The Regeneration of a Limb Muscle in the Axolotl from Minced Fragments [1]

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ABSTRACT   In over 50 cases the pubo-ischio-tibialis muscle in mature axolotls was removed, minced and the minced fragments replaced into the site from which the muscle was removed. In 13 control animals the same muscle was removed but nothing was replaced. Regenerates were studied at post-operational intervals of up to 150 days. Both grossly and histologically the regeneration of muscles from minced fragments in the axolotl follows a course very similar to that observed in frogs and rats. There is an initial period of destruction of the sarcoplasm of the minced muscle fragments. This is characterized by intense phagocytic activity. Following this, a population of myoblasts is established and the differentiation of mature muscle fibers ensues. The regeneration of muscles from minced fragments is compared with the formation of muscles in regenerating limbs. Following mincing, new muscle fibers develop rapidly and without the mediation of a blastema. Following limb amputation, a blastema is established before any differentiation of muscle occurs in the regenerate. This occurs more slowly than the differentiation of muscle fibers following mincing. The implications of these differences are discussed.

Several investigators (Studitsky, '59; Zhenevskaya, '62; Gallucci et al., '66; Carlson, '68) have demonstrated that completely removed muscles in higher vertebrates (frogs, chickens and rats) can be reconstituted from re-implanted minced muscle fragments by a regenerative process histologically similar to that which occurs after many types of minor trauma. This type of regeneration is often referred to as tissue regeneration and consists of the appearance of a population of myoblastic cells, their fusion into multinucleated strap-like elements called sarcoblasts or myotubes and the differentiation of the elements of the contractile apparatus within these regenerating fibers. Such muscle regeneration proceeds without the early appearance of a blastema, but neither the cellular origin of the myoblastic cells nor the factors which control the differentiation and ultimate morphogenesis of minced muscle regenerates are known.

The regeneration of muscle as a tissue has been studied extensively in mammals (reviewed by Betz et al., '66; Field, '60; Studitsky, '59; Zhenevskaya, '62) and to a lesser extent in birds (Studitsky, '54) and adult anuran amphibians (Carlson, '68; Samsonenko, '56), none of which forms are normally capable of participating in complex epimorphic regenerative processes such as the restoration of an amputated limb. In salamanders and tadpoles, however, the regeneration of muscle has been studied as a part of the overall process of limb regeneration (De Haan, '56; Hay, '59, '62; Laufer, '59; Thornton, '38; Towle, '01), but the regeneration of muscle outside the overall context of limb regeneration has received little attention.

The research presented below was designed to answer the following question: In an animal with a high capacity for limb regeneration, what would happen if one removed a muscle, minced it and reimplanted the minced fragments? Would a new muscle regenerate in the manner which has been described for higher vertebrates or would the extensive damage to the muscle be a sufficient stimulus to elicit an epimorphic regenerative response in the form of a supernumerary limb?

MATERIALS AND METHODS

This experiment was conducted upon two year old black axolotls (*Siredon mexicanum*) ranging in length from 195–225

mm. After anesthetization in 1:1000 MS 222 (Sandoz) or 1:1000 ethyl m-aminobenzoate methane-sulfonate (Eastman), the pubo-ischio-tibialis (p.i.t.) muscle (Francis, '34) was removed through a skin incision as indicated in figure 1A. Although the p.i.t. muscle arises close to the midline, the muscle fibers fan out into a thin sheet proximal to the dotted line in figure 1A. The gross architecture of the muscle also undergoes a rather consistent and abrupt change at this level. This anatomical peculiarity plus the frequent presence of ectopic mucous glands in this region prompted the decision to remove only the distal portion of the muscle. After its removal, the muscle was minced by scissors into 1 mm³ fragments (fig. 1B), and the minced muscle fragments were then replaced into the site from which they were removed (fig. 1C). The incision in the overlying skin

was tightly sutured with 7-0 silk in order to prevent a wound epidermis from forming over the mass of minced muscle. The control experiment consisted of removal of the p.i.t. muscle as was done in the experimental series, but nothing was replaced. Sterile operating conditions were not necessary. Following the operations, the animals were kept in individual glass bowls and maintained at 21°C for the duration of the experiment. About 50 muscle regenerates were examined at intervals up to 150 days after the mincing operation. All regenerates were fixed in Bouin's, serially sectioned at 7 μ and stained with Ehrlich's hematoxylin and eosin. The histological course of muscle regeneration in this experimental system was compared with that seen in post-amputational limb regeneration in axolotls of the same size and maintained at the same temperature, feeding and environmental conditions.

RESULTS

Gross results. Experimental series. Grossly the development of minced muscle regenerates follows the pattern observed in other vertebrates. By the end of the first week the overall form of the mass of minced fragments is molded to an approximate model of the removed muscle. Because of early regenerative events and the proliferation of connective tissue at the periphery, the form of the regenerate is quite well maintained even though the central area contains little more than muscle fragments held together by fibrin. During the second and third week the total mass of the regenerate decreases because of the breakdown of the originally implanted muscle fragments. After the first month the size of the regenerate increases, the amount being proportional to the percentage of regenerating muscle fibers as opposed to connective tissue in the regenerates. Figure 2 illustrates two gross regenerates at 37 days.

Control series. In 13 cases the p.i.t. muscle was removed in the usual fashion, and in two cases the entire p.i.t. muscle was excised. The limbs were then examined at post-operative intervals of up to 82 days. Grossly, muscle regeneration was not observed from the cut ends of the
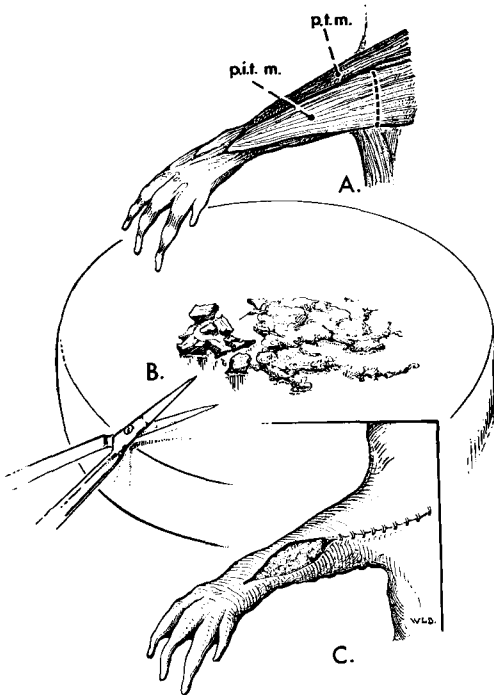

Fig. 1 Diagram of operation. A. Normal relationships of pubo-ischio-tibialis and pubo-tibialis muscles. All tissue of the p.i.t. muscle distal to the dotted line is removed. B. The muscle is minced on the back of a Petri dish. C. Reimplantation of the minced fragments and suturing of the skin.
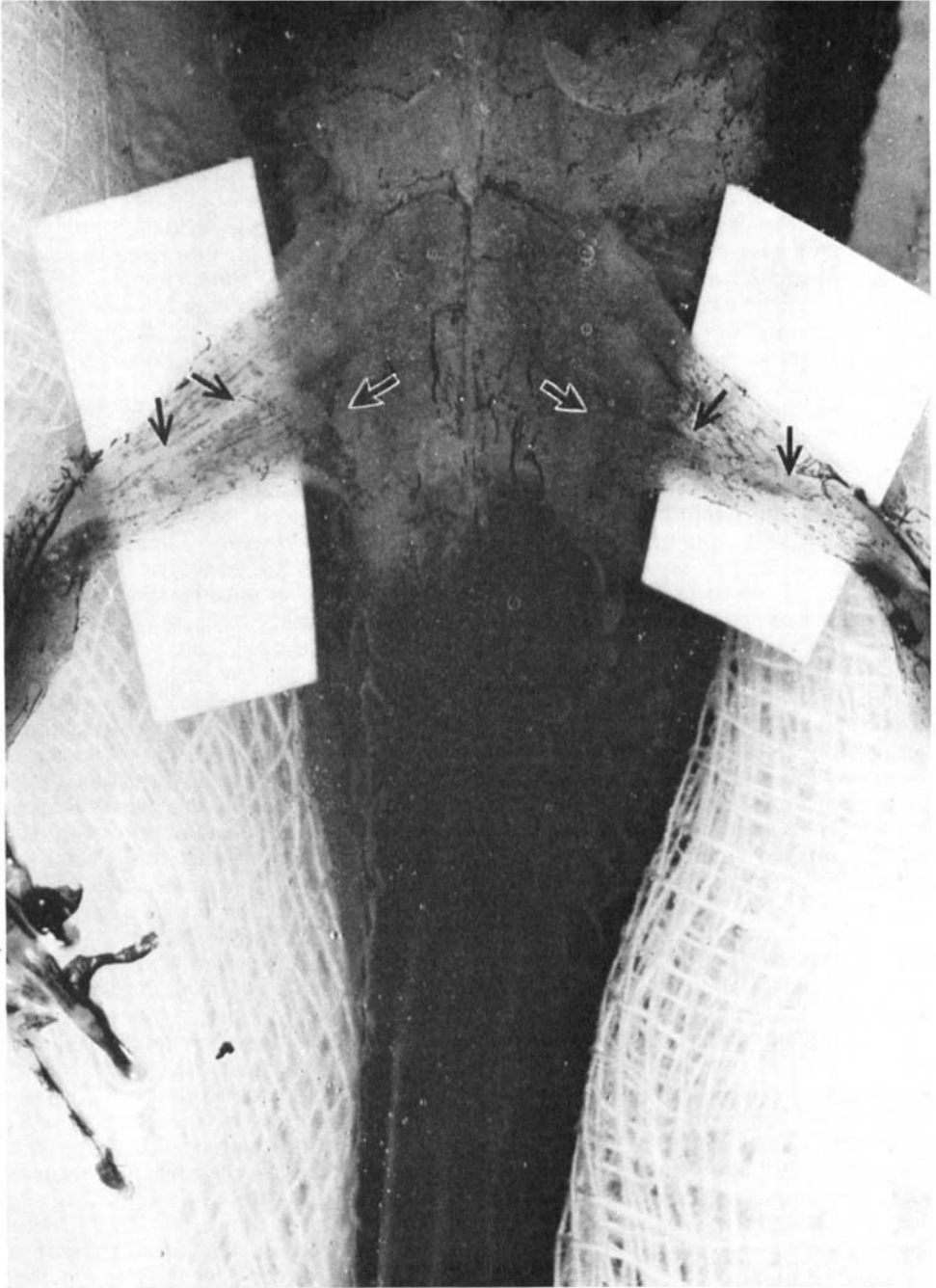
Fig. 2   Thirty-seven day muscle regenerate. The size and organization of the regenerate on the right limb is exceptional. That on the left is more typical. The extent of regenerates is indicated by arrows.

p.i.t. muscles. In two cases thin bands of connective tissue were observed connecting the stump of the excised muscle to the distal femur. Histologically, these did not contain muscle. Hypertrophy of the pubo-tibialis, the caudo-femoralis and the pubo-femoralis muscles is pronounced after 20 days. In one case a hypertrophied caudo-femoralis muscle was mistakenly thought to be a regenerate. This prompted a gross examination of two control limbs at five or six day intervals for 33 days. This procedure clearly indicated that no gross regeneration occurs from the distal cut end of the p.i.t. muscle. Figure 3 illustrates a control limb and shows the relationships of the surrounding muscles to the stump of the p.i.t. muscle.

Microscopic results. Experimental series. The general pattern of minced muscle regeneration in the axolotl is very similar to that described for rats and frogs (Carlson, '68). Throughout most of the first week pockets of erythrocytes and leukocytes may be seen scattered among the degenerating muscle fragments (fig. 4). Infiltration by macrophages and the subsequent degeneration of the sarcoplasm of the implanted muscle is well underway by the end of the first week. At this time the first cells which resemble myoblasts are visible. These may be either free spindle-shaped cells with very basophilic cytoplasm or similarly shaped cells located at the periphery of the degenerating muscle fibers (fig. 5). At this stage light microscopic observations do not permit greater specificity concerning the origin, identity or fate of these cells. Cuffs of basophilic cells lining the insides of basement membranes have been observed, but their frequency is considerably less than that observed in frogs or rats. Most of the spindle-shaped myoblastic cells are found in loose aggregates scattered throughout the developing connective tissue. Pigment and debris-laden macrophages form a prominent cellular component of the early regenerate. Fusion of the myoblasts begins at about ten days. Figure 6 illustrates several band-like aggregates of mononucleated myoblasts which have just begun to fuse. Mitotic figures among both the fibroblasts and myoblasts are not infrequently seen. In these

early stages of regeneration, the mass occupied by the developing connective tissue is greater than the mass of regenerating muscular elements. By the beginning of the third week, multinucleated myotubes with peripheral myofibrillae are present (fig. 7). The cytoplasm of the myotubes is still highly basophilic, but with a slight eosinophilic cast. The maturation of individual muscle fibers follows a course which differs little from that described in the frog (Carlson, '68). During the fourth week, the muscle fibers are quite thin, and the nuclei tend to be centrally located (fig. 8). They are not, however, arranged in the highly regular "nuclear chains" which are so characteristic of regenerating muscle in higher vertebrates. Early in the second month the muscle fibers have assumed all the histological characteristics of normal muscle except for diameter (fig. 9). The time required for the regenerating muscle fibers to attain normal diameters is quite long as may be seen in figure 10, which contrasts normal to regenerating muscle fibers at 81 days. Sections taken through a 150 day regenerate (fig. 11) show a return to normal diameter of the regenerated muscle fibers. In axolotls, as is the case with minced muscle regenerates in other vertebrates, the ratios of connective tissue to muscle are usually considerably greater than those seen in either normal muscle or muscles found in limb regenerates. The regenerate depicted in figure 11 is typical in terms of the ratio of muscle to connective tissue.

Comparison between the regeneration of muscle in an epimorphic process and the regeneration of a muscle from minced fragments. Limb regeneration in the axolotl has been described previously (Carlson, '69; DeHaan, '56; Hellmich, '30; Kazancev, '34) and will not be redescribed here. Instead, the time sequences and major morphological stages of muscle regeneration following mincing and limb amputation are compared in figure 12. The illustrations on the left hand side of the figure summarize the data presented above for the regeneration of minced muscle. The regeneration of muscle after limb amputation follows basically the same histological course as that described by Thornton ('38)
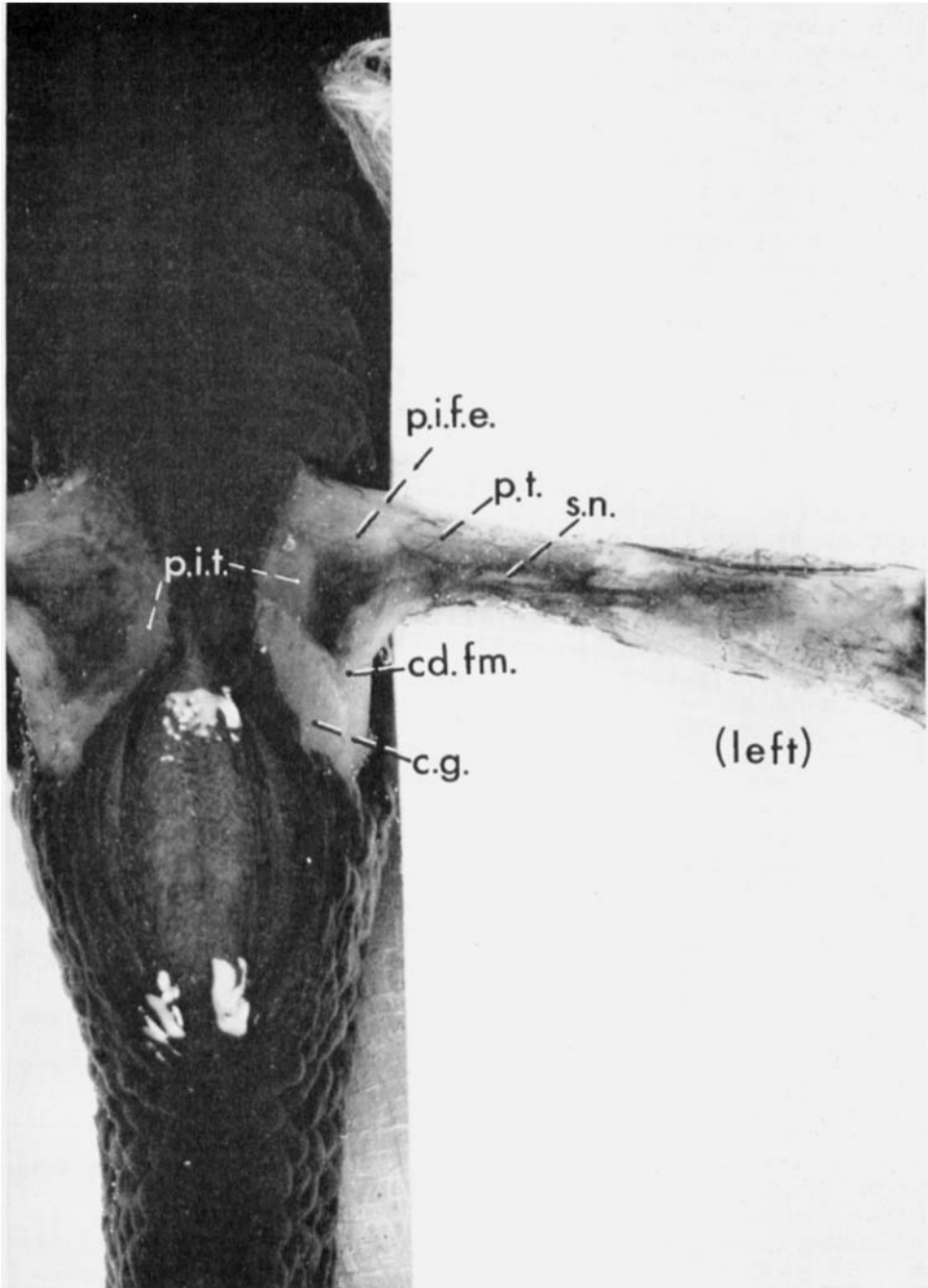
Fig. 3 Sixteen day controls showing relationships of other muscles to the stump of the p.i.t. muscle. The left limb is an exact control for the experimental series. In the right leg, the p.i.t. muscle was removed almost back to its origin. Cd. fm. (caudo-femoralis m.); p.t. (pubo-tibialis m.; p.i.f.e. (pubo-ischio-femoralis externus m); s.n. (sciatic nerve); c.g. (edge of cloacal gland).

for *Amblystoma punctatum* larvae. As the period of dedifferentiation comes to a close and the blastema is established, a certain amount of regeneration occurs as a direct outgrowth from the ends of the dedifferentiated muscle fibers in the limb stump. This regeneration, called sarcoplasmic budding by Thornton ('38), follows a morphological course very similar to that occurring in regeneration from minced muscle fragments. In the mature axolotl, however, this type of muscle regeneration in the amputated limb appears limited to the limb stump and the immediate vicinity of the level of amputation. Most of the muscle in the regenerating limb arises from cells located laterally to the newly differentiating cartilage. Before these cells have differentiated to a stage at which they can definitely be called myoblastic elements, they are histologically indistinguishable from those cells making up the rest of the blastema (fig. 12, 34 days). Under the conditions of the present experiment, aggregation of a dense-central core of pre-cartilaginous cells is just beginning during the fifth post-amputational week. After the cartilaginous digital primordia have differentiated, groups of spindle-shaped myogenic cells with basophilic cytoplasm become aligned parallel to one another. These cells then fuse lateral to the skeletal elements (fig. 12, 44 days). It was at this stage when DeHaan ('56) was first able to detect muscle proteins in the regenerate by the use of serological methods. It is important to note that the first masses of differentiating muscle fibers often appear in distal regions of the regenerate before they are distinguishable proximally. After this stage the individual muscle fibers undertake a morphological course of differentiation which is histologically indistinguishable from that occurring in minced muscle regenerates (fig. 12; 49 and 91 days). The outstanding feature of muscles formed in limb regenerates is their great similarity to normal muscles, both grossly and histologically. The architectural regularity of the muscle fibers and the extremely small amount of interspersed connective tissue offer a striking contrast to most minced muscle regenerates.

DISCUSSION

The results of this experiment clearly show that a given muscle in the limb of an axolotl can be regenerated under two sets of experimental circumstances — (1) as part of an overall epimorphic process occurring after limb amputation and (2) as an isolated process, the stimulus for which is the mincing of a muscle.

Although there are distinct similarities in the morphological end products of these two processes, some of the differences between them are even more striking. The differences which seem to be the most important are (1) the presence or absence of a blastema, (2) the rates of the regenerative processes and (3) the degree of morphological perfection of the regenerated muscles. An adequate interpretation of these differences is difficult because of the many factors which still remain unknown. Primary among the unknown factors remains the origin of the moygenic cells in either of the two regenerating systems. Although the majority of those who have studied limb regeneration in amphibians have supported the concept of morphological dedifferentiation whereby relatively unspecialized appearing cells are derived from nucleated elements broken off from multinucleated skeletal muscle fibers (DeHaan, '56; Hay, '59, '62; Lentz, '69; Thornton, '38; Towle, '01; Trampusch and Harrebomée, '65), many of those who have studied muscle regeneration similar to the type seen after mincing have implicated the satellite cell as the most likely source of myoblasts (Allbrook et al., '66; Muir et al., '65; Shafiq and Gorycki, '65; Shafiq et al., '67).

Despite our lack of definite *information* about the origin of the myogenic cells, it is apparent that histologically there is little or no difference in the subsequent differentiation of individual muscle fibers regenerating in either of the two experimental systems. Thus at the cellular level, the greatest known difference between the two regenerating systems is the relatively long delay in the onset of differentiation of muscle in epimorphic regeneration. The presence of a blastema in epimorphic regeneration seems to provide a key to our understanding of the delay. Whatever the

origin, new muscle fibers do not appear distal to the level of amputation during the period of the undifferentiated blastema. Only after the cartilaginous skeletal primordia have been laid down does muscle begin to differentiate. There is presently available little information which would be useful in interpreting the significance of this delay, but it appears to be reflected at the level of tissue or organ morphogenesis rather than in the differentiation of individual muscle fibers. In terms of gross morphology and internal architecture, a muscle in a regenerated limb is usually a perfect replica of the original. It forms in the virtual absence of mechanical function of the regenerating limb. In minced muscle regenerates, on the other hand, both gross form and internal architecture seem to depend upon the presence of mechanical forces (i.e., tension and pressure) in the limb (Carlson, unpublished; Studitsky, '63). It is possible that the delay in redifferentiation of muscle fibers in an epimorphic system of regeneration represents a period of time during which the myogenic cells are acquiring morphogenetic information which is needed for them to correctly associate with one another and form an entire muscle. In the tissue regeneration of muscle (after mincing) the conditions leading to the acquisition of such information may not be present. Thus the differentiating muscle fibers may have to depend upon mechanical factors alone in order to form a complete muscle.

Finally, the fact that after the mincing of a muscle in the axolotl a supernumerary limb does not form indicates that severe tissue damage alone does not in itself constitute an adequate stimulus for the initiation of an epimorphic regenerative process, in this case supernumerary limb formation.

LITERATURE CITED

Allbrook, D. B., W. deC. Baker and W. H. Kirkaldy-Willis 1966 Muscle regeneration in ex-

perimental animals and in man. J. Bone Joint Surg., 48B: 153–169.

Betz, E. H., H. Firket and M. Reznik 1966 Some aspects of muscle regeneration. Int. Rev. Cytol., 19: 203–227.

Carlson, B. M. 1968 Regeneration of the completely excised gastrocnemius muscle in the frog and rat from minced muscle fragments. J. Morph., 125: 447–472.

———— 1969 Inhibition of limb regeneration in the axolotl after treatment of the skin with actinomycin D. Anat. Rec., 163: 389–402.

DeHaan, R. L. 1956 The serological determination of developing muscle protein in the regenerating limb of Amblystoma mexicanum. J. Exp. Zool., 133: 73–85.

Field, E. J. 1960 Muscle regeneration and repair. In: Structure and Function of Muscle. III. G. H. Bourne, ed. Academic Press, N. Y., pp. 139–170.

Francis, E. T. R. 1934 The Anatomy of the Salamander. Oxford, 381 pp.

Gallucci, V., F. Novello, A. Margreth and M. Aloisi 1966 Biochemical correlates of discontinuous muscle regeneration in the rat. Brit. J. Exp. Path., 47: 215–227.

Hay, E. D. 1959 Electron microscopic observations of muscle dedifferentiation in regenerating Amblystoma limbs. Devel. Biol., 1: 555–585.

———— 1962 Cytological studies of dedifferentiation and differentiation in regenerating amphibian limbs. In: Regeneration. D. Rudnick, ed. Ronald Press, N. Y., pp. 177–210.

Hellmich, W. 1930 Untersuchungen über Herkunft and Determination des regenerativen Materials bei Amphibien. Arch. f. Entwmech., 121: 135–203.

Kazancev, W. 1934 Histologische Untersuchungen des Regenerationsprozesse an amputierten Extremitäten beim Axolotl hauptsächlich zweicks Klarung der Frage nach der Herkunft der Zellen des Regenerats. Trav. Lab. Zool. Exp. Leningrad, 3: 29–54.

Laufer, H. 1959 Immunochemical studies of muscle proteins in mature and regenerating limbs of the adult newt, Triturus viridescens. J. Embryol. Exp. Morph., 7: 431–458.

Lentz, T. L. 1969 Cytological studies of muscle dedifferentiation and differentiation during limb regeneration of the newt, Triturus. Am. J. Anat., 124: 447–480.

Muir, A. R., A. H. M. Kanji and D. Allbrook 1965 The structure of satellite cells in skeletal muscle. J. Anat., 99: 435–444.

Samsonenko, R. V. 1956 Development of muscular tissue in transplanted minced muscle in the place of completely removed muscular organs in frogs (in Russian). Arkh. Anat. Gist. Embryol., 33: 56–64.

Shafiq, S. A., and M. A. Gorycki 1965 Regeneration in skeletal muscle of mouse: some electron microscope observations. J. Path. Bact., 90: 123–127.

Shafiq, S. A., M. A. Gorycki and A. T. Milhorat 1967 An electron microscopic study of regen-

eration and satellite cells in human muscle. Neurology, *17:* 567–575.

Studitsky, A. N. 1954 Laws of restoration of muscle in higher vertebrates (in Russian). Trudy Inst. Morph. Zhivotnikh im. A. N. Severtsova, *11:* 225–264.

—— 1959 Experimental Surgery of Muscle (in Russian). Izdatel. Akad. Nauk SSSR, Moscow, 338 pp.

—— 1963 Dynamics of the development of myogenic tissue under conditions of explantation and transplantation. In: Cinemicrography in Cell Biology. G. G. Rose, ed. Academic Press, N. Y., pp. 171–200.

Thornton, C. S. 1938 The histogenesis of muscle in the regenerating forelimb of larval *Amblystoma punctatum.* J. Morph., *62:* 17–47.

Towle, B. W. 1901 On muscle regeneration in the limbs of *Plethodon.* Biol. Bull., 2: 289–299.

Trampusch, H. A. L., and A. E. Harrebomée 1965 Dedifferentiation a prerequisite of regeneration. In: Regeneration in Animals and Related Problems. V. Kiortsis and H. A. L. Trampusch, eds. North-Holland Publ. Co., Amsterdam, pp. 341–374.

Zhenevskaya, R. P. 1962 Experimental histologic investigation of striated muscle tissue. Rev. Canad. Biol., *21:* 457–470.

PLATE 1

EXPLANATION OF FIGURES

4 Five day regenerate. Pockets of erythrocytes and leukocytes are scattered among the degenerating muscle fibers. Note the irregular orientation of the minced muscle fragments. There is little peripheral regenerative activity. $\times$ 31.

5 Six day regenerate. Macrophages (M) may be seen within the degenerating sarcoplasm of the old muscle fibers. Spindle-shaped myoblastic cells (Mb) with basophilic cytoplasm are seen at the periphery of degenerating muscle fibers or as free cells. $\times$ 236.

6 Ten day regenerate. Aggregation and early fusion of myoblasts into bands. These bands are located in well vascularized early connective tissue along with many macrophages. $\times$ 124.

7 Sixteen day regenerate. Early myotube with longitudinal myofibrils (arrow) present. The granule containing cells are pigment-laden macrophages. $\times$ 513.
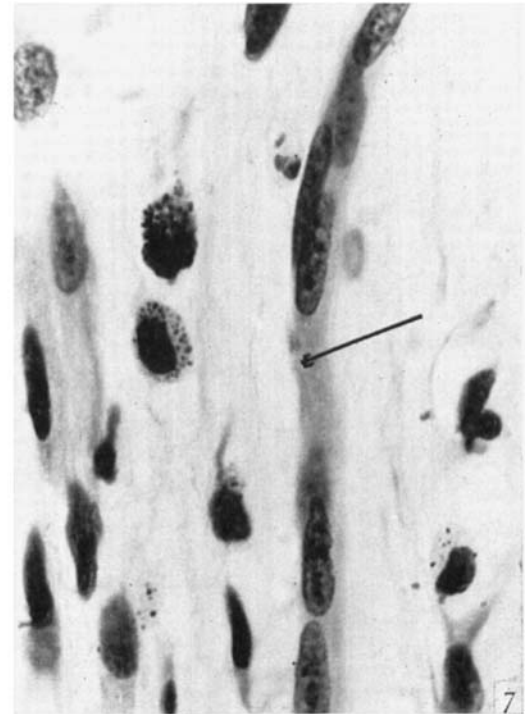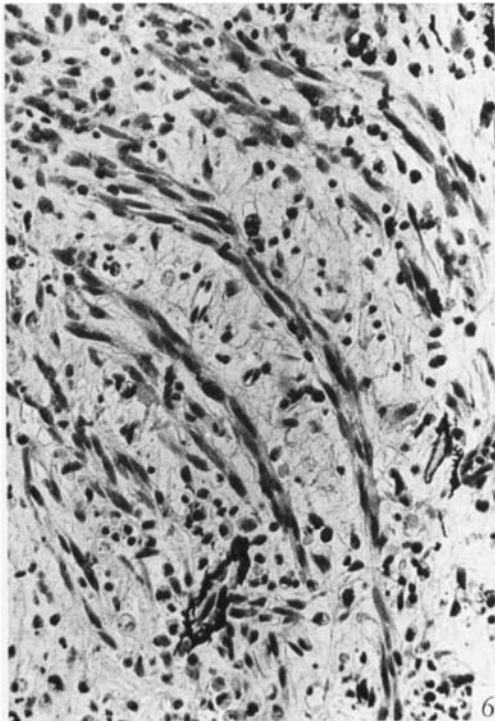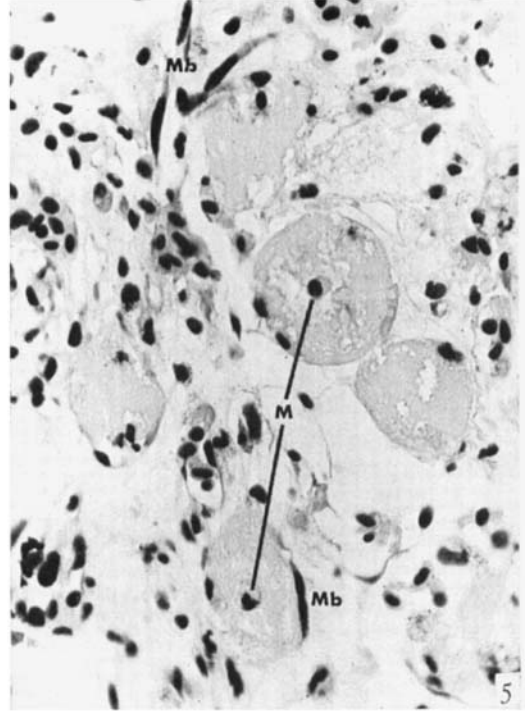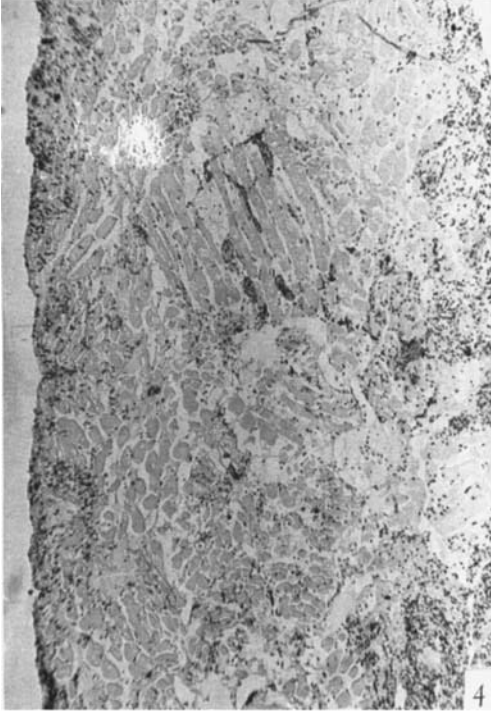
8   Twenty-six day regenerate. A field of young muscle fibers in early stages of differentiation. Scattered among them are macrophages. × 124.

9   Thirty-four day regenerate. A field of regenerating muscle. Most nuclei have moved to the periphery of the muscle fibers, and except for the diameters, the fibers appear to be normal. × 124.

10   Eighty-one day regenerate. A comparison between normal muscle fibers of the adjacent p.t. muscle and muscle fibers of the regenerating p.i.t. muscle. × 70.

11   One hundred and fifty day regenerate. The longitudinally sectioned fibers belong to the regenerated p.i.t. muscle. Note the diameter of the fibers and the amount of interspersed connective tissue. The cross sectioned muscle fibers belong to the normal caudo-femoralis muscle which meets the regenerate at right angles. Some vacuolization of a regenerated muscle fiber is present (arrow). × 31.
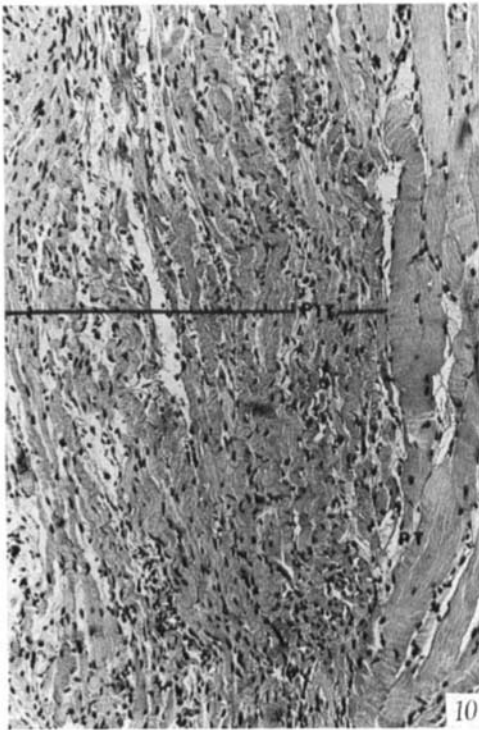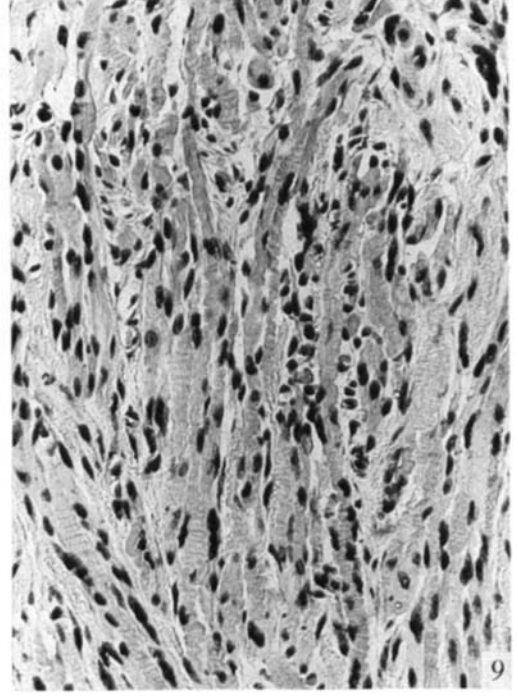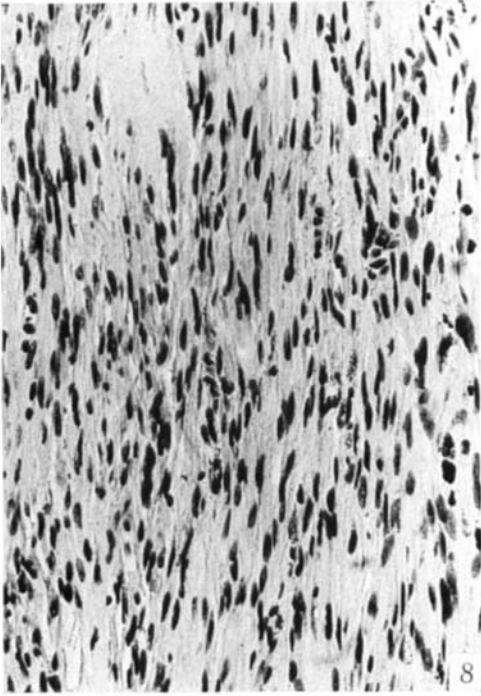
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