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

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MANIPULATIONS OF AVIAN DYSTROPHIC MUSCLE
TITLE: Experimental Manipulation Which Results in the Phenotypic Expression of the Dystrophic Process: Cross-reinnervation of a Slow Tonic Muscle by the Nerve of a Fast Twitch Muscle in Chickens With Hereditary Muscular Dystrophy

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ABSTRACT

In chickens afflicted with hereditary muscular dystrophy, the two major types of muscle present respond dissimilarly to the disease process: fast twitch glycolytic muscles possess and express the dystrophic gene overtly during ex ovo development while the genotypically dystrophic slow tonic muscles fail to express dystrophic phenotypes. Therefore, in chickens, muscular dystrophy is muscle fibre type specific.

The primary goal of this thesis was to experimentally alter the genetically dystrophic slow tonic muscle in an attempt to induce this muscle to express dystrophic phenotypes. Since motor nerves influence the phenotypes of skeletal muscles, it was decided to replace the motor innervation of a slow tonic muscle with that of a fast twitch muscle within a dystrophic chicken. The surgical cross-reinnervation between the ALD muscle and the 'fast' nerve was performed at hatching by transposing the right ALD muscle to the left side of the back in order to prevent selective self-reinnervation by the severed ALD nerve. The experimental muscles were examined at various time intervals from 2 to 104 weeks postoperatively and compared to age-matched ALD muscles in genetically normal chickens. In addition, denervation of ALD muscles, with and without transposition, served as control experiments.

Selected histochemical and structural properties of unoperated ALD and fast twitch posterior latissimus dorsi (PLD) muscles of normal and dystrophic chickens were compared between 2 and 32 weeks ex ovo to
provide criteria for the analysis of muscles cross-reinnervated by 'fast' nerves and to determine which of these parameters were altered as a result of the dystrophic process. Normal and dystrophic ALD muscles exhibited similar phenotypes: acid and alkaline stable myosin ATPase activity, 'en grappe' endplates, weak glycolytic and strong oxidative capacities, and peripheral location of nuclei. Furthermore, the growth rate, size, and shape of fibres in the normal and dystrophic ALD muscles were similar. In contrast, the myosin ATPase and innervation patterns of normal and dystrophic PLD muscles differed from those of ALD muscles: PLD fibres of either genotype exhibited alkaline stable, acid labile myosin ATPase activity and focal 'en plaque' innervation. Normal and dystrophic PLD muscles also exhibited different phenotypes: dystrophic muscles had a lower muscle weight, abnormal size, shape, and growth rate of fibres, increased number of scattered nuclei, and abnormal glycolytic and oxidative capacities.

The results presented from this work indicate that the genetically dystrophic ALD muscles respond differently to cross-reinnervation than do normal ALD muscles. The cross-reinnervated muscles in normal birds demonstrated all of the characteristics of an unoperated ALD muscle with the exception of the presence of isolated groups of fibres exhibiting a fast twitch type of myosin ATPase response. This result suggests that the principle response of normal ALD muscles to a foreign 'fast' nerve is one of resistance to alteration. In contrast, data from the experimental ALD muscles of dystrophic genotype support the conclusion that these muscles are dramatically remodelled after cross-reinnervation. An augmented regenerative response within the dystrophic muscles...
muscles resulted in hyperplasia which, in turn, led to a complete restructuring of these muscles. Therefore, the present cross-reinnervation experiments demonstrate, for the first time, that a phenotypically normal muscle of dystrophic genotype can be induced to express a dystrophic characteristic: an augmented regenerative response after an experimentally-induced injury. It is important to note that the initial induction of this dystrophic phenotype was demonstrated in the absence of the foreign 'fast' nerve and seemed to be due to the response of this muscle to the severe injury imposed upon the muscle during the initial operation.
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LIST OF ABBREVIATIONS

ACh  - acetylcholine
AChE - acetylcholinesterase
AChR - acetylcholine receptor
ALD  - anterior latissimus dorsi
ATPase, - adenosinetriphosphatase
da  - day
DLD  - dorsocutaneous latissimus dorsi
DNA  - deoxyribonucleic acid
epp  - end plate potential
H+  - acid
Hz   - hertz (cycle per second)
i.e.  - that is
LC   - light chains of myosin
LD   - latissimus dorsi
mg   - milligram
msec - millisecond
mV   - millivolt
MLD  - metapatagial latissimus dorsi
NMJ  - neuromuscular junction
OH-  - alkaline
Pase - phosphorylase
PLD  - posterior latissimus dorsi
RNA  - ribonucleic acid

(xii)
SB - superior brachialis
SDH - succinic dehydrogenase
S.E.M. - standard error of the mean
St - stage
\( \mu m \) - micrometer
wk - week
X-RI - cross-reinnervated
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). In the chicken, the anterior latissimus dorsi (ALD) muscle consists almost entirely of slow tonic fibres. This circumstance provides an ideal opportunity to study not only the in ovo differentiation of these fibres within a single muscle but to follow the ex ovo maturation of these same cells. Therefore, the following review is divided into two sections: the first deals with the in ovo differentiation of ALD muscle fibres during embryogenesis and is followed by an investigation of the maturation of these fibres ex ovo.

DEVELOPMENT OF THE ALD MUSCLE IN OVO

Development of the ALD muscle during embryogenesis can arbitrarily be divided into three different stages. In the first stage, between one and seven days in ovo, the ALD muscle, along with the other brachial muscles within the dorsal muscle mass, forms and differentiates a specific myosin ATPase profile. As will be discussed, these phenomena can occur independent of any neuronal input. The second stage, between eight and thirteen days in ovo, is characterized by the initiation and development of innervation on embryonic ALD myogenic cells. During the
third and final stage, from day 14 to hatching, the electrical properties of ALD muscle fibres are recorded for the first time, contractile responses first elicited, and the synthesis of metabolic enzymes first noticed within embryonic ALD muscles (see Figure 1).

Stage 1 - Formation of ALD Muscles (1-7 days in ovo)

Using intraspecific chick-quail chimaeras, Beresford (1983) has shown that the ALD muscle originates from myogenic cells located in primary somites 18 through 20. Between 2 to 3.5 days in ovo, these pre-ALD myogenic cells migrate from the appropriate somites to form a part of the dorsal muscle mass within the anterior limb bud (Chevalier, 1978). It is during this period that spinal nerves forming the brachial plexus, follow the migrating cells, but only up to the base of the limb bud (Butler and Cosmos, 1981). Within the next twelve to twenty-four hours (4.0 to 4.5 days), two major events occur: the nerves of the brachial plexus continue their migration into the wing bud and the formation of myotubes is initiated within the now lobulated dorsal and ventral muscle masses by the fusion of myogenic cells (Hilfer et al., 1973). It is possible, but not proven, that the myosin synthesized by these newly-formed myotubes is responsible for the alkali stable myosin ATPase activity detected within the anterior limb bud at 4.5 days in ovo (Butler and Cosmos, 1981).

Cleavage of the primary muscle masses begins at 5.0 days in ovo and by 5.5 days individual muscle primordia are observed (Grim, 1971; Butler and Cosmos, 1981). At this stage, the common latissimus dorsi primordium, the anterior part of which will eventually give rise to the
Figure 1. Diagrammatic summary of the development of various properties of the ALD muscle during in ovo growth of the chick embryo. Adapted from Butler and Cosmos (1981). It should be emphasized that the placement of some of these properties represents the time when they are first detected but not necessarily when they first form.
ALD muscle, is now separated from the dorsal muscle mass and demonstrates an alkali stable myosin ATPase activity only (Butler and Cosmos, 1981). Twelve hours later (6.0 days), a physical division of the common latissimus dorsi primordium begins to become apparent. This cleavage continues to 6.5 days and is completed by seven days in ovo. Concomitant with the cleavage of the common latissimus dorsi primordium, Sullivan (1962) has demonstrated that the nerve which innervates this primordium also bifurcates between 6.0 and 6.5 days. Furthermore, it is during this period (at 6.5 days), that cells within the anterior region of the common latissimus dorsi primordium begin to express a myosin ATPase profile similar to that observed in ALD muscles ex ovo, i.e., an acid and alkali stable myosin ATPase activity (Butler and Cosmos, 1981). That the ALD muscle expresses a mature myosin ATPase profile early within in ovo development has also been observed by Toutant et al. (1979) who demonstrated the acid and alkali stable response of the ALD muscle to the myosin ATPase reaction at eight days in ovo.

Two points regarding the formation and differentiation of the ALD muscle should be emphasized at this time. The first is that the events described above precede the formation of permanent neuromuscular junctions on ALD muscle cells which begins at about eight days in ovo (Atsumi, 1977). Before 8 days, pioneering axon tips freely explore and, at times, seem to 'palpate' the surface of the myogenic cells which they encounter in their path. These transient nerve–muscle interactions may be observed as early as the 6th day but occur with a much higher frequency after the 8th day (Sisto Daneo and Filogamo, 1976). The
second point is that the formation and differentiation of the brachial musculature, including the ALD muscle, proceeds on schedule independent of any neuronal input from the brachial plexus (Butler et al., 1982). These investigators showed that removal of the brachial neural tube at two days in ovo had no effect on the formation of individual brachial muscles and their initial differentiation of appropriate myosin ATPase profiles. Furthermore, this work also implies that the fusion of myogenic cells into myotubes is unaffected by the aneural condition in vivo.

Stage 2 - Establishment of Innervation on ALD Muscles (8-14 days in ovo)

At the beginning of the second week of embryogenesis, the ALD muscle consists of a band of longitudinally-oriented myotubes. Situated within this band are individual myoblasts as well as myoblasts undergoing fusion with other myoblasts and with myotubes (Bennett and Pettigrew, 1974a). An electron microscopic examination of these myogenic cells in vivo by Lentz and associates (Jacob and Lentz, 1979; Burrage and Lentz, 1981) has yielded the following results. Soon after myotube formation, the sarcolemma of these cells begins to change. Whereas most of the myotube cell surfaces remain unspecialized and underdeveloped, small areas of dense material begin to arise within the sarcolemma. These focal 'surface patches' are morphologically specialized, mobile, and can be identified as slightly elevated ridges of the sarcolemma. Associated with each ridge is an external layer of amorphous material and an internalized layer of submembranous dense material. The submembranous material consists of cytoplasmic
microfilaments, microtubules, and coated vesicles while the extraneous coating is thought to be the precursor of the basement membrane. In addition, nicotinic acetylcholine receptors (AChR) and acetylcholinesterase (AChE) are associated with these surface patches.

Whether or not these surface patches represent recognition sites on uninnervated ALD myotubes for incoming nerves or represent the response of the myotube sarcolemma to the neuronal influences arising from innervating axon terminals is unknown at this time. Recent experiments by Sohal and co-workers on another slow tonic muscle, the superior oblique muscle of the Peking duck embryo, suggest that these surface patches may act as targets for incoming nerves. In these experiments, superior oblique muscles were rendered aneural by the permanent destruction of the innervating motoneurons prior to the entry of these motoneurons into the muscle. When the myotubes of the aneural muscles were examined, clusters of AChR (Creazzo and Sohal, 1983), patches of sarcolemmal specializations (Sohal and Wrenn, 1983), and distinct spots of AChE (Sohal and Wrenn, 1984) could be identified on the surfaces of these myotubes. These results imply that specializations of the surface of myotubes in vivo can form in the absence of, and therefore prior to, any neuronal input. Similar conclusions can be drawn from the work of Bekoff and Betz (1976). In the latter experiments, lumbosacral spinal cord segments 21 to 30 were surgically extirpated from stage 15 to 16 chicks. Explant cultures of mesodermal cores of hindlimb buds taken from 3 to 4 day old aneural embryos were analyzed after 6 to 7 days in culture and the myotubes were found to have synthesized 'hot' spots or clusters of AChR on their surfaces. Clusters of AChR have also been
described on the surface of non-innervated myotubes in primary cultures raised from dissociated 10- to 12-day old embryonic chick pectoralis muscle (Fishbach and Cohen, 1973; Prives et al., 1976; Betz and Osborn, 1977). Whether or not the results of all these experiments can be extrapolated to the development of surface patches on ALD myotubes is unknown at the present time. However, analyses of myotubes within the aneural ALD muscles produced by Butler et al. (1982) would definitely answer this question.

Recapping, by eight days in ovo, the ALD muscle is composed of an array of myogenic cells. On the surface of many of the newly-formed myotubes are small but highly specialized regions of membrane distributed randomly throughout the sarcolemma. However, the distribution of these surface patches does not remain scattered but is altered upon innervation of the myotubes.

According to Atsumi (1977), undifferentiated axon terminals begin to form junctions with ALD myotubes at about 8 days in ovo. Soon after contact has been established between an axon terminal and the sarcolemma, surface patches begin to accumulate and become co-extensive within the region of the synapse and its immediate surroundings (Sisto Daneo and Filogamo, 1975; Jacob and Lentz, 1979; Burrage and Lentz, 1981). Simultaneously, the density of surface patches declines in the extrajunctional regions and eventually these patches disappear from these regions. Therefore, it seems that nerves are involved in stability of these surface patch clusters. Further evidence for this has been demonstrated in vitro where it has been shown that clusters of AChR form within 7 days of plating but completely disappear from the
surface of uninnervated myotubes (Prives et al., 1976) by 10 days. As outlined in a review by Bennett (1983), nerves could influence the surface patches through one of several mechanisms: 1) via a direct interaction of surface molecules within the membrane of the axon terminal with the myotube sarcolemma; 2) by secreting a trophic factor, or 3) by establishing a localized but steady synaptic potential thereby inducing the diffusion of surface patches to the junction and acting to stabilize the clusters while at the synapse. In support of this latter mechanism, Gordon and Vrbová (1974) have shown that blockage of synaptic transmission by the administration of curare to embryos from day-11 to day-14, prevents the establishment of multiple deposits of AChE along the length of developing ALD muscle fibres.

By the use of intracellular recordings, Bennett and Pettigrew (1974a) have shown that many of the junctions between incoming axon terminals and ALD myotubes become functional by nine days in ovo; while spontaneous electrical activity, recorded as miniature end plate potentials could be recorded in a few myotubes at different locations within the ALD muscle, the detection of evoked potentials was more consistent and could be recorded in most of the myotubes impaled. In addition, morphological analyses of the nerve terminals supports the electrophysiological evidence that these endplates are becoming functional; small agranular vesicles, coated vesicles and large dense-cored vesicles have been identified in many of the terminals (Bennett and Pettigrew, 1974a; Atsumi, 1977).

The establishment of polyneuronal innervation on the ALD myotubes has been detected electrophysiologically at nine days in ovo by Bennett
and Pettigrew (1974a). However, before their evidence is presented, it is necessary to clarify what is meant by 'multiple' and 'polyneuronal' innervation. When a muscle fibre is 'multiply' innervated this means that there are two or more neuromuscular junctions along the longitudinal axis of the fibre. A muscle fibre is 'polyneuronal' if two or more nerve axons are involved in the formation of a single neuromuscular junction. In order to quantify the number of nerve terminals innervating a single endplate, Bennett and Pettigrew gradually increased the stimulus intensity to the primary ALD nerve trunk in an attempt to recruit an increasing number of axons within the nerve trunk. By doing this, these investigators interpreted each consistent change in the end plate potential response (i.e., the time course and amplitude of the end plate potential response) to the activation of an additional nerve terminal synapsing on the muscle cell. When this type of approach was used on impaled myotubes in nine day old ALD muscles, changes in the response of the end plate potentials in most cells indicated that they received polyneuronal innervation. Polyneuronal innervation of 9 to 11 day old ALD muscles has since been implied by the morphological analyses of Sisto Daneo and Filogamo (1976) and confirmed by Atsumi (1977) in eleven and eighteen day old preparations. Additional experiments performed by Bennett and Pettigrew (1974a) show that the number of terminals innervating an endplate increases during the last week of embryogenesis to a maximum of approximately ten terminals per endplate. After hatching, this number drops to approximately four terminals per endplate. However, even though the number of terminals per endplate changes during development, polyneuronal innervation of the ALD muscle
persists even in adult birds (Bennett and Pettigrew, 1974a).

Identification of multiple innervation, on the other hand, can be made with the AChE reaction soon after detection of polynervous innervation. Bennett and Pettigrew (1974a) have shown that between eleven to thirteen days in ovo, when about 50% of the ALD muscle cells are myofibres, localized AChE deposits can be observed occurring in register across individual fascicles of muscle cells and at regular intervals.

In summary, initial contacts between nerves and the embryonic ALD muscle cells are established at about eight days in ovo. Polynervous and functional innervation of the ALD muscle cells begins at nine days in ovo while the establishment of multiple innervation on muscle fibres cannot be detected with the AChE reaction until day-11.

It has been hypothesized that the multiple innervation of developing ALD muscles is attributable to the size of the end plate potentials produced by nerve terminals on the ALD muscle fibres (Gordon et al., 1974). To test this hypothesis, these investigators attempted to decrease the size of the end plate potentials (epp's) produced on embryonic ALD muscle fibres to see whether or not there would be a subsequent decline in the distance between individual endplates. In order to interrupt the interaction of acetylcholine with the muscle membrane, curare was injected into chick embryos between 11 and 14 days in ovo and the ALD muscles were examined a week after the final injection. When compared to 21-day old control ALD muscles, the distance between the endplates on curare-treated muscle fibres was found to be significantly smaller. These results implied that the local
depolarization produced by an epp was sufficient to make the muscle refractory within a limited area. However, outside this area, the muscle fibre membrane remained sensitive to acetylcholine and therefore receptive to additional incoming nerves.

The interpretation of this experiment by its authors, however, is limited in several ways. Unfortunately, the amplitudes of the epp's in curarized muscles were never measured in order to determine whether or not they had actually been altered. In addition, Gordon and Vrbová assumed that the innervation of an ALD muscle was initiated at about eleven days in ovo and therefore began the administration of curare at this time. However, as discussed previously, Atsumi (1977) and Bennett and Pettigrew (1974a) have both presented morphological and electrophysiological evidence that innervation is initiated at about 8–9 days in ovo. Therefore, at the time when Gordon and Vrbová initiated the curare injections, endplates had already formed on the ALD muscle fibres. Perhaps the strongest evidence against the hypothesis of Gordon and Vrbová is presented by an analysis of the cable properties of the developing ALD muscle fibres. According to the recent experiments of Poznansky and Steele (1984), an ALD muscle fibre isolated from a 14-day old embryo has an average length constant of 1.7 mm. This means that the amplitude of an epp will have declined by 67% after it has travelled 1.7 mm away from its point of origin (i.e., from an endplate).

Furthermore, from the work of Bennett and Pettigrew (1974a), it is known that the mean distance between endplates on a 14 day old embryonic ALD muscle fibre is about 164 μm's. With this information, we can predict the percent drop in the amplitude of an epp 164 μm's away from its point
of origin by the following equation:

\[ \frac{V_x}{V_0} = e^{-x/\lambda} \]

where \( V_x \) \( / \) \( V_0 \) = the percent drop in the amplitude of the epp
\( x \) = the distance along the membrane (\( \mu \)m)
\( \lambda \) = the length constant (\( \mu \)m)

Solving for \( V_x \)/\( V_0 \) shows that the amplitude of an epp initiated at an endplate will have dropped off by only ten percent by the time it reaches the next endplate. This implies that the amplitude of an epp has to drop by only 10% before the muscle fibre membrane becomes receptive to further synaptic input. Direct electrophysiological evidence for this is still lacking. In a later study, Gordon and co-workers injected hemicholinium into chick embryos between days 11 and 14 of incubation and examined the distribution of endplates on the ALD muscles at day-21 (Gordon et al., 1975). Hemicholinium is a drug which prevents or reduces the amount of transmitter released from nerve terminals and will therefore affect the size of the epp. However, upon examination of the ALD muscles, these investigators found that treatment with hemicholinium had no effect on the distance between successive endplates. Finally, when Oppenheim et al. (1978) attempted to replicate the findings of Gordon and Vrbová, they found that curarized embryos developed more slowly than untreated embryos; treated embryos showed a distinct developmental lag of approximately one day and curarized ALD muscle fibres were smaller than control ALD fibres. Since the distance between endplates is directly related to the length of ALD
muscle fibres (Ginsborg and Mackay, 1961; Bennett and Pettigrew, 1974a), the developmental lag expressed by the curarized embryos could account for the decrease in the distance between endplates observed by Gordon and Vrbová. In light of these discrepancies, the work of Gordon et al. (1974) is inconclusive and the mechanism(s) responsible for the separation of endplates on multiply-innervated ALD muscle fibres remains to be elucidated.

Before the last week of embryogenesis is discussed, a final point must be made. Interaction between the ALD muscle and its nerve is critical during the second week of embryogenesis for the growth and survival of this muscle is severely impaired if the ALD muscle is rendered aneurogenic early in embryogenesis (Buțler et al., 1982). This dependency is based to a large degree on the ability of the nerves to electrically stimulate the ALD muscle. In support of this statement, Bloom et al. (1985) have shown that direct stimulation alone serves to enhance significantly the growth and survival of embryonic ALD muscles which were rendered aneural early in embryogenesis.

In summary, stage 1 can be characterized as that period of time in embryogenesis when the ALD muscle forms and the myogenic cells within the muscle begin to express a myosin ATPase activity. The incipient expression of the myosin ATPase activity is correlated with the formation of myotubes and is neurally-independent. The second stage is characterized as a period of time when there is a long period of intense and dynamic contact between the nerves and myogenic cells in the ALD muscle. Myotubes begin to differentiate into myofibres during this time. As will be seen, the third stage is characterized by the
maturation of the ALD neuromuscular junctions, the formation of muscle spindles, the synthesis of metabolic enzymes, and the differentiation of contractile properties.

Stage 3 - Differentiation of ALD Muscles (15–21 days in ovo)

The maturation of synapses innervating ALD muscle fibres takes place during the last week of embryogenesis so that by 18 days in ovo, the morphological features of the endplates are similar to those of mature 'en grappe' endings (Atsumi, 1977). A mature 'en grappe' ending on a slow tonic muscle fibre is composed of a number of axon terminals synapsing as a cluster over a limited region of the muscle membrane. When stained with the cholinesterase reaction, each endplate appears as a cluster of darkly-staining droplets (Hess, 1961). During the course of the last week, each axon terminal within an endplate region evolves into an individual entity: not only does each axon terminal become ensheathed by a Schwann cell process, but several long and thin processes transiently protrude from the surface of the junctional sarcoplasm to separate each axon terminal. Although these sarcoplasmic protrusions disappear by day-18, the axon terminals within an endplate region remain distinct from one another (Atsumi, 1977). In addition, during this time, each axon terminal increases in size and the organelles normally associated with undifferentiated terminals (microtubules, smooth endoplasmic reticulum, mitochondria) are replaced by structures characteristic of mature nerve terminals. Within these mature terminals, synaptic vesicles increase greatly in number and the axoplasm becomes electron-lucent (Jacob and Lentz, 1979).
Changes can be observed within the synaptic cleft and on the postsynaptic membrane as well. A layer of filamentous material is deposited within the synaptic cleft and becomes continuous with the basal lamina of the muscle membrane. Within the sarcoplasm, surface patches fuse and form a continuous band which extends well beyond the immediate region of the nerve-muscle contact. However, as the zone of synaptic contact lengthens, the extent of the surface band decreases so that by 19 days in ovo, the band becomes restricted to the immediate synaptic surface. Junctional folds do not become apparent until after hatching (Jacob and Lentz, 1979).

As stated previously, the polyneuronal and multiple innervation of ALD muscle fibres persists during the last week of embryogenesis. During this time the number of axon terminals innervating a single endplate declines to a mean of four per ending (Bennett and Pettigrew, 1974a) while the average distance between endplates increases to approximately 200 μm (Bennett and Pettigrew, 1974a; Burden, 1977).

The morphological and histochemical differentiation of muscle spindles in the developing ALD muscle has also been investigated during the last week of embryogenesis (Toutant, 1982). Muscle spindles within the ALD muscle are first detected on the 13th day of incubation. The number of intrafusal fibres per spindle and the number of spindles per muscle increase up to hatching and during the first few weeks of ex ovo development. The mean number of intrafusal fibres per spindle increases from 1.8 at 13 days in ovo to 4.3 by 18 days in ovo with only a slight increase to 5.4 by 6 weeks ex ovo. The average number of spindles per muscle increases dramatically from 2.6 at 13 days in ovo to 38 at day-16
then levels off to approximately 54 muscle spindles per ALD muscle at 6 weeks ex ovo. In an attempt to follow the histochemical differentiation of the intrafusal fibres, determination of the type of intrafusal fibre was performed at the level of the encapsulated polar zone near the juxtaequatorial region. Toutant (1982) used this type of approach since the myosin ATPase profile of an intrafusal fibre apparently changes along its longitudinal axis. All ALD intrafusal fibres display an acid and alkali stable (or slow tonic) myosin ATPase profile when sampled in the end regions of a muscle spindle. What is interesting about this observation is that the B-skeletofusimotor axons innervating the end regions also innervate the extrafusal fibres, all of which display a slow tonic myosin ATPase profile. However, when sampled in the equatorial region, intrafusal fibres can display more than one type of response to the myosin ATPase reaction. While some intrafusal fibres express a slow tonic response, others express a fast twitch response (acid labile and alkali stable). This means that an intrafusal fibre can express a homogeneous myosin ATPase profile along its entire longitudinal axis or a heterogeneous response, i.e., one where the ends of an intrafusal fibre stains differently than at its centre. Since the equatorial and end regions of a muscle spindle are innervated from two different sources, the histochemical properties of an intrafusal fibre may be related to the type of innervation it receives. Finally, the ratio of fast twitch to slow tonic intrafusal fibres in multifibril spindles is 2:1 whereas most monofibril spindles contain only one fast twitch intrafusal fibre (Toutant, 1982). This ratio is not a reflection of the situation regarding the extrafusal fibres since the ALD muscle is
nearly entirely composed of slow tonic extrafusal fibres.

Soon after the establishment of reflex activity, the ALD muscle begins to synthesize metabolic enzymes. As early as 15 (Toutant et al., 1979) to 16 (Gordon et al., 1977a) days in ovo, succinic dehydrogenase (SDH) activity can be demonstrated histochemically within developing ALD muscle fibres. This SDH activity becomes well developed and uniform throughout the ALD muscle by days 17-18 (Gordon et al., 1977a; Toutant et al., 1979) and continues to increase up until hatching. Whether or not the synthesis of SDH in the ALD muscle during embryogenesis is activity-dependent is unknown at the present time. It is true that the synthesis of SDH commences one to two days after peak wing motility has been reached, i.e., at 14 days in ovo, (Cauwenbergs et al., 1983). However, during the latter portion of embryogenesis, (a time when wing motility drastically declines due to the growth of the embryo within the confines of the egg), the activity of SDH continues to increase. Phosphorylase (Gordon et al., 1977a) and aldolase (Gutmann and Syrový, 1967) activities can also be measured from ALD muscle preparations late in embryogenesis. In conclusion, the synthesis of enzymes associated with the glycolytic and oxidative pathways can first be detected during the last week of embryogenesis in ALD muscles.

It is well established that the speed of contraction and myosin ATPase activity of a mature muscle are highly correlated (Bárány, 1967). Since the type of myosin light chains is also correlated with the myosin ATPase activity of a muscle, it follows that a correlation exists between the type of myosin light chains within a muscle and the speed of contraction of that muscle. For example, fast twitch muscles possess
'fast' myosin light chains, express a high myosin ATPase activity and rapidly contract. Whether or not this correlation also resides in developing ALD muscles has been examined by several investigators.

To determine the types of myosin light chains present in embryonic ALD muscles, these investigators have used one or two dimensional SDS gel electrophoretic techniques. Those light chains isolated from embryonic ALD muscles which co-migrate with the light chains extracted from adult fast twitch muscle have been routinely classified as 'fast' myosin light chains. Similarly, those which co-migrate with light chains from adult slow tonic or twitch muscles have been classified as 'slow' myosin light chains. When the developmental expression of myosin light chains was examined from the 10th day of incubation to the 21st day, ALD muscles were found to synthesize both 'slow' and 'fast' myosin light chains (Rubinstein et al., 1977; Pette et al., 1979; Stockdale et al., 1981; Gauthier et al., 1982). Since it has recently been reported that between 0.5% to 5.0% of the total fibre population of developing ALD muscles is comprised of fast twitch fibres (Toutant et al., 1980), those 'fast' myosin light chains identified electrophoretically are probably synthesized by these fibres. This also implies that since a majority of the muscle fibres in embryonic ALD muscles synthesize 'slow' myosin light chains, these muscles should be slow contracting.

The most detailed study examining the contractile characteristics of developing ALD muscles has been performed by Gordon and Vrbová (1975b). In this study, ALD muscles were excised from embryos between the 13th and 21st days of incubation. Contractions were elicited via stimulating electrodes applied directly to the muscles in vitro.
Contractions could be recorded in ALD muscles in response to single and repetitive stimuli as early as 14 days in ovo. At 14 days, ALD muscles responded to a direct single shock by a contracture. This response was not maintained though, and by 18 days ALD muscles twitched in response to a single stimuli. When the twitch contraction and relaxation times were examined, these times increased between 14 to 16 days but then suddenly dropped after 16 days. Similarly, contraction times in response to repetitive stimulation (at 20 and 40 Hz), increased up to 16 days then declined afterwards. When similar parameters were examined in age-matched fast twitch posterior latissimus dorsi (PLD) muscles, it was found that after 18 days in ovo, ALD muscles demonstrated slower contraction and relaxation times. It is evident from these results that the contractile characteristics of ALD and PLD muscles become clearly distinguishable during the last week of embryogenesis; the ALD muscles being about three times slower than PLD muscles (Gordon and Vrbová, 1975b). Therefore, as predicted from the myosin ATPase analyses, developing ALD muscles are slowly-contracting and the correlation between myosin light chain type and the speed of contraction persists in these muscles.

In a recent study (Poznansky and Steele, 1984), isolated fibres from 14- and 21-day old ALD muscles were used to study the in vivo development of membrane electrical properties during the last week of embryogenesis. This study yielded the following results. One week prior to hatching (day-14), ALD muscle fibres have high membrane resistances, long time and length constants, and no detectable resting Cl\(^-\) conductance. As the ALD muscle fibres increase slowly in size
during the last week of embryonic life, the membrane resistances (internal resistance ($r_i$) and transmembrane resistance ($r_m$)) decrease slightly as expected since these resistances vary inversely with the size of the fibres. However, since the ratio of $r_m$ to $r_i$ remains constant, the length constant ($\lambda$) remains long (since $\lambda = \sqrt{r_m/r_i}$). Similarly, the time constant continues to remain long up to the 21st day of incubation. In physiological terms, long time and length constants increase the extent to which nerve impulses coming into a muscle fibre can temporally and spatially summate. Further evidence that the electrical properties of the ALD muscle fibres do not change during the last week of embryogenesis is indicated by the non significant drop in the membrane potential from $-56mV$ at day-14 to $-65mV$ at day-21. This indicates that embryonic ALD fibres do not have a measurable conductance to $Cl^-$. 

The evolution of satellite cells in fast twitch muscles have been investigated recently by Armand et al. (1983). A satellite cell is a mononucleated cell which resides between the basal lamina and the plasma membrane of a skeletal muscle fibre. These cells are capable of producing myoblasts by mitosis which are, in turn, capable of either entering into a quiescent state until needed for muscle growth or repair, or withdrawing from the cell cycle and differentiating into postmitotic myogenic cells. Using chick-quail chimaeras, Armand and co-workers showed that satellite cells are of somitic origin. Extrapolating these results to the ALD muscle, it is most likely that satellite cells residing in the ALD muscle, arise from primary somites 18 to 20 and are subsequently deposited in this muscle sometime during
embryogenesis.

DEVELOPMENT OF THE ALD MUSCLE EX OVO

Historically, the primary reason for studying chicken muscles ex ovo has been to compare the properties of ALD and PLD muscles since each muscle is essentially composed of a specific fibre type. Those characteristics or properties which have been examined can arbitrarily be divided into six groups: 1) innervation, 2) myofibrillar ATPase, 3) metabolic, 4) structural, 5) electrical, and 6) mechanical. In the following discussion, properties specific to the ALD muscle will be examined. Each characteristic will be examined systematically by identifying who first studied the property, their results, and whether or not these results have been confirmed. In addition, in each section there will be a brief discussion on whether or not these properties change during ex ovo development and how these properties differ from those measured in PLD muscles.

Innervation Characteristics

The multiple innervation of ALD muscle fibres, established during the second week of embryogenesis, is not a transient phenomenon but persists throughout the ex ovo period. The first investigators to present histochemical evidence that ALD muscle fibres have multiple 'en grappe' nerve endings ex ovo were Ginsborg and MacKay (1961). In earlier studies, these investigators had noticed that the spontaneous electrical activity recorded intracellularly in certain fibres of the biventer cervicis muscle (Ginsborg, 1960a) and in all the fibres of the
ALD muscle (Ginsborg and MacKay, 1960) resembled that seen in the slow fibres of specific frog muscles; specifically, there were large variations in the time course and rise times of the miniature end plate potentials. This suggested to them that these miniature end plate potentials originated from a series of endplates at varying distances from the site of recording. To confirm this interpretation, ALD muscles were taken from two to sixteen week old chickens and stained for cholinesterase activity (Ginsborg and MacKay, 1961). Subsequent examination of the surfaces of stained whole muscles, frozen sections, or single fibres revealed many neuromuscular junctions situated at short intervals along individual fibres. When the distance between endplates was measured, it was found that this distance was directly proportional to the length of the muscle up to 15 weeks ex ovo, i.e., the shorter the muscle fibre the shorter the distance between the endplates on that fibre. Bennett and Pettigrew (1974a) have confirmed this observation and have found that it persists even after 15 weeks ex ovo. Measurements taken by Ginsborg and MacKay (1961) indicated that the mean distance between endplates increased from about 225 μm at 2 weeks to approximately 790 μm at 15 weeks. In adult chickens, this distance increases further to a mean of approximately 1000 μm between endplates (Hess, 1961; Bennett and Pettigrew, 1974a). Since the number of endplates varies directly with the length of the muscle fibre, this suggests that the number of neuromuscular junctions on a fibre remains constant during in ovo and ex ovo development. This was estimated to be about 80 junctions per muscle fibre by Ginsborg and MacKay (1961). Since this early investigation by Ginsborg and MacKay (1961), the
identification of multiple 'en grappe' endplates has been confirmed by many investigators on maturing (Hess, 1961; Bennett and Pettigrew, 1974a; Ashmore et al., 1978) and adult (Silver, 1963; Nene and Chinoy, 1965; Mazliaha, 1980) ALD muscle fibres using various cholinesterase techniques. Moreover, to confirm these histochemical findings, multiple sensitivity peaks to iontophoretically applied acetylcholine (ACH) have been found to be distributed along ALD muscle fibres (Fedde, 1969; Bennett et al., 1973). In conclusion, the innervation of ALD muscle fibres is always multiple with a tendency toward regular spacing of terminals.

Each fibre of the PLD muscle is innervated by a single 'en plaque' motor nerve ending. Not only can PLD 'en plaque' and ALD 'en grappe' endings be distinguished histochemically with the cholinesterase reaction, but differences can be detected biochemically and morphologically as well. Histochemically, each 'en plaque' ending appears as a large discrete droplet of AChE-staining material as opposed to the diffuse staining of an 'en grappe' ending. Biochemically, the endings on ALD and PLD fibres differ with respect to the activities of cholinesterase (Buckley and Heaton, 1971) and choline acetyltransferase (O'Brien and Vrbová, 1978); these activities are higher in 'en plaque' endings. In addition, greater amounts of transmitter are released per impulse at an 'en plaque' ending (Vyskočil et al., 1971). As yet, no difference has been detected between the mean channel open time of Ach receptors at ALD and PLD endplates (Schuetze, 1980). At the ultrastructural level, while few junctional sarcolemmal infoldings occur under the nerve terminals on ALD fibres, many more appear under PLD nerve.
terminals (Zelená and Sobotková, 1973).

Myofibrillar ATPase Characteristics

Once established, the myosin ATPase profile of ALD muscle fibres does not change after the first week of embryogenesis, i.e., the ATPase staining pattern of ALD muscle fibres is not influenced by preincubation in either high or low pH solutions (Butler and Cosmos, 1981) even when these fibres are taken from adult chickens. The first investigators to show that ALD muscles ex ovo are acid and alkali stable were Asiedu and Shafiq (1972). Just three years before this study, Guth and Samaha (1969) had introduced a histochemical myofibrillar ATPase reaction which used acid and alkali preincubations with which they were able to differentiate mammalian type 1 (red) muscle fibres from type 2 (white) muscle fibres. When Asiedu and Shafiq performed this same histochemical reaction on ALD muscles excised from adult chickens, they found that the ALD fibres did not stain as typical mammalian twitch fibres in that, unlike type 1 and type 2 fibres, they lacked a differential pH stability to the myosin ATPase reaction. In addition, these investigators were able to demonstrate two subpopulations of fibres within the ALD muscle; the first type showed a more intense or darker reaction for myofibrillar ATPase activity after both acid and alkali preincubation while the second type stained less intense. In a later study, the dark staining fibres were classified as b' fibres while the light staining fibres were classified as a' fibres (Ashmore et al., 1978). Both a' and b' fibres are multiply-innervated (Ashmore et al., 1978). Since the original study by Asiedu and Shafiq (1972), many other investigators have
confirmed their findings (Koenig and Fardeau, 1973; Ashmore and Doerr, 1976; Mazliah and Cosmos, 1979; Mazliah, 1980).

Although the myofibrillar ATPase profile of ALD muscles does not change during in ovo and ex ovo development, the relative percentages of the two subpopulations of fibres does vary. Toutant et al. (1980) demonstrated that embryonic ALD muscles are composed entirely of b' fibres. During the first week of ex ovo growth, a' fibres begin to differentiate from this pool of b' fibres (Ashmore et al., 1978) so that by eight weeks ex ovo between 60-70% of the ALD muscle consists of a' fibres (see Results).

Metabolic Characteristics

In the mid sixties, it was discovered that not only did mammalian fast and slow twitch fibres exhibit structural and physiological differences but differed metabolically as well. These findings prompted Gutmänn and Syrový (1967) to examine the metabolism of fast twitch and slow tonic fibres of the chicken during the early stages of ontogenetic development. Subsequently, the glycogen content and aldolase activity of the slow tonic ALD and fast twitch PLD muscles were compared in newly-hatched, 8- and 30-day old chicks. Differences between the ALD and PLD muscles were found to be well established in newly-hatched chicks and to persist up to 30 days ex ovo. The glycogen content of ALD muscles remained low and stable compared to the PLD muscles while aldolase activity was consistently greater in PLD muscles. These results suggested that the PLD muscle had adopted by hatching, an anaerobic metabolism compatible with the quick and repetitive
contractions performed by this muscle. They also implied that since the ALD muscle did not differentiate an anaerobic metabolic profile, this muscle must use another energy-supplying system. Since it was known that ALD muscles were required for posture and for the maintenance of tension for long periods of time, this suggested that these muscles used a more aerobic type of metabolism.

This prediction was confirmed three years later by Bass et al. (1970). In this study, ALD muscles were excised from chicks 1 through 39 days of age and the activity patterns of key enzymes in various energy-supplying systems were examined. During this time period, no significant changes could be found in the absolute and specific activities of enzymes representing glycolysis (triosephosphate dehydrogenase), glycogenolysis (glycogen phosphorylase), and glucose phosphorylation (hexokinase) which remained low and the enzymes representing the citric acid cycle (citrate synthase) and fatty-acid oxidation (3-hydroxyacyl-CoA dehydrogenase) which remained high. These investigators were, therefore, the first to show that the enzyme activity pattern of an ALD muscle did not significantly change during postnatal development; the aerobic type of metabolic profile established at hatching was maintained throughout ex ovo development.

In contrast to the work of Gutmann and Syrový (1967), Bass et al. (1970) found that the metabolic profile of the PLD muscle was essentially aerobic at hatching and that during the first few weeks of ex ovo development, this metabolism switched to become more anaerobic. These findings confirmed the earlier work of Cosmos (1966a) and Cosmos and Butler (1967) who had demonstrated histochemically and biochemically
these same results in the slow tonic soleus muscle and the fast twitch pectoralis muscle during early ex ovo development. In turn, the findings of Bass et al. (1970) on the ALD and PLD muscles have been confirmed biochemically by Straley (1975) and histochemically by Cosmos and co-workers (Butler et al., 1978; Cosmos et al., 1979b; Mazliah, 1980). In conclusion, ontogenetic analyses of the biochemical and histochemical characteristics of the ALD and PLD muscles have shown that while ALD fibres achieve adult metabolic characteristics by hatching, PLD fibres differentiate an adult metabolic profile only after hatching.

Whether or not the differentiation of the vascularization of ALD and PLD muscles occurs simultaneously with or secondarily to the differentiation of ALD and PLD metabolic properties is undecided at the present time. An earlier study by Hudlická (1969) reported that blood flow did not differ between ALD and PLD muscles by 8 days ex ovo even though these two muscles had distinct metabolic profiles by this time. By 10 days ex ovo, a slightly higher blood flow was observed in ALD muscles but the blood flow did not become completely differentiated until 28 days when it was 60% higher in the ALD muscle. These results suggested that the vascular changes within these muscles were secondary to the differentiation of characteristic metabolic profiles. More recently, though, Cotter (1976) has shown that by seven days ex ovo, the capillary density is greater in the ALD muscle than in the PLD muscle. This is at a time when the SDH activity, as demonstrated histochemically by this investigator, is consistently higher in ALD than PLD muscles. Therefore, these results suggest that the differentiation of metabolic and vascular characteristics occur simultaneously early ex ovo.
Even though a' and b' fibres exhibit an aerobic metabolism, these two subpopulations of fibres can be distinguished histochemically. The b' fibres exhibit stronger phosphorylase and SDH activities than do a' fibres (Nene and Chinoy, 1965; Asmussen et al., 1969; Ovalle, 1978; Cosmos et al., 1979b). Histochemically, the b' fibres exhibit a lavender to purple iodine colour indicating the presence of intermediate length chain polysaccharides while the a' fibres exhibit a pink to red iodine colour indicative of short chain polysaccharides (Cosmos, 1966a; Mazliah, 1980).

Structural Characteristics

During the 1950's, a German scientist by the name of Krüger reported on the structural properties of different muscles in a variety of species of bird. It is since these studies that the terms 'felderstruktur' and 'fibrillenstruktur' have been used to differentiate between slow tonic and fast twitch fibres, respectively. In 1961, Hess decided to study further the structure of these muscle fibres in the ALD and PLD muscles in chickens at varying ages (Hess, 1961). The results of this study have been confirmed at the light and electron microscopic levels and form the basis on which ALD and PLD fibres are structurally identified (Page and Slater, 1965; Hess, 1967; Page, 1969; Shear and Goldspink, 1972). Basically, the two fibre types differ in their degree of organization within the muscle; the ALD fibres being less organized than the PLD fibres. The ALD fibres have a typical 'felderstruktur' arrangement in which the myofilaments are disorganized thereby giving the fibrils an irregular appearance and causing the Z-disk to appear
zig-zag. Although the sarcoplasmic reticulum around ALD fibrils is capable of accumulating calcium, it is poorly developed. Diads or triads are relatively rare in that there is usually only one per sarcomere and this diad is located randomly within the sarcomere. In contrast, the 'fibrillenstruktur' muscle fibres of the PLD have myofilaments organized into discrete evenly distributed myofibrils each surrounded by sarcoplasm and granules. In these muscles, the Z-band, which is thinner than the Z-disk in ALD fibres, runs straight across the width of the fibril. The appearance of both T-system and sarcoplasmic reticulum is very regular with frequent regions of contact between them forming triads. Frequently, two series of triads are present in each sarcomere and are located near the level of the A-I boundary.

According to Shear and Goldspink (1972), embryonic and young ALD muscle fibres exhibit a 'fibrillenstruktur' type of arrangement. Then the ALD fibres shift to a 'felderstruktur' type of arrangement after approximately 3 months ex ovo due to the incomplete splitting of the myofibrils. This observation, however, has yet to be confirmed in White Leghorn chickens which are the focus of this present study.

Although ultrastructural differences between a' and b' fibres in ALD muscles have not been identified, differences in the sizes of the two fibre types have been recorded from histological cross-sections (Ovalle, 1978; Toutant et al., 1980). In these reports, a' fibres are consistently found to be larger than b' fibres.

Two other major structural differences have also been reported between ALD and PLD muscle fibres; fibre diameter of ALD fibres is larger than PLD fibres during in ovo and ex ovo development (Shear and
Goldspink, 1972; Gordon et al., 1977a); and nuclei in the ALD fibres are at the periphery while they are scattered in the PLD (Cosmos et al., 1979b).

**Electrical Properties**

Since the electrical properties of twitch and tonic fibres of the frog were known to differ, Fedde (1969) decided to examine whether or not this phenomenon was true in chickens as well. The ALD and PLD muscles were removed from 3 to 6 month old male chickens and when various membrane constants were compared, striking differences were found between ALD and PLD muscle fibres. Most notably, the time and length constants were much longer in ALD fibres while the transmembrane resistance was approximately eight times greater in the ALD fibres. The ALD muscle fibres had a mean length constant of 1.78 mm (0.68 mm in the PLD), an average time constant of 35 msec (3.6 msec in the PLD), and a mean transverse resistance of 4388 Ω cm² (561 Ω cm² in the PLD). That the length and time constants are greater in ALD than PLD muscle fibres after hatching has since been confirmed by other investigators (Gordon et al., 1977b; Gordon et al., 1981; Huerta and Stefani, 1981). Furthermore, Gordon et al. (1981) and Huerta and Stefani (1981) have shown that in ALD muscle fibres, the transverse resistance is approximately 7 times greater than in PLD fibres.

Whether or not the passive electrical properties of ALD muscle fibres change during ex ovo development has not been decided. Poznansky and Steels (1984) have recently found that 14- and 21-day old embryonic ALD fibres have length and time constants (i.e., 1.7 mm and 35 msec,
respectively) similar to those fibres analyzed by Fedde (1969). This
would suggest that once established, these two constants do not change
during in ovo or ex ovo development. However, a report by Gordon et
al. (1977b) has shown that not only does the length constant remain
below 1.0 mm up until two weeks ex ovo, but the time constant remains
below 10 msec up until the same time. This would suggest that the
length and time constants of ALD fibres increase after two weeks.
Further support for this interpretation has come from a study by
Korenaga (1980) who showed that the length and time constants of ALD
fibres increase with age up to 30 weeks ex ovo. Resting membrane
potentials have consistently ranged between 50-70 mV for ALD fibres
during in ovo and ex ovo development (Fedde, 1969; Gordon et al.,

Mechanical Properties

This section is divided into two parts. The first examines the
electrophysiology of ALD muscle fibres recorded in vitro and the second
part reviews the contractile properties of ALD muscles measured in vitro
and in vivo.

Since frog tonic fibres do not generate action potentials, it was
an unexpected finding by Ginsborg (1960b) that the ALD muscle, isolated
from 3 week old chickens, could propagate an action potential. When
elicited by a direct stimulation of ALD muscles in vitro and recorded
extracellularly, these action potentials had small amplitudes (14 mV)
and were propagated at low conduction speeds (0.41 - 0.71 m/sec)
relative to the amplitudes and conduction speeds of action potentials
recorded in 'fast' muscles. Ginsborg also reported that in a small proportion of multiply-innervated fibres isolated from another slow tonic muscle, the biventer cervicis, action potentials could be recorded intracellularly upon nerve stimulation. However, it wasn't until the study of Cullen et al. (1975) that intracellular recordings of action potentials generated by individual ALD fibres were made. Working in vitro, Cullen and co-workers, using current-pulse stimulation of ALD fibres isolated from 4-8 week old muscles, found small and slow action potentials in almost all of the fibres tested. Since the generation of these action potentials could be blocked by tetrodotoxin, saxitoxin, and when the external sodium was replaced by choline, they concluded that these action potentials were sodium dependent. Furthermore, both temperature and denervation were shown to affect the maximum rate of rise of the action potential. Korenaga (1980), again using intracellular current pulses, confirmed that action potentials could be generated in ALD muscle fibres stimulated in vitro but only up to 12 weeks ex ovo. Slow tonic ALD fibres isolated from muscles older than 12 weeks failed to generate an action potential in response to a current pulse and developed a graded response instead. From these studies it can be concluded that ALD fibres, isolated from young birds, have the innate ability to generate and propagate action potentials when stimulated directly or intracellularly in vitro.

The ALD muscle is commonly referred to as a 'slow tonic' muscle. The word 'tonic' has come from those experiments which have shown that ALD muscles give a maintained contraction in response to either repetitive nerve stimulation (tetanic stimulation) or to depolarizing
agents. This latter property of the ALD was first described by Ginsborg (1960b) who showed that when drugs known to cause depolarization at the motor endplate (i.e., decamethonium) were added to the fluid bathing the ALD muscle, a prolonged increase in tension of the muscle resulted. Similar results were observed when the muscles were bathed in a solution rich in potassium. These results have since been confirmed by Page (1969), Melichna et al. (1974) and Huerta and Stefani (1981). Apparently, completely sustained contractures in the presence of KCl are not observed in ALD muscles until 3 days ex ovo (Melichna et al., 1974). Potassium-induced contractures in the ALD typically show an initial transient component followed by a maintained tension. Page (1969) was able to demonstrate that these phases could be modified by altering the concentrations of sodium and calcium. The sodium-independence of the contractures was established when muscles, pre-soaked in sodium-free solutions or in fluids containing tetrodotoxin, responded normally when exposed to a solution rich in KCl. However, the application of calcium-free fluids caused the contracture tension of the initial and late phases to decline (Page, 1969). Furthermore, blockers of Ca\textsuperscript{2+} channels such as D600 and Mn\textsuperscript{2+} greatly diminished the maintained phase of the potassium contracture (Huerta and Stefani, 1981). These results indicate that the sustained phase of tension in ALD potassium contractures is dependent upon the presence of external calcium. Results showing that caffeine-induced contractures of ALD muscles are reduced by the absence of external calcium and by the addition of blocking agents of Ca\textsuperscript{2+} channels are consistent with this observation (note: caffeine elicits tension in skeletal muscles by releasing
calcium ions from the sarcoplasmic reticulum). As a rule, prolonged potassium-induced contractures cannot be elicited from the PLD muscle (Ginsborg, 1960b).

A twitch response in ALD muscles can be elicited in several ways: 1) in vitro – by directly or indirectly stimulating the isolated muscle (nerve) preparation, and 2) in vivo – by directly or indirectly stimulating the ALD muscle or its nerve. Unlike action potentials, the generation of twitches is unrelated to the age of the preparation, that is, a twitch response can be elicited in ALD muscles isolated from embryonic, young, and adult birds.

The majority of investigators examining the twitch characteristics of the ALD muscle have done so in vitro. Ginsborg (1960b) was the first investigator to show that isometric twitches could be elicited from young ALD muscles in response to nerve stimulation in vitro. Similarly, Rall and Schotelius (1973) using ALD muscles isolated from adult chickens were able to elicit twitches in response to nerve stimulation. Many more investigators have elicited twitch responses by direct stimulation of embryonic and young ALD muscles (Gutmann and Syrový, 1967; Canfield, 1971; Melichna et al., 1974; Gordon and Vrbová, 1975a; Pette et al., 1979; Gordon et al., 1981) and adult ALD muscles (Page, 1969; Melichna et al., 1974). However, in these experiments, stimulation through intramuscular nerves cannot be ruled out since the introduction of curare into the bath solution can reversibly block contractions (Gordon and Vrbová, 1975a).

Elicitation of twitch responses has also been performed in vivo. Although Shear and Goldspink (1972) found that they could elicit a
twitch by directly stimulating the ALD muscle in situ, this has not been confirmed by either Hnůk and co-workers (Hnůk et al., 1967; 1977; Jirmanová and Zelená, 1973) or by Mazliah (1980). Instead, these investigators found that a twitch response could be elicited only after a prolonged tetanus, a phenomenon referred to as post tetanic potentiation.

Regardless of the protocol used, the response of ALD muscles to a single stimuli is a slow twitch which has contraction and relaxation times longer than the twitch responses evoked in PLD muscles. The contraction time is 2.4 to 8 times as long while the relaxation time is 5 to 10 times longer. The range of contraction (time to peak) and half relaxation times reported for the ALD muscle vary from 224 to 550 msec and 87 to 700 msec, respectively. They vary from 62 to 90 msec and 17 to 82 msec, respectively, in PLD muscles (Gutmann and Syrový, 1967; Page, 1969; Canfield, 1971; Rall and Schottelius, 1973; Melichna et al., 1974; Gordon and Vrbová, 1975a; Mazliah, 1980; Gordon et al., 1981).

As early as 18 days in ovo, the contractile characteristics of ALD and PLD muscles differ; ALD muscles being about three times slower than PLD muscles (Shear and Goldspink, 1972; Gordon and Vrbová, 1975a). These differences persist after hatching and show only slight changes during development ex ovo (Shear and Goldspink, 1972; Melichna et al., 1974; Gordon and Vrbová, 1975a). Therefore, these results suggest that the contractile properties of ALD and PLD muscles are established before hatching and are modified only slightly after hatching.

Two factors have been proposed which may contribute to the greater
speed of contraction displayed by PLD muscles. The biochemically determined myosin ATPase activity is greater in PLD muscles than in ALD muscles (Syrový, 1973; Reasons and Hikida, 1973) and the sarcoplasmic reticulum and T system are much more extensive in PLD muscles (Page, 1969). In addition, an increased density of intramembranous particles (considered to represent Ca$^{2+}$-dependent ATPase transfer protein) within the sarcoplasmic reticulum isolated from PLD muscles may contribute to the faster relaxation characteristics of this muscle (Bray and Rayns, 1976; Ryan and Shafiq, 1980).

In conclusion, most of the properties of the ALD muscle are established before the end of embryogenesis. The innervation pattern, myofibrillar and metabolic profiles, and contractile characteristics have all been established by hatching and are only slightly modified during ex ovo development. The fine structure and passive membrane properties of the ALD fibres may be altered after hatching but this has not been demonstrated conclusively. However, the ex ovo period is characterized by the growth of ALD fibres and the expression of a fibres within the ALD muscle.

EXPERIMENTAL MANIPULATIONS OF THE ALD MUSCLE

Effects of Stretch

In chickens, two ALD muscles are located superficially on the back, one on either side of the dorsal midline. Each ALD muscle originates from the dorsal midline and inserts into the humerus. When activated, each ALD muscle acts to hold the posterior tip of the humerus to the
vertebral column in the middle of the back. This action holds the wings against the trunk and prevents the wings from drooping. Any manipulation which actively extends the wings will, therefore, stretch the ALD muscles.

Active stretch of the ALD muscle has been achieved by either attaching weights (Feng et al., 1963; Sola et al., 1973; Laurent and Sparrow, 1977; Laurent et al., 1978; Gollnick et al., 1983) or a spring-loaded device (Holly et al., 1980) to one of the wings. In this way, the ALD muscle in the unstretched wing acts as an internal control. In response to being stretched, an ALD muscle rapidly gains weight. It has been observed consistently that ALD muscles which have been stretched for only one week weigh between 40-80% more than contralateral unstretched muscles (Feng et al., 1963; Sola et al., 1973; Holly et al., 1980). Moreover, ALD muscles which have been stretched for as long as eight weeks can weigh 179% more than unstretched muscles (Sola et al., 1973). This suggests that so long as the wing is extended, the wet weight of the ALD muscle will be elevated.

Increases in water content, longitudinal and circumferential fibre growth rates, and the activation of new fibres have all been proposed as factors which contribute to the observed wet weight increases. The increased water content within stretched muscles is a transient phenomenon taking place during the first week of stretch. During this time, Laurent et al. (1978) found that the wet weight of stretched muscles increased faster than did the protein content. However, by seven days, the increases in wet weight and protein content were proportional indicating that any excess of water had been lost by this
time. Another transient phenomenon is the rate of longitudinal growth of the muscles. Holly et al. (1980) showed that during the first week of stretch, there was a 24% increase in the length of stretched ALD muscles when compared to unstretched control muscles. Thereafter, both the stretched and their contralateral control muscles grew in length at similar rates. These investigators presumed that the initial increase in muscle length was due to the accelerated addition of sarcomeres in series to the stretched muscle fibres.

Unlike the transient increases in the water content and the longitudinal growth rate, the rate of circumferential growth of individual fibres within stretched muscles remains elevated as long as the stretch is maintained (Holly et al., 1980). Therefore, it is not unexpected that non-collagen protein synthesis and content have been reported to be elevated in stretched ALD muscles (Laurent and Sparrow, 1977; Laurent et al., 1978). In these studies, Laurent and co-workers show that the non-collagen protein content increases markedly during the first week of stretch; a time when the longitudinal and circumferential growth rates of ALD fibres are accelerated. Since an accelerated longitudinal growth rate is essentially complete by the end of the first week, the elevated non-collagen protein content observed after the first week may be responsible for the continued circumferential growth of stretched fibres.

At the present time it is not known whether stretching leads to the de novo synthesis of new fibres in ALD muscles. Histological analyses have demonstrated the presence of new small fibres in ALD muscles which have been stretched for two weeks (Sola et al., 1973). However,
hyperplasia through fibre splitting or satellite cell activation has not been confirmed in muscles stretched up to seven weeks (Holly et al., 1980; Gollnick et al., 1983). Although Laurent and Sparrow (1977) suggest that an increase in the DNA content of stretched ALD muscles reflects an increase in the number of nuclei within these muscles, they are hesitant in stating exactly which type of cell is undergoing division since both satellite cells and interstitial cells have been shown to proliferate during compensatory hypertrophy of rat soleus muscle (Schiaffino et al., 1972).

There is as yet no suitable explanation of how stretch is transduced into muscle fibre growth. Laurent et al. (1978) speculate that stretch-mediated transport of amino acids or stretch-induced alterations of membrane structures may activate the anabolic processes present in ALD fibres responsible for their growth. In addition to affecting anabolic processes within the muscle, stretching of the ALD muscle, from one to five weeks, causes a reduction in (Sola et al., 1973) or complete loss of (Holly et al., 1980; Gollnick et al., 1983) the ability to differentiate between a' and b' fibres within the ALD muscle.

**Effects of Denervation**

Denervation, in the absence of stretch, also induces growth of maturing (Hájek et al., 1966; Shafiq et al., 1974; Gordon et al., 1981) or matured (Feng et al., 1963; Žímanová and Želená, 1970; Sola et al., 1973; Šyrový, 1976; Feng et al., 1981) ALD muscles. It is a common observation that when an ALD muscle is denervated, the wing of
the operated side droops thereby passively stretching the ALD muscle. Therefore, to study the effects of denervation per se, investigators have either used bands to prevent wing droop (Feng et al., 1963; Hájek et al., 1966; Feng et al., 1981) or analyzed the experimental muscles after the wing has reverted back to its original position (Feng et al., 1963; Jirmanová and Zelená, 1970; Sola et al., 1973; Syrovy, 1976); a process which normally takes about one to two weeks (Feng et al., 1963). A problem with the latter approach is reflected in the works of Feng et al. (1965) and Bennett and Pettigrew (1973) who demonstrated that denervated ALD muscles become self-reinnervated within 16 to 184 days postoperatively. Most investigators do state that their denervated ALD muscles have not become reinnervated but conclusive histological analyses have rarely been provided. Furthermore, Hněk et al. (1967) showed that a slight hypertrophy persists in an ALD muscle even up to four months after reinnervation has been demonstrated. In light of this observation, denervated ALD muscles which remain hypertrophied are not necessarily denervated. Unfortunately, those investigators who have failed to prevent drooping of the wing and have examined the ALD within the one to two week postoperative period, cannot make statements about the effects of denervation per se on the ALD muscle (Malvey et al., 1971; 1973; Harris et al., 1973; Cullen et al., 1975; Staubert and Schottelius, 1975a,b).

The response of an ALD muscle to denervation is similar to its response to stretch; in both cases the wet weight of the ALD muscle increases. When denervated muscles have been compared to their control muscles two to twenty weeks postoperatively, the per cent difference in
wet weights increases although it has never been found to exceed 60% (Feng et al., 1963; Jirmanová and Zelená, 1970; Sola et al., 1973). Since the degree of wet weight increase observed in stretched muscles can range from 40% at one week to as high as 179% by eight weeks, with respect to the wet weight increase, it seems that denervation of the ALD muscle has less of an effect than does stretching of this muscle.

An increase in the cross-sectional size of individual fibres accounts for some of the wet weight increase. Gordon et al. (1981), performing denervation experiments on three week old chickens, found that the mean fibre diameter of denervated fibres was 19% larger than in innervated control ALD fibres after four weeks of denervation. When denervation operations were performed in adult birds, the degree of fibre hypertrophy was found to be slightly higher, attaining a level of 34% after three weeks of denervation (Jirmanová and Zelená, 1970). Recently, Feng et al. (1981) re-examined the phenomenon of post-denervation hypertrophy in adult chickens and found that there was a differential response of the two subpopulations of fibres within the ALD muscle; while the $a'$ fibres hypertrophied the $b'$ fibres remained unaltered two to three weeks postoperatively.

The differential response of the two subpopulations of fibres to denervation observed by Feng and co-workers was an unexpected finding since previous experiments had shown that denervated muscles became homogeneous for one type of fibre (Sola et al., 1973; Shafiq et al., 1974). Therefore, how were Feng and associates able to determine the specific effects of denervation on the $a'$ and $b'$ fibres? If the age of the chickens at the time of the operation and the length of the post-
operative period are taken into consideration, this discrepancy can be accounted for. Shafiq et al. (1974) performed their denervation experiments within newly-hatched chicks and therefore at a time preceding the differentiation of a' fibres (Ashmore et al., 1978). It is conceivable that denervation at this time interferes with this process of differentiation and subsequently prevents the ALD muscle from expressing a heterogeneous population of fibres. This implies that the differentiation of a' fibres in the ALD muscle is neurally-dependent and that ALD muscles, denervated at hatching, consist of b' fibres only. On the other hand, Feng et al. (1981) performed their denervation experiments on adult chickens and therefore at a time after the differentiation of a' fibres (which takes place immediately after hatching). According to Sola et al. (1973) when denervation is performed at this time, the b' fibres are preferentially lost so that denervated ALD muscles eventually consist of a' fibres only. However, this did not occur until four months postoperatively. Therefore, since Feng et al. (1981) examined their muscles two to three weeks postoperatively, it is quite likely that a' and b' fibres could still be identified.

The de novo synthesis of new fibres contributes only slightly to the wet-weight increases of denervated ALD muscles. Some new fibres have been observed in adult ALD muscles twenty weeks after denervation (Sola et al. 1973), but so far the formation of new fibres has not been observed in ALD muscles denervated early ex ovo (Shafiq et al., 1974).

Some properties of the ALD muscle remain unaltered after denervation. The peptidase and catheptic activities of ALD muscles are
not significantly altered by denervation (Hájek et al., 1966). Furthermore, a conversion of slow tonic to fast twitch fibres has not been detected histochemically (Sola et al., 1973; Shafiq et al., 1974; Feng et al., 1981). This latter finding correlates well with the observation that the light chains of myosin remain the same after denervation (Syrový, 1976). However, a decrease in the myosin ATPase activity (Syrový, 1976) and changes in the passive membrane properties of ALD muscle fibres (Gordon et al., 1981) have been observed after denervation.

Effects of Cross-reinnervation

In an attempt to examine how nerves influence muscle phenotypes, cross-reinnervation experiments were first performed in the early sixties. In these studies (Buller et al., 1960a, b) nerves from one type of muscle were severed and transferred to another type of muscle within newborn kittens. At the same time that these studies were being performed, Ginsborg and co-workers were publishing work on the properties of two muscles found in the back of chickens, the ALD and PLD muscles (Ginsborg 1960a, b). This prompted a Czechoslovakian group to investigate whether or not the transformation of these two very different types of muscles could be induced by cross-reinnervation.

While the transformation of one of the muscles, the PLD, was relatively simple, the acceptance of a 'fast' nerve by the other muscle, the ALD, proved to be more difficult. In fact, in their first report on such an attempt (Hník et al., 1967), the authors concluded that cross-reinnervation of the ALD muscle had been unsuccessful. They
speculated that either the 'fast' nerve which they had used was incompetent or that the ALD muscle fibres were unable to accept this foreign innervation. Credence was given to the former interpretation since it was known by this time that the 'fast' PLD nerve was a mixed nerve containing a 'fast' component, which innervated fast twitch muscles, and a 'slow' component, which innervated slow tonic muscles. From the earlier studies of Feng et al. (1965), it had been shown that if given the choice of a PLD nerve or its original nerve, a denervated ALD muscle would accept its original nerve. Therefore, the failure of the ALD muscle to become transformed could have been due to the selective reinnervation of the large 'slow' component of the PLD nerve or due to self-reinnervation by the ALD nerve. Alternatively, Hník and co-workers suggested that the membrane of mature ALD muscle fibres was not 'plastic' enough to respond to the 'fast' nerve. Therefore, this same group proceeded to perform cross-reinnervation experiments in newly-hatched chicks with a different 'fast' nerve. The results of these experiments were first reported in 1968 (Jirmanová et al., 1968) but not published in detail until later (Jirmanová et al., 1971) and were the first to demonstrate a successful transformation of the ALD muscle by a 'fast' nerve. In these experiments, operations were performed on seven 2-13 day old chicks using the superior brachialis, 'a predominantly 'fast' nerve'. Ten months after the operations, innervation patterns and contractile properties of the experimental muscles were examined. With the cholinesterase reaction/‘en plaque’ endplates with strong AChE activity could be observed in the vicinity of the implanted foreign nerve. When the ratio between muscle fibres with
slow or fast types of innervation was determined in two of the seven cross-reinnervated muscles, the degree of conversion was estimated as 7% in one muscle and 42% in the other, i.e., in the latter muscle, 21 of 50 muscle fibres examined had 'en plaque' endings. Normally, only 'fast' muscles twitch in response to a single indirect shock in vivo. However, when a single supramaximal nerve volley was elicited, a twitch response could be recorded in all seven of the experimental muscles. On the basis of these results, Jirmanová and co-workers concluded that young ALD muscles were capable of accepting and responding physiologically to the influences of a 'fast' nerve.

In a second series of experiments by the same group, cross-reinnervations were performed immediately after hatching on five chicks (Zelená and Jirmanová, 1973a, b, c). However, to avoid self-reinnervation, ALD muscles were first transposed to the contralateral side and then supplied with the superior brachialis nerve. Six to eight months after the operation, the innervation patterns and ultrastructural characteristics of the experimental muscles were examined. This time, between 60-80% of the 550 fibres examined were found to be innervated focally by 'en plaque' endings. It was assumed that the 'en grappe' endplates identified on the remaining fibres originated from the 'slow' component of the implanted nerve. In order to establish the degree of ultrastructural alterations of cross-reinnervated ALD fibres, the incidence of triads, the width of the Z-lines, and the mean mitochondrial contents were determined. When quantified, transformed fibres had a greater number of triads, thinner Z-lines, and a lower mean mitochondrial content; each one of these changes were indicative of a
transformation towards the fast fibre type. Therefore, not only did this series of experiments confirm the findings that ALD muscles could accept 'fast' motoneurons, but they suggested that these incoming nerves could influence the ultrastructural differentiation of the slow tonic fibres as well.

The aim of the third and final cross-reinnervation study by this group, was to determine the time of onset and the progression of muscle transformation postoperatively (Syrový and Zelená, 1975). These experiments were performed exactly as described in the second series of experiments. Cross-reinnervated muscles were examined from 2-15 months postoperatively for histochemical, ultrastructural, and biochemical changes. Histochemically, with the myosin ATPase reaction, fast twitch fibres were identified in cross-reinnervated muscles as early as two months postoperatively. Ultrastructural changes were also detected by two months. However, the light chains of myosin and the myosin ATPase activity did not change until 3 months postoperatively. At this time, 'fast' myosin light chains could be identified in the electrophoretic gels of myosin prepared from transformed muscles. Moreover, the myosin ATPase activity increased significantly to about 62% of the myosin ATPase activity found in FLD muscles at 3 months. These results indicated that changes within cross-reinnervated ALD muscles could be detected as early as two months postoperatively by some criteria but not by others.

The following summary reiterates several of the important points stressed by Jirmanová, Zelená and associates: 1) slow tonic ALD muscles can accept a 'fast' motoneuron as early as two months
postoperatively and can respond to this innervation as indicated by a transformation of the functional, ultrastructural, biochemical, and histochemical properties of the reinnervated ALD fibres; 2) the transformation of these properties is time-dependent and onset of these changes do not occur simultaneously; 3) the initial transformation of ALD muscle fibres is restricted to the region surrounding the implanted nerve but later this area of transformation expands; 4) the degree of transformation increases when young ALD muscles are used and when these muscles are transposed prior to cross-reinnervation; 5) considering the relative maturity of ALD muscles at hatching, it is not surprising that a complete changeover of properties to the fast type is never observed; 6) unconverted regions are either reinnervated by the original ALD nerve or by the 'slow' component of the superior brachialis nerve; and 7) it is a distinct possibility that converted fibres do not arise from pre-existing fibres, but, instead, form from newly-formed fibres which arose from the degeneration and regeneration of muscle fibres caused by a transient ischemia during the initial operative procedure (Zelená and Jirmanová, 1973a).

Using a similar strategy as Jirmanová, Zelená and associates, another group attempted to cross-reinnervate the ALD muscle with a 'fast' nerve (Gordon and Vrbová, 1975c; Gordon et al., 1977b). In this set of experiments, ALD muscles were first removed from 4–6 week old chickens, minced, and then returned to the chicken in the place usually occupied by the contralateral FLD muscle. Six to seventeen weeks later, the innervation patterns, contractile properties, metabolic enzyme patterns, and myosin ATPase profiles of the experimental muscles were
examined. In six to eight successfully regenerated muscles, Gordon and associates showed that every property examined changed towards the 'fast' type. From these results, these investigators concluded that the four properties examined were not predetermined by the muscle but determined by the motor nerve ex ovo therefore confirming the earlier work of Jirmanová, Zelená and associates.

To investigate the time course of transformation, Ashhurst and Vrbová (1979) repeated the surgical protocol used by Gordon and associates but examined the regenerating muscles at various periods from four days postoperatively and onwards. In the first few days after the operation, undifferentiated cells containing poorly differentiated cytoplasm and some rough endoplasmic reticulum were identified. Since these cells were located within the basement membrane surrounding degenerating muscle fibres, they were classified as satellite cells. Between 8-14 days postoperatively, the regenerating muscle fibres displayed the ultrastructural features of typically 'fast' muscle fibres. Furthermore, by 14 days, while the regenerating muscles did not contract as rapidly as the control PLD muscles, they did contract more rapidly than the control ALD muscles. Moreover, nerve endings were identified within the muscle regenerates at this time. Although there is no mention of the degree of conversion, these results indicate that within the first two weeks of the operation, the regenerating muscle fibres differentiate, become reinnervated, and begin to acquire the physiological and morphological properties of the muscle that they replace. In addition, they indicate that satellite cells can differentiate to form myoblasts and subsequently differentiate into
fibres appropriate to the new site.

In two detailed studies examining the ability of 'fast' motoneurons to reinnervate ALD muscles, Bennett and associates (Bennett et al., 1973; Bennett and Pettigrew, 1974b) were able to confirm and extend several of the early findings of Čirmanová, Zelená and co-workers. In their initial study, Bennett et al. (1973) showed that mature ALD muscles cross-reinnervated with the PLD nerve retained their original pattern of innervation. In this study, cross-reinnervation experiments were performed in five adult birds using the same technique as Hník et al. (1967), i.e., the ALD muscle was maintained in its original position. Since two of the five muscles showed no evidence of being reinnervated after 1-2 months, analyses were performed on the remaining three muscles. When the PLD nerve was repetitively stimulated, contractions were elicited in the ALD muscles. However, this response was restricted to a small region within each muscle. Analyses of the innervation pattern within these restricted areas revealed that a hybrid pattern of innervation had formed. For example, the ALD fibres within this region were multiply-innervated by regularly spaced 'en plaque' endings superimposed on degenerating 'en grappe' terminals. These results suggested that even though the mature ALD muscles had accepted and could respond to the implanted PLD nerve, this nerve was unable to alter the original multiple innervation pattern of the ALD muscle fibres. Taken together with the observation that multiple endings were re-established on self-reinnervated mature ALD muscle fibres, these investigators concluded that the pattern of innervation on mature ALD muscles is determined by the muscle fibres and not by the type of nerve
supply.

In a subsequent experiment, Bennett and Pettigrew (1974b), found the opposite result when synapse formation was examined in self- and cross-reinnervated developing ALD muscles. After being denervated at hatching, self-reinnervating ALD muscles became multiply-innervated as early as two weeks postoperatively. However, in 4 of 8 successfully cross-reinnervated muscles (using the technique of Želená and Jirmanová (1973a)), a singular 'en plaque' ending was found on 80% of the muscle fibres examined. Therefore, these results indicated that it is the nerve which determines the pattern of innervation on ALD fibres during development.
THE RESEARCH PROBLEM

SPECIFIC BACKGROUND

Domestic fowl afflicted with hereditary muscular dystrophy are homozygous for an autosomal gene (Julian and Asmundson, 1963). Many studies have focused on the biochemical, electrophysiological and electromyographical properties of the affected muscles in an attempt to understand the influence of dystrophy on the functional parameters of the muscle. Other studies have concentrated on the abnormal structural features of the dystrophic muscles. Moreover, these characteristics have been studied in ovo and ex ovo in an attempt to understand the progression of the dystrophic process within affected muscles.

In chickens there are primarily two types of muscle fibres: fast twitch and slow tonic. Both fast twitch and slow tonic fibres express a similar enzymatic profile during the last week of embryogenesis, i.e., high and low levels of oxidative and glycolytic enzymes, respectively. This aerobic type of metabolism is maintained in slow tonic fibres after hatching and throughout the entire ex ovo period. In contrast, the metabolism of fast twitch fibres does not remain aerobic but switches after hatching to become highly anaerobic (Cosmos, 1966a; Cosmos and Butler, 1967; Bass et al., 1970). In genetically dystrophic chickens, fast twitch fibres are unable to differentiate a mature anaerobic type of metabolism (Cosmos, 1966a; Cosmos and Butler, 1967; Cosmos, 1970; Mazlieh et al., 1976; Mazlieh, 1980; Cosmos et al., 1980). It is when
the dystrophic fast twitch fibres are attempting to differentiate this mature metabolic profile that the anomalies normally associated with the dystrophic process are first expressed, i.e., fibre degeneration, fibre regeneration, lipid infiltration, increases in the number of nuclei. Since slow tonic fibres of dystrophic genotype do not express these anomalies (Julian and Asmundson, 1963; Holliday et al., 1965; 1968; Cosmos and Butler, 1967; Cosmos, 1970; Mazliah et al., 1976; Mazliah, 1980; Barnard et al., 1982; Pizzey et al., 1983), this implies that the expression of dystrophy is target specific, i.e., affecting only the fast twitch fibres. Furthermore, these findings implicate those mechanisms controlling the differentiation of fast twitch fibres as being defective in birds carrying the dystrophic gene.

Since motor neurons are able to influence muscle fibre type properties it seems reasonable to implicate a defective neuronal factor in the etiology of the disease. However, one study in particular has demonstrated that the peripheral motor neurons in dystrophic birds are fully competent and are able to support the complete differentiation of fast twitch muscles (Cosmos and Butler, 1972). In this study, pectoralis muscles were transplanted from newly-hatched normal chicks to age-matched dystrophic chicks. Analyses of the transplanted muscles examined up to 10 months postoperatively revealed that these genetically normal muscles could grow and differentiate in the environment of the dystrophic host. Similarly, when the same pectoralis nerve was forced to reinnervate both normal and dystrophic regenerates in a dystrophic environment simultaneously, each regenerate expressed its own inherent phenotype. These results imply that genotypically dystrophic 'fast'
motoneurons are competent since these nerves can support the proper
growth and differentiation of transplanted genetically normal muscles.

In another set of experiments, Cosmos and Butler (1972) performed
similar manipulations to those just described except that dystrophic
pectoralis muscles were implanted into newly-hatched normal chicks.
Detailed analyses of these muscles showed that even in a normal
environment, dystrophic transplants were unable to differentiate mature
fast twitch characteristics. On the basis of these results Cosmos and
Butler concluded that fast twitch fibres of dystrophic genotype seemed
unable to respond to the dictates of the 'fast' motoneuron to properly
differentiate the mature characteristics of glycolytic muscles. In more
general terms, these results suggest that genetically dystrophic fast
twitch fibres express an inability to respond to, or to interact with,
factors in their environment that influence or control their differenti-
tation (Cosmos et al., 1979b).

Based on the results cited above, it seemed reasonable to postulate
that if a fast twitch muscle of dystrophic genotype is not asked to
alter its embryonic aerobic type of metabolism, it should not express
dystrophic phenotypes. In order to test this hypothesis, Mazliah (1980)
decided to disrupt the normal process of differentiation in a dystrophic
fast twitch muscle by replacing its original motor neuron with the motor
innervation of a slow tonic muscle. In this way the fast twitch muscle
would be 'held back' from differentiating a mature anaerobic type of
metabolism, (determined, not prevent fast twitch muscles from
differentiating - ; 1966b)). When examined from 1 to 27 months
postoperatively, successfully cross-reinnervated fibres of both normal
and dystrophic PLD muscles acquired the mechanical, structural, and histochemical properties of slow tonic fibres. These results established that PLD muscles of dystrophic chickens were able to accept new innervation and were capable of responding to the influences of the new nerve as a normal PLD muscle would. More importantly, though, these experiments showed that when cross-reinnervated dystrophic fast twitch fibres were prevented from fully differentiating, these fibres did not express dystrophic phenotypes.

THE HYPOTHESIS

Based on the results of the experiments cited above, it seemed reasonable to hypothesize that if a genetically dystrophic slow tonic muscle is asked to differentiate phenotypes of mature fast twitch fibres, the dystrophic gene should be activated and dystrophic phenotypes subsequently expressed.

EXPERIMENTAL STRATEGY

Since it has been demonstrated with cross-reinnervation experiments that nerves can influence the phenotype of muscles, it was decided to replace the motor innervation of a slow tonic muscle with the motor innervation of a fast twitch muscle within a dystrophic chicken. In this way a genotypically dystrophic slow tonic muscle, a muscle which never expresses dystrophic phenotypes, will be asked to differentiate phenotypes characteristic of fast twitch fibres.

The anterior latissimus dorsi (ALD) muscle in the dystrophic chicken was selected to represent a slow tonic muscle. The superficial
position of the ALD muscle in the back of the chicken permitted easy access to this muscle and its nerve. On the basis of a number of earlier reports in the literature it became clear that to successfully cross-reinnervate the ALD muscle, several guidelines had to be established.

1. **Nerves:** In various attempts to cross-reinnervate the ALD muscle, both the PLD and superi pr brachi alis (SB) nerves were used in this study. Both of these nerves occupy superficial positions and are therefore accessible to surgical manipulation. The PLD nerve was first used by Gordon and Vrbová (1975c) to successfully cross-reinnervate the ALD muscle while Zelená and Jirmanová (1973a) were the first to use the SB nerve.

2. **ALD Manipulation:** In those papers reporting a successful transformation of the ALD muscle, this muscle was always manipulated prior to being cross-reinnervated. In the case of Gordon and Vrbová (1975c), the ALD muscle was minced before being cross-reinnervated with the PLD nerve. On the other hand, Zelená and Jirmanová (1973a) transposed the ALD muscle away from its original position before cross-reinnervating it with the SB nerve. In an attempt to cross-reinnervate the dystrophic ALD muscle, both techniques were used in the present study.

3. **Age:** Thus far, all attempts to cross-reinnervate the ALD muscle in adult chickens have been unsuccessful (Hník et al., 1967; Bennett et al., 1973). Since both Zelená and Jirmanová
(1973a) and Bennett and Pettigrew (1974b) cross-reinnervated the ALD muscle in newly-hatched chicks, operations were always performed within the first few days after hatching.

4. **Time Course:** According to the work of Syrový and Zelená (1975), the transformation of ALD fibres to fast twitch fibres is time dependent. Therefore, cross-reinnervated muscles were examined at different times, usually between 2 and 32 weeks postoperatively in the present study.

**CONTROL ASPECTS**

The present study is the first attempt to cross-reinnervate a slow tonic muscle with a motor nerve to a fast twitch muscle in the dystrophic chicken. In order to properly assess the cross-reinnervated ALD muscles, it became necessary to perform the following control studies:

1. **Developmental Study:** The aim of this study was to examine selected structural and histochemical properties of ALD and PLD muscles of normal chickens to determine which of these properties were altered in the ALD and PLD muscles of dystrophic chickens during early ex ovo development. The data established in this section were used as baseline for the analyses of ALD muscles cross-reinnervated by "fast" nerves as well as for other experimental manipulations of the ALD muscle.

2. **Denervation Study:** Since the experimental ALD muscles go through a period of denervation prior to cross-reinnervation, the aim of this study was to examine the effects of denervation on selected structural and histochemical properties of the ALD
muscle.—Since denervated ALD muscles, left in their original position, have a tendency to become self-reinnervated, this phenomenon was also examined in normal and dystrophic chickens.

3. **Pilot Cross-Reinnervation Studies:** The aim of this study, in which three different techniques were used to cross-reinnervate the ALD muscle, was to see which technique yielded the greatest degree of slow tonic to fast twitch fibre type conversion.

4. **Transposition Study:** The purpose of this study was to act as a sham control for cross-reinnervation experiments where the ALD muscle was transposed away from its original position prior to being cross-reinnervated by the SB nerve.

5. **Cross-Reinnervation Study with Normal Birds:** Since in these experiments, a slow tonic muscle under the influence of a 'fast' nerve will be asked to continue to differentiate characteristics of a fast twitch muscle, it was important to establish the maximal capabilities of a normal ALD muscle subjected to this experimental manipulation. Once this is established, then additional alterations which may occur within the dystrophic ALD muscle could be attributed to the expression of dystrophic phenotypes.
METHODS

ANIMALS

All fertilized eggs from chickens homozygous for dystrophy were obtained from Dr. Louis Pierro from the Department of Animal Genetics at the University of Connecticut at Storrs. Fertilized White Leghorn chicken eggs of normal genotype were purchased from Martindale Hatcheries in Hamilton and received on a weekly basis. On delivery, all experimental eggs were either incubated immediately or stored in a cooler at 12°C for up to one week and subsequently incubated. The eggs were incubated in a Petersime incubator (37°C, 56% humidity) allowing the eggs to be periodically rotated. Incubation in this manner took place for nineteen days after which time the eggs were removed and placed in cages at the bottom of the incubator. All birds hatched within the next two to three days. Within one to three days of hatching, the chicks were removed from the incubator and housed in small circular cages in our laboratory. Each cage was illuminated and heated by a single 100 watt light bulb. All experimental manipulations of the ALD muscle were performed within the first week ex ovo. The operated birds were allowed to recover in the laboratory for two or three days after which time an identification band was attached securely to the unoperated wing of each bird. The birds were then housed in temperature-controlled brooder cages in the animal quarters at the McMaster University Health Sciences Centre. The birds were maintained in cages set at 35°C during the first week, at 32°C during the second, at
29°C during the third, and then maintained at 24°C for three weeks. At this time the birds were transferred to larger growing cages. While housed in the brooder and growing cages, the birds were exposed to a constant light source. At eight weeks of age, the birds were transferred to larger adult cages and placed in rooms maintained at 16°C with alternating light-dark cycles. Chickens were placed in couples with one male and one female sharing a cage. This arrangement was maintained unless the two proved to be incompatible whereupon they were separated. Food and water were supplied daily and provided ad lib during the entire ex ovo period. Any bird which appeared to be in poor health and lost more than one hundred grams of body weight within a single week was immediately sacrificed.

**SURGICAL PROCEDURES**

**Anaesthesia**

The anaesthetic used throughout the operations was Combuthal, an anaesthetic prepared by dissolving 1.0 gram of Pentothal Sodium (Abbott, N. 8670) with 5.2 cc of sterile Sodium Pentobarbital (M.T.C. Pharmaceuticals, DIN 141690) and diluting with 45 cc of sterile water. Every injection was made intramuscularly into the right leg of each bird. The average dosage administered to induce a deep and maintained anaesthesia was 0.09±0.02 cc (mean ± S.D.) for dystrophic chicks and 0.10±0.02 cc (mean ± S.D.) for normals or 2.6 µl per gram body weight for dystrophic birds and 2.5 µl per gram body weight for normals. The anaesthetic took effect within five minutes of being injected and lasted
for approximately four hours.

Intact Muscle Position and Innervation

In chickens, the latissimus dorsi complex is composed of four muscles which form the most superficial muscle layer in the back. Each muscle lies on either side of the dorsal mid-line (Figure 2). The anterior aspect, or ALD, originates from the neural spines of the caudal cervical vertebrae and inserts on to the upper third of the humerus. The PLD originates posterior to the ALD origin from the spines of the lateral thoracic vertebrae. Superficial to the ALD and PLD muscles and lying next to the skin, are the dorsocutaneous (DLD) and metapataqial (MLD) latissimus dorsi muscles. These two muscles have the same origin as the PLD muscle and insert into the skin at different points.

The complete latissimus dorsi complex is innervated via the brachial plexus through the brachiales dorsales nerve. Distal to the brachial plexus, this nerve divides into four branches, one of which is the musculi latissimi dorsi which subsequently divides into two rami: the cranialis and caudialis. The ramus cranialis (or ALD nerve) innervates the ALD muscle specifically while the ramus caudialis sends branches to all of the latissimus dorsi muscles. The ramus caudalis (normally referred to as the PLD nerve), runs under and along the tendon of the PLD muscle before it enters this muscle. Whilst in the tendon, the ramus caudialis gives off a small branch which innervates the ALD and DLD muscles. Distal to this branch, the main division of the ramus caudalis enters the PLD muscle where it subdivides sending one small branch to the MLD. Since the ALD, DLD, and MLD muscles consist of slow
Figure 2. Schematic drawings showing the relative positions of various muscles and nerves in the back of the chicken following the surgical manipulation at hatching. As outlined in the text, the right ALD muscle was severed from its insertion on the humerus, denervated, flipped over the midline (M) of the back and sutured into the left triceps brachii (TB) muscle. The superior brachialis nerve (N.SB) was severed and then brought over to the transposed ALD muscle and loosely tied to the distal stump of the ALD nerve (N.ALD). In the sham controls, the N.SB was not tied to the N.ALD but returned to its original position. The PLD muscles and nerves were not manipulated and the left ALD muscle and nerve were also left intact.
a. anterior, p. posterior.

Figure 3. (Insert) Schematic drawing showing the nerve cross-union technique used in these experiments. The distal end of the ALD nerve was placed beside the proximal stump of the N.SB and the two nerves tied loosely together with 9-0 suture. Tying of the nerves together was necessary to prevent these nerves from being pulled apart postoperatively. The nerve union was then covered by a small piece of steri-strip.
tonic fibres, the ramus caudialis contains a mixed population of axons supplying the focally-innervated fibres of the PLD and the multiply-innervated fibres of the other muscles.

The distal branch of the brachiales dorsales nerve (also referred to as the superior brachialis nerve), forms, the radialis nerve. This nerve branch lies proximally at the humerus under the triceps muscle and crosses the dorsal side of the upper wing, more or less on a slant on the second third of the humerus. At this point, the radialis nerve is easy to locate as it lies on the bone and is covered only by the skin. Furthermore, it is at this point where the radialis nerve is sectioned and the proximal portion brought over to the transposed ALD muscle. The muscles innervated by the radialis nerve are the extensors of the wing and are composed primarily of fast twitch fibres.

**Surgical Manipulation**

After removal of the feathers from the dorsal skin, the anaesthetized chick was placed on a styrofoam dissecting board in which was carved a groove to fit the wings and the ventral side of the chick. This allowed the wings to be extended throughout the operation and the dorsal side of the chick to lie flat. When pinching of the toes with a pair of forceps failed to elicit a withdrawal reflex of the leg, the operation was begun. In all operations, the surgical manipulations were performed on the right ALD muscle with the left ALD serving as a control. During the course of the operation, if a chick began to experience difficulty with the anaesthetic, a gas mixture (95% oxygen, 5% carbon dioxide) was administered manually to the chick. This gas
mixture was kept in a cylinder obtained from the Canadian Liquid Air Limited and was passed to the chick through a plastic tube (90 cm in length). A rubber bulb was attached to the distal end of the tube and was cut in such a way as to form a seal over the beak of the chick. The gas mixture was administered until the breathing pattern was stabilized. Upon completion of the operation, the dorsal skin of the chick was closed using black 5-0 surgical thread (Ethicon, No.WU 146) and covered with tetraleanB -55 (M.T.C. Pharmaceuticals). Protocols for each of the surgical procedures performed are outlined below.

1. Cross-reinnervation of the ALD Muscle by the Superior Brachialis Nerve: To cross-reinnervate the ALD muscle by the superior brachialis (SB) nerve, a modification of the technique of Zelenā and Jimanovā (1973a) was used (Figure 3). One hundred and thirty-two operations were performed on normal and dystrophic birds. Operations were performed on sixty-four normal chicks having an average body weight of 39.7±0.68 grams (mean ± S.E.M.) from one to five days _ex ovo_. Further experimental manipulations were performed on sixty-eight dystrophic chicks having an average body weight of 30.3±0.45 grams (mean ± S.E.M.) from one to four days _ex ovo_. The right ALD muscle was exposed by an incision made along the dorsal midline and separated from the surrounding connective tissue. The triceps brachii muscles, underwhich the ALD muscle passes, were elevated and the ALD muscle then dissected from its insertion on the humerus. At this point, the ALD muscle contracted down to approximately one quarter of its resting _in situ_ length. The ALD nerve was separated from the FLD nerve and then tied off with suturing thread (Ethilon 9-0 black monofilament nylon, No. 2809
G). The ALD nerve was then severed, a deep incision was made into the underlying musculature and the proximal ALD nerve bundle was implanted into the incision. Since no attempt was taken to separate the ALD nerve from the major blood vessel supplying the ALD muscle, this blood vessel was implanted with the nerve into the underlying musculature thereby rendering the ALD muscle ischemic. The small nerve branch which separates from the ramus caudialis within the tendon of the PLD muscle, was severed in all operations. With the ALD muscle now in a 'supercontracted' state, an incision was made from the center of the initial incision along the midline of the back to a point about two thirds of the way down the left humerus. With its origin intact, the right tenotomized and denervated ALD muscle was flipped over the dorsal midline and transposed to the left side where it was stretched and then sutured in two places to the left triceps brachii muscles with blue 9-0 monofilament nylon thread (Ethilon, No. 2809 G). This step allowed at least some of the resting tension within the ALD muscles to be maintained. In this position, the ventral surface of the transposed ALD muscle became the dorsal surface. This allowed easy access to the distal ALD nerve stump. Next, the left SB nerve was exposed, separated from the surrounding connective tissue and tied off by two knots of suture (black 9-0 monofilament nylon). Between the knots the nerve was sectioned. Tying off of the SB nerve in this way was done so as to prevent excessive bleeding since a major blood vessel of the wing runs alongside the SB nerve. The section was made at the boundary between the proximal and middle third of the humerus so that the proximal nerve stump was sufficiently long enough to avoid traction when transported to
the transposed ALD muscle. The SB nerve was then turned back underneath the triceps brachii muscle and made to form a union with the ALD nerve stump as follows: the two nerves were placed side-by-side so that the end of the nerves overlapped, the nerves were then joined together by a piece of suturing thread, and the union was covered with a piece of Steri-Strip (3M Company), (see Fig.3). No manipulations were performed on the left ALD or FLD muscles and the bird was closed with surgical thread. Therefore, in all cross-reinnervation experiments, the left ALD muscle was used as an unoperated control muscle to identify changes due solely to the experimental manipulation.

2. Transposition of the ALD Muscle: To test specifically for the effects of transposition on the ALD muscle, the following sham operations were performed. The exact same protocol as outlined above was used to transpose the ALD muscle. Similarly, the left SB nerve was isolated, severed, and then turned back underneath the triceps brachii muscles. However, instead of suturing the proximal branch of the SB nerve to the distal branch of the transposed ALD nerve bundle, the SB nerve was fed back underneath the triceps brachii muscles and returned to its original position in the wing after which the bird was closed. In total, 38 sham operations were performed on normal and dystrophic birds. Operations were performed on twenty-four normal chicks having an average body weight of 40.1±0.65 grams (mean ± S.E.M.) from one to four days ex ovo and on fourteen dystrophic chicks having an average body weight of 38.3±1.55 grams (mean ±S.E.M.) from one to six days ex ovo.

3. Denervation of the ALD Muscle: To test for the effects of denervation on normal and dystrophic ALD muscles and to examine the time
course of self-reinnervation of these muscles, short-term denervation experiments were performed. Twenty-four operations were executed on normal and dystrophic birds. Operations were carried out on twelve normal chicks having an average body weight of 40.6±0.83 grams (mean ± S.E.M.) at one day ex ovo and on twelve dystrophic chicks having an average body weight of 34.5±0.67 grams (mean ± S.E.M.) at two days ex ovo. In all operations, the right ALD muscle was denervated while the left ALD muscle served as an unoperated control. The right ALD muscle was exposed by an incision made along the dorsal midline and separated from the surrounding connective tissue. The ALD and PLD nerve branches, which run together after they divide from the musculi latissimi dorsi nerve, were separated by cutting the connective tissue between the two nerves back to the point where the two nerves branch. With forceps, the ALD nerve was pulled from its ventral insertion into the ALD muscle. Major bleeding was always encountered during this stage as no precautions were taken to prevent the muscle from becoming ischemic by the separation of the ALD nerve from the major blood vessel supplying the ALD muscle prior to denervation. A knot was tied to the end of the ALD nerve with suturing thread, a deep incision was made into the underlying musculature, and the ALD nerve plus blood vessel were implanted into the incision. Except for severing the small nerve branch which separates from the PLD nerve, the right PLD muscle and nerve were left intact. The incision was then closed with sutures.

4. Cross-reinnervation of the ALD Muscle (not transposed): To examine the ability of the SB nerve to alter the fibre type of a non-transposed ALD muscle, the following experiments were performed. The right ALD
muscle was first denervated as outlined in the previous section. The proximal ALD nerve stump was implanted into an incision made into the underlying musculature and covered with Gelfoam (Upjohn). The right SB nerve was isolated, severed, and then turned back underneath the triceps brachii muscle. As outlined in Figure 3, the distal ALD nerve stump was then tied to the proximal stump of the right SB nerve. Therefore, in this operation, the ALD muscle was not tenotomized and transposed to the left side of the bird but maintained on the right side. However, the ALD muscle was denervated and made ischemic by the surgical manipulation. Except for severing the small nerve branch which separates from the PLD nerve, the right PLD muscle and nerve were left intact. Twenty-nine operations were performed on normal and dystrophic chicks. Twenty-five were carried out on normal chicks having a mean body weight of 39.5±0.88 grams (mean ± S.E.M.) from two to three days ex ovo. Only four operations were performed on dystrophic chicks between two and three days ex ovo.

5. Ipsilateral Cross-Reinnervation of Injured ALD Muscles:

Newly-hatched dystrophic chicks were obtained from fertilized eggs homozygous for the dystrophic gene (University of Connecticut, Storrs Line). Operations were performed on ten male chicks having an average body weight of 32.8±0.80 grams (mean ± S.E.M.) and on ten female chicks having an average body weight of 32.4±0.80 grams (mean ± S.E.M.) between 1 and 4 days ex ovo. In all operations, the right ALD muscle was cross-reinnervated while the left ALD muscle served as an unoperated control. The right ALD muscle was denervated as previously described and then injured in situ. Injury involved mincing the ventral and
dorsal surfaces of the muscle and then pinching the muscle with forceps. The origin and insertion of the muscles were left intact and none of the muscles were tenotomized. The right posterior latissimus dorsi (FLD) muscle was then removed from the chick and the FLD tendon, in which the FLD nerve runs, was inserted into a hole cut into the centre of the injured ALD muscle. It was secured with fibrin clot and the chick closed. This technique is a modification of the procedure developed by Gordon and Vrbová, (1975c).

METHODS FOR ANALYSIS OF MUSCLE PROPERTIES

Unoperated and experimental ALD muscles of both normal and dystrophic chickens were analyzed similarly. Mechanical, histochemical, and structural properties were examined according to the procedures outlined below. These analyses were carried out on muscles from chickens 2 to 104 weeks ex ovo.

Mechanical Analyses

The protocol used to quantify the contractile properties of control and experimental ALD muscles was developed by Mazliah (1980) and are reiterated below. On a predetermined day, an experimental chicken was anaesthetized with Combuthal administered to the musculature of the right leg. Although the dosage required to induce a deep anaesthesia was variable between chickens, the average dose found to produce satisfactory anaesthesia was 0.35 cc per 100 gram body weight (concentration 20mg/ml). Only when pinching of the toes failed to elicit a withdrawal reflex of the leg were the feathers covering the dorsal
surface of the back and wings plucked and the bird prepared for surgery. During surgery, the chicken was placed on a custom made styrofoam base in which was carved a groove to fit the wings and the ventral side of the chicken. This allowed the wings to be extended and the dorsal side of the chicken to lie flat. Throughout the experiment, the chicken was maintained on a conventional small animal respirator (Harvard 681) using a tracheal cannula inserted through the pharynx. The flow rate was adjusted to maintain a normal respiratory rate of between 300-500 cc per minute. An incision was made along the dorsal midline between the shoulders along the thoracic vertebrae to the pelvic region. A second incision was made laterally from the dorsal midline to the wing within the thoracic region. The skin was then deflected back to expose the experimental ALD muscle. Paraffin oil saturated with Hanks' balanced salt solution heated to 40°C (chicken body temperature) was dropped continually on the muscle to keep it wet and at the proper temperature. A heating lamp was placed beside the chicken in order to keep it warm. Under these conditions, a drop in body temperature of only 1-2°C was observed during the course of the operation (Mazliah, 1980).

Because of the anatomical differences between the positioning of the transposed and non-transposed ALD muscles, it was necessary to use two different approaches when recording the contractile properties of these muscles. However, in both cases either the insertion or origin of the muscle was freed and tied to a mechanical transducer while the other end was maintained with its normal anatomical connection. Further details of the measurement of the mechanical properties of the ALD muscles in vivo are now given.
Non-transposed ALD: To prepare the ALD muscle for the in vivo measurement of its isometric contractions, the muscle was freed from its origin on the cervical vertebrae. The free end of the muscle was then tied to a custom made light weight stainless steel hook (Maziah, 1980). The ALD muscle was separated from the surrounding connective tissue and the triceps brachii muscles lifted with hooks away from the ALD muscle. The wing was then immobilized by a clamp rigidly fixed to the humerus. In turn, this clamp was attached to a retort stand with a magnetic base. Since the whole experimental set-up was supported by a heavy metal plate, the magnetic bases attached to the retort stand and the styrofoam dissecting board ensured that all parts of the chicken remained stationary. The ALD nerve was then freed from its surrounding connective tissue and the PLD nerve. The ALD blood vessel was not severed thereby ensuring a constant blood flow to the muscle. This set-up was used to record the contractile responses of the contralateral left ALD as well, i.e., the origin was freed instead of the insertion.

Transposed ALD: In these experiments, the transposed ALD muscle was severed from its insertion into the triceps brachii muscles. In normal birds, it was easy to distinguish between the distal end of the transposed ALD muscle and the triceps brachii muscles. However, because of the accumulation of connective tissue around the transposed ALD muscle in dystrophic chickens, severing of the distal end of the ALD muscle at the location of the two blue 9-0 sutures was more difficult. Regardless of this difficulty, once the distal end of the transposed ALD was freed, it was tied to the lightweight hook. The muscle was then separated from the surrounding connective tissue and the trunk and wing
of the chicken secured. In order to stimulate the muscles indirectly, the SB nerve was then isolated. This set-up was used to record the mechanical properties of the underlying left ALD muscle as well, i.e., the insertion was freed instead of the origin.

**Mechanical Recording:** The small hook attached to either the distal or proximal end of the muscle was, in turn, connected to an isometric strain gauge transducer (BLH, SR-4 semi-conductor). To maintain the natural line of pull of the muscle, the transducer was adjusted so that the muscle was only slightly raised from the body, yet still able to contract at right angles to the transducer. The transducer was connected to a bridge amplifier (operational amplifier LI445J5 siliconix) and the output of this amplifier was displayed on a dual beam cathode ray storage oscilloscope (taktronic 5113).

The ALD and SB nerves were stimulated with a pair of platinum electrodes. The intensity and duration of the supramaximal rectangular pulse (1-50 volts, 0.02-0.1 msec) were determined and supplied by a stimulator (Digitimer LTD, Model DS2). The timing and triggering of the selected stimulus pulses was controlled by a Digitimer D4030. Through this system, a single pulse, a train of stimuli ranging from 10-1000 Hz, or any combination of the two could be delivered to the nerve.

The optimum initial length was determined by stimulating the muscle indirectly with a train of stimuli (100 Hz) and the length of the muscle adjusted until it gave its maximal tetanic response. This resting tension was maintained throughout the duration of the experiment. Each muscle was tested for its tetanic response to a train of stimuli at 50, 70, and 100 Hz. Preliminary studies by Mazliah (1980) indicated that
with these frequencies, between 80-100% of the maximum tetanic tension of an ALD muscle could be generated. Since an unoperated ALD muscle does not consistently respond to a single stimuli in vivo, the experimental muscle was also tested to determine its twitch response following a single pulse and a post tetanic stimulation. In the latter case, a tetanic stimulation at a frequency of 70 Hz was applied to the nerve for 7 seconds and a single pulse applied 150 msec later.

The tetanic and twitch responses of the muscle were displayed on a storage cathode ray oscilloscope. Photographs of these responses were taken during the experiments using an oscilloscope polaroid camera (Tektronix C-5A). These photographs were then used to measure specific parameters of the responses (Figure 4): Twitch tension (TWT), tetanic tension (TT), time to half contraction (T1/2C), contraction time (Tc), and time to half relaxation (T1/2R). Subsequent to the mechanical analyses, the muscle was removed from the bird, the wet weight determined, and then prepared for histochemical examination.

After analyzing many of these experiments, though, it was noticed that the histochemical staining pattern for the metabolic enzymes was being disrupted by the physiological measurements. This was not unexpected since the ALD muscle is a highly aerobic muscle and severing of the distal or proximal end of the muscle always caused excessive bleeding. Since the operative procedures lasted for as long as 1-1 1/2 hours, this was sufficient time for the muscle to become ischemic. Therefore, quantitative physiological analyses were discontinued and replaced by more rapid qualitative analyses. In these analyses, the response of the muscle to an indirect or direct single pulse or a train
Figure 4. 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 (ThC) 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 (ThR) 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. (From Mazliah, 1980).
of pulses was noted but not recorded. In this way, analyses were performed within fifteen minutes and the histochemical detection of the metabolic enzymes was minimally affected.

Histochemical Analyses

Established techniques for the histochemical analysis of avian muscle were followed (Cosmos, 1966a, 1970; Cosmos and Butler, 1967; Cosmos et al., 1979b). After the physiological tests were performed, the chicken was sacrificed by an injection of 2.0 cc of Sodium Pentobarbital into a major wing vein. After the bird had been bled, the ALD and PLD muscles were removed, trimmed of blood and connective tissue, and then weighed.

After being weighed, each muscle was sectioned into two halves; one half representing the origin or proximal end of the muscle and the second half representing the insertion or distal end. Each block was then mounted in cross-section with the aid of mounting media (O.C.T. Compound) on small cork blocks. In most cases, similar regions of both the control and experimental ALD muscles were mounted together on the same cork to permit a simultaneous exposure to the same histochemical procedure. Similarly, blocks from the right and left PLD muscles were mounted in this way. Each block was then deep-frozen for 30-60 seconds in isopentane (Practical grade, J.T. Baker) precooled between -150° to -160°C by liquid nitrogen. The frozen blocks were subsequently placed in precooled polystyrene culture dishes (60 mm) and then the dishes were stored in a deep freezer (Revco) maintained at -70°C.

On the day of cryostat sectioning, a block of tissue was removed
from the freezer and mounted on a chuck with the aid of mounting media. This step was performed in a dewar flask maintained at approximately -70°C by dry ice. The mounted tissue was then placed in a cryostat maintained at -27°C and at least twenty serial sections were cut from each block. Each section was cut at a thickness of 14 μm, picked up on a microscope slide and air dried at room temperature for at least 1/2 hour. Prior to mounting of a section from the PLD muscle, the microscope slide was covered with a thin coat of egg albumin to prevent the PLD sections from contracting off the slide during the histochemical reactions. The following histochemical reactions were done on consecutive serial sections:

1. Myosin ATPase reaction after acid (pH = 4.35) and alkali (pH = 10.0) preincubation to examine the fibre type composition of avian muscles. While slow tonic fibres possess an acid and alkali stable myosin ATPase activity, fast twitch fibres show an alkali stable activity only, that is, the myosin ATPase activity of fast twitch fibres is lost after exposure to an acid medium. The reaction used in this work was developed by J. Butler (Butler and Cosmos, 1979) and was modified for avian muscle after a technique developed by Guth and Samaha (1970) for distinguishing fibre types in mammalian muscles.

2. Phosphorylase (Pase) and succinic dehydrogenase (SDH) reactions to examine the metabolic state of a muscle. The activities of these enzymes indicate whether or not a muscle has an energy-supplying system based on an aerobic or anaerobic metabolism (Cosmos, 1966a).

3. Silver cholinesterase (AChE) reaction to examine the innervation status of a muscle and to determine its type of innervation. With this
reaction, muscle fibre endplates, nerve bundles and axons can be identified (Toop, 1976). In some cases, the Köelle reaction was also used to identify endplates (Koelle, 1950).

4. Oil Red O reaction to demonstrate the extra- and intracellular accumulation of neutral lipids within muscles (Barka and Anderson, 1963).

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

After exposure of the various histochemical reactions, the stained cross-sections were examined. Specific sections were then selected and photographed with a Zeiss Photomicroscope. Black and white (Kodak Panatomic-X) photographs and 35 mm colour slides (Kodachrome 64) were taken by Dr. E. Cosmos.

Structural Analysis

The size and shape of individual muscle fibres was quantified by an Image Analysis System (Carl Zeiss Videoplan). These parameters were measured after projecting a magnified image (100–400 times) of a muscle cross-section through a camera lucida onto a piece of drawing paper (see Figure 5). This piece of paper was laid over the surface of a digitizing tablet. With this set-up an individual fibre was simultaneously viewed under the microscope and its image traced out on the digitizing tablet with a stylus. By simply tracing around the circumference of a muscle fibre, the area, perimeter, maximum, minimum and mean diameters, and the shape of that fibre were automatically
Figure 5. A drawing of the basic set-up for the quantification of the structural properties of individual muscle fibres using an Image Analysis System. A muscle cross section (M) was projected from the microscope (MICRO), through a camera lucida (CL) and onto the surface of a digitizing tablet (TAB). The outline of a fibre was then traced out by a pen (STYLUS). The information was transferred from the stylus to the computer console (CONSOLE) where it was stored. Using the KEYBOARD, various programs stored on 5" floppy disks, could be activated to analyze the stored data. Analysis results were displayed on the video monitor (MONITOR) or PRINTER.
compiled. In the latter case, the computer calculated the ratio of the minimum (b) to maximum (a) diameter. With this calculation, if b/a = 1, the cross section of the fibre is a circle and, similarly, if b/a < 1, the cross section of the fibre is more elliptical in shape. Data obtained in this way was entered into the memory of the computer and then stored on 5" floppy disks. To examine the distribution of a specific parameter, 'canned' programs were available which, when activated, automatically constructed a histogram showing the percentage distribution of that parameter for a given muscle or a group of muscles. Furthermore, statistical programs were also available through this system.

All measurements were taken from muscle cross sections which had been stained for the myosin ATPase reaction (alkaline preincubation). When sampling a muscle, an area in the central portion of the muscle was selected and then 200 fibres within that area analyzed. The results obtained by this type of selected procedure did not significantly differ from the results obtained through random sampling of the central regions of the muscle since the fibres of the ALD and PLD muscles are homogeneous in size and shape throughout the central regions of the muscle. Sampling of the fibres around the edge of the muscles was avoided since the periphery was often damaged during the initial operative procedures.

Estimation of Acid Labile Fibres

Normally, ALD muscles of either normal or dystrophic genotype are composed entirely of slow tonic fibres. These fibres synthesize a type
of myosin which expresses an ATPase activity after exposure to acid and alkali preincubation solutions. After cross-reinnervating ALD muscles with a nerve which normally innervates a fast twitch muscle, fibres which synthesize a different type of myosin can be identified. This myosin retains its ATPase activity after alkali preincubation but loses it after acid preincubation. Therefore, the fibres synthesizing this 'fast' type of myosin can be referred to as 'acid labile' fibres inferring that the myosin synthesized by these fibres is labile after acid preincubation. To quantify the percentage of acid labile fibres within cross-reinnervated muscles, three approaches were used in the present study:

1. By counting the total number of unconverted and acid labile fibres within a cross section of an experimental muscle and then expressing the percentage of acid labile fibres as: number of acid labile fibres/total number of fibres x 100. The percentage of acid labile fibres in two week old cross-reinnervated normal muscles was quantified in this manner. Although this approach was the most accurate one to determine the percentage of acid labile fibres within a muscle, it was not practical to continue with this approach as the ALD muscles grew larger. Therefore, a second, and more rapid approach was adopted;

2. From a sample of 200 fibres taken from the central region of each muscle, the percentage of acid labile fibres within this sample was calculated. Although accurately depicting the percentage of acid labile fibres within a small central region of each experimental muscle, if the distribution of acid labile fibres was not randomly distributed throughout the muscle then the estimates of the percentage of acid
labile fibres could not be applicable to the muscle as a whole. This was not a problem in the dystrophic preparations since in most of the cross-reinnervated muscles, the acid labile fibres were randomly scattered throughout the muscle. However, in many of the cross-reinnervated normal muscles the acid labile fibres were not randomly scattered but biased in their distribution. To overcome this limitation, a third approach was adopted;
3. The percentage of acid labile fibres was estimated visually by two investigators, (myself and Dr. E. Cosmos). The average of the two estimates was calculated and used as the percentage of acid labile fibres for that muscle.
RESULTS

PART 1: DEVELOPMENTAL STUDY

INTRODUCTION

The aim of this study is to examine selected structural and histochemical properties of the ALD and PLD muscles of normal chickens and to determine which of these parameters are altered in the ALD and PLD muscles of dystrophic chickens during ex ovo development. The data established in this section will be used as baseline data for the analysis of muscles cross-reinnervated by 'fast' nerves as well as for other experimental manipulations of the ALD muscle.

WEIGHT CHANGES

Body Weight of Normal and Dystrophic Chickens

The growth rate of normal (White Leghorn) and dystrophic (Storrs) unoperated roosters and hens is biphasic; an initial period of growth is followed by a second period where all birds achieve a steady state level with no further weight gain (Maziah, 1980). Transition from the first period to the second takes place at approximately 160 days, a time when chickens reach sexual maturity (Cosmos, 1970). Regression analyses were used to compare the growth rates of experimental normal and dystrophic hens and roosters during the first 160 days after
hatching (Table 1). In these analyses, scatter plots of body weights up to 160 days were fitted with a regression line, the slope of which reflects the rate of growth of chickens during this time. As shown in Table 1, in both normal and dystrophic birds, roosters grow at a significantly faster rate than hens (p<0.001). Furthermore, roosters and hens of dystrophic genotype grow less rapidly than do roosters and hens, respectively, of normal lineage (p<0.001). Table 1 also shows that the correlation between body weight and age is less in dystrophic birds than measured in normal birds.

Muscle Wet Weight

After the experimental birds had been subjected to physiological analyses, the unoperated ALD and PLD muscles were excised from the birds and weighed prior to freezing. Due to the differences noted in body weight between hens and roosters, ALD and PLD muscle weights were related to body weight rather than to age. Scatter plots were subsequently prepared comparing muscle wet weight to body weight in both normal and dystrophic chickens; the slope of which represents the wet weight to body weight ratio.

ALD Muscle Weight: Table 2 records the ALD muscle wet weight to body weight ratio for normal (n = 89) and dystrophic (n = 73) chickens from 6 to 300 days of age. Regression analyses of the data presented in this table indicate:

1. a positive correlation exists between ALD muscle weight and body weight in both genotypes although the correlation is greater in normal birds; and
### TABLE 1. RATE OF GROWTH OF NORMAL AND DYSTROPHIC CHICKENS BETWEEN 20 AND 160 DAYS OF AGE

#### a. The regression lines \( y = mx + b \)

<table>
<thead>
<tr>
<th>Group</th>
<th>Slope</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>N - ♂ (39)</td>
<td>15.2</td>
<td>0.98</td>
</tr>
<tr>
<td>N - ♀ (44)</td>
<td>10.9</td>
<td>0.97</td>
</tr>
<tr>
<td>D - ♂ (44)</td>
<td>11.1</td>
<td>0.91</td>
</tr>
<tr>
<td>D - ♀ (54)</td>
<td>8.0</td>
<td>0.92</td>
</tr>
</tbody>
</table>

#### b. Test for difference between slopes

<table>
<thead>
<tr>
<th>Group</th>
<th>t</th>
<th>df</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N - ♂ vs N - ♀</td>
<td>9.43</td>
<td>81</td>
<td>0.001</td>
</tr>
<tr>
<td>D - ♂ vs D - ♀</td>
<td>4.91</td>
<td>96</td>
<td>0.001</td>
</tr>
<tr>
<td>N - ♀ vs D - ♀</td>
<td>6.52</td>
<td>96</td>
<td>0.001</td>
</tr>
<tr>
<td>N - ♂ vs D - ♂</td>
<td>6.14</td>
<td>81</td>
<td>0.001</td>
</tr>
</tbody>
</table>

( ) = number of chickens

♂ = roosters; ♀ = hens; m = slope; b = intercept

df = degree of freedom

* two tail probability
TABLE 2. WET WEIGHT TO BODY WEIGHT RATIO OF NORMAL AND DYSTROPHIC MUSCLES BETWEEN 6 AND 300 DAYS OF AGE

a. The regression lines \( y = mx + b \)

<table>
<thead>
<tr>
<th>Group</th>
<th>Slope</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>N - ALD (89)</td>
<td>0.33</td>
<td>0.97</td>
</tr>
<tr>
<td>D - ALD (73)</td>
<td>0.40</td>
<td>0.94</td>
</tr>
<tr>
<td>N - PLD (85)</td>
<td>0.61</td>
<td>0.97</td>
</tr>
<tr>
<td>D - PLD (71)</td>
<td>0.37</td>
<td>0.90</td>
</tr>
</tbody>
</table>

b. Test for differences between slopes

<table>
<thead>
<tr>
<th>Group</th>
<th>t</th>
<th>df</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N - ALD vs D - ALD</td>
<td>5.23</td>
<td>160</td>
<td>0.001</td>
</tr>
<tr>
<td>N - PLD vs D - PLD</td>
<td>12.83</td>
<td>154</td>
<td>0.001</td>
</tr>
<tr>
<td>N - ALD vs N - PLD</td>
<td>21.71</td>
<td>172</td>
<td>0.001</td>
</tr>
<tr>
<td>D - ALD vs D - PLD</td>
<td>1.54</td>
<td>142</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

( ) = number of muscles
N.S. = not significant
* two tail probability
2. the slopes of the regression lines are significantly different (p<0.001) with the wet weight to body weight ratio being significantly greater in dystrophic birds.

**PLD Muscle Weight**: Table 2 also shows the PLD muscle wet weight to body weight ratio for normal (n = 85) and dystrophic (n = 71) chicks aged between 6 and 300 days ex ovo. The regression lines indicate a positive correlation exists between PLD muscle weight and body weight for both normal and dystrophic chickens; this correlation is less in dystrophic birds. When tested statistically for the difference between the slopes, a highly significant difference (p <0.001) was obtained where the dystrophic PLD wet weight to body weight ratio was lower than that of its normal counterpart.

**Ratio Between the Weight of ALD and PLD Muscles**: When the wet weight to body weight ratio of ALD muscles was compared to that of PLD muscles in normal chickens a highly significant difference was found (Table 2); the ratio is greater in PLD muscles. When the same comparison was made in dystrophic birds a similar relationship was not noted. Instead, the dystrophic ALD and PLD muscles had similar ratios. Since the dystrophic ALD and PLD muscles have the same wet weight at hatching (Mazliah, 1980), this means that for a given body weight, the wet weight of the ALD and PLD muscles are similar.

**STRUCTURAL ANALYSES**

Several structural parameters of ALD and PLD muscle fibres were calculated from traces made of normal and dystrophic muscles, i.e., fibre area, shape and minimum diameter. The minimum fibre diameter is
defined as the maximum diameter across the lesser aspect of the muscle fibre and was first suggested to correct for any distortion which occurs when a muscle fibre is cut obliquely (Dubowitz and Brooke, 1973).

**ALD Muscles**

Figures 6 and 7 represent composite drawings of fibres taken from age-matched normal and dystrophic ALD muscles, respectively. These drawings are presented to emphasize similarities in the general architecture of normal and dystrophic ALD muscles. As shown in these figures, the fascicles and fibres of normal and dystrophic ALD muscles are elliptical in shape and large extracellular spaces exist between these structures. The histograms accompanying the two drawings demonstrate that the distribution of minimum fibre diameters is normal. In a small percentage of normal and dystrophic muscles, the distribution is not normal but slightly bimodal. This bimodality is due to a slight difference in the diameters of $a'$ and $b'$ fibres within the ALD muscles of either genotype. Past the onset of sexual maturity, the fibres in the ALD muscles of roosters are consistently larger in size than those fibres in the ALD muscles of hens.

Figure 8 correlates the minimum fibre diameter of normal and dystrophic ALD muscles with age up to 32 weeks ex ovo. It is clear from this histogram that an initial period of rapid growth is followed by a second period where the minimum fibre diameters stabilize and do not continue to increase. This biphasic pattern of growth is characteristic of fibres from both normal and dystrophic ALD muscles. At every time period examined, no significant difference was noted between the mean
Figure 6. A composite drawing showing 200 individual muscle fibres in cross section drawn from a normal ALD muscle excised from a 24 week old rooster. Each clear fibre represents an a' fibre while those fibres with a dot represent b' fibres. Accompanying the drawing is a histogram showing the relative distribution of minimum diameters (μm) of the fibres in the drawing.
Figure 7. A composite drawing showing 200 individual muscle fibres in cross section drawn from a dystrophic ALD muscle excised from a 24 week old rooster. Each clear fibre represents an a' fibre while those fibres with a dot represent b' fibres. Accompanying the drawing, is a histogram showing the relative distribution of minimum diameters (μm) of the fibres in the drawing.
Figure 8. The relationship between the minimum fibre diameter of normal and dystrophic ALD muscle fibres with age ex ovo. This histogram shows that the minimum fibre diameter of fibres in normal and dystrophic ALD muscles change similarly with time.
fibre diameter of normal and dystrophic ALD muscles.

Table 3 shows the fate of small diameter fibres in normal and dystrophic ALD muscles. Those fibres which have a minimum fibre diameter of less than 20 μm were defined as 'small diameter fibres'. Within the first four weeks after hatching there is a precipitous drop in the relative percentage of small diameter fibres in the ALD muscles of either genotype so that after four weeks, these fibres never comprise more than 5% of the total population of fibres within the ALD muscles.

In an attempt to quantify the shape of ALD muscle fibres, the minor and major fibre axes of individual muscle fibres were compared in a ratio (minor axis/major axis). With this analysis, a ratio of 1.0 indicates that a fibre is circular in shape; the minor axis length being equal to the major axis length. Table 4 shows that normal ALD muscle fibres throughout the ex ovo period, are elliptical in shape since the minor axis length is between 0.65 to 0.70 times shorter than the major axis length. Similarly, dystrophic ALD muscle fibres are also elliptical throughout the same period. The shape of normal and dystrophic ALD fibres do not differ significantly over any of the time periods analyzed.

PLD Muscles

The approach used to analyze the structural properties of ALD muscle fibres was also used to analyze PLD fibres. Several structural parameters of PLD muscle fibres were calculated from traces made of normal and dystrophic PLD muscles excised from chickens aged between two and thirty-two weeks. Of these parameters, only three will be
### TABLE 3. CHANGE IN THE PERCENTAGE OF SMALL DIAMETER FIBRES IN NORMAL AND DYSTROPHIC ALD MUSCLES

<table>
<thead>
<tr>
<th>AGE (Weeks)</th>
<th>SMALL DIAMETER FIBRES (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>15.7 (15)</td>
</tr>
<tr>
<td>4</td>
<td>3.9 (16)</td>
</tr>
<tr>
<td>8</td>
<td>0.4 (12)</td>
</tr>
<tr>
<td>12</td>
<td>1.0 (12)</td>
</tr>
<tr>
<td>16</td>
<td>0.4 (10)</td>
</tr>
<tr>
<td>20</td>
<td>0.1 (7)</td>
</tr>
<tr>
<td>24</td>
<td>0.3 (11)</td>
</tr>
<tr>
<td>28</td>
<td>1.7 (8)</td>
</tr>
<tr>
<td>32</td>
<td>0.2 (5)</td>
</tr>
</tbody>
</table>

( ) = number of muscles analyzed
TABLE 4. SHAPE OF FIBRES IN NORMAL AND DYSTROPHIC ALD MUSCLES

<table>
<thead>
<tr>
<th>AGE (Weeks)</th>
<th>MINOR/MAJOR AXIS LENGTH</th>
<th>Normal</th>
<th>Dystrophic</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.66 ± 0.01a (15)</td>
<td>0.66 ± 0.01  (14)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.70 ± 0.01 (16)</td>
<td>0.68 ± 0.01  (11)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.68 ± 0.01 (12)</td>
<td>0.66 ± 0.01  (11)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.66 ± 0.01 (12)</td>
<td>0.66 ± 0.01  (10)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.65 ± 0.01 (10)</td>
<td>0.63 ± 0.01  (9)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.66 ± 0.01  (7)</td>
<td>0.64 ± 0.01  (8)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.67 ± 0.01 (11)</td>
<td>0.64 ± 0.02  (5)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>0.65 ± 0.01  (8)</td>
<td>0.62 ± 0.01  (5)</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>0.68 ± 0.01  (5)</td>
<td>0.63 ± 0.02  (4)</td>
<td></td>
</tr>
</tbody>
</table>

a. Mean ± S.E.M.

() = number of muscles analyzed
presented, i.e., the minimum fibre diameter, area, and shape of individual fibres.

Figures 9 and 10 represent composite drawings of PLD fibres taken from mature normal and dystrophic PLD muscles, respectively. These drawings are presented to emphasize the differences which exist in the size and shape of fibres in both normal and dystrophic PLD muscles. As demonstrated in Figure 9, when viewed in cross section, the fascicles of normal PLD muscles are elongated in shape and compact in appearance. Each fascicle is tightly-packed with fibres. While most of these fibres are similar in size, a low percentage of the fibres are smaller. This distribution of fibre sizes is accurately depicted in the histogram accompanying Figure 9. While a majority of fibres have diameters distributed normally about a mean value, a population of smaller fibres also exists. This distribution, then, results in a histogram of minimum fibre diameters which is slightly skewed to the left.

In contrast to the highly organized appearance of the normal PLD muscles, mature dystrophic PLD muscles contain fascicles and fibres which are highly variable in both size and shape. When viewed in cross section, the fascicles within mature dystrophic PLD muscles have rounded shapes and are not compact in appearance; large spaces separate individual fascicles (Figure 10). Each fascicle is loosely-packed with large extracellular spaces present between the fibres. Most of the fibres are either very large or very small. This distribution is accurately reflected in the skewed histogram accompanying Figure 10.

To investigate when structural differences first appear between normal and dystrophic PLD muscles, the PLD muscles of both genotypes
Figure 9. A composite drawing showing 200 individual muscle fibres in cross section drawn from a normal PLD muscle excised from a 16 week old rooster. Accompanying the drawing is a histogram showing the relative distribution of minimum diameters (μm) of the fibres in the drawing.
Figure 10. A composite drawing showing 200 individual muscle fibres in cross section drawn from a dystrophic PLD muscle excised from a 24 week old rooster. Accompanying the drawing, is a histogram showing the relative distribution of minimum diameters (µm) of the fibres in the drawing.
were examined developmentally. Figures 11 and 12 show how the minimum diameter of fibres sampled from normal and dystrophic muscles change during the early ex ovo period. In Figure 11 it is quite clear that by two weeks after hatching, the minimum fibre diameters compiled from normal PLD muscles are distributed normally about a mean value. As the normal PLD muscles mature from 4 through 24 weeks, the mean size of the fibres increases. However, the left-handed skewness of the histograms indicates that from 4 weeks and on, a population of small fibres persists within the normal PLD muscles.

A similar analysis of dystrophic PLD fibres (Figure 12), during the same time period reveals that structural differences between normal and dystrophic PLD fibres can be detected as early as two weeks after hatching. At two weeks ex ovo, the minimum diameter of dystrophic PLD fibres are distributed normally about a mean value; a distribution similar to that observed at 2 weeks in normal PLD muscles. However, close examination of the two week normal and dystrophic PLD graphs shows that the dystrophic fibres are slightly larger than their normal counterparts. Differences in the size distribution of normal and dystrophic PLD fibres become more evident with further maturation. In contrast to normal PLD fibres, the majority of fibres in dystrophic PLD muscles continue to remain small. The right-handed skewness of the histograms at 4, 12, and 24 weeks in Figure 12, however, indicates that a population of dystrophic fibres do increase in size. In fact, some of these growing fibres are hypertrophic when compared to age-matched PLD fibres.

Early differences in the size distribution of normal and dystrophic
Figure 11. Minimum fibre diameter distribution of muscle fibres sampled from normal PLD muscles at 2, 4, 12, and 24 weeks ex ovo. Five or more PLD muscles were taken from birds of both sexes at each time period and 200 fibres were sampled from each muscle, i.e., total is 1000 plus fibres for each age indicated. These histograms emphasize the steady increase in the size of normal PLD muscle fibres during the ex ovo period and demonstrate the development of the slightly skewed distribution of minimum fibre diameters observed in mature PLD muscles.
Figure 12. Minimum fibre diameter distribution of muscle fibres sampled from dystrophic PLD muscles at 2, 4, 12, and 24 weeks ex ovo. Five or more PLD muscles were taken from birds of both sexes at each time period and 200 fibres were sampled from each muscle, i.e., total is 1000 plus fibres for each age indicated. These histograms clearly demonstrate the maintenance of a large number of fibres with a small fibre diameter throughout the ex ovo period in dystrophic PLD muscles.
PLD fibres are re-emphasized in a more precise form in Figure 13. In this figure, histograms are presented of the frequency distribution of fibre area for normal and dystrophic PLD fibres from 2 to 24 weeks ex ovo. Analysis of these histograms indicate that there is a steady increase in the average area of normal PLD fibres from 2 to 24 weeks although skewing of the histograms at 4, 12 and 24 weeks indicate the maintenance of a population of small fibres in these muscles. By two weeks, the distribution of dystrophic PLD fibres is unimodal with a slight skewing to the right. By four weeks, the distribution of dystrophic PLD fibres becomes flattened. This type of distribution persists at 12 weeks and by 24 weeks, it is clear that two populations of fibres are present: a population of small fibres and another of larger fibres. Although the distribution of fibre areas in normal and dystrophic PLD muscles is similar at 2 weeks, it is clear that the dystrophic fibres are larger than the normal PLD fibres by this time. With further growth the dystrophic PLD fibres become more variable in size relative to the normal PLD fibres. In fact, by 4 weeks a population of fibres is established and maintained in dystrophic PLD muscles which are hypertrophic compared to normal PLD fibres. The maintenance of a large population of small fibres also contributes to the increased variability evident in 4 to 24 week old dystrophic muscles. Although not shown, the bimodal distribution of fibre areas persists in dystrophic PLD muscles up to 32 weeks ex ovo.

Table 5 emphasizes the fact that a population of small fibres persists in dystrophic PLD muscles. This table shows that within the first four weeks after hatching, there is a sharp decrease in the
Figure 13. Fibre area distribution of muscle fibres sampled from normal and dystrophic PLD muscles at 2, 4, 12, and 24 weeks ex ovo. Five or more PLD muscles were taken from birds of both sexes at each time period and 200 fibres were sampled from each muscle. These histograms emphasize the steady increase in the size of normal PLD muscle fibres during the ex ovo period and clearly demonstrate the development of the skewed distribution of fibre areas observed in mature PLD muscles. In contrast, two populations of fibres form in dystrophic PLD muscles during the first 6 months of ex ovo development. One population consists of small fibres while the other consists of larger fibres.
TABLE 5. CHANGE IN THE PERCENTAGE OF SMALL DIAMETER FIBRES IN NORMAL AND DYSTROPHIC PLD MUSCLES

<table>
<thead>
<tr>
<th>AGE (Weeks)</th>
<th>SMALL DIAMETER FIBRES (%)</th>
<th>Normal</th>
<th>Dystrophic</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>80.8 (7)</td>
<td></td>
<td>55.0 (7)</td>
</tr>
<tr>
<td>4</td>
<td>30.7 (5)</td>
<td></td>
<td>57.9 (5)</td>
</tr>
<tr>
<td>8</td>
<td>12.9 (4)</td>
<td></td>
<td>52.0 (3)</td>
</tr>
<tr>
<td>12</td>
<td>17.6 (5)</td>
<td></td>
<td>48.3 (5)</td>
</tr>
<tr>
<td>16</td>
<td>6.7 (5)</td>
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<td>53.6 (6)</td>
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<td>20</td>
<td>4.1 (4)</td>
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</tr>
<tr>
<td>32</td>
<td>4.1 (5)</td>
<td></td>
<td>44.5 (5)</td>
</tr>
</tbody>
</table>

( ) = number of muscles analyzed
relative percentage of small fibres in normal PLD muscles. From four to
twelve weeks, the percentage of these fibres continues to decline;
after 16 weeks, small fibres represent less than 10% of the total PLD
fibre population. In contrast, never during the same period does the
percentage of small fibres in dystrophic PLD muscles decline to, less
than 40%, i.e., at least four out of every ten fibres will have a
minimum fibre diameter less than 20 μm.

Table 6 demonstrates that normal and dystrophic PLD fibres can be
differentiated on the basis of shape during the early ex ovo period.
This difference is significant (p < 0.01) by 8 weeks and persists until
at least 32 weeks. Throughout this period the dystrophic PLD fibres are
more elliptical in shape than the normal PLD fibres.

HISTOCHEMICAL ANALYSES

Structural analyses, therefore indicate that while normal and
dystrophic ALD muscles are similar, the PLD muscles of normal and
dystrophic chickens are dissimilar. These results correlate well with
the structural analyses performed by Mazlia (1980) on developing normal
and dystrophic ALD and PLD muscles. In addition, Cosmos and co-workers
(Cosmos et al., 1979b; Cosmos et al., 1980) were unable to detect
differences in the histochemical properties of normal and dystrophic ALD
muscles although differences were noted between normal and dystrophic
PLD muscles. The histochemical findings reported in the present study
confirm these histochemical observations and were included to be used as
a baseline for the analyses of ALD muscles cross-reinnervated by 'fast'
nerves.
TABLE 6. SHAPE OF FIBRES IN NORMAL AND DYSTROPHIC PLD MUSCLES

<table>
<thead>
<tr>
<th>AGE (Weeks)</th>
<th>MINOR/MAJOR AXIS LENGTH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>0.68 ± 0.01 (7)</td>
</tr>
<tr>
<td>4</td>
<td>0.68 ± 0.01 (5)</td>
</tr>
<tr>
<td>8</td>
<td>0.72 ± 0.01 (4)</td>
</tr>
<tr>
<td>12</td>
<td>0.70 ± 0.02 (5)</td>
</tr>
<tr>
<td>16</td>
<td>0.70 ± 0.01 (5)</td>
</tr>
<tr>
<td>20</td>
<td>0.61 ± 0.02 (4)</td>
</tr>
<tr>
<td>24</td>
<td>0.68 ± 0.01 (5)</td>
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<tr>
<td>28</td>
<td>0.67 ± 0.01 (5)</td>
</tr>
<tr>
<td>32</td>
<td>0.67 ± 0.02 (5)</td>
</tr>
</tbody>
</table>

a. Mean ± S.E.M.

( ) = number of muscles analyzed

*Statistical significance (P < 0.01) between normal and dystrophic PLD muscles
ALD Muscles

The ALD muscle fibres of either genotype are multiply-innervated by ‘en grappe’ endings and demonstrate an alkali- and acid-stable myosin ATPase response (Figures 14a and 14b). These fibres have a low phosphorylase and high SDH activities indicative of the aerobic metabolism characteristic of slow tonic muscles (Figure 15). The response of ALD muscle sections to the histochemical test for DNA-RNA shows that the nuclei of ALD fibres are peripherally located. Moreover, intracellular lipid droplets are readily observed in these fibres with the Oil Red O reaction.

As shown in Table 7, the a' and b' fibres within the normal and dystrophic ALD muscles can be differentiated on the basis of several criteria. These two fibre sub-types are distributed randomly throughout the ALD muscle and give the muscle a mosaic appearance. The b' fibres demonstrate a stronger (darker) myosin ATPase activity particularly after acid preincubation. These fibres exhibit a lavender to purple iodine colour with the phosphorylase reaction while the a' fibres exhibit a red-iodine colour, indicative of the relatively stronger phosphorylase activity of the b' fibres. In addition, the b' fibres exhibit a relatively stronger SDH activity.

Previous analyses indicates that early ex ovo, ALD muscles are essentially mature at hatching (Cosmos et al., 1979b; 1980). There is, however, a slight modification of the histochemical profile of ALD muscles after hatching. This modification involves the expression of the a' and b' fibres. To evaluate the relative percentage of these two fibre types, analyses were performed on four muscles between 1 and 4
Figure 14a. Photomicrographs of PLD and ALD muscle fibres following the 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. (Taken from Mazliah, 1980)
Figure 14b. Photomicrographs of frozen sections of muscles subjected to the myosin ATPase reaction after acid (pH = 4.35) and alkaline (pH = 10.0) preincubation (×10). 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 the myosin ATPase reaction. (From Maziliah, 1980).
Figure 15. Photomicrographs of frozen cross sections of ALD and PLD muscles following the Pase reaction. These photomicrographs demonstrate the homogeneous strong (blue) response of normal PLD muscle (A x25), the cellular variability and the wasting characteristics of dystrophic PLD muscle (B x25), and the weak (red) Pase activity of the a' fibres of the ALD muscle (C x100). The darker b' fibres in C demonstrate an intermediate (purple) Pase response. (From Mazliah, 1980).
TABLE 7. Comparison of selected fibre types in mature ALD muscles of either genotype. For the myosin ATPase reaction, one '+' means that these fibres stain with relatively less intensity than those fibres with two (++) . With the phosphorylase reaction, a' fibres exhibit a red-iodine colour (weak) while b' fibres exhibit a lavender-purple colour (intermediate). With the SDH reaction, a' fibres have less distinct mitochondria but more background staining than do b' fibres.

<table>
<thead>
<tr>
<th>Histochemical Criteria</th>
<th>a'</th>
<th>b'</th>
</tr>
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<tbody>
<tr>
<td>Fibre innervation</td>
<td>multiple</td>
<td>multiple</td>
</tr>
<tr>
<td>ATPase (pH = 10.0)</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>ATPase (pH = 4.35)</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Phosphorylase</td>
<td>weak</td>
<td>intermediate</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>intermediate</td>
<td>strong</td>
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<tr>
<th>Histological Criteria</th>
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<tbody>
<tr>
<td>Relative fibre size</td>
<td>large</td>
<td>medium</td>
</tr>
<tr>
<td>Relative fibre shape</td>
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<td>elliptical</td>
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<tr>
<td>Myonuclei distribution</td>
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<td>peripheral</td>
</tr>
<tr>
<td>Intracellular lipid droplets</td>
<td>low</td>
<td>high</td>
</tr>
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</table>
weeks ex ovo. The results are shown in Table 8 and indicate that there is a gradual increase in the relative percentage of a' fibres during the early post-hatch period. Also shown in this Table is a small population of intermediate (i) fibres which represent those fibres which could not be classified as being an a' or b' fibres. These fibres persist for 2 weeks and only by 3 weeks ex ovo can every fibre in the ALD muscle be identified as being an a' or b' fibre.

PLD Muscles

Assessment of the type of innervation and the myosin ATPase profile of normal and dystrophic PLD muscles reveals that the PLD fibres of either genotype are focally-innervated by 'en plaque' nerve endings and demonstrate an alkali-stable, acid-labile myosin ATPase response (Figures 14a and 14b). However, when the metabolic profiles of normal and dystrophic muscles are compared, differences are observed. In general, normal PLD muscle fibres have high phosphorylase and low SDH activities. When viewed in cross section, a majority of these fibres exhibit a homogeneous strong (blue) response to the phosphorylase reaction and a weak response to the SDH reaction. In contrast, the Pase and SDH activities of dystrophic PLD muscles are highly variable. When examined after the phosphorylase reaction, a majority of dystrophic fibres exhibit a dull purple colour, a colour reaction which is specific to dystrophic fast twitch muscles. Similarly, dystrophic muscles respond uniquely to the SDH reaction; distinctive mitochondria are present and there is an increased background staining with the SDH reaction. Photomicrographs of normal and dystrophic PLD muscles stained
TABLE 8. Comparison of the relative distributions of selected fibre types in normal ALD muscles during the first four weeks of ex ovo development. At each age indicated, one ALD muscle was taken and a majority of the fibres within an entire cross section staining with the myosin ATPase reaction (pH = 4.35) were typed as either a', b', or intermediate (i) fibres. Intermediate fibres had staining properties intermediate between a' and b' fibres and may represent fibres in the process of transforming from b' to a' fibres.

<table>
<thead>
<tr>
<th>AGE (Weeks)</th>
<th>NUMBER OF FIBRES COUNTED</th>
<th>RELATIVE PERCENTAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a'</td>
</tr>
<tr>
<td>1</td>
<td>2800</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>2400</td>
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<td>3</td>
<td>2958</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>2800</td>
<td>63</td>
</tr>
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</table>
for the phosphorylase reaction are presented in Figure 15.

In normal PLD muscles, while a majority of fibres exhibit a high anaerobic profile, there are some fibres which display an intermediate (purple) response to the phosphorylase reaction and a slightly higher SDH activity. These fibres comprise between 1 to 36% ($\bar{X} = 11\%$) of the total fibre population of the mature PLD muscles sampled ($n = 21$). Furthermore, these fibres contain very small and discrete intracellular lipid droplets while the majority of PLD fibres do not. In both fibre types though, nuclei are randomly scattered within each fibre when viewed in cross section. Therefore, in normal PLD muscles, there exists an inverse correlation between the oxidative and glycolytic capacities of the fibres. As a rule, the higher the phosphorylase activity of a fibre, the lower the SDH activity and intracellular lipid content of that fibre.

In dystrophic PLD muscles, this inverse relationship between phosphorylase and SDH activities within fibres breaks down. For instance, there are many fibres in these muscles which exhibit a high SDH activity but display a variable phosphorylase response. These fibres also contain variable amounts of intracellular lipid. The relative percentage of fibres with a strong SDH activity is much greater in dystrophic than normal PLD muscles. In 18 muscles sampled, these fibres comprised between 5 to 61% ($\bar{X} = 21\%$) of the total fibre population.

Not only is the metabolic profile of dystrophic PLD muscles disrupted but evidence of destruction and regeneration is present throughout the muscle. Replacement of fibres by lipid can also be
observed with the Oil Red O stain. A characteristic of dystrophic PLD muscles is the increased number of nuclei within individual fibres. When 1000 fibres from five PLD muscles of 5 normal chickens at 24 weeks of age were sampled, the number of nuclei per fibre was found to average 2.7 ± 0.14. In contrast, a sample of 292 fibres from one dystrophic PLD at 24 weeks ex ovo, gave a mean number of nuclei per fibre at 4.2.

SIGNIFICANCE

The development of normal and dystrophic fast twitch and slow tonic muscles in chickens was first examined by Cosmos and Butler nearly twenty years ago (Cosmos, 1964; Cosmos, 1966a; Cosmos and Butler, 1967; Cosmos, 1970). These histochemical and biochemical ontogenetic analyses revealed that the slow tonic soleus muscle of dystrophic lineage failed to express overt signs of dystrophy while there was a dramatic expression of dystrophic characteristics in the fast twitch pectoralis muscle of dystrophic genotype. The dystrophic pectoralis muscles demonstrated an impaired ability to differentiate, completely, a mature metabolic profile.

Using physiological criteria, Mazliah et al. (1976) extended the finding that avian muscular dystrophy is target specific. These investigators found that the mechanical properties of normal and dystrophic slow tonic ALD muscles were similar while the mechanical properties of dystrophic fast twitch PLD muscles differed from those of their normal counterparts.

As an extension of this line of research into the development of dystrophic phenotypes during ontogeny, the present study shows, through
quantitative structural analyses, that the slow tonic ALD muscle of
dystrophic genotype is phenotypically normal while the fast twitch PLD
muscle of dystrophic lineage is phenotypically abnormal. From two to
twenty-two weeks, there is a linear and rapid increase in the wet weight
of normal and dystrophic ALD muscles. Subsequent to this period, the
wet weight of these muscles plateaus. Detailed structural analyses
indicates that this biphasic growth pattern is reflected at the level of
individual fibres in ALD muscles of both genotypes. A consistent and
rapid increase in the size of ALD fibres is followed by a period where
the average size of these fibres does not increase. These results
indicate that dystrophic ALD fibres are able to grow as well as normal
fibres. Furthermore, they reveal that the growth of fibres is
asymptotic with both normal and dystrophic ALD muscles able to maintain
their fibres at a constant size and shape.

Histochemical analyses of normal and dystrophic ALD muscles in the
present study support earlier reports that the slow tonic muscles of
either genotype possess mature characteristics early in ex ovo
development (Cosmos and Butler, 1967; Gutmann and Syrový, 1967; Bass
et al., 1970; Melichna et al., 1974; Mazliah et al., 1976; Cosmos et
al., 1979b; Mazliah, 1980; Cosmos et al., 1980). Specifically, the
present analyses show that the histochemical properties of normal and
dystrophic ALD muscles do not change from two to thirty-two weeks ex
ovo. These properties include low Pase activity, high SDH activity,
acid and alkaline stable myosin ATPase activity, multiple endplates
with low ACHe activity, and the peripheral localization of nuclei.
Therefore, there seems to be no difference in the ability of normal and
dystrophic ALD fibres to grow and differentiate mature characteristics and, at least until 32 weeks ex ovo, dystrophic slow tonic muscles are spared disease phenotypes.

Another important phenotype is the demonstration of two classes of fibres in the ALD muscles as shown by the myosin ATPase reaction. Asideu and Shafiq (1972) were the first to show that the b' fibres stain more intensely with the myosin ATPase reaction after both acid and alkaline preincubation while a' fibres stain less intensely. The present study demonstrates that the relative percentage of these two fibre types changes during the first four weeks of ex ovo ontogeny. Accompanying a decrease in the relative percentage of b' fibres there is an increase in the relative percentage of a' fibres so that from eight weeks and on, the relative percentage of a' fibres consistently ranges between 60 to 70% in the ALD muscles of both lineages.

In contrast to the similarities noted with normal and dystrophic slow tonic muscles, dystrophic fast twitch (PLD) muscles deviate considerably from the growth pattern characteristic of normal fast twitch muscles. Whereas the latter demonstrate a consistent increment in fibre size as development proceeds, the dystrophic fibres not only fail to maintain such a consistency but, instead, demonstrate a large variability in fibre sizes. An examination of fibre areas at two weeks posthatching reveals primarily a unimodal distribution for both normal and dystrophic PLD fibres with the exception that the histogram for the dystrophic fibres is shifted slightly to the right. This early hypertrophy has been noted previously with other dystrophic fast twitch fibres (Cosmos, 1966a) and may indicate a faster growth rate of fibres
early in the developmental period. As development proceeds, however, there is a dramatic alteration in the distribution of fibre sizes; the growth pattern of dystrophic fast twitch muscles is disrupted by the emergence of a significant population of small fibres. With time, the unimodal distribution characterizing events of normal growth slowly disappears to be replaced by a bimodal distribution which demonstrates large populations of both small fibres and fibres with larger than normal size. Indeed, the percentage of fibres with areas similar to the majority of normal fibres is greatly diminished. In the dystrophic fast twitch muscle, the complete change in the distributions of fibre areas noted as development proceeds, i.e., a shift from a unimodal distribution at 2 weeks to a bimodal one at 24 weeks, probably reflects a variety of simultaneously occurring events in the diseased muscle. The abrupt appearance of a significant population of small fibres during the early phase of rapid muscle growth may indicate a burst of regenerative activity; the maintenance of this population of fibres may reflect an inability of this group of fibres to increase in size, i.e., an abortive type of regeneration. In addition, small-sized fibres could also represent degenerating, dying fibres. Evidence for this possibility may be derived from the observation that the relative frequency of the seemingly hypertrophied fibres noted at 2 weeks decreases with age. The event leading to this observed decrease may either be due to the degeneration of the enlarged fibres and/or a splitting of these fibres; both phenomena would yield a population of small-sized fibres. Perhaps, all these events do occur in the dystrophic muscle simultaneously, as has been demonstrated recently by
Miike (1983) using the acridine-orange fluorescent technique with human dystrophic muscle. It must be emphasized that the ability of dystrophic muscle to sustain high levels of regenerative activity has been characterized in both animal models of dystrophy (Cosmos, 1966a; Cosmos et al., 1979a; Nichols and Shafiq, 1979; Yorita et al., 1980; Johnson et al., 1983) and humans (Pearce and Walton, 1963; Pearson, 1962; Mastaglia and Kakulas, 1969); the stimulus for regeneration has been associated with a response to muscle injury or degeneration due to the dystrophic process.

Histochemical analyses of normal and dystrophic PLD muscles in the present study support earlier reports that the fast twitch muscle of dystrophic genotype is unable to differentiate, completely, a mature metabolic profile. In normal chickens, the PLD muscle at hatching contains two fibre types; fast twitch oxidative and fast twitch glycolytic (Cosmos et al., 1979b; Mazliah, 1980; Cosmos et al., 1980). While fast twitch oxidative fibres are characterized by high oxidative (SDH) but intermediate glycolytic (Pase) enzymic activities, fast twitch glycolytic fibres reveal a weak oxidative but strong glycolytic enzymic levels. As these muscles mature, there is a steady decline in the relative number of fast twitch oxidative fibres and an increase in the percentage of fast twitch glycolytic fibres so that by approximately four weeks ex ovo, the normal PLD muscle contains a majority of fast twitch glycolytic fibres. Dystrophic PLD muscles also demonstrate a mixed fibre population immediately after hatching; however, unlike the homogeneity noted with normal PLD muscles, dystrophic PLD muscles maintain a heterogeneous response to the metabolic enzymes throughout
subsequent development. This response is further complicated by the observation that the dystrophic fibres can no longer be reliably typed as oxidative or glycolytic since the inverse relationship normally present between the SDH and Pase activities breaks down.

CONCLUSION

The developmental assessment described above for slow tonic (ALD) and fast twitch (PLD) muscles will form the basis for analyses of slow tonic muscles cross-reinnervated by nerves of fast twitch muscles in both normal and dystrophic newly-hatched chickens. Based on the conclusions of this and previous studies that the ALD muscle of dystrophic birds exhibits the same phenotype characteristics of the ALD muscle of normal birds, the primary objective of the cross-reinnervation experiments will be to determine if experimentally-manipulated dystrophic and normal ALD muscles will still exhibit similar properties or, instead, will the response of the cross-reinnervated dystrophic muscles be influenced by the presence of the dystrophic gene.
RESULTS

PART 2: COMPARATIVE ASSESSMENT OF CROSS REINNERVATION TECHNIQUES

RATIONALE

The experiments described in this section were performed to evaluate a technique for cross-reinnervating normal and dystrophic ALD muscles which would yield maximal success. Prior to the onset of this work, three groups had reported the successful cross-reinnervation of normal ALD muscles by foreign 'fast' nerves (Zelená and Jímanová, 1973a,b,c; Bennett and Pettigrew, 1974b; Gordon and Vrbová, 1975c). Therefore the strategies developed by these investigators were tested, with slight modifications, with both normal and dystrophic ALD muscles.

METHODS

Detailed descriptions of the different cross-reinnervation techniques are outlined in the Methods section. In the first series of experiments, ALD muscles were denervated, minced in situ, and cross-reinnervated with the PLD nerve in twenty newly-hatched dystrophic chickens (Group 1). In a second series, ALD muscles were denervated and cross-reinnervated with the superior brachialis (SB) nerve in twenty-five newly-hatched normal chickens (Group 2). In the third series, ALD muscles were denervated, transposed away from their original
position over the dorsal midline to the contralateral side and cross-reinnervated with the SB nerve in five newly-hatched normal chickens (Group 3). The cross-reinnervated muscles in each Group were analyzed physiologically and histochemically to determine the success of cross-reinnervation at various intervals between 2 and 52 weeks postoperatively.

COMPARATIVE ANALYSES OF EXPERIMENTAL MUSCLES

Degree of hypertrophy: After most experimental manipulations, ALD muscles increase in wet weight. This postoperative hypertrophy has been observed after denervation, stretch, and reinnervation (Feng et al., 1963; Hněk et al., 1967; Sola et al., 1973; Holly et al., 1980) but not after tenotomy (Jímanová and Zeleň, 1970). To see whether this phenomenon also occurs after cross-reinnervation, the wet weight/body weight ratio of each experimental (cross-reinnervated) ALD muscle was compared to the wet weight/body weight ratio of its contralateral unoperated muscle. As shown in Table 9, for a given body weight, experimental muscles in every Group have a greater wet weight than their unoperated counterparts. In Group 1, experimental muscles remained hypertrophied up to 52 weeks after the operation.

Physiological alterations: One of the ways to determine the success of cross-reinnervation is to examine the contractile characteristics of the experimental muscles. Normally, muscles composed entirely of slow tonic fibres, like the ALD, give a sustained contraction in response to a repetitive nerve stimulation but twitch only if a single pulse is preceded by a prolonged tetanus in situ (post-tetanic potentiation). In
<table>
<thead>
<tr>
<th>GROUP (n)</th>
<th>AGE (Weeks)</th>
<th>WET WEIGHT/BODY WEIGHT (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (20)</td>
<td>9-52</td>
<td>$0.38 \pm 0.01$\textsuperscript{a}</td>
</tr>
<tr>
<td>2 (25)</td>
<td>2-20</td>
<td>$0.32 \pm 0.01$</td>
</tr>
<tr>
<td>3 (5)</td>
<td>3-39</td>
<td>$0.27 \pm 0.02$</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mean ± S.E.M.

* Statistical significance ($P<0.01$) between the experimental and contralateral muscle groups.
contrast, muscles composed of fast twitch fibres will twitch irrespective of a preceding tetanus. Therefore, if an experimental muscle twitches in response to a single pulse which has not been preceded by a tetanus, this should indicate that some degree of fibre type conversion has taken place.

Of the 12 experimental muscles analyzed physiologically in Group 1, 10 responded as if no fibre type conversion had taken place; these muscles could sustain a contraction in response to an indirect repetitive stimulation and twitched only after a prolonged tetanus. In only two muscles could a twitch be elicited in response to an isolated single pulse but in both cases the amplitude of the twitch was too small to record. On the basis of these observations, the degree of fibre type conversion was predicted to be minimal in the muscles of this Group. Myosin ATPase analyses of these muscles indicated this to be true (see next section).

In Group 2, the experimental muscles were analyzed at 2 weeks, 4 weeks and every fourth week thereafter to a maximum of 20 weeks postoperatively. None of the 2 week old (n = 5) muscles responded to direct or indirect stimulation; it was not until 8 weeks postoperatively that contractions could consistently be elicited in the experimental muscles. While all of the experimental muscles examined from 8 to 20 weeks postoperatively contracted in response to repetitive stimulation of the superior brachialis nerve, a twitch response to an isolated pulse could be elicited in only four of these muscles, i.e., 4 of 15 muscles tested. In addition, in nine birds of Group 2, aged between 4 and 20 weeks, the transected ALD nerve was located and
stimulated. In every case, a tetanus could be elicited in the experimental ALD muscle. On the basis of these results, the per cent conversion was predicted to be low in the majority of the Group 2 muscles.

Physiological analyses were performed on three of the five muscles of Group 3. All three muscles responded to an indirect repetitive stimulation and elicited a twitch in response to an isolated pulse. Therefore, these muscles displayed the contractile characteristics of a muscle composed of twitch and tonic fibres. The degree of fibre type conversion within the muscles of Group 3 as well as in the other two groups will now be assessed.

Fibre type composition: Different fibre types within a muscle can be identified with the myosin ATPase reaction. The ATPase staining pattern of slow tonic muscle fibres is not influenced by preincubation in either high or low pH solutions (Butler and Cosmos, 1981); these slow tonic fibres can be identified by their acid and alkaline stable myosin ATPase activity. In contrast, the myosin ATPase activity of fast twitch fibres is maintained after alkaline preincubation but lost after acid preincubation, i.e., acid labile. When the cross-reinnervated muscles of each Group were examined using the myosin ATPase reaction, the following results were obtained.

Normally, unoperated ALD muscles are devoid of acid labile fibres. In the experimental muscles of Group 1, the number of fibres displaying a fast twitch myosin ATPase profile (acid labile, alkali stable), ranged from less than 1% to a maximum of 5% of the total fibre population. Therefore, as predicted from the physiological analyses, the degree of
fibre type conversion within the muscles of this group is negligible.

The degree of fibre type conversion was even less in the experimental muscles of Group 2. There was not one muscle in which the number of acid labile fibres exceeded 2% of the total fibre population. Unlike the muscles of Group 1, however, the fibres within the muscles of Group 2 could be differentiated into a' and b' fibres after 4 weeks. In 11 of these experimental muscles, the mean relative percentage of b' fibres was 30.5 ± 3.2 (mean ± S.E.M.).

There was an improvement in the percentage of acid labile fibres within the cross reinnervated muscles of Group 3. While three of the five muscles displayed 3% fibre type conversion or less, the remaining two muscles were 10 to 20% converted. The muscle from Group 3 displaying approximately 10% acid labile fibres is presented in Figure 16.

SIGNIFICANCE

The present investigation concerning the response of normal and dystrophic slow tonic muscles to cross reinnervation was undertaken as a step in the analysis of the nerve-muscle interaction in chickens afflicted with hereditary muscular dystrophy. The primary aim of the study was to select a technique which would yield the greatest degree of slow tonic to fast twitch fibre type conversion in the ALD muscle. The results show that when the ALD muscle is transposed away from its original position, i.e., away from the transected ALD nerve, the degree of fibre type conversion after cross reinnervation with a foreign nerve is enhanced.
Figure 16. A composite drawing showing 200 individual muscle fibres in cross section drawn from a normal ALD muscle 38 weeks after cross-reinnervation. Each clear fibre represents a slow tonic fibre while the dark fibres represent acid labile fibres. Accompanying the drawing is a histogram showing the relative distribution of minimum diameters (μm) of the fibres in the drawing.
The technique used in the Group 1 experiments was adapted from a cross reinnervation technique used by Gordon and Vrbová (1975c). In that study, the ALD muscle of 4 to 6 week old normal chickens was removed, minced, and the mince was implanted into the position of the contralateral PLD muscle. Six to 17 weeks later, six of the eight transposed ALD muscles were found to have taken on the physiological properties and the innervation pattern of a fast twitch muscle. These results implied that the PLD nerve was able to alter the physiological and morphological properties of damaged ALD fibres. In light of this finding, why is it that the PLD nerves used in the present experiments failed to change the myosin ATPase pattern of the minced muscles of Group 1? There are two possible explanations which both involve selective reinnervation of the ALD fibres by 'slow type' nerves. The first involves selective reinnervation by the severed ALD nerve. This phenomenon of self-reinnervation has already been observed both in young and old chickens when the denervated ALD has been left in situ (Feng et al., 1965; Bennett and Pettigrew, 1973; Bennett and Pettigrew, 1974a; next section). The second involves selective reinnervation by 'slow' motoneurons present in the mixed PLD nerve. Normally, the PLD nerve contains a mixed population of axons supplying the focally-innervated fibres of the PLD muscle and the multiply-innervated fibres of the dorsocutaneous and metapatalagial latissimus dorsi muscles. Therefore, selective reinnervation by the ALD nerve and/or the 'slow' component of the mixed PLD nerve may account for the absence of PLD type fibres in the muscles of Group 1.

A similar explanation may account for the poor degree of conversion
within the experimental muscles of Group 2. On the basis of physiological evidence both the superior brachialis and ALD nerves are able to innervate the intact denervated ALD muscle. Therefore, selective reinnervation by the ALD nerve and/or the 'slow' component of the superior brachialis nerve may account for the failure to alter the type of myosin synthesized by the intact ALD fibres in muscles of Group 2. Alternatively, because the ALD muscles are not damaged prior to cross reinnervation, it is possible that the superior brachialis nerve successfully reinnervates the ALD muscle but that the intact ALD fibres are unresponsive to this new type of innervation. Evidence for this explanation comes from the work of Bennett and Pettigrew (1973). These investigators showed that the multiple innervation pattern of individual ALD fibres was restored as a result of incoming PLD nerve axons growing to the original synaptic regions on the surface of the fibres 4 weeks after cross reinnervation in adult chickens. Based on this observation, they concluded that the type of innervation on mature intact ALD fibres was not determined by the nerve but by the muscle itself. Although these experiments were done in adult birds, the results can be extrapolated to ALD muscles in newly-hatched birds since ALD fibres are essentially mature at the time of hatching. Therefore, while it is evident that the superior brachialis nerve is able to reinnervate the experimental ALD muscles of Group 2, it appears as though this nerve is unable to repress the synthesis of the myosin type normally expressed in the fibres which make up this muscle.

The results of the Group 3 experiments show that the resistance of normal ALD muscles to alteration diminishes when the ALD is removed from
its in situ position. There are two possibilities to be considered: 1) The risk of self-reinnervation by the transected ALD nerve has been eliminated since the ALD muscle has been transposed away from its in situ position, and, 2) the technique of transplantation causes extensive damage to the ALD muscle resulting in the de novo synthesis of myofibres and such newly-formed fibres are being innervated for the first time by the foreign nerve and therefore selective reinnervation is not a problem. Even with this method, however, the degree of conversion as determined in the present experiments was still limited. Of the three approaches tested, however, the method used in the Group 3 experiments was chosen for a larger series of cross reinnervation experiments in both normal and dystrophic newly-hatched chickens.
RESULTS

PART 3: DENERVATION STUDY

RATIONALE

Cross-reinnervation procedures require that a muscle is first denervated prior to being cross-reinnervated by a foreign nerve. Thus, from the time when the operation is completed to the period of reinnervation by the foreign nerve, the experimental muscle experiences a period of denervation. The purpose of this study was to investigate the effects of denervation on selected properties of normal and dystrophic ALD muscles.

METHODS

A detailed description of the denervation procedure is outlined in the Methods section. In brief, nerves were severed from normal \( n = 12 \) and dystrophic \( n = 12 \) ALD muscles immediately after hatching. In every case, the right ALD (experimental) muscle was denervated, the ALD nerve deflected into the underlying musculature, and the left ALD (control) muscle was left intact. At the end of selected postoperative periods, the denervated muscles were removed and analyzed histochemically by the silver cholinesterase reaction to detect the presence of nerves and endplates and by the myosin ATPase reaction to determine their fibre type composition. Succinic dehydrogenase (SDH) and phosphorylase (Pase) reactions were used to examine the metabolic
status of the experimental muscles and structural analyses were performed to note changes in muscle and fibre size during the postoperative period. Experimental and control ALD muscles were examined at 1, 2, 3, and 4 weeks postoperatively.

RESULTS

Gross examination of both normal and dystrophic experimental chickens revealed a drooping of the right wing immediately after the operation which persisted for 1 week postoperatively. During the course of the second week, the wing reverted back to its original position. Therefore, not only were the experimental muscles denervated during the first week postoperatively, but they were stretched as well. Since other investigators have shown that normal ALD muscles respond with a wet weight increase to denervation and/or stretch (Feng et al., 1963; Jirmanová and Zelená, 1970; Malvery et al., 1971, 1973; Sola et al., 1973; Cullen et al., 1975; Laurent et al., 1978; Holly et al., 1980), the wet weight of each experimental muscle was measured.

Table 10 shows that significant wet weight increases occur at 1 week when the experimental muscles are both denervated and stretched. In subsequent weeks, the wet weight of normal experimental muscles steadily approaches control ALD values. In contrast, not only does the wet weight of the dystrophic experimental muscles remain significantly elevated above their unoperated counterparts throughout the entire postoperative period, but these muscles weigh significantly more than the normal experimental muscles at 4 weeks.

Since an accumulation of connective tissue around the periphery of
<table>
<thead>
<tr>
<th>AGE (Weeks)</th>
<th>GENOTYPE</th>
<th>CONTROL ALD</th>
<th>EXPERIMENTAL ALD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MUSCLE WET WEIGHT (mg)</td>
<td>FIBRE CROSS-SECTIONAL AREA ((\mu m^2))</td>
</tr>
<tr>
<td>1</td>
<td>N</td>
<td>12.9* ±0.7</td>
<td>284 ±11.7</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>15.8 ±3.5</td>
<td>459 ±103.0</td>
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<tr>
<td>2</td>
<td>N</td>
<td>29.8 ±3.3</td>
<td>609 ±91.6</td>
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<tr>
<td></td>
<td>D</td>
<td>36.0 ±2.6</td>
<td>881 ±129.7</td>
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<td>N</td>
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<td>748 ±99.7</td>
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<tr>
<td></td>
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<td>882 ±114.8</td>
</tr>
<tr>
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<td>N</td>
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<tr>
<td></td>
<td>D</td>
<td>102.7 ±16.9</td>
<td>1592 ±127.5</td>
</tr>
</tbody>
</table>

*a.* Mean ± S.E.M.

* Significant difference (P<0.05) between experimental and unoperated ALD muscles.

≠ Significant difference (P<0.05) between normal (N) and dystrophic (D) experimental ALD muscles.
the experimental muscles invariably contributes to their wet weight, individual fibre size measurements were done to test for true muscle hypertrophy. In normal birds, there was a significant increase in fibre size in 1 and 2 week old preparations (Table 10). By 3 and 4 weeks, however, the fibres in the experimental muscles were similar in size to control ALD fibres. Experimental dystrophic fibres showed the same response as normal fibres up to 2 weeks postoperatively but then increased significantly above the normal experimental ALD fibre values at 3 and 4 weeks. Therefore, cellular hypertrophy takes place in both normal and dystrophic muscles in response to the operation. When compared to their contralateral controls, dystrophic experimental fibres at 1 and 2 weeks of age were not significantly larger in size due to abnormally high values of the former. At this time there is no explanation for why the fibres in the control dystrophic ALD muscles are so large.

Not only did denervation affect the structural properties of normal and dystrophic ALD muscles but the histochemical properties of these muscles were also affected. Normally, two populations of fibres can be identified in unoperated ALD muscles; each distinguished by a differential response to the myosin ATPase reaction. The lighter staining fibres are the a' fibres while the b' fibres stain relatively darker. This typical staining pattern, which becomes apparent soon after hatching and persists throughout ontogeny, is altered in the denervated muscles. Up to three weeks postoperatively, both normal and dystrophic experimental muscles expressed a homogeneous response to the myosin ATPase reaction. However, while reliable identification of a'
and b' fibres was possible at 4 weeks in normal birds (Figure 17), the
dystrophic experimental muscles failed to differentiate these fibres by
4 weeks (Figure 18). In both normal and dystrophic experimental
muscles, small acid labile fibres could be identified in areas which had
been damaged during the initial operative procedure. These small areas
of damage were always confined to the periphery of the muscle. The
central portion of each experimental muscle maintained a typical ALD
type of topography; fascicles and individual muscle fibres were
elliptical in shape and the fascicles were loosely-packed (see Figures
17 and 18).

The histochemical tests for Pase and SDH show that unoperated ALD
muscles contain two distinct fibre types. The a' fibres show weak Pase
and intermediate SDH activity while the b' fibres show intermediate Pase
and strong SDH activity. In experimental muscles from 1 to 3 weeks
postoperatively, this heterogeneous staining pattern is replaced by a
homogeneous response in which all of the fibres stain with the same
intensity after both the Pase and SDH reactions. By 4 weeks, the
metabolic profile of the normal experimental ALD muscles returns to that
of an unoperated ALD muscle. In contrast, the normal heterogeneous
pattern of staining never returns in any of the dystrophic experimental
muscles.

The results thus far have shown that while normal and dystrophic
muscles respond similarly to denervation initially, this similarity in
response does not persist throughout the entire postoperative period.
By 4 weeks, several differences become apparent. In an attempt to
account for these differences, the innervation status of the
DENERVATED NORMAL ALD

Figure 17. A composite drawing showing 200 individual muscle fibres in cross section drawn from a normal ALD muscle 4 weeks after denervation. Each clear fibre represents an a' fibre while those fibres with a dot represent b' fibres. Accompanying the drawing is a histogram showing the relative distribution of minimum diameters (µm) of the fibres in the drawing.
Figure 18. A composite drawing showing 200 individual muscle fibres in cross section drawn from a dystrophic ALD muscle 4 weeks after denervation. All of the fibres give a homochromatic response to the myosin ATPase reaction. Accompanying the drawing is a histogram showing the relative distribution of minimum diameters (μm) of the fibres in the drawing.
experimental muscles was examined. To determine the presence or absence of nerves and endplates in these muscles, random transverse sections were assessed by the silver-cholinesterase reaction. An experimental muscle was designated as denervated if detailed microscopic analysis revealed a significant loss of nerve axons and endplates and if the endplates which persisted were faintly-staining. An experimental muscle was considered reinnervated if its innervation pattern was similar to that of an unoperated ALD muscle. Using these criteria, it was determined that from 1 to 3 weeks, normal experimental muscles were denervated but became reinnervated by 4 weeks. In contrast, eleven of the twelve dystrophic experimental muscles remained denervated throughout the postoperative period. Only one muscle was reinnervated (by 3 weeks). Since this is the only muscle to show extensive innervation by 3 weeks it is possible that this muscle was not completely denervated during the initial operative procedure.

SIGNIFICANCE

When denervation experiments were performed in normal birds, ALD muscles showed extensive reinnervation four weeks after denervation. During the early (1 week) postoperative period, ALD muscles displayed a loss of nerve axons, a few remaining endplates with weak cholinesterase activity, wet weight hypertrophy, and an increase in the mean fibre cross-sectional area. As nerves began slowly to re-enter the experimental muscles over the next 3 weeks, the wet weight hypertrophy of the experimental muscles diminished and fibre sizes approached control levels. Throughout the entire postoperative period, the experimental
muscles maintained the typical topographical appearance of ALD muscles; signs of acid labile fibres or regeneration were exceedingly rare. Differentiation of a' and b' fibres, absent in 1-3 week old experimental muscles, was expressed concomitantly with the re-establishment of extensive nerve-muscle contacts at 4 weeks. Thus, distinct changes noted with denervated ALD muscles of normal chickens were present by 1 week postoperatively; however, by 4 weeks, the muscles became successfully reinnervated and demonstrated all phenotypes characteristic of unoperated ALD muscles.

When the same experiments were performed in dystrophic birds, the ALD muscles responded normally to denervation in that each of the changes detected in denervated ALD muscles of normal birds could also be identified in denervated dystrophic ALD muscles. However, once established, these changes persisted throughout the postoperative period in dystrophic birds only. It is likely that the distinct changes associated with denervation persisted in the experimental dystrophic muscles because of a delay in the reinnervation of these muscles; muscle and fibre hypertrophy and a homogeneous response to the myosin ATPase, SDH, and Pase reactions persisted in all of the 4 week old dystrophic preparations. While it is evident that the affects of denervation on the properties of ALD muscles are reversible upon reinnervation in normal birds, more extensive and longer-term experiments are necessary to determine if the same phenomenon occurs in ALD muscles of dystrophic birds.
CONCLUSION

In summary, the present study shows the following:

1. Both normal and dystrophic ALD muscles denervated at hatching demonstrate increases in wet weight, signs of fibre hypertrophy, a loss of cholinesterase-staining endplates, and a failure to differentiate a' and b' fibres; however, denervated muscles maintain the topographical appearance of unoperated ALD muscles.

2. Self-reinnervation of denervated ALD muscles of normal birds is completed by 4 weeks postoperatively; at this time, all alterations attributed to the denervation procedure are reversed and muscles assume all characteristics of unoperated ALD muscles.

3. Self-reinnervation of denervated ALD muscles of dystrophic birds is absent by 4 weeks postoperatively; at this time, all alterations associated with the denervation process persist.

The relevance of these findings with respect to the cross-reinnervation studies will be discussed in a later chapter.
RESULTS

PART 4: TRANSPOSITION STUDY

RATIONALE

In an effort to facilitate a successful cross-reinnervation of the ALD muscle and to avoid selective self-reinnervation, the ALD must be transposed to the contralateral side away from the severed ALD nerve. Before the ALD muscle is transposed, it is first denervated, severed from its insertion on the humerus and freed from surrounding connective tissue. The origin on the spine is left intact. Following this procedure, the ALD muscle typically supercontracts. In order to accomplish transposition to the contralateral side, the contracted ALD muscle must be forcibly stretched, pulled across the dorsal midline and sutured into the contralateral wing musculature where cross-reinnervation by the superior brachialis (SB) nerve is accomplished. Since the entire procedure involves a severe manipulation of the ALD muscle, it was deemed necessary to examine the effects of transposition alone, before cross-reinnervation by the SB nerve was accomplished, on the subsequent survival and differentiation of both normal and dystrophic denervated ALD muscles.

METHODS

A description of the transposition procedure is outlined in detail in the Methods section. In brief, the right ALD muscle was denervated,
severed at its insertion, stretched over the dorsal midline, and sutured into the musculature of the left wing. The left SB nerve was severed, extended to the transposed ALD muscle but returned to its original position in the wing. Operations were performed on 24 normal and 14 dystrophic chickens soon after hatching. The right transposed (experimental) and left contralateral (control) ALD muscles were examined between 2 and 28 weeks, postoperatively.

RESULTS

ALD Muscles of Normal Birds

Support of the right wing was effectively removed when the right ALD muscle was transposed to the left side in experimental birds. Subsequently, gross examination of the experimental birds immediately following the operation consistently revealed a drooping of the right wing. This droop persisted throughout the entire postoperative period as the right PLD muscle was unable to compensate for the removal of its synergistic ALD muscle; regeneration of the right ALD muscle was never observed. At no time during the postoperative period did the left wing droop since it was supported by the left ALD muscle. This prevented the transposed ALD muscle from being stretched.

As described in the preceding study, denervation alone induces specific alterations of the ALD muscle. Thus the denervated muscles fail to differentiate a' and b' fibres, increase their glycolytic capacity and demonstrate an absence of acetylcholinesterase activity at the endplate regions. Furthermore, muscles show both an increase in
weight and mean fibre size (Figure 19). Since the experimental muscles in the present study were denervated prior to transposition, these muscles were examined for denervation characteristics. Indeed, of the five denervation characteristics just described, four were apparent in the transposed muscles at 2 weeks postoperatively. Only fibre hypertrophy was absent. Instead, as illustrated in Figure 20, fibres in these muscles were smaller and more variable in size relative to fibres in age-matched denervated muscles. An unexpected finding, however, was the presence of a population of fibres expressing an acid labile myosin ATPase activity. In 2 of the 3 muscles examined at 2 weeks, the percentage of these fibres ranged from 15 to 30% of the total fibre population. In these muscles, the acid labile fibres were located in small clusters scattered randomly throughout the muscle cross section. In the third 2 week old muscle, less than 1% of the muscle was made up of acid labile fibres. Although excessive amounts of connective tissue surrounded all three muscles, each muscle retained a normal ALD type of topography. Therefore, not only do transposed, denervated ALD muscles at 2 weeks postoperatively display several phenotypes normally associated with denervation, but two novel phenotypes as well.

As demonstrated in the previous study, when left in situ, ALD muscles of normal genotype become extensively reinnervated as early as 4 weeks after denervation and subsequently assume a normal appearance. In contrast, 4 week old transposed muscles remain denervated and retain all of the phenotypes expressed at 2 weeks except one; the muscles return to control wet weight levels. In 2 of the 3 muscles examined at 4 weeks, the range and distribution of acid labile fibres was greater than
Figure 19. A composite drawing showing 200 individual muscle fibres in cross section drawn from a normal ALD muscle two weeks after denervation. All of the fibres give a homochromatic response to the myosin ATPase reaction. Accompanying the drawing is a histogram showing the relative distribution of minimum diameters (μm) of the fibres in the drawing. The fibres in this muscle have an average minimum diameter of 29.2 μm while fibres in its contralateral control muscle have an average minimum diameter of 26.0 μm.
Figure 20. A composite drawing showing 200 individual muscle fibres in cross section drawn from a normal ALD muscle 2 weeks after transposition. The dark fibres represent acid labile fibres while the clear fibres represent slow tonic fibres. Accompanying the drawing is a histogram showing the relative distribution of minimum diameters (µm) of the fibres in the drawing.
observed in any of the 2-week-old muscles; the percentage of acid labile fibres increased to 40-50% and these were more extensively distributed throughout the cross section of each muscle. Acid labile fibres were absent in the third muscle. Irrespective of the number of acid labile fibres, however, each 4-week-old muscle retained the topographical appearance of a control ALD muscle.

In these experiments, no attempt was made to cross-reinnervate the transposed muscles with a foreign nerve. As a consequence, 10 of the 18 muscles examined after 4 weeks were denervated. The fibres in this group of muscles remained both acid and alkali stable, i.e., slow tonic, but failed to differentiate into a' and b' fibres. Acid labile fibres were absent in all of these muscles. Figure 21 shows that the denervated ALD muscles retain the normal ALD topography where individual fascicles remain distinct and are elliptical in shape. Furthermore, individual fibres remain intact and demonstrate the ability to increase in size during the postoperative period. Therefore, despite being denervated for as long as 7 months, ALD muscles survive and are spared extensive degenerative changes.

In contrast, those muscles which became randomly reinnervated (8 of 18 muscles) displayed major structural changes. At 16 weeks postoperatively, there was extensive replacement of individual fibres and entire fascicles by lipid and the fibres which remained were smaller and more variable in size than fibres in control ALD muscles. Examination of this group of muscles with the silver cholinesterase reaction revealed that 3 preparations were extensively innervated by 'en grappe' endings. The fibres in this group of muscles demonstrated both
Figure 21. A composite drawing showing 200 individual muscle fibres in cross section drawn from a normal denervated ALD muscle 16 weeks after transposition. All of the fibres give a homochromatic response to the myosin ATPase reaction. Accompanying the drawing is a histogram showing the relative distribution of minimum diameters (µm) of the fibres in the drawing.
acid and alkali stable myosin ATPase reactions and, in 2 of the 3 preparations, differentiated into a' and b' fibres. Examination of the remaining 5 muscles revealed that these muscles were innervated by both 'en gruppe' and 'en plaque' endings. In addition, these 5 muscles contained acid labile fibres which were confined primarily to the central region of each muscle and ranged between 25-80% of the total fibre population. Figure 22 shows that not only are these acid labile fibres maintained for as long as 16 weeks after transposition but tend to be larger in size than the remaining acid stable fibres.

**ALD Muscles of Dystrophic Birds**

All of the characteristics expressed by normal ALD muscles 2 weeks after transposition are also expressed by dystrophic muscles. Therefore, not only do transposed dystrophic muscles increase in wet weight, fail to differentiate a' and b' fibres, lose acetylcholinesterase activity from the endplate regions, and increase in glycolytic capacity, but the fibres within these muscles are smaller and more variable in size than control ALD fibres. In addition, acid labile fibres appear as early as 2 weeks after transposition and are randomly scattered throughout the cross section of each of the three muscles examined. While the response of normal and dystrophic ALD muscles to transposition is similar in several aspects, there are also distinct differences. The number of acid labile fibres in 2 week old dystrophic muscles is greater than in their normal counterparts and ranges from 35 to 75% of the total fibre population. While both normal and dystrophic transposed muscles become surrounded by excessive amounts of connective
Figure 22. A composite drawing showing 200 individual muscle fibres in cross section drawn from a normal re-innervated ALD muscle 16 weeks after transposition. The dark fibres represent acid labile fibres while the clear fibres represent slow tonic fibres. Accompanying the drawing is a histogram showing the relative distribution of minimum diameters (µm) of the fibres in the drawing.
tissue, only the normal muscles retain a normal ALD type of architecture; the fascicles in the dystrophic preparations are more angular in shape and more densely-packed with muscle fibres. In addition to the striking alteration in the overall topographical appearance of transposed dystrophic ALD muscles relative to their normal counterparts, the cross-sectional area of the former is 2-3 times larger than the unoperated ALD muscle. Since the fibres present in these preparations are smaller in size than those in unoperated ALD muscles but the muscle cross-sectional area is greatly increased and tightly-packed with muscle fibres, one must conclude that there has been an extensive proliferation of new cells. This phenomenon was not apparent in the transposed normal muscles.

The differences noted between normal and dystrophic preparations at 2 weeks were greatly enhanced in 2 of the 3 preparations at 4 weeks. The rounded loosely-packed appearance which normally characterizes ALD muscles is now completely replaced by one in which elongated fascicles are tightly-packed with irregularly-shaped fibres, 70% to 80% of which are acid labile (Figure 23). Furthermore, the wet weight remains elevated above control values and the accumulation of connective tissue around the periphery of each muscle persists. The muscles remain devoid of innervation and a' and b' fibres are not differentiated. Unlike the first 2 muscles, the third muscle at 4 weeks retained the architecture of an unoperated ALD muscle. Small acid labile fibres were found scattered throughout the muscle and made up between 30-40% of the total fibre population. This muscle was also devoid of innervation and failed to express a' and b' fibres.
Figure 23. A composite drawing showing 200 individual muscle fibres in cross section drawn from a denervated dystrophic ALD muscle 4 weeks after transposition. The dark fibres represent acid labile fibres while the clear fibres represent slow tonic fibres. Accompanying the drawing is a histogram showing the relative distribution of minimum diameters (µm) of the fibres in the drawing.
As the postoperative period progressed from 8 to 20 weeks, the acid labile fibres in transposed muscles failed to increase in size and were eventually replaced by lipid. Despite this eventual loss of acid labile fibres, most of the older dystrophic preparations continued to display a highly disorganized appearance; fibres were abnormally abundant, small and highly variable in size. Furthermore, not all of the fibres were grouped into fascicles and many fibres were found between fascicles. Disorientation of fibres was evident and dying fibres could be identified within large areas of connective tissue which continued to surround and infiltrate into the interior of each muscle. Muscle fascicles continued to demonstrate a large variability in size and shape.

Source of Innervation: In the present experiments, no attempt was made to cross-reinnervate the transposed muscles with a foreign nerve. As indicated, however, in some preparations examined after 4 weeks postoperatively, endplates were demonstrated. Reinnervation of the transposed muscles could either stem from the cut superior brachialis nerve or from nerves originating from the wing musculature to which the severed ALD muscle was attached. The latter seems unlikely, however, since analyses of the attached area demonstrated the presence of a heavy layer of connective tissue; a continuum of fibres between the two muscles was never noted.

SIGNIFICANCE

The purpose of the present study was to examine the effects of the transposition technique on the development of normal and dystrophic ALD.
muscles. Since each ALD muscle was denervated prior to transposition, it was predicted and confirmed that the transposed muscles expressed many of the phenotypes normally associated with denervation. In addition, both normal and dystrophic transposed muscles demonstrated important new phenotypes. Normally, the ALD muscle is composed entirely of slow tonic fibres which express both acid and alkali stable myosin ATPase activities. This fibre type composition of ALD muscles is not altered by denervation or subsequent selective self-reinnervation by the severed ALD nerve (this thesis). The results of the transposition studies, however, demonstrate clearly that following this procedure, a new type of fibre appears which expresses a type of myosin ATPase activity characteristic of fast twitch, not slow tonic muscles. Such fibres, demonstrating alkali stable but acid labile myosin ATPase activity are noted by 2 weeks postoperatively. Since acid labile fibres first appear in transposed muscles devoid of innervation, i.e., 2 and 4 week old preparations, their formation is not dependent on the presence of nerves. In contrast, their maintenance over a prolonged period is dependent on the presence of nerves: acid labile fibres persist only in transposed muscles which demonstrate 'en plaque' endings. Such fibres are absent in muscles which demonstrate only 'en grappe' endings and in older preparations which demonstrate an absence of endplates.

Another important difference between denervated muscles left in situ and denervated muscles transposed to the contralateral side is the individual fibre sizes. Whereas ALD fibres normally hypertrophy after denervation, fibres in the transposed muscles are smaller in size than fibres in unoperated muscles. The hypertrophy noted in the denervated
muscles left in situ was greatest during the first week postoperatively and decreased in subsequent weeks. Following denervation an immediate drooping of the wing occurs which persists for 7 days. Following this period, the wing gradually returns to its normal position. It is possible that during the 7 day period, a stretching of the denervated ALD muscle is the trigger for the fibres hypertrophy noted since the denervated plus transposed muscles are not subjected to stretch and thus do not hypertrophy.

The results of the present study also demonstrate clearly that ALD muscles survive denervation; ALD muscles are maintained for as long as 7 months after denervation and remain organized into distinct fascicles. The maintenance of slow tonic ALD fibres after denervation is in marked contrast to the post-denervation atrophy of fast twitch PLD fibres (unpublished observations). Furthermore, the observation that a' and b' fibres remain undifferentiated in denervated muscles yet return upon reinnervation lends support to the hypothesis that the differentiation of a' and b' fibres is neurally-dependent.

While similarities exist in the response of normal and dystrophic muscles to transposition, differences are also evident. A comparison of the response to denervation plus transposition between ALD muscles of normal and dystrophic birds emphasizes a greater plasticity of the experimental muscles of dystrophic genotype following the surgical procedure. Thus, the latter demonstrated a remarkable transformation in topographical appearance, the number and size and shape of acid labile fibres and their mode of distribution. The normal muscles maintained the structural appearance of an unoperated ALD muscle; acid labile
fibres were clustered in groups varying in size from several fibres to the entire central core of the cross-sectional area of the muscle. In contrast, the dystrophic experimental ALD muscles did not resemble structurally its unoperated counterpart. Instead, fascicles showed a variety of shapes and sizes and were tightly-packed with variable-shaped fibres. Acid labile fibres were scattered throughout the muscle cross-sectional area and were not clustered into distinct groups. The most striking observation was the muscle hyperplasia first noted in one preparation at 2 weeks. Muscles demonstrating hyperplasia throughout the remaining postoperative period demonstrated a cross-sectional area which was as much as 2 1/2 times that of contralateral control ALD muscles. Since the acid labile fibres in these muscles did not appear to increase in size but only in number from 2 to 4 weeks, it was concluded that extensive proliferation of fibres was triggered as a result of the transposition technique.

The aim of this group of experiments was to identify changes occurring in denervated ALD muscles subjected to the technique of transposition to the contralateral side without the additional procedure of cross-reinnervation. Any reinnervation which subsequently occurred in some of these preparations was random and its course and origin unpredictable. Experiments focussing on the cross-reinnervation of transposed ALD muscles, both normal and dystrophic, by the predominantly 'fast' SB nerve will be presented in the following section. To be emphasized here is the appearance of acid labile fibres in denervated plus transposed ALD muscles and the differential responses of normal and dystrophic muscles to the surgical procedure.
RESULTS

PART 5: CROSS REINNERVATION STUDY

RATIONALE

In the two preceding sections, normal and dystrophic ALD muscles were examined after denervation per se and denervation plus transposition. By doing so, the development of normal and dystrophic ALD muscles in the absence of a nerve both in situ and after transposition could be examined. In the present study, an examination is made of the effect of a foreign 'fast' nerve coupled to an ALD muscle previously denervated and transposed to the contralateral side. The question being addressed relates to the ability of an avian slow tonic muscle (the ALD), which has already expressed mature characteristics at hatching, to accept and respond to the influences of a 'fast' nerve. When fast twitch muscles are coupled to nerves of slow tonic muscles, the results noted indicate an arrest in differentiation, or the maintenance of a fetal enzymic profile (Cosmos et al., 1979b; Mrazil, 1980; Cosmos et al., 1980). In sharp contrast, the present experimental procedure of a nerve of a fast twitch muscle coupled to a denervated slow tonic muscle will attempt to impose upon this muscle a command to differentiate further to a fast twitch muscle—a developmental event not experienced by an ALD muscle. Since the main objective of this
thesis is to examine the response of the ALD muscle of dystrophic genotype but normal phenotype, to an experimental manipulation, it is essential to establish first the maximal capabilities of a normal slow tonic muscle to cross-reinnervation by a foreign 'fast' nerve. Once established with normal ALD muscles, additional altered phenotypes unique to cross-reinnervated ALD muscles of dystrophic origin might be attributed to the expression of dystrophic phenotypes.

METHODS

In this series of experiments, the right ALD muscle was denervated, severed from its insertion on the humerus, stretched over the dorsal midline and sutured into the musculature of the left wing. The left superior brachialis (SB) nerve was severed, extended to the transposed ALD muscle and made to form a union with the distal branch of the transposed ALD nerve (for further details see the Methods section). The operations were performed on newly-hatched normal (n = 64) and dystrophic (n = 68) birds. Experiments were terminated at 2 weeks, 4 weeks and at 4 week intervals thereafter up to 32 weeks. A few experiments were also analyzed up to 2 years postoperatively. Physiological, histochemical, and structural analyses were performed on all cross-reinnervated (experimental) and contralateral unoperated (control) ALD muscles.

CROSS REINNERVATED ALD MUSCLES OF NORMAL BIRDS

Gross Examination

Of the 64 normal chickens used in this study, 10 were subsequently
eliminated from the study due to poor health. Forty-nine of the 54 remaining birds were terminated between 2 and 32 weeks and 5 between 52 and 104 weeks postoperatively.

Prior to sacrifice, the wing position and righting ability of each bird was examined. Transposition of the right ALD muscle to the left contralateral position effectively removed support for the right wing. Therefore, experimental birds of all ages exhibited a drooping right wing. Normally, when placed on its back, a chicken will immediately extend its wings and resume a standing position. All of the experimental birds in which this righting ability was tested were able to resume a standing position in 5 out of 5 attempts (Rudecki et al.; 1978; 1979; 1980).

Once these examinations had been carried out, each chicken was anaesthetized, the back of each chicken opened, and the cross-reinnervated ALD muscle exposed. The threads used to suture the right ALD muscle into the left triceps brachii muscle failed to hold in only one of the 49 birds examined. The tranposed ALD muscle in this one bird had contracted to approximately one quarter of its resting length and had fused with the underlying ALD muscle. In none of the remaining 48 birds was there any evidence that the transposed muscle had become self-reinnervated, i.e., the proximal end of the right ALD nerve was never observed transversing the midline of the back towards the transposed ALD muscle. Furthermore, the nerves of the left ALD and PLD muscles appeared to be intact and no branches were observed travelling from these nerves towards the transposed ALD muscle. In all 48 birds, it was easy to identify the point of entry of the transposed ALD muscle into
the triceps brachii muscle. Sections of the junctional area showed that the two muscles were separated by a continuous band of connective tissue. Furthermore, a fine layer of connective tissue separated the transposed ALD muscle from the underlying muscle. There was no evidence in any of the preparations examined that the transposed and contralateral ALD muscles had fused and it was always easy to separate the two muscles. In addition, the absence of connective tissue accumulation around the transposed ALD muscle facilitated the localization and isolation of the transposed SB nerve. In all cases, the SB nerve was seen penetrating into the transposed muscle.

**Innervation Characteristics**

In the preceding study, denervated ALD muscles were transposed to the contralateral side but no attempt was made to cross-reinnervate these muscles with a foreign nerve. In the present study, the denervated ALD muscles were transposed to the contralateral side and cross-reinnervated by a foreign 'fast' nerve. In order to monitor the innervation status of the cross-reinnervated muscles, physiological and histochemical analyses were performed.

**Histochemical analyses:** The silver-cholinesterase reaction was used to determine when the SB nerve begins to penetrate and form endplates with the cross-reinnervated ALD muscles. Normally, the ALD muscle is multiply-innervated and exhibits the 'en grappe' type of endplates. In contrast, twitch muscles are focally-innervated and demonstrate the 'en plaque' type of endplate. Using this staining technique on frozen sections the onset of reinnervation and the type of endplates formed on
ALD muscles was determined.

Examination of the innervation pattern of the cross-reinnervated ALD muscles revealed that by 2 weeks postoperatively, nerves could be identified in every muscle examined. On the other hand, endplates were detected in only 2 of the 6 muscles examined at this time and in these two muscles the number of endplates present was significantly lower than normally observed in unoperated ALD muscles. Nerves continued to be conspicuous in all of the 4 week old preparations but now a small number of endplates could be identified in every muscle. From 8 weeks and on, reinnervation of the experimental muscles appeared to be complete as nerves and a normal compliment of endplates could be identified in every preparation. In all of the reinnervated muscles in which nerve-muscle contacts had been established, the majority of endplates were of the 'en grappe' type.

Physiological analyses: Physiological analyses were performed for two specific reasons: a) to establish when functional contact is made between the SB nerve and the transposed ALD muscle, and b) to qualify and quantify the response of the experimental ALD muscles when the SB nerve is stimulated.

An examination of the mechanical properties of the cross-reinnervated ALD muscles demonstrated that the experimental muscles were functionally innervated by 4 weeks; at 2 weeks postoperatively, no response could be elicited following either single or repetitive stimulation of the SB nerve. In vivo, ALD muscles respond only to a series of repetitive indirect stimuli and do not respond to a single pulse applied to the nerve. In the present experiments, the mechanical
properties of the responsive experimental muscles revealed that these muscles could be divided into two groups:

1. muscles which responded to indirect repetitive stimulation only;

2. muscles which responded to both single and repetitive stimulation of the SB nerve.

Of the forty muscles tested, 15% responded only to an indirect repetitive stimulation. This response was not noted in muscles of any one age group but in preparations examined throughout the postoperative period, i.e., from 4 to 28 weeks. The majority of experimental muscles, however, responded to both indirect single and repetitive stimulation. Figure 24 gives representative recordings of the contractile responses of a cross-reinnervated muscle of the second group and its contralateral control muscle. These recordings indicate that both the contralateral control and the cross-reinnervated ALD muscles sustain a tetanus following a train of repetitive stimuli and elicit a twitch in response to a single pulse after a train of stimuli (post-tetanic potentiation). However, only the cross-reinnervated ALD muscle twitches when a single pulse is applied to the nerve.

Not only were the contractile properties of both the contralateral and experimental muscles tested qualitatively, but in 8 of 40 birds, the mechanical properties were quantified as described previously (Figure 4, Methods). As shown in Table 11 the tetanic tensions elicited by the experimental muscles are lower than those measured from age-matched contralateral ALD muscles. The half-relaxation time is also consistently longer in the experimental muscles. Furthermore, it is
Figure 24.

Examples of isometric tetanic, post tetanic twitch and twitch responses of contralateral (a,b,c) and experimental (d,e,f) ALD muscles of a 12 week old normal bird. The tetanic contractions (a,d) were evoked in response to indirect stimulation at 50, 70, and 100 Hz. The post tetanic twitches (b,e) were evoked after 7 sec of 70 Hz nerve stimulation followed by a single stimulus. While the contralateral ALD muscle did not respond to a single indirect stimulus (c), the experimental muscle responded with a twitch (f). Upon histochemical analysis, the experimental muscle was found to contain 20% adx labile fibres.
TABLE 11. Analysis of the mechanical properties of contralateral (C) and experimental (E) ALD muscles in normal birds at 4, 8, and 12 weeks postoperatively. Tetanic tension (TT), time to half contraction (T\(_{1/2}C\)), and time to half relaxation (T\(_{1/2}R\)) were measured in response to indirect stimulation for 7 sec at 100 Hz. In those cases where a twitch response could be elicited, twitch tension (TWT), full contraction time (Tc), and time to half relaxation (T\(_{1/2}R\)) were measured. The post tetanic twitches were evoked as outlined in Figure 19. All tension measurements are expressed in grams while all of the time measurements are expressed in msec.
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<td>TT</td>
<td>T1C</td>
<td>T1R</td>
</tr>
<tr>
<td>4</td>
<td>C (3)</td>
<td>27.0</td>
<td>173</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±3.7</td>
<td>±42.8</td>
<td>±20.5</td>
</tr>
<tr>
<td>8</td>
<td>E (4)</td>
<td>12.4</td>
<td>153</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±6.4</td>
<td>±20.1</td>
<td>±23.3</td>
</tr>
<tr>
<td>12</td>
<td>C (3)</td>
<td>46.7</td>
<td>169</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±11.4</td>
<td>±15.0</td>
<td>±13.3</td>
</tr>
<tr>
<td></td>
<td>E (3)</td>
<td>24.4</td>
<td>106</td>
<td>249</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±12.2</td>
<td>±31.7</td>
<td>±121.7</td>
</tr>
<tr>
<td></td>
<td>C (3)</td>
<td>87.4</td>
<td>199</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±3.9</td>
<td>±47.2</td>
<td>±12.4</td>
</tr>
<tr>
<td></td>
<td>E (1)</td>
<td>86.3</td>
<td>202</td>
<td>193</td>
</tr>
</tbody>
</table>

a. ( ) = number of muscles analyzed

b. Mean ± S.E.M.
evident from this data that the time-to-half-contraction of the experimental muscles is not altered from that recorded for control muscles. Analysis of the twitch elicited following a prolonged tetanus (PTP) indicates that twitch tensions generated by contralateral and experimental muscles are similar at each time period examined. The twitch contraction and half-relaxation times are longer in the experimental muscles except at 4 weeks when the full contraction time is shorter in the experimental muscles. Twitches in response to an isolated pulse applied to the nerve could consistently be recorded only in the experimental muscles. It should be noted that whenever tested (in 5 out of 40 birds), the cross-reinnervated muscles did not respond when either the left ALD or FLD nerves were stimulated.

In summary, these results show that the SB nerve does functionally innervate a denervated, transposed ALD muscle. They also show that while all of the responsive experimental ALD muscles still retain the capacity to elicit a tetanus in response to an indirect repetitive stimulus, a majority of these muscles show a new property: they can now twitch when a single pulse is applied to the SB nerve.

Fibre Type Analyses

Slow tonic fibres of the ALD muscle demonstrate a positive myosin ATPase response after both acid and alkaline preincubation and are distinctly different than fast twitch fibres which stain positively only after alkaline preincubation. Therefore, the myosin ATPase histochemical reaction was used to determine the fibre type composition of the experimental muscles. When this type-of analyzes was performed,
two populations of fibres were noted in the experimental muscles:

1. fibres which demonstrated myosin ATPase activity following both acid and alkaline preincubation; and

2. fibres which demonstrated myosin ATPase activity following alkaline preincubation only.

The photomicrographs in Figure 25 show the response of experimental and control ALD muscles to the myosin ATPase reaction, following acid and alkaline preincubation. As shown in Figure 25a, slow tonic fibres in unoperated ALD muscles differentiate into lighter (a') and darker (b') staining fibres following acid preincubation. In contrast, the slow tonic fibres in 81% of the cross-reinnervated muscles demonstrate a homogeneous response after acid preincubation (Figure 25b). As shown in Figures 25c and 25d, acid labile fibres stain with a greater intensity after alkaline preincubation than do acid- and alkali-stable fibres. Figures 25e and 25f show that there is a good correlation between the type of endplate and the fibre type; those fibres on which 'en plaque' endings terminate are acid labile.

It was shown in the previous section that acid labile fibres are present in transposed ALD muscles as early as 2 weeks postoperatively. Since the presence of these fibres was detected in transposed muscles depleted of nerves, their formation does not appear to be nerve dependent. Indeed, in the present set of experiments, acid labile fibres were also found in experimental muscles within 2 weeks of the operation (Figure 26). Of 6 muscles examined at this time, the percentage of acid labile fibres was greater than 40% in 3 muscles but less than 2% in the other 3 muscles. All 6 muscles, however, gave no
Figure 25. Photomicrographs of frozen sections of unoperated and cross-reinnervated normal ALD muscles demonstrating the myosin ATPase reaction after acid and alkaline preincubation and the silver cholinesterase reaction.

Photomicrographs a and b show an unoperated left ALD muscle and a cross-reinnervated right ALD muscle, respectively, at 2 weeks ex ovo, after acid preincubation. While it is easy to identify a' (lighter-staining) and b' (darker-staining) fibres within the unoperated ALD muscle, slow tonic fibres within the cross-reinnervated muscle cannot be differentiated into a' and b' fibres.

Photomicrographs c and d show a cross-reinnervated muscle at 20 weeks ex ovo after acid and alkaline preincubation, respectively. From these two micrographs, it is evident that acid labile fibres express a differential pH stability to the myosin ATPase reaction. (The arrows are for orientation.)

Photomicrographs e and f show a cross-reinnervated muscle at 2 years postoperatively after the myosin ATPase (acid pre-incubation) and silver cholinesterase reactions, respectively. These two micrographs demonstrate the presence of 'en plaque' endplates on acid labile fibres (see arrows).
Figure 26. A composite drawing showing 200 individual muscle fibres in cross section drawn from a normal experimental ALD muscle 2 weeks after the operation. The dark fibres represent acid labile fibres while the clear fibres represent slow tonic fibres. Accompanying the drawing is a histogram showing the size distribution of 255 acid labile fibres taken from this muscle which was made up of approximately 51% acid labile fibres.
response to indirect stimulation. Thus, as in the denervated plus transposed muscles just described, the formation of acid labile fibres in the experimental ALD muscles does not appear to be nerve dependent.

The percentage of acid labile fibres estimated for each experimental ALD muscle older than 2 weeks is presented in the scatter diagram of Figure 27. This Figure shows that acid labile fibres persist in all cross-reinnervated muscles, albeit at a low level in many of these muscles. This observation is emphasized by noting that a majority of the dots in Figure 27 fall below the arbitrary border drawn between 25-30%. This Figure also shows that there is no correlation between the percentage of acid labile fibres in cross-reinnervated muscles and the postoperative time. Therefore, from the time that functional innervation is first recorded (4 weeks), the percentage of acid labile fibres does not consistently increase or decrease.

Physical Properties

Wet Weight: It has been shown in previous sections that denervated ALD muscles show a transient increase in wet weight irrespective of whether they remain in situ or are transposed to a contralateral site. Since denervation per se seems to be responsible for the wet weight increase and since experimental muscles in the present study go through a period of denervation immediately after the operation, wet weight analyses were performed on the experimental muscles. Indeed, at 2 and 4 weeks postoperatively, the wet weight to body weight ratio of the experimental muscles increases significantly above that of the contralateral control muscles (Table 12). Furthermore, concomitant with the re-establishment
Figure 27. The relative percentage of acid labile fibres to total number of fibres in normal experimental ALD muscles from 4 weeks, when functional innervation is first noted, through 32 weeks. Each filled-in circle (●) represents the percentage of acid labile fibres estimated in a single experimental muscle.
TABLE 12. MEAN MUSCLE WET WEIGHT TO BODY WEIGHT RATIO OF CONTRALATERAL AND EXPERIMENTAL A LD MUSCLES

<table>
<thead>
<tr>
<th>AGE (Weeks)</th>
<th>WET WEIGHT/BODY WEIGHT (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contralateral</td>
</tr>
<tr>
<td>2 (6)</td>
<td>0.34 ± 0.05a</td>
</tr>
<tr>
<td>4 (7)</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td>8 (6)</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>12 (5)</td>
<td>0.35 ± 0.03</td>
</tr>
<tr>
<td>16 (5)</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>20 (5)</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>24 (5)</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>28 (5)</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>32 (4)</td>
<td>0.21 ± 0.02</td>
</tr>
</tbody>
</table>

( ) = number of muscles analyzed

a. Mean ± S.E.M.

*Statistical significance (P<0.05) between experimental and contralateral muscle groups.
of innervation (by 8 weeks), the wet weight to body weight ratio of the experimental muscles returns to control values.

Structural analyses: Table 13 correlates the size of fibres in contralateral and experimental ALD muscles with age up to 32 weeks, postoperatively. It is clear that in contralateral unoperated muscles, an initial period of rapid fibre growth is followed by a second period where fibre size stabilizes. This normal pattern of ALD fibre growth was shown to be disrupted by transposition as described in the previous section; fibres in the transposed muscles grew at a slower rate up to 12 weeks postoperatively and then remained approximately 30% smaller than fibres of the contralateral muscles throughout the remaining postoperative period. Comparative structural analyses between the fibres in these denervated transposed muscles and fibres in the successfully cross-reinnervated muscles of the present study show that both groups of fibres have a similar pattern of growth. That is, fibres in the cross-reinnervated ALD muscles remain 18-39% smaller than the fibres in their contralateral counterparts (Table 13).

Previous analyses of fast twitch fibres indicated that there is a steady increase in the average area of normal PLD fibres from 2 to 24 weeks ex ovo although skewing of the distribution of individual fibre areas suggested a maintenance of a population of small fibres throughout ontogeny (see Figure 13). In the present study, a similar type of analysis was performed in order to determine whether or not acid labile fibres in cross-reinnervated muscles have a size distribution similar to that of fibres in normal PLD muscles. The distribution of individual fibre areas for two successfully cross-reinnervated muscles are
TABLE 13. MINIMUM DIAMETER OF FIBRES IN NORMAL CONTRALATERAL AND EXPERIMENTAL ALD MUSCLES

<table>
<thead>
<tr>
<th>AGE (Weeks)</th>
<th>MINIMUM FIBRE DIAMETER (µm)</th>
<th>Experimental</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (6)</td>
<td>25.5 ± 1.5(^a)</td>
<td>19.5 ± 0.5</td>
<td>76(^b)</td>
</tr>
<tr>
<td>4 (6)</td>
<td>33.4 ± 3.0</td>
<td>27.3 ± 1.9</td>
<td>82</td>
</tr>
<tr>
<td>8 (5)</td>
<td>46.4 ± 1.1</td>
<td>32.6 ± 2.1</td>
<td>70</td>
</tr>
<tr>
<td>12 (5)</td>
<td>56.4 ± 4.9</td>
<td>43.2 ± 3.4</td>
<td>77</td>
</tr>
<tr>
<td>16 (5)</td>
<td>61.2 ± 2.7</td>
<td>40.6 ± 1.0</td>
<td>66</td>
</tr>
<tr>
<td>20 (4)</td>
<td>63.2 ± 5.5</td>
<td>49.1 ± 4.8</td>
<td>78</td>
</tr>
<tr>
<td>24 (5)</td>
<td>61.0 ± 2.5</td>
<td>37.0 ± 3.8</td>
<td>61</td>
</tr>
<tr>
<td>28 (5)</td>
<td>54.3 ± 5.4</td>
<td>41.2 ± 3.7</td>
<td>76</td>
</tr>
<tr>
<td>32 (4)</td>
<td>61.8 ± 1.9</td>
<td>37.9 ± 3.0</td>
<td>61</td>
</tr>
</tbody>
</table>

( ) = number of muscles analyzed
a. Mean ± S.E.M.
b. Experimental/Contralateral X 100
presented as histograms in Figures 28 and 29. These two muscles were chosen because each demonstrated a high percentage of acid labile fibres. Both histograms show that the acid labile fibres in these two cross-reinnervated muscles have a similar size distribution pattern as fibres in normal PLD muscles (compare these histograms to those presented in Figure 13).

Metabolic Profile

In order to follow the metabolic changes in the experimental muscles throughout the postoperative period, histochemical analyses were performed. Unoperated ALD- and PLD muscles, analyzed concurrently with the cross-reinnervated muscles, acted as reference muscles; ALD muscles are highly oxidative while PLD muscles are highly glycolytic. By comparing the metabolic profile of the cross-reinnervated muscles to those of the reference muscles, the type of metabolism characterizing the cross-reinnervated muscles could be elucidated.

In response to histochemical tests for Pase, the a' and b' fibres in unoperated ALD muscles exhibit different iodine-based colours. The a' fibres exhibit a red colour indicative of short chain polysaccharides while the b' fibres are lavender to purple, indicating the presence of intermediate length chain polysaccharides. Mature fast twitch fibres, on the other hand, demonstrate the dark blue iodine colour characteristic of long chain polysaccharides (Cosmos, 1966a). In cross-reinnervated muscles a range of colour reactions was noted from which only the dark blue colour was absent. Although the acid labile fibres in the cross-reinnervated muscles did not stain with a dark blue
Figure 28. A composite drawing showing 200 individual muscle fibres in cross section drawn from a normal cross-reinnervated ALD muscle 12 weeks postoperatively. The dark fibres represent acid labile fibres while the clear fibres represent slow tonic fibres. Accompanying the drawing is a histogram showing the size distribution of 1036 acid labile fibres taken from this muscle which was made up of approximately 50% acid labile fibres.
Figure 29. A composite drawing showing 200 individual muscle fibres in cross section drawn from a normal cross-reinnervated ALD muscle 32 weeks postoperatively. The dark fibres represent acid labile fibres while the clear fibres represent slow tonic fibres. Accompanying the drawing is a histogram showing the size distribution of 690 acid labile fibres taken from this muscle which was made up of approximately 50% acid labile fibres.
colour, the blue colour was of strong enough intensity to distinguish these fibres from the slow tonic fibres. Thus, the Pase reaction correlated well with the myosin ATPase results and could be used to differentiate slow tonic and acid labile fibres. Although the slow tonic fibres in the cross-reinnervated muscles displayed a metabolic profile similar to fibres in unoperated ALD muscles, it was still very difficult to differentiate these fibres into a' and b'. In addition, the oxidative enzymic (SDH) response was highly variable in all of the cross-reinnervated muscles examined and therefore could not be used as a reliable marker to identify specific fibre types.

In unoperated ALD muscles, lipids are observed both intracellularly as discrete droplets and extracellularly around blood vessels and nerves. As these muscles mature, the amount of extracellular lipid deposition and connective tissue accumulation increase between the fascicles. While the distribution of lipid was not extensively altered in the cross-reinnervated muscles, there was a slight decrease in the deposition of lipid both intracellularly and extracellularly. Connective tissue accumulation was slightly elevated around the cross-reinnervated muscles but did not change within these muscles when compared to age-matched controls.

In summary, it appears as though all of the experimental muscles examined, regardless of the number of acid labile fibres present, show an alteration in metabolic enzymes relative to contralateral ALD muscles. This metabolic response is a hybrid of the typical metabolic profiles demonstrated by slow tonic and fast twitch muscles. Therefore, it appears as though the experimental muscles make an attempt to
differentiate a highly glycolytic profile in response to dictates from the SB nerve; however, this ability to respond is limited.

Further evidence that an incomplete response to the SB nerve characterizes experimental muscles is shown by an analysis of the distribution of nuclei. Slow tonic fibres in control ALD muscles and in the experimental muscles exhibit nuclei around the periphery of each fibre. This distribution was maintained in acid labile fibres and only occasionally were scattered nuclei, characteristic of fast twitch fibres, noted. Furthermore, irrespective of the degree of conversion or the postoperative age, the experimental muscles of normal birds never took on the general appearance of a normal fast twitch muscle. Instead, these muscles retained the general topography and cell structure of unoperated slow tonic muscles, i.e., both fascicles and fibres ranged from being round to slightly elliptical in shape and large extracellular spaces were maintained between fascicles and fibres.

CROSS REINNERVATED ALD MUSCLES OF DYSTROPHIC BIRDS

Gross Examination

In a total of 68 dystrophic birds, the right ALD muscle was denervated, transposed to the contralateral side and cross-reinnervated with the left SB nerve. Of these experimental birds, 16 were eliminated from the study due to poor health. Forty-nine of the remaining birds were sacrificed between 2 and 32 weeks and 3 were left for 1 to 2 years.

As in normal chickens, prior to sacrifice, the wing position and flipping ability of each bird was tested. Dystrophic experimental birds could always be identified by a drooping right wing. Furthermore, while
every bird tested at 2 weeks could flip over in 5 out of 5 attempts, none of the birds tested from 4 to 32 weeks could right themselves from the supine position.

In only 2 of the 49 birds examined between 2 and 52 weeks had the threads used to suture the transposed muscle to the musculature of the left wing not held. In none of the remaining 47 birds was there any anatomical evidence that the transposed ALD muscle had become self-reinnervated or reinnervated by the nerves of the left ALD or PLD muscles.

One outstanding difference between the experimental ALD muscles of normal and dystrophic birds was the excessive amount of connective tissue surrounding the transposed ALD muscle of the latter. As a consequence, it was difficult to determine the boundary between the distal end of the transposed ALD muscle and the belly of the left triceps brachii muscle to which it was sutured. Furthermore, locating and isolating the SB nerve was difficult and, in a few cases, the SB nerve was accidentally severed prior to physiological analysis. Although it was often difficult to separate the transposed ALD muscle from the overlying skin due to the excessive formation of connective tissue, fusion of the dystrophic experimental ALD muscle with the underlying left ALD muscle was never observed.

**Innervation Characteristics**

**Histochemical analyses:** The location of nerves and endplates in the dystrophic experimental muscles was determined from the distribution of silver-impregnated axons and cholinesterase-staining endplates. At 2
weeks postoperatively, single axons were observed running between fascicles in all of the experimental muscles examined. However, nerve-muscle contact was exceedingly rare. At this time as endplates could be identified in only 1 of the 6 experimental muscles examined. By 4 weeks, the incidence of nerve-muscle contacts increased as endplates were found in 4 of the 6 muscles examined. From 8 weeks and on, it was possible to locate endplates in every experimental muscle examined. In these older preparations, both 'en grappe' and 'en plaque' types of endplates were observed.

Physiological analyses: In vivo analyses of the physiological response of cross-reinnervated dystrophic muscles to stimulation of the SB nerve were performed on 41 out of 47 muscles between 2 and 32 weeks postoperatively. As noted in the normal preparations, none of the experimental dystrophic muscles tested at 2 weeks responded to stimulation. In fact, the first response to indirect stimulation was not detected until 4 weeks postoperatively. From 4 weeks and on, all of the dystrophic preparations tested gave a tetanic response when repetitively stimulated and a majority (56%) twitched in response to a single isolated pulse applied to the SB nerve. An example of the contractile properties of a 12 week old cross-reinnervated dystrophic ALD muscle is presented in Figure 30. This figure shows that the cross-reinnervated muscle, similar to its control muscle, gives a tetanic response following a train of repetitive stimuli and a twitch in response to a single pulse after a train of repetitive stimuli (PTP). In contrast, only the cross-reinnervated muscle twitches when stimulated indirectly with a single pulse. (Note: The 'sag' phenomenon seen in Figure 30d
Figure 30.

Example of isometric tetanic, post tetanic twitch and twitch responses of contralateral (a,b,c) and experimental (d,e,f) ALD muscles of a 12 week old dystrophic bird. The tetanic contractions (a,d) were evoked in response to indirect stimulation at 50, 70, and 100 Hz. The post tetanic twitches (b,e) were evoked after 7 sec of 70 Hz nerve stimulation followed by a single stimulus. While the contralateral ALD muscle did not respond to a single indirect stimulus (c), the experimental muscle responded with a twitch (f). Upon histochemical analysis, the experimental muscle was found to contain over 70% acid labile fibres.
characterize only a small number of dystrophic cross-reinnervated muscles.)

In summary, evidence has been presented which shows that the SB nerve reinnervates transposed normal and dystrophic ALD muscles at approximately the same rate; axons are present in the muscles by 2 weeks postoperatively and form extensive connections by 8 weeks. Functional innervation of both types of muscles is initiated between 2 and 4 weeks postoperatively and in a majority of these muscles a twitch response can be elicited when a single pulse is applied to the SB nerve.

Fibre Type Analyses

The myosin ATPase profiles of ALD and PLD muscles are not altered by the dystrophic process in that the slow tonic fibres of an ALD muscle continue to exhibit an acid and alkali stable myosin ATPase activity while the fast twitch fibres of the PLD remain alkali stable but acid labile. Figure 31 shows the myosin ATPase profile of several experimental dystrophic ALD muscles at various postoperative ages. It is evident from these photomicrographs that each one of the experimental muscles presented contains two types of fibres.

To determine the distribution of acid labile fibres within dystrophic muscles, all 47 muscles were examined and subsequently divided into two groups:

1. muscles in which the acid labile fibres are randomly scattered throughout the muscle cross section; and

2. muscles in which the acid labile fibres are not randomly scattered throughout the muscle but show a bias in their
Photomicrographs of frozen sections of unoperated and cross-reinnervated dystrophic ALD muscles demonstrating the myosin ATPase reaction after acid and alkaline preincubation.

Photomicrograph a shows a 12 week old right cross-reinnervated ALD muscle (top) with its left unoperated ALD muscle (bottom) after the myosin ATPase reaction (alkaline preincubation). Note the dramatic difference in architecture between the two muscles.

Photomicrograph b shows a cross-reinnervated ALD muscle (right) with its unoperated ALD muscle (left) after the myosin ATPase reaction (acid preincubation) taken from a 32 week old chicken. Note that all of the fibres in the unoperated ALD muscle are acid stable while a majority of the fibres in the cross-reinnervated ALD muscle are acid labile. The percentage of acid labile fibres was estimated to be 80% in the cross-reinnervated muscle.

Photomicrographs c and d are cross-reinnervated ALD muscles stained with the myosin ATPase reaction (acid preincubation) taken from 8 and 20 week old dystrophic chickens, respectively. Both micrographs show that the light-staining acid labile fibres are distributed throughout the entire muscle cross section. The percentage of acid labile fibres was estimated to be 93% in the cross-reinnervated muscle in c and 70% in the cross-reinnervated muscle in d.

Photomicrographs e and f are cross-reinnervated ALD muscles stained with the myosin ATPase reaction (alkaline preincubation) taken from a 20 week old normal chicken and a 20 week old dystrophic chicken, respectively. Note that the normal cross-reinnervated muscle maintains a typical ALD type of architecture despite containing 40% acid labile fibres. In contrast, the topographical appearance of the dystrophic cross-reinnervated muscle is altered. The percentage of acid labile fibres was estimated to be 57% in the dystrophic cross-reinnervated muscle.
distribution.

In the dystrophic birds, 42 muscles (or 88%) fall into Group 1 whereas only 6 muscles (or 12%) fall into Group 2. This is in marked contrast to the distribution noted with the experimental ALD muscles in normal chickens where a majority of these muscles demonstrated a biased distribution (Group 2) of acid labile fibres. Fibre type grouping was not detected in any of the dystrophic experimental muscles analyzed.

In an attempt to quantify the effects of the operation on fibre type, the number of acid labile fibres relative to the total number of fibres was determined for each experimental dystrophic ALD muscle. Within 2 weeks of transposition, the percentage of acid labile fibres in the experimental muscles ranged from 4 to 74%. This observation is consistent with the results presented in the previous section on denervated and transposed dystrophic ALD muscles. Furthermore, this result demonstrates that the formation of acid labile fibres occurs prior to the establishment of functional contacts between the SB nerve and the transposed dystrophic muscle.

The percentage of acid labile fibres estimated for experimental dystrophic muscles older than 2 weeks is presented in the scatter diagram of Figure 32. Not only does this figure show that acid labile fibres persist in all of the cross-reinnervated muscles but that the percentage of acid labile fibres is greater than 30% in a majority of muscles. This latter observation is emphasized by noting that a majority of the dots in Figure 32 lie above the arbitrary border drawn between 25-30%.

A comparison of Figures 21 and 32 shows that, on average, cross-
Figure 32. The relative percentage of acid labile fibres to total number of fibres in dystrophic cross-reinnervated muscles from 4 weeks, when functional innervation is first noted, through 32 weeks. Each filled-in circle (●) represents the percentage of acid labile fibres estimated in a single cross-reinnervated muscle.
reinnervated dystrophic muscles contain a greater number of acid labile fibres than do cross-reinnervated normal muscles. This point is emphasized in Figure 33 which compares the percentage of experimental muscles of each genotype displaying a particular percentage of acid labile fibres. It is evident from this figure that a high percentage of experimental muscles of dystrophic lineage contain a large number of acid labile fibres. For example, nearly all of the cross-reinnervated dystrophic muscles are made up of at least 10% acid labile fibres while 38% of the experimental normal muscles contain less than 10% acid labile fibres. Furthermore, while 43% of the dystrophic muscles contain a majority of acid labile fibres (>60%), none of the cross-reinnervated normal muscles contain a comparable number of acid labile fibres.

Physical Properties

Wet Weight: As shown in Figure 34, the initial response of normal and dystrophic ALD muscles to transposition is similar; there is a significant increase in the wet weight of both types of muscles above their respective contralateral control values up to 4 weeks postoperatively. However, while the wet weight to body weight ratio of the experimental normal muscles returns to control values by 8 weeks, in the dystrophic preparations this ratio remains significantly elevated above normal. Cross-reinnervated ALD values throughout most of the remaining postoperative period, i.e., not significant at 12 and 24 weeks.

Structural Analyses: It was established in an earlier section that there is no difference in the growth pattern of unoperated normal and
Figure 33. Since the percentage of acid labile fibres appears to be independent of the postoperative age beyond the two week period, all values for each genotype were pooled. Data were segregated according to the percentage of acid labile fibres (acid labile fibres/total fibres X 100) and the frequency of the percent recorded. This analysis emphasizes the large percentage of cross-reinnervated muscles of normal birds which responded poorly, or not at all, to the 'fast' nerve: only 62% of the normal muscles show greater than 10% conversion, whereas, 100% of the dystrophic muscles fall within this category. Furthermore, 43% of the experimental dystrophic muscles show greater than 60% conversion, (>60%), yet none of the normal experimental muscles attain this level of conversion.
X-Ri Anterior Latissimus Dorsi

Frequency of Muscles (%)

Acid Labile Fibres/Total Fibres (%)
Figure 34. The wet weight to body weight ratio of normal and dystrophic cross-reinnervated ALD muscles with ex ovo age. The bars represent the standard error of the mean.
dystrophic ALD fibres up to 32 weeks ex ovo. Both normal and dystrophic ALD fibres are characterized by an initial period of rapid fibre growth followed by a second period where the fibre size stabilizes (see Figure 8). However, transposition of either a normal or dystrophic ALD muscle disrupts the normal growth pattern of its fibres; the fibres in these muscles remain approximately 30% smaller in size than the fibres in their respective contralateral counterparts. Consistent with these observations, Table 14 shows that the muscle fibres in the experimental dystrophic muscles of the present study are significantly smaller than fibres in their contralateral controls from 8 to 32 weeks postoperatively.

The fact that the fibres in the transposed muscles are consistently smaller than the fibres in age-matched control muscles is common to both normal and dystrophic preparations and persists despite the fact that both groups become reinnervated by the SB nerve. A direct comparison between normal and dystrophic transposed muscles shows that their fibres grow at the same rate up to 12 weeks postoperatively. However, between 12 and 20 weeks, the fibres in the dystrophic muscles fail to keep pace with the increment noted with normal ALD fibre size so that during this period there is a significant difference in the size of fibres in normal and dystrophic transposed muscles. The difference in the size of the fibres in normal and dystrophic cross-reinnervated muscles does not persist after 20 weeks and therefore appears as only a transient phenomenon.

Earlier analyses of normal and dystrophic acid labile fibres of PLD muscles revealed that a significant difference in the size distribution
TABLE 14. MINIMUM DIAMETER OF FIBRES IN DYSTROPHIC CONTRALATERAL AND EXPERIMENTAL ALD MUSCLES

<table>
<thead>
<tr>
<th>AGE (Weeks)</th>
<th>MINIMUM FIBRE DIAMETER ((\mu m))</th>
<th>CONTRALATERAL</th>
<th>EXPERIMENTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (6)</td>
<td>24.1 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.9 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>4 (5)</td>
<td>33.1 ± 3.0</td>
<td>24.4 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>8 (5)</td>
<td>49.9 ± 2.1</td>
<td>29.1 ± 2.9&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>12 (5)</td>
<td>51.6 ± 3.5</td>
<td>32.6 ± 2.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>16 (6)</td>
<td>58.6 ± 0.8</td>
<td>30.7 ± 2.7&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>20 (5)</td>
<td>54.1 ± 2.1</td>
<td>28.3 ± 0.8&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>24 (5)</td>
<td>55.5 ± 4.6</td>
<td>35.1 ± 4.1&lt;sup&gt;p&lt;/sup&gt;</td>
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<tr>
<td>28 (5)</td>
<td>61.1 ± 4.0</td>
<td>34.1 ± 4.5&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>32 (5)</td>
<td>60.5 ± 3.5</td>
<td>37.8 ± 3.6&lt;sup&gt;*&lt;/sup&gt;</td>
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<sup>a</sup> = number of muscles analyzed

<sup>p</sup>, <sup>*</sup> = Mean ± S.E.M.

Statistical significance (<sup>p</sup> < 0.05; <sup>*</sup> < 0.01) between the experimental and contralateral muscle groups.
of normal and dystrophic PLD fibres becomes apparent as early as 4 weeks ex ovo and persists up to at least 28 weeks (see Figure 13). While the size distribution of normal PLD fibres remains normal with a slight skewing to the left, the size distribution of dystrophic PLD fibres becomes bimodal indicative of the formation of both large and small fibres in the dystrophic muscles. In the present study, a similar type of analyses was performed in order to determine whether the acid labile fibres noted in the cross-reinnervated muscles have a normal or bimodal distribution. In Figures 35 and 36, histograms are presented of the frequency distribution of fibre area in two successfully cross-reinnervated dystrophic muscles which exhibit a high percentage of acid labile fibres. Both Figures show that the acid labile fibres in these two muscles tend to have a normal size distribution although skewing to the left is evident in both preparations.

A direct comparison of the architecture of normal and dystrophic experimental muscles revealed outstanding differences between the two groups. While the normal preparations retained a normal type of ALD topography, the general architecture of the dystrophic preparations was drastically altered. From 4 weeks and on, the fascicles in the dystrophic preparations were angular in shape and densely-packed with fibres. There was little evidence of destruction and the dystrophic preparations were relatively free of interfascicular lipid and connective tissue. In addition, a reduction in interfascicular space gave each muscle a highly compact appearance. These drastic changes in topography are most likely due to the extensive proliferation of cells which occurs in dystrophic ALD muscles soon after transposition (see
Figure 35. A composite drawing showing 332 individual muscle fibres in cross section drawn from a dystrophic cross-reinnervated ALD muscle excised from a 24 week old rooster. Each dark fibre represents an acid labile fibre while the clear fibres represent slow tonic fibres. Accompanying the drawing is a histogram showing the relative distribution of fibre areas (um²) of 961 acid labile fibres sampled from the same muscle.
Figure 36. A composite drawing showing 200 individual muscle fibres in cross section drawn from a dystrophic cross-reinnervated ALD muscle excised from a 28 week old hen. Each dark fibre represents an acid labile fibre while the clear fibres represent slow tonic fibres. Accompanying the drawing is a histogram showing the relative distribution of fibre areas ($\text{um}^2$) of 1087 acid labile fibres sampled from the same muscle.
Metabolic Profile

Normal and dystrophic unoperated slow tonic muscles respond similarly to the Pase reaction. In contrast, normal and dystrophic fast twitch muscles respond dissimilarly. While normal fast twitch muscles stain with a homogeneous dark blue colour, fascicles in dystrophic fast twitch muscles contain cells of varying sizes with mixed iodine colours indicating the formation of a mixture of polysaccharides of varying chain lengths.

The metabolic profile of fibres in experimental dystrophic muscles could be characterized as follows. The slow tonic fibres showed a similar response to the Pase reaction when compared to slow tonic fibres in unoperated ALD muscles although it was difficult to differentiate these into a' and b'. These fibres, therefore, retained a low glycolytic capacity. Acid labile fibres, on the other hand, showed a variable response to the Pase reaction but, in general, stained with a more intermediate iodine reaction (purple) in contrast to the blue colour noted with the cross-reinnervated fibres in normal muscles. As in normal preparations, the response of the experimental dystrophic muscles to the SDH reaction was highly variable and therefore could not be used as a reliable marker to identify specific fibre types.
Long-Term Experiments

To observe the long-term effects of cross-reinnervation, several normal and dystrophic ALD muscles were examined from 1 to 2 years postoperatively. Examination of these muscles revealed that differences in the response of normal and dystrophic ALD muscles to cross-reinnervation continued to persist 1 to 2 years after the initial operation. The cross-reinnervated dystrophic muscles continued to weight significantly more than their normal counterparts and the cross-sectional area of the dystrophic muscles was greater than that of the cross-reinnervated normal muscles. In addition, fascicles within the dystrophic preparations continued to be angular in shape and tightly-packed with fibres. Therefore, even 1 to 2 years postoperatively, the general architecture of the cross-reinnervated normal muscles remained altered while the topography of the cross-reinnervated normal muscles continued to resemble that of an unoperated ALD muscle. Despite the fact that significant differences persisted in the architecture of the normal and dystrophic preparations, the percentage of acid labile fibres was similar: all of the cross-reinnervated normal and dystrophic muscles examined contained between 15 to 25% acid labile fibres.
NORMAL CROSS REINNERVATED MUSCLES

During the uninterrupted development of the ALD muscle of dystrophic genotype, all of the parameters examined have indicated that overt phenotypes of this muscle mimic those of the same muscle of normal lineage (Linkhart and Wilson, 1975; Mazliah et al., 1976; Cosmos et al., 1980; Barnard et al., 1982). In the present study, these observations have been confirmed using structural and histochemical parameters on ALD muscles from newly-hatched chicks up to 32 weeks ex ovo. From these studies it was concluded that in the chicken with hereditary muscular dystrophy, slow tonic muscles are spared disease characteristics.

Such observations are in sharp contrast to observations made with avian fast twitch muscles which do express dystrophic characteristics notably from early posthatching throughout maturity. In this laboratory, experiments have been designed to investigate properties of fast twitch and slow tonic muscles in an effort to understand why two genetically dystrophic muscles are affected differently by the dystrophic gene. Of the many experimental approaches tested, (for a review see Cosmos et al., 1980), the only one successful in altering the phenotype of a dystrophic muscle was the cross-reinnervation of the fast twitch PLD muscle by the nerve of the slow tonic ALD muscle (Mazliah, 1980). The rationale for the design of this experiment was based on the observation that slow tonic muscles maintain embryonic enzymic profiles, established during embryogenesis, throughout development. Unlike twitch
muscles, once established, these profiles are never altered. As a working hypothesis, it was reasoned that twitch muscles of dystrophic birds could not successfully complete those metabolic changes necessary for the proper maturation of this muscle type. Evidence to support this hypothesis was obtained from the examination of fibres of the fast twitch PLD muscles successfully cross-reinnervated by the nerves of the slow tonic ALD muscle, i.e., they maintained fetal enzymic profiles and were spared overt signs of the disease. Furthermore, cross-reinnervated fibres of PLD muscles of both normal and dystrophic birds responded similarly.

The present experiments were designed to examine the effects of cross-reinnervation on the slow tonic ALD muscle. Since in the dystrophic bird this muscle expresses normal phenotypes, experiments were designed to examine whether or not it would respond similarly to the normal ALD muscle when forced to alter its developmental pattern. Individual fibres of the ALD muscle of both normal and dystrophic birds cross-reinnervated by a nerve of a fast twitch muscle, histochemically demonstrated acid labile myosin ATPase activity, focal innervation, increased glycolytic enzymic activity and variable oxidative enzymic levels, i.e., characteristics of fast twitch fibres. Striking differences were noted, however, in the response of the entire muscles to the operative procedure. Whereas the cross-reinnervated ALD muscles of normal birds showed limited alterations in muscle size, structure, and enzymic profiles when compared to the unoperated muscle, experimental ALD muscles of dystrophic birds revealed many alterations in these same parameters. Of the changes noted in the latter, the most
revealing were the high percentage of acid labile fibres, the increase in both fibre number and total muscle weight, and the tightly-compact appearance of the experimental muscles. Such observations led to the conclusion that whereas the normal ALD muscles coupled to a 'fast' nerve attempted to resist changes imposed by the foreign nerve and maintain many phenotypes of its unoperated control muscle, the dystrophic ALD muscle could not respond similarly.

In the present experiments, a consideration of the extent to which slow tonic muscles of normal birds can be altered in the cross-reinnervation experiments must be considered. Since the pioneering cross-reinnervation studies of Buller et al. (1960a,b), it has been known that denervated twitch muscles can accept and respond to a foreign nerve. Thus, a fast twitch muscle, reinnervated by a nerve which normally supplies a slow twitch muscle demonstrates contractile, structural, metabolic, and biochemical characteristics of a slow twitch muscle (Dubowitz, 1967; Guth et al., 1968). Such results indicate that a nerve placed on a foreign target has the capacity to alter established phenotypes either by functional demands imposed or trophic mechanisms exerted. Thus, there appears to be an inherent flexibility built into the genetic machinery of twitch muscle fibres enabling them to express different phenotypes in accordance with the neuronal demands placed on them. Even though it has been established that such pleiotropism pertains to twitch muscles, it has not been demonstrated clearly to characterize slow tonic muscles as well.

Slow tonic fibres are distinct from twitch muscle fibres in that they are multiply-innervated, synthesize a unique isoform of myosin and,
at hatching, express mature enzymic profiles. Cross-reinnervations performed with slow tonic ALD muscles of normal chickens have been designed to test the ability of these muscles both to accept and respond to the influences of a foreign 'fast' nerve; results of these experiments, however, are inconclusive. While the transformation of the fast twitch PLD muscle by a foreign nerve in chickens has proven successful (Jirmanová and Zelená, 1973a), the acceptance of a 'fast' nerve by the slow tonic ALD muscle has presented many problems. In fact, initial attempts to cross-reinnervate the ALD muscle maintained in situ in adult chickens with 'fast' nerve failed to demonstrate cross-reinnervation effects (Hník et al., 1967). In an attempt to interpret this result, Hník and co-workers speculated that the 'fast' PLD nerve which they had used had been incompetent. Credence was given to this interpretation since it was known by this time that the 'fast' PLD nerve was a mixed nerve containing a 'fast' component, which innervated fast twitch muscles, and a 'slow' component, which innervated slow tonic muscles. From the earlier studies of Feng et al. (1965), it had been known that if given the choice of a PLD nerve or its original nerve, a denervated ALD muscle would accept its original nerve. Therefore, the failure of the ALD muscle to become transformed could have been due to the selective reinnervation of the large 'slow' component of the PLD nerve or due to self-reinnervation by the ALD nerve. These same investigators then proceeded to perform cross-reinnervation experiments on ALD muscles maintained in situ in newly-hatched chickens with a different 'fast' nerve (Jirmanová et al.; 1971). In these experiments, operations were performed on 7 chickens
ranging in age from 2-13 days posthatching using the superior brachialis nerve, "a predominantly 'fast' nerve". Ten months after the operations, innervation patterns and contractile properties of the experimental muscles were examined. With the cholinesterase reaction, 'en plaque' endplates with strong AChE activity could be observed but only in the vicinity of the implanted foreign nerve.

In a second series of experiments by the same group, cross-reinnervations were performed immediately after hatching on 5 chicks (Zelenš and Jirmanová, 1973a,b,c). To avoid the problem of self-reinnervation, the ALD muscles were first transposed to the contralateral side and then supplied with the superior brachialis nerve. Six to 8 months after the operation, the innervation patterns and ultrastructural characteristics of the experimental muscles were examined. This time, between 60-80% of the 550 fibres examined in the vicinity of the nerve implant were found to be innervated focally by 'en plaque' endings. It was assumed that the 'en grappe' endplates identified on the remaining fibres originated from the 'slow' component of the implanted nerve. In order to establish the degree of ultrastructural alterations within the cross-reinnervated ALD muscles, the incidence of triads, the width of the Z-lines, and the mean mitochondrial contents were determined. When quantified, transformed fibres had a greater number of triads, thinner Z-lines, and a lower mean mitochondrial content; each of these changes were indicative of a transformation towards the fast fibre type. Therefore, the results of this series of experiments suggested that ALD muscles could accept 'fast' nerves and that these incoming nerves could influence the ultrastructural differentiation of the ALD muscles.
The aim of the third and final cross-reinnervation study by this group was to determine the time of onset and the progression of muscle transformation postoperatively (Sycová and Zelená, 1975). These experiments were performed exactly as described in the second series of experiments. Cross-reinnervated muscles were examined from 2-15 months for histochemical, ultrastructural, and biochemical changes. Histochromically, with the myosin ATPase reaction, fast twitch fibres were identified in cross-reinnervated muscles as early as 2 months postoperatively. However, the light chains of myosin and the biochemically-determined myosin ATPase activity did not change until 3 months. At this time, 'fast' myosin light chains could be identified in the electrophoretic gels of myosin prepared from transformed muscles.

Moreover, the myosin ATPase activity increased significantly to about 68% of the myosin ATPase activity found in PLD muscles at 3 months. These results extended earlier results by showing that fibres within the cross-reinnervated expressed altered histochemical and biochemical properties in addition to altered ultrastructural characteristics.

In a detailed study designed to examine the ability of 'fast' nerves to reinnervate ALD muscles, Bennett and Pettigrew (1974b) were able to confirm and extend several of the early findings of Jirmanová and co-workers. Using the same cross-reinnervation technique as Jirmanová et al. (1973a), these investigators found a singular 'en plaque' ending on 80% of the muscle fibres examined in the vicinity of the implanted foreign nerve.

Implicit in all of these reported studies is the interpretation that the foreign 'fast' nerve is responsible for the histochemical, bio-
chemical, physiological, and ultrastructural changes which characterize the cross-reinnervated muscles; the extent of this effect is dependent upon such factors as pre-treatment of the ALD muscle, postoperative time, age of the muscle, and the ability of the nerve to penetrate into the cross-reinnervated muscle. One of the more important findings of the present study is that the foreign 'fast' nerve plays no role in the initial change in cross-reinnervated muscle phenotypes but is critical to the continued maintenance of these changes.

In the preliminary stages of this thesis, an attempt was made to cross-reinnervate ALD muscles maintained in situ. The ability of the cross-reinnervated muscles to interact with the foreign 'fast' nerve was monitored primarily by alterations in the physiological response, the characteristic myosin ATPase profile, and the acetylcholinesterase pattern of nerve endings. These three parameters were selected because they are distinctly different between ALD and PLD muscles. Examination of the ALD muscles cross-reinnervated in situ revealed that none of the above parameters were altered; these results confirmed the results of others (Feng et al., 1963; Hněk et al., 1967); that ALD muscles, which are left in situ, are highly resistant either to the acceptance of a foreign nerve or to alterations imposed by a transposed nerve, and, support the theory originally proposed by Feng et al. (1965) that, if given the choice, slow tonic muscles have a preference for their own nerve. In an effort to avoid self-reinnervation, denervated ALD muscles were transposed to the contralateral side away from the severed ALD nerve prior to cross-reinnervation by a 'fast' nerve.

When an ALD muscle of normal genotype is denervated in situ, it
takes between 2–4 weeks before the ALD nerve begins to re-enter the muscle and form connections with the denervated fibres (Bennett and Pettigrew, 1974b; this thesis). It takes another 1–2 weeks before these connections become functional (Bennett and Pettigrew, 1974b). Similarly, as shown in the present study, when a normal ALD muscle is denervated, transposed, and then cross-reinnervated with the ‘fast’ superior brachialis nerve, it takes approximately 2–4 weeks for this nerve to enter the muscle and form contacts with the ALD fibres. It takes another 1–2 weeks before these connections become functional. Therefore, it seems as though the type of incoming nerve presents no barrier to the ability of the denervated ALD muscle to recapture a nerve; both the original ALD nerve and the foreign superior brachialis nerve can reinnervate and form functional connections with these fibres at approximately the same rate. Furthermore, once captured the nerve is not rejected. Cross-reinnervated muscles consistently contract in response to stimulation of the superior brachialis nerve for as long as 8 months after the operation.

It is evident from the examination of both transposed and cross-reinnervated muscles 2 weeks postoperatively that the foreign nerve is not responsible for the initial formation of acid labile fibres; acid labile fibres are already present at 2 weeks, a time when nerves could not be detected in the experimental muscles either physiologically or histochemically. In fact, more recent studies done to investigate the appearance of acid labile fibres in the absence of nerves verified the presence of acid labile fibres as early as 1 week postoperatively in ALD muscles which were transposed but not cross-reinnervated. Furthermore,
in transposed ALD muscles which were not cross-reinnervated there is an eventual loss of acid labile fibres from these muscles within 12 weeks of the operation. In contrast, acid labile fibres persist in the cross-reinnervated muscles throughout the postoperative period. Therefore, while the 'fast' nerve appears to play no role in the initial formation of acid labile fibres in the experimental muscles, the maintenance of these fibres seems to depend upon this nerve.

The distribution of acid labile fibres in the cross-reinnervated muscles also gives some indication of how the superior brachialis nerve and the ALD muscle interact during the postoperative period. Clusters of acid labile fibres were found in only 11 of the 45 cross-reinnervated muscles examined. This fibre type grouping has been reported to occur in cross-reinnervated mammalian twitch muscles (Romanul and Van Der Meulen, 1967) and is thought to be due to the restricted ability of the incoming nerves to penetrate a tightly-packed muscle. As both the fibres and fascicles in twitch muscles are compact with very little extracellular space existing between either the fibres or fascicles, fibre grouping in cross-reinnervated twitch muscles is not unexpected. The absence of fibre type grouping observed with a majority of the cross-reinnervated ALD muscles could be due to the large extracellular spaces between fascicles and fibres; this would permit the incoming superior brachialis nerve to course through the muscle and establish contacts in different areas. A bias in the distribution of acid labile fibres was noted in 89% of the muscles examined, however, which suggests that some restriction may have been placed on the incoming nerve.

Another observation which has been made is that the relative
percentage of acid labile fibres does not increase or decrease after the establishment of functional innervation; their numbers are independent of the postoperative age past 2 weeks. These results suggest that there is not a competitive displacement of nerve terminals formed between the superior brachialis nerve and the ALD fibres by reinnervating 'slow' nerves nor is there a selective degeneration of innervated acid labile fibres during the postoperative period. Therefore, once formed, the connections between the superior brachialis nerve and the ALD fibres appear to be compatible and stable.

How does the relative percentage of acid labile fibres found in the cross-reinnervated muscles in this study compare to the relative degree of transformation found by other investigators? Unfortunately, a direct comparison is difficult for the present study is the only one to quantify the number of acid labile fibres within the cross-reinnervated muscles. Other investigators have used different criteria to monitor the degree of transformation. In addition, the reported studies have examined only a small number of muscles and at only one postoperative period; have usually sampled in an area restricted to the vicinity of the implanted nerve; and have never sampled more than 4% of the total fibre population. In the present study, a greater number of muscles were analyzed and over a longer period of time than in any of the previous studies: up to 50 chickens were examined between 2 and 104 weeks postoperatively. Furthermore entire cross sections of muscle were analyzed and thousands of fibres were reliably identified histochemically as being either acid labile or acid- and alkali-stable. Yet, of the 50 cross-reinnervated muscles examined in this study only 5
showed between 40-70% transformation. This is in marked contrast to the 68-80% transformation for all of the cross-reinnervated muscles reported by Jirmanová and co-workers and Bennett and Pettigrew. A reason for this discrepancy may reside in the sampling protocols used. While the formation of acid labile fibres in the cross-reinnervated muscles appears to occur in the absence of a nerve, the maintenance of these fibres is neurally-dependent. Therefore, acid labile fibres formed within the vicinity of the incoming nerve would have a better chance of surviving than those formed in areas away from the nerve. This interpretation is supported by Zelená and Jirmanová (1973a) who reported that the percentage of fibres supplied with 'en plaque' endplates was greatest in the middle third of the cross-reinnervated muscles, the middle third being the area of nerve implantation. In addition, Syrový and Zelená (1975) reported that while acid labile fibres were present in regions surrounding the implanted nerve, regions far from the site of nerve implantation did not show any acid labile fibres. Thus, it is not surprising that studies sampling in the vicinity of the nerve should report higher degrees of transformation than those observed in the present study where the percentage of acid labile fibres within an entire cross section of muscle was considered.

Syrový and Zelená (1975) were the first to show that acid labile fibres can form in cross-reinnervated ALD muscles and the present study confirms this observation. However, these investigators also reported that the histochemical transformation of the ALD muscles did not occur until 8 weeks postoperatively. The results of the present study strongly disagree with this interpretation and show that acid labile
fibres are present within the cross-reinnervated muscles by 2 weeks postoperatively.

There is a strong indication that the acid labile fibres present in the transposed ALD muscles do not arise from slow tonic fibres which have been transformed but represent fibres arising de novo. Early attempts to cross-reinnervate uninjured ALD muscles in adult birds resulted in a very limited degree of transformation (Hněk et al., 1967). However, these investigators did note that in a small group of experiments, 'fast' nerves could form connections on ALD fibres following injury to the fibres or if the operation was performed in young birds, i.e., if the membrane of the ALD fibres was made plastic enough to respond to the influences of the nerve. Subsequently, procedures were developed to increase the plasticity of the ALD fibres (Zelená and Jirmanová, 1973a,b,c). Not only were the cross-reinnervated experiments now performed in newly-hatched chicks, but the ALD muscles were injured prior to cross-reinnervation by transposition of these muscles. With this procedure, Zelená and Jirmanová (1973a) observed areas of regeneration in the cross-reinnervated muscles which they suggested were due to a temporary ischemia of the muscle during the operation. They hypothesized that the new fibres formed from myoblasts in these areas were more liable to submit to neural influences than those fibres already present in the ALD muscles at the time of the operation. Similarly, Gordon and Vrbová (1975c) reported that muscle regeneration from minced muscle tissue induced a neurally-dependent transformation of ALD muscles.

If regenerating fibres do, indeed, arise in transposed ALD muscles
then there must be an event during the initial operative procedure which activates a regenerative response. A recent study in our laboratory was designed specifically to examine whether some initial event in the transposition technique per se caused excessive damage to the ALD muscle (Moore et al., 1984); this damage acting as a trigger for the proliferation of new fibres. For this study, eight 2-day old normal chicks were anaesthetized and the right ALD muscle in each bird was severed from its insertion to the humerus. When freed from its insertion and surrounding connective tissue, the tenotomized ALD contracted to approximately a quarter of its normal resting length and had to be forcibly extended in order to suture the distal end of the muscle into the wing musculature. Ultrastructural analyses revealed extensive muscle damage immediately following the operation. By 24 hours, regeneration in response to this injury had commenced. Mitotic figures both within and adjacent to regions of disruption were observed and many mononucleated cells with prominent nucleoli and rich in cytoplasmic ribosomes were present. By 48 hours, these mononucleated cells, interpreted to be satellite daughter cells, had aggregated linearly along the muscle axis and myotubes containing myofilaments were observed. On the basis of these observations, it is clear that de novo formation of muscle fibres occurs subsequent to the operative procedure for transposition.

Another way of demonstrating that acid labile fibres in the cross-reinnervated muscles arise from regenerating fibres is to show that these fibres do not form in cross-reinnervated muscles which have not been injured. As alluded to above, Hník et al. (1967) have already shown this to be true in adult birds and an experiment performed as a
part of the 'Comparative Assessment of Cross Reinnervation Techniques' confirms this in younger birds. In this experiment, 25 ALD muscles were cross-reinnervated in situ with the superior brachialis nerve. The ALD muscles were not minced or tenotomized prior to cross-reinnervation and care was taken not to damage these muscles. When analyzed from 2 to 20 weeks after the operation, the percentage of acid labile fibres, if present, did not exceed 2% of the total fibre population in any muscle. This result also precludes any role of ischemia in the initial formation of acid labile fibres since the major blood supply to the experimental muscles was removed during the initial operative procedure.

There is also the possibility that ALD muscle fibres, in response to denervation, begin to synthesize the acid labile isoform of myosin. To test this, 12 ALD muscles were denervated in newly-hatched normal chicks and analyzed from 1 to 4 weeks postoperatively (see the 'Denervation Study'). In none of the muscles analyzed, were acid labile fibres observed. Furthermore, by 3 to 4 weeks, the ALD muscles began to show signs of self-reinnervation. Yet, in none of the muscles analyzed was there any indication that the reinnervated fibres had changed their myosin ATPase properties. Therefore, on the basis of these observations, it is likely that the acid labile fibres present in the tranposed ALD muscles do not arise from slow tonic fibres which have been transformed but represent fibres arising de novo which synthesize an acid labile isoform of myosin.

Implications: Since the pioneering work of Buller et al. (1960a,b), it has been known that a nerve can alter the phenotypes of twitch fibres. At the present time, however, it is not known whether this pleiotropism
is specific to twitch muscle fibres or characterizes tonic fibres as well. Cross-reinnervation experiments with the slow tonic ALD muscle of chickens have been performed in the past in an attempt to test the pleiotropism of these muscles but the results of these experiments are inconclusive. Although several studies have demonstrated the presence of focally-innervated acid labile fibres within cross-reinnervated ALD muscles (Zelená and Jirmanová, 1973a, b, c; Bennett and Pettigrew, 1974b; Syrový and Zelená, 1975), whether these fibres represent slow tonic fibres which have been converted by the incoming nerve or simply represent regenerating fibres synthesizing an acid labile isoform of myosin has not been convincingly demonstrated. Evidence presented in this thesis support the latter interpretation and subsequently implies that mature slow tonic fibres are resistant to fibre type conversion and therefore are non-pleiotropic in nature.

Resistance to change is not only reflected at the level of individual fibres but at the level of the ALD muscle as well. This is particularly evident when examining the fibre type composition of the cross-reinnervated ALD muscles. In 38% of the muscles analyzed between 2 and 32 weeks postoperatively, acid labile fibres made up less than 10% of the total fibre population. Furthermore, while 44% of the cross-reinnervated muscles contained between 10-30% acid labile fibres, only 8 of 45 muscles were made up of more than 30% acid labile fibres.

There are criteria other than fibre type which also suggest that ALD muscles are highly resistant to change. This is particularly evident when examining the topographical structure of the cross-reinnervated muscles. Examination of the experimental muscles
reveals that their general architectural appearance, irrespective of the number of acid labile fibres, remain similar to that of an unoperated ALD muscle throughout the postoperative period; both fascicles and fibres of these muscles remain loosely-packed and rounded in shape. Furthermore, the amount of lipid and connective tissue infiltration in the cross-reinnervated muscles is not significantly different from that observed in the unoperated ALD muscles and there is no change in the position of nuclei within the fibres of these muscles. In addition, while the metabolic profile did change towards that characteristic of fast twitch muscles, this change was incomplete. Therefore, while it is evident that the ALD muscles can make contact with the superior brachialis nerve, the cross-reinnervated muscles are resistant to change and do not assume the general architecture and complete properties of a fast twitch muscle. The cross-reinnervated ALD muscles of normal genotype, examined up to 104 weeks postoperatively, never resembled a fast twitch muscle; instead, overall, they resembled slow tonic muscles.

**DYSTROPHIC CROSS REINNERVATED MUSCLES**

Before discussing the cross-reinnervated ALD muscle of dystrophic birds, it is important to compare the distinct developmental patterns characteristic of fast twitch and slow tonic muscles from the viewpoint of how they might affect cross-reinnervation studies. Such a distinction is important since the rationale underlying cross-reinnervation experiments performed neonatally is to investigate the ability of a foreign nerve to alter phenotypes of its new target to mimic those of its original partner. For the present experiments, the focus was an
alteration of slow tonic phenotypes to mimic those of fast twitch muscles.

Fast twitch muscles immediately posthatching exhibit fetal enzymic profiles of high oxidative and weak glycolytic enzymes; further differentiation to achieve the adult enzymic profile occurs during the *ex ovo* period (Cosmos, 1966; Cosmos and Butler, 1967). When the nerve of a slow tonic muscle cross-reinnervates a fast twitch muscle at hatching, it functions to arrest further differentiation of fibres which have yet to complete the maturation process both histochemically and physiologically (Mazliah, 1980). In sharp contrast to this experimental condition, is that noted with slow tonic muscles. The latter differentiate their adult metabolic profile of low glycolytic and high oxidative enzymic activity during the last week of embryogenesis (Cosmos and Butler, 1967; Cosmos et al., 1979); at hatching, when the cross-reinnervation is performed, the slow tonic muscle has already established adult characteristics. The role of a foreign 'fast' nerve coupled to a slow tonic muscle would be one of forcing an 'adult' muscle to differentiate characteristics never experienced previously. Unlike the arrest in differentiation associated with cross-reinnervation of a fast twitch muscle, the experimental slow tonic muscle would be challenged to differentiate new phenotypes. Under the latter conditions, one should observe a complete switch in the enzymic profile from that characteristic of tonic muscles, i.e., weak glycolytic and strong oxidative, to that characteristic of twitch muscles, i.e., strong glycolytic and weak oxidative enzymic reactions. The myosin ATPase profile would show alkali stability only; acid stability would be lost. Myonuclei would
reposition themselves from a peripheral to an internal, scattered location and the rounded appearance of individual fibres loosely-packed in similarly shaped fascicles would be replaced by elongated fibres within tightly-packed, elongated fascicles. Interfascicular lipid, normally characteristic of ALD muscles, would disappear. In other words, this alteration would constitute a complete remodelling of the muscle.

As noted previously, such a restructuring of the ALD muscle in normal birds did not occur. Instead, the experimental muscles in these birds demonstrated all characteristics of an ALD unoperated muscle with the exception of the presence of isolated groups of fibres exhibiting an acid labile myosin ATPase reaction. Therefore, we can conclude that in normal ALD muscle coupled to a 'fast' nerve, the response was mainly a resistance to conversion.

Whereas these data support the observation that the ALD muscle of normal genotype is resistant to change, data for the experimental ALD muscle of dystrophic genotype support the conclusion that this muscle is almost completely remodelled both structurally and histochemically. This was an unexpected observation since normally the dystrophic ALD muscle demonstrates phenotypes characteristic of the normal ALD muscle.

When the developmental pattern of ALD muscles of both genotypes was interrupted by the experimental procedure, acid labile fibres were observed in both as early as 2 weeks postoperatively. In fact, in a group of experiments done more recently, such fibres were observed as early as 7 days. Although a differential response to the operative procedure was identifiable in some preparations by 2 weeks, by 4 weeks
postoperatively, differences noted between the two types of experimental muscles were pronounced (Fig. 33). A quantification of acid labile fibres revealed that the experimental dystrophic muscle contained a large percentage of these fibres dispersed evenly throughout the cross-sectional area. Furthermore, topographically the structure of the experimental dystrophic muscle was altered: fascicles lost their rounded appearance and became tightly-packed with fibres; interfascicular lipid and connective tissue were virtually non-existent; but, despite these changes, the cross-sectional area of the muscle actually increased. The above alterations were maintained throughout the two-year postoperative period analyzed.

An examination of both normal and dystrophic experimental muscles at 2 weeks postoperatively revealed that the transposed superior brachialis nerve was not responsible for the initial appearance of acid labile fibres since these fibres were observed at a time when innervation was undetectable either histochemically or physiologically; however, the subsequent maintenance of acid labile fibres was neurally dependent [See Transposition Study].

Experiments designed to identify the origin of acid labile fibres revealed that these fibres represented regenerative phenomena associated with muscle repair in response to severe damage. Such damage was incurred during the operative procedure necessary to transpose the ALD muscles to the contralateral side (Moore et al., 1984). An ultrastructural analysis at varying periods from 6 hours to 7 days postoperatively verified the presence of extensive damage and indicated that the repair phenomena were initiated within 48 hours of the imposed
trauma. As noted earlier, the newly formed fibres characteristically synthesized an acid labile isoform of myosin and were completely independent of neuronal influences for their formation. Initially, experimental ALD muscles of both genotypes exhibited the presence of newly-regenerated fibres demonstrating an acid lability. During subsequent periods, however, the experimental dystrophic muscle not only maintained a high percentage of these fibres but also their formation appeared to be augmented leading to a hyperplasia of the entire muscle. Even in the experimental ALD muscles of normal birds which demonstrated the highest percent of acid labile fibres, hyperplasia was never observed.

It is unlikely that the extent of damage incurred during the initial operative procedure was greater in dystrophic preparations since both normal and dystrophic muscles were treated similarly. In fact, at 2 weeks postoperatively, the percentage of acid labile fibres ranged from 2 to 68% in the normal preparations; in the dystrophic preparations, the range was from 4 to 74%. It was primarily after 2 weeks that significant differences in the percentage of acid labile fibres were observed. This difference was, in part, due to a decrease in the percentage of acid labile fibres noted in the normal preparations. This was probably due to the inability of the superior brachialis nerve to form contacts with all of the newly-formed acid labile fibres: those that failed to become innervated subsequently degenerated. Evidence for this statement was noted with muscles which were transposed but not cross-reinnervated. In these aneural preparations, although acid labile fibres did appear initially, they subsequently degenerated in the
absence of innervation.

In contrast to the decrease noted in the experimental normal muscles was the increase in acid labile fibres observed in dystrophic muscles following their initial appearance at 2 weeks postoperatively. The relative increase in the number of acid labile fibres was not due to a loss of slow tonic fibres since it was shown earlier that ALD fibres persist for many weeks after denervation [See Denervation and Transposition studies]. Instead, the increase in number of acid labile fibres was probably due to their continued formation between 2 and 4 weeks. Since the number of acid labile fibres in the cross-reinnervated dystrophic muscles continued to increase with further development, this suggested that the regenerative process resulting from the initial damage continued throughout the postoperative period; as noted, a similar phenomenon was not observed in the normal preparations.

This interpretation is supported by an additional observation: the random distribution of acid labile fibres in the dystrophic preparations. As discussed previously, a biased distribution of acid labile fibres characterized the normal cross-reinnervated ALD muscles and these appeared clustered within the vicinity of the implanted nerve. In contrast, acid labile fibres were distributed throughout the cross section of 88% of the cross-reinnervated dystrophic muscles examined; indeed, many fascicles were completely filled with these fibres. Such a distribution pattern would be expected if acid labile fibres in the vicinity of the implanted nerve co-existed with the acid labile fibres which were continuously forming peripheral to the vicinity of the implanted nerve.
Two additional observations with the cross-reinnervated dystrophic ALD muscle are in support of an augmented proliferation of fibres: increased cross-sectional area or hyperplasia and the tightly-packed appearance of muscle fibres and fascicles. As a result of the latter, fascicles and fibres assumed elongated shapes and interfascicular lipid and connective tissue were virtually absent. The topographical appearance of the experimental dystrophic muscle was completely altered to resemble that of a normal fast twitch muscle: a highly-compact muscle with very little interfascicular or intercellular spacing.

An integral feature of the histopathology of dystrophic muscles is the ability of the afflicted muscles to regenerate after injury. Experimentally-induced regeneration of avian dystrophic muscles has been limited to fast twitch muscles and was first described by Cosmos and Butler (1972). In this study, minced pectoral muscles were transplanted between normal and dystrophic birds between 1 and 3 days ex ovo, i.e., the normal mince was placed into the dystrophic host whose excised muscle was in turn minced and placed into the normal host. Microscopic analyses at 2 weeks postoperatively indicated that the dystrophic and normal transplants showed large areas of regeneration. In a second series of experiments, these same investigators removed the right pectoral muscle in both normal and dystrophic chicks but did not replace it. Examination of the experimental chicks revealed that muscles grew back from muscle buds attached to the clavicle in both normal and dystrophic hosts. However, the regenerate in the dystrophic host was twice the size of its normal counterpart. In a third series of experiments, the pectoral muscle was removed, minced, and then returned
to its original position. When examined, the weight of the regenerated muscle in the dystrophic birds was 1.5 times greater than its unoperated contralateral muscle whereas the comparable normal regenerate never attained the size of its unoperated control muscle. These experiments, therefore, demonstrate that fast twitch dystrophic muscles have the capacity to regenerate excessively after an experimentally-induced injury.

Several other laboratories have compared the regenerative capacity of myogenic cells in cultures derived from dystrophic and normal fast twitch muscles. The first investigators to examine normal and dystrophic cells in culture were Herrmann et al. (1964). These investigators cultured 8 to 15 day old pectoralis muscles from both normal and dystrophic chickens and found that the dystrophic extracts grew better than their normal counterparts under many different medium conditions. Several years later, Askanas et al. (1971) established cultures of pectoral muscles obtained from 10 to 15 day old chick embryos and found that the dystrophic cultures contained a greater number of myofibres which appeared 'thicker' than those noted in normal cultures. They concluded that dystrophic muscles initially grow at a faster rate than do normal muscles in culture. Similar results were reported more recently by Johnson et al. (1983). In these experiments, cultures were established from 8 to 10 week old pectoral muscles extracted from normal and dystrophic chickens. When compared to normal cultures, the dystrophic preparations were found to have proliferated and differentiated at a more rapid rate prompting Johnson and co-workers to propose that satellite cells from dystrophic muscles may be in a state
of stimulated proliferation and differentiation in vitro and, perhaps, in vivo. Therefore, it has been consistently observed, both in vitro and in vivo, that dystrophic fast twitch muscles can regenerate after an experimentally-induced injury. The present experiments represent the first report of a similar phenomenon occurring in a slow tonic muscle of dystrophic genotype.

PRÉCIS

The major findings of the present experiments concern, first, fibre type conversion in the cross-reinnervated ALD muscles of both normal and dystrophic genotype and, second, the unique response of the ALD muscles of dystrophic birds to an experimental injury. Although the following comments concerning the issue of fibre type conversion refer primarily to analyses of cross-reinnervated ALD muscles of normal genotype, they are, in general, also applicable to the experimental muscles of dystrophic genotype. The disparity between the experimentally altered ALD muscles of normal and dystrophic genotype will be emphasized in the second issue.

Using the classical cross-reinnervation technique of Buller et al. (1960a,b), it has not been possible to alter the phenotypes of the ALD muscle (Feng et al., 1965; Hník et al., 1967; Bennett and Pettigrew, 1973). With this method, the experimental surgery is performed in situ; only the nerve of the ALD muscle is severed and a foreign nerve of a fast twitch muscle is then coupled to the denervated ALD muscle. In contrast, however, when both the nerve and muscle are severed and the
ALD muscle is transposed to the contralateral side, alterations of muscle phenotypes have been observed (Zelená and Jirmanová, 1973a,b,c; Bennett and Pettigrew, 1974b). In the latter, the degree of conversion reported varied and this variability was probably due to differences in both the sampling technique and the actual parameters used to assess conversion. For the present experiments, which entailed an examination of experimental muscles in over 100 experimental birds over a two year postoperative period, entire cross sections of experimental muscles were monitored using the myosin ATPase and silver cholinesterase histochemical reactions. Both were excellent markers to identify the presence of fast twitch fibre type characteristics, i.e., the loss of acid myosin ATPase stability instead of the dual stability of ALD type fibres and the presence of focal innervation instead of the multiple innervation of slow tonic fibres. Using these markers, it was evident that not only was conversion per se limited in degree in the normal ALD muscles, but that alterations were present before reinnervation had occurred. Indeed, analyses of a series of control experiments where the ALD muscle was denervated and stretched but not transposed to the contralateral side revealed extensive damage to myofibres within 24 hours postoperatively which was followed by the initiation of a regenerative phase within 48 hours. Regenerated muscle fibres examined at 1 week postoperatively demonstrated a myosin ATPase profile characteristic of fast twitch fibres, i.e., acid lability. Thus, we can conclude that although the cross-reinnervated ALD muscle was altered phenotypically, albeit to a limited degree, individual muscle fibres were not. Instead, the acid labile fibres observed actually represented newly-formed fibres
which had regenerated in response to the injury inflicted on the ALD muscle by the operative technique per se. It should be emphasized, however, that the normal ALD muscle tended to repair itself more rapidly than did the dystrophic ALD muscle and, in most cases, recaptured the phenotypes characteristic of the unoperated ALD muscle.

Previously, our laboratory demonstrated that the myosin ATPase profiles of the ALD and PLD muscles are established during the first week of embryogenesis and remain essentially unaltered during further development, even in an aneurogenic environment (Butler and Cosmos, 1981; Butler et al., 1982). These observations would imply that during regeneration ex ovo, myosatellite cells stimulated to reduplicate in response to injury (Konigsberg et al., 1975), should also eventually differentiate myosin ATPase profiles appropriate for the parent muscle. Indeed, the majority of fibres of the cross-reinnervated muscles of normal genotype did express dual myosin ATPase reactivity of unoperated ALD muscles, i.e., alkali and acid stability, as the postoperative period increased. Thus, this population of daughter satellite cells retained the capacity to develop similar to myogenic cells of the developing embryo (Matsuda et al., 1983). In contrast, a variable percentage of muscle fibres observed in cross-reinnervated ALD muscles of normal genotype remained as acid labile fibres. This particular class of muscle fibres may represent either satellite cells which did not retain the capacity to differentiate normally (arrested development) or cells which were delayed in forming and, thus, were innervated by neurons of fast nerves before the normal transition was completed. In favour of the latter interpretation is the observation that during
embryogenesis initially (4 to 4.5 days in ovo) all myogenic cells of the dorsal and ventral muscle masses of the wing bud are alkali stable but acid labile; the profile of dual stability is not expressed by the ALD muscle until approximately 2 days later (Butler and Cosmos, 1981).

What remains unresolved is the source of multiple innervation associated with the cross-reinnervated ALD fibres which demonstrated ALD phenotypes. The superior brachialis nerve is essentially a 'fast' nerve (Zelená and Jirmanová, 1973a); however, a 'slow' type component is also present, as verified by histochemical examination both in ovo (Butler et al., 1982) and ex ovo (Gandy, unpublished observations) of wing muscles normally innervated by this nerve. Either there is a sufficient number of slow motoneurons to allow selective reinnervation of the ALD muscle fibres or the ALD type of muscle fibres command multiple innervation from the 'fast' motoneurons. Studies are now in progress to test experimentally the slow tonic component of the superior brachialis nerve by coupling this nerve to the fast twitch PLD muscle.

Several investigators have indicated that the ALD muscle of dystrophic genotype is phenotypically normal (Linkhart and Wilson, 1975; Mazliah et al., 1976; Cosmos et al., 1979b; 1980; Barnard et al., 1982). The present experiments represent the first attempt to alter the developmental sequence of this muscle. It was reasoned that if the dystrophic slow tonic ALD muscle was challenged to alter its developmental pattern, perhaps it would express abnormal characteristics. Before assessing the response of the dystrophic ALD muscle, it was necessary to monitor the capabilities of a normal ALD muscle to respond to a cross-reinnervation experiment. Alterations unique to the ALD
muscle of dystrophic birds could be interpreted as the expression of dystrophic phenotypes. In other words, the design was to impose upon the ALD muscle of dystrophic genotype but normal phenotype, a similar event which occurs during development with the PLD muscle of dystrophic genotype and dystrophic phenotype. The results indicated that the experimental ALD muscle of dystrophic birds did not respond similarly to ALD muscles of normal birds. Not only did the former demonstrate a larger percentage of acid labile (fast twitch type) fibres but, also, large increases in muscle volumes 2 to 3 times the cross-sectional area of experimental normal muscles. These two phenomena are characteristic of fast twitch muscles of dystrophic birds when stimulated to regenerate after mincing (Cosmos and Butler, 1972) and represent a dystrophic phenotype now being expressed by the slow tonic ALD muscle in response to mechanical injury.

In all preparations examined, experimental and control, in addition to structural and histochemical analyses with the silver cholinesterase and myosin ATPase reactions, all muscles were tested physiologically in vivo and histochemically for the metabolic enzymes phosphorylase and succinic dehydrogenase and stained for the detection of lipid infiltration. The latter were performed since it has been demonstrated previously that these reactions are abnormal in dystrophic fast twitch muscles (Cosmos, 1966; Cosmos and Butler, 1967; as reviews, Cosmos et al., 1979b, 1980). Unfortunately, with the experimental muscles these proved to be inadequate markers to identify either normal or dystrophic phenotypes. In all cases, the metabolic patterns were altered from those observed with unoperated ALD muscles, i.e., phosphorylase activity
increased and succinic dehydrogenase activity decreased. The reactions, however, were variable and under no conditions did the cross-reinnervated ALD muscles of normal birds resemble that of fast twitch muscle. In the experimental muscles of the dystrophic bird, one should have expected a greater demonstration of enzymic responses similar to those of the dystrophic fast twitch muscles since the percentage of acid labile fibres was extremely high. Again, the enzymic patterns noted were too variable to permit conclusions to be drawn with confidence. Instead, the characteristic which could be associated with the presence of the dystrophic gene was the augmented regeneration phenomenon, an event initiated in response to mechanical injury.

What might explain the observations that the experimental ALD muscle of dystrophic birds exhibited regeneration and hyperplasia whereas dystrophic fast twitch muscle exhibits regeneration followed by degeneration, lipid infiltration, i.e. all phenotypes associated with the disease process (as a review, Cosmos et al., 1979b; 1980). One possible factor may be the physiological demands normally placed on a dystrophic fast twitch muscle, demands to which this muscle is not equipped metabolically to respond. It is conceivable that such a physiological stress results in the breakdown of muscle fibres. It is interesting that in experiments where the fast twitch pectoral muscle of dystrophic birds was tenotomized, a retardation of the dystrophic process was reported (Julian and Asmundson, 1963). Since the transposed ALD muscles in the present experiments were positioned on the dorsal surface of the intact, contralateral ALD muscle and sutured perpendicular to the triceps brachii muscles of the wing, the work demanded of
this muscle was not normal. Many of its movements could be predicted as passive ones in response to the contractions and relaxations of the muscle to which it was sutured. How this passive physiological function affects the development of regenerating cells is difficult to gauge. When a majority of the experimental muscles were stimulated indirectly, a large twitch response to a single stimulus was elicited, indicating that the implanted foreign nerve was viable and that the muscles could respond when driven through indirect stimulation. To predict the daily functional activity of the experimental muscles would be purely speculative; however, to predict the effect of activity on the expression of metabolic profiles is possible since this is known to be influenced by the function of muscle (Pette, 1984). Indeed, the possibility that the daily function of the experimental muscle during the postoperative period was erratic may account for the variable enzymic metabolic profiles noted in muscles of both genotypes and the absence of degeneration noted in that of dystrophic lineage.

The primary goal of these experiments was to monitor the response of the ALD muscles of dystrophic genotype but normal phenotype to surgical manipulation which would alter its normal developmental status. The results of this manipulation indicated that not only did this muscle not express phenotypes similar to those expressed by its normal counterpart but, in addition, it expressed the dystrophic phenotype of augmented regeneration resulting in hyperplasia. Consequently, unlike the cross-reinnervated normal ALD muscle which demonstrated a limited alteration, the experimental dystrophic muscle was completely remodelled. These observations can only be attributed to the presence of the
dystrophic gene and, thus, represent the first documentation that a phenotypically normal muscle of dystrophic genotype can be forced to express overtly dystrophic characteristics.
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