# ANEUROGENIC LIMB DEVELOPMENT AND MUSCLE DEGENERATION

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# ABSTRACT

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Out of a need for a quantitative study on aneurogenic limb development and muscle degeneration the present study was undertaken. Early tailbud (Harrison's stage 24) Amblystoma maculatum embryos had their entire presumptive nervous system excised. For controls, embryos of the same age were operated in an identical manner except that about a one mm segment of neural tube dorsal to the presumptive limb buds was left intact. Operated embryos were reared in Steinberg's solution at 18°C until processed for light microscopy and electron microscopy at selected ages. Plastic embedded, mid-stylopodium regions of limbs were sampled and the tissues histometrically measured with a TTMC particle counter. The data were statistically analysed.

The results indicated that the humerus was entirely stable to the absence of nerves whereas aneurogenic limb myofibers initially developed almost normally followed by degeneration and muscle disappearance. In 13 day aneurogenic and control limbs the muscle area (mostly myoblasts) per limb cross section was determined to be equal; there was then a two to five fold increase in muscle area in control limbs whereas the aneurogenic muscle area gradually decreased as the animal grew older. Myofiber differentiation was measured by counting the number of peripherally located nuclei per total number of muscle fiber nuclei. The number of peripheral nuclei reached a peak at 31 days after the operation in aneurogenic limbs. The number of peripheral nuclei in the controls also peaked at the same time but remained at that maximum. With the exception of day 13, the number of peripheral nuclei was always greater in control than in aneurogenic muscle.

Total number of muscle nuclei per limb cross section gradually decreased in aneurogenic muscle reaching a number significantly lower than in controls by 31 days after the operation and thereafter. The cross sectional size of aneurogenic muscle nuclei seemed to decrease at a faster rate than control nuclei. Reflecting nuclear density, the myonuclear area per arbitrary muscle area in aneurogenic limbs reached its lowest value by 21 days

after the operation. Beyond 21 days aneurogenic nuclear densities progressively increased whereas control densities remained at the low, day 21 value. From day 13 to day 21 the muscle nuclear density in control and aneurogenic limbs was statistically equal.

Ultrastructurally, control larvae were seen to be normally innervated by the "isolated spinal cord" and walking movements were observed in some animals. In aneurogenic animals nerves were never found in electron microscopic thin sections. Aneurogenic myofibers in an advanced state of degeneration incorporated more $^{3}$H-leucine or $^{3}$H-uridine than fibers where cross-striations were still visible with the light microscope. With the electron microscope acid phosphatase stained lysosomes were found adjacent to disintegrating myofibrillar material in degenerating aneurogenic myofibers. Ruthenium red staining indicated that myofiber basement laminae developed normally in aneurogenic muscle and persisted for some time. Ruthenium red staining assured the identification of muscle satellite cells which were routinely detected in aneurogenic, control and normal muscle. Muscle tendons persisted in aneurogenic limbs although atrophic in appearance.

# ANEUROGENIC LIMB DEVELOPMENT AND MUSCLE DEGENERATION

By

Heinz Popiela

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DEDICATION

In memory of the late Dr. Charles Stead Thornton who, unfortunately, did not witness the completion of this work.

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# INTRODUCTION

The idea of studying body parts separated from the presence of the nervous system has been pursued for a long time (Zelená, 1962, review). Harrison in 1904 concluded that the axial muscle develops "perfectly" normally in the aneurogenic region of a frog embryo. His experimental strategy was to cut .... "a narrow strip extending from the region of the pronephros to the tip of the tail" off the dorsal side of early tailbud embryos just above the notochord, thus removing the developing spinal cord. Six to seven days later he examined the larval myotome in section. Harrison wrote that the operated embryos developed at a slower rate than normal ones and that the larvae are often edematous. Operated larvae remained motionless even if "strongly" stimulated but the muscle contracted locally if stimulated electrically. The spinal cord was, of course, absent but he did observe longitudinal nerve bundles to pass posteriorly into the chordless tissues. The arrangement of myofibers was normal, however he often saw large clear spaces separating the fibers. Besides clear spaces, he also found vacuoles along the axes of myofibers which he attributed to "unfavorable accidents of the operation" instead of to the nerveless condition. In a brief account he mentioned having instructed a Mr. Langnecker to make longitudinal cuts between presumptive limb buds and the

notochord in order to separate the buds from their nerve source. However, he experienced difficulties in keeping the embryos alive until metamorphosis. Of all operated embryos only one specimen survived. In this specimen the hindlimb had an atrophic appearance. The hindlimb cartilage, bone and muscle were observed to have differentiated normally but myofiber diameters were smaller than in muscle of normal forelimbs.

A detailed study on aneurogenic or sparsely innervated frog hindlimb development was done by Hamburger (1928). He removed the right posterior segment of the neural folds in frog embryos in which the neural folds had just closed. The purpose of the experiments was the study of nerveless hindlimb development. In another series, he inserted a piece of mica longitudinally between the hindlimb bud and the neural tube in tailbud embryos in order to isolate the bud from its nerve source. In experiments in which the neural fold was removed he experienced difficulties in obtaining a nerveless condition since the neural tube regenerated in 70% of the cases, and in mica implantations nerves often grew around the slit created by the mica. In the few successful cases he noted that the right hindlimbs were paralysed, markedly atrophic but perfectly normal in appearance although miniatures of normal limbs. Morphological development from limb bud to digit formation occurred normally. However, at the time when the knee was visible and the hindlimb in the early five digit stage, growth was gradually retarded

although external morphology (Formbildung) occurred synchronously with the control side. As the animals grew, the leg muscles on the operated side became progressively narrower but showed great variation from animal to animal. In some cases only one muscle remained behind in their growth in thickness and in more severe cases the legs were nothing but "skin and bones" at the start of metamorphosis. In these severe cases the leg skeletal system, although smaller, was entirely normal except for a couple of cases in one of which a few distal tarsals were fused and in the other case the head of the femur was grown to the hipjoint. All other limb tissues such as skin, skin glands and blood vessels developed normally. In most cases Hamburger found thin nerves either coming from the other side or from remaining nerves 11 and 12. Hamburger found the musculature to be the most sensitive component to the absence of nerves. He did find normal, cross-striated fibers but the number of fibers and their diameter were smaller. Fibers were often separated by clear spaces, had a wavy appearance and their nuclei were distributed randomly in uneven distances from one another. Many fibers had degenerated the signs of which were dissolution of cross-striations, bloated fibers and thicker nuclei. More advanced signs of degeneration were the wax-like appearance of fibers in cross section (hyaline degeneration) and disintegration of fibers where the "... contents of fibers has fallen apart in large clumps, the sarcolemmal tubes are torn or

missing."* Sometimes fibers were normal looking but ended in thick, stained clumps. In one case, he fixed a younger animal in which the musculature had not completely differentiated. The muscle was largely "im Stadium der Myoblasten" and only few cross-striations existed. In this particular case he found no signs of degeneration whatsoever. As to the causes of muscle degeneration Hamburger mentioned that he could not tell whether interference with the nervous system, nutrition or lack of function led to the poor condition of muscle.

Another cited investigator, Wintrebert (1903), repeatedly denervated the hindlimbs of 50 frog larvae in the early hindlimb digit formation stage and observed the continued gross development of the limbs. However, he did not examine the operated animals for the presence of nerves nor did he check on the histological condition of tissues. According to Wintrebert, the hindlimbs developed normally in general form and proportion and with such meagre data to go by he wrote: "Nous pouvons donc conclure que le système nerveux n'est pas nécessaire dans la génération du membre, ni pour sa croissance, ni pour sa morphogénie generale, ni pour sa differentiation." In a later experiment (Wintrebert, 1905) he excised a piece of the neural tube from frog embryos at the closed neural fold stage and noted that larvae developed all right but were paralysed.

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* This author's translation.

Again the observations made were superficial and contained no histological data.

Hooker (1911), a student of Harrison, examined the development of aneurogenic voluntary and cardiac muscle in frog embryos by removing the presumptive spinal cord, brain, cranial ganglia, and head skin from closed neural fold stage embryos. He observed that the myotome developed normally and contracted upon electrical stimulation or by pricking with a needle between 1 to 3 days after the operation; spontaneous movement he never detected. The irritability in a majority of animals lasted only for 1 or 2 days after the operation and no reaction was elicited after the third day.

Other techniques of isolating organs or tissues from the influence of the nervous system included transplantation of somites to the chorio-allantoic membrane of chicks (Hoadley, 1925), transplantation of limb buds to the chorio-allantoic membrane of chicks (Hunt, 1932), transplantation of chick limb buds to the coelom (Eastlick, 1943), and transplantation of somite to ventrolateral positions in Amblystoma embryos (Muchmore, 1968). In all of these cases early myofibers developed normally at first followed by atrophy and degeneration. In the chick embryo, degeneration of muscle manifested itself by fat replacement. More modern techniques employed to study the influence of nerves on muscle tissue include tissue culture methods (Shimada et al., 1967), explant culture

methods (Peterson and Crain, 1970; Lentz, 1971, 1972; Askanas et al., 1972; Crain and Peterson, 1974) and minced muscle implants (Hsu, 1974; Carlson, 1973, review). These studies also indicate that muscle does not need nerve until the late myotube to early myofiber stage after which times it will not differentiate (Askanas et al., 1972). Nerves are absolutely required for the continued maintenance of muscle tissue. Emphasizing the importance of nerve for muscle development and maintenance it was shown that the degenerative process of nerveless muscle explants in culture is reversed when pieces of embryonic spinal cord are presented and neuromuscular connections are established (Peterson and Crain, 1970; Crain and Peterson, 1974).

By far the most extensive literature exists on <u>in vivo</u> denervated adult muscle (Gutmann and Zelená, 1962; Guth, 1968, review). The term, denervated, denotes a tissue which has had received innervation during ontogeny but in which the nerve supply was destroyed. One may add that the observed processes of muscle degeneration in denervated muscle are quite comparable to the processes of degeneration in aneurogenic muscle, a muscle which had never been innervated during ontogeny (Tweedle et al., 1974). If adult cat forelimb muscle is kept denervated for 2 months one sees a 40% to 50% loss of muscle weight. By 2 months after denervation myofibers are reduced in diameter by 1/3 to 1/2, by 4 months the muscle appears vacuolated and granular

and by one year muscle fibers are "reduced to the status of fibrous tissue" (Tower, 1935). In the denervated rat leg muscle Pellegrino and Franzini (1963) described two major, overlapping phases of muscle atrophy. In the first phase they recognize a degenerative autolysis which manifests itself by a loss of striations and an almost 50% weight loss. In the second phase, myofibrils are reduced in diameter, lose their parallel arrangement and break down within the interfibrillar space. The Z-lines become irregular and the number of nuclei seem more numerous and are centrally located. Possibly, the increased number of nuclei is an illusion due to the disappearance of contractile material since Cardasis (1975) found no change in the number of nuclei per isolated myofiber following denervation. Surprisingly, the sarcoplasmic reticulum is still well preserved and relatively overdeveloped. Mitochondria disappear in parallel with the contractile material. The reduction in cross sectional fiber area and myofibrillar area per fiber was confirmed quantitatively in the adult rat (Engel and Stonnington, 1974). Furthermore, Engel and Stonnington observed a transient increase in mitochondrial area and total sarcotubular surface followed by a decrease. In fact, Muscatello et al. (1965) believe that an increase in sarcoplasmic reticulum and number of mitochondria in response to denervation reflects change to a new metabolic pattern for the survival of cells and muscle repair. The number of sarcomeres is

reduced by 35% in denervated muscle (Goldspink et al., 1974). Schiaffino and Settembrini (1970) in the neonatally denervated rat leg also noted hypertrophy of the sarcotubular system and concluded that the continued differentiation of the sarcotubular system is not impaired by neonatal denervation. Muscle spindles never differentiate in rat muscle denervated during the myotube stage (Zelená, 1957) the cause of which is the absence of sensory innervation (Zelená and Soukup, 1974). In rat leg muscle, denervation 3 days before birth was found to result in unaffected growth and development initially, but the differential Z-band pattern of white and red muscle never developed in extensor digitorum longus and soleus fibers (Hanzlíková and Schiaffino, 1973). Furthermore, neonatally denervated rat muscle developed histochemically identifiable type I fibers but not type II fibers (Engel and Karpati, 1968). Apparently, fiber types do not differentiate in the absence of nerves (Shafiq et al., 1972a) and mature fibers degenerate upon denervation in a pattern characteristic of their type (Tomanek and Lund, 1973).

Physiologically, denervated muscle shows extrajunctional sensitivity to acetylcholine but by 24 hours miniature endplate potentials are no longer detected (Albuquerque and Mc Isaac, 1970). As to the mechanism of contractile protein removal lysosomes have been detected among myofibrils in dystrophic muscle (Shafiq et al.,

1972b; Milhorat et al., 1966), acid hydrolases (Pollack and Bird, 1968) and lysozyme (Krishnamoorthy, 1972) have been seen to increase in denervated muscle. However, Schiaffino and Hanzlíková (1972) conclude that myofibril disintegration in denervated muscle is not a result of lysosomal activation but is initiated by an extralysosomal mechanism. In corroboration, Kohn (1964, 1966) does not find enzymes capable of degrading myosin in denervated muscle.

The denervated rat diaphragm hypertrophies before it shows the usual symtoms of atrophy (Sola and Martin, 1953). The parameters are an initial increase in wet weight, amino acid incorporation, protein synthesis and DNA synthesis 3 to 6 days after denervation followed by a decrease below control levels at 12 days after denervation (Zak et al., 1969; Turner and Manchester, 1973). Ultrastructurally the number of actin and myosin filaments increase during the hypertrophic period followed by the usual signs of atrophy (Miledi and Slater, 1969). The insulin accelerated uptake of $^{14}$C-leucine or aminoisobutyric acid (AIB) into control muscle did not occur in denervated hemidiaphragms (Buse et al., 1965; Manchester, 1974). When ribosomal activity was studied <u>in vitro</u>, it was found that the number of nascent peptide chains and the specific activity in terms of amino acid incorporation did not differ in denervated and control diaphragms; however, the ribosomal yield increased by 80% in diaphragms 3 days after denervation

(Manchester, 1974). The increase in ribosomal number was ultrastructurally confirmed by Manolov and Ovtscharoff (1974).

Insect musculature, in addition to the usual signs of muscle degeneration upon denervation (Teutsch-Felber, 1970; Rees and Usherwood, 1972) has the unusual feature of containing normally degenerating muscle during development (Nüesch, 1968, review). In the bug Rhodnius, the intersegmental muscle repeatedly breaks down after each moulting cycle (Wigglesworth, 1956) although normal neuromuscular junctions are maintained during the breakdown process (Anwyl and Finlayson, 1973). In the cricket the axon terminals looked normal even after the contractile system had disappeared (Gutmann et al., 1974); and the normal degeneration of muscle is retarded upon injection of ecdysterone but no effect is observed in denervated muscle (Srihari et al., 1975). In the moth lysosomes appear in normally degenerating muscle (Lockshin and Beaulaton, 1974a,b), but myoprotein is thought not to be digested within lysosomal membranes. However, muscle proteins do appear in the hemolymph (Lockshin, 1975). The control over insect muscle degeneration is hypothesised to occur via combined hormonal and nervous means (Srihari, 1974). The development of denervated cricket thorax muscle was quantitatively studied by Thommen (1974); the results indicate that growth is interfered with but not stopped. Growth in fiber thickness was found to be the most sensitive aspect to

denervation. The number of muscle nuclei were calculated to be more than 100% over normal. Thommen explained, that because of denervation growth in thickness is interfered with much more strongly than nuclear proliferation thereby creating a higher nuclear number per fiber volume.

In Amblystoma larvae the development and subsequent degeneration of limb musculature in aneurogenic or sparsely innervated larvae were studied with the electron microscope by Tweedle et al. (1974). The results indicate that early development and differentiation of this muscle occurs in a comparable manner to normal larvae. Muscle differentiated to an early myofiber stage but soon atrophied in a characteristic pattern similar to the one described above.

The present study was undertaken out of a need for a quantitative description of aneurogenic limb development and muscle degeneration. The results showed, in addition, that the aneurogenic larvae in this study indeed had no nerves eliminating doubts about "sparsely innervated" conditions (Egar et al., 1973). More over, further details of ultrastructural and cytochemical characteristics of deterioration in aneurogenic muscle are presented.

## MATERIAL AND METHODS

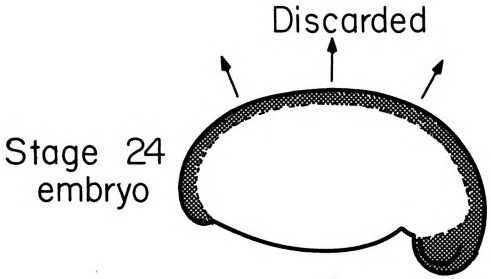
1. Surgical procedures.

Amblystoma maculatum embryos, obtained from Tennessee, were allowed to develop at 18°C. At Harrison's stage 24 the embryos had their entire presumptive nervous system removed, down to the notochord, by excision with modified watchmaker's forceps. Any remaining presumptive nerve cells could be easily detected due to their different pigmentation and were therefore removed with a Spemann hairloop. The operative procedure is schematically shown in Figure 1. The stippled region indicates the level of excision of presumptive neural tissue. Figure 2 schematically shows the operation performed to produce control animals. Here, the excision of presumptive neural tissue paralleled that of the aneurogenic embryos except that a segment of neural tissue about 1 mm long dorsal to the limb bud anlagen was left intact. Control larvae had to be produced experimentally since normal, unfed and freely walking and swimming larvae deplete their yolk supply at a faster rate than aneurogenics, develop only three digit forelimbs, and died at an earlier time than aneurogenics. Stage 24 embryos were chosen because they permitted easy removal of the neural tube and assured removal of the neural crest cells before their beginning migration (Detwiler, 1937).

Figure 1

Surgical procedure to produce aneurogenic larvae. Harrison's stage 24 embryos had their presumptive neural tissue, including the neural crest, excised (stippled region). Operated embryos were allowed to develop immersed in Steinberg's saline. Photograph (a) shows a 38 days (Stage 24 + 38 days) old aneurogenic larva. Arrow in (a) indicates mid-stylopodium region of forelimb where cross section samples were taken for measurement.
Photograph (a) 15X

Fig. 1

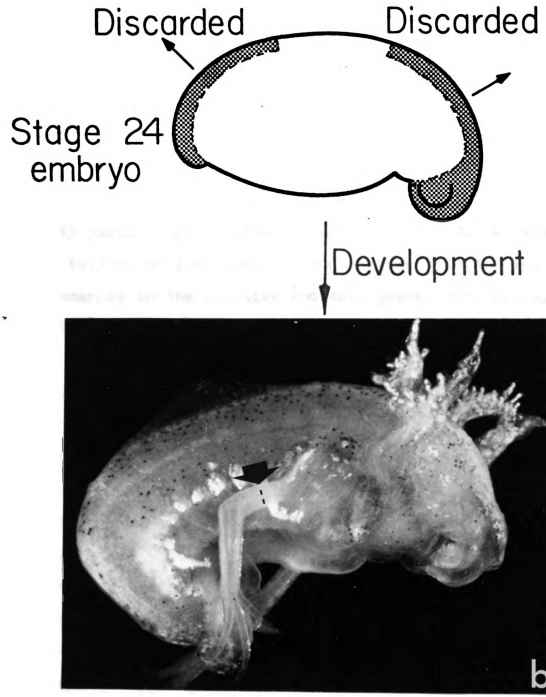



Aneurogenic Animal (38 days)
Four digit forelimbs

Figure 2

Surgical procedure to produce control larvae. Harrison's stage 24 embryos had their presumptive neural tissue excised (stippled region) except for an about 1 mm segment dorsal to the limb bud anlagen. Operated embryos were allowed to develop immersed in Steinberg's saline. Photograph (b) shows a 38 days old (Stage 24 + 38 days) control larva. Arrow in (b) indicates mid-stylopodium region of forelimb where cross section samples were taken for measurement.
Photograph (b) 15X

# Fig. 2




Control Animal (38 days)
Four digit forelimbs

After the operation, embryos were placed in previously aerated sterile Steinberg's solution containing 0.25 g of Na-sulfadiazene, 0.2 mg of penicillin and 0.5 mg of streptomycin per liter. Operated embryos were allowed to develop at 18°C in an incubator. Clearly, aneurogenic and control animals had to rely on their stored yolk for food; they were unable to feed even when mature. Normal animals were fed brine shrimp, Daphnia, and immature snails.

2. Tissue processing.

At appropriate times whole animals were killed in ice cold, 2% purified glutaraldehyde solution in a 0.1 M cacodylate buffer, pH 7.4. Limbs were dissected with the animals immersed in the fixative and then placed into fresh, ice cold fixative solution for 1 to 2 hours. Entire limbs were postfixed in ice cold 2% osmium tetroxide in a 0.1 M cacodylate buffer, pH 7.4, containing 0.15 M sucrose for 3 hours. After fixation, tissues were washed three times in cacodylate buffer containing 0.2 M sucrose. Next, the tissues were dehydrated in ethanol, treated with 1% p-phenylenediamine in 70% ethanol (Ledingham and Simpson, 1972) for 10 minutes and stained <u>en bloc</u> with 2% uranyl acetate in 100% ethanol. Following dehydration and prestaining, the tissues were infiltrated with Spurr's plastic (Spurr, 1969). For light microscopy, micrometer thick sections were cut on a LKB or Porter Blum MT2 ultramicrotome, mounted on microscope slides, and stained with 1% azure B containing 1% sodium borate. For electron

microscopy, grey sections were cut with the same instruments, placed on copper grids and stained with lead citrate for about 20 minutes. Grids were viewed with a Hitachi 11E or a Zeiss 9A electron microscope.

3. Ruthenium red staining (myofiber basement laminae).

For ruthenium red staining, the method of Luft (1971a, b) was employed except that ruthenium red was not added to the glutaraldehyde fixative solution. Prior to ruthenium red - osmium tetroxide fixation, the limb skin was peeled from fixed limbs since the skin blocks penetration of ruthenium red. The dehydration and infiltration procedure was followed as described above.

4. Acid phosphatase staining (lysosomes).

Glutaraldehyde fixed limbs denuded of their skin were incubated for 15 minutes according to the method of Novikoff et al. (1971). For control incubations, 10 mmol sodium fluoride was added to the incubation mixture or the substrate was omitted. Sodium fluoride was shown quantitative-histochemically (Pfeifer and Witschel, 1972) and biochemically (Boyer et al., 1961, 1971) to be an extremely potent (98%) inhibitor of acid phosphatase.

5. Autoradiography.

Aneurogenic animals, whose forelimbs were in the early four digit stage of development, were treated with 0.02 mCi of tritiated uridine or 0.001 mCi of tritiated leucine in a volume of 0.02 ml. The tritiated precursors were injected intraperitoneally with a

Hamilton syringe to which a glass micropipette was mounted with paraffin to the syringe needle. After 3 hours of incorporation the animals were killed while immersed in the fixative and processed for light microscopy and electron microscopy as described above. Stained sections were dipped in Ilford K-5 emulsion diluted 3:1 with distilled water. After a 3 week exposure time the stained slides were photographically processed.

6. Quantitative procedure.

Plastic embedded limbs were marked at the proximal-distal center of the upper forelimb and several 1 um cross sections per slide were mounted and stained as described above. Before the measurement, all slides with 13 to 65 day aneurogenic and control as well as 43 day and 65 day normal section samples were shuffled for randomization. Cross sectional limb tissue areas were measured with a TTMC particle measurement computer system (Millipore Corp.) which consisted of a light microscope with an attached television scanner, and a small electronic computer. Whole limb areas and solid tissue areas (whole limb minus clear spaces) were measured at 10X; muscle and humerus areas were measured at 40X. For myonuclear area measurements a region of a muscle bundle for each limb was selected. Measurements were performed under oil (100X) and the electronically selected muscle frame was placed at random near the periphery of a muscle bundle (C, Table 13). The same muscle bundle was chosen

for each limb. The total number of myonuclei per limb and the peripheral or central location of myonuclei within a myofiber were determined with a tally. Lengths of myonuclei were measured with an ocular micrometer.

7. Statistics.

For statistical calculations program BMD07M, stepwise discriminant analysis, and program BMD01D, single data description, from the Health Sciences Computing Facility, University of California (Dixon, 1973) were used. In addition, t-tests were done on a programmable Olivetti calculator.

# RESULTS

In contrast to previous studies (Thornton and Steen, 1962; Egar et al., 1973; Tweedle et al., 1974), the methodology employed in this investigation produced true aneurogenic limbs; i.e. nerves were never (even with the electron microscope) seen in these aneurogenic creatures. The control animals, on the other hand, developed nerve strands; and typical neuromuscular junctions were detected (Plate 1, Arrows). Furthermore, several mature control animals were seen to use their forelimbs in walking movements. The development of aneurogenic and control forelimbs occurred at exactly the same rate (Tweedle, personal communication).

Table 1 (Appendix) shows the cross sectional areas, in squared micrometers, of the various aneurogenic, control and normal limb tissues and Table 2, which was derived from Table 1, shows the average limb tissue areas including their standard errors. A statistical time series analysis (Table 3, 4, 5) by computer revealed that during the 13 to 65 day time span (Table 3) the whole aneurogenic limb underwent the greatest area change; less but still statistically significant change is seen in muscle and in the number of muscle nuclei during this time. The least change in time is seen in the solid tissue area (all stained tissues exclusive unstained clear

spaces). In fact, the change is statistically insignificant even at $P<0.05$. That the solid tissue area does indeed show an insignificant change during the 13 to 65 day period is graphically obvious in Figure 4. In comparing the Y-axis positions of the 13 day to the 65 day plot, one appreciates the negligible distance between the two points on the Y-axis. The control whole limb tissue area also shows the greatest change during the 13 to 65 day time period immediately followed by muscle. However, the change in total number of muscle nuclei per limb cross section is barely significant at $P<0.01$, and the change in time of solid tissue and humerus area is not significant at all at the 5% and 1% level of significance.

Table 4 shows a time series statistical analysis of the various aneurogenic limb tissues added sequentially. For example, aneurogenic muscle (M) alone shows no statistically significant change (at $P<0.01$) during 13 to 43 days except for the day 21 to day 31 time period. The least change occurs during 54 to 65 days followed by 31 to 43 days. The greatest change occurs in aneurogenic muscle during the day 21 to day 54 time period. If two aneurogenic variables, muscle area and limb area, are considered together (Table 4, M+L) no statistically significant change is apparent during the day 13 to day 43 time period. However, from

day 21 to day 31 the change in muscle plus limb area is statistically significant. The greatest change in the muscle plus limb area is seen during the day 54 to day 65 time period. The bottom horizontal segment of Table 4 considers all five variables together (M+L+S+H +N), i.e. whole limb (L), solid tissue (S), humerus (H), muscle area (M) and number of muscle nuclei (N). This entire aneurogenic limb system, defined by the five variables, shows the greatest single time period (54 to 65 days) change during 54 to 65 days. The greatest overall change occurs from 21 to 54 days and statistically insignificant change at the 1% level of significance is seen during the 13 to 31 and 31 to 43 day time periods.

Table 5 shows the same statistical time analysis for control limb tissues. Here, the muscle alone changes the most during 13 to 31 days and least during 43 to 54 days. Considering the entire control limb system, one finds the greatest area change to occur during 13 to 21 days and statistically insignificant changes at $P<0.01$ are seen during the day 21 to day 43 time span.

The gross, cross sectional limb areas for aneurogenic, control and normal animals presented in Table 2 are graphically shown in Figure 3. At 13 days there is no statistical difference between aneurogenic and control means (Table 6, Figure 3). In fact, the two

means are statistically equal even at the 5% level of significance. Beginning with 21 days after the operation aneurogenic and control limb areas are different; the aneurogenic limbs remain smaller in cross sectional area beyond 13 days in comparison to control limbs. Both kinds of limbs, however, seem to increase in area from day 13 to day 21 and then gradually decrease in size except for aneurogenic limbs which drastically increase in cross sectional area from day 54 to day 65. The control solid limb tissue, measured as the cross sectional limb area minus the clear spaces, shows a gradual increase in area from 13 days to 31 days (Figure 4, Table 1, 2) followed by a decline. Aneurogenic solid areas seem to diminish in time from day 13 except for the period of days 54 to 65 where the solid areas seem to return to day 13 levels. The control and aneurogenic humeri (Table 1, 2, Figure 5) gradually increase in cross sectional area in time reaching a maximum at 43 days. Statistical comparison of aneurogenic with control humeri (Table 6) indicates no difference between means at the 5% level of significance throughout the entire time period. Another structure exhibiting a remarkable stability inspite of the absence of nerves is the muscle tendon. With each limb cross section a single tendon is seen (Plate 3). Even 65 day old aneurogenic limbs contain one clearly visible tendon which becomes more obvious since all muscle had virtually

disappeared. Plate 4 shows an electronmicrograph of a tendon from a 65 day aneurogenic forelimb. For comparison, a tendon from a normal forelimb is shown in Plate 5 and further magnified in Plate 5a. However, one does notice that the aneurogenic tendon is somewhat reduced in diameter and that the amount of collagen, which fills the intercellular spaces, is reduced.

The greatest change within the limb area occurs in the muscle (Plate 2, 3, Figure 6, Table 3, 4, 5). In fact, two aneurogenic animals have no light microscopically detectable muscle whatsoever at 54 and 65 days respectively (Table 1). However, small remnants of myofibers could always be found with the electron microscope (Plate 6). The aneurogenic limb musculature at day 13 is not significantly different from controls (Table 6). At this stage of development the upper arm limb muscle is in the early myotube stage. But beginning with day 21, Figure 6 and Table 6 show a statistically significant separation between control and aneurogenic muscle. The control muscle reaches a plateau at the four digit stage (31 days) of development and decreases in area from 54 to 65 days (Figure 6). Aneurogenic muscle seems to increase from 13 to 21 days, then gradually diminishes with time. The great difference between aneurogenics and controls in cross sectional limb muscle area is readily apparent in Plates 2 and 3. Furthermore,

Plates 2b, 3b and 3c show large nerve bundles, whereas the aneurogenic limbs do not have any nerves whatsoever (Plates 2a, 3a). Far from being metabolically inactive, degenerating aneurogenic limb muscle readily incorporates tritiated leucine (Plate 7) or tritiated uridine. In fact, by visual inspection one notices in autoradiographic preparations the presence of more disintegration silver grains over partially degenerated muscle than over muscle where cross striations are still visible (Plate 7, arrows). The number of aneurogenic peripheral myonuclei, as a measure of myofiber differentiation, reaches its peak at the four digit stage in the upper arm (Tables 7, 8, Figure 7). In the control larvae, peripheral nuclear numbers plateau at the four digit stage. The plateau is statistically sound taking into consideration that at the 5% level of significance the control 31 and 43 day means are equal whereas for aneurogenics they are not (Figure 7, Table 8). At 13 days after the operation there is no statistical difference between aneurogenic and control groups at 1% or 5% level of significance (Table 8). However, at 21, 31 and 43 days nuclear numbers are statistically quite different between aneurogenic and control larvae.

In addition to the disappearance of muscle, the total number of muscle nuclei (myonuclei and satellite cell nuclei) declines also with time in control and

aneurogenic limb muscle (Figure 8, Tables 1, 2). Initially, at days 13 and 21 one does not detect a statistical difference between aneurogenic and control mean number of muscle nuclei, but from day 31 on a separation between controls and aneurogenics at the 0.1% level of significance becomes established. Also, beginning with day 31 the total number of muscle nuclei per limb section in aneurogenic limbs diminishes from less than two thirds to as much as one fourth as compared to control limbs. The cross sectional area of muscle nuclei also diminishes with time (Table 9 - Appendix, Table 10, Figure 9). However, only on days 43 and 54 does one see any statistical difference between aneurogenic and control groups. From day 13 to day 31 and on day 65 one finds no statistical difference between the two groups. The length of aneurogenic and control myonuclei at 43 days of development is presented in Table 11 (Appendix) and Table 12. Analysis of variance (Table 12) indicates that all myonuclei are statistically equal at the 1% level of significance. The control sum nuclear area per selected muscle area (Table 13) reaches a level minimum by 21 days (Figure 10), whereas the aneurogenic muscle attains a peak at 54 days. This peak sum nuclear area is approximately at the same level as in day 13 muscle. At day 13 and 21 no statistical difference between aneurogenic and control sum nuclear area is found (Table

14). However, from day 31 on the difference between aneurogenics and controls is significant at the 1% and 5% level of significance.

In a previous study (Tweedle et al., 1974) it was suggested that macrophages may engulf degenerating aneurogenic muscle fragments thereby removing waste sarcoplasmic material. In corroboration Plate 8 is presented showing a macrophage in the process of engulfing a muscle fragment (Arrows). Plate 9 shows an acid phosphatase (Acpase) preparation depicting a macrophage with several digestion vacuoles and lysosomes. Employing the Acpase technique several lysosomes were found next to myofibrillar material within degenerating aneurogenic muscle. Plate 10 shows a sample electron-micrograph of such an Acpase preparation. Here, a lysosome is situated adjacent to some myofibrillar material. Phagosomes, similar to the ones seen in Plate 9 were never found in aneurogenic myofibers or remnants of myofibers. Also, developing aneurogenic muscle or control muscle never exhibited Acpase stained lysosomes. In ruthenium red stained muscle it was found that aneurogenic myofibers developed basement laminae similar in appearance as in normal animals. Plate 11 shows basement laminae of normal muscle and Plate 12 depicts a portion of a 21 day aneurogenic myofiber with its ruthenium red stained basement lamina. Ruthenium red

stain was also effective in the identification of muscle satellite cells. Plate 13 is an example of the numerous satellite cells seen in normal and aneurogenic Amblystoma muscle. In addition to limb musculature, the axial musculature also disappears in time although the degeneration of axial muscle was not specifically studied here.

## Table 2

## AVERAGE LIMB TISSUE AREAS

| | | Means ± standard error | | | | | |
|---|---|---|---|---|---|---|---|
| Age of animals | Number of limbs | Whole limb area ($um^2$) | Solid tissue area ($um^2$) | Humerus area ($um^2$) | Total muscle area ($um^2$) | Total number of myonuclei | |
| 43 | 5 | 174792±7495 | 123103±8758 | 15457±1861 | 30577±2603 | 132.8±3.6 | Norm. |
| 65 | 5 | 232956±28940 | 174894±25300 | 14903±1795 | 59389±10081 | 175.2±29.8 | |
| 13 | 5 | 81116±2034 | 63094±2804 | 10144±321 | 6715±836 | 47.2±4.7 | Anurogenic |
| 21 | 5 | 99910±8238 | 57072±3956 | 12187±634 | 8154±1184 | 47.8±1.7 | |
| 31 | 5 | 79420±4686 | 47268±7252 | 13257±786 | 4329±387 | 40.2±3.2 | |
| 43 | 5 | 84789±10291 | 54018±4515 | 16666±897 | 5180±883 | 38.6±3.3 | |
| 54 | 5 | 62350±7085 | 37662±3941 | 15733±1064 | 621±173 | 18.2±5.4 | |
| 65 | 5 | 150905±4662 | 62192±13042 | 15399±1489 | 717±207 | 11.4±3.8 | |
| 13 | 5 | 83670±4161 | 65843±4954 | 11774±671 | 8356±752 | 71.4±9.7 | Control |
| 21 | 5 | 131886±4512 | 79580±5867 | 14156±1023 | 16854±1703 | 62.6±6.7 | |
| 31 | 6 | 116345±3668 | 81875±3078 | 12897±1095 | 21383±2168 | 66.2±4.3 | |
| 43 | 5 | 118278±11143 | 72061±9690 | 15208±1297 | 18363±969 | 69.6±3.3 | |
| 54 | 5 | 100222±3823 | 70112±7731 | 14495±1187 | 18662±5052 | 53.2±4.2 | |
| 65 | 5 | 102702±5130 | 61113±6550 | 14490±834 | 9717±849 | 44.8±4.1 | |

Table 3

STATISTICS FOR TIME SERIES

| | | 13 day old limb tissues | | | | | | 43 day old normal limb tissues | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Aneurogenic | | | Control | | | | | |
| | | F | $P_{.05}$ | $P_{.01}$ | F | $P_{.05}$ | $P_{.01}$ | F | $P_{.05}$ | $P_{.01}$ |
| 65 day old limb tissues | Whole limb (L) | 20.90 | 2.62 | 3.90 | 8.15 | 2.60 | 3.86 | 3.78 | 5.32 | 11.30 |
| | Solid tissue (S) | 2.00 | ↓ | ↓ | 1.56 | ↓ | ↓ | 3.74 | ↓ | ↓ |
| | Humerus (H) | 6.98 | ↓ | ↓ | 1.50 | ↓ | ↓ | 0.04 | ↓ | ↓ |
| | Muscle (M) | 18.48 | ↓ | ↓ | 4.98 | ↓ | ↓ | 7.66 | ↓ | ↓ |
| | Myonuclei (N) | 15.85 | ↓ | ↓ | 3.24 | ↓ | ↓ | 1.99 | ↓ | ↓ |

Statistics: BMD07M-stepwise discriminant analysis - Health Sciences Computing Facility, UCLA, 1973

P-values: Freund, Livermore, Miller - Manual of Experimental Statistics, 1960.

Table 4

STATISTICS FOR TIME SERIES, ANEUROGENICS

| Age of animals (days) | 13 | 21 | 31 | 43 | 54 | P<.05 | P<.01 |
|---|---|---|---|---|---|---|---|
| | | | | | | 4.26 | 7.82 |
| M | 1.99 | 14.14 | | | | | |
| 21 | 5.50 | 8.54 | 0.70 | | | | |
| 31 | 2.28 | 54.83 | 13.28 | 20.08 | | | |
| 43 | 35.88 | 53.44 | 12.60 | 19.25 | 0.01 | | |
| 54 | 34.76 | | | | | | |
| | | | | | | 3.42 | 5.66 |
| M+L | 3.63 | 11.38 | | | | | |
| 21 | 2.90 | 6.68 | 0.62 | | | | |
| 31 | 1.09 | 42.41 | 9.87 | 15.43 | | | |
| 43 | 22.87 | 32.44 | 28.71 | 27.06 | 43.99 | | |
| 54 | 34.73 | | | | | | |
| | | | | | | 3.05 | 4.82 |
| M+L+S | 3.89 | 7.96 | | | | | |
| 21 | 2.02 | 5.12 | 0.41 | | | | |
| 31 | 0.80 | 29.50 | 6.82 | 10.25 | | | |
| 43 | 14.68 | 21.24 | 18.31 | 17.29 | 28.74 | | |
| 54 | 22.41 | | | | | | |
| | | | | | | 2.82 | 4.37 |
| M+L+S+N | 2.78 | 5.71 | | | | | |
| 21 | 1.45 | 3.80 | 0.54 | | | | |
| 31 | 0.73 | 22.80 | 6.94 | 8.21 | | | |
| 43 | 12.28 | 22.00 | 20.65 | 17.41 | 22.29 | | |
| 54 | 23.02 | | | | | | |
| | | | | | | 2.71 | 4.10 |
| M+L+S+H+N | 2.60 | 4.36 | | | | | |
| 21 | 1.73 | 4.72 | 2.00 | | | | |
| 31 | 4.75 | 18.09 | 5.86 | 6.50 | | | |
| 43 | 11.74 | 16.76 | 15.74 | 15.12 | 17.73 | | |
| 54 | 18.01 | | | | | | |
| | | | | | | F-values | |

← Age of animals (days) →

Statistics: BMD07M-Stepwise discriminant analysis--Health Sciences Computing Facility, UCLA, 1970.

P-values: Freund, Livermore, Miller--Manual of Experimental Statistics, 1960.

M=muscle; L=whole limb; S=solid tissue; N=number of muscle nuclei; H=humerus.

Table 5

STATISTICS FOR TIME SERIES, CONTROLS

F-values

| Age of animals (days) | 13 | 21 | 31 | 43 | 54 | P .05 | P .01 |
|---|---|---|---|---|---|---|---|
| M | 6.18 | 1.91 | 0.85 | 0.01 | 6.84 | 4.24 | 7.77 |
| 21 | 15.83 | 0.19 | 0.69 | 6.39 | | | |
| 31 | 8.57 | 0.28 | 12.70 | | | | |
| 43 | 9.08 | 4.36 | | | | | |
| 54 | 0.16 | | | | | | |
| M+L | 17.42 | 3.08 | 0.47 | 2.29 | 3.47 | 3.40 | 5.61 |
| 21 | 13.86 | 1.46 | 2.10 | 4.24 | | | |
| 31 | 11.04 | 7.33 | 6.87 | | | | |
| 43 | 5.62 | 7.16 | | | | | |
| 54 | 2.48 | | | | | | |
| M+L+S | 13.57 | 2.23 | 0.72 | 1.79 | 2.24 | 3.03 | 4.76 |
| 21 | 10.11 | 0.95 | 1.35 | 2.90 | | | |
| 31 | 9.91 | 4.88 | 4.43 | | | | |
| 43 | 4.83 | 4.67 | | | | | |
| 54 | 3.16 | | | | | | |
| M+L+S+N | 9.86 | 1.65 | 0.67 | 2.92 | 1.61 | 2.82 | 4.31 |
| 21 | 7.60 | 0.71 | 1.85 | 3.80 | | | |
| 31 | 7.14 | 4.74 | 4.12 | | | | |
| 43 | 5.62 | 4.67 | | | | | |
| 54 | 4.52 | | | | | | |
| M+L+S+H+N | 8.49 | 2.61 | 1.79 | 2.56 | 1.89 | 2.68 | 4.04 |
| 21 | 5.83 | 0.54 | 1.70 | 2.96 | | | |
| 31 | 6.36 | 3.98 | 5.08 | | | | |
| 43 | 4.44 | 3.61 | | | | | |
| 54 | 4.89 | | | | | | |

Age of animals (days)

Statistics: BMD07M-Stepwise discriminant analysis--Health Sciences Computing Facility, UCLA, 1970.

P-values: Freund, Livermore, Miller--Manual of Experimental Statistics, 1960.

M=muscle; L=whole limb; S=solid tissue; N=number of muscle nuclei; H=humerus.

## Table 6

STATISTICS, ANEUROGENIC vs. CONTROL

| | Age of animals (days) | | | | | | Age 13, 21 43, 54, 65 | | Age 31 only | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 13 | 21 | 31 | 43 | 54 | 65 | P .05 | P .01 | P .05 | P .01 |
| Whole limb (L) | 0.30 | 11.59 | 43.15 | 4.87 | 22.13 | 48.35 | 5.32 | 11.30 | 5.12 | 10.60 |
| Solid tissue (S) | 0.23 | 10.12 | 22.81 | 2.86 | 13.98 | 0.01 | | | | |
| Humerus (H) | 4.80 | 2.68 | 0.07 | 0.85 | 0.60 | 0.28 | | | | |
| Muscle (M) | 2.13 | 17.59 | 59.23 | 101.06 | 12.73 | 105.93 | | | | |
| Myonuclei (N) | 5.01 | 4.59 | 25.48 | 43.05 | 26.19 | 35.53 | | | | |
| M+N | 3.37 | 8.31 | 36.75 | 47.35 | 11.75 | 48.77 | 4.74 | 9.55 | 4.46 | 8.65 |
| M+N+L | 2.95 | 24.52 | 28.44 | 37.64 | 8.48 | 41.82 | 4.76 | 9.78 | 4.35 | 8.45 |
| M+N+L+S | 1.93 | 16.20 | 18.29 | 42.80 | 5.31 | 27.37 | 5.19 | 11.40 | 4.53 | 9.15 |
| M+N+L+S+H | 3.15 | 10.40 | 25.96 | 27.98 | 8.67 | 19.01 | 6.26 | 15.50 | 5.05 | 11.00 |

P-values

Statistics: BMD07M-Stepwise discriminant analysis--Health Sciences Computing Facility, UCLA, 1970.

P-values: Freund, Livermore, Miller-- Manual of Experimental Statistics, 1960.

Table 7 LOCATION OF MYONUCLEI

| Age of animals (days) | Animal I.D. | A Number of centrally located nuclei | B Number of peripherally located nuclei | $\frac{B}{A+B}$ | |
|---|---|---|---|---|---|
| 13 | 45-15s | 8 | 3 | 0.27 | Aneurogenic animals |
| | 45-17s | 17 | 3 | 0.15 | |
| | 45-16s | 11 | 1 | 0.08 | |
| | 45-15s | 3 | 2 | 0.40 | |
| | 45-18s | 4 | 2 | 0.33 | |
| 21 | 46-3 | 24 | 5 | 0.17 | |
| | 46-6 | 21 | 3 | 0.12 | |
| | 46-10 | 16 | 8 | 0.33 | |
| | 46-4 | 20 | 9 | 0.31 | |
| | 46-5 | 12 | 10 | 0.45 | |
| 31 | 47-2 | 6 | 8 | 0.57 | |
| | 47-3 | 1 | 5 | 0.83 | |
| | 47-5 | 6 | 6 | 0.50 | |
| | 47-8 | 10 | 10 | 0.50 | |
| | 47-12 | 10 | 7 | 0.41 | |
| 43 | 48-1 | 3 | 2 | 0.40 | |
| | 48-16 | 18 | 5 | 0.21 | |
| | 48-6 | 15 | 7 | 0.32 | |
| | 48-15 | 13 | 7 | 0.35 | |
| | 48-9 | 11 | 6 | 0.35 | |
| 13 | 45-1 | 10 | 6 | 0.37 | Control animals |
| | 45-3 | 12 | 9 | 0.42 | |
| | 45-5 | 15 | 5 | 0.25 | |
| | 45-9 | 14 | 6 | 0.30 | |
| | 45-13 | 18 | 10 | 0.36 | |
| 21 | 46-9s | 11 | 26 | 0.70 | |
| | 46-8s | 4 | 29 | 0.88 | |
| | 46-6s | 8 | 39 | 0.83 | |
| | 46-5s | 10 | 30 | 0.75 | |
| | 46-4s | 12 | 24 | 0.66 | |
| 31 | 47-7s | 5 | 34 | 0.87 | |
| | 47-15s | 8 | 30 | 0.79 | |
| | 47-14s | 4 | 35 | 0.90 | |
| | 47-4s | 4 | 27 | 0.87 | |
| | 47-13s | 2 | 36 | 0.95 | |
| 43 | 48-3s | 4 | 32 | 0.88 | |
| | 48-10s | 4 | 30 | 0.88 | |
| | 48-5s | 4 | 34 | 0.89 | |
| | 48-15s | 4 | 29 | 0.88 | |
| | 48-9s | 9 | 26 | 0.74 | |

Table 8

LOCATION OF MYONUCLEI

| Age of animals (days) | Experimental state of limbs | Number of limbs | $\frac{\overline{X}_B}{A+B}$ | Std. error | t |
|---|---|---|---|---|---|
| 13 | control<br>aneurogenic | 5<br>5 | 0.338<br>0.246 | 0.029<br>0.058 | 1.4290 |
| 21 | control<br>aneurogenic | 5<br>5 | 0.764<br>0.276 | 0.040<br>0.060 | 6.9024 |
| 31 | control<br>aneurogenic | 5<br>5 | 0.876<br>0.562 | 0.026<br>0.071 | 4.1978 |
| 43 | control<br>aneurogenic | 5<br>5 | 0.854<br>0.326 | 0.029<br>0.032 | 13.200 |
| 31<br>43 | control<br>control | | | | 0.5882 |
| 31<br>43 | aneurogenic<br>aneurogenic | | | | 3.0490 |

$\frac{\overline{X}_B}{A+B}$ = Average fraction of number of peripheral myonuclei per total

$P<.05 = 2.306$

$P<.01 = 3.355$

Statistics: t-test for equality of two means.

## Table 10

## MYONUCLEAR AREA

| Age of animals (days) | Number of limbs | Number of nuclei | Nuclear area | | D/C | | |
|---|---|---|---|---|---|---|---|
| | | | Mean ($um^2$) | Std. error | Mean | Std. error | |
| 13 | 4 | 26 | 53.454 | 10.133 | 0.502 | 0.028 | Aneurogen. |
| 21 | 5 | 43 | 46.669 | 4.591 | 0.276 | 0.051 | |
| 31 | 5 | 49 | 34.254 | 4.128 | 0.376 | 0.035 | |
| 43 | 5 | 51 | 28.201 | 1.187 | 0.422 | 0.033 | |
| 54 | 4 | 42 | 18.382 | 1.257 | 0.497 | 0.062 | |
| 65 | 5 | 27 | 27.363 | 1.354 | 0.423 | 0.044 | |
| 13 | 5 | 32 | 43.833 | 4.341 | 0.399 | 0.033 | Control |
| 21 | 4 | 36 | 40.336 | 2.972 | 0.154 | 0.020 | |
| 31 | 6 | 53 | 37.240 | 2.540 | 0.156 | 0.018 | |
| 43 | 5 | 50 | 32.518 | 2.513 | 0.153 | 0.025 | |
| 54 | 5 | 43 | 33.826 | 1.860 | 0.177 | 0.047 | |
| 65 | 5 | 45 | 28.113 | 1.826 | 0.196 | 0.018 | |
| 43 | 5 | 51 | 41.257 | 2.172 | 0.217 | 0.019 | Nor-mal |
| 65 | 5 | 41 | 36.401 | 4.974 | 0.110 | 0.018 | |

D/C : See Table 13

Table 12

MYONUCLEAR LENGTH

| Animal I.D. | Mean length of myonuclei (um) | Standard error | Standard deviation | |
|---|---|---|---|---|
| 48-10 | 46.408 | 2.000 | 6.341 | Aneurogenic 43 day old animals |
| 48-2 | 37.430 | 1.945 | 6.160 | |
| 48-13 | 36.694 | 2.328 | 7.427 | |
| 48-3 | 40.820 | 1.238 | 3.949 | |
| 48-4 | 39.641 | 2.181 | 6.897 | |
| 48-8s | 36.694 | 1.591 | 5.570 | Control 43 day old animals |
| 48-6s | 39.494 | 1.739 | 5.511 | |
| 48-14s | 38.757 | 1.415 | 4.539 | |
| 48-12s | 38.020 | 1.356 | 4.362 | |
| 48-2s | 40.378 | 1.238 | 3.949 | |

F=2.66 for equality of all means
P<.01=2.80
P<.05=2.00

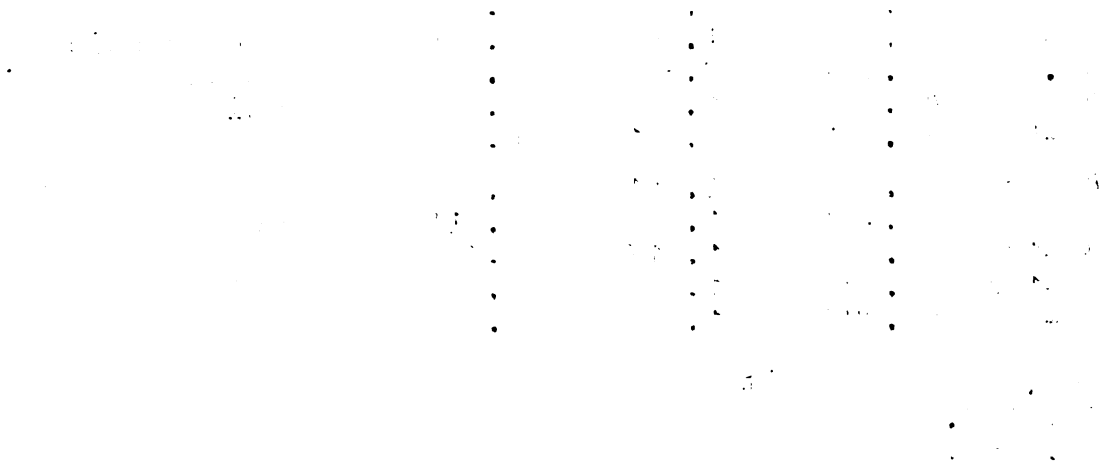


Table 13

MYONUCLEAR AREA

| Age of animals (days) | Animal I.D. | C Selected muscle area ($um^2$) | Number of nuclei per C | D Sum nuclear area ($um^2$) | D/C | |
|---|---|---|---|---|---|---|
| 13 | 45-15d | 440.0 | 5 | 194.3 | 0.442 | Aneurogenic muscle |
| | 45-18s | 480.0 | 6 | 266.8 | 0.556 | |
| | 45-16s | 695.0 | 8 | 376.6 | 0.542 | |
| | 45-17s | 1248.3 | 7 | 583.9 | 0.468 | |
| 21 | 46-3 | 2810.1 | 10 | 443.5 | 0.158 | |
| | 46-6 | 1585.7 | 6 | 370.5 | 0.234 | |
| | 46-5 | 593.9 | 8 | 271.4 | 0.457 | |
| | 46-4 | 2459.5 | 13 | 559.5 | 0.227 | |
| | 46-10 | 983.8 | 6 | 301.7 | 0.307 | |
| 31 | 47-2 | 1834.2 | 11 | 468.2 | 0.255 | |
| | 47-12 | 474.6 | 6 | 193.4 | 0.408 | |
| | 47-5 | 853.5 | 12 | 308.8 | 0.362 | |
| | 47-3 | 765.0 | 14 | 357.8 | 0.468 | |
| | 47-8 | 700.0 | 6 | 271.1 | 0.387 | |
| 43 | 48-16 | 726.8 | 11 | 274.6 | 0.378 | |
| | 48-1 | 579.0 | 10 | 313.1 | 0.541 | |
| | 48-9 | 892.2 | 13 | 369.5 | 0.414 | |
| | 48-6 | 867.0 | 10 | 301.5 | 0.348 | |
| | 48-15 | 425.2 | 7 | 183.1 | 0.431 | |
| 54 | 43-9 | 564.9 | 17 | 361.0 | 0.639 | |
| | 43-14 | 409.6 | 15 | 229.7 | 0.561 | |
| | 43-3 | 259.8 | 5 | 96.6 | 0.372 | |
| | 43-6 | 211.4 | 5 | 88.3 | 0.418 | |
| | 43-13 | 0.0 | 0 | 0.0 | 0.0 | |
| 65 | 50-2 | 405.7 | 5 | 126.2 | 0.311 | |
| | 50-5 | 397.8 | 6 | 169.1 | 0.425 | |
| | 50-9 | 438.5 | 5 | 161.3 | 0.368 | |
| | 50-7 | 258.7 | 6 | 149.1 | 0.576 | |
| | 50-18 | 301.4 | 5 | 131.4 | 0.436 | |
| 43 | 48-12r | 1620.0 | 10 | 416.3 | 0.257 | Normal muscle |
| | 48-10r | 1905.5 | 12 | 428.1 | 0.225 | |
| | 48-7r | 2006.5 | 7 | 340.2 | 0.170 | |
| | 48-3r | 2282.1 | 14 | 588.6 | 0.258 | |
| | 48-4r | 1746.7 | 8 | 306.7 | 0.176 | |
| 65 | 50-8r | 2485.6 | 13 | 390.0 | 0.157 | |
| | 50-10r | 3233.5 | 9 | 337.1 | 0.104 | |
| | 50-15r | 2842.8 | 6 | 187.7 | 0.066 | |
| | 50-9r | 2552.0 | 7 | 196.0 | 0.077 | |
| | 50-11r | 2233.0 | 6 | 331.6 | 0.148 | |

Table 13(cont.)

MYONUCLEAR AREA

| Age of animals (days) | Animal I.D. | C Selected muscle area ($um^2$) | Number of nuclei per C | D Sum nuclear area ($um^2$) | D/C | |
|---|---|---|---|---|---|---|
| 13 | 45-1 | 1533.9 | 10 | 583.8 | 0.381 | Control muscle |
| | 45-9 | 490.0 | 7 | 232.9 | 0.475 | |
| | 45-5 | 467.0 | 6 | 221.0 | 0.473 | |
| | 45-13 | 632.3 | 5 | 225.4 | 0.356 | |
| | 45-3 | 593.7 | 4 | 182.4 | 0.307 | |
| 21 | 46-4s | 3811.0 | 10 | 391.7 | 0.103 | |
| | 46-5s | 3330.0 | 11 | 532.4 | 0.160 | |
| | 46-9s | 1776.8 | 8 | 272.6 | 0.153 | |
| | 46-6s | 1387.2 | 7 | 277.9 | 0.200 | |
| 31 | 47-7s | 5590.0 | 15 | 558.2 | 0.100 | |
| | 47-14s | 2394.2 | 10 | 297.8 | 0.124 | |
| | 47-13s | 2659.9 | 9 | 341.0 | 0.128 | |
| | 47-8s | 1245.5 | 7 | 240.5 | 0.193 | |
| | 47-15s | 991.2 | 6 | 214.2 | 0.216 | |
| | 47-4s | 1664.3 | 6 | 291.0 | 0.175 | |
| 43 | 48-3s | 2385.7 | 16 | 428.4 | 0.180 | |
| | 48-15s | 2640.7 | 8 | 228.3 | 0.086 | |
| | 48-5s | 2316.2 | 13 | 478.4 | 0.207 | |
| | 48-9s | 2366.4 | 6 | 239.7 | 0.101 | |
| | 48-10s | 1120.0 | 7 | 213.7 | 0.191 | |
| 54 | 49-10 | 3318.0 | 14 | 533.3 | 0.161 | |
| | 49-14 | 5354.0 | 9 | 307.5 | 0.057 | |
| | 49-9 | 3528.1 | 9 | 336.8 | 0.095 | |
| | 49-2 | 549.0 | 5 | 156.0 | 0.284 | |
| | 49-13 | 587.7 | 6 | 169.5 | 0.288 | |
| 65 | 50-7s | 1960.9 | 10 | 330.3 | 0.168 | |
| | 50-16s | 1749.3 | 14 | 422.3 | 0.241 | |
| | 50-4s | 824.5 | 7 | 159.9 | 0.194 | |
| | 50-14s | 763.1 | 7 | 175.9 | 0.231 | |
| | 50-1s | 1422.0 | 7 | 205.8 | 0.145 | |

Table 14

STATISTICS FOR MYONUCLEAR AREA

| Age of animals (days) | t for mean nuclear area | t for mean D/C | P<.05 | P<.01 | |
|---|---|---|---|---|---|
| 13 | 0.95 | 2.30 | 2.36 | 3.50 | |
| 21 | 1.08 | 2.02 | ▼ | ▼ | |
| 31 | -0.63 | 5.85 | 2.26 | 3.25 | Aneurogenic vs. control |
| 43 | -2.64 | 6.54 | 2.31 | 3.36 | |
| 54 | -6.49 | 4.18 | 2.36 | 3.50 | |
| 65 | -0.33 | 4.75 | 2.31 | 3.36 | |
| 43 | -5.28 | 5.39 | | | Aneurogenic vs. normal |
| 65 | -1.75 | 6.51 | | | |
| 43 | 2.63 | -2.05 | | | Control vs. normal |
| 65 | -1.57 | 3.29 | | | |

Statistics: Two tailed t-test for equality of two means

P-values: Freund, Livermore, Miller- Manual of Experimental Statistics, 1960.

Figure 3

Gross cross sectional limb area (see Table 2, 6).
n.d. = No statistical difference between aneurogenic and control means at $P<0.05$
Numbers between graphs = Difference between aneurogenic and control means at the indicated level of significance.

Figure 3

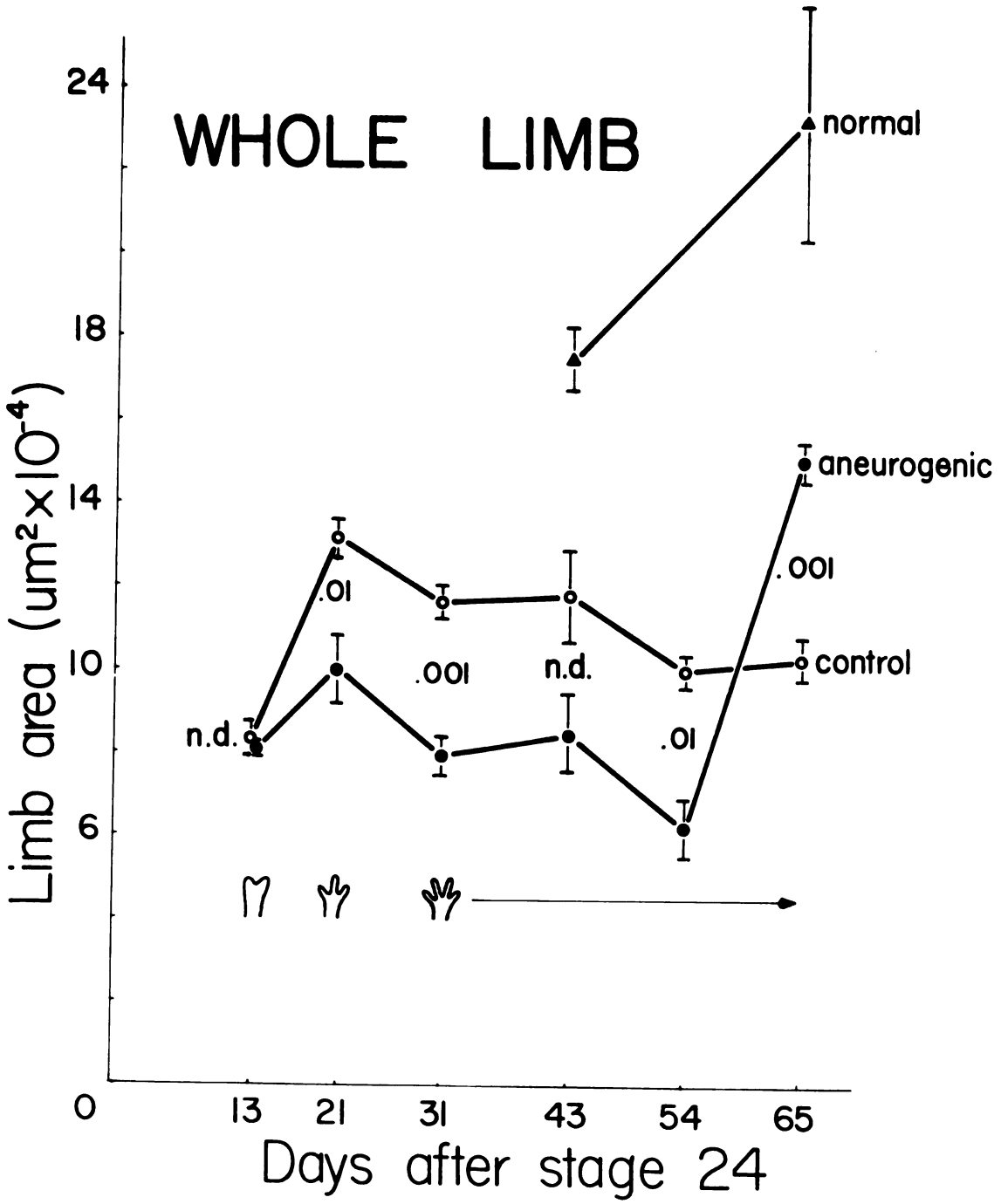

WHOLE LIMB
normal
aneurogenic
control
.01
.001
n.d.
.001
n.d.
.01
Limb area (um²x10⁻⁴)
24
18
14
10
6
0
13
21
31
43
54
65
Days after stage 24

Figure 4

Gross cross sectional limb area minus clear spaces (see Tables 2, 6).
● = Aneurogenic
O = Control
▲ = Normal
n.d. = No statistical difference between aneurogenic and control means at $P<0.05$.
Numbers between graphs = Statistical difference between aneurogenic and control means at indicated level of significance.

Figure 4

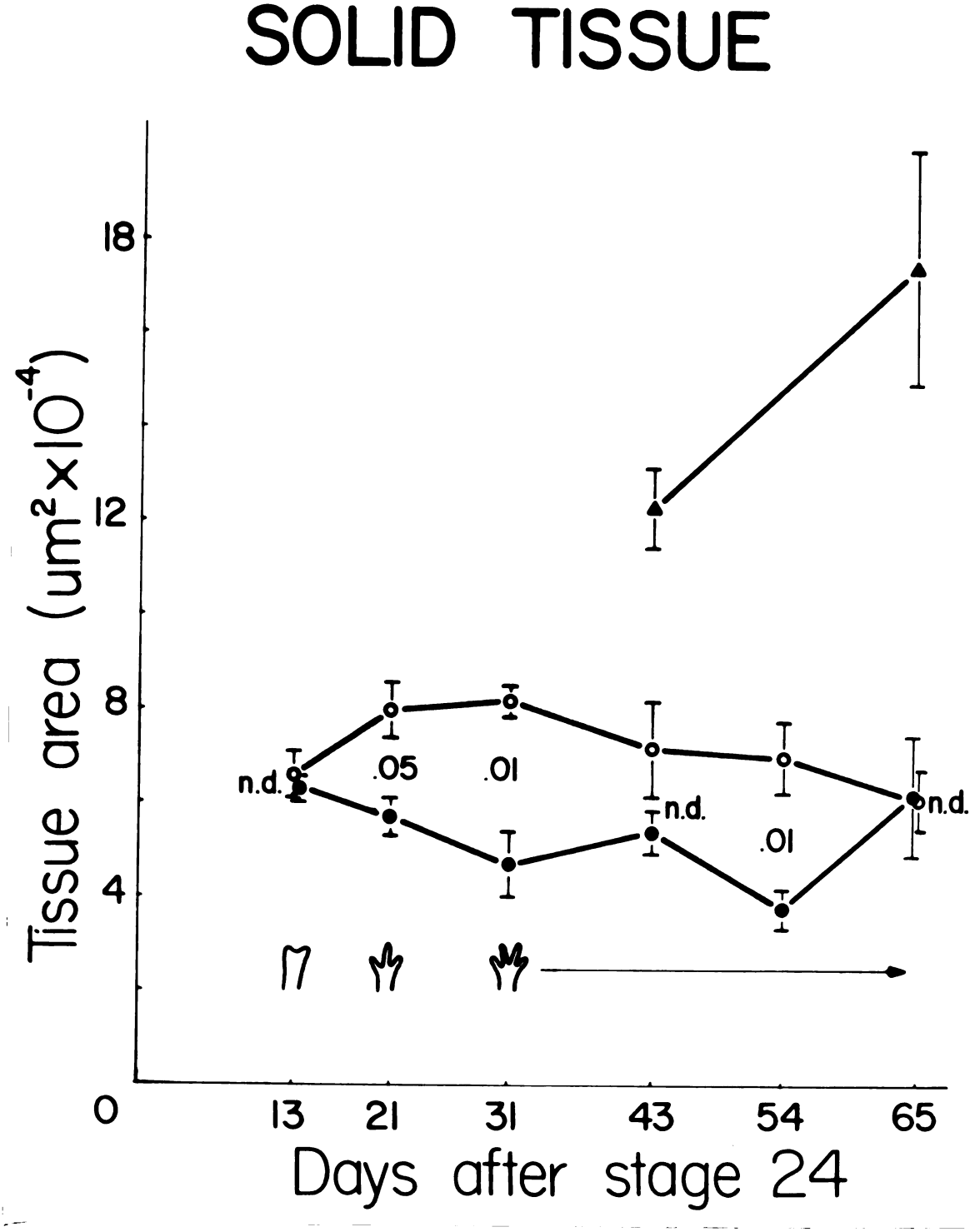

SOLID TISSUE
Tissue area (um²x10⁻⁴)
18
12
8
4
0
n.d.
.05
.01
n.d.
.01
n.d.
13
21
31
43
54
65
Days after stage 24

Figure 5

Cross sectional area of the humerus (see Tables 2, 6).
● = Aneurogenic
○ = Control
▲ = Normal
n.d. = No statistical difference between aneurogenic and control humeri at $P<0.05$.

**Figure 5**

# HUMERUS IN CROSS SECTION

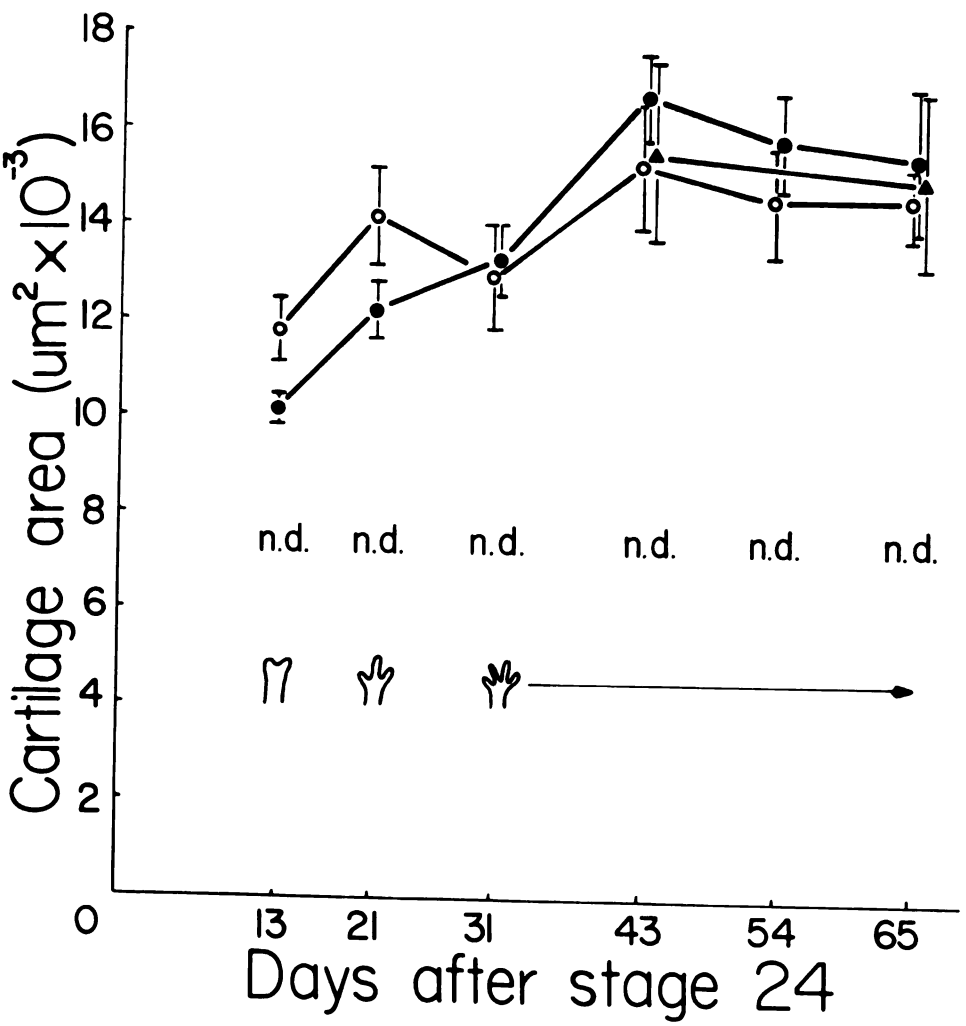
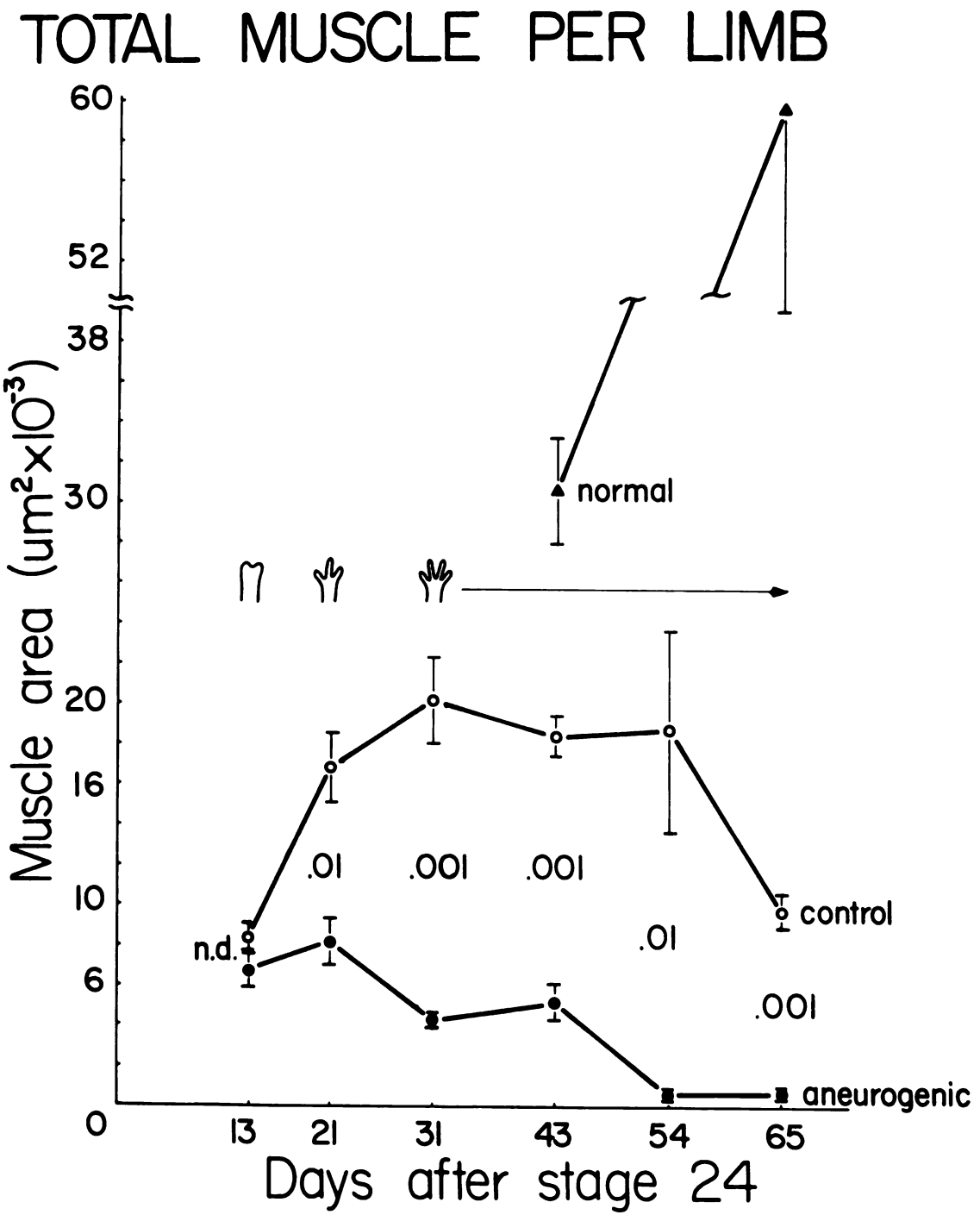


Figure 6

Cross sectional total upper forelimb muscle area (see Table 2, 6).
n.d. = No statistical difference between aneurogenic and control means at $P<0.05$
Other numbers within graphs = Difference between aneurogenic and control means at indicated level of significance.

**Figure 6**


TOTAL MUSCLE PER LIMB
Muscle area ($um^2 \times 10^{-3}$)
60
52
38
30
20
16
10
6
0
normal
control
aneurogenic
n.d.
.01
.001
.001
.01
.001
13
21
31
43
54
65
Days after stage 24

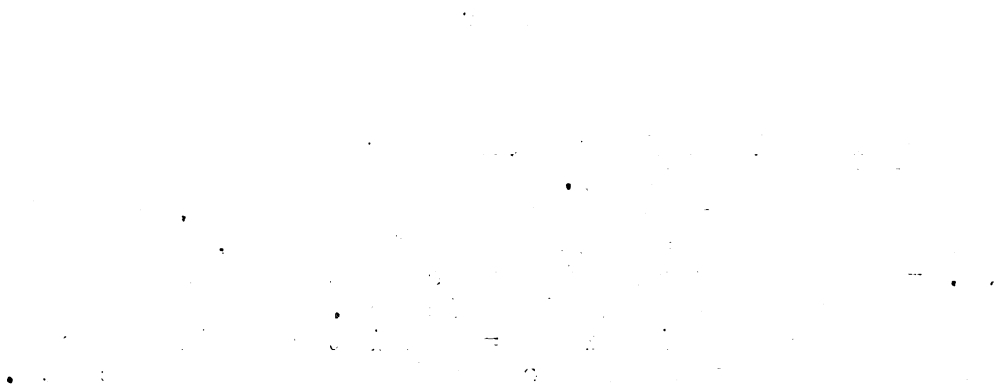


Figure 7

Number of peripheral myonuclei per total number of myonuclei (see Table 8).
vertical arrows = statistics aneurogenic vs. control.
horizontal arrows = statistics 31 days vs. 43 days.
n.d. = No statistical difference between means at the indicated level of significance.
Other numbers within graph = Statistical difference between means at the indicated level of significance.

Figure 7

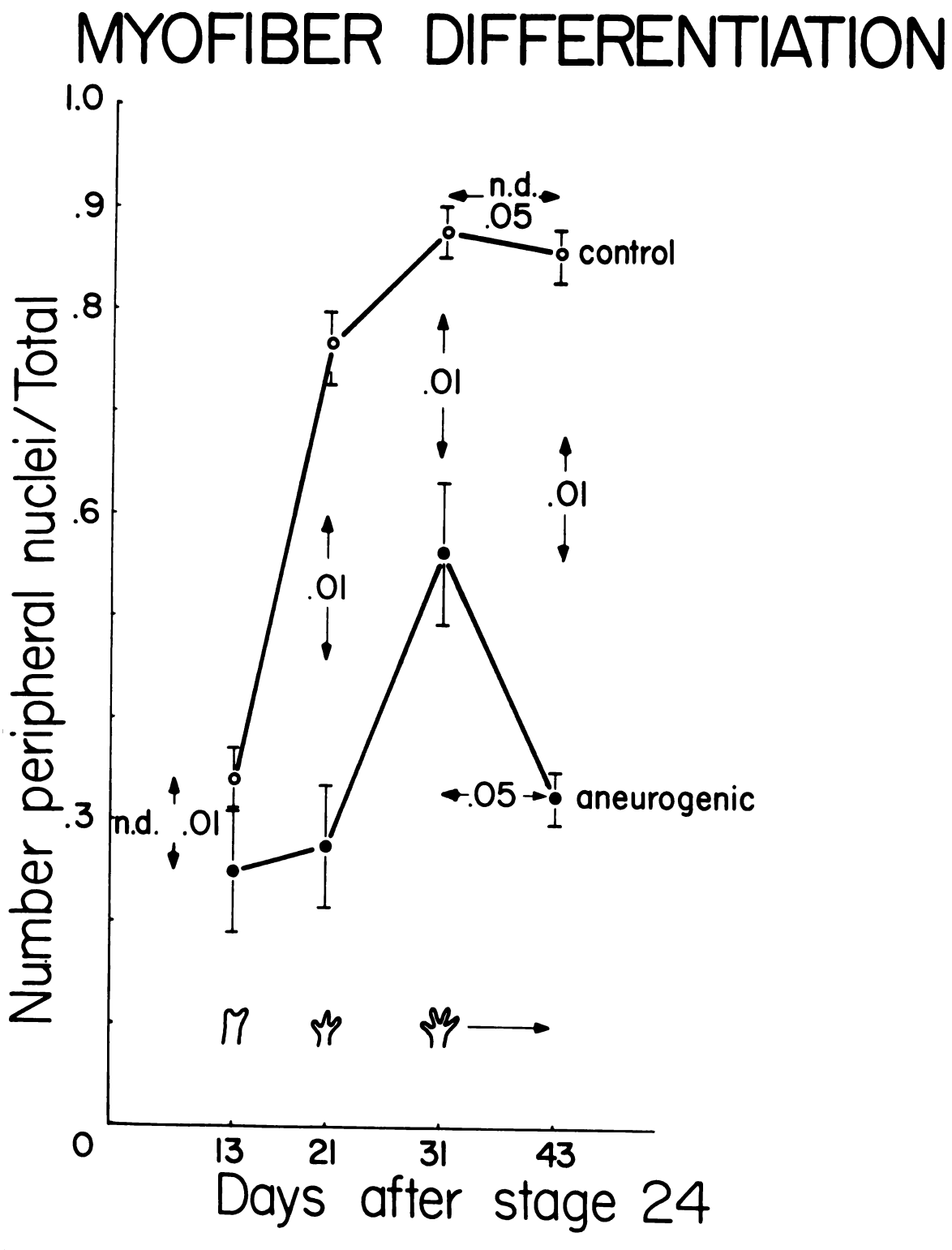

MYOFIBER DIFFERENTIATION
Number peripheral nuclei/Total
1.0
.9
.8
.6
.3
0
n.d.
.05
control
.01
.01
.01
n.d.
.01
.05
aneurogenic
13
21
31
43
Days after stage 24

Figure 8

Total number of muscle nuclei per limb cross section (see Table 2, 6).
n.d. = No statistical difference between aneurogenic and control number of muscle nuclei at $P<0.05$
Other numbers between graphs = Statistical difference between aneurogenics and controls at indicated level of significance.

Figure 8

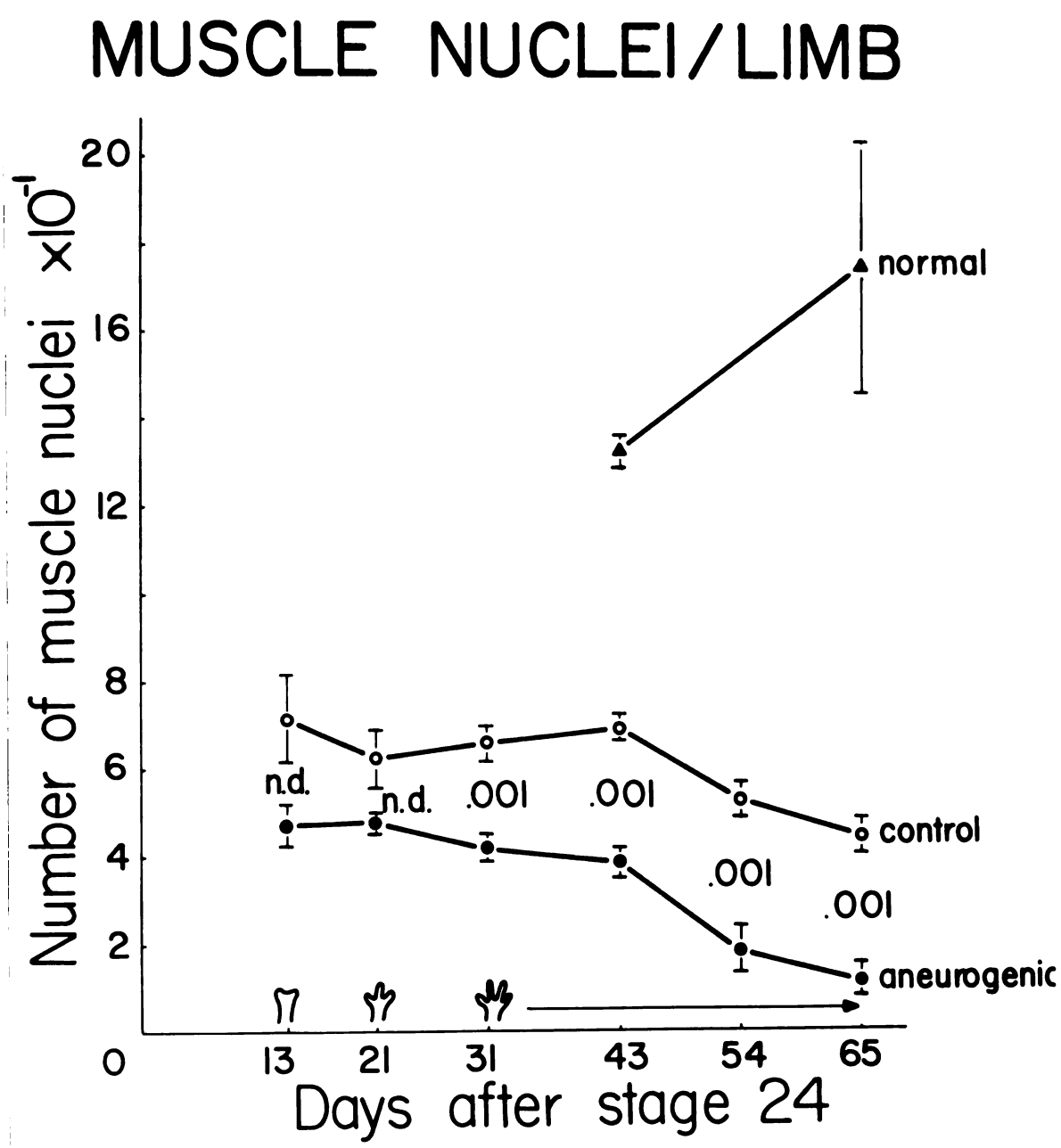

MUSCLE NUCLEI/LIMB
Number of muscle nuclei x10⁻¹
20
16
12
8
6
4
2
0
normal
control
aneurogenic
n.d.
n.d.
.001
.001
.001
.001
13
21
31
43
54
65
Days after stage 24

Figure 9

Cross sectional area of muscle nuclei (see Tables 10, 14).
●= Aneurogenic
O= Control
▲= Normal
n.d. = No statistical difference between control and aneurogenic means at $P<0.05$.
Numbers between graphs = Statistical difference between aneurogenic and control means at indicated level of significance.

**Figure 9**

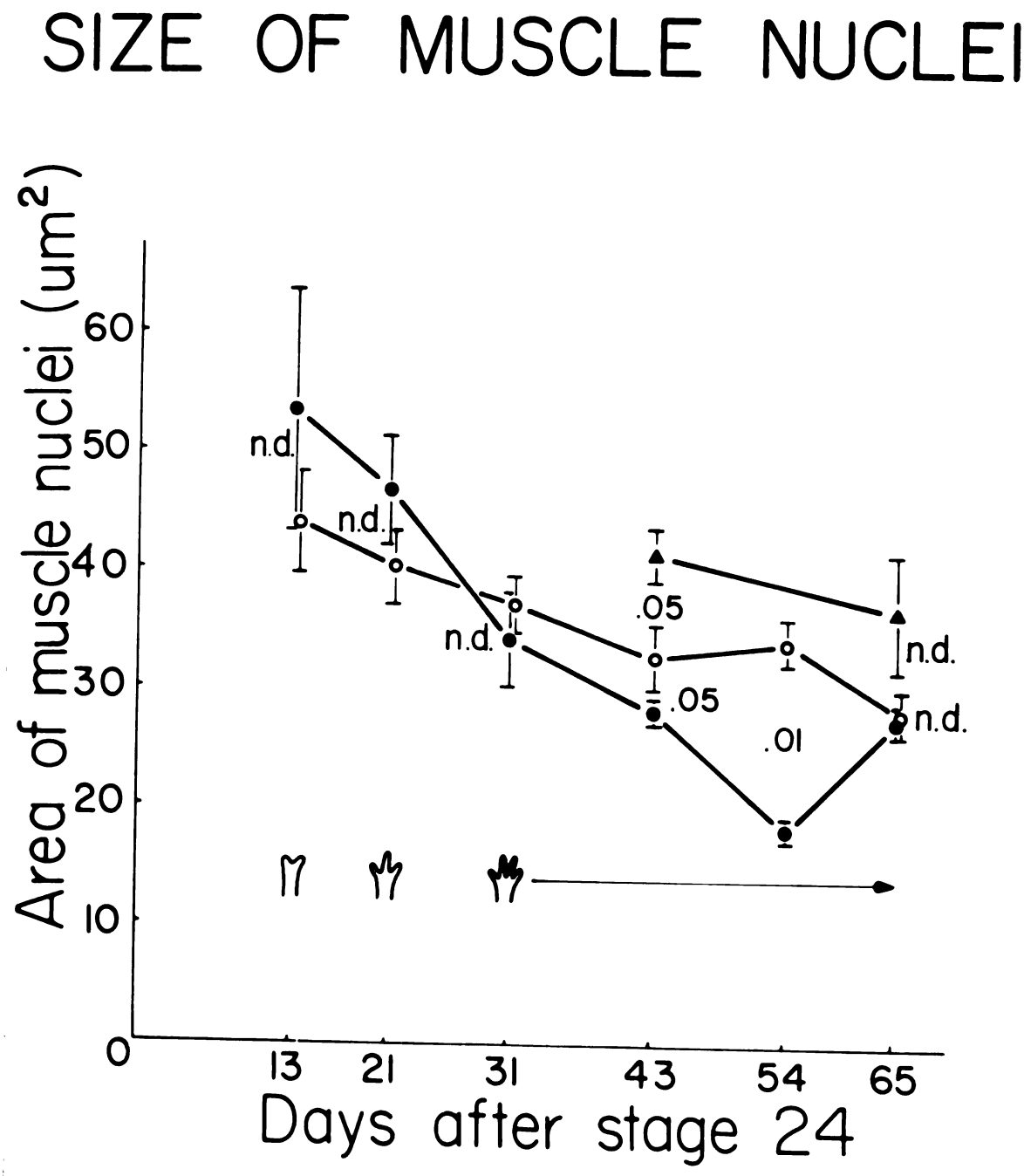

SIZE OF MUSCLE NUCLEI
Area of muscle nuclei (um²)
0
10
20
30
40
50
60
13
21
31
43
54
65
Days after stage 24
n.d.
n.d.
n.d.
.05
.05
.01
n.d.
n.d.

Figure 10

Sum myonuclear area per selected muscle area (see Table 10, 14).
Myonuclear area/Muscle area = D/C in Table 10
n.d. = No statistical difference between control and aneurogenic means at $P<0.05$
Numbers between graphs = Difference between aneurogenic and control means at indicated level of significance.

**Figure 10**

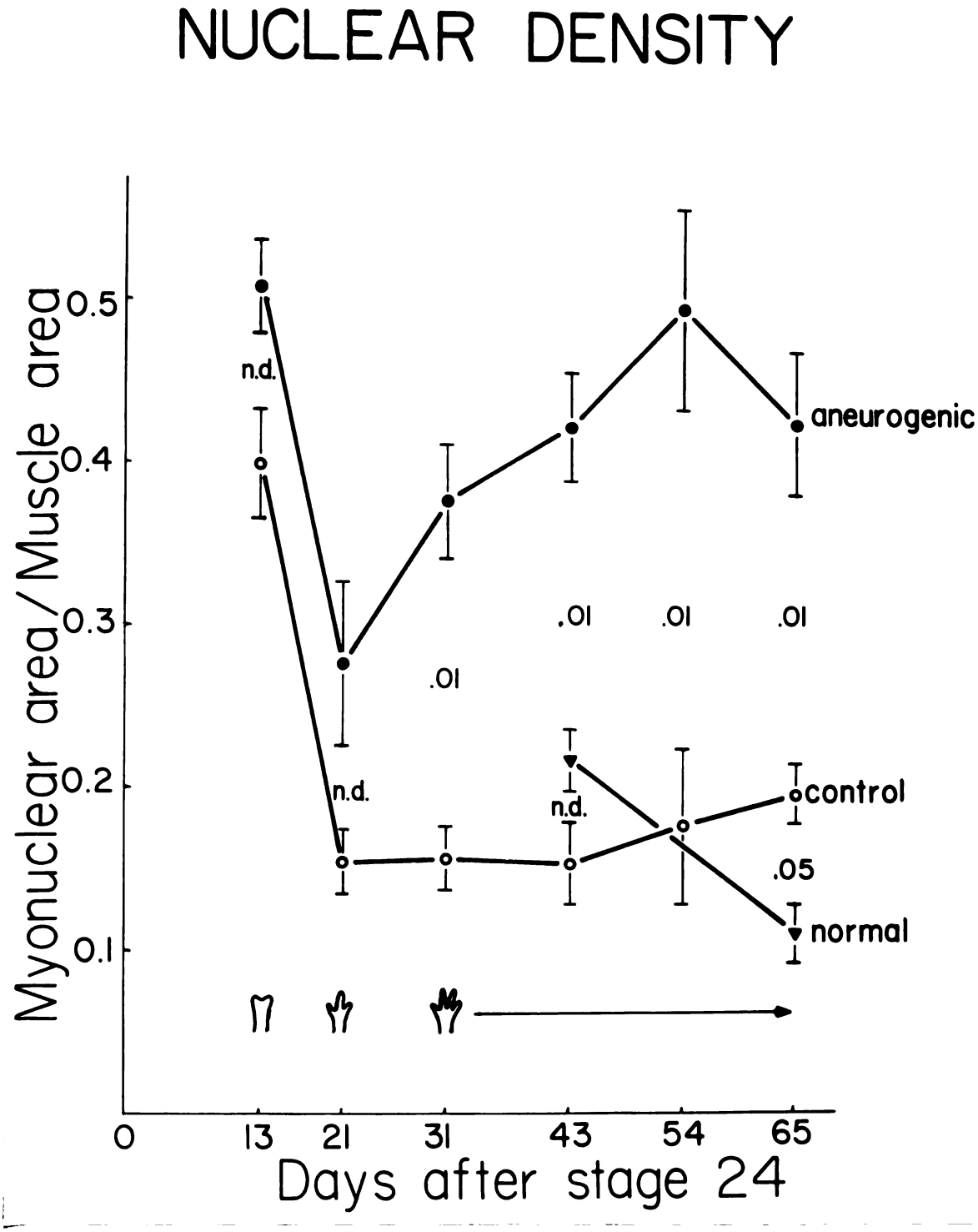

NUCLEAR DENSITY
Myonuclear area/Muscle area
0.5
0.4
0.3
0.2
0.1
n.d.
n.d.
n.d.
.01
.01
.01
.01
.05
aneurogenic
control
normal
0
13
21
31
43
54
65
Days after stage 24

Plate 1

Control three digit (21 days) forelimb muscle.
Two axons are seen to contact a myofiber (Arrows).
Electronmicrograph 13680X

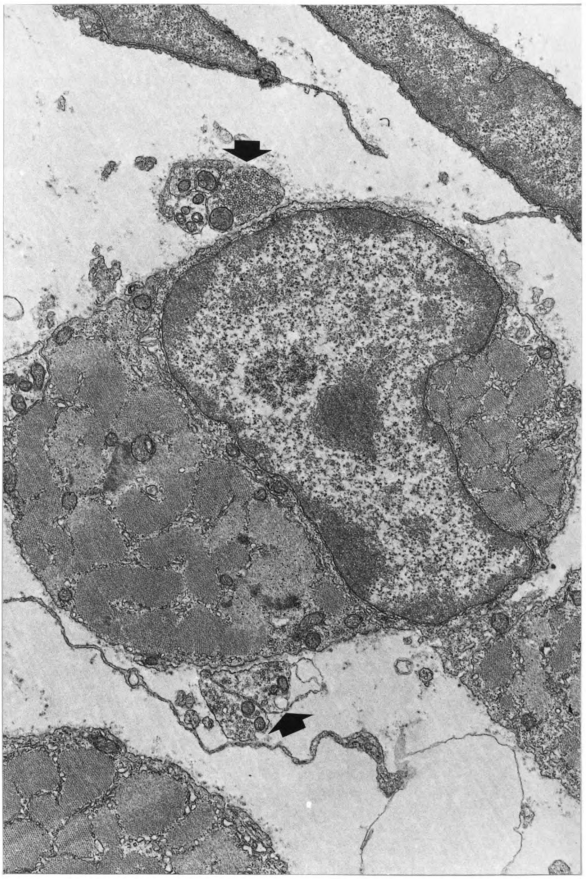


Plate 2

Cross section at mid-stylopodium region at 54 days of development.
a = Aneurogenic forelimb
b = Control forelimb
Note absence of nerves in aneurogenic limb and virtual absence of muscle.
B = Blood vessel
E = Epidermis
H = Humerus
M = Muscle
N = Nerve bundle
T = Tendon
Photomicrograph 80X

Plate 2

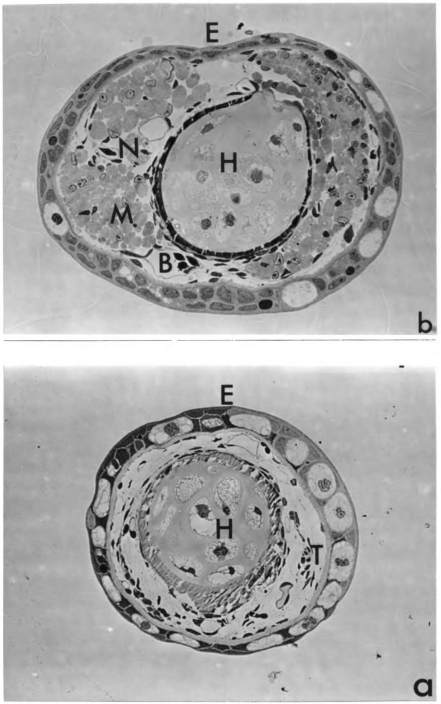
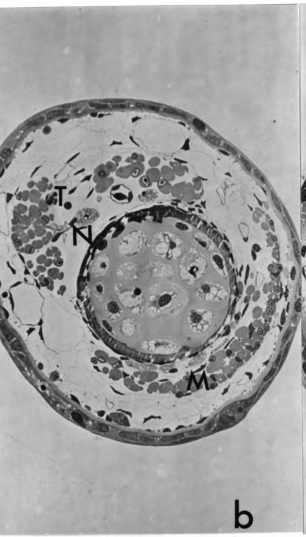
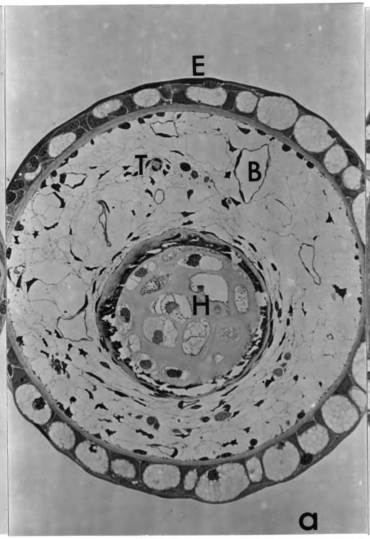


Plate 3

Mid-stylopodium cross section at 65 days of development.
a = Aneurogenic forelimb
b = Control forelimb
c = Normal forelimb
Note virtual absence of muscle in aneurogenic limb.
B = Blood vessel
E = Epidermis
H = Humerus
M = Muscle
N = Nerve bundle
T = Tendon
Photomicrograph 80X


T
N
M
b


E
T
B
H
a

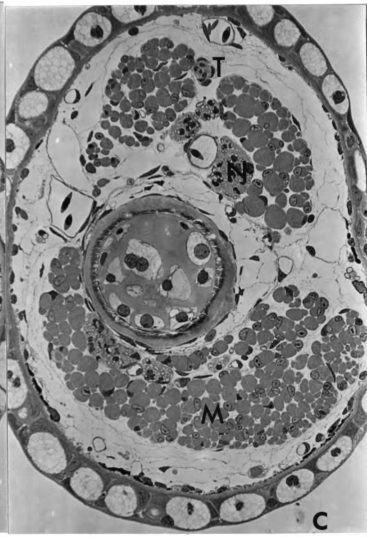

T
N
M
c

Plate 4

Tendon from 65 day aneurogenic forelimb muscle in cross section.
Electronmicrograph 12300X

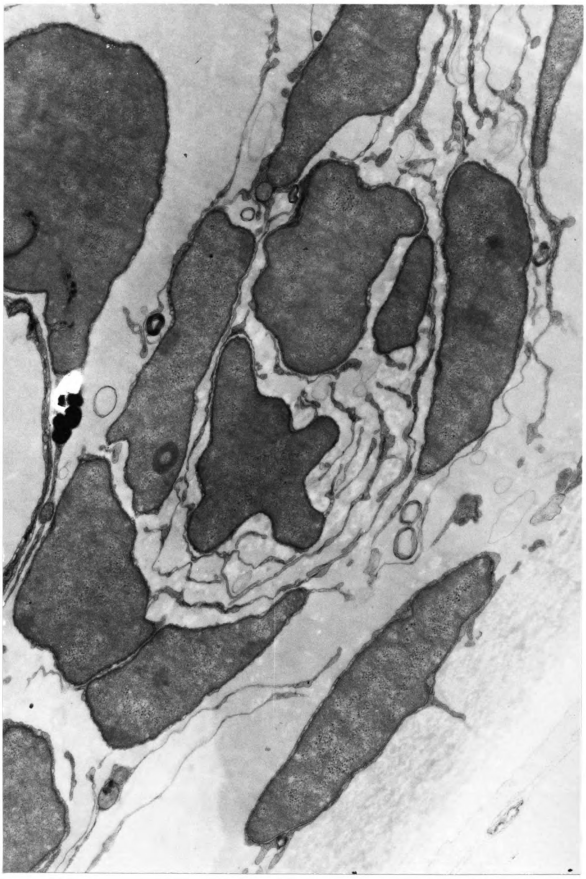


Plate 5

Tendon from normal forelimb muscle in cross section.
Electronmicrograph 4980X

Plate 5

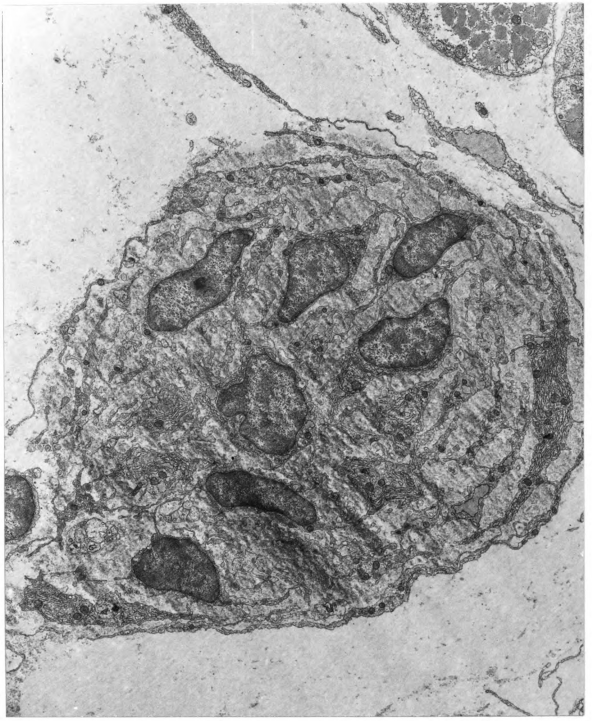


Plate 5a

Higher magnification of Plate 5.
C = Collagen
Note collagen fibrils within intercellular spaces.
Electronmicrograph 77200X

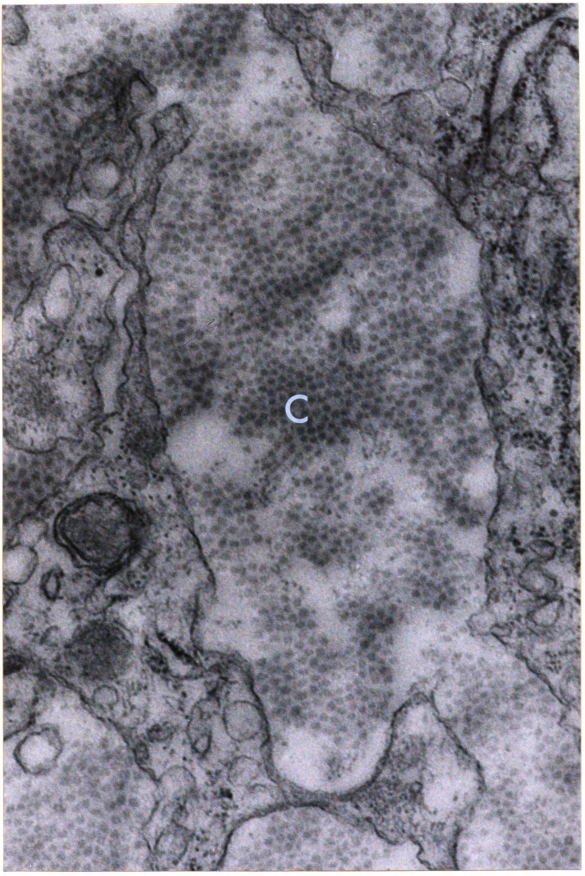

C

Plate 6

Remnants of forelimb muscle after 65 days in aneurogenic condition. This thin section was taken from a specimen in which muscle could no longer be detected with the light microscope.
Electronmicrograph 12890X

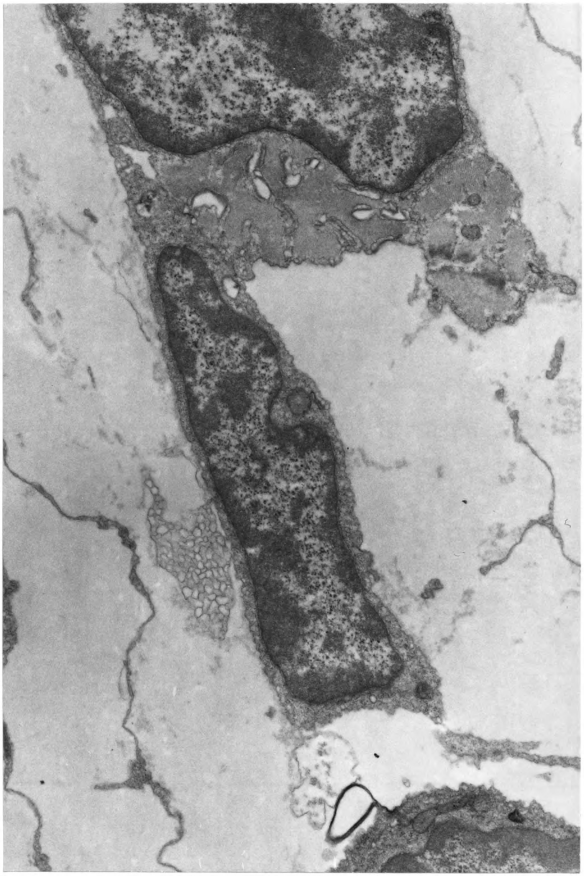


Plate 7

Longitudinal forelimb section from 26 days old aneurogenic larva. Grains are sites of disintegrations from incorporated tritiated leucine. Tritiated uridine incorporations show a similar grain pattern.
E = Epidermis
H = Humerus
M = Muscle
Note that more grains are visible over degenerated myofibers (arrows) than over fibers where cross striations are still visible.
Photomicrograph 2250X

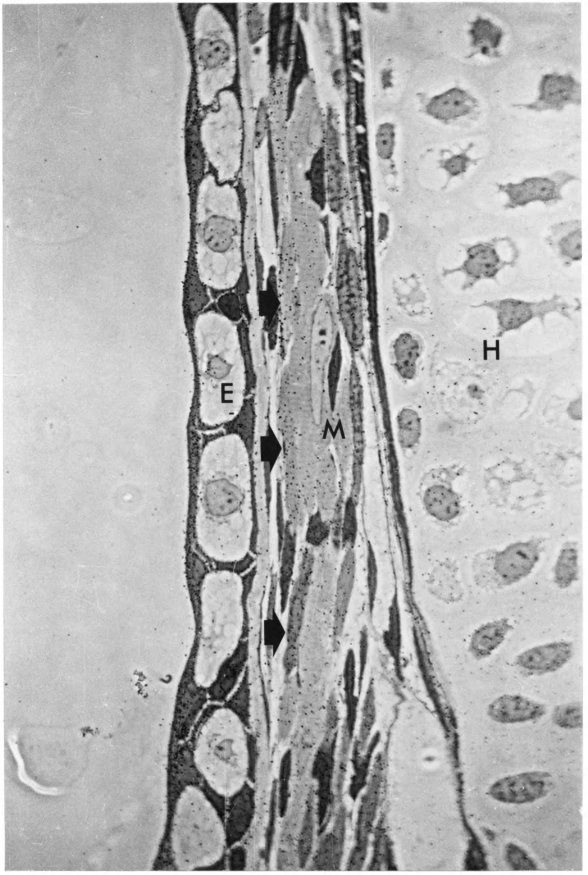

H
E
M

Plate 8

Macrophage from aneurogenic four digit forelimb with muscle fragment in the process of being engulfed (arrows).
MaN = Macrophage nucleus
Electronmicrograph 25800X

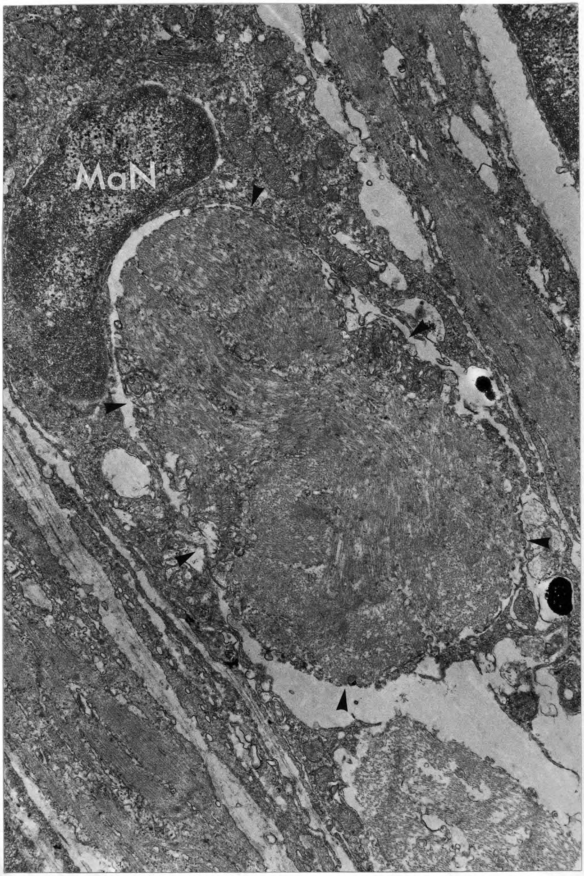

MaN

Plate 9

Acid phosphatase preparation of a portion of a macrophage found among degenerating four digit aneurogenic muscle.
D = Digestion vacuole
L = Lysosome
Electronmicrograph 45000X

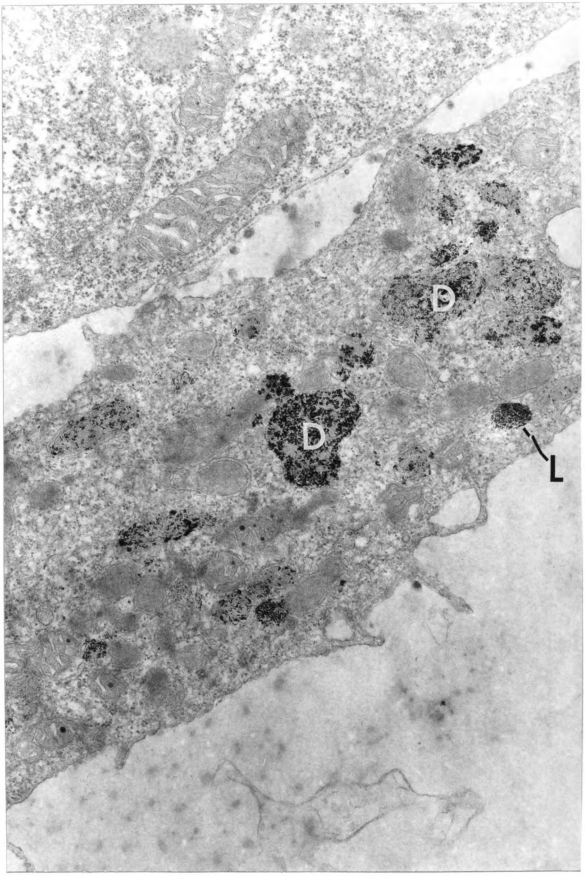

D
D
L

Plate 10

Acid phosphatase preparation of degenerating aneurogenic muscle.
L = Lysosome
MN = Myofiber nucleus
My = Myofibrillar material
Note the position of the lysosome adjacent to some disintegrating myofibrillar material.
Electronmicrograph 45000X

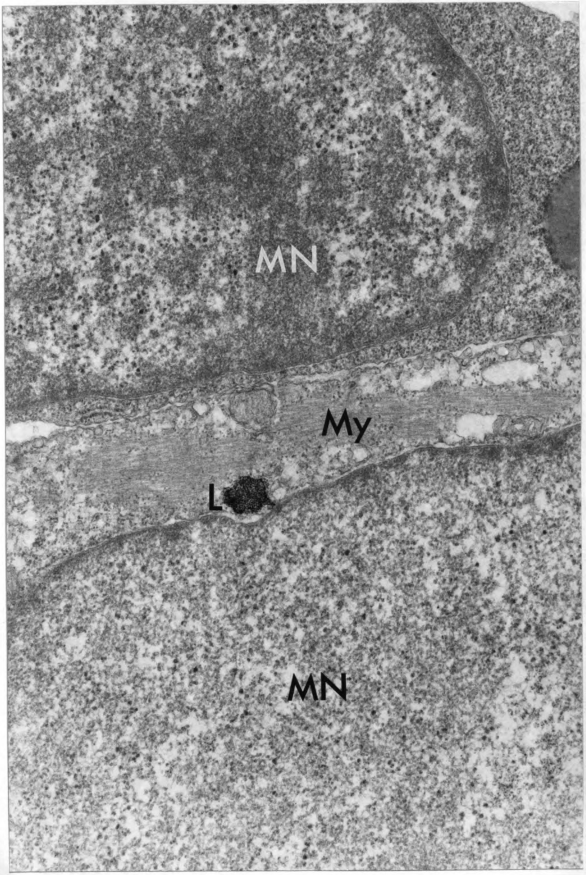

MN
My
L
MN

Plate 11

Portion of normal myofibers.
BL = Basement lamina
F1, F2 = Myofiber
Ruthenium red stain
Compare basement lamina with aneurogenic in Plate 12.
Electronmicrograph 23760X

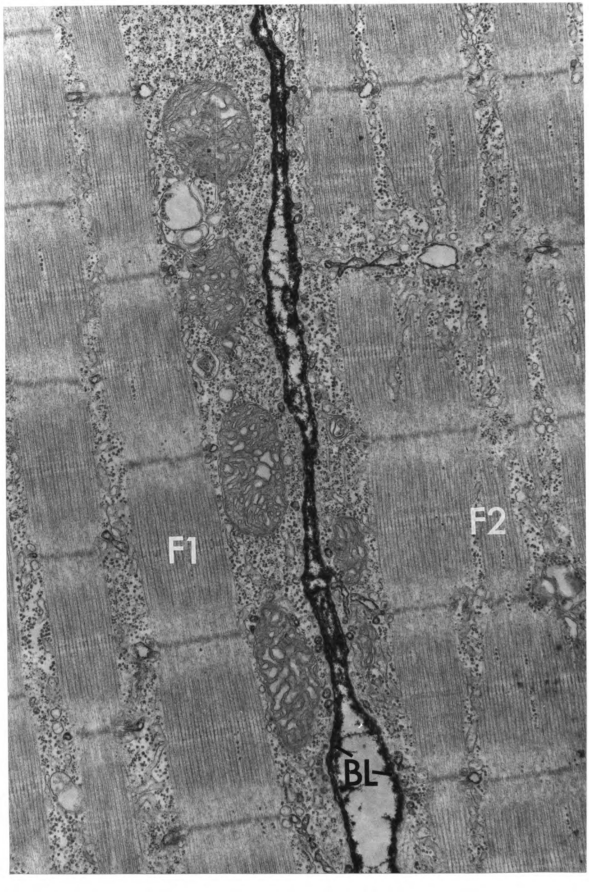

F1
F2
BL

Plate 12

Portion of a three digit (21 days) aneurogenic myofiber.
BL = Basement lamina
F = Myofiber
Ruthenium red stain
Compare basement lamina with that in Plate 11.
Electronmicrograph 40000X

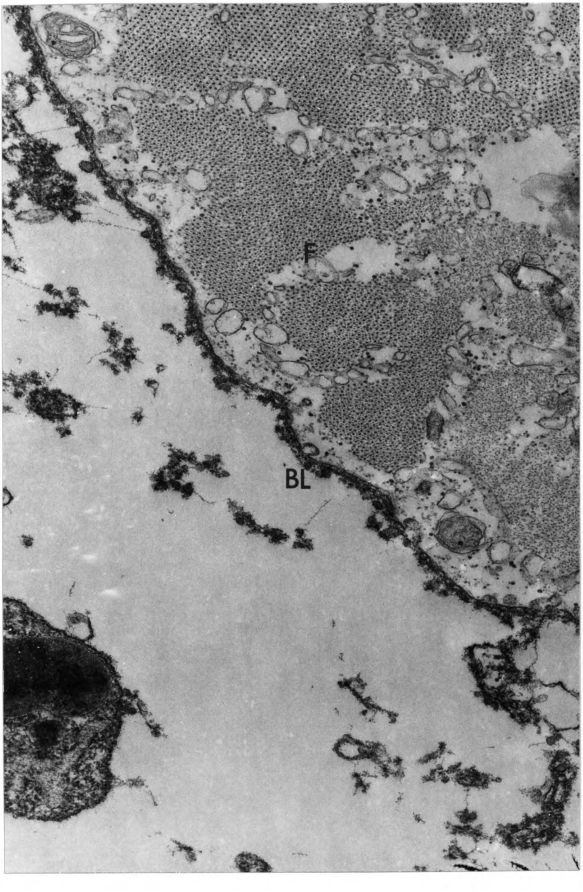
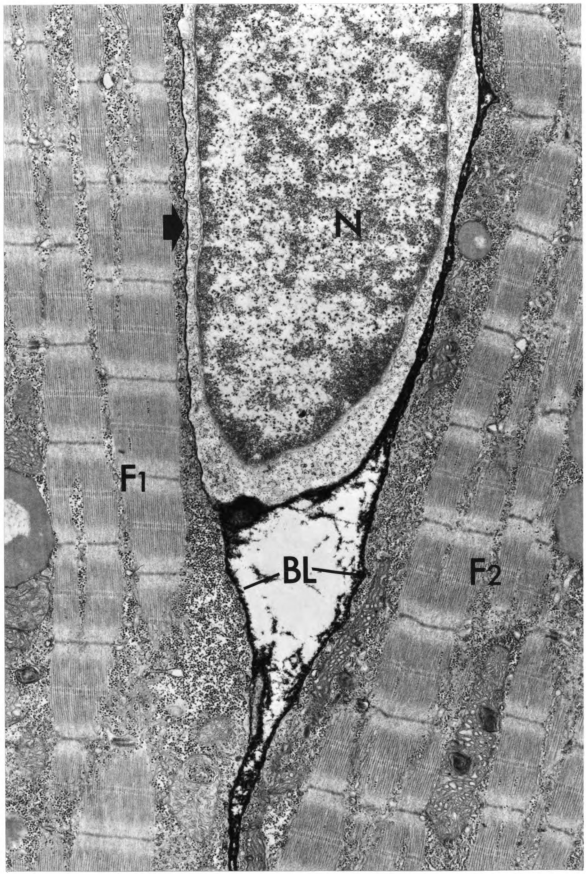

F
BL

Plate 13

Satellite cell in normal Amblystoma forelimb muscle.
BL = Basement lamina
F1, F2 = Myofiber
N = Satellite cell nucleus
Ruthenium red stain
Note that the basement lamina envelops the myofiber and its satellite cell. The satellite cell cytoplasm is separated from the sarcoplasm by a double plasma membrane (arrow) (Mauro, 1961).
Electronmicrograph 25080X


N
F1
BL
F2

# DISCUSSION

The most striking visual difference between aneurogenic and control larvae is the complete absence of pigmentation in the aneurogenic ones (Figure 1a) which is not surprising since melanocyte precursors were removed with the neural crest (Detwiler, 1937; Balinsky, 1970). Control larvae are pigmented (Figure 2b). As in previous studies (Wintrebert, 1905; Hooker, 1911), aneurogenic larvae were never seen to move spontaneously and nerves were never detected in the aneurogenics even with the electron microscope. Aneurogenic limbs extended from the body and the only movement visible was in the heart and circulating blood. However, control larvae were seen to use their forelimbs spontaneously in walking movements attesting to the functional innervation of these limbs by the "isolated spinal cord". Furthermore, control limbs and control mid-trunk regions showed reflex movements upon stimulation by touch. The control limb musculature was seen in the electron microscope to be normally innervated although a myofiber has been seen with two neuromuscular junctions (Plate 1). It is not known whether or not multiple innervation in Amblystoma is the rule or appeared only under these experimental conditions. With the exception of one case (Plate 1),

control sections showed one neuromuscular junction per fiber which, of course, does not rule out the possibility of further innervation elsewhere along the myofiber.

Aneurogenic and control limbs developed at exactly the same rate. Notch forelimbs developed at 13 days after the operation with the musculature largely in the early myotube stage of development, three digits developed at 21 days and four digits at 31 days (note developmental stages schematically indicated on all figures). On the other hand, normal, unfed larvae never developed beyond the three digit stage and were observed to have depleted their yolk supply at that time. Presumably lack of food arrested the development of normal, unfed larvae at the three digit stage. The total aneurogenic and control larvae in this study had an adequate yolk supply until the four digit stage and beyond and presumably quiescence in aneurogenics and reduced movement in controls conserved food for complete development. It is for these reasons that normal, unfed larvae were not used as controls and experimental controls were produced. Also, control larvae were more equal to aneurogenics since their nervous system was removed in a similar fashion (brain, pituitary etc.) except for the segment of spinal cord dorsal to the limbs.

The entire limbs (Table 2, Figure 3) in control and aneurogenic larvae were identical in thickness (Table 6)

at 13 days after the operation. Starting with day 21, aneurogenic limbs were thinner except for day 65 when aneurogenic limbs were actually thicker than control limbs. Possibly the sharp increase in limb thickness from day 54 to 65 was due to a general edema as seen previously for whole animals (Hooker, 1911). Surprisingly, even if the fluid (unstained clear spaces) was electronically subtracted from limb cross sections one sees an increase in aneurogenic limb tissues from day 54 to 65 (Figure 4). Muscle nuclei increased in cross sectional size also during the same time (Figure 9). Possibly, in addition to the influx of more intercellular fluid during the last nine days of the experimental series, intracellular fluid increased also.

The aneurogenic humeri grew in size from day 13 to 43 but remained constant in size from day 43 on (Figure 5, Table 2). It is important to note that the humeri were completely insensitive to the absence of nerves either during the humerus growth period (day 13 to 43) or during the maintenance period (day 43 to 65, Table 6). Even limbs from normally feeding animals, where limb tissues were twice as abundant as in aneurogenics, contained humeri of aneurogenic or control size. Apparently, even normalcy and feeding had no effect on the cross sectional size of humeri.

The tendons also showed a remarkable insensitivity to

the absence of nerves (Plate 4). This insensitivity was dramatized when tendons became extremely conspicuous in limbs where the muscle had almost completely disappeared (Plates 2, 3). Normalcy, on the other hand, did have an effect on tendons as for example in 65 day normal tendons (compare Plate 4 with Plates 5, 5a). The normal tendons are much thicker at day 65, contain more collagen within intercellular spaces and have more nuclei than aneurogenic tendons.

A single limb tissue most susceptible to the aneurogenic condition was musculature. In two cases all traces of muscle, as seen with the light microscope, had disappeared by 54 and 65 days (Table 1, Appendix). Although Table 3 indicates that whole aneurogenic limbs underwent a slightly greater change during the 13 to 65 day period than muscle, it probably is a reflection of the additive effects of all limb tissues. Figure 6 indicates an insignificant (Table 4) increase in aneurogenic muscle mass from 13 to 21 days whereas ultrastructural studies have shown deposition of contractile material at that time (Tweedle et al., 1974). In the neonatally denervated rat limb muscle Zelena (1962) observed an initial three to five fold increase in weight. Cricket thorax muscle denervated at juvenile stages also grew slightly in cross sectional area and fiber number (Thommen, 1974). The fact that the nuclear

density (Figure 10) decreased by about 50% in aneurogenic muscle from day 13 to 21 (D/C ratio, Table 10) combined with the statistical probability that the muscle area per limb remained constant during this time period leads one to conclude that some growth of aneurogenic muscle must have occurred from day 13 to 21. As mentioned above, aneurogenic muscle growth was detected with the electron microscope; aneurogenic early myotubes possessed much less contractile protein than fully differentiated, four digit, aneurogenic myofibers (Tweedle et al., 1974). Control muscle grew most rapidly during the 13 to 31 day period (Table 5, Figure 6) but also declined later in time. Aneurogenic and control larvae had depleted their yolk supply by approximately 54 days. Therefore, one could assume that the decline of control muscle from 54 to 65 days was due to starvation. Aneurogenic muscle disappeared in time beginning with day 21 and finally reached amounts one tenth of controls at 54 days. A couple of reference plots for normal muscle at 54 and 65 days indicate the enormous effect of normalcy and feeding on muscle growth. By 65 days normal muscle was six times the amount of control muscle. Statistically, the initial amounts of control and aneurogenic muscle per limb were equal at 13 days but soon thereafter the amounts of aneurogenic and control muscle diverged and remained statistically separated throughout the

remaining time period. Except on day 13, aneurogenic muscle was always much less than control muscle. Control muscle grew rapidly whereas aneurogenic muscle grew only feebly and then degenerated soon. Clearly, the only difference between the two groups of animals were the pieces of spinal cord left in control embryos in time innervating control limbs.

The total number of muscle nuclei per limb cross section gradually declined in aneurogenic limbs (Figure 8) shown statistically in Table 3. However, such a decline during the 13 to 65 days period was not evident at the 1% level of significance in control muscle. Statistical equality between aneurogenic and control number of muscle nuclei from 13 to 21 days suggest that total number of muscle nuclei in aneurogenics and controls are the same during that time. Beginning with day 31, however, total number of muscle nuclei are different in control versus aneurogenic limbs (Table 6) suggesting that survival and possibly proliferation of nuclei was interfered with in aneurogenics but less in controls. By counting the number of muscle nuclei in the denervated thorax musculature of the cricket, Thommen (1974) also saw a reduction in the number of nuclei per individual muscle. For example, two of the thorax muscles contained 6.4% and 15% of normal numbers of nuclei after denervation at stage 3 or 5 and measurement at adult stages. However, a shorter denervation period

led to a survival of a higher number of nuclei. Gutmann and Zelená (1962) wrote that nuclear changes are still controversial but claim a temporary 40% increase in DNA during the first few months of denervation in adult rat limb muscle followed by a return to normal levels thereafter. In the present study, an increase in total number of nuclei per aneurogenic limb even from notch to the three digit stage, a time when myotubes differentiate, is doubtful.

The amount of nucleoplasm in cross section per arbitrary aneurogenic muscle area shows an increase to notch stage levels by day 54 (Figure 10, Tables 10, 13, 14). This increase in nuclear density does not seem to have occurred by swelling of nuclei (Gutmann and Zelená, 1962) since the size of muscle nuclei actually decreased with developmental age (Figure 9, Table 10). Apparently, the increase in nuclear density of aneurogenic muscle is a reflection of greater rate of disappearance of myofibrillar material relative to nuclear numbers combined with a greater survival rate for myonuclei. A similar survival of muscle nuclei relative to denervated muscle was hinted at by Gutmann and Zelená (1962). If one rearranges the data obtained by Thommen (1974) into a form similar to the one employed in this study one sees the same increased number of muscle nuclei per muscle area in all of the denervated cricket thorax

muscles. Figure 10 furthermore shows a parallel decrease in nuclear density in control and aneurogenic limb muscle from 13 to 21 days of development. During this period, the difference between aneurogenics and controls is not statistically significant (Table 14) allowing one to conclude that nuclear densities are the same in aneurogenic and control muscle at 13 and 21 days. Such a conclusion implies some growth of aneurogenic muscle during the early part of limb development. Starting with day 21, control nuclear density reaches a plateau of about 15% nucleoplasmic area per muscle area whereas aneurogenic muscle shows a sharp increase in nuclear density as mentioned previously. From the three digit stage of development until the termination of the experimental series aneurogenic nuclear densities were distinctly and statistically significantly (Table 14) separated from control densities.

It was previously mentioned that individual nuclear areas decrease with progressing developmental age (Figure 9, Table 10). Since no statistical difference between aneurogenic and control muscle is apparent until the four digit stage (Table 14) it seems that nuclei decrease in cross sectional size regardless of whether the muscle is innervated or not during this time. However, at 43 and 54 days aneurogenic nuclei are statistically smaller in cross section than control nuclei. A decrease in cross

sectional area, of course, does not necessarily mean a decrease in volume since it is well known that myonuclei elongate as muscle differentiates. However, Table 12 shows the length of muscle nuclei for aneurogenics and controls at 43 days of development and analysis of variance indicates at the 1% level of significance that all nuclei (aneurogenic and control) are of the same length. So, one can conclude that at least 43 day aneurogenic nuclei are smaller in volume than control nuclei making it improbable that a differential elongation of aneurogenic vs. control muscle nuclei occurs. Armed with this piece of information, one could interpret the steeper slope of aneurogenic nuclear size as compared to the control slope (Figure 9) to mean a greater rate of nuclear shrinkage for aneurogenic muscle nuclei. At 65 days aneurogenic, control, and normal muscle nuclei are statistically the same; the apparent size increase in aneurogenic nuclei from day 54 to 65 is probably due to a general limb edema as discussed previously. When the number of peripherally located nuclei were counted as a measure of myofiber differentiation (Figure 7, Table 8), it was found that aneurogenic myofibers from the upper forelimb reached their peak differentiative state at the four digit stage. Control myofibers differentiated at the same time and remained differentiated whereas aneurogenic

fibers appeared to return to an undifferentiated state. Possibly the apparent return of aneurogenic myofibers to an undifferentiated state is an illusion since, as already discussed, contractile material disappears at a greater rate than nuclei leaving nuclei with a thin rim of cytoplasm in the latter part of the experimental series (Plate 6). That control fibers reached a plateau in their differentiation and aneurogenic fibers did not is indicated by the statistical comparison of aneurogenic and control fibers (Table 8) during the 31 to 43 day time span. Control fibers do not differ statistically from 31 to 43 days at the 5% level of significance whereas aneurogenic fibers do during the same time period and at the same level of significance. At notch stages of development control and aneurogenic fibers were equally undifferentiated; however, subsequent to day 13 control fibers were always more differentiated than aneurogenic fibers. Both conclusions are confirmed by statistical inference (Table 8). Even at their peak differentiative state, the aneurogenic myofibers were less differentiated than control fibers.

In a previous study (Tweedle et al., 1974) ultrastructural changes during aneurogenic muscle degeneration were described. In the present examination of aneurogenic muscle degeneration it was found that the ultrastructural characteristics of degeneration as

described by Tweedle et al. applied here also, although the methodology employed differed and larvae were totally aneurogenic. The most striking characteristics of aneurogenic muscle at its peak differentiative period is the abnormal dilation of the sarcoplasmic reticulum. Otherwise, aneurogenic myofibers looked quite normal at that time.

Basement laminae developed in aneurogenic myofibers in a similar fashion as in normal limbs (compare Plate 11 with 12) indicating that the absence of nerves did not affect the development of fiber sheaths. Typical muscle satellite cells (Mauro, 1961) were detected in aneurogenic, control, and normal Amblystoma muscle although satellite cells are characteristically found in mammalian muscle only and have not been seen heretofore in amphibian muscle except in Amblystoma mexicanum (Flood, 1964; Flood, 1971).

The mechanism by which muscle atrophies and contractile protein disappears has remained a mystery to this day. Thus, Kohn (1964, 1966) did not find enzymes capable of digesting myosin in denervated muscle nor has contractile protein been seen within lysosomal membranes. In the aneurogenic limb muscle stained for acid phosphatase, lysosomes were seen between disintegrating myofibrils (Plate 10) but as others have noted (Lockshin and Beaulaton, 1974a,b; Kohn, 1966) besides

the presence of lysosomes no evidence of lysosomal digestion of contractile material has been observed. Some macrophages were seen to ingest muscle fragments (Plate 8) but did not seem numerous enough to account for the massive disappearance of aneurogenic muscle. Interestingly, Lockshin (1975) observed the appearance of muscle antigens in the hemolymph of silkmoths during normal degeneration of intersegmental muscle. Concomitantly, enzymes, which have been shown to appear in dystrophic muscle, also appeared in hemolymph. In addition, Goldberg et al. (1974) claim an increased rate of protein degradation and decreased rate of synthesis in denervated or disused muscle. The reverse is claimed to take place during work induced muscle hypertrophy i.e. synthesis increases and degradation decreases; however, no values are given. Possibly, as in other muscle systems (Goldberg et al., 1974), so in aneurogenic Amblystoma larvae the homeostatic turnover balance between catabolism and metabolism of myoprotein is shifted towards the catabolic mechanism; protein and amino acids liberated from aneurogenic muscle end up in the bloodstream to be reutilized or metabolized elsewhere. At this point it is interesting to note that aneurogenic muscle was far from being metabolically inactive but incorporated tritiated leucine (Plate 7) and tritiated uridine vigorously. In fact, by visual inspection more

disintegration grains were found over myofibers where cross-striations had disappeared than over fibers where cross-striations were still clearly visible. Possibly myofibers well underway in their atrophy synthesize more protein and RNA than relatively unatrophied fibers.

That aneurogenic muscle degeneration is readily reversible was shown in experiments in which 70 to 90 day old aneurogenic limbs were orthotopically transplanted. Most animals regenerated a new limb next to the transplanted one but in one case the animal failed to regenerate its own limb and the transplanted limb became entirely functional. Piatt (1942) determined that aneurogenic limbs became approximately normally innervated in a three dimensional pattern after orthotopic transplantation to normal larvae and in the one successful case in this study normal use of the transplanted forelimb spoke for normal innervation. Fifty days after transplantation the larva was fixed and the limb examined in cross section. The muscle tissue was almost as extensive as in the contralateral normal limb indicating that aneurogenic, greatly atrophied muscle had grown back to almost normal cross sectional dimensions after innervation and use. Another indication of easy reversibility of aneurogenic muscle degeneration has come from regeneration studies. Aneurogenic limbs were amputated and regenerated normally (Yntema, 1959a,

b; Thornton, 1969). When the limb muscle was studied in longitudinal section it appeared as if proximal to the amputation plane muscle continued to atrophy but that distal to the amputation plane muscle had regenerated from degenerating muscle remains. However, the results were not clear cut and therefore are not discussed further in this report.

# APPENDIX

Table 1

LIMB TISSUE AREAS

| Age of animals (days) | Animal I.D. | Whole limb area ($um^2$) | Solid tissue area ($um^2$) | Humerus area ($um^2$) | Total muscle area ($um^2$) | Total number of myonuclei | |
|---|---|---|---|---|---|---|---|
| 13 | 45-15s | 82100 | 66372 | 9443 | 8767 | 57 | Aneurogenic animals |
| | 45-15d | 87172 | 69685 | 10201 | 6779 | 60 | |
| | 45-17s | 78275 | 53816 | 9685 | 8239 | 41 | |
| | 45-18s | 75260 | 59900 | 11306 | 4253 | 40 | |
| | 45-16s | 82772 | 65696 | 10087 | 5539 | 38 | |
| 21 | 46-5 | 123840 | 63510 | 11454 | 6081 | 49 | |
| | 46-6 | 115340 | 42940 | 13385 | 5089 | 41 | |
| | 46-4 | 87460 | 65510 | 10787 | 10910 | 51 | |
| | 46-10 | 90300 | 57130 | 11301 | 10746 | 49 | |
| | 46-3 | 82610 | 56270 | 14009 | 7944 | 49 | |
| 31 | 47-8 | 87020 | 73800 | 12765 | 5172 | 51 | |
| | 47-5 | 86870 | 40500 | 14671 | 3039 | 32 | |
| | 47-3 | 70510 | 36970 | 11110 | 4082 | 41 | |
| | 47-12 | 65680 | 33840 | 12325 | 4271 | 36 | |
| | 47-2 | 87020 | 51230 | 15414 | 5080 | 41 | |
| 43 | 48-15 | 57686 | 46952 | 15584 | 5645 | 40 | |
| | 48-1 | 102300 | 53860 | 19843 | 2114 | 29 | |
| | 48-6 | 92370 | 58710 | 14953 | 7318 | 44 | |
| | 48-9 | 108350 | 68180 | 15521 | 6221 | 33 | |
| | 48-16 | 63240 | 42390 | 17430 | 4603 | 47 | |
| 54 | 43-13 | 44270 | 27040 | 13910 | 0 | 0 | |
| | 43-3 | 61770 | 29960 | 15324 | 664 | 12 | |
| | 43-6 | 62460 | 41770 | 17041 | 1050 | 23 | |
| | 43-9 | 87510 | 47980 | 19111 | 782 | 28 | |
| | 43-14 | 55740 | 41560 | 13280 | 610 | 28 | |
| 65 | 50-5 | 158866 | 69560 | 10291 | 655 | 11 | |
| | 50-8 | 150180 | 11200 | 16278 | 0 | 0 | |
| | 50-7 | 163000 | 80660 | 19332 | 1170 | 21 | |
| | 50-9 | 145580 | 81480 | 16537 | 675 | 7 | |
| | 50-2 | 136900 | 68060 | 14557 | 1085 | 18 | |
| 43 | 48-4r | 173350 | 140367 | 14998 | 25572 | 126 | Normal animals |
| | 48-7r | 181020 | 95870 | 11485 | 28492 | 125 | |
| | 48-3r | 197640 | 134960 | 18874 | 39898 | 145 | |
| | 48-12r | 151520 | 108890 | 11431 | 26571 | 135 | |
| | 48-10r | 170430 | 135430 | 20499 | 32355 | 133 | |
| 65 | 50-11r | 188260 | 143700 | 12419 | 38915 | 130 | |
| | 50-9r | 267120 | 206940 | 11194 | 82417 | 200 | |
| | 50-8r | 236430 | 146640 | 20252 | 54882 | 187 | |
| | 50-10r | 318790 | 257980 | 18115 | 83395 | 266 | |
| | 50-15r | 154180 | 119210 | 12538 | 37335 | 93 | |

**Table 1**

LIMB TISSUE AREAS (Cont.)

| Age of animals (days) | Animal I.D. | Whole limb area ($um^2$) | Solid tissue area ($um^2$) | Humerus area ($um^2$) | Total muscle area ($um^2$) | Total number of myonuclei | |
|---|---|---|---|---|---|---|---|
| 13 | 45-5 | 71900 | 56260 | 12639 | 6965 | 106 | Control animals |
| | 45-13 | 88946 | 82312 | 12233 | 6375 | 56 | |
| | 45-1 | 80520 | 66900 | 9446 | 9917 | 67 | |
| | 45-3 | 96350 | 68770 | 13304 | 10092 | 51 | |
| | 45-9 | 80635 | 54972 | 11248 | 8432 | 77 | |
| 21 | 40-6s | 125310 | 84860 | 14800 | 18405 | 81 | |
| | 46-5s | 120900 | 83520 | 12701 | 16519 | 49 | |
| | 46-4s | 128420 | 95690 | 17473 | 22494 | 76 | |
| | 46-8s | 140140 | 61230 | 11443 | 13100 | 50 | |
| | 46-9s | 144660 | 72600 | 14366 | 13754 | 57 | |
| 31 | 47-4s | 103140 | 79922 | 11490 | 16518 | 68 | |
| | 47-15s | 111580 | 94500 | 14100 | 25381 | 51 | |
| | 47-13s | 116350 | 75180 | 15467 | 32259 | 61 | |
| | 47-8s | 126530 | 78420 | 8768 | 14310 | 66 | |
| | 47-7s | 121870 | 78720 | 14599 | 26138 | 78 | |
| | 47-14s | 118600 | 84510 | 12958 | 22691 | 73 | |
| 43 | 48-10s | 110200 | 81765 | 17873 | 19602 | 65 | |
| | 48-5s | 88250 | 41300 | 12056 | 14897 | 70 | |
| | 48-15s | 104930 | 64490 | 18674 | 19611 | 79 | |
| | 48-9s | 141310 | 72910 | 13413 | 17588 | 60 | |
| | 48-3s | 146700 | 99840 | 14026 | 29119 | 74 | |
| 54 | 49-13 | 104370 | 75050 | 10570 | 9096 | 41 | |
| | 49-2 | 95280 | 73220 | 13850 | 9792 | 48 | |
| | 49-14 | 89710 | 55310 | 17900 | 22713 | 62 | |
| | 49-9 | 112050 | 94930 | 15267 | 36354 | 63 | |
| | 49-10 | 99700 | 52050 | 14890 | 15356 | 52 | |
| 65 | 50-1s | 94180 | 38014 | 15969 | 7299 | 36 | |
| | 50-16s | 112020 | 71130 | 13181 | 11908 | 57 | |
| | 50-14s | 116540 | 74120 | 12816 | 8909 | 48 | |
| | 50-4s | 101230 | 66390 | 13496 | 9096 | 35 | |
| | 50-7s | 89540 | 55910 | 16987 | 11375 | 48 | |

## Table 9

MYONUCLEAR AREA ($um^2$)

Aneurogenic muscle

| | 13 days | | | | 21 days | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Animal I.D. | 45-15d | 45-18s | 45-16s | 45-17s | 46-3 | 46-6 | 46-5 | 46-4 | 46-10 |
| | 33.0 | 25.0 | 34.5 | 82.9 | 44.2 | 44.7 | 29.1 | 33.6 | 70.5 |
| | 36.7 | 50.0 | 55.8 | 70.9 | 40.0 | 50.7 | 49.4 | 56.0 | 28.7 |
| | 50.0 | 52.9 | 81.0 | 93.0 | 54.2 | 34.9 | 43.9 | 35.6 | 72.6 |
| | 37.3 | 46.0 | 48.0 | 85.0 | 31.8 | 65.0 | 31.0 | 40.6 | 57.6 |
| | 37.3 | 46.0 | 48.0 | 47.0 | 14.1 | 70.0 | 37.3 | 20.8 | 36.3 |
| | | 46.9 | 50.1 | 76.0 | 64.3 | | 23.0 | 36.6 | 36.0 |
| | | | 29.6 | 130.0 | 62.1 | | 28.7 | 58.1 | |
| | | | 29.6 | | 40.0 | | 29.0 | 58.0 | |
| | | | | | 61.0 | | | 44.2 | |
| | | | | | 31.8 | | | 32.5 | |
| | | | | | | | | 43.5 | |
| | | | | | | | | 44.0 | |
| | | | | | | | | 56.0 | |

| | 31 days | | | | | 43 days | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Animal I.D. | 47-2 | 47-12 | 47-5 | 47-3 | 47-8 | 48-16 | 48-1 | 48-9 | 48-6 | 48-15 |
| | 47.0 | 22.4 | 23.8 | 15.0 | 51.7 | 37.4 | 63.2 | 31.2 | 34.5 | 40.9 |
| | 30.0 | 46.8 | 6.2 | 25.0 | 42.1 | 27.6 | 24.2 | 34.5 | 35.8 | 36.2 |
| | 46.5 | 26.1 | 27.4 | 32.0 | 66.3 | 14.3 | 35.2 | 32.1 | 34.5 | 15.0 |
| | 41.4 | 50.6 | 27.4 | 39.0 | 46.0 | 23.4 | 28.2 | 22.0 | 29.4 | 27.2 |
| | 44.3 | 23.5 | 28.0 | 35.0 | 40.0 | 43.8 | 26.2 | 30.2 | 26.2 | 31.3 |
| | 21.8 | 24.0 | 28.0 | 20.3 | 35.0 | 26.9 | 26.0 | 8.0 | 32.7 | 16.5 |
| | 57.4 | | 28.0 | 13.0 | | 32.2 | 21.6 | 68.5 | 27.4 | 16.0 |
| | 65.4 | | 28.0 | 27.0 | | 28.6 | 21.0 | 28.8 | 27.0 | |
| | 33.0 | | 28.0 | 32.0 | | 12.2 | 33.5 | 21.3 | 28.0 | |
| | 38.9 | | 28.0 | 15.5 | | 14.2 | 34.0 | 21.3 | 26.0 | |
| | 42.5 | | 28.0 | 15.0 | | 14.0 | | 21.3 | | |
| | | | 28.0 | 30.0 | | | | 21.3 | | |
| | | | | 29.3 | | | | 29.0 | | |
| | | | | 30.0 | | | | | | |

Table 9 (cont.)

MYONUCLEAR AREA ($um^2$)

| | 54 days | | | | | 65 days | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Animal I.D. | 43-9 | 43-14 | 43-3 | 43-6 | 43-13 | 50-2 | 50-5 | 50-9 | 50-7 | 50-18 | |
| | 24.5 | 18.2 | 15.2 | 16.8 | 0.0 | 27.6 | 37.0 | 32.3 | 53.5 | 24.5 | Aneurogenic muscle |
| | 35.6 | 16.9 | 23.3 | 20.4 | | 28.4 | 28.6 | 28.1 | 7.5 | 32.2 | |
| | 28.3 | 18.9 | 16.9 | 26.7 | | 12.8 | 29.7 | 59.8 | 19.7 | 29.0 | |
| | 17.6 | 12.2 | 20.6 | 12.4 | | 29.6 | 25.0 | 20.0 | 25.4 | 18.8 | |
| | 25.2 | 13.7 | 20.0 | 12.0 | | 27.8 | 24.4 | 21.1 | 13.1 | 26.9 | |
| | 17.3 | 13.0 | | | | | 24.4 | | 29.9 | | |
| | 33.9 | 13.0 | | | | | | | | | |
| | 22.8 | 16.7 | | | | | | | | | |
| | 12.7 | 13.9 | | | | | | | | | |
| | 20.3 | 13.2 | | | | | | | | | |
| | 19.7 | 17.6 | | | | | | | | | |
| | 15.1 | 13.4 | | | | | | | | | |
| | 16.0 | 21.0 | | | | | | | | | |
| | 18.0 | 11.0 | | | | | | | | | |
| | 18.0 | 17.0 | | | | | | | | | |
| | 18.0 | | | | | | | | | | |
| | 18.0 | | | | | | | | | | |

Table 9 (cont.)

MYONUCLEAR AREA ($um^2$)

Control muscle

| | 13 days | | | | | 21 days | | | |
|---|---|---|---|---|---|---|---|---|---|
| Animal I.D. | 45-1 | 45-9 | 45-5 | 45-13 | 45-3 | 46-4 | 46-5 | 46-9 | 46-6 |
| | 70.8 | 40.0 | 61.0 | 47.0 | 45.6 | 29.1 | 42.2 | 68.6 | 31.7 |
| | 60.9 | 40.1 | 50.0 | 47.0 | 45.6 | 46.9 | 28.9 | 44.0 | 33.7 |
| | 77.1 | 40.1 | 40.1 | 47.0 | 45.6 | 18.1 | 41.5 | 18.0 | 49.2 |
| | 48.8 | 30.0 | 30.0 | 42.0 | 45.6 | 68.3 | 58.4 | 25.0 | 41.2 |
| | 35.2 | 30.9 | 20.0 | 42.4 | | 60.8 | 49.6 | 22.0 | 21.9 |
| | 36.0 | 25.0 | 20.0 | | | 50.6 | 54.4 | 35.0 | 50.1 |
| | 54.0 | 26.7 | | | | 55.6 | 22.6 | 30.0 | 50.1 |
| | 54.0 | | | | | 20.0 | 25.0 | 30.0 | |
| | 73.0 | | | | | 20.0 | 46.4 | | |
| | 74.0 | | | | | 22.3 | 47.4 | | |
| | | | | | | | 116.0 | | |

| | 31 days | | | | | | 43 days | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Animal I.D. | 47-7s | 47-14s | 47-13s | 47-8s | 47-15s | 47-4s | 48-3s | 48-15s | 48-5s | 48-9s | 48-10s |
| | 50.3 | 55.7 | 49.3 | 39.3 | 54.4 | 40.9 | 19.9 | 22.6 | 41.8 | 87.9 | 32.8 |
| | 34.5 | 37.8 | 23.3 | 45.6 | 47.6 | 37.9 | 37.2 | 22.0 | 49.5 | 33.9 | 24.1 |
| | 40.6 | 29.0 | 23.3 | 40.0 | 28.5 | 45.5 | 13.2 | 27.1 | 28.5 | 23.8 | 29.7 |
| | 25.9 | 23.0 | 34.7 | 10.8 | 30.0 | 55.0 | 15.5 | 11.8 | 42.4 | 26.3 | 16.3 |
| | 37.2 | 13.7 | 40.0 | 31.6 | 43.8 | 49.1 | 29.9 | 25.1 | 18.3 | 33.9 | 34.3 |
| | 34.8 | 33.1 | 38.7 | 31.6 | 25.0 | 49.1 | 25.5 | 37.4 | 47.7 | 33.9 | 28.7 |
| | 18.2 | 20.8 | 34.7 | 31.6 | | | 26.1 | 42.1 | 61.1 | | 47.8 |
| | 29.7 | 32.1 | 25.5 | | | | 46.2 | 40.2 | 34.5 | | |
| | 36.2 | 29.0 | 65.1 | | | | 14.5 | | 36.4 | | |
| | 48.5 | 29.0 | | | | | 26.9 | | 20.4 | | |
| | 58.7 | | | | | | 18.8 | | 44.3 | | |
| | 23.0 | | | | | | 43.7 | | 26.5 | | |
| | 27.0 | | | | | | 26.0 | | 27.0 | | |
| | 57.0 | | | | | | 18.0 | | | | |
| | 46.0 | | | | | | 43.0 | | | | |
| | | | | | | | 34.0 | | | | |

Table 9 (cont.)

MYONUCLEAR AREA ($um^2$)

| | 54 days | | | | | 65 days | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Animal I.D. | 49-19 | 49-14 | 49-9 | 49-2 | 49-13 | 50-7s | 50-16s | 50-4s | 50-14s | 50-1s | |
| | 34.5 | 26.0 | 40.0 | 26.7 | 39.5 | 15.4 | 19.8 | 25.4 | 50.7 | 38.7 | Control muscle |
| | 27.8 | 66.6 | 39.1 | 40.5 | 26.0 | 38.3 | 28.6 | 26.6 | 24.8 | 14.3 | |
| | 53.9 | 36.5 | 52.0 | 23.0 | 26.0 | 59.9 | 19.2 | 31.2 | 30.0 | 38.5 | |
| | 38.9 | 30.2 | 50.0 | 22.0 | 26.0 | 36.0 | 31.5 | 19.7 | 15.5 | 15.5 | |
| | 50.6 | 39.9 | 33.7 | 43.8 | 26.0 | 25.0 | 23.4 | 20.3 | 25.8 | 29.4 | |
| | 22.5 | 29.2 | 47.0 | | 26.0 | 33.3 | 28.0 | 5.7 | 10.8 | 51.9 | |
| | 39.4 | 25.6 | 25.0 | | | 36.2 | 33.9 | 31.0 | 18.3 | 17.5 | |
| | 26.6 | 26.5 | 25.0 | | | 36.2 | 39.2 | | | | |
| | 55.7 | 27.0 | 25.0 | | | 25.0 | 39.6 | | | | |
| | 54.7 | | | | | 25.0 | 25.0 | | | | |
| | 44.5 | | | | | | 25.1 | | | | |
| | 22.2 | | | | | | 37.0 | | | | |
| | 31.0 | | | | | | 36.0 | | | | |
| | 31.0 | | | | | | 36.0 | | | | |

| | 43 days | | | | | 65 days | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Animal I.D. | 48-12r | 48-10r | 48-7r | 48-3r | 48-4r | 50-8r | 50-10r | 50-15r | 50-9r | 50-11r | |
| | 60.9 | 32.5 | 42.6 | 45.5 | 28.7 | 48.9 | 27.4 | 12.0 | 20.0 | 57.6 | Normal muscle |
| | 44.8 | 40.8 | 39.8 | 21.5 | 43.8 | 26.2 | 37.4 | 21.7 | 20.0 | 33.3 | |
| | 46.9 | 58.5 | 57.7 | 55.3 | 76.3 | 20.9 | 37.4 | 36.9 | 45.0 | 65.1 | |
| | 40.0 | 30.7 | 59.6 | 46.9 | 49.4 | 19.4 | 29.5 | 18.8 | 26.0 | 60.0 | |
| | 18.0 | 29.4 | 55.0 | 53.1 | 27.4 | 55.2 | 30.4 | 55.1 | 27.4 | 65.2 | |
| | 32.7 | 33.2 | 55.0 | 40.9 | 31.1 | 39.1 | 66.7 | 43.2 | 15.1 | 50.4 | |
| | 50.0 | 27.7 | 30.5 | 53.0 | 25.0 | 35.6 | 44.3 | | 42.5 | | |
| | 32.0 | 34.9 | | 12.0 | 25.0 | 22.5 | 44.0 | | | | |
| | 57.0 | 33.8 | | 44.2 | | 23.0 | 20.0 | | | | |
| | 34.0 | 49.6 | | 44.2 | | 16.1 | | | | | |
| | | 44.0 | | 50.0 | | 32.6 | | | | | |
| | | 13.0 | | 62.0 | | 27.5 | | | | | |
| | | | | 30.0 | | 23.0 | | | | | |
| | | | | 30.0 | | | | | | | |

Table 11

MYONUCLEAR LENGTH

| | Myonuclear length (in ocular units) of 43 days old animals | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | | | | | Aneurogenic | | | | |
| | 1.82 | 1.55 | 0.80 | 1.40 | 1.15 | 1.10 | 1.75 | 1.55 | 1.10 | 1.30 |
| | 1.50 | 1.55 | 1.55 | 1.30 | 1.00 | 1.20 | 1.25 | 1.10 | 1.25 | 1.40 |
| | 1.60 | 1.50 | 1.00 | 1.40 | 1.55 | 1.30 | 1.30 | 1.50 | 1.15 | 1.20 |
| | 1.68 | 1.25 | 1.10 | 1.30 | 1.25 | 1.00 | 1.15 | 1.50 | 1.20 | 1.35 |
| | 1.64 | 1.15 | 1.45 | 1.10 | 1.20 | 1.30 | 1.20 | 1.15 | 1.35 | 1.30 |
| | 1.20 | 1.00 | 1.40 | 1.35 | 1.50 | 1.40 | 1.15 | 1.25 | 1.10 | 1.65 |
| | 1.20 | 1.25 | 1.45 | 1.60 | 1.50 | 1.10 | 1.40 | 1.20 | 1.35 | 1.35 |
| | 1.72 | 0.95 | 1.45 | 1.50 | 1.80 | 1.00 | 1.25 | 1.35 | 1.50 | 1.25 |
| | 1.70 | 1.30 | 1.25 | 1.40 | 1.25 | 1.35 | 1.45 | 1.30 | 1.45 | 1.55 |
| | 1.72 | 1.20 | 1.00 | 1.50 | 1.25 | 1.20 | 1.50 | 1.25 | 1.45 | 1.35 |
| | | | | | | 1.65 | | | | |
| | | | | | | 1.35 | | | | |
| Animal I.D. | 48-10 | 48-2 | 48-13 | 48-3 | 48-4 | 48-8s | 48-6s | 48-14s | 48-12s | 48-2s |

1 ocular unit = 29.473 um

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