinnervated muscles is 0.17 ± 0.02 for the dystrophic muscle and 0.40 ± 0.02 for the normal, which agrees with the ratio obtained for the unoperated PLD muscles (Fig. 20). The unusually high muscle weight of one of the self reinnervated dystrophic PLD muscles (marked with an asterisk) is from a

Figure 24: Recording of indirectly evoked isometric twitch and tetanic (50 Hz) contractions from self reinnervated PLD muscles of normal and dystrophic chickens, over six months postoperatively. These recordings show that both normal and dystrophic self reinnervated PLD muscles have fast twitch characteristics, but the tension developed by the dystrophic muscle is lower than that of the normal. See Figure 17 for isometric contraction of unoperated PLD muscles. Note difference in calibration of vertical scale between normal and dystrophic recordings.

SELF REINNERVATED PLD MUSCLES
ISOMETRIC CONTRACTIONS

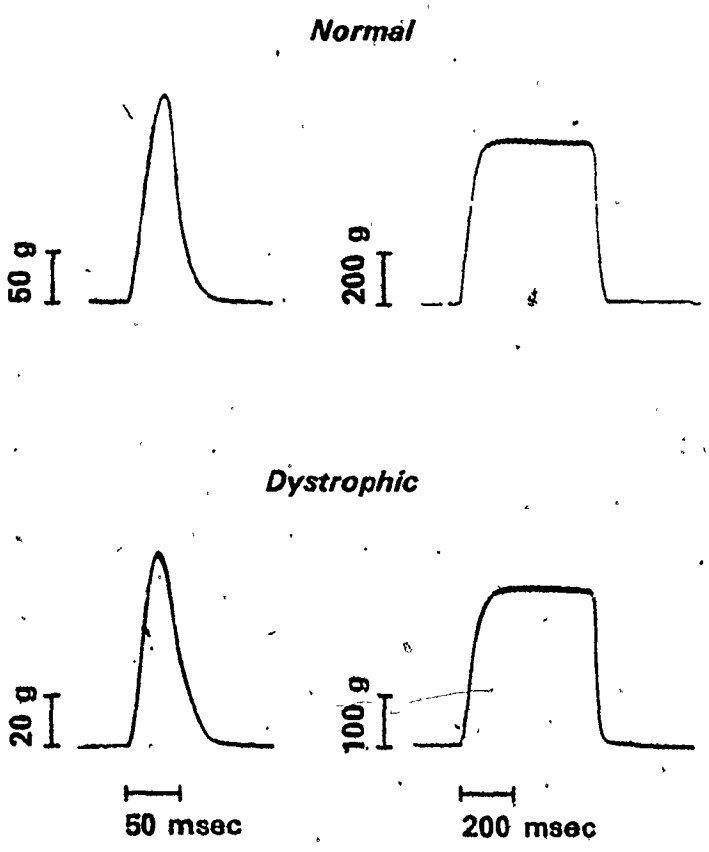


Table 11:

$T_{\frac{1}{2}}C$ = time to half contraction; Tc = contraction time; $T_{\frac{1}{2}}R$ = time to half relaxation; S-RI = self reinnervated; df = degree of freedom; N.S. = not significant.

The values of the unoperated PLD muscles of normal and dystrophic chickens are taken from Table 9.

* mean ratio is antilog of the difference in mean log, thus it represents the proportional change between these muscles.

** although the mean $\pm$ S.D. of the actual observation are tabled, the Student's t-test is calculated on log transformed data in order to stabilize the variance. Such a calculation represents a test of the proportional change between the values.

The df is not constant because a complete set of measurements was not always available from each muscle.

TABLE 11 COMPARISON OF THE CONTRACTION AND RELAXATION TIMES OF THE SELF REINNERVATED POSTERIOR LATISSIMUS DORSI TO THE UNOPERATED POSTERIOR LATISSIMUS DORSI MUSCLES

NORMAL CHICKENS

Mechanical Properties		PLD S-RI mean ± S.D.	PLD msec ± S.D.	PLD S-RI/PLD mean ratio *	Student's t** t	df	p
Twitch	T½C	16.8 ± 3.1	14.2 ± 2.5	1.19	2.71	46	N.S.
	TC	37.6 ± 6.0	33.3 ± 5.0	1.13	2.15	46	N.S.
	T½R	22.9 ± 6.1	22.5 ± 5.0	1.01	0.15	46	N.S.
Tetanus 50 Hz	T½C	38.5 ± 6.9	33.1 ± 7.1	1.17	1.90	41	N.S.
	T½R	45.9 ± 9.1	40.3 ±10.1	1.15	1.37	41	N.S.
Tetanus 70 Hz	T½C	38.5 ± 7.8	32.8 ± 7.5	1.18	1.85	39	N.S.
	T½R	48.9 ± 8.0	41.8 ± 9.2	1.18	1.96	39	N.S.
Tetanus 100 Hz	T½C	35.0 ± 6.7	29.8 ± 6.2	1.18	1.97	38	N.S.
	T½R	49.1 ± 6.7	42.4 ± 9.6	1.18	1.91	38	N.S.

DYSTROPHIC CHICKENS

Twitch	T½C	15.5 ± 2.7	13.4 ± 2.4	1.16	2.34	74	N.S.
	TC	32.1 ± 4.7	30.3 ± 4.1	1.06	1.20	74	N.S.
	T½R	24.2 ± 4.8	25.5 ± 5.7	0.95	0.65	74	N.S.
Tetanus 50 Hz	T½C	48.0 ± 8.7	42.1 ± 8.7	1.15	1.73	70	N.S.
	T½R	42.6 ±12.1	40.0 ±11.1	1.07	0.65	70	N.S.
Tetanus 70 Hz	T½C	42.9 ± 8.0	39.8 ± 8.7	1.09	1.06	69	N.S.
	T½R	40.4 ±13.5	42.0 ±13.4	0.96	0.36	69	N.S.
Tetanus 100 Hz	T½C	39.9 ± 7.2	47.9 ±10.2	1.07	0.78	69	N.S.
	T½R	43.1 ±13.5	43.3 ±14.2	0.99	0.01	69	N.S.

TABLE 12 TWITCH AND TETANIC TENSIONS OF THE SELF REINNERVATED POSTERIOR LATISSIMUS DORSI MUSCLES

I - Normal Chickens

Age (days)	Body wt (g)	Muscle wt (g)	P_t (g)	P_o (g)	P_t/P_o Ratio
110	1577	0.840	110	340	0.32
181	1960	1.158	261	--	--
272	1490	0.900	207	490	0.42
423	1360	0.782	209	482	0.43
441	2230	1.430	350	660	0.53
469	1604	0.866	179	541	0.33
469	1973	1.174	288	648	0.44
545	2520	1.176	336	911	0.37
mean					0.40
S.E.					0.03

II - Dystrophic Chickens

107	1637	0.650	--	--	--
154	1256	0.501	--	--	--
207	1380	0.610	87	341	0.26
251	1611	0.952	85	--	--
448	1036	3.100*	110	672	0.16
515	1627	0.914	70	432	0.16
520	1998	1.461	62	468	0.13
537	1400	0.982	86	492	0.17
671	1716	0.640	35	228	0.15
mean					0.17
S.E.					0.02

P_t = maximum twitch tension; P_o = maximum tetanic tension.

* unusually high muscle weight of a dystrophic rooster.

dystrophic rooster. This high muscle weight is occasionally seen in unoperated PLD muscles of dystrophic roosters.

Histochemical Analyses

The self reinnervated PLD muscles of both genotypes were analyzed with the same histochemical procedures as were the unoperated contralateral PLD and ALD muscles. The self reinnervated PLD muscles were composed of the same types of fibres, noted with the contralateral unoperated PLD muscles. The majority of fibres exhibited the histochemical properties of fast twitch fibres while a small population demonstrated characteristics of slow tonic ALD fibres. The latter, identified as "ALD type" fibres, were not detected in all muscles, and were not detected physiologically during isometric contractions. In only one self reinnervated PLD muscle of a normal chicken and in three dystrophic chickens, the proportion of "ALD type" fibres was 3-10%. This range exceeds the 2% levels detected occasionally in unoperated PLD muscles. In these 4 chickens, the initial operation included the removal of the ALD muscle, which might explain the higher proportion of "ALD type" fibres noted (see Discussion). The "ALD type" fibres were identical histochemically to ALD fibres, i.e. they exhibited acid and alkali stable myosin ATPase activity, "en grappe" innervation, weak Pase and strong SDH enzymic activity and peripheral location of nuclei. In cross section, the "ALD type" fibres were rounded in shape and enlarged in size compared to both the surrounding fast twitch fibres and the fibres of the contralateral ALD muscle.

As indicated, the majority of the fibres noted in the self

reinnervated PLD muscles demonstrated histochemical properties similar to the unoperated fast twitch PLD fibres. The self reinnervated fibres of both normal and dystrophic chickens showed only alkaline stable myosin ATPase activity. They exhibited focal "en plaque" endplate with strong AChE reaction characteristic of fast twitch fibres. AChE activity was also detected both at the extrajunctional membrane and intracellularly in the self reinnervated fast twitch fibres of the dystrophic chickens.

The two fibre types identified in the unoperated PLD muscles of normal genotype with the Pase and SDH reactions were also present in the self reinnervated PLD muscles of normal chickens. That is, most fibres demonstrated strong Pase and weak SDH activities and the rest (about 7%) showed intermediate Pase and strong SDH activities. Although the latter were usually scattered throughout the muscle cross section, in 3 of the self reinnervated PLD muscles of normal chickens, they tended to be clustered in groups. The fast twitch self reinnervated PLD fibres of dystrophic chickens demonstrated abnormal Pase and SDH enzymic activities and a mixed fibre type distribution characteristic of fast twitch fibres of dystrophic genotype. In 2 self reinnervated muscles, fibre type grouping was also detected. Such fibres demonstrated intermediate Pase and strong SDH activities.

The number and distribution of nuclei in the fast twitch self reinnervated fibres were also found to be similar to those of the unoperated PLD fibres. In the normal muscle, the nuclei were located both at the periphery and inside of the fibres. In the dystrophic muscle the number of interiorally located nuclei was higher than normal. In the grouped fibres which were characterized by intermediate Pase and strong

SDH activities, the nuclei were located only at the periphery of individual fibres in both normal and dystrophic muscles. Seven out of the 9 self reinnervated muscles of the normal chickens revealed a fibre structure in cross section similar to that of the unoperated PLD muscles. The size and shape of both fibres and fascicles were not altered by the surgical manipulation of self reinnervation. In the other 2 muscles (251 and 469 days postoperatively) in which the PLD was injured during the initial operation at hatching, small areas (2-3 fascicles) with an abnormal orientation of fibres were detected. In these areas both cross and longitudinal orientation of fibre were noted. All self reinnervated muscle of the dystrophic chickens showed the structural abnormalities which characterize the unoperated PLD muscles of dystrophic genotypes, i.e. variability in size, shape and compactness of both fibres and fascicles, splitting of fibres and lipid accumulation.

SECTION 2: CROSS-REINNERVATION OF PLD MUSCLES BY THE ALD NERVE

Number of Experiments

Cross reinnervation of the PLD muscle by the ALD nerve was performed on newly hatched normal and dystrophic chickens. The operation included the following surgical manipulations: 1. removal of the ALD muscle; 2. denervation of the PLD muscle; 3. implantation of the PLD nerve inside underlying muscles; and 4. loose attachment of the ALD nerve to the PLD tendon (Methods p42). The PLD muscles were either injured (normal, n = 12; dystrophic, n = 20) to facilitate reinnervation or left intact (normal, n = 8; dystrophic, n = 31). In total 20 normal and 51 dystrophic PLD muscles were cross reinnervated by the ALD nerve.

The cross reinnervated muscles were analyzed at various time intervals from 1 to 27 months postoperatively. Since some chickens died from oversensitivity to the anesthesia, their muscles could not be tested for contraction characteristics; however, all cross reinnervated PLD muscles were analyzed histochemically.

Since many properties of the cross reinnervated muscles of normal and dystrophic chicken were similar, the following results refer to both genotypes except where indicated.

Gross Examination

The PLD muscles were exposed for mechanical testing and were examined for the success of cross reinnervation. As noted with the self reinnervated PLD muscles, more connective tissue was found around PLD muscles which had been injured at hatching. This was true in both normal and dystrophic chickens, but was more apparent in the dystrophic chickens. In both normal and dystrophic cross reinnervated PLD muscles, the foreign nerve was located underneath the tendon and entered the muscle at the site usually seen in the unoperated PLD muscles. In all operated dystrophic chickens due to the presence of excessive amounts of connective tissue and the progression of wing stiffness the PLD nerve which had been deflected into underlying muscles could not be located. In contrast, the deflected PLD nerve was located in 6 of the 20 normal chickens and when stimulated, elicited a response from the underlying muscles. In one preparation only the PLD muscle also contracted.

In the cross reinnervation experiments in which the ALD had been removed at hatching, the wing drooped and the metapatagial latissimus

dorsi (MLD) muscle caused the formation of a groove along the cross reinnervated PLD muscle in both normal and dystrophic chickens, as was also seen in the self reinnervated experiments. In one preparation, a piece of the MLD was cut from the operated side and its weight compared to the weight of the same length of MLD muscle from the contralateral side. The MLD from the operated side weighed 0.160 g and the MLD from the contralateral side weighed 0.061 g. The ratio of the weights of these muscles is 2.6:1 which indicates that the MLD of the operated side increased its weight as a result of the operative manipulation performed. Although it was weighed in only one preparation, the MLD muscle was often observed to be larger on the experimental side (see Discussion).

Mechanical Analysis

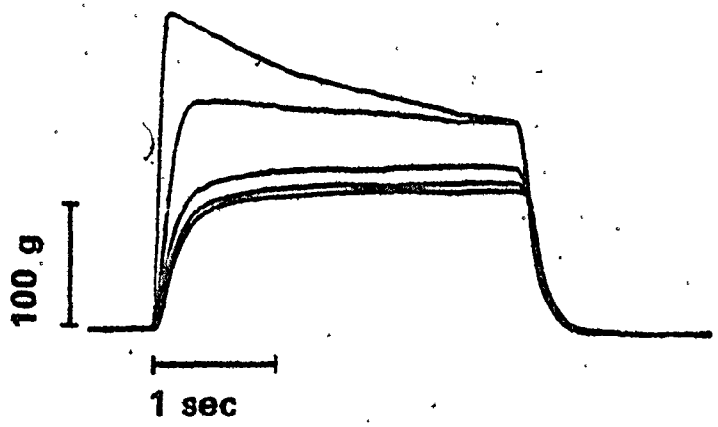
Contraction characteristics: The cross reinnervated PLD muscles were tested in vivo for their isometric contraction in response to nerve stimulation. The muscles were tested for their response to a single stimulus and to a train of stimuli 10-200 Hz. The contraction responses obtained from the cross reinnervated muscles indicated that the muscles could be divided into:

1. muscles which contracted like self reinnervated muscles;
2. muscles which exhibited no response to a single stimulus but a slow tonic response to a train of stimuli from 10-100 Hz. However, when the stimulus frequency was increased beyond 100 Hz, the contraction speed increased and the tension became greater.

Figure 25 shows an example of such a response evoked by a cross reinnervated muscle of dystrophic genotype 538 days postoperatively. However,

Figure 25: Recording of indirectly evoked isometric tetanic contractions from a cross reinnervated PLD muscle of dystrophic genotype 539 days post-operatively. The tetanic contractions were evoked in response to stimulation at 50, 70, 100, 200 and 300 Hz. When the frequency of stimulation was increased beyond 100 Hz the muscle response became faster and the tension higher. At 300 Hz the tension decayed during 3 seconds of stimulation, probably due to fatigue of the fast twitch fibres (Hoekman, 1977).

CROSS REINNERVATED PLD MUSCLE ISOMETRIC CONTRACTIONS



one cross reinnervated PLD muscle of a dystrophic chicken showed only slow tonic contraction at all frequencies of stimulation. This muscle was examined 504 days postoperatively and, as will be discussed later (p185), was comprised exclusively of converted fibres.

The number of cross reinnervated muscles subjected to mechanical analyses and the type of response evoked by indirect stimulation of these muscles are shown in Table 13. From these data, it is apparent that when the cross reinnervated PLD muscles of both genotypes were not injured during the initial operation (at hatching) more muscles responded with a slow tonic contraction to indirect stimulation. Figure 26 compares the slow tonic responses of cross reinnervated PLD muscles of normal and dystrophic genotypes to that of the ALD muscle stimulated indirectly at 50 Hz. Also shown are the responses of a cross reinnervated muscle at 10-70 Hz.

Time parameters of contraction: The contraction and relaxation times of the slow tonic responses evoked in the cross reinnervated muscles of both genotypes were measured as described in Methods (Fig. 6). The values of the time to half contraction ($T_{\frac{1}{2}}C$) and half relaxation ($T_{\frac{1}{2}}R$) of the slow tonic responses at 50 and 70 Hz are presented in Appendix S. Tables 14 and 15 show the analyses of these parameters for the normal and dystrophic muscles respectively. The time parameters of the slow tonic responses evoked in the cross reinnervated PLD muscles were compared statistically with the time parameters of the responses obtained from unoperated PLD (Tables 14 and 15, I) and unoperated ALD (Tables 14 and 15, II) muscles. It is evident from these Tables that the time to half contraction and half relaxation of the slow tonic responses evoked in

TABLE 13 CROSS REINNERVATION

TYPE OF RESPONSE TO INDIRECT STIMULATION

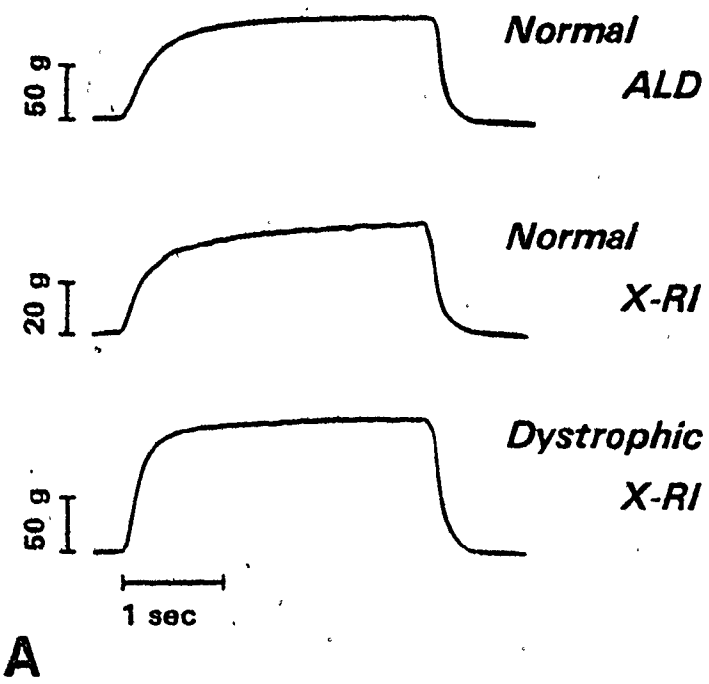
	Mechanically analyzed	Response to Indirect Stimuli	
	Number	Fast Twitch only	Slow Tonic and Fast Twitch
Normal:			
PLD injured	8	6 (75%)	2 (25%)
PLD intact	6	1 (17%)	5 (83%)
Total	14	7 (50%)	7 (50%)
Dystrophic:			
PLD injured	15	11 (73%)	4 (27%)
PLD intact	24	7 (29%)	17 (71%)
Total	39	18 (46%)	21 (54%)

Figure 26:

A Recordings of indirectly evoked isometric tetanic contraction (70 Hz) from ALD and cross reinnervated PLD muscles of normal and dystrophic chickens. Postoperative time for normal is 193 days and for dystrophic, 509 days. ALD muscle is from the same normal chickens.

B Recordings of indirectly evoked isometric tetanic contraction at 10 to 70 Hz from cross reinnervated PLD muscle of a dystrophic chicken 538 days postoperatively. These recordings emphasize the similarity between the time parameters of contraction of cross reinnervated PLD muscles and unoperated ALD muscles (see also Fig. 15).

CROSS REINNERVATED PLD MUSCLES
ISOMETRIC CONTRACTIONS



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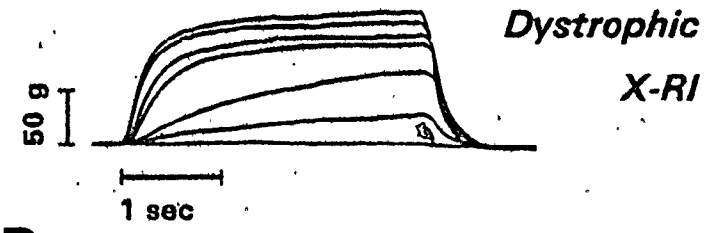


B

Table 14:

$T_{\frac{1}{2}}C$ = time to half contraction; $T_{\frac{1}{2}}R$ = time to half relaxation;

X-RI = cross reinnervated

The values of the unoperated ALD and PLD msucles of normal chickens are taken from Tables 6 and 9 respectively.

* mean ratio is antilog of the difference in mean log, thus it represents the proportional change between these muscles

** although the mean $\pm$ S.D. of the actual observation are tabled, the Student's t-test is calculated on log transformed data in order to stabilize the variance. Such a calculation represents a test of the proportional change between the values.

The df is not constant because a complete set of measurements was not always available from each muscle.

TABLE 14 NORMAL CHICKENS
ANALYSIS OF THE CONTRACTION AND RELAXATION TIMES OF THE CROSS REINNERVATED POSTERIOR LATISSIMUS DORSI MUSCLES

I - Comparison with Unoperated Posterior Latissimus Dorsi Muscles

Mechanical Properties		PLD X-RI mean ± S.D.	PLD mean ± S.D.	PLD X-RI/$_{PLD}$ mean ratio*	Student's t** t	df	P
Tetanus 50 HZ	T½C	185.7 ± 58.7	33.1 ± 7.1	5.49	17.26	40	<0.001
	T½R	145.6 ± 79.1	40.3 ± 10.1	3.31	8.99	40	<0.001
Tetanus 70 Hz	T½C	135.8 ± 33.9	32.8 ± 7.5	4.13	14.06	37	<0.001
	T½R	91.8 ± 35.6	41.8 ± 9.2	2.11	6.54	37	<0.001
Tetanus 100 Hz	T½C	118.0 ± 60.8	29.8 ± 6.2	3.61	10.18	36	<0.001
	T½R	74.8 ± 13.9	42.4 ± 9.6	1.78	5.71	36	<0.001

II - Comparison with Unoperated Anterior Latissimus Dorsi Muscles

Mechanical Properties		PLD X-RI msec ± S.D.	ALD msec ± S.D.	PLD X-RI/$_{ALD}$ mean ratio*	Student's t** t	df	P
Tetanus 50 Hz	T½C	185.7 ± 58.7	365.4 ± 116.3	0.51	4.58	25	<0.001
	T½R	145.6 ± 79.1	189.6 ± 55.9	0.71	2.10	25	N.S.
Tetanus 70 Hz	T½C	135.8 ± 33.9	294.9 ± 93.7	0.47	4.81	23	<0.001
	T½R	91.8 ± 35.6	181.8 ± 50.1	0.49	4.73	23	<0.001
Tetanus 100 Hz	T½C	118.0 ± 60.8	238.4 ± 72.4	0.46	4.15	23	<0.001
	T½R	74.8 ± 13.9	187.7 ± 57.1	0.41	6.48	23	<0.001

TABLE 15 DYSTROPHIC CHICKENS
ANALYSIS OF THE CONTRACTION AND RELAXATION TIMES OF THE CROSS REINNERVATED POSTERIOR LATISSIMUS DORSI MUSCLES

I - Comparison with Unoperated Posterior Latissimus Dorsi Muscles

Mechanical Properties		PLD X-RI msec ± S.D.	PLD msec ± S.D.	PLD X-RI/PLD mean ratio *	Student's t** t	df	P
Tetanus 50 Hz	T½C	225.7 ± 62.0	42.1 ± 8.7	5.28	25.51	82	<0.001
	T½R	152.3 ± 45.9	40.0 ± 11.1	3.78	18.55	82	<0.001
Tetanus 70 Hz	T½C	185.6 ± 56.9	39.8 ± 8.7	4.53	21.95	80	<0.001
	T½R	134.5 ± 38.6	42.0 ± 13.4	3.20	14.39	80	<0.001
Tetanus 100 Hz	T½C	161.0 ± 52.1	37.9 ± 10.2	4.14	20.53	81	<0.001
	T½R	114.0 ± 44.5	43.3 ± 14.2	2.54	10.48	81	<0.001

II - Comparison with Unoperated Anterior Latissimus Dorsi Muscles

Mechanical Properties		PLD X-RI msec ± S.D.	ALD msec ± S.D.	PLD X-RI/ALD mean ratio*	Student's t** t	df	P
Tetanus 50 Hz	T½C	225.7 ± 62.0	363.9 ± 113.8	0.63	4.97	46	<0.001
	T½R	152.2 ± 45.9	200.8 ± 56.6	0.75	3.32	46	<0.002
Tetanus 70 Hz	T½C	185.6 ± 56.9	302.5 ± 86.2	0.61	5.26	44	<0.001
	T½R	134.5 ± 38.6	204.5 ± 59.1	0.65	4.69	44	<0.001
Tetanus 100 Hz	T½C	161.0 ± 52.1	246.5 ± 57.1	0.63	5.18	45	<0.001
	T½R	114.0 ± 44.5	201.3 ± 60.7	0.54	5.71	45	<0.001

See Glossary for Table 14.

the cross reinnervated muscles are 2 to 5 times longer ($p < 0.001$) than the time parameters of the responses evoked from the unoperated PLD muscles. These Tables also indicate that these time parameters are significantly different when compared to those of unoperated ALD muscles. However, although they are significantly different statistically, the mean ratio and the t-values indicate that the responses of the cross reinnervated PLD muscles are similar to the response of the ALD muscle. When the contraction and relaxation times of the cross reinnervated PLD muscles of normal and dystrophic chickens were compared (Table 16) no significant difference was observed. This indicates that fibres within the cross reinnervated PLD muscles of both genotypes acquired slow tonic contraction characteristics that were similar and resembled those of unoperated ALD muscles.

Tetanic tension: Table 17 gives the tetanic tensions measured from the slow tonic contraction of the cross reinnervated PLD muscles in response to nerve stimulation at 50, 70 and 100 Hz. Also tabled for each chicken analyzed is the age, body weight and muscle weight. No correlation could be made between these parameters and the tension obtained.

Histochemical Analysis

Myosin ATPase: The cross reinnervated PLD muscles were tested histochemically for myosin ATPase activity following both acid and alkaline preincubation. In the cross reinnervated PLD muscles which displayed both slow tonic and fast twitch contraction to nerve stimulation, two populations of muscle fibres were noted:

1. Fibres which show myosin ATPase activity following acid and

TABLE 16 ANALYSIS OF THE CONTRACTION AND RELAXATION TIMES OF THE CROSS REINNERVATED POSTERIOR LATISSIMUS DORSI BETWEEN NORMAL AND DYSTROPHIC CHICKENS

Mechanical Properties		N/D Mean Ratio*	Student's t** t	df	P
Tetanus 50 Hz	$T_{\frac{1}{2}}C$	0.82	1.42	26	N.S.
	$T_{\frac{1}{2}}R$	0.88	0.76	26	N.S.
Tetanus 70 Hz	$T_{\frac{1}{2}}C$	0.75	1.82	24	N.S.
	$T_{\frac{1}{2}}R$	0.67	2.50	24	N.S.
Tetanus 100 Hz	$T_{\frac{1}{2}}C$	0.69	1.99	25	N.S.
	$T_{\frac{1}{2}}R$	0.70	1.84	25	N.S.

$T_{\frac{1}{2}}C$ = time to half contraction; $T_{\frac{1}{2}}R$ = time to half relaxation; N = normal; D = dystrophic. df = degree of freedom; N.S. = not significant.

The mean $\pm$ SD of the observation are available in Tables 14 and 15. See Appendix S for the individual values.

* mean ratio is antilog of the difference in mean log, thus it represents the proportional change between these muscles.

** although the mean $\pm$ SD of the actual observation are tabled, the Student's t-test is calculated on log transformed data in order to stabilize the variance. Such a calculation represents a test of the proportional change between the values.

The df is not constant because a complete set of measurements was not always available from each muscle.

TABLE 17 TETANIC TENSION OF THE CROSS REINNERVATED POSTERIOR LATISSIMUS DORSI MUSCLES

I - Normal Chickens

Age (days)	Body wt (g)	Muscle wt (g)	Tetanic Tension (g) 50 Hz	70 Hz	100 Hz
28	298	0.112	14	28	41
28	294	0.144	10	14	15
92	1100	0.499	56	98	116
114	2323	1.772	--	--	--
193	1386	0.596	28	64	118
511	1971	1.211	21	35	--
522	2884	1.551	65	88	177

II - Dystrophic Chickens

92	1264	0.672	7	16	36
92	1160	0.534	16	30	56
151	1705	1.238	24	70	84
167	1589	1.092	121	146	200
169	1390	0.784	50	--	--
232	1182	0.629	24	29	47
247	1388	0.736	70	85	150
257	2220	1.588	155	240	310
362	1209	0.458	92	103	118
376	1205	0.355	112	112	118
504	1297	0.264	64	71	75
509	2520	1.062	112	124	159
511	1420	1.166	138	144	180
538	1646	0.600	114	132	156
572	2700	1.882	132	222	288
581	2460	0.691	67	78	90
641	2690	2.294	72	74	108
672	2370	2.780	43	55	72
686	2029	0.747	108	120	132
800	2256	1.446	66	83	115
800	2404	1.705	84	90	120

alkaline preincubation, characteristic of slow tonic fibres; and

2. Fibres which show myosin ATPase activity following alkaline preincubation only, characteristic of fast twitch fibres.

The slow tonic fibres present in the cross reinnervated PLD muscles (1) will be referred to as converted fibres, while the rest of the fibres (2) will be referred to as unconverted fibres. The photomicrographs in Figures 27 and 28 show the myosin ATPase activity of cross reinnervated PLD muscles following acid and alkaline preincubation compared to the activity of unoperated ALD and PLD muscles. The converted fibres of both genotypes and the ALD muscle fibres show activity following both acid and alkaline preincubation. The unconverted fibres and the unoperated PLD fibres show activity following alkaline preincubation only. Unlike the ALD muscle which reveals two fibre types following myosin ATPase activity (Fig. 22), the converted fibres of the cross reinnervated PLD muscles are of one type only and closely resemble the predominant ALD fibre type. Fibres which revealed acid and alkaline stable myosin ATPase activity were also detected in cross reinnervated PLD muscles which were not tested for contraction responses and in cross reinnervated PLD muscles which exhibited only fast twitch contraction in response to nerve stimulation.

Acetylcholinesterase: Frozen cross sections of the cross reinnervated PLD muscles revealed that the converted fibres have the finely structured deposits of AChE reaction products characteristic of "en grappe" type of innervation (Fig. 29A). The converted fibres were also shown to be multiply innervated in teased fibre preparations (Fig. 29B). However, the endplates were not as regularly distributed as noted with the multiply

Figure 27: Photomicrographs of frozen cross sections of muscles demonstrating myosin ATPase reaction after acid preincubation (pH = 4.35).

A In a dual preparation of the ALD (top) and PLD (bottom) muscles from an 84-day old chicken, no reaction is noted in PLD muscles, while all fibres of ALD muscle show enzymic activities (see also Figure 22). Due to the contrast between the dark staining ALD muscle and the non-reactive PLD muscle, the area of the PLD muscle appears blank (x100).

B and C. Section of a cross reinnervated PLD muscle of normal chicken 92 days postoperatively. Only converted fibres show acid stable myosin ATPase activity. The nonreactive PLD muscle shown in A, B and C illustrate the large percentage of converted fibres that have acquired the acid stable reaction characteristics of the ALD fibres. (B x100; C x25). See Figure 28 for myosin ATPase activity after alkaline preincubation.

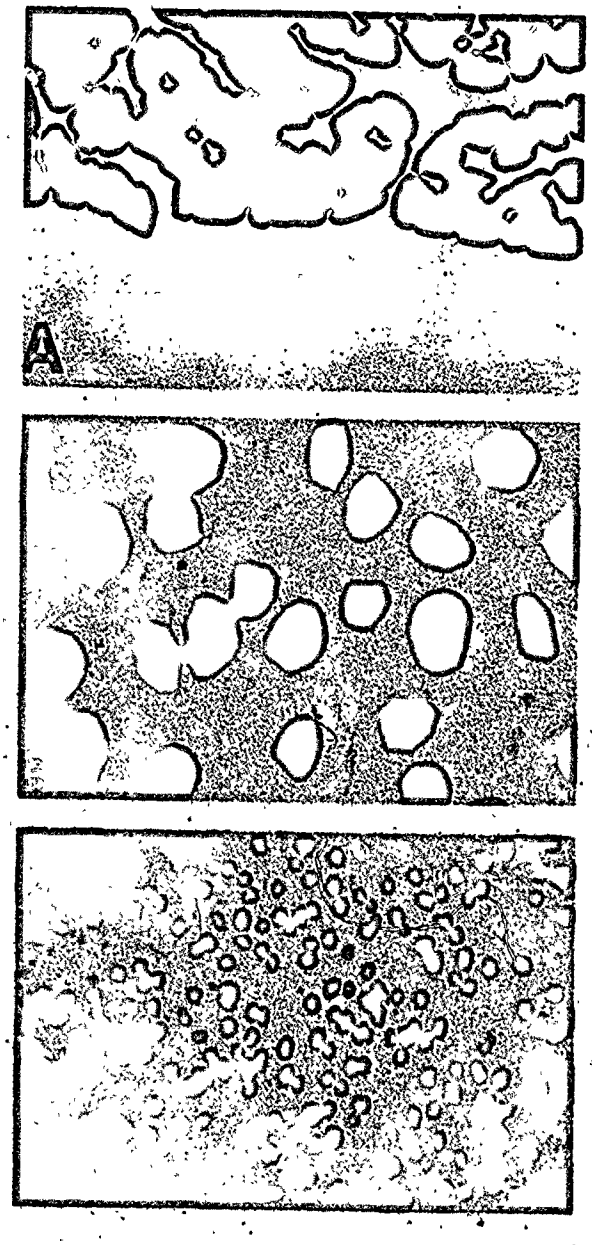

A

Figure 28: Photomicrographs of frozen cross section of muscles demonstrating myosin ATPase activity after alkaline (pH = 10.0) preincubation (A and B) and acid (pH = 4.35) preincubation (C).

A Both the ALD (top) and the PLD (bottom) muscles exhibit myosin ATPase activity in all fibres (x100). Compare with Figure 27A.

B Section from the same cross reinnervated PLD muscle shown in Figure 27B, showing that both the converted fibres (lighter and larger) and the unconverted fibres (darker and smaller) exhibit activity after alkaline preincubation. These sections indicate that the converted fibres (like ALD fibres) show myosin ATPase activity following both acid and alkaline preincubation (x100).

C Low power micrograph (x25) of a section from a cross reinnervated PLD muscle of a dystrophic chicken 376 days postoperatively. This section and the section of Figure 27C indicate that the converted fibres of both normal (27C) and dystrophic (28C) genotype respond similarly.

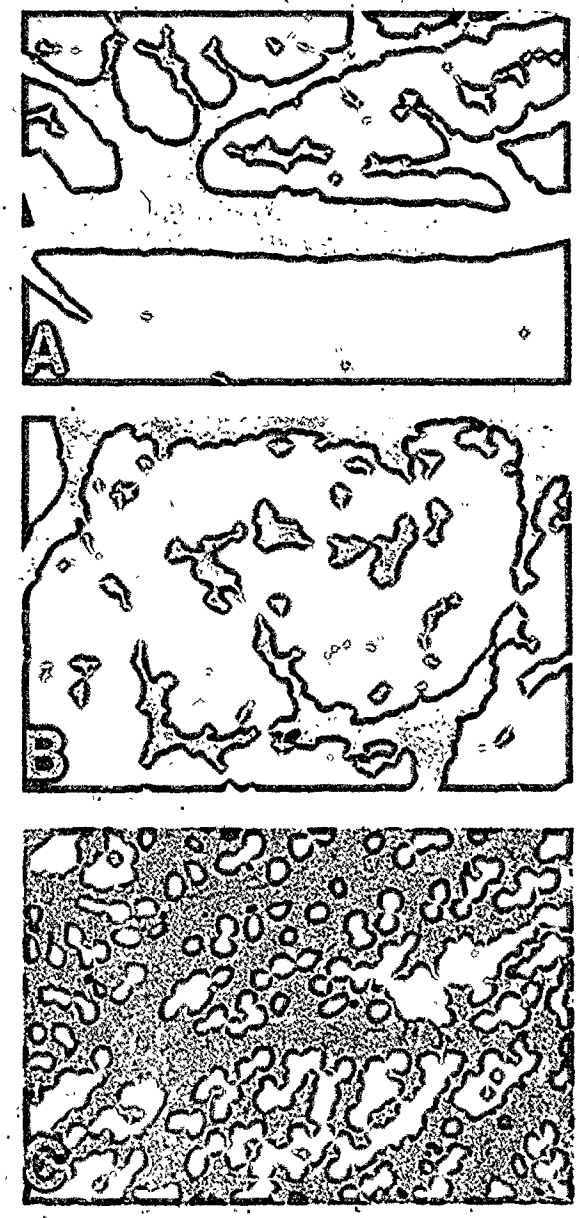
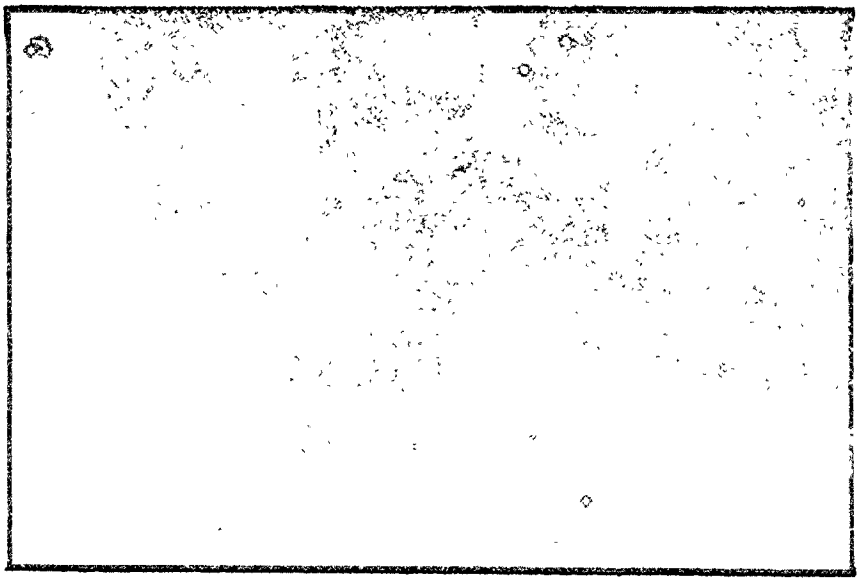

A
B
C

Figure 29:

A Photomicrograph of a frozen cross section of a cross reinnervated PLD muscle of a dystrophic chicken, 539 days postoperatively demonstrating AChE reaction. (Its isometric contraction is shown in Fig. 25). The arrows indicate "en grappe" type endplates in converted fibres.

B AChE reaction demonstrates the endplate distribution on teased fibres from ALD (top), and PLD (middle) and cross reinnervated PLD (bottom) muscles.



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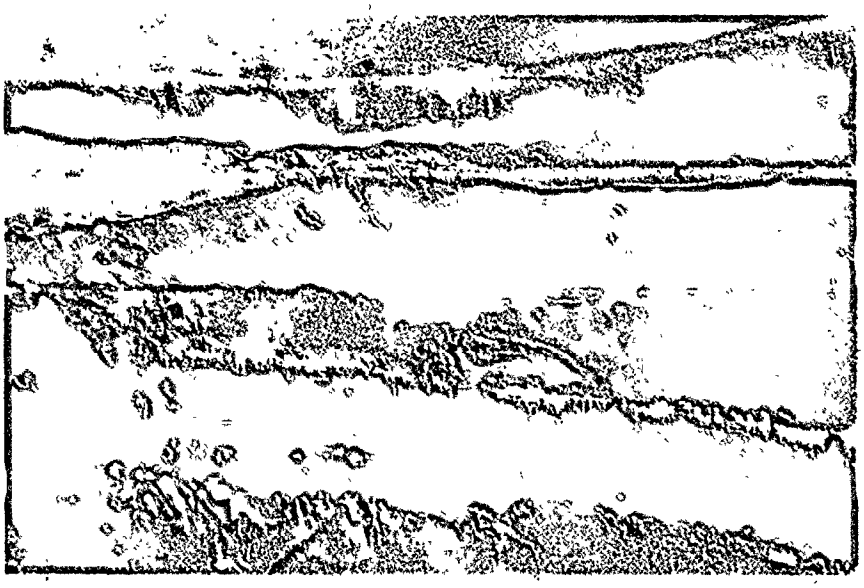


B

innervated ALD fibres.

The innervation of the unconverted fibres could not always be clearly defined in muscle cross sections. With the AChE reaction some fibres showed the endplates structure of "en plaque" innervation while others showed variation in shape and in the degree of AChE activity which could not be clearly defined as being "en plaque" or "en grappe".

The unconverted fibres of dystrophic genotype also showed AChE activity within the cytoplasm and at the extrajunctional membrane.

Phosphorylase and succinic dehydrogenase: The converted fibres in all the cross reinnervated PLD muscles showed weak Pase and strong SDH enzymic activity. Figures 30 and 31 show that the converted fibres of both genotypes demonstrate the same Pase (Fig. 30) and SDH (Fig. 31) enzymic activities as do ALD muscle fibres. However, unlike the ALD muscle which reveals two fibre types following Pase and SDH activities (Fig. 21), the converted fibres of the cross reinnervated PLD muscle of both genotypes show only one type. As noted with the myosin ATPase reaction, the converted fibres closely resemble the larger ALD fibres with the weaker myosin ATPase and Pase reactions.

The unconverted fibres of the cross reinnervated PLD muscles of normal genotype exhibited mixed activity following Pase and SDH reactions. The majority of the fibres gave strong Pase and weak SDH responses; the remaining fibres exhibited intermediate Pase and either weak or strong SDH enzymic activities. As with self reinnervated PLD muscles, fibre type grouping was also noted (Fig. 32A).

The unconverted fibres of the cross reinnervated PLD muscles of dystrophic genotype exhibited mixed Pase and SDH activities with many

Figure 30: Photomicrograph of frozen cross sections of cross reinnervated PLD muscles of a a normal chicken 92 days postoperatively (A and C) and a dystrophic chicken 376 days postoperatively (B) demonstrating Pase reaction.

A The cross reinnervated PLD muscle of normal genotype exhibits a loss of homogeneity (compare to Fig. 21) due to the presence of the converted fibres which show weak Pase reaction (x40).

B In the cross reinnervated PLD muscle of dystrophic genotype, the converted fibres also exhibit weak Pase reaction similar to that noted in the normal muscle (x40).

C A higher magnification (x100) of the preparation shown in A emphasizes the similarity in Pase reaction between the converted fibres and the predominant ALD fibre type shown in Figure 21C.

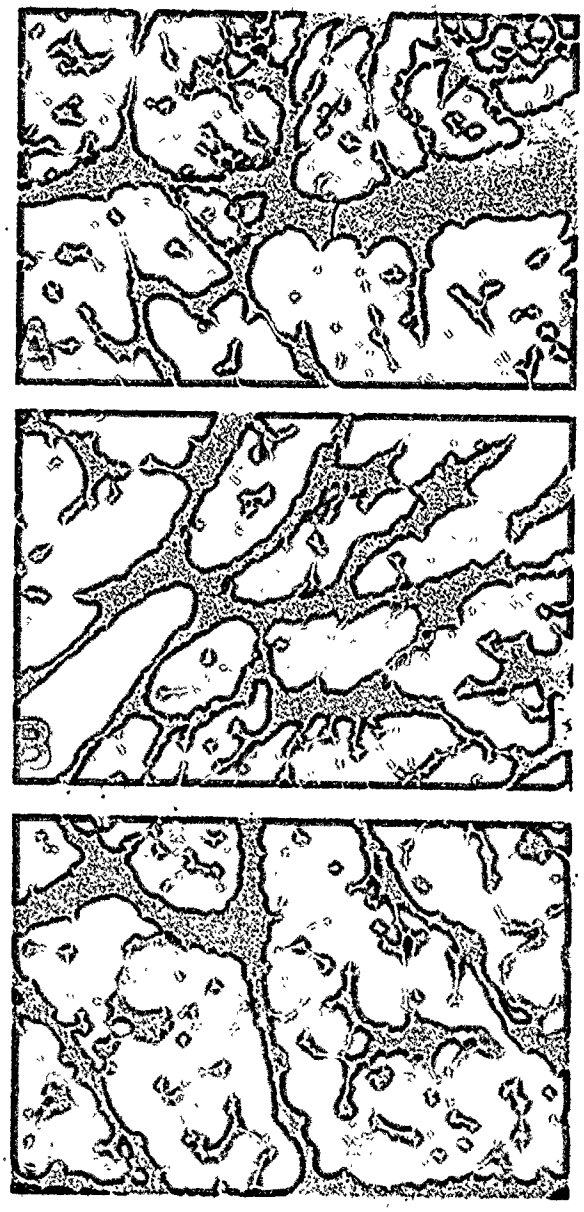


Figure 31: Photomicrographs of frozen cross sections of ALD (A) and cross reinnervated PLD (B) muscles of normal chickens 92 days post-operatively, and of cross reinnervated PLD muscle of dystrophic chicken 376 days postoperatively (C), demonstrating SDH reaction (x100). The converted fibres (rounded, dark reacting fibres) in the cross reinnervated PLD muscles of both normal and dystrophic chickens demonstrate strong SDH reaction similar to that of the ALD fibres.

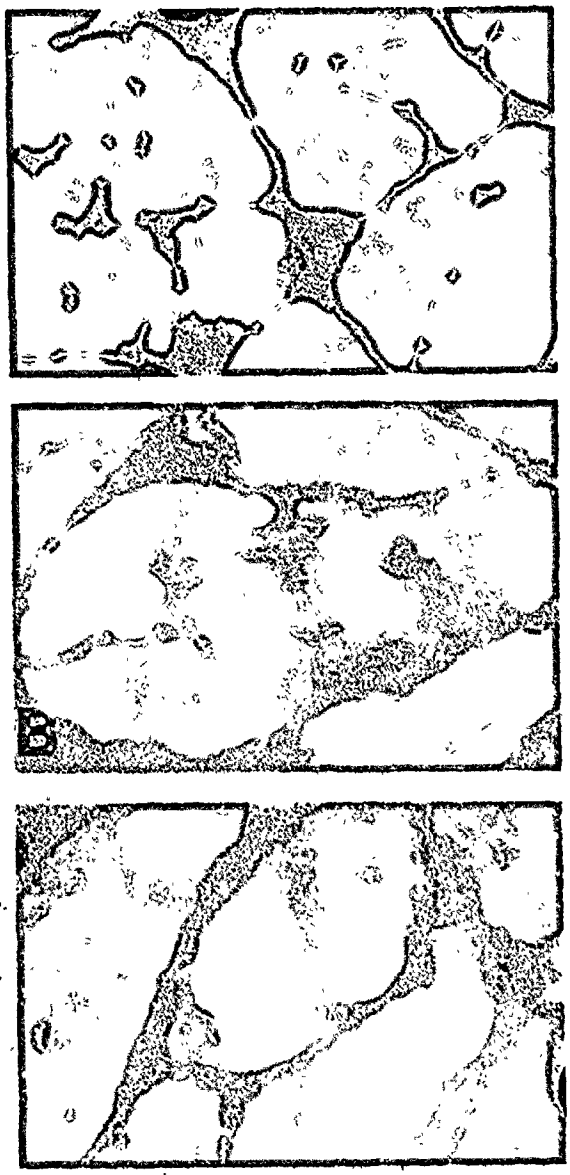

B

Figure 32: Photomicrographs of frozen cross sections of cross reinnervated PLD muscles of normal (A, right) and of dystrophic chickens (B), demonstrating SDH reaction (x10). Following reinnervation, both normal and dystrophic PLD muscles show fibre type grouping. The striking groups showing strong SDH reaction also show weak Pase reaction. Compare to the unoperated contralateral normal PLD muscle (A, left) which shows no fibre type grouping. Postoperative times are 147 days for normal and 107 days for dystrophic chickens.

A

B

abnormalities associated with the disease (e.g. uneven enzymic reaction within individual fibres, clumping of mitochondria, etc.). As noted with the self reinnervated PLD muscles, clusters of homogeneously reacting fibres which show intermediate Pase and strong SDH enzymic activities were also detected among these unconverted fibres (Fig. 32B). Furthermore, both normal and dystrophic cross reinnervated PLD muscles showed some exceptionally small unconverted fibres, which were either grouped separately or surround the converted fibres. In some dystrophic muscles these clusters of fibres were especially obvious since they exhibited homogeneous enzymic activities.

Fibre type grouping was detected at various ages postoperatively in cross reinnervated PLD muscles which displayed both slow tonic and fast twitch responses or a fast twitch response only. Of the 20 cross reinnervated PLD muscles of normal genotype, and the 51 muscles of dystrophic genotype, 7 muscles of normal and 11 muscles of dystrophic genotypes showed fibre type groupings.

In 4 cross reinnervated PLD muscles of dystrophic genotype, older than 12 months postoperatively, which responded as slow tonic muscles and histochemically demonstrated a large number of converted fibres, unconverted fibres were rare. Instead, areas of lipid were present between converted fibres.

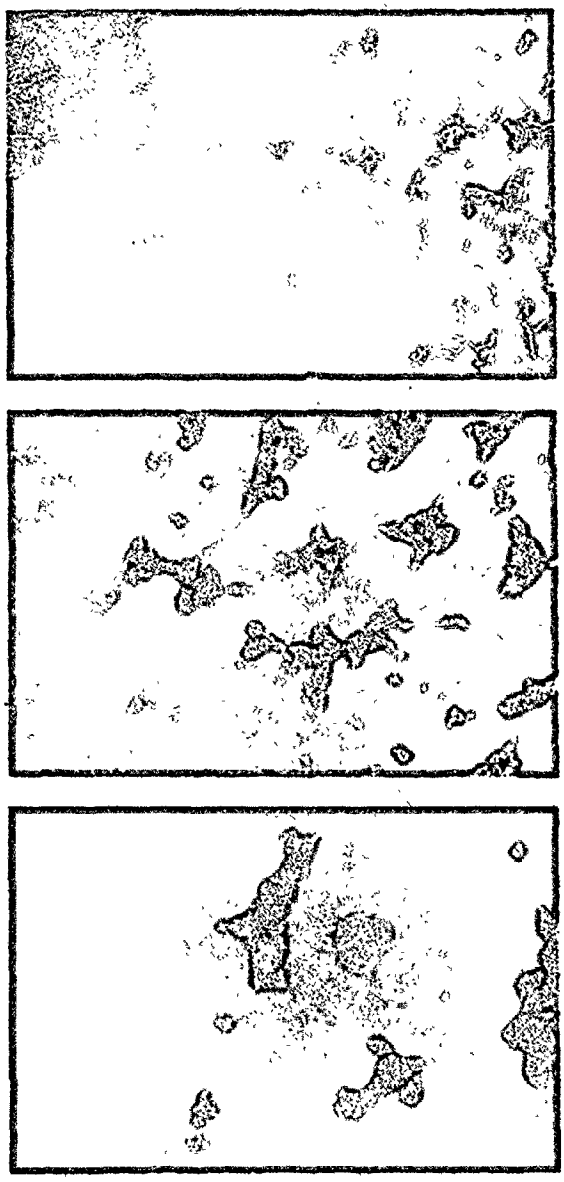
Position of nuclei: The converted fibres of all cross reinnervated muscles exhibited nuclei mainly at the periphery; a characteristic of slow tonic ALD fibres (Fig. 33). Some individual fibres also showed internal nuclei. This is observed occasionally with ALD muscle fibres and with "ALD type" fibres in the self reinnervated PLD muscles. The

Figure 33: Photomicrographs of frozen cross sections of muscles demonstrating the distribution of myofibre nuclei.

A In ALD muscle all nuclei are located at the periphery of the fibres (RNA-DNA x100).

B In cross reinnervated PLD muscle of dystrophic chickens 376 days postoperatively, all converted fibres (large and rounded) have peripheral nuclei (RNA-DNA x40).

C In unoperated PLD muscle of dystrophic genotype the number of internally located nuclei are increased compared to the unoperated PLD muscle of normal chickens shown in C, insert (Oil red O x100).



internal location of nuclei was associated with unusually large fibres or fibres which had undergone structural changes (e.g. splitting).

The unconverted fibres in the cross reinnervated PLD muscles of normal genotypes showed the usual number and location of nuclei as seen in unoperated PLD fibres. The nuclei were scattered, located at the periphery or internally. The groupings of fibres which exhibited intermediate Pase and strong SDH showed mainly peripheral nuclei.

Most of the unconverted fibres of cross reinnervated PLD muscles of dystrophic genotype exhibited high numbers and scattered localization of nuclei, characteristic of unoperated PLD muscles of dystrophic genotype. The group of fibres which exhibited intermediate Pase and strong SDH activities showed mainly peripheral nuclei.

Structural Analysis

When the cross reinnervated PLD muscles of both genotypes were examined histochemically one of the features noted was the overall change in the appearance of the muscles in cross section. Unlike the elongated fascicles characterizing the unoperated PLD, the fascicles of the cross reinnervated PLD muscles were rounded with increased extracellular spacing between both the fascicles and fibres. The converted fibres were, in general, larger than the fibres surrounding them and the unconverted fibres sometimes showed grouping of fibre types.

Figures 34 and 35 show tracings of fibres in a cross section of cross reinnervated muscles of normal and dystrophic genotypes; post-operative times were 3 months and 12 months respectively. Shading identifies converted fibres; all other fibres are identified as

Figure 34: Tracing of projected micrographic cross section from a representative area of cross reinnervated PLD muscle of a normal chicken 3 months postoperatively. This drawing emphasizes the difference in size between the converted (shaded fibres) and the unconverted fibres. Although the converted fibres represent only 8% of the total number of fibres present in this section, they cover 42% of the total muscle-fibre cross sectional area.

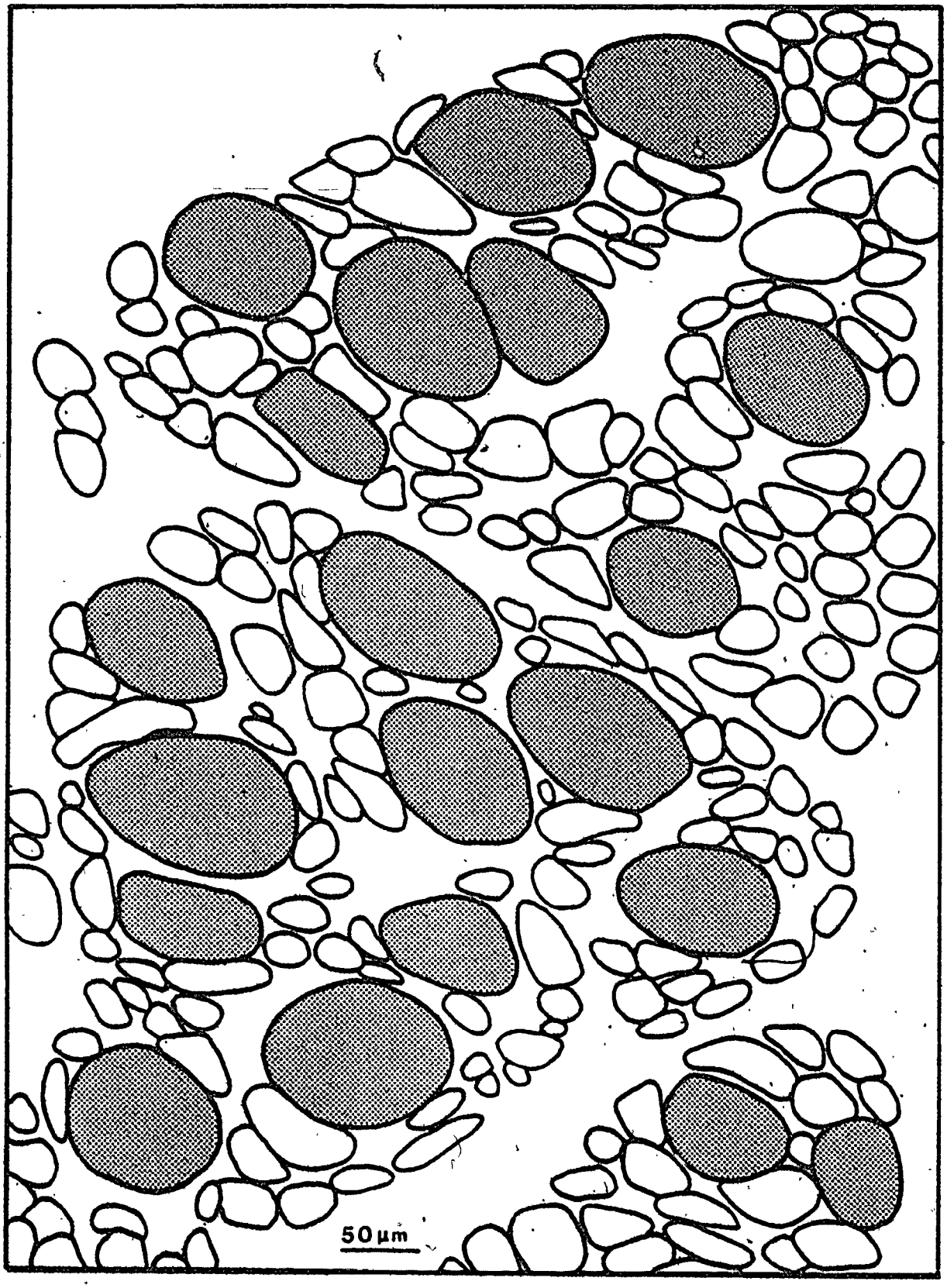
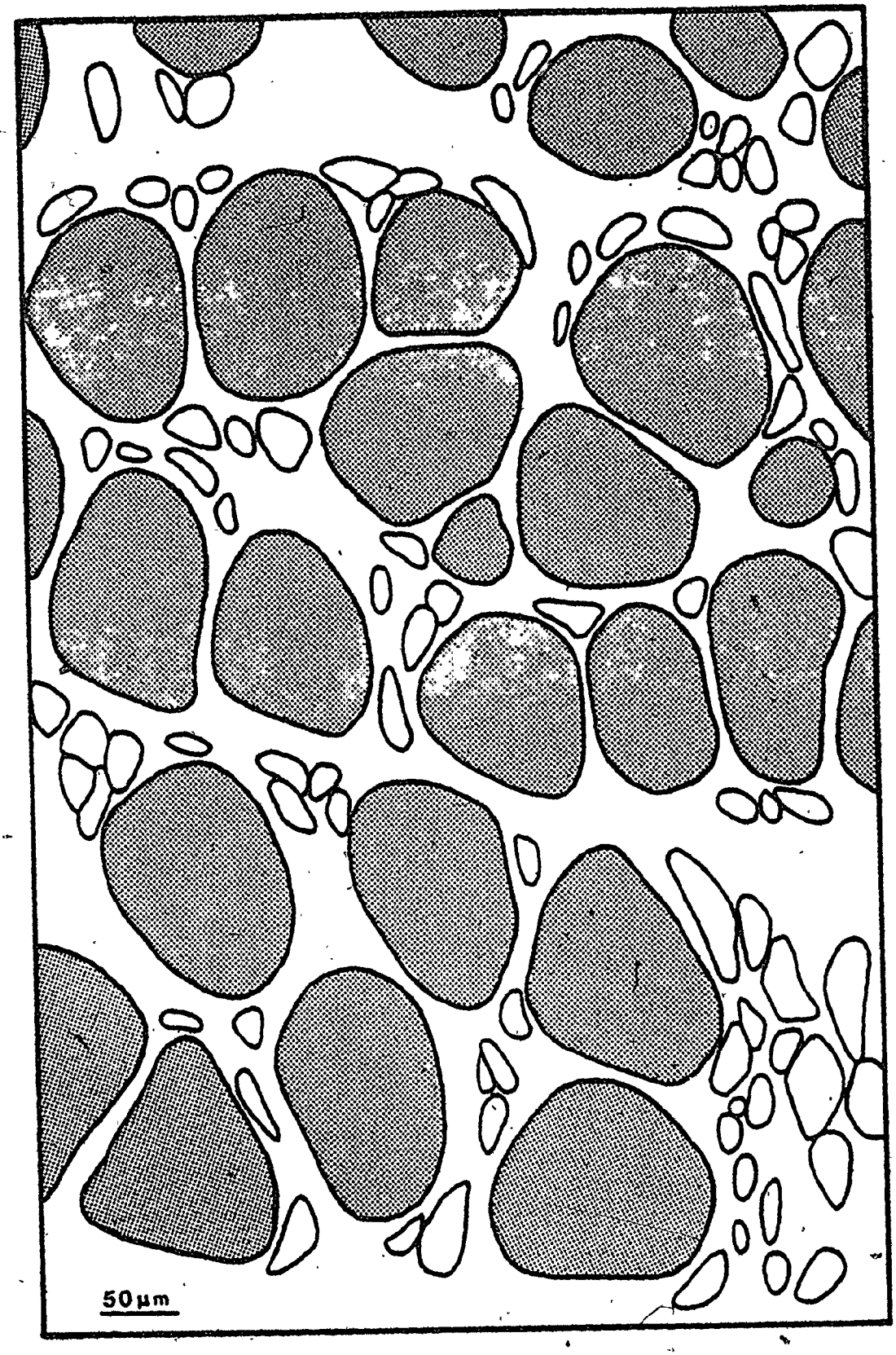

50µm

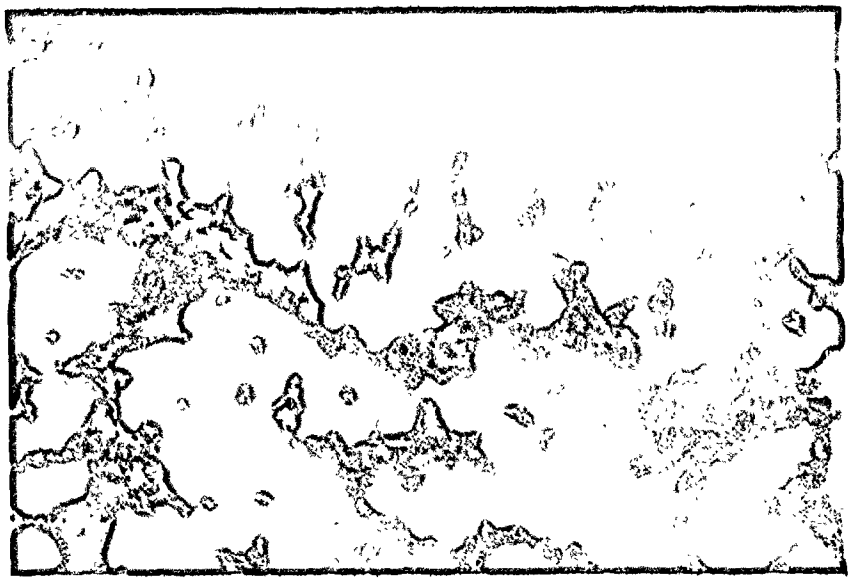
Figure 35: Tracing of projected micrographic cross section from a representative area of cross reinnervated PLD muscle of a dystrophic chicken 12 months postoperatively. This drawing emphasizes the difference in size between the converted (shaded fibres) and the unconverted fibres. Although the converted fibres represent only 23% of the total number of fibres present in this section, they cover 81% of the total muscle-fibre cross sectional area.


50 µm

unconverted fibres. In both muscles the converted fibres are larger and more or less rounded, while unconverted fibres are small and variable in shape.

The proportion of the converted fibres: In the cross reinnervated PLD muscles which responded with slow tonic contractions to nerve stimulation, the proportion of the converted fibres, although variable, was larger than the proportion of "ALD type" fibres detected in unoperated PLD muscles. Within a cross reinnervated muscle, the number of converted fbres was usually lower than the number of unconverted fibres. However, the area occupied by the converted fibres was greater than their actual number would imply. The drawings in Figures 34 and 35 emphasize this observation and demonstrate that the converted fibres cover 42% (normal) and 81% (dystrophic) of the total muscle-fibre cross-sectional area. In contrast, these same fibres represent only 8% (normal) and 23% (dystrophic) of the total number of fibres present in the sections. Furthermore, in the dystrophic chickens as a result of muscle fibre destruction, presumably of the unconverted fibres, the number of converted fibres relative to unconverted fibres could increase, although their absolute number may not have changed. In one cross reinnerved PLD muscle of a dystrophic chicken which was examined 504 days postoperatively and exhibited only slow tonic contraction, 99% of its surviving fibres were converted (Fig. 36). The absolute number of these converted fibres was approximately 1200 which was less than the number of fibres in the unoperated PLD muscle of the contralateral side (>1500). For these reasons, the absolute number of the converted fibres was not counted in every cross reinnervated muscle. Instead the cross sectional areas of

Figure 36: Photomicrograph of frozen cross ection of a cross reinnervated PLD muscle of a dystrophic chicken 17 months postoperatively, demonstrating myosin ATPase reaction after acid preincubation. 99% of the surviving fibres of this muscle were converted.



converted fibres was assessed visually and the accuracy of this estimation was demonstrated by measurements of cross sectional areas in a small sample.

Diameter of the converted fibres: The mean fibre diameter of the converted fibres was measured using the same technique applied to the unoperated PLD and ALD muscle fibres (Methods, Fig 7). Figure 37 shows a histogram of the mean fibre diameter of the converted fibres in cross reinnervated PLD muscle of normal genotype 3 months postoperatively, and histograms of the unoperated PLD and ALD muscles. These histograms emphasize the similarity between the mean fibre diameter of converted fibres and ALD fibres. Figure 38 shows a histogram of the mean fibre diameter of the converted fibres in the cross reinnervated PLD of 2 dystrophic chickens at 3 and 12 months postoperatively and a histogram of the unoperated contralateral PLD muscle of the 3-months old chicken. Although the converted fibres show as broad a distribution of fibre diameters as the unoperated PLD fibres, there are differences in the frequency distribution between the two fibre populations. While the unoperated PLD fibres have a flattened distribution, the converted fibres show maxima and have a tendency toward a distribution similar to that of normal fibres (Fig. 37). Figure 39 shows the histograms of mean fibre diameters of the converted fibres from cross reinnervated muscle of both genotypes at 18 months postoperatively and a histogram of an unoperated ALD muscle of normal genotype of the same age. These histograms clearly display the similarities between the mean fibre diameters of the converted fibres of both genotypes and the ALD fibres. Appendix T presents the data shown in the histograms in Figures 37, 38

Figure 37: Frequency distribution of fibre diameter (μm) of the converted fibres in the cross reinnervated (X-RI) PLD muscle of normal chicken 3 months postoperatively and of fibre diameters of unoperated PLD and ALD muscles. The PLD muscle was taken from the same chicken as the cross reinnervated muscle. The ALD muscle was taken from another chicken of the same age (Fig. 13) to emphasize the similarity between the fibre diameters of the converted fibre and the ALD fibres. The number of fibres measured from each muscle, the calculated means and the statistical differences between the histograms are listed in Appendix T.

NORMAL CHICKENS

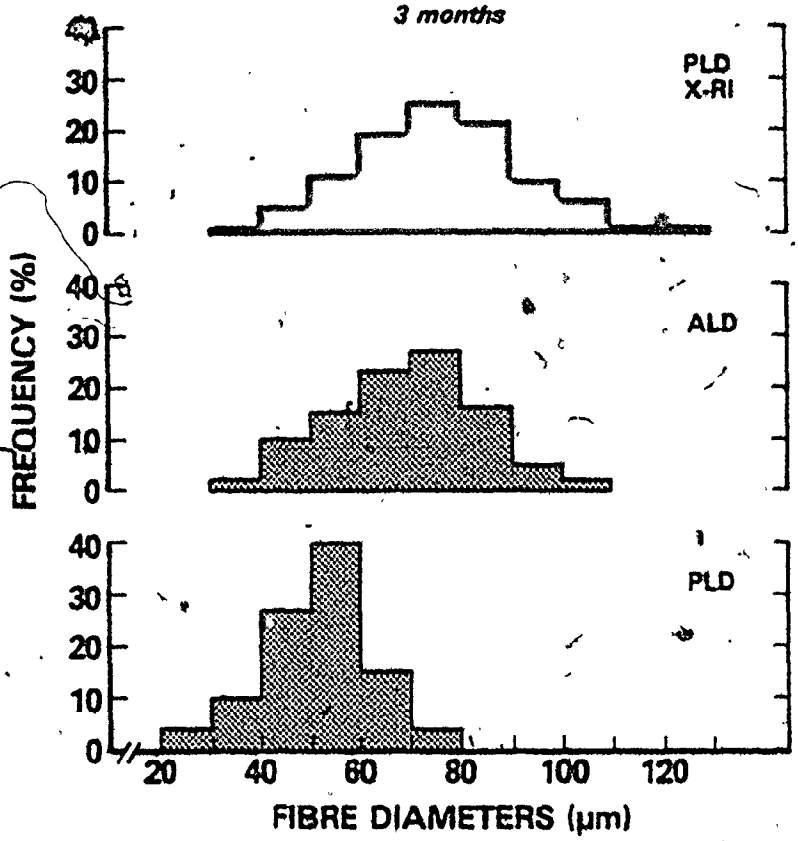


Figure 38: Frequency distribution of fibre diameter (µm) of the converted fibres in the cross reinnervated (X-RI) PLD muscles of two dystrophic chickens 3 and 12 months postoperatively and of fibre diameter of the unoperated contralateral PLD muscle of the 3-month old dystrophic chicken. While the unoperated PLD fibres have a flattened distribution with no apparent maxima, the converted fibres show a tendency toward a distribution similar to that of normal fibres (Fig. 37). The exact number of fibres measured for each muscle, the calculated means and the statistical differences between the histogram are listed in Appendix T.

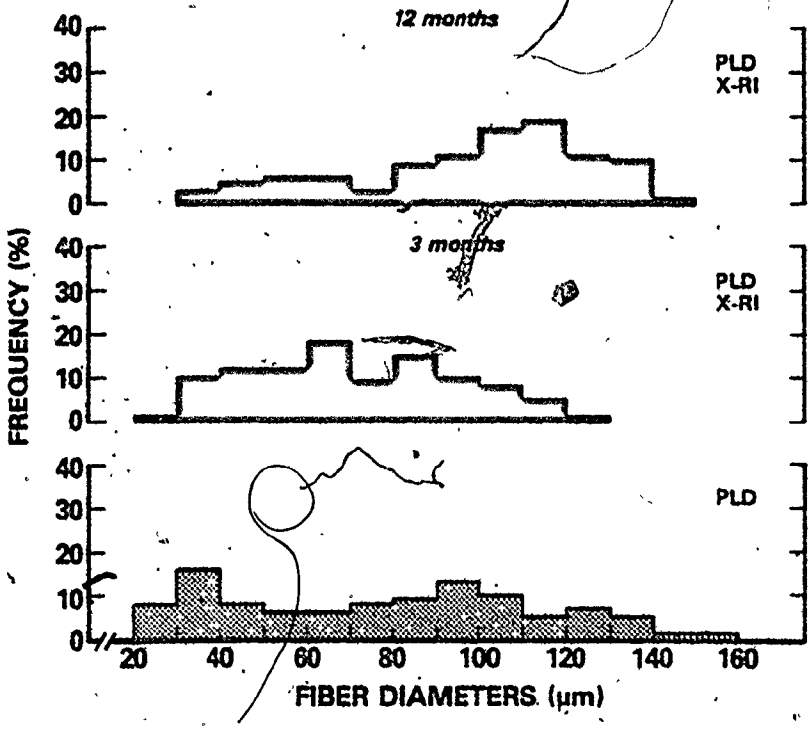
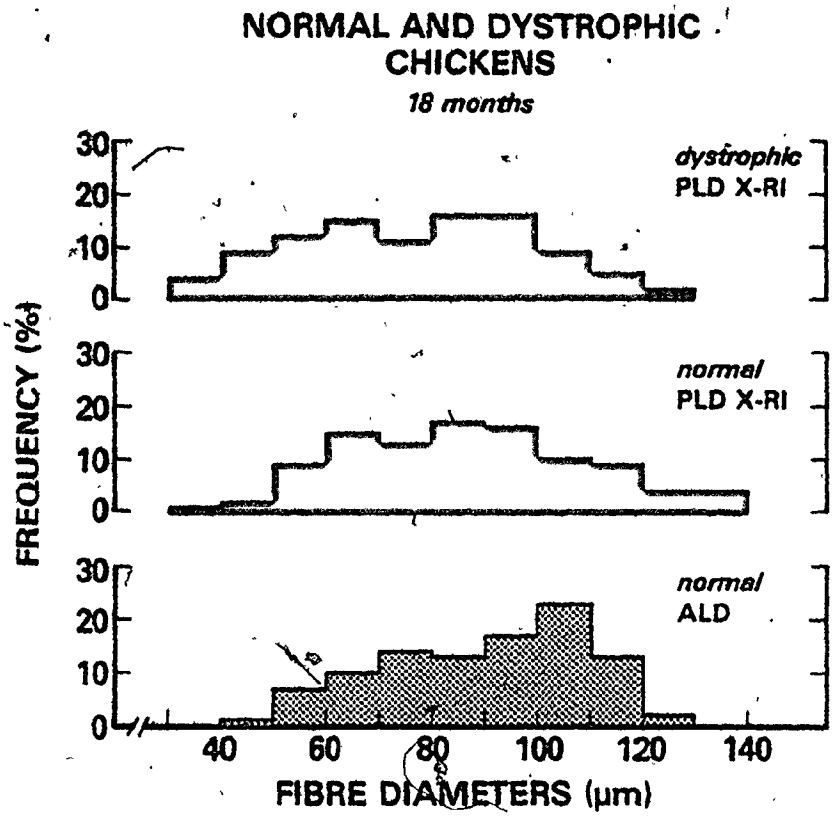

DYSTROPHIC CHICKENS
12 months
3 months
PLD
X-RI
PLD
X-RI
PLD
FREQUENCY (%)
40
30
20
10
0
20
40
60
80
100
120
140
160
FIBER DIAMETERS (µm)

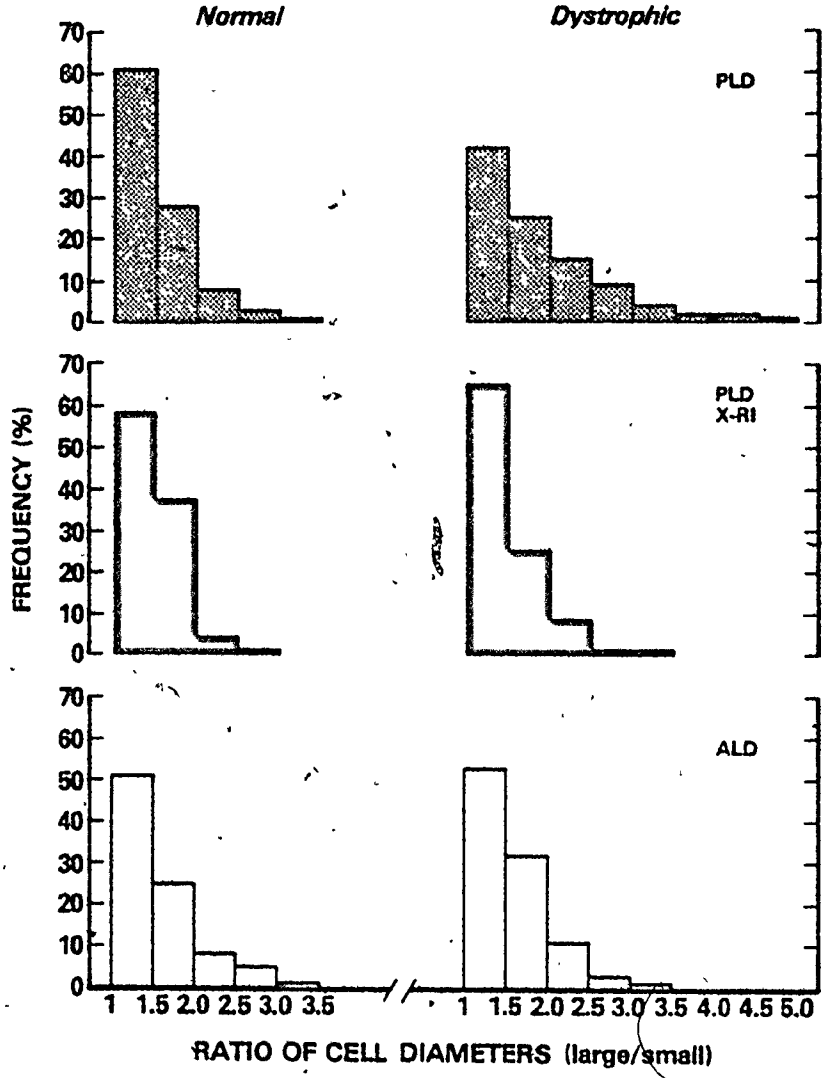
Figure 39: Frequency distribution of fibre diameters (μm) of the converted fibres in the cross reinnervated (X-RI) PLD muscles of normal and dystrophic chickens 18 months postoperatively, and of unoperated ALD fibres of normal chicken of the same age. These histograms show the similarity between the mean fibre diameter of the converted fibres of both genotypes and the ALD fibres. The exact number of fibres measured for each muscle, the calculated means and the statistical differences between the histograms are listed in Appendix T.


NORMAL AND DYSTROPHIC CHICKENS
18 months
dystrophic
PLD X-RI
normal
PLD X-RI
normal
ALD
FREQUENCY (%)
30
20
10
0
40
60
80
100
120
140
FIBRE DIAMETERS (µm)

and 39, and gives the statistical analysis of these data. The Student's t-test analyzes the difference between the means of the histograms, while the chi square (x^2) tests assess the difference between the distribution of the histograms. These tests emphasize the difference already noted visibly in the histograms.

As indicated in the Methods (p 58), the mean fibre diameter does not necessarily correlate well with the shape of the fibres. Thus, although the converted fibres within the cross reinnervated PLD muscles of dystrophic genotype look rounded in shape (Fig. 35), they cover a wide range of mean fibre diameters (Fig. 38). In order to quantitatively express the shape of the fibres, the ratios of the individual fibre diameters was calculated, and the histogram of the frequency of such ratios is presented in Figure 40. Since a ratio of 1 indicates equal diameters, a high percentage of fibres with values close to 1 will indicate a greater uniformity of fibre shape. As can be seen from the histogram in Figure 40 and from Appendix U, which gives the data presented in the histogram, 90-95% of the converted fibres of both genotypes have ratio of cell diameters between 1 and 2.

Figure 40: Frequency distribution of the ratio of the large and small diameters of individual muscle fibres (large/small) from muscles of normal and dystrophic chickens. The unoperated PLD and ALD muscles of both genotypes are from chickens 12 months of age. The postoperative times for the cross reinnervated (X-RI) muscles were 3 months for the normal and 12 months for the dystrophic. The number of fibres measured for each muscle and the calculated ratios are listed in Appendix U. These histograms show that 90-95% of the converted fibres of both genotypes have ratios of fibre diameter between 1 and 2, indicating more or less rounded shapes.


Normal
Dystrophic
PLD
PLD
X-RI
ALD
FREQUENCY (%)
0
10
20
30
40
50
60
70
1 1.5 2.0 2.5 3.0 3.5
1 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0
RATIO OF CELL DIAMETERS (large/small)

DISCUSSION

INTRODUCTION

Cross reinnervation experiments have demonstrated the ability of the peripheral motor nerve to alter the properties of fast and slow twitch muscles in mammals. This effect is obtained in muscles of adult, as well as younger animals (Buller et al., 1960a,b). In chickens, however, the transformation of fast twitch muscles into slow tonic muscles as a result of a nerve alteration is limited in muscles of adult chickens (Hník et al., 1967; Koenig, 1970; Bennett et al., 1973; Koenig and Fardeau, 1973), but can be achieved in developing muscles of newly hatched chickens (Jirmanová and Zelená, 1973; Bennett and Pettigrew, 1974; Hník et al., 1977). The reason may be that the differences between fast and slow muscle of the chicken (PLD and ALD muscles are the only muscles in the chicken which have been cross reinnervated) are more complex. Unlike mammals, where both fast and slow muscles are focally innervated twitch muscles, the chicken PLD muscle is a fast twitch focally innervated muscle while the ALD is a slow tonic multiply innervated one.

In neither mammals nor birds have all the fibres in the cross reinnervated muscles been successfully converted. One of the reasons may be related to imperfection in the surgical techniques which encourage self reinnervation of denervated fibres. More fundamental reasons, however, may include nerve muscle incompatibilities (Cosmos et al., 1979b),

selective reinnervation (Feng et al., 1965), or the developmental state of individual fibres at the time of reinnervation by the foreign nerve, i.e. the "younger" the fibre, the more responsive it seems to be to foreign neuronal influences. In the chicken specifically, the ability of the ALD nerve to multiply innervate each individual PLD muscle fibre must also be taken into account.

The present study shows alterations of the PLD muscle of dystrophic genotype that was cross reinnervated by the ALD nerve at hatching. The rationale behind the design of this experiment is as follows:

1. Fast twitch muscles of dystrophic genotype are specifically affected by the disease in that they cannot differentiate characteristics of mature glycolytic muscles (Cosmos, 1966; Cosmos and Butler, 1967).
2. Fast twitch muscles of dystrophic genotype seem unable to respond to the appropriate neuronal demand to successfully complete the transition from embryonic to adult metabolism (Cosmos and Butler, 1972; Cosmos, 1973).
3. Slow tonic oxidative muscles of dystrophic genotype, never challenged to alter their enzymic profile during development, do not express disease phenotypes (Cosmos, 1966; Cosmos and Butler, 1967; Linkhart and Wilson, 1975).

It thus seemed reasonable to assume that by cross reinnervating the PLD muscle with the ALD nerve in the dystrophic chicken, the fast twitch PLD muscle would not be challenged to alter its metabolism during development, and would then, like the slow tonic muscle, be spared disease phenotypes.

Since this study is the first attempt to cross reinnervate the

PLD muscle by the ALD nerve in dystrophic chickens, it was not known if the PLD of dystrophic genotype would accept foreign innervation, would respond by changing its mechanical and/or histochemical properties and, if successfully cross reinnervated, would be spared expression of disease phenotypes. To answer these questions, cross reinnervated PLD muscles of dystrophic chickens were compared to self reinnervated muscles of the same genotype and with cross reinnervated PLD muscles of normal genotype. Analyses of the mechanical response, pH specific myosin ATPase activities and the innervation of the experimental muscles were used as tests for successful cross reinnervation of the dystrophic PLD. Analyses of Pase and SDH enzymic activities, nuclear distribution, lipid accumulation and muscle structure were used as additional criteria to test for the success of the cross reinnervation, and as tests for the expression of disease phenotypes.

This study presents evidence that cross reinnervated PLD muscle fibres of both normal and dystrophic genotype responded similarly to the ALD nerve. Although not all fibres in cross reinnervated muscles were converted, the converted fibres in both normal and dystrophic muscles showed phenotypic characteristics of ALD fibres.

MECHANICAL RESPONSE

As expected from the results reported by others (Hník et al., 1967; Bennett et al., 1973), self reinnervated PLD muscles of both genotypes exhibited contraction properties characteristic of unoperated PLD muscle with respect to their genotypes. Self reinnervated PLD muscles of both genotypes responded as fast twitch muscle to indirect stimulation.

Their contraction and relaxation times were not significantly different from those of the unoperated PLD muscles, and their tension responses in most cases fell within the range of tensions developed by the unoperated muscles. As seen in unoperated muscles, self reinnervated PLD muscles of dystrophic genotype evoked lower twitch and tetanic tensions compared to the self reinnervated PLD of normal genotype. These observations indicate that although the muscles go through denervation and reinnervation processes, the reinnervation of the muscle by its own nerve restores its typical mechanical response.

In contrast, of the 39 cross reinnervated PLD muscles which were subjected to mechanical testing, about 50 percent demonstrated isometric contraction properties characteristic of ALD muscle, i.e. no response to single nerve stimulation, but a slow tonic response to a train of stimuli. The other 50 percent showed contraction properties characteristic of unoperated PLD muscles when stimulated indirectly. The latter have been considered as self reinnervated PLD muscles.

The contraction and relaxation times of the slow tonic response evoked in the cross reinnervated PLD muscles were not significantly different between normal and dystrophic genotypes and were very similar to the time parameters of the slow tonic response of ALD muscles.

During the mechanical testing, it was noted that slow tonic contraction of the cross reinnervated PLD muscles could be elicited in response to a low frequency (10-100 Hz) and low excitatory current ($t < 0.1$ msec) of nerve stimulation. However, when the frequency of stimulation was increased the contraction speed of the muscle increased and the tension became greater (Fig. 25). This observation, coupled

with histochemical examination of the cross reinnervated PLD muscles revealed the existence of two fibre populations; converted and unconverted. The slow tonic contraction recorded at low frequencies was attributed to the excitation of converted fibres multiply innervated by the ALD nerve and the fast contraction recorded at higher frequencies to the excitation of unconverted fibres. Although it might be assumed that the unconverted fibres were self reinnervated by PLD nerve axons, a possibility exists that at least some of them received a focal innervation from the ALD nerve. In an attempt to clarify this possibility the following arguments can be considered:

It is possible that the unconverted fibres were innervated by the PLD axons only. Support for this can be drawn from one experiment in which excitation of the deflected PLD nerve elicited a contraction from the cross reinnervated PLD muscle and from the studies of others which reported the possibility of self reinnervation of fibres in the cross reinnervated PLD muscle (Bennett et al., 1973). Kuffler and Vaughan Williams (1953) reported that the axons innervating the fast twitch muscles of the frog have larger diameters and lower excitatory thresholds than the axons innervating the slow tonic muscles. In addition, Hess (1961) reported that the axons innervating the PLD muscles of the chicken have larger diameters than the axons innervating the ALD muscles. Thus, if during the mechanical testing of the cross reinnervated PLD muscles, both ALD and PLD axons were stimulated, one would expect the fast contraction to be recorded in response to low frequency and low excitatory current. Since, in the present experiments, the slow tonic contraction was recorded in response to low frequency and low excitatory current, it

may indicate that the ALD axons had a larger diameter and/or lower threshold than the PLD axons. This reversal in axonal properties could be due to the influence of the PLD muscle on the properties of the regenerating ALD axons as demonstrated in the toad by Close and Hoh (1968). However, rather than assuming changes in axonal properties, it can be postulated that the dual recorded response of the cross reinnervated PLD muscles was due to recruitment of converted and unconverted fibres which were both innervated by ALD nerve axons, while other unconverted fibres innervated by the PLD nerve axons were not stimulated. It is possible that the deflected PLD axons, in an attempt to self reinnervate the cross reinnervated PLD muscle, had to create new pathways and new entrance sites to the muscle. As a result, PLD nerve axons could not be stimulated by the excitatory electrodes positioned on the ALD nerve (underneath the PLD tendon) either because of location or because they were cut while preparing the muscle for contraction recordings.

In contrast, the regenerating ALD nerve which had been placed underneath the PLD tendon during the initial operation at hatching possibly followed the original pathway of the degenerating PLD nerve and thus entered the PLD muscle at the same site (Guth, 1956). Not all muscle fibres received the same number of endings from the reinnervating ALD nerve (Jirmanová and Zelená, 1973). Perhaps only those fibres which received many endings were converted to slow tonic fibres. As such, they responded to the low frequency of excitation by summation of the many local graded responses. However, those fibres which were innervated by the ALD nerve at only one or two points may not have been

converted and would not respond to low frequencies of excitation (< 100 Hz) because the amount of ACh released (Vyskočil and Vyklický, 1974) was not sufficient to elicit an action potential or to permit summation of local responses. When the frequency of the excitatory current was increased (> 100 Hz) enough ACh was available to bring the fibres to threshold. This argument suggests that it is more likely that some unconverted fibres were self reinnervated by PLD axons while others were reinnervated focally by the ALD axons. Those self reinnervated by the PLD axons where not excited during the mechanical test. While those reinnervated by the ALD axons were excited during high frequency stimulation.

In any case, the observed slow tonic contraction of the cross reinnervated PLD muscles in response to nerve stimulation indicated that functional innervation did occur and that the normal and dystrophic PLD fibres both accepted the foreign innervation and responded to this innervation by changing their contractile properties.

HISTOCHEMICAL ANALYSES

Histochemical analyses of the self reinnervated PLD muscles of both genotypes demonstrated alkaline stable myosin ATPase activity and focal "en plaque" innervation. These properties correlate well with the fast twitch contraction elicited in response to nerve stimulation. Furthermore, the metabolic differentiation, as well as the nuclei and lipid distribution, correlate well with the above characteristics with respect to genotype of the self reinnervated muscles. The restoration of the inherent properties of the PLD muscles following self reinnervation

agrees with the results obtained from self reinnervated PLD muscles of normal chickens (Hník et al., 1967; Koenig, 1970; Koenig and Fardeau, 1973; Bennett et al., 1973) and from transplantation of fast twitch muscle to their original site in dystrophic chickens (Cosmos and Butler, 1972).

Aggregations of enzymatically similar muscle fibres were noted in some self reinnervated PLD muscles. This phenomenon, known as fibre type grouping, was reported to occur in reinnervated mammalian muscles following either self reinnervation or cross reinnervation (Romanul and Van Der Meulen, 1967; Yellin, 1967) and in self reinnervated PLD muscle of mature normal chickens (Koenig and Fardeau, 1973). The current explanation for fibre type grouping is based on the observation that muscle fibres of a single motor unit demonstrate a uniform histochemical enzymic activity (Edström and Kugelberg, 1968). Additional factors include the rate of growth of regenerating axons, the pathway they follow and the restricting influence of the connective tissue of the denervated muscles. It is thus suggested that fibre type grouping results from collateral sprouting of the fastest growing nerve axons (Romanul and Van Der Meulen, 1967; Yellin, 1967; Kugelberg et al., 1970). These axons reinnervate adjacent muscle fibres of differing histochemical characteristics which under the common influence of one motor nerve are transformed into histochemically uniform muscle fibres. The exact distribution and number of fibres in a motor unit cannot, however, be determined, since units of similar histochemical types may intermingle or lie in contact with one another (Burke, 1978).

Fibres which demonstrated acid and alkaline stable myosin ATPase

activity were also detected in self reinnervated PLD muscles of both genotypes, as was noted in both unoperated PLD muscles (Jirmanová and Zelená, 1973; Cosmos et al., 1979b; Toutant, 1979), and in self reinnervated PLD muscles of mature chickens (Koenig and Fardeau, 1973). In the present study, only 4 self reinnervated PLD muscles (1 normal and 3 dystrophic) revealed 3-10% of "ALD type" fibres. These self reinnervated PLD muscles developed in chickens in which the ALD muscle was removed during the initial operation at hatching. Thus, it is possible that the removal of the ALD muscle permitted innervation of PLD fibres by ALD nerve axons. However, it is also possible that PLD nerve axons originally innervating "ALD type" fibres were able to increase the number of fibres they innervated during the process of regeneration. Interestingly, these muscles, although containing more "ALD type" fibres than the contralateral unoperated PLD, did not exhibit slow tonic contraction in response to nerve stimulation. In this respect too, they resembled the unoperated PLD muscles. It is possible that the characteristic contraction response of "ALD type" fibres was masked by the predominant response of the majority of fast twitch fibres and/or while preparing the muscle for recording the small branches of the ALD nerve were damaged and thus could not be stimulated properly.

In conclusion, the reinnervation of the fast twitch PLD muscle of both genotypes by its own original nerve results in the return of the phenotypic characteristics of its fibres, including the small number of "ALD type" fibres. Removal of the ALD muscle, although intended to provide a control for the similar removal of the ALD muscle in the cross reinnervation experiments, may have permitted partial cross reinnervation

of the self reinnervated muscles.

The cross reinnervated PLD muscles of both genotypes, exhibited slow tonic contraction in response to nerve stimulation, and a higher proportion of fibres with acid and alkaline stable myosin ATPase activity relative to the unoperated contralateral PLD muscles. However, the cross reinnervated muscles also showed fibres which exhibited only alkaline stable myosin ATPase. Using biochemical analyses of cross reinnervated PLD muscle of normal chickens, Hník et al. (1977) reported that the myosin ATPase activity of the cross reinnervated PLD muscles was reduced but did not reach the low activity level of the unoperated ALD muscles. This intermediate myosin ATPase activity noted with a biochemical analysis of entire cross reinnervated muscles probably reflected the existence of both populations of fibres; converted and unconverted.

The myosins of slow and fast twitch mammalian muscles are not identical proteins that differ only in their enzymic activity, but are qualitatively distinct proteins (Guth and Samaha, 1969). After cross reinnervation there is an alteration in the histochemical reaction of myosin ATPase and in the type of myosin synthesized by the muscle, and it has been suggested that the nerve is influencing gene expression (Guth et al., 1970; Samaha et al., 1970). It is possible to apply the same argument to the present experiments with avian muscle. The myosins of fast twitch and slow tonic muscles in the chicken differ in the electrophoretic mobility of their light chains (Lowey and Risby, 1971; Syrový, 1973; Hoh, 1978), their myosin ATPase activities (Syrový, 1973) and in their stability at various pH's (Asiedu and Shafiq, 1972; Butler et al.,

1978; Cosmos et al., 1979b), and therefore are thought to be two distinct proteins. It was also demonstrated that PLD muscles never expressed the slow myosin light chains found in ALD fibres (Rubinstein et al., 1977; Hoh, 1978; Pette et al., 1979) nor did the PLD of both genotypes express acid stable myosin ATPase activity in histochemical analyses during in ovo development (Butler et al., 1978; Butler and Cosmos, 1979a,b). Thus, the present observation that a considerable number (over 50 percent) of fibres in the cross reinnervated PLD of both genotype expressed myosin ATPase activity following both acid and alkaline preincubation, and that total myosin ATPase activity was reduced in cross reinnervated PLD of normal genotype (Hník et al., 1977), suggests that the ALD nerve can influence gene expression in these fibres. For such a reason the term "converted fibres" has been used in referring to the cross reinnervated PLD fibres which demonstrate ALD muscle fibre phenotypes.

Converted fibres in all of the successfully cross reinnervated muscles exhibited the "en grappe" type of innervation with low AChE activity, the oxidative type of metabolism characterized by weak Pase and strong SDH enzymic activities, peripheral localization of nuclei and high lipid content. All of these properties are characteristic of slow tonic fibres. The achievement of these properties probably involves different levels of regulation. For example, the metabolic differentiation of the converted fibre may indicate a nerve influence which affects the rates of synthesis or the rates of degradation of proteins. The number of endplates per muscle fibre may depend not only upon the ability of a nerve to multiply or focally innervate a fibre, but also

upon the number of fibres which the nerve must reinnervate (normal PLD muscle contains about 9,000 fibres, while an ALD muscle contains about 4,000 fibres p 122). Alternatively, it may depend upon the ability of all muscle fibres to accept one or many endplates.

Unlike the ALD muscles which are characterized by two types of fibre, the converted fibres of the cross reinnervated PLD muscles were homogeneous for one fibre type similar to the predominant ALD fibres. The selectivity of one fibre type only following cross reinnervation of the PLD muscles is of interest, since Koenig and Fardeau (1973) reported that following denervation of the ALD muscle only one type of fibre (the larger predominant one) showed hypertrophy, while the other type did not change significantly. The reason for such selectivity is unknown.

The unconverted fibres of the cross reinnervated PLD muscles of both genotypes exhibited alakali stable myosin ATPase only, as is characteristic of fast twitch PLD fibres. However, the AChE reactions revealed variability in shape and activity of AChE stained endplates. It is possible that this variability is due to the reinnervation of the unconverted fibres by PLD and ALD nerves, as suggested from the mechanical testing. Jirmanová and Zelená (1973) reported that all nerve terminals in the cross reinnervated PLD muscles of normal genotype were of the slow type. Yet, they also reported some variation in shape and in the degree of AChE activity of the endplates. Of the 407 cross reinnervated fibres that they examined, 64% showed only one or two endplates. In addition, the degree of ultrastructural transformation differed from fibre to fibre. Only about 30% of the cross reinnervated PLD fibres had

transformed ultrastructurally to closely resemble ALD fibres. The majority of the cross reinnervated fibres were partly transformed with ultrastructural characteristics carying from the fast toward the slow type. Benhett and Pettigrew (1974) reported that both "en plaque" and "en grappe" endplate configurations were identified in the cross reinnervated PLD muscle of normal genotype. All of these observations strongly suggest that some unconverted fibres were innervated focally by the ALD nerve and others by the PLD nerve.

The metabolic enzymic activity of the unconverted fibres, the nuclear number and the nuclear distribution, all reflect their specific genotype. The cross reinnervated PLD muscle of normal genotype showed unconverted fibres with strong Pase and weak SDH activities with both internal and peripheral localization of nuclei. The cross reinnervated PLD muscles of the dystrophic genotype show unconverted fibres with variable (weak to strong) activities for both Pase and SDH. Their nuclei were located both internally and peripherally but the number of their internal nuclei increased. Fibres which showed intermediate Pase, strong SDH, peripheral localization of nuclei and focal "en plaque" innervation were usually scattered throughout the muscle in small numbers (7%) in unoperated self reinnervated and cross reinnervated PLD muscles of both genotypes. However, in some self reinnervated and cross reinnervated muscles they were clustered in small groups. Because of their small number and scattered distribution, these fibres were not conspicuous in the unoperated PLD muscle of dystrophic genotype. However, they were very obvious when they appeared as clusters of homogeneous

fibres amidst the heterogeneous mixture characteristic of the dystrophic muscle. It is not understood how these fibres maintain a normal phenotype in muscles of dystrophic genotype. This is obviously an important problem for further research. The exceptionally small fibres noted among the unconverted fibres mainly in the cross reinnervated muscles of dystrophic genotype may be regenerating fibres at early stages of differentiation, or perhaps atrophic fibres. Jirmanová and Zelená (1973) reported that cross reinnervated PLD muscle of normal chickens contained approximately 10 percent of atrophic fibres which had ultrastructural features of chronically denervated fast fibres.

The existence of cross reinnervated fibres which are maybe focally innervated by the ALD nerve, but which show characteristics of fast twitch PLD fibres, may support the suggestion that complete transformation of the PLD fibre can only be brought about after cross reinnervation of fibres at early stages of development, whereas the more developed fibres are resistant (Jirmanová and Zelená, 1973). The present findings that more converted fibres exist in cross reinnervated PLD muscles of dystrophic genotype at later stages postoperatively, than seen with cross innervated PLD muscles of normal genotype may support the above suggestion. The cyclic degeneration and regeneration phenomena of muscle fibres known to occur throughout immaturity in dystrophic fast twitch muscle (Cosmos, 1970) may have stimulated the existing ALD axons to sprout (Desment and Borenstein, 1973) and to innervate the new developing fibres.

If indeed the inability of select PLD fibres to be converted by the ALD nerve does not reflect fundamental mechanism(s), the following

procedures may allow a more complete transformation:

1. cross reinnervating the PLD muscle during in ovo development (Presently performed in Dr. Cosmos' laboratory);
2. allow longer time to elapse between the time of denervation and reinnervation (Studitsky, 1974);
3. a month or so following reinnervation by the ALD nerve, injure the cross reinnervated PLD in situ (Riley, 1974); and
4. when performing the cross reinnervation operation, remove half of the PLD muscle in an effort to reduce the total number of fibres to be reinnervated.

From the above discussion it is possible to conclude that PLD fibres of both genotypes which were successfully cross reinnervated by the ALD nerve not only responded with slow tonic contraction, but also exhibited histochemical properties characteristic of slow tonic oxidative ALD fibres. These observations support the result obtained for the cross reinnervated PLD muscle of normal genotype (Jirmanová and Zelená, 1973; Bennett and Pettigrew, 1974; Hník et al., 1977) and indicate that the cross reinnervated PLD fibres of dystrophic genotype not only accept the new innervation, but also respond to it by changing their mechanical and histochemical properties.

STRUCTURAL ANALYSIS

The most notable change in appearance of a frozen cross section of the cross reinnervated PLD muscle was the large rounded converted fibres which were distributed throughout the section and were surrounded by smaller unconverted fibres. Histogram analyses of fibre diameters

indicated that there was a shift in the diameter of the converted fibres toward the size of the ALD fibre; however, in many cross reinnervated muscles converted fibres were also larger than the ALD fibres. An explanation for the hypertrophy of the converted fibres may be related to stretch of the reinnervated PLD muscles as a result of the removal of the ALD muscle at hatching. The observed enlargement of slow tonic metapatagial latissimus dorsi muscle (MLD) in operated animals (p 139 and p 149) can support such explanation. Both the hypertrophy of the cross reinnervated converted fibres and the enlargement of the MLD muscle probably relate to the known hypertrophy of the slow tonic fibres of the chicken as a response to overload or stretch (Gutmann et al., 1970; Sola et al., 1973). As a result of the wing droop caused by the removal of the ALD muscle, both the PLD and MLD were subjected to overload or stretch. Only the slow tonic fibres respond to this wing droop with hypertrophy.

Other structural changes noted in the cross reinnervated PLD muscle of both genotypes were the rounded shape and the small size of the fascicles. In the cross reinnervated muscle of dystrophic genotype, the unconverted fibres exhibited all the structural characteristics of dystrophic PLD fibres as shown by their size and shape and destruction of fibres. In some cross reinnervated muscles of dystrophic genotype, examined more than one year postoperatively, destruction of the unconverted fibres was so complete that only converted fibres survived; these were surrounded by fat which had replaced the dying fibres.

CONCLUSION

The present study demonstrates the ability of the ALD nerve to influence the phenotypic expression of the PLD muscle during development ex ovo. Similar conclusions have been reached by others in experiments with normal chickens (Jirmanová and Zelená, 1973; Bennett and Pettigrew, 1974; Hník et al., 1977); however, the present study is the first to report , within the dystrophic chicken, successful cross reinnervation of the PLD muscle with the ALD nerve .

The experiments performed with dystrophic chickens were designed to focus on three important questions:

1. What is the ability of the fast twitch muscle of dystrophic genotype to accept the innervation of a slow tonic muscle?
2. Once reinnervated, could the fast twitch muscle respond to the dictates of the "slow tonic" nerve by expressing the appropriate phenotypes?
3. Would the dystrophic phenotype also be expressed?

Results of the present study indicate clearly that all changes noted with the cross reinnervated PLD muscle of normal genotype were also found to occur in the cross reinnervated PLD muscle of dystrophic genotype. Thus, muscles of both genotypes responded similarly to the ALD nerve: Fibres of both muscles which had been successfully cross reinnervated acquired mechanical, structural and histochemical characteristics of the ALD muscle. These results establish that the PLD muscle of dystrophic genotype is (1) able to accept the new innervation and (2) is as capable of responding to the influence of the ALD nerve as is the normal PLD.

The third question with which the present study was concerned was whether the new innervation would permit the expression of disease phenotypes. A property of a muscle can be identified as being abnormal only when it is noted to deviate from the same property of a normal muscle. In the present studies, normal and dystrophic fast twitch muscles were compared structurally, physiologically and histochemically from hatching through maturity. Such studies identified abnormalities in the dystrophic fibres with all parameters examined and established a baseline for the analyses of cross reinnervated PLD muscles of both genotypes. Thus, in a comparison of the phenotypes of a cross reinnervated PLD muscle with those of a self reinnervated PLD muscle, it was noted that none of the characteristics of the self reinnervated muscles were present in successfully cross reinnervated PLD fibres. This was demonstrated with experiments of both normal and dystrophic chickens. Instead, the cross reinnervated fibres demonstrated phenotypes of the ALD muscle. Since ALD muscles of both genotypes are spared dystrophic phenotypes throughout development (present study), the cross reinnervated fibres of dystrophic lineage which also demonstrate these characteristics do not express dystrophic phenotypes. Thus, it can be concluded that PLD fibres of dystrophic genotype which have been both reinnervated successfully by the ALD nerve and converted to slow tonic oxidative fibres do not express dystrophic characteristics.

The present cross reinnervation experiments demonstrate, for the first time, an experimental manipulation which interferes with the expression of the phenotypic characteristics of dystrophy during development ex ovo. These findings support the rationale underlying the

present study: the PLD muscle of dystrophic genotype is unable to respond appropriately to the demand from its own motor nerve to complete successfully the transition from embryo to adult metabolism (thus expressing the dystrophic phenotypes). However, when this challenge is removed by cross reinnervating the PLD muscle with the ALD nerve, the dystrophic phenotype is not expressed. As a consequence of the cross reinnervation, presumptive fast twitch glycolytic PLD fibres lose their identity by being converted to slow tonic oxidative PLD fibres, but, gain the ability to express normal phenotypes. These findings strongly suggest that regardless of the time during development when slow tonic characteristics are achieved, i.e. either during development in ovo or by surgical manipulation ex ovo, slow tonic fibres are spared dystrophic phenotypes.

Problems for future research

1. Further evidence to substantiate the hypothesis stated in the present study may be obtained by forcing the slow tonic fibres of dystrophic genotype to differentiate fast twitch glycolytic properties and, thus, to express dystrophic phenotypes. Cross reinnervation of the ALD muscle with nerves innervating fast twitch muscles has demonstrated that ALD fibres can be forced to differentiate properties of fast twitch muscles of normal chickens (Zelená and Jirmanová, 1973; Syrový and Zelená, 1975). In preliminary studies of similar cross reinnervated experiments with both normal and dystrophic chickens, I have found that ALD fibres can respond to a "fast" nerve with a both a twitch response and the appropriate myosin ATPase activity (alkaline stable only). Although such experiments established the feasibility

of this type of cross reinnervation, much difficulty was experienced in the analysis of the newly acquired phenotypes. Alternative techniques must be developed to permit an accurate identification of dystrophic phenotypes since abnormalities were noted in preparations done with both normal and dystrophic chickens.

2. Muscle activity and/or trophic substances are the proposed mechanisms by which a nerve can directly influence the expression of specific phenotypes of skeletal muscles from a variety of normal animals (Guth, 1969; Gutmann, 1976; Purves, 1976). The present study indicates that muscles of dystrophic genotype can also be influenced to alter diseased phenotypes when coupled to a foreign nerve. Whatever the mechanisms involved in the influence of nerve on the differentiation of muscle (normal or dystrophic) following cross reinnervation are, a study of such mechanisms is not only important for the understanding of nerve muscle relationship during development ex ovo, but also may lead to an understanding of the etiology of dystrophy. The present experimental techniques of crossing a focally innervated muscle by the nerve of a multiply innervated muscle offer the opportunity to explore not only the effect of a specific type of nerve on the transformation of muscle phenotypes but also the influence of the number of endplates formed by this nerve on each fibre. In the present experiments, the possibility existed that the ALD nerve innervated PLD fibres both focally and multiply. In each case the muscle phenotypes induced were strikingly different. The multiply innervated fibres acquired all characteristics of ALD muscles; the focally innervated ones remained unconverted. The existence of unconverted fibres of uncertain focal innervation (PLD or ALD nerve)

focuses on the importance of establishing a reliable marker to distinguish between the two nerves. One such potential marker is the difference in the diameter of nerve axons innervating fast twitch and slow tonic fibres (Kuffler and Vaughan Williams, 1953; Hess, 1961; Close and Hoh, 1968). Two other possible markers are the use of horseradish peroxidase to locate motoneurons in the spinal cord (DeSantis et al., 1977) or the use of AChE molecular forms to distinguish between the nerves themselves (Di Giamberardino et al., 1979). If these markers can be used, they would provide a means of testing the concept that multiple versus focal innervation as a key factor in the expression of dystrophic or normal phenotypes.

3. Another approach which might help to clarify the mechanisms underlying the changes noted in the cross reinnervated PLD muscles, would be to correlate changes in the muscle with a functional nerve-muscle interaction at short intervals postoperatively. For example, if one were to observe the initiation of fibre conversion prior to the formation of a functional neuromuscular junction or the initiation of muscle activity, then trophic substances might be implicated in the conversion process. Preliminary experiments (not reported here) showed that there is significant fibre conversion in cross reinnervated PLD muscles of both genotypes by 15 days postoperatively, a time when a functional nerve muscle contact is first being observed. Such an observation implies a conversion of fibres prior to the establishment of functional activity and offers the opportunity to examine separately trophic versus activity influences on the conversion of fibres types.

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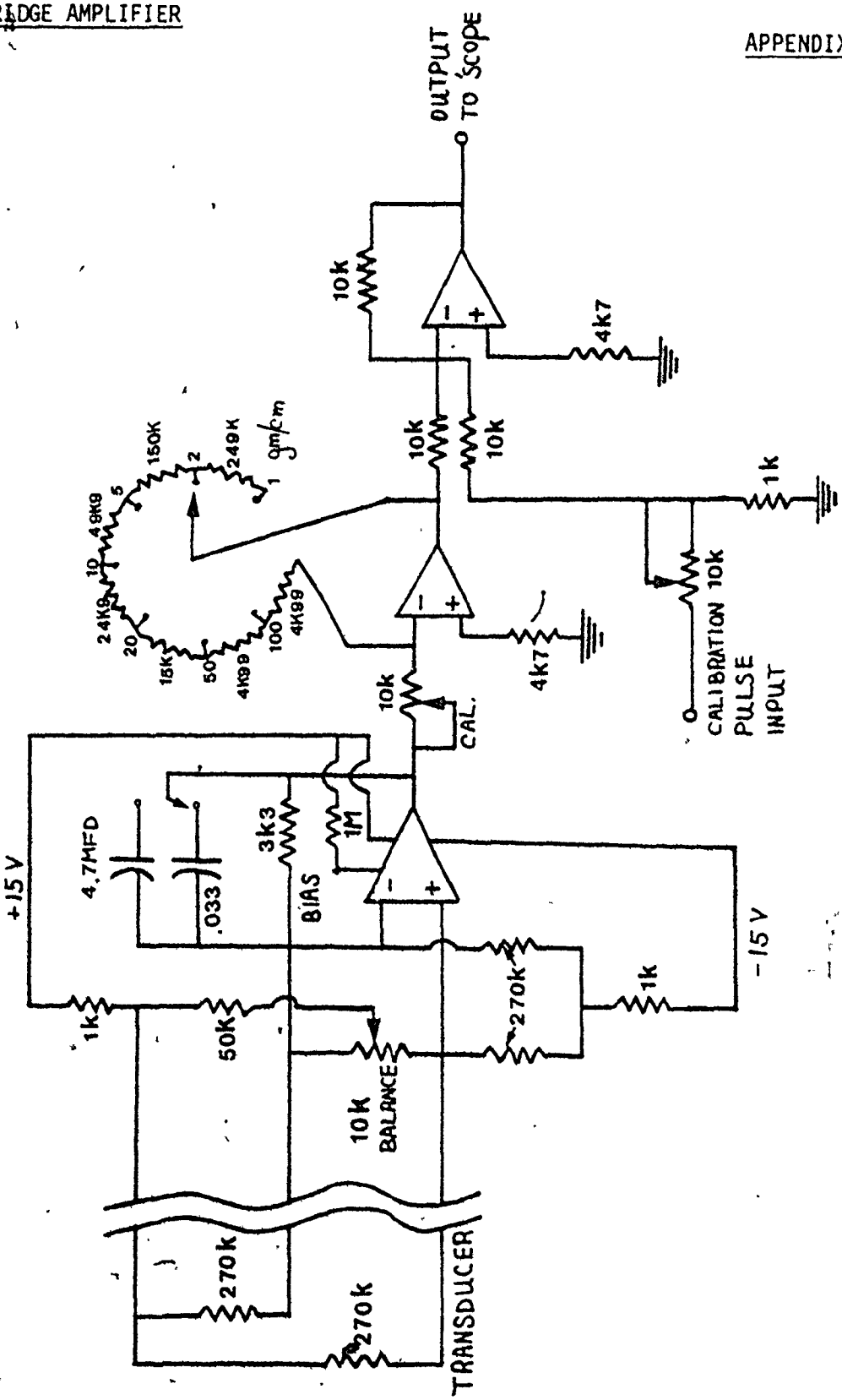
BRIDGE AMPLIFIER

APPENDIX A



Designed and constructed by G.L. Shine, McMaster University

APPENDIX B

PROCEDURE FOR THE HISTOCHEMICAL DEMONSTRATION OF MYOSIN ATPASE ACTIVITY

PROCEDURE

I Demonstration of Alkali-stable myosin ATPase activity

1. Fix sections for 2 min in buffered formalin solution at 4°C.
2. Wash in H_2O for 2 min.
3. Preincubate in alkali preincubation solution for 10 min at room temperature.
4. Rinse in H_2O (x2)
5. Wash in H_2O for 1 min.
6. Rinse in H_2O.
7. Incubate sections for 30 min in incubation solution at 37°oC.
8. Rinse in H_2O. (x2).
9. Wash in H_2O for 1 min.
10. Rinse in H_2O.
11. Place in 1% $CaCl_2$2 (dihydrate)* for 3 min.
12. Rinse in H_2O.
13. Wash in H_2O for 1 min.
14. Rinse in H_2O.
15. Place in 2% $CoCl_2$ for 3 min.
16. Rinse in H_2O (x3).
17. Place in 1% $(NH_4)_2S$ for 1 min.
18. Rinse in H_2O (x4).
19. Mount with Farrant's solution.

II Demonstration of Acid-stable myosin ATPase activity

1. Preincubate the unfixed sections in acid preincubation solution for 1.5 min. (no longer).
2. Complete the procedure as per steps 4-19 above.

SOLUTIONS

Buffered formalin (10%)*

Formaldehyde solution (37%)	25.0 ml
Na Cacodylate	15.5 gm
$CaCl_2$ (dihydrate)	5.0 gm
Sucrose	57.5 gm

bring to final volume of 500 ml with H_2O and adjust to pH 7.6 with 6 N HCl (approximately 4-6 drops).

Alkali preincubation solution

A.	glycine	0.751 gm
	NaCl	0.585 gm
	$CaCl_2$ (dihydrate)	0.800 gm

bring to final volume of 100 ml with H_2O.

B. 0.1 N NaoH*

C. 50 ml of solution A plus 45 ml of solution B

adjust 25.0 of solution C to pH 10.0 with 1.0 N HCl (approximately 3-4 drops).

Incubation solutions

To 60 ml of solution C, add 100 mg ATP; adjust to pH 9.4 with 6.0 N and 1.0 N HCl. Filter and place in H_2O bath at 37°C.

Acid preincubation solution

Adjust 25.0 ml of acetate buffer (0.1 N) to pH 4.35 with glacial acetic acid (approximately 1 drop).

Acetate Buffer (0.1 N)*

A.	0.1 N sodium acetate (NaAC)	0.8203 gm/100 ml H_2O
B.	0.1 N acetic acid (HAC)	1.2 ml/200 ml H_2O
C.	Mix 74.0 ml of A with 126.0 ml of B and add 1.48 gm of KCl	

* stock solutions can be stored at 4°C for several months.

APPENDIX C

PROCEDURE FOR THE HISTOCHEMICAL DEMONSTRATION OF ACETYLCHOLINESTERASE ACTIVITY IN WHOLE MUSCLE PREPARATION

PROCEDURE

1. Cut fresh blocks of muscle (approximately 1 x 1 cm and 2 mm thick)
2. Fix tissue in formol saline for 1 hr at room temperature.
3. Wash in sodium acetate buffer for ½ hr.
4. Preincubate in preincubation solution for 1-2 hrs (until the tissue turns a light blue) at 32°C.
5. Incubate in incubation solution for 5-7 hrs (until white spots appear on the tissue) at 32°C.
6. Wash in sodium acetate buffer for ½-1 hr.
7. Differentiate in 10% $(NH_4)_2$ S for ½ hr.
8. Rinse in H_2O.
9. Store in 20% glycerol solution at 4°C.

SOLUTIONS

Formal saline 10%*

Formaldehyde (37%)	100 ml
NaCl	8.5 gm

bring to final volume of 1.0 litre with H_2O

Sodium acetate buffer (0.1 M)*

Solution A:

sodium acetate (anhydrous)	6.805 gm/500 ml H_2O

Solution B:

glacial acetic acid (16.7 N) 0.6 ml/100 ml H_2O

Add approximately 452 ml of solution A to 48 ml of solution B, adjust to pH 6.0.

Copper glycine solution*

Glycine	3.75 gm
$CuSO_4.5H_2O$	2.50 gm

bring to final volume of 100 ml with H_2O.

Preincubation solution

To 18.0 ml of sodium acetate buffer add 2.0 ml of copper glycine solution.

Incubation solution

To 10 ml of preincubation solution, add 30.0 mg of acetylthiocholine iodide.

* stock solutions - can be stored at 4^oC for several months

APPENDIX D

DATA RECORD FORMAT FOR COMPUTER ANALYSIS

The data from individual chickens were keypunched and stored on disc in the HP 3000 computer. A single chicken's data comprised of four sections: (1) PLD; (2) ALD; (3) X-RI PLD; and (4) S-RI PLD. For each section, the following data were available:

	Columns
chicken I.D. number	1 - 4
muscle name (PLD, ALD, X-RI PLD, S-RI PLD)	5
chicken strain (N or D)	6
Sex	7
Age (days)	8 - 10
Body weight (g)	11 - 14
muscle weight (g)	15 - 18
twitch tension (g)	19 - 21
time to half peak for twitch (msec)	22 - 24
time to peak for twitch (msec)	25 - 27
time to half relaxation for twitch (msec)	28 - 30
tetanic tension at 50 Hz	31 - 33
time to half peak	34 - 36
time to half relaxation	37 - 39
tetanic tension at 70 Hz	40 - 42
time to half peak	43 - 45
time to half relaxation	46 - 48
tetanic tension at 100 Hz	49 - 51
time to half peak	52 - 54
time to half relaxation	55 - 57
max tetanic tension	58 - 60

In this way, for each chicken, 4 cards were available. Although an individual chicken's data would only consist of at most three sections, all data was maintained in a four-section (i.e. fixed length) format and values indicating unavailable data were inserted where necessary. The statistical package for the social sciences Nie et al. (1975)was used throughout for analysis.

APPENDIX E

CALCULATION OF MEAN AND STANDARD DEVIATION (INDIVIDUAL OBSERVATIONS)

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

$$SD\ (x) = \sqrt{\frac{\sum_{i=1}^{n} X_i^2 - \left(\frac{\sum_{i=1}^{n} X_i}{n}\right)^2}{n-1}}$$

$\bar{X}$ = mean

X_i = the ith observation

n = number of observations

SD = standard deviation

CALCULATION OF MEAN AND STANDARD DEVIATION FROM GROUPED DATA

$$\bar{X} = \frac{\sum_{i=1}^{k} f_i X_i}{\sum_{i=1}^{k} f_i}$$

X_i = mid point of the ith interval

fi = frequency of observations in the ith interval

k = the number of intervals

similarly

$$SD(x) = \sqrt{\frac{\sum_{i=1}^{k} f_i X_i^2 - \frac{\left(\sum_{i=1}^{k} f_i X_i\right)^2}{\sum_{i=1}^{k} f_i}}{\sum_{i=1}^{k} f_i - 1}}$$

(Ferguson, 1966)

APPENDIX F

NORMAL CHICKENS
RELATIONSHIP OF BODY WEIGHT TO AGE

Age (days)	n(♀)	Body wt (g) mean ± S.E.	Age (days)	n(♂)	Body wt (g) mean ± S.E.
2	24	44 ± 2	2	20	45 ± 2
11	16	84 ± 7	10	22	84 ± 5
20	15	180 ± 4	20	12	198 ± 5
28	7	274 ± 13	27	9	305 ± 18
33	14	348 ± 12	33	10	374 ± 25
39	9	430 ± 20	39	7	477 ± 14
51	9	595 ± 27	51	11	678 ± 32
57	21	654 ± 14	61	10	863 ± 35
69	4	804 ± 30	74	11	1218 ± 32
75	20	946 ± 22	99	12	1498 ± 48
92	20	1084 ± 33	108	14	1710 ± 33
107	17	1229 ± 28	132	4	2003 ± 54
124	9	1355 ± 44	143	19	2130 ± 38
144	14	1625 ± 57	182	21	2343 ± 54
166	14	1635 ± 50	206	20	2382 ± 52
220	10	1696 ± 78	233	14	2398 ± 61
244	5	1657 ± 57	262	11	2338 ± 74
269	5	1631 ± 53	290	5	2402 ± 55
295	5	1799 ± 101	336	10	2397 ± 73
341	7	1805 ± 63	378	8	2467 ± 94
409	11	1701 ± 70	432	6	2609 ± 81
551	6	1777 ± 137			

APPENDIX G

DYSTROPHIC CHICKENS
RELATIONSHIP OF BODY WEIGHT TO AGE

Age (days)	n(♀)	Body wt (g) mean ± S.E.	Age (days)	n(♂)	Body wt (g) mean ± S.E.
3	49	35 ± 1	1	27	34 ± 1
7	7	85 ± 4	6	21	42 ± 1
17	12	111 ± 8	14	9	90 ± 7
26	25	213 ± 5	19	7	149 ± 7
31	19	258 ± 17	26	20	246 ± 7
41	5	360 ± 28	31	14	316 ± 22
45	10	467 ± 11	44	19	519 ± 26
53	13	564 ± 13	52	10	708 ± 31
60	15	652 ± 22	60	11	875 ± 32
71	19	829 ± 17	70	15	1021 ± 43
84	28	965 ± 19	83	24	1262 ± 41
100	20	1157 ± 27	95	12	1501 ± 71
120	22	1281 ± 36	107	13	1745 ± 63
135	21	1430 ± 36	121	17	1914 ± 45
150	22	1477 ± 50	135	15	2049 ± 40
163	14	1568 ± 53	151	20	2101 ± 42
177	21	1567 ± 40	164	15	2230 ± 47
189	16	1535 ± 41	176	23	2205 ± 40
203	19	1575 ± 39	188	16	2245 ± 56
218	16	1605 ± 42	202	20	2294 ± 42
233	11	1613 ± 55	218	20	2340 ± 53
247	15	1619 ± 45	233	20	2326 ± 49
270	15	1596 ± 48	248	16	2331 ± 88
291	24	1640 ± 36	271	23	2389 ± 61
312	12	1580 ± 66	290	31	2426 ± 49
334	22	1635 ± 40	312	19	2510 ± 53
370	23	1627 ± 42	334	20	2576 ± 50
401	11	1704 ± 44	354	18	2519 ± 58
430	20	1756 ± 30	376	23	2580 ± 42
465	12	1713 ± 47	399	21	2558 ± 43
489	14	1782 ± 51	419	21	2601 ± 44
			438	18	2619 ± 45
			461	20	2621 ± 48
			484	14	2540 ± 97
			523	21	2617 ± 38
			560	10	2541 ± 56

APPENDIX H

MULTIPLE REGRESSION

Regression analysis has been used where it was of interest to investigate the relationship between two or more variables. The simplest form is the straight line in which a "dependent" variable y is related to an "independent" variable x through a linear relationship of the form

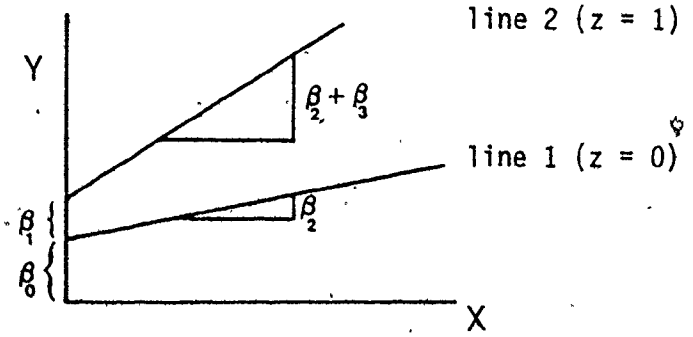
$$y = \beta_0 + \beta_1 x$$

The estimation procedure of least squares provides best estimates for β_0 and β_1 based on the data and provides a framework for testing statistical hypothesis of the form

$H_0 : \beta_1 = 0$ (null hypothesis of no relationship)

The approach is easily extended to more than one x value and is then called multiple linear regression. This is a very general technique and of fundamental importance in the statistical analysis of experimental data (Kleinbaum and Kupper, 1978).

Comparison of two lines



Y = the response of interest (e.g. muscle weight)

X = the independent variable (e.g. age) thought to affect the level of Y

Z = an indicator variable to distinguish between 2 sets of points (e.g. normal, z = 0, or dystrophic z = 1)

In order to compare the position of the regression lines between the two sets of data the following models must be fitted and statistically compared in their ability to fit the data.

The full model allowing separate intercepts and separate slopes is:

$$Y = \beta_0 + \beta_1 Z + \beta_2 X + \beta_3 ZX$$

This is depicted in the diagram above. When Z = 0 and Z = 1 are put into equation of the full mode, β_1 is the difference in intercepts and β_3 the difference in slopes between the two sets of data. Using the general theory of multiple regression it is possible to test the difference in intercept by dividing β_1 by its standard error to produce a t-statistic. This tests the null hypothesis that β_1 is in fact zero.

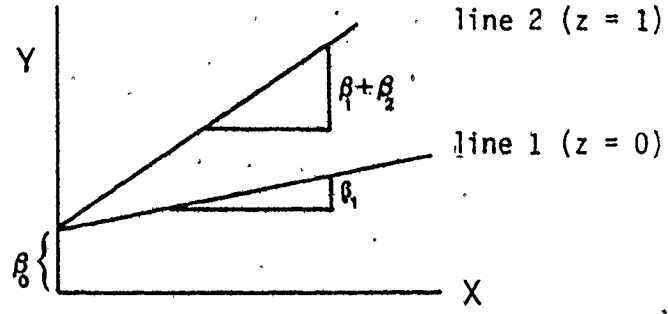
If the lines have the same intercept then the differences in slope can be tested. The restricted model is:

$$Y = \beta_0 + \beta_1 X + \beta_2 ZX$$

where β_0 is the common intercept

β_1 is the slope for the z = o line

β_2 is the difference in slopes between the z = 0 and z = 1 lines



Again, using the general theory of multiple regression, the coefficient β_2 can be tested by dividing it by its estimated standard error to form a t-statistic. This tests the hypothesis that the two sets of data have the same slope (i.e. $\beta_2 = 0$). Since the lines have already been con-

strained to have a common intercept, this means that the lines are coincident.

NORMAL CHICKENS
ANTERIOR AND POSTERIOR LATISSIMUS DORSI:
CHANGE IN MUSCLE WEIGHT WITH BODY WEIGHT
(AGE 1-180 DAYS)

	Age (days)	Body wt (g)	Muscle wet weight (g) PLD	ALD	$ALD/_{PLD}$
	1	39	0.006	0.010	1.667
	1	39	0.009	0.012	1.333
	1	41	0.009	0.012	1.333
	1	42	0.008	0.013	1.625
	1	43	0.009	0.011	1.222
	1	43	0.011	0.016	1.455
	1	44	0.009	0.013	1.444
	1	44	0.009	0.012	1.333
mean		42	0.009	0.012	1.426
S.E.		1	0.0005	0.0006	0.06
	9	63	0.017	0.017	1.000
	9	66	0.018	0.018	1.000
	10	72	0.022	0.019	0.864
	10	74	0.020	0.018	0.900
	10	86	0.026	0.021	0.808
	10	88	0.031	0.026	0.839
	10	90	0.029	0.024	0.828
	10	93	0.028	0.024	0.857
mean		79	0.024	0.021	0.887
S.E.		4	0.002	0.001	0.03
	15	115	0.041	0.035	0.854
	15	120	0.050	0.042	0.840
	15	120	0.060	0.050	0.833
	15	132	0.057	0.045	0.789
	15	140	0.052	0.038	0.731
mean		125	0.052	0.042	0.809
S.E.		5	0.003	0.003	0.02
	21	160	0.049	0.034	0.694
	21	188	0.056	0.057	1.018
	21	197	0.065	0.052	0.800
	21	197	0.071	0.060	0.845
	21	211	0.077	0.050	0.649
mean		191	0.064	0.051	0.801
S.E.		8	0.005	0.004	0.06

	Age (days)	Body wt (g)	Muscle wet weight (g) PLD	ALD	ALD/PLD
	28	254	0.095	0.071	0.747
	28	294	0.130	0.126	0.969
	30	296	0.138	0.092	0.667
	28	298	0.143	0.102	0.713
	28	300	0.113	0.100	0.885
	30	303	0.130	0.090	0.692
	30	344	0.172	0.116	0.674
mean		298	0.132	0.100	0.764
S.E.		10	0.009	0.008	0.04
	42	550	0.307	0.250	0.814
	42	550	0.325	0.230	0.708
	51	570	0.257	0.195	0.759
	56	610	0.349	0.219	0.628
	63	637	0.372	0.288	0.774
	60	650	0.338	0.266	0.787
	87	680	0.386	0.280	0.725
	57	700	0.325	0.300	0.923
	56	705	0.318	0.255	0.802
mean		628	0.331	0.254	0.769
S.E.		20	0.01	0.01	0.03
	92	1100	0.585	0.329	0.562
	120	1150	0.721	0.437	0.606
	89	1160	0.568	0.389	0.685
	85	1429	0.893	0.596	0.667
	110	1577	0.955	0.574	0.601
	148	1600	0.780	0.556	0.713
	110	1660	0.814	0.676	0.830
	112	1660	0.970	0.626	0.645
	149	1740	1.109	0.765	0.690
mean		1454	0.821	0.550	0.667
S.E.		83	0.06	0.05	0.03

	Age (days)	Body wt (g)	Muscle wet weight (g) PLD	ALD	$ALD/_{PLD}$
	153	1864	1.105	0.672	0.608
	112	1874	1.070	0.695	0.650
	149	1876	0.996	0.697	0.700
	156	1890	1.027	0.717	0.698
	138	1910	1.111	0.764	0.688
	180	1960	1.268	0.681	0.537
	147	2025	1.346	0.786	0.584
	156	2065	1.120	0.744	0.664
	146	2086	1.121	0.731	0.652
mean		1950	1.129	0.720	0.642
S.E.		29	0.04	0.01	0.02
	146	2276	1.352	0.743	0.550
	154	2300	1.319	0.909	0.689
	114	2323	1.104	0.958	0.868
	180	2355	1.256	0.933	0.743
	180	2428	1.559	0.882	0.566
	154	2450	1.380	1.100	0.797
	180	2476	1.600	1.015	0.634
	180	2484	1.478	0.969	0.656
	170	2488	1.378	0.903	0.655
	170	2496	1.428	0.948	0.664
mean		2408	1.385	0.936	0.680
S.E.		27	0.05	0.03	0.03

APPENDIX J

DYSTROPHIC CHICKENS
ANTERIOR AND POSTERIOR LATISSIMUS DORSI:
CHANGE IN MUSCLE WEIGHT WITH BODY WEIGHT
(AGE 1-180 DAYS)

	Age (days)	Body wt (g)	Muscle wet weight (g) PLD	Muscle wet weight (g) ALD	$ALD/_{PLD}$
	1	28	0.005	0.007	1.400
	1	31	0.007	0.010	1.429
	1	33	0.005	0.009	1.800
	1	34	0.006	0.010	1.667
	1	37	0.009	0.010	1.111
mean		33	0.006	0.009	1.480
S.E.		2	0.001	0.001	0.12
	15	86	0.038	0.030	0.789
	15	96	0.032	0.034	1.063
	15	99	0.035	0.032	0.914
	15	102	0.033	0.034	1.030
	15	104	0.046	0.034	0.739
	15	110	0.041	0.035	0.854
	17	151	0.058	0.044	0.759
	17	156	0.040	0.051	1.275
mean		113	0.040	0.037	0.928
S.E.		23	0.003	0.025	0.65
	28	223	0.064	0.066	1.031
	28	244	0.089	0.124	1.393
	28	262	0.085	0.082	0.965
	32	310	0.127	0.103	0.811
	36	344	0.103	0.100	0.971
	32	347	0.116	0.121	1.043
mean		288	0.973	0.099	1.030
S.E.		22	0.009	0.009	0.79
	50	506	0.198	0.205	1.035
	57	578	0.132	0.215	1.629
	63	617	0.131	0.195	1.489
	56	720	--	0.293	--
	85	860	0.231	0.311	1.346
	85	885	0.277	0.340	1.227
mean		694	0.194	0.260	1.345
S.E.		63	0.028	0.025	0.10

	Age (days)	Body wt (g)	Muscle wet weight (g) PLD	ALD	ALD/PLD
	121	1115	0.418	0.453	1.084
	70	1118	0.416	0.545	1.310
	92	1160	0.476	0.504	1.059
	107	1171	0.518	0.385	0.743
	154	1256	0.368	0.429	1.166
	92	1264	0.464	0.594	1.280
	156	1350	0.607	0.793	1.306
	169	1390	0.561	0.743	1.324
mean		1228	0.479	0.556	1.159
S.E.		37	0.028	0.052	0.07
	167	1589	0.780	0.809	1.037
	110	1594	0.500	0.688	1.376
	107	1637	0.479	0.784	1.637
	110	1677	0.490	0.544	1.110
	151	1705	0.855	0.966	1.130
	110	1725	0.654	0.700	1.070
mean		1655	0.626	0.749	1.227
S.E.		23	0.067	0.058	0.10
	155	1918	1.099	0.980	0.892
	107	1920	0.718	1.300	1.811
	170	1936	0.516	0.772	1.496
	154	1947	0.891	1.052	1.181
	133	1994	0.683	0.680	0.996
	154	2002	0.759	0.903	1.190
	180	2020	0.883	0.679	0.769
	159	2050	0.694	0.744	1.072
	180	2085	0.728	1.010	1.387
	180	2282	0.852	0.815	0.957
	170	2416	0.657	0.829	1.262
mean		2052	0.771	0.888	1.183
S.E.		48	0.046	0.056	0.09

APPENDIX K

NORMAL CHICKENS
ANTERIOR AND POSTERIOR LATISSIMUS DORSI:
CHANGE IN MUSCLE WEIGHT WITH BODY WEIGHT
(AGE >180 DAYS)

Age (days)	Body wt (g)	Muscle wet weight (g) PLD	Muscle wet weight (g) ALD	$ALD/_{PLD}$
251	1090	0.642	0.334	0.520
198	1150	0.720	0.275	0.382
215	1322	0.531	0.330	0.621
511	1324	0.845	0.327	0.387
373	1383	0.744	0.400	0.538
476	1435	0.560	0.315	0.563
295	1454	0.668	0.334	0.500
272	1490	0.958	0.520	0.543
390	1496	0.815	0.484	0.594
mean	1349	0.720	0.369	0.516
S.E.	48	0.05	0.03	0.03
385	1500	0.971	0.157	0.532
476	1512	0.690	0.352	0.510
258	1536	0.740	0.371	0.500
490	1590	0.838	0.438	0.523
469	1604	0.893	0.509	0.570
238	1652	1.136	0.486	0.428
230	1697	0.876	0.407	0.465
205	1700	0.973	0.481	0.494
333	1740	0.915	0.443	0.484
230	1756	1.086	0.428	0.394
373	1766	1.030	0.500	0.463
230	1823	1.050	0.493	0.469
248	1900	1.262	0.821	0.651
386	1909	1.225	0.560	0.457
497	1915	1.000	0.500	0.500
263	1940	1.171	0.790	0.675
490	1943	1.046	0.655	0.626
350	1963	1.200	0.915	0.762
301	1966	1.018	0.610	0.599
469	1973	1.133	0.832	0.734
mean	1769	1.013	0.537	0.542
S.E.	37	0.03	0.04	0.02

Age (days)	Body wt (g)	Muscle wet weight (g) PLD	ALD	ALD/PLD
214	2019	1.430	0.853	0.597
322	2039	0.988	0.640	0.648
330	2051	1.273	0.727	0.571
295	2053	1.033	.602	0.556
353	2066	1.290	0.850	0.659
318	2094	1.237	0.853	0.690
391	2135	1.195	0.736	0.616
268	2137	1.125	0.693	0.616
322	2159	1.107	0.679	0.613
300	2210	1.346	0.840	0.624
268	2230	1.573	0.809	0.514
441	2230	1.317	1.070	0.812
220	2246	1.200	0.860	0.717
230	2246	1.233	0.428	0.347
263	2260	1.440	0.897	0.623
538	2265	1.346	0.756	0.562
198	2275	1.419	0.776	0.547
226	2295	1.333	0.822	0.617
365	2300	1.500	0.877	0.585
226	2315	1.424	0.935	0.657
196	2375	1.637	0.639	0.390
365	2380	1.317	0.900	0.683
446	2432	1.433	1.153	0.805
217	2480	1.353	0.871	0.644
mean	2220	1.314	0.805	0.612
S.E.	26	0.03	0.03	0.02

Age (days)	Body wt (g)	Muscle wet weight (g) PLD	ALD	$ALD/_{PLD}$
210	2510	1.690	1.095	0.648
545	2520	1.174	1.093	0.931
420	2542	1.433	1.011	0.760
267	2552	1.431	0.761	0.514
378	2556	1.613	0.823	0.510
226	2570	1.478	0.998	0.675
420	2599	1.756	1.162	0.662
198	2650	1.910	1.193	0.625
405	2656	1.222	1.080	0.884
196	2660	1.300	0.838	0.645
378	2676	1.373	0.878	0.639
198	2695	1.642	0.872	0.531
350	2718	1.857	1.245	0.670
378	2748	1.500	1.395	0.930
405	2764	1.650	1.517	0.919
405	2830	1.453	1.000	0.688
522	2884	1.477	1.164	0.788
391	2887	1.658	1.377	0.831
365	3000	1.457	1.170	0.803
mean	2685	1.530	1.088	0.719
S.E.	32	0.05	0.05	0.03

APPENDIX L

DYSTROPHIC CHICKENS
ANTERIOR AND POSTERIOR LATISSIMUS DORSI:
CHANGE IN MUSCLE WEIGHT WITH BODY WEIGHT
(AGE >180 DAYS)

	Age (days)	Body wt (g)	Muscle wet weight (g) PLD	ALD	$ALD/_{PLD}$
	365	1137	0.333	0.337	1.012
	232	1182	0.540	0.800	1.481
	376	1205	0.468	0.437	0.934
	423	1284	0.785	0.630	0.803
	532	1285	0.900	0.437	0.486
	322	1290	0.762	0.630	0.827
	454	1358	0.838	0.353	0.421
	207	1380	0.526	0.349	0.663
	537	1400	0.834	0.397	0.476
	399	1470	0.567	0.654	1.153
mean		1299	0.655	0.502	0.826
S.E.		33	0.06	0.05	0.10
	321	1508	0.657	0.588	0.895
	330	1552	0.478	0.490	1.025
	301	1585	0.944	0.540	0.572
	538	1646	0.960	0.596	0.621
	454	1656	0.672	0.560	0.833
	463	1698	0.507	0.400	0.789
	671	1716	0.760	0.560	0.737
	330	1834	0.802	0.461	0.575
	286	1895	0.501	0.721	1.439
	303	1900	1.455	1.157	0.795
	490	1932	0.690	0.605	0.877
	520	1998	1.159	1.018	0.878
mean		1743	0.799	0.641	0.836
S.E.		47	0.08	0.06	0.07

Age (days)	Body wt (g)	Muscle wet weight (g) PLD	ALD	ALD/PLD
686	2029	0.728	0.713	0.979
230	2175	1.039	0.811	0.781
311	2199	1.907	1.525	0.800
257	2220	1.342	1.592	1.186
665	2243	1.327	0.845	0.637
800	2256	0.844	1.170	1.386
368	2272	0.783	0.872	1.114
307	2290	1.730	1.391	0.804
386	2290	1.122	0.756	0.674
547	2310	0.984	1.082	1.100
390	2330	0.983	1.111	1.130
230	2350	1.234	0.998	0.809
672	2360	1.639	1.148	0.700
672	2370	1.771	1.165	0.658
529	2373	1.445	1.549	1.072
299	2387	0.841	0.885	1.016
311	2388	1.045	0.907	0.868
800	2404	1.400	1.251	0.894
654	2450	2.236*	1.726*	0.772
581	2460	0.905	0.841	0.929
214	2465	1.228	1.043	0.849
311	2480	0.803	1.137	1.416
445	2496	1.274	1.190	0.934
mean	2330	1.244	1.118	0.935
S.E.	24	0.08	0.06	0.04

Age (days)	Body wt (g)	Muscle wet weight (g) PLD	ALD	ALD/PLD
286	2550	1.045	0.888	0.850
368	2510	1.162	1.179	1.015
334	2517	1.310	1.335	1.019
287	2520	1.488	1.133	0.761
509	2520	1.376	1.224	0.890
230	2564	1.263	1.045	0.827
451	2565	1.027	1.307	1.273
667	2577	1.699	1.061	0.624
277	2678	1.330	1.300	0.977
583	2680	1.952	1.070	0.548
641	2690	1.950	1.060	0.545
391	2695	1.823	1.420	0.779
572	2700	2.400*	1.446	0.602
310	2730	1.434	0.933	0.651
613	2730	1.808	1.660	0.918
451	2746	1.220	1.547*	1.268
311	2770	1.478	1.183	0.800
460	2780	1.157	1.211	1.047
725	2817	1.024	1.501	1.466
447	2820	1.985	1.838*	0.926
522	2884	2.254*	1.654*	0.734
448	2888	2.378*	1.467	0.617
214	2967	1.317	1.736*	1.318
299	2993	1.704	1.569*	0.921
mean	2703	1.566	1.323	0.891
S.E.	29	0.09	0.05	0.05

APPENDIX M

TESTS OF SIGNIFICANCE

t Test

Student t-test is used for comparing means (usually between two samples, e.g. normal and dystrophic) of data which is measured on a continuous numerical scale. In addition, the variation within the sample should follow a normal (i.e. Gaussian) distribution although the t-test is relatively insensitive to non-normality.

The following is an example of a statistical analysis using the Student's t-test:

Comparison of fibre diameters of the anterior latissimus dorsi between normal and dystrophic chicken at 15 days of age. The results obtained from the measurement of the cell diameters (see Table 5) were,

	number of fibres (n)	mean diameter ($\bar{X}$)	variance (s^2)
Normal	636	28.1	25.2
Dystrophic	1198	28.5	26.1

Comparison of the mean (independent two-sample Student's t-test)

(a) calculation of the pooled standard deviation (Sp)

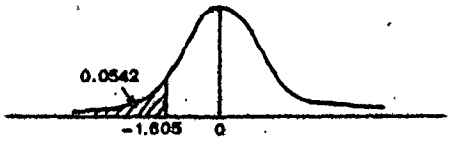
$$S_p = \sqrt{\frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{n_1+n_2-2}} = \frac{635\times25.5 + 1197\times26.1}{636+1198-2} = 5.08$$

(b) calculation of the t value

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_p\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}} = \frac{28.1-28.5}{5.08\sqrt{\frac{1}{636}+\frac{1}{1198}}} = -1.605$$

The degrees of freedom associated with the t statistic are n_1+n_2-2, which for this example is 1832. The significance of the observed value of t is usually obtained from tables of Student's t but with the large number of observations available here the normal distribution is approp-

riate. Thus from tables of the normal distribution for a random variable z, 5.42% of the distribution falls to the left of Z = -1.605



For a two-tailed test the p-value for this observed mean difference is doubled, 0.1084. The p value is a measure of the strength of evidence in support of the null hypothesis of "no difference in population means".

Small p values indicate that the observed data are unlikely to have arisen if the null hypothesis were true and thus more consistent with an alternative hypothesis of a real difference existing. Throughout this work a p-value of 1% or less is considered to be statistically significant and thus an indication of the existence of a real difference. In the example above, there is little evidence to suggest differences in the mean fibre diameter between ALD muscle of normal and dystrophic chicken at 15 days of age.

Note

Where there was a statistical difference between the standard deviations of the groups being compared, Welch's t statistic was used (Armitage, 1971).

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{s_1}{n_1} + \frac{s_2}{n_2}}} \qquad df = \frac{\left[s_1^2/n_1 + s_2^2/n_2\right]^2}{(s_1^2/n_1)^2/(n_1-1) + (s_2^2/n_2)^2/(n_2-1)}$$

In situations where a clear relationship between mean and standard deviation across samples existed, a logarithmic transformation was used. This tended to stabilize the variation from sample to sample and improve the assumption of normality. A test based on log transormed data can be thought of as testing differences in population medians rather than means.

Chi square (x^2) is a test applied to frequency data, i.e. counts of the number of observations falling in a particular range as opposed to records of the actual values. This test is used to compare the shape of two sample histograms.

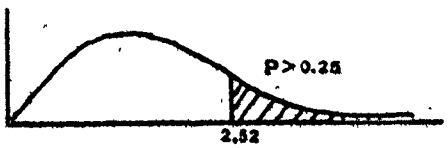
Comparison of fibre distribution

		10 - 19 µm	20 - 29 µm	30 - 39 µm	total %
Normal	n	12	415	209	636
	%	1.9	65.2	38.9	100
Expected		11.1	400.5	224.1	
Dystrophic	n	20	740	438	1198
	%	1.7	61.8	36.5	100
Expected		20.9	754.5	422.6	
Total		32	1155	647	1834
	%	1.7	63.0	35.3	100

Null hypothesis: % distribution same for normal and dystrophic

Observed O	Expected E	Chi Square-Contribution $(O-E)^2/E$
12	11.1	0.07
20	20.9	0.04
415	400.5	0.52
740	754.5	0.28
209	224.4	1.05
438	422.6	0.56

$$x^2 = \sum \left(\frac{O-E}{E}\right)^2 = 2.52 \text{ with. 2 df}$$



From tables of the Chi square distriution for 2 df we see that

$$P\left[\chi_2^2 \geq 2.52\right] > 0.25 \text{ (the closest tabulated value)}$$

It can be concluded that there is little evidence to suggest differences in fibre diameter distribution between ALD fibre of normal and dystrophic chickens at 15 days of age.

APPENDIX N

NORMAL CHICKENS
TETANIC TENSION OF THE ANTERIOR LATISSIMUS DORSI
DURING DEVELOPMENT EX OVO

Age (days)	Muscle wet wt (g)	Tetanic Tension (g) 50 HZ	70 HZ	100 HZ
28	0.126	16	18	23
56	0.219	41	44	47
42	0.230	37	41	48
42	0.250	53	56	60
mean	0.206	36.8	39.8	44.5
S.E.	0.027	7.7	7.9	7.8
56	0.255	33	36	37
60	0.266	42	46	51
63	0.288	58	67	71
57	0.300	70	76	78
511	0.327	50	58	62
mean	0.287	50.6	56.6	59.8
S.E.	0.013	6.4	7.2	7.3
120	0.437	87	73	180
193	0.473	94	100	106
205	0.481	84	90	93
390	0.484	80	88	88
469	0.509	97	--	--
385	0.517	89	99	105
272	0.520	73	80	95
mean	0.489	86.0	88.4	111.2
S.E.	0.011	3.1	4.3	14.1
148	0.556	147	158	165
318	0.853	147	156	172
441	1.071	200	212	210
mean	0.827	164.7	175.3	182.3
S.E.	0.149	17.7	18.3	14.0

APPENDIX O

DYSTROPHIC CHICKENS
TETANIC TENSION OF THE ANTERIOR LATISSIMUS DORSI
DURING DEVELOPMENT EX OVO

	Age (days)	Muscle wet wt (g)	Tetanic Tension (g) 50 HZ	70 HZ	100 HZ
	63	0.195	53	56	62
	50	0.205	40	41	50
	57	0.215	53	55	55
mean		0.205	48.7	50.7	55.7
S.E.		0.006	4.3	4.8	3.5
	196	0.343	97	100	103
	209	0.400	68	72	74
	362	0.403	129	144	150
	154	0.429	58	70	79
	376	0.437	65	71	76
	121	0.453	114	120	129
mean		0.411	88.5	96.2	101.8
S.E.		0.016	11.9	12.6	12.9
	92	0.504	101	101	106
	220	0.505	65	74	76
	70	0.545	171	176	180
	671	0.560	117	126	132
	92	0.594	85	109	121
	247	0.620	160	160	165
	399	0.654	168	174	186
mean		0.569	123.9	131.4	138.0
S.E.		0.022	16.2	14.9	15.4
	169	0.743	133	150	167
	156	0.793	108	138	175
	232	0.800	141	118	106
	151	0.966	176	148	192
	155	0.980	108	122	156
mean		0.856	133.2	135.2	159.2
S.E.		0.049	12.6	6.6	14.5

	Age (days)	Muscle wet wt	Tetanic response (g) 50 HZ	70 HZ	100 HZ
	390	1.111	240	288	312
	368	1.179	348	384	384
	509	1.224	212	224	235
	334	1.335	276	300	300
	391	1.420	408	432	476
mean		1.254	296.8	325.6	341.4
S.E.		0.055	35.9	36.8	41.1

APPENDIX P

NORMAL CHICKENS
TWITCH AND TETANIC TENSIONS OF THE POSTERIOR LATISSIMUS DORSI
DURING DEVELOPMENT EX OVO

Age (g)	Muscle wet wt (g)	P_t (g)	P_o (g)	P_t/P_o Ratio
15	0.060	14	24	0.58
28	0.143	47	111	0.42
51	0.257	93	168	0.55
42	0.307	120	276	0.43
56	0.318	132	228	0.58
42	0.325	88	189	0.47
57	0.325	139	276	0.50
60	0.338	108	227	0.48
56	0.349	162	276	0.59
63	0.372	113	336	0.34
mean	0.324	119.4	247.0	0.49
S.E.	0.012	8.7	19.3	0.03
198	0.720	138	--	--
120	0.721	252	528	0.48
120	0.726	252	--	--
148	0.780	182	459	0.40
mean	0.737	206	493.5	0.44
S.E.	0.014	28.0	34.5	0.04
390	0.815	342	600	0.57
542	0.836	228	672	0.34
511	0.845	288	828	0.35
138	0.879	233	483	0.48
85	0.893	213	--	--
469	0.893	224	553	0.41
mean	0.860	254.7	627.2	0.43
S.E.	0.013	20.5	58.8	0.04
385	0.900	255	--	--
423	0.933	235	541	0.43
385	0.971	270	720	0.38
205	0.973	252	648	0.39
mean	0.944	253	636.4	0.40
S.E.	0.017	7.2	52.0	0.02

Age (g)	Muscle wet wt (g)	P_t (g)	P_o (g)	P_t/P_o Ratio
301	1.018	378	600	0.63
149	1.109	252	768	0.33
149	1.110	228	816	0.28
469	1.133	336	600	0.56
545	1.174	300	864	0.35
511	1.207	241	506	0.48
318	1.237	396	648	0.61
441	1.317	290	606	0.49
300	1.346	384	756	0.51
446	1.433	384	720	0.53
365	1.457	360	998	0.36
522	1.477	218	707	0.31
323	1.637	353	800	0.44
mean	1.274	316.9	722.2	0.45
S.E.	0.053	18.1	36.7	0.03

P_t = maximum twitch tension; P_o = maximum tetanic tension

APPENDIX Q

DYSTROPHIC CHICKENS
TWITCH AND TETANIC TENSION OF THE POSTERIOR LATISSIMUS DORSI
DURING DEVELOPMENT EX OVO

	Age (days)	Muscle wet wt (g)	P_t (g)	P_o (g)	P_t/P_o Ratio
	15	0.038	5	13	0.38
	15	0.040	4	14	0.29
	15	0.041	4	11	0.36
	15	0.046	12	25	0.48
	17	0.058	7	--	--
	28	0.064	6	13	0.46
	28	0.089	14	34	0.41
mean		0.054	7.4	18.3	0.40
S.E.		0.007	1.5	3.7	0.03
	56	0.106	18	--	--
	32	0.116	24	65	0.37
	32	0.127	38	72	0.53
	63	0.131	20	95	0.21
	57	0.132	19	89	0.21
	50	0.198	19	46	0.41
mean		0.135	23.0	73.4	0.35
S.E.		0.013	7.6	8.8	0.06
	365	0.333	17	94	0.18
	154	0.368	20	131	0.15
	70	0.416	85	255	0.33
	121	0.418	41	255	0.16
	209	0.452	41	215	0.19
	92	0.464	24	--	--
	376	0.468	22	118	0.19
	92	0.476	35	151	0.23
mean		0.424	35.6	174.1	0.20
S.E.		0.018	7.8	25.2	0.02

	Age (days)	Muscle wet wt (g)	P_t (g)	P_o (g)	P_t/P_o Ratio
	362	0.516	44	294	0.15
	232	0.540	94	382	0.25
	141	0.541	73	329	0.22
	196	0.557	70	329	0.21
	169	0.561	69	--	--
	399	0.567	113	396	0.29
	156	0.607	30	120	0.25
	247	0.623	--	320	--
	321	0.657	43	253	0.17
mean		0.574	67.0	302.9	0.22
S.E.		0.015	9.8	30.6	0.02
	154	0.759	110	480	0.23
	671	0.760	62	264	0.23
	322	0.762	56	250	0.22
	423	0.785	80	306	0.26
mean		0.766	77.0	324.0	0.23
S.E.		0.006	12.1	53.0	0.01
	515	0.829	120	490	0.24
	537	0.834	101	552	0.18
	800	0.844	100	324	0.31
	151	0.855	80	300	0.27
	251	0.871	99	400	0.25
	581	0.905	108	480	0.22
	301	0.944	156	528	0.30
	538	0.960	101	408	0.25
mean		0.880	108.1	422.7	0.25
S.E.		0.018	7.9	29.9	0.01
	155	1.099	50	--	--
	520	1.159	63	576	0.11
	368	1.162	168	600	0.28
	445	1.274	46	408	0.11
	334	1.310	99	576	0.17
	665	1.327	138	855	0.16
	257	1.342	96	529	0.18
	509	1.376	88	565	0.16
	800	1.400	108	258	0.42
	303	1.455	130	706	0.18
mean		1.290	98.6	563.7	0.20
S.E.		0.037	12.4	56.0	0.03

Age (days)	Muscle wet wt (g)	P_t (g)	P_o (g)	P_t/P_o Ratio
672	1.771	108	528	0.21
391	1.823	224	792	0.28
641	1.950	126	887	0.14
583	1.952	75	720	0.10
447	1.985	84	624	0.13
448	2.378	96	360	0.27
572	2.400	114	648	0.18
mean	2.037	118.1	651.3	0.19
S.E.	0.095	18.8	65.6	0.03

P_t = maximum twitch tension; P_o maximum tetanic tension

APPENDIX R

CONTRACTION AND RELAXATION TIMES OF THE SELF REINNERVATED POSTERIOR LATISSIMUS DORSI MUSCLES

I - Normal Chickens

Age (days)	Twitch T½C	Twitch Tc	Twitch T½R	Tetanic 50 Hz T½C	Tetanic 50 Hz T½R	Tetanic 70 Hz T½C	Tetanic 70 Hz T½R
110	13	29	18	35	59	35	59
181	22	45	22	--	--	--	--
257*	14	30	19	26	45	39	52
272	15	35	27	39	26	45	43
423	17	38	29	42	47	35	47
441	15	39	18	47	47	47	59
469	21	47	35	47	47	47	35
469	18	36	20	36	48	24	48
545	16	39	18	36	48	36	48
mean	16.8	37.6	22.9	38.5	45.9	38.5	48.9
S.E.	1.0	2.0	2.0	2.5	3.2	2.8	2.8

II - Dystrophic Chickens

107	15	29	17	--	--	--	--
141*	12	29	25	35	35	35	29
154	10	22	19	39	39	39	26
207	15	35	26	47	29	47	29
251	18	38	24	42	59	42	59
448	18	33	30	48	60	36	48
515	18	33	21	48	43	48	48
520	17	36	32	60	53	60	60
537	17	36	27	53	29	43	29
671	15	30	21	60	36	36	36
mean	15.5	32.1	24.2	48.0	42.6	42.9	40.4
S.E.	2.7	1.5	1.5	2.9	4.0	2.7	4.5

T½C = time to half contraction; Tc = contraction time; T½R = time to half relaxation

* maximum twitch tension measurements were not obtained from these chickens.

APPENDIX S

CONTRACTION AND RELAXATION TIMES OF THE CROSS REINNERVATED POSTERIOR LATISSIMUS DORSI MUSCLES

I - Normal Chickens

Age (days)	Tetanic 50 Hz		Tetanic 70 Hz	
	$T_{\frac{1}{2}}C$	$T_{\frac{1}{2}}R$	$T_{\frac{1}{2}}C$	$T_{\frac{1}{2}}R$
28	113	94	94	71
28	129	59	106	47
92	194	129	129	90
114	223	94	--	--
193	265	155	151	78
511	235	294	188	118
522	141	194	147	147
mean	185.7	145.6	135.8	91.8
S.E.	22.2	29.9	13.9	14.6

II - Dystrophic Chickens

92	335	206	258	116
92	290	155	239	90
151	235	165	212	165
167	206	77	90	64
169	161	96	--	--
232	235	176	147	176
247	90	103	77	103
257	238	97	194	65
362	188	122	176	118
376	132	147	118	176
504	147	124	118	118
509	206	176	147	147
511	252	120	180	120
538	255	195	226	170
572	240	150	240	150
581	270	278	240	180
641	270	180	210	180
672	330	120	240	90
686	180	150	150	132
800	270	180	270	150
800	210	180	180	180
mean	225.7	152.2	185.6	134.5
S.E.	13.5	46.0	12.7	8.6

$T_{\frac{1}{2}}C$ = time to half contraction; $T_{\frac{1}{2}}R$ = time to half relaxation

MEAN FIBER DIAMETERS OF EXPERIMENTAL AND UNOPERATED MUSCLES

I - Frequency distribution

Range of Fiber diameter (μm)	Frequency									
	Normal 3 mos			Dystrophic 3 mos			12 mos	Normal 18 mos		Dystrophic 18 mos
	PLD	ALD	PLD X-RI	PLD	ALD	PLD X-RI	PLD X-RI	ALD	PLD X-RI	PLD X-RI
20- 29	8	--	--	13	1	2	--	--	--	--
30- 39	21	7	4	26	12	18	11	--	1	14
40- 49	59	34	17	13	53	22	20	2	2	34
50- 59	88	53	34	10	66	22	23	15	10	47
60- 69	33	84	59	10	250	33	24	22	16	57
70- 79	9	97	79	11	224	17	13	30	14	44
80- 89	--	59	68	15	67	28	39	27	18	61
90- 99	--	18	31	21	11	18	46	35	17	60
100-109	--	6	20	17	1	15	71	48	11	36
110-119	--	1	2	8	1	10	77	27	10	21
120-129	--	--	4	12	--	2	45	4	5	8
130-139	--	--	--	8	--	--	40	--	5	2
140-149	--	--	--	2	--	1	6	--	--	2
150-159	--	--	--	1	--	--	1	--	--	--
	218	359	318	167	686	188	416	210	109	386
$\bar{X}$	51.6	69.3	75.8	76.9	67.7	71.9	98.9	89.8	87.3	78.4
S.D.	10.9	14.9	17.0	35.0	11.9	24.6	27.4	19.2	23.1	23.1

Cont'd

II - Comparative Analysis

Age (mos)	genotype	Muscles Compared		mean diameter (X̄) t	P*	Frequency Distribution χ^2	df**	P*
3	N	PLD;	PLD X-RI	18.61	0.001	241.68	4	0.001
3	N	ALD;	PLD X-RI	5.42	0.001	29.49	7	0.001
3	D	PLD;	PLD X-RI	1.57	N.S.	48.75	10	0.001
3	D	ALD;	PLD X-RI	3.29	0.001	202.8	7	0.001
18	N	ALD;	PLD X-RI	1.03	N.S.	19.57	8	N.S.
18	N, D	PLD X-RI;	PLD X-RI	3.55	0.001	14.47	9	N.S.

* two-tail probability

** ranged combined where appropriate

APPENDIX U

RATIO OF CELL DIAMETERS OF INDIVIDUAL MUSCLE FIBERS

Ratio of cell diameters	Frequency					
	Normal			Dystrophic		
	PLD	ALD	PLD X-RI	PLD	ALD	PLD X-RI
1.0	30	17	25	9	16	47
1.1	45	35	45	26	27	76
1.2	35	27	34	23	24	54
1.3	42	23	41	25	23	50
1.4	31	19	41	20	13	45
% of Total	60	51	58	42	53	65
1.5	32	20	36	17	13	29
1.6	14	23	38	12	14	29
1.7	18	20	18	14	14	25
1.8	6	11	14	8	7	12
1.9	15	10	11	10	14	11
% of Total	28	35	37	25	32	25
2.0	8	6	6	9	9	16
2.1	7	5	3	10	6	5
2.2	7	2	1	6	3	3
2.3		3	1	7	3	6
2.4	2	2	1	4		5
% of Total	8	8	4	15	11	8
2.5	3	4	2	6	2	2
2.6	3	4		3	2	
2.7	1	1	1	9	2	1
2.8	1	1		2	1	
2.9	1	1		2		
% of Total	3	5	1	9	3	1
3.0				3		1
3.1				3		1
3.2	1	1			1	
3.3		1		2	1	
3.4	1			13		
% of Total	1	1		9	1	1
Total no.	303	236	318	243	195	418