The Regeneration of Skeletal Muscle — A Review [1]

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Regeneration of skeletal muscle has often been described during the past century, but only during the past 25 years have attempts been made to uncover causal mechanisms underlying the process. The historical and classical histological aspects of skeletal muscle regeneration have been reviewed several times (Studitsky and Striganova, '51; Godman, '57; Field, '60; Betz et al., '66). Here I shall attempt to synthesize the results of the major experimental studies as well as place into sharper focus some of the new problems which have stemmed from electron microscopic investigations of the past decade.

With respect to both control mechanisms and certain morphological aspects of regeneration, considerable confusion has reigned in the past because events occurring in the repair of damaged mammalian muscle have been equated with those which take place in the regenerating amphibian limb. One of the major functions of this review will be to point out where similarities between these two processes may exist and where the systems are not directly comparable.

The review will conclude with a short discussion about the clinical implications of recent progress in the field of muscle regeneration.

CELLULAR ASPECTS OF MUSCLE REGENERATION

Mammalian muscle

After almost all forms of injury, the damaged skeletal muscle fiber goes through a characteristic degenerative reaction before the regenerative response becomes obvious. These changes have been summarized by Field ('60), Denny-Brown ('62) and Price et al. ('64a). It is important to recognize that the basement membrane of the injured muscle fiber remains intact during the early degenerative reaction and that almost no phagocytic cells are yet found within the muscle fiber.

Within a day or two the degenerating muscle fibers that are close to functioning blood vessels become heavily infiltrated with phagocytic cells which rapidly ingest the degenerating sarcoplasm. Almost concurrent with the phase of sarcoplasmic removal is the appearance of enlarged oval nuclei surrounded by a small amount of basophilic cytoplasm between the basement membrane and the degenerating muscle fiber. In areas far removed from a blood supply, necrosis sets in and sarcoplasmic degeneration occurs over a period of several days or weeks in the absence of an intracellular phagocytic reaction. No demonstrable nuclei remain under these conditions. In damaged muscle placed in filter chambers (O'Steen, '63; Aloisi, '70; Carlson, unpublished) or in vitro (Peterson and Crain, '72), sarcoplasmic degeneration is extremely slow, and although activated nuclei appear beneath the basement membrane, further progressive changes occur only slowly, if at all.

The viable-appearing nuclei lying between the degenerating muscle fiber and its basement membrane are surrounded by a thin rim of cytoplasm bounded by a discrete plasma membrane. They are assumed to be myoblastic cells or their precursors. The origin of these cells has been the subject of considerable debate during the past decade. According to some they arise by the separation of nuclei from the degenerating muscle fiber whereas another school contends that they are direct descendants of satellite cells. Details of this controversy will be presented later.

Not only the origin of myogenic cells,

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but also the nature of the conditions leading to their activation remains wholly obscure. The first question to be answered is whether the myogenic precursor cells or nuclei must be specifically stimulated to begin the regenerative response or whether they are always potentially active but inhibited from regenerating by the presence of the normal muscle fiber or the environment associated with it. Until the origin of myoblasts in regeneration is established, however, it will be extremely difficult to focus critical experiments or hypothetical reasoning upon factors which may stimulate or release the regenerative response.

Myoblasts. Whatever their origin, myoblasts are normally first apparent as spindle-shaped mononuclear cells located between the degenerating muscle fiber and its basement membrane. Myoblasts can not always be identified with certainty under the light microscope, but they have been described as having basophilic cytoplasm and oval nuclei, larger than those in uninjured muscle. The finely clumped heterochromatin and prominent nucleoli attest to the considerable synthetic activity of these cells. Autoradiographic observations support this impression. Cells identified as myoblasts actively incorporate precursors of both DNA (Bintliff and Walker, '60; Walker, '62; Zhinkin and Andreeva, '63; Reznik, '68) and RNA (Carlson, '72a).

Electron microscopic studies of myoblasts confirm the impression that they are highly active cells. The cytoplasm is dominated by large quantities of free ribosomes. Some of the ribosomes are aggregated in groups of 25 to 60 as long polyribosome spirals. There are associated with the production of myosin (Heywood and Rich, '68; Galavazi, '71). Larson et al. ('69) have observed polyribosomes closely associated with newly formed myosin filaments in regenerating mouse muscle. Rough endoplasmic reticulum is sparse, and the Golgi apparatus poorly developed. Mitochondria are few. Short pseudopodia occasionally project from the surface of myoblasts, and there is some evidence that myoblasts may engage in limited phagocytic activity (Pfühl, '37; Allbrook, '62; Trupin, '72). Several investigators who have made detailed electron microscopic studies of muscle regeneration have described the formation of myofilaments in the mononuclear myoblastic cell (Allbrook, '62; Price et al., '64b).

The nomenclature applied to mononuclear myogenic cells has varied, due largely to an incomplete knowledge of the sequence of cellular changes during the early stages of regeneration. Nevertheless, the following terms have proven to be useful in recent years: mononuclear cells possessing recognizable myofilaments at the electron microscopic level are commonly called *myoblasts* (myocytes). Cells with approximately the same size, shape and complement of cytoplasmic organelles, but lacking recognizable myofilaments are best termed *presumptive myoblast* (see discussions in Mauro et al., '70). This term is appropriate not only because one cannot predict whether such a cell will ultimately contribute to the formation of a muscle fiber or remain in reserve, but also because in some animals, particularly the frog (Trupin, '72), it is sometimes difficult to distinguish between myoblastic cells and other mononuclear cells, such as some macrophages, which are present within the basement membrane of a recently damaged muscle fiber. The distinction between myoblast and presumptive myoblast is obviously inappropriate at the light microscopic level of observation, which seldom enables one to identify any cells as myoblasts with complete assurance. Although some attempts have been made to equate "activated satellite cells" with presumptive myoblasts, it seems best to wait until the relationship between satellite cells and regeneration is unequivocally proven or disproven before including or excluding satellite cells or their activated stages in the same series as recognizable myoblastic cells.

Myotubes. Following most types of simple injury to mammalian muscle, the numbers of myoblastic cells reach a peak within 48–72 hours. During the next few days the population of myoblasts undergoes a steady decline, and in their stead long syncytial straps with intensely basophilic cytoplasm and central chains of nuclei appear beneath the basement membranes of the endomysial tubes (Reznik, '70). These straps are commonly known

as myotubes or sarcoblasts (myosymplast is the synonym commonly used in the Russian literature).

It is now apparent that the stages of development in a regenerating muscle fiber are quite similar to those which have been described during embryonic development and that myotubes are formed by the fusion of myoblasts. In a study of embryonic and regenerating muscle in the rat, Zhinkin and Andreeva ('63) found the mitotic cycle in embryonic muscle to be 20 hours and the duration of mitosis about 2.5 hours. These authors implied that in regenerating muscle the length of the cell cycle was the same. Although the complex nature of regenerating muscle has not allowed careful analysis of the fusion cycle, such as that done in vitro by Bischoff and Holtzer, (69; Bischoff, '70), morphological and cytochemical evidence (Lash et al., '57; Allbrook, '62; Price et al., '64b) as well as autoradiographic studies (Bintliff and Walker, '60; Reznik, '68; Carlson, '72a) have left little doubt that myoblastic fusion is, indeed, primarily responsible for the formation and growth of myotubes.

Mitotic divisions are not seen in nuclei of either normal or colchicine-treated myotubes (Pietsch, '61a), and short term labeling studies with ^{3}H-thymidine have shown no incorporation of the isotope into myotube nuclei (Bintliff and Walker, '60; Carlson, '72a). The older concept that the multinucleated condition of myotubes is due to amitotic division of nuclei within myoblasts or an injured muscle fiber has largely been abandoned. Klishov ('72, pp. 86–94), however, still feels that the evidence against amitosis in muscle cells is not sufficient to exclude the possibility of its occurrence.

The earliest multinucleated structures to appear in regenerating muscle are often highly basophilic cuffs lying inside the basement membranes of the original muscle fibers. These cuffs, in turn, may encircle masses of degenerating sarcoplasm and macrophages which are almost invariably associated with the degenerating muscle. Although there have been no direct observations on the transition from muscle in the basophilic cuff stage to typical solid myotubes, it is likely that where basophilic cuffs are prominent, they eventually collapse to form solid structures. Myotubes of large diameter, which contain several chains of nuclei, are likely to have formed in this manner. That the lateral membranes of multinucleated forms of regenerating muscle can fuse was indicated in a recent response by Jirmanová and Theslaff ('72). Other myotubes, particularly in areas where there is no old basement membrane material to serve as a scaffold, form initially as solid structures from chain-like aggregations of fusing myoblasts. Peterson and Crain ('72) have observed in vitro the swelling of myoblastic cells beneath the basement membranes of muscle fibers and their subsequent fusion into myotubes.

When seen at either the light or the electron microscopic level, the early myotube gives the impression of considerable synthetic activity. The nuclei are large and contain loosely scattered chromatin and very prominent nucleoli. The cytoplasm is intensely basophilic and contains large numbers of ribosomes. The majority of ribosomes are free, often in the form of long polyribosomes, but some membrane-bound ribosomes are present throughout the myotube stage (Allbrook, '62). The incorporation of ^{3}H-uridine in early myotubes is intense (Carlson, '72a). The rough endoplasmic reticulum and the Golgi apparatus may be involved in the synthesis and transport of materials used in building up the new basement membrane which surrounds the myotube, but evidence is needed to confirm this point. Despite the formation of a new basement membrane, the highly convoluted old basement membrane still surrounds it throughout most of the myotube stage. Numerous small mitochondria with well-defined cristae are present.

Recently Vracko and Benditt ('72) have investigated the basement membrane (basal lamina) in regenerating rat and rabbit muscle. They confirmed the previous observations by others that the early stages of muscle regeneration occur within the highly pleated basement membranes of the original muscle fibers. New basement membrane material is first seen in areas where folding has removed the old basement membrane from close association with the myotube. This implies that the original basement membrane lying next to the

myotube may inhibit the formation of a new basement membrane by the myotube. A double basement membrane, consisting of a new one lying within the old one, is the rule during the second week of regeneration. From that time until the fifth week the old basement membranes gradually disappear.

Although myofilamentogenesis has been described in the myoblast, by far the greatest synthesis and assembly of myofilaments occurs in the myotube. Myofilaments first appear in the subsarcolemmal cytoplasm (Allbrook, '62). Peripheral aggregates containing evenly spaced filaments are held together at the Z lines. Even in these the A, I, H, and M bands are apparent, and the sarcomere lengths are the same as those of normal adult muscles. More centrally, discrete myofibrils containing numerous regularly arranged filaments can be distinguished. According to Allbrook ('62) new filaments are added to the periphery of the young myofibrils, and new fibrils are formed peripherally to the preexisting ones. Thus, within a myoblast or early myotube there is a gradient of myofibrillar size, ranging from the oldest and thickest in the central part of the cell to the thinnest and most newly formed ones at the periphery. Myofilamentogenesis in early regenerating muscle does not appear to differ substantially from that in the normal embryogenesis of muscle (reviewed by Fischman, '70).

The number of ribosomes (and consequently the cytoplasmic basophilia) gradually decreases as the number of myofilaments increases. As the myofilaments become organized into myofibrils and the numbers of the latter increase, crossstriations are visible in the cytoplasm with light microscopy. Vesicular elements appear, and the sarcoplasmic reticulum takes shape. One of the most prominent features of maturation is a breaking up of the central nuclear chains and the migration of the nuclei to the periphery of the muscle fiber. The nuclear size diminishes, the nucleoli lose prominence and the nuclear chromatin becomes more condensed. When these changes have occurred, the myotube has matured into a young muscle fiber which, although still thin, does not differ greatly from the original fiber which was damaged.

The regenerating amphibian limb

In the amputated limb, all muscle fibers which crossed the plane of amputation are, of course, cleanly transected. During the first few days after amputation the distal ends of the transected muscle fibers undergo a sarcoplasmic degenerative phase which differs little from that of newly transected mammalian muscle (Thornton, '38; Trampusch and Harrebomée, '65; Schmidt, '68). The participation of phagocytic cells in sarcoplasmic removal, however, is considerably less than that which occurs in damaged mammalian muscle.

There next occurs a process which is characteristic of the regenerating limb. Most of the tissues in the terminal part of the limb stump undergo an exaggerated loss of their differentiated structure which extends farther proximad than would be the expected reaction to simple transection alone. This process is called morphological dedifferentiation, and it has been described several times for the muscular component of the limb stump (Towle, '01; Thornton, '38; Hay, '59; Lentz, '69). Interpretations of the cellular events which occur during the phase of dedifferentiation vary considerably. It is agreed by almost all workers that the terminal ends of the muscle fibers undergo pronounced fragmentation, with subsequent liberation of nucleated and non-nucleated fragments of muscle. Whether or not the nucleated fragments survive remains a moot point. The basement membranes around the muscle fibers become highly convoluted early in the dedifferentiative phase (Norman and Schmidt, '67; Hay, '70), but remain essentially intact. As dedifferentiation progresses, the distal basement membranes disappear (Hay, '62, '70). The cause of their disappearance has not been determined, but there is a pronounced increase in collagenase activity during the late dedifferentiative phase of limb regeneration (Grillo et al., '68).

During the period of dedifferentiation, unspecialized mononuclear cells, lacking any distinctive features of skeletal muscle or any other mature cell of the limb, appear in the space left by the dedifferen-

tiated muscle (Hay, '58; Salpeter and Singer, '60; Lentz, '69a). These cells, normally called blastemal cells, migrate apically and accumulate beneath the wound epithelium of the limb stump to form what is called the regeneration blastema. At this point in the limb regenerative process there is a large void in our knowledge of the cellular reactions of the muscular component of the limb. To date it has been impossible to trace the fate of the fragmented muscle fibers once their unique cytoplasmic markers are lost. Thus it cannot be said with certainty that any of the blastemal cells were derived from the damaged muscle fibers themselves although the majority of those working on limb regeneration believe it to be the case.

Because of the difficulty in tracing the precursor cells of muscle (or any other mesenchymal tissue) during the blastemal phase of limb regeneration, nothing definitive can be said about regeneration of the muscle fiber until overt differentiation within the regeneration blastema has begun. In order to provide continuity for the reader who is not familiar with the process of limb regeneration, a brief overview of the major events occurring during the blastemal period of limb regeneration will be presented here. The phase of dedifferentiation involves essentially all of the mesodermal cells in the stump. The morphologically unspecialized cells arising from the dedifferentiative process migrate apically under the influence of the considerably thickened apical wound epithelium (Thornton, '65) to form the regeneration blastema. Under the influence of both the nerves and the thickened apical epidermal cap, the regeneration blastema undergoes a period of rapid cell proliferation and growth. Redifferentiation of cartilaginous skeletal elements occurs first in the blastema and is followed shortly by the redifferentiation of muscles. For details of the early and blastemal phases of limb regeneration, the reviews of Goss ('61), Rose ('64), Schmidt ('68), Thornton ('68) and Carlson (in press) should be consulted.

Cellular aspects of muscle redifferentiation in the regenerating limb are similar to those which occur in embryonic myogenesis (Hay, '62, '63; Grim and Carlson, unpublished). Mononucleated myoblasts accumulate in a highly organized fashion first as common muscle blastemas (not to be confused with the limb regeneration blastema) and then as anlagen of discrete muscles. The myoblasts fuse into myotubes, and the myotubes then differentiate into mature cross-striated muscle fibers. A primary morphological distinction between differentiation of muscle fibers in the regenerating limb and that following damage to mammalian muscle is that in the former system the myoblasts are not bounded by or constrained by basement membranes left over from the original muscle. Except for species differences, the differentiation of muscle fibers from myoblasts within the regenerating limb does not differ significantly in morphology from that in regenerating mammalian muscle.

ORIGIN OF THE MYOGENIC CELL

During the past decade more effort has been directed toward defining the origin of the myogenic cell than any other question related to the regeneration of skeletal muscle. The fact that there is still no general consensus of opinion on the matter attests to the technical difficulty of the problem, and the great interest in it reflects its theoretical importance. Current interest in this problem can be traced back to Mauro's ('61) description of the satellite cell in muscle of the frog. The satellite cell, which has since been found in a wide variety of vertebrates, is a mononuclear cell located between a muscle fiber proper and its basement membrane. Satellite cells represent a population of cells which theoretically could serve as a cellular reserve and could be mobilized to repair damage to the muscle fibers themselves. Recognition of satellite cells in skeletal muscle has rekindled the old controversy of whether the cells of highly differentiated structures can act in their own behalf in posttraumatic repair or whether an infusion of new cells from a normally dormant reserve population is necessary. According to the former viewpoint nuclei, surrounded by a thin rim of cytoplasm, break off from damaged muscle fibers and after becoming completely invested with a cell membrane, serve as the cellular source of the regenerating muscle fibers. This is called

the dedifferentiation of muscle. According to the latter viewpoint, nuclei of the damaged muscle fiber are unable either to reactivate the synthetic processes required of a myogenic cell or even to survive. Therefore they would be excluded from participation in the subsequent regenerative process. In their stead a population of undifferentiated cells with myogenic potential (satellite cells) is activated to proliferate and later differentiate into muscle. Historically, cells other than satellite cells have been considered to represent reserve populations. These have been primarily cells of the endomysial or perivascular connective tissue and, at times, even nucleated blood cells.

Satellite cells

Normal morphology and distribution. As originally described (Mauro, '61), the satellite cell of mature muscle is a mononucleated cell lying between the basement membrane and the sarcolemma of a muscle fiber. Satellite cells are normally spindle-shaped and often tend to be flattened along one axis. The dimensions of a mammalian satellite cell are about $25 \times 4 \times 5\ \mu$ (Muir et al., '65; Muir, '70). Flood ('71) has estimated that in trunk muscle of the axolotl, cytoplasmic projections may extend the length of satellite cells to more than 600 μ. Satellite cells with this enormous length have not been found in limb muscles of the axolotl (Carlson, unpublished). The long axis of these cells is normally oriented parallel to that of the muscle fiber and they are frequently embedded in depressions in the surface contour of the muscle fiber itself.

According to most authors (Ishikawa, '66; Reger and Craig, '68) the plasmalemma of the satellite cell is separated from that of the muscle fiber by a space of 150–200 Å. The intercellular space looks empty, but short projections of basement membrane material may be interposed between the two plasmalemmae at the periphery of the satellite cell (Reger and Craig, '68). Typical junctional complexes have not been seen between satellite cells and muscle fibers. Teräväinen ('70, fig. 2) described possible localized areas of cytoplasmic continuity between the two, but another interpretation is that some apparent cytoplasmic bridges might represent cytoplasmic processes with obliquely sectioned membranes.

The satellite cell nucleus in the bat ranges from 8–12 μ in length and is about 3 μ in width (Muir et al., '65), and in the mouse the average satellite cell nucleus is $13 \times 4.5 \times 2.8\ \mu$ (Venable, '66). Those of the frog are of similar dimensions (Trupin, '72), but in the bird they are considerably more flattened (Muir, '70). Seen through the light microscope, satellite cell nuclei are difficult to distinguish from myonuclei but some differences are detectable with the electron microscope. Commonly, the nuclear chromatin of the satellite cell tends to be somewhat more densely clumped around the inner face of the nuclear membrane than that of the corresponding myonucleus. Occasional binucleated satellite cells have been described (Shafiq et al., '67), but examination of the evidence (e.g., their fig. 10) indicates that the "double" nuclei might instead be a single nucleus sectioned through a deep indentation of the nuclear membrane.

There have been some discrepancies in published electron microscopic descriptions of the cytoplasmic constituents of satellite cells, but considering the range of mammalian forms examined as well as the maturity of the specimens, the descriptions have,on the whole, been surprisingly similar. The cytoplasm is scanty and contains no distinctive organelles. Mitochondria are few, are smaller than those of the muscle fiber, and possess less well developed cristae. Free ribosomes are common. Descriptions of the relative amounts of rough endoplasmic reticulum vary, but generally it is more prominent in satellite cells of embryonic or perinatal muscle (Ishikawa, '66). As muscle matures, the rough endoplasmic reticulum of the satellite cells is reduced to a minor cytoplasmic constituent. The Golgi apparatus is visible, but is not well developed. Neither glycogen nor lysosomes have been reported. Numerous pinocytotic vesicles may be associated with the plasma membrane. Centrioles are present along the midregion of the nucleus and occasionally atypical cilia are found (Muir et al., '65; Conen and Bell, '70).

Most investigators agree that a cell can-

not possess myofilaments in order to conform to the original definition of a satellite cell. There is still some uncertainty about the proper terminology for cells resembling satellite cells but possessing filamentous structures in their cytoplasm. They are considered by some to be residual myoblasts.

Despite the presence of centrioles there has been little direct evidence of mitotic activity in satellite cells of normal adult muscle. In three week-old rats, Shafiq et al. ('68) found several arrested mitotic figures in satellite cells following colchicine treatment. There is now a considerable body of evidence indicating that the satellite cells of growing muscle undergo mitotic divisions.

The unremarkable appearance of satellite cells makes their identification with the light microscope in ordinary paraffin sections extremely difficult. This is due primarily to the similarity in size and shape of myonuclei and satellite nuclei, the scanty cytoplasm and the intercellular space which is below the limit of resolution of the light microscope. On the basis of the more compact nuclear chromatin and a clear space between satellite cell and fiber cytoplasm seen in cross-sections, both Venable and Lorenz ('70) and Church ('69, '70a) have claimed to distinguish satellite cell nuclei from myonuclei by high power light microscopy. In certain systems fairly accurate estimates may be obtained in this manner, but the difficulty in discriminating between satellite cells proper and endomysial nuclei or nuclei recently incorporated into growing muscle fibers, would preclude the possibility of making accurate cell counts without electron microscopic control. In normal and regenerating muscle of the frog, Trupin ('72) demonstrated the difficulty in identifying cell types even with the electron microscope. Thus, unless good statistical correlation between alternating sections at the light and electron microscopic level is obtained, electron microscopy should remain the accepted means of identifying satellite cells.

Since first described, satellite cells have been found in a wide variety of muscles in both vertebrates and invertebrates (Muir, '70). Their frequency of occurrence in adults of most species has been found to be in the range of 5–10% of the nuclei present within the basement membranes of the muscle fibers, but in muscles of newborn animals, up to one third of the nuclei may belong to satellite cells (Allbrook et al., '71). It is generally agreed that the percentage of satellite cell nuclei declines rapidly during the perinatal period and then tends to level off as maturity is reached.

The role of satellite cells in growth of skeletal muscle. The early literature on muscle development was replete with speculations on the mode of muscle growth, but few attempts to amass quantitative supporting data were made until the early 1960's. Chiakulas and Pauly ('65) demonstrated a pronounced increase in the numbers of fibers in several muscles of the rat during the first three postnatal weeks (from 42% in the extensor carpi radialis longus to 83% in the plantaris). The total number of muscle fibers remains relatively stable or may gradually decrease during the remainder of the animal's lifetime. Later growth of muscle is accomplished by an increase in length and diameter of existing muscle fibers. Accompanying the increase in sarcoplasmic mass is a pronounced increase in the number of nuclei. This was first clearly demonstrated by Enesco and Puddy ('64), according to whose calculations the total number of nuclei in several limb muscles of the rat increases by factors of two to four times from the third week to the third month of life. Similar results have been obtained by Allbrook et al. ('71) and Galavazi and Szirmai ('71). After colchicine treatment, arrested mitotic figures are seen within the basement membranes of muscle fibers, the incidence declining with increasing age (MacConnachie et al., '64). Although the latter study was conducted at the light microscopic level, the dividing nuclei were suspected to belong to satellite cells. This was confirmed by Moss and Leblond ('70, '71), who examined these dividing nuclei with electron microscopic autoradiography. After pulse labelling with tritiated thymidine, these authors found that during the first ten hours only satellite cell nuclei were labeled. In the interval from 24 to 72 hours after injection they

found increasing numbers of labeled myonuclei and decreasing numbers of labeled satellite cell nuclei. They concluded that the nuclei which first pick up the label belong to satellite cells and that these in time become incorporated into the growing muscle fibers. Similar results were obtained by Allbrook et al. ('71). In the course of their studies, Moss and Leblond ('71) noted that labeled nuclei, newly incorporated into muscle fibers, possessed densely clumped nuclear chromatin like that of the free satellite cells. This latter observation underscores the need for making satellite cell counts with the electron microscope, or at a high enough magnification so that cytoplasmic discontinuity between the satellite cell nucleus and the muscle fiber proper can be demonstrated. Otherwise, in a rapidly growing muscle, estimations of satellite cell percentages are likely to be skewed upwards.

Satellite cells and mammalian muscle regeneration. From the time of their original description by Mauro ('61), satellite cells have been considered to be a potential source of myogenic cells in the post traumatic regeneration of skeletal muscle. A lively controversy still exists concerning the role of satellite cells in regeneration. This question was discussed at length in a symposium largely devoted to the origin of the myogenic cell, and the reader is referred to the symposium report (Mauro et al., '70) for details of the descriptive material and theoretical discussions.

Early reports linking satellite cells to the regenerative process were largely descriptive, and the evidence was circumstantial. It was noted, for instance, that regeneration was possible in skeletal muscle, which possesses satellite cells, whereas at that time regeneration was not generally recognized in cardiac muscle in which satellite cells have not been found [Midzukami ('64), however, has described satellite cells in cardiac muscle of a crab]. Studies of normal growth (vide supra) and hypertrophy (Reger and Craig, '68) of skeletal muscle have left little doubt that satellite cells can be incorporated into enlarging muscle fibers. From this finding, it has been inferred that satellite cells should be similarly capable of participating in myogenesis during post-traumatic regeneration.

Church et al. ('66) and Shafiq et al. ('67) studied the early stages of regeneration of mammalian muscle by electron microscopy. Both of these groups noted the persistence of satellite cells in areas of severe trauma as well as an increase in their numbers in the days immediately following injury. Morphological transitions between undoubted satellite cells and cells possessing the characteristics of myoblasts have been described (Shafiq et al., '67; Shafiq, '70). In addition to finding transitional forms, Church et al. ('66) indicated that during the first two days intact satellite cells could be found in regions of maximal damage to the muscle fiber. After the third day, the number of satellite cells rapidly declined, but from three to six days cells possessing the features of early myoblasts were frequently seen instead. In addition, mitotic figures were present within sarcolemmal tubes containing myoblasts from four to seven days following injury. Although other alternatives were offered, these authors favored the possibility that satellite cells do transform into myoblasts following injury to the muscle fiber.

In a quantitative study of regeneration three months after making discrete crush lesions in the web muscles of fruit bats, Church ('70a) found increased numbers of satellite cells in the area of the lesion. He suggested that satellite cells present at the time of injury not only give rise to myoblasts capable of restoring the number of myonuclei, but that some of their descendents remain as satellite cells to the newly regenerated muscle fibers, thereby ensuring a potential supply of myoblasts in the event of repeated trauma.

The satellite cell population of a muscle fiber appears to respond to trauma by an increase in numbers. A striking example of this phenomenon was provided by Teräväinen ('70), who by means of gentle compression, damaged the superior rectus muscle of the eye in adult rats. Although the damage was so slight that degenerative changes were not seen upon electron microscopic examination of the muscle fibers themselves, the numbers of satellite cells were noticeably increased during the

first 24 to 48 hours following compression. Unfortunately, quantitative evidence was not provided. The satellite cells observed during this period differed in morphology from those of normal muscle. Cytoplasmic projections of these cells extended for considerable distances along the muscle fibers, and the cytoplasm contained progressively increasing amounts of ribosomes, polysomes, rough endoplasmic reticulum, mitochondria and Golgi complexes. A further indication of activity in these cells was the appearance of prominent nucleoli.

Most investigators now believe that these mononuclear cells (often called "activated" satellite cells or presumptive myoblasts) which become prominent within the first days after muscle damage will become definitive myoblasts. These "activated" cells, which are found beneath the basement membranes of traumatized muscle, possess microfilaments but not a lattice array of myofilaments. They are assumed to go on to form definitive muscle and will be referred to as presumptive myoblasts in this review. Because their origin is still in dispute, it is unwise to call them activated satellite cells. Reznik ('69a, '70) has shown that these cells can incorporate tritiated thymidine and also undergo mitotic division, leaving little doubt that this population of cells is an expanding one.

Despite general agreement that presumptive myoblasts of newly injured mammalian muscle go on to form muscle, their origin has not been satisfactorily determined. Contrary to the opinions of those who believe that presumptive myoblasts are derived from the satellite cells of uninjured skeletal muscle, several investigators contend that the presumptive myoblasts are derived from myonuclei which have broken off from damaged muscle fibers.

Reznik ('69b, '70) studied regeneration of muscles in the mouse and rabbit subsequent to injury by cold and crushing. He presented a sequence of descriptive morphological observations at the ultrastructural level which, he claims, demonstrate that myogenic cells arise by the breaking off of myonuclei from the damaged muscle fiber. According to Reznik's interpretation, some of the nuclei in injured muscle become partially segregated from the remainder of the muscle fiber by underlying accumulations of vesicles and clefts. The vesicles fuse in time to separate completely the nucleus, surrounded by a thin rim of cytoplasm, from the muscle sarcoplasm. Although the origin of the clefts and vesicles is unknown, they may be derived from the transverse tubular system or the sarcoplasmic reticulum. The "broken off" mononucleated cells have been called satellite cells or presumptive myoblasts by Reznik ('70) and are equivalent in morphology to the cells referred to as presumptive myoblasts in this review. It is noteworthy that Reznik claims not to have seen satellite cells in normal muscle fibers of the tibialis anterior of adult rabbits or the gastrocnemius of the mouse. Recently, similar findings and conclusions have been reported by Elyakova ('72) for regenerating rat muscle.

Thus with respect to descriptive morphological observations, discrete presumptive myoblasts have been found beneath the basement membranes of newly damaged muscle by all who have studied early regeneration, and there is general agreement on the subsequent fate of these cells. Those investigators who have recognized well defined populations of satellite cells in normal undamaged muscle have almost uniformly considered presumptive myoblasts to represent satellite cells which have become activated by injury to the muscle fiber. In contrast those who have found few or no satellite cells in normal muscle have interpreted presumptive myoblasts as former myonuclei which have broken off from the damaged muscle fiber.

Recently Walker ('72) attempted to determine the origin of myoblasts during regeneration of the tibialis anterior muscle of 30 gm rats (subadult and still growing) by means of light microscopic autoradiography. The experiment consisted of two major series. One, a single injury experiment, involved the administration of tritiated thymidine three times a day for three days and an incision injury 12 days after the last injection of isotope. The muscle was fixed four days after injury. In the other series, a double injury experiment, muscle was injured and the isotope was administered two days later. This

procedure was assumed to result in the labeling of myoblasts as well as other cell types. The myoblasts were incorporated into the regenerating muscle fiber and could be recognized as labeled central nuclei of myotubes in succeeding days. Then the muscle was re-injured in the region of the original injury 12 days after the injection of isotope and fixed four days after the second injury.

The single injury experiment was designed so that before the injury satellite cells would be selectively labeled. Four days after injury Walker found few labeled central nuclei in proportion to the number of peripherally situated nuclei associated with the newly regenerated muscle fibers. In the double injury experiment both central and peripheral nuclei were labeled at the time of the second injury. The myotubes examined four days after re-injury contained label in both central and peripheral nuclei. These results were interpreted by Walker to mean that nuclei of regenerating muscle fibers are derived mainly from pre-existing myonuclei.

A comparison of Walker's experimental design with other autoradiographic studies on the muscle of growing rats lends some doubt to his interpretations. In an electron microscopic autoradiographic study of normal muscle in 30 gm rats, Moss and Leblond ('71) found that one hour after injection of ^{3}H-thymidine 100% of labeled nuclei belonged to satellite cells and no myonuclei were labeled. By 48 hours, however, satellite cells comprised only 51% of the labeled nuclei, with the remainder of the labeled nuclei (49%) being found in the population of myonuclei. If in normal growing muscle the population of labeled satellite cells is halved in each 48-hour period, then in Walker's single injury experiment one would expect that 12 days following the last injection of isotope only 1–2% of labeled nuclei would belong to satellite cells and the remainder would be incorporated into the muscle fibers as true myonuclei. An alternative explanation for the lack of labeling of myonuclei in Walker's experiment could be that non-labeled satellite cell nuclei become incorporated into the regenerating muscle fibers and the labeled peripheral myonuclei do not. Resolution of this apparent discrepancy awaits an electron microscopic demonstration of the relative proportions of labeled myonuclei vs satellite cell nuclei.

Origin of myoblastic cells in regenerating extremities

The origin of myoblastic cells is a much more complex problem in the regenerating limb or tail than in the case of damage to a muscle alone. This is due primarily to the greater variety of cells which could possibly serve as myoblastic precursors.

Most workers believe that the damaged ends of the muscle fibers transected by amputation undergo fragmentation and that nucleated fragments of muscle become completely invested in a new cell membrane and then migrate toward the tip of the stump. There they join the blastema and represent precursor cells of the regenerating limb or tail. This concept, put forth long ago by Towle ('01) and Naville ('22), has been supported primarily by descriptive morphological evidence. The dedifferentiation of muscle in larval *Amystoma* was reported at the light microscopic level by Thornton ('38) and at the electron microscopic level by Hay ('59, '62, '68). Lentz ('69a) described a similar phenomenon in the adult newt.

Most descriptions of the early events in muscle following limb amputation have been remarkably similar. The degenerative reaction in the terminal portions of the transected muscle fibers does not appear to be causally related to the subsequent regenerative events because it also occurs under conditions in which limb regeneration is inhibited. Morphological dedifferentiation follows the period of initial degeneration of muscle. Morphological aspects of the dedifferentiative reaction were described above. Dedifferentiation of limb muscle in adult amphibians is the result of a complex set of tissue interactions; in larvae, the process is subject to different controls. The tissue interactions involved in dedifferentiation and redifferentiation of muscle in the amphibian limb have recently been reviewed (Carlson, '72c, in press) and will not be reiterated here.

There is no doubt that a phase of histological dedifferentiation occurs in the regenerating amphibian limb, but cytological interpretations of this phenomenon are not

unanimous. According to Thornton ('38), striations in the distal ends of the damaged muscle fibers undergo a progressive disappearance. The nuclei increase in thickness and show no signs of degeneration, but rather appear to break off from the end of the muscle fiber. Surrounded by a thin rim of cytoplasm, they lose any morphological indication of their former phenotype and intermingle with other cells arising from similar dedifferentiative changes occurring in the skeleton and connective tissues. These cells are then called blastemal cells.

Descriptions based upon light microscopy have been to a large extent supported by electron microscopic observations. In early dedifferentiating muscle, Hay ('68) described dissolution of the myofibrils as well as the acquisition of a substantial population of free ribosomes and segments of rough endoplasmic reticulum. These findings were confirmed by Lentz ('69a). During early dedifferentiation, myonuclei are frequently partially separated from the bulk of the sarcoplasm by a region apparently containing a sheet of small membrane-bound vesicles (Hay, '59, '70; Barberie, '70). The origin of the vesicles has not been ascertained, but the sarcoplasmic reticulum or Golgi vesicles have been prime candidates. It has been assumed that the vesicles eventually coalesce to form a membrane-lined cleft between the nucleated fragment and the muscle fiber. The nucleated fragments are then released.

Uniformly autoradiographic studies have demonstrated considerable synthetic activity in the region of muscle fragmentation. Incorporation of ^{3}H-thymidine into the nuclei of dedifferentiating muscle has been observed at both the light (Hay and Fischman, '61) and electron microscopic levels (Barberie, '70; Hay, '70). Labeling of nuclei not yet separated from the muscle fiber has not been reported. The uptake of ^{3}H-uridine into the sarcoplasmic fragments of dedifferentiating muscle is particularly intense, and dedifferentiation of muscle does not occur in newts treated with actinomycin D (Carlson, '66, '67). There is also considerable protein synthesis in dedifferentiating muscle (Bodemer and Everett, '59; Anton, '65, '68). The zone of highest activity is located in the distal fragmenting ends of the muscle fibers and follows the proximal wave of dedifferentiation.

The overall phase of dedifferentiation of muscle and other internal tissues of the limb continues to involve successively more proximal regions of the limb stump until a distal accumulation of blastemal cells occurs. At least in larvae, the presence of a regeneration blastema appears to provide the signal for the dedifferentiative process to cease (Schotté et al., '41). Although the stages of dedifferentiation, blastema formation and redifferentiation of muscle are quite discrete in larval limbs, the situation in adults is more complex because deep in the limb stump the simultaneous dedifferentiation and redifferentiation of muscle may occur in different fibers in the same area. The significance of this phenomenon will be discussed later in this review.

The descriptions of muscle dedifferentiation in the degenerating limb as presented above have been severely criticized by some (Schmidt, '62; Holtzer in discussions of Hay, '70 and Steen, '70), primarily because the methods used have not permitted the exact tracing of cells. A number of the arguments against this interpretation have been summarized by Schmidt ('68). Alternative suggestions for sources of myoblastic cells during limb regeneration have been undefined "reserve cells" (Weiss, '39), fibroblasts or endomysial cells (Manner, '53; Schmidt, '62, '68; Toto and Annoni, '65) and more recently, satellite cells. To date, little concrete evidence in support of these viewpoints has been presented.

SUMMARY

In no system of muscle regeneration has the origin of the myogenic cell been proven beyond a doubt. Circumstantial evidence, particularly that relating to the normal growth of muscle, suggests that satellite cells play a role in the regeneration of mammalian muscle. Even if satellite cells are unquestionably demonstrated to participate in mammalian muscle regeneration, separate experiments would have to be designed to rule in or out the participation of myonuclei. The role of myonuclei could be ascertained by labeling growing animals in such a way that ^{3}H-

thymidine would be retained in the myonuclei but greatly diluted in the dividing satellite cells. Following injury to these fibers and their subsequent regeneration, the presence of labeled myonuclei in the new fibers would indicate the participation of old myonuclei whereas the absence of labeled myonuclei would provide evidence against the dedifferentiation of muscle fibers.

At this point it is unwise to assume that myoblasts in the regenerating amphibian limb are established in the same manner as they are in damaged mammalian muscle. The descriptive evidence indicates enough differences between the two that considerable caution should be exercised in the extrapolation of data or conclusions from the injured mammalian muscle to the regenerating limb or vice versa.

MORPHOGENETIC PROCESSES IN REGENERATING MUSCLES

The posttraumatic regeneration of mammalian muscle and muscle formation in the regenerating amphibian limb appear to be examples of two distinct modes of skeletal muscle regeneration (Carlson, '70a), and the differences between them are particularly pronounced when the mechanisms of morphogenetic control are examined. One has commonly been called the tissue mode of regeneration, and this is the type of restorative process that typically occurs in a damaged mammalian muscle. Tissue regeneration is a rapid process involving local repair. It does not involve the massive loss of structure (dedifferentiation) of muscle adjacent to the wound, and a blastema is not formed. Morphogenesis of the regenerated tissue is not perfect. The other mode of regeneration is called epimorphic regeneration, and it is best represented by the regenerating amphibian limb. Epimorphic regeneration is a very complex process involving all tissues of the limb. After amputation a series of interactions of several limb tissues brings about a general dedifferentiative process followed by the formation of a regeneration blastema. The blastema then increases in size, and in a manner morphologically similar to that occurring in the embryo, the blastemal cells differentiate into nearly perfect replicas of the missing structures of the amputated limb. Although important aspects of both of these processes remain poorly or not at all understood, it is now possible to present a rough comparison for the regeneration of muscle by each of these modes.

The tissue mode of regeneration

This is the reparative response which follows damage to muscle alone or which occurs after limb amputation if the limb as a whole fails to regenerate. Because there is such great variation in the kind and degree of muscle damage which is followed by the tissue mode of regeneration, underlying similarities in the morphological development and the control factors governing morphogenesis have often been overlooked.

In tissue regeneration, the initial trauma to the muscle is followed by degeneration of the sarcoplasm in the area of damage and the subsequent rapid appearance of a recognizable population of myoblasts. The myoblasts, at least in the initial phases of regeneration, are closely associated with the inner surface of the basement membrane of the original muscle fiber, and their differentiation into myotubes usually begins two to four days following trauma. Because of their location, the orientation of the early myotubes is largely determined by that of the old basement membranes. In experimental systems which involve no mechanical dislocation of muscle fibers (e.g., following thermal injury or ischemia) the initial orientation of myotubes is almost perfect whereas in other systems, such as a minced muscle, architectural disorder prevails. Because of the persisting basement membranes, communication among myoblasts in different muscle fibers is limited. Frequently, outgrowth of a regenerating muscle fiber occurs from the end of a damaged sarcolemmal tube. In this case the initial direction of growth normally follows the long axis of the original fiber. Thus, the early steps in restoration of the internal architecture of regenerating muscle are determined to a great extent by the pattern of damage to the original muscle. Much of the cytodifferentiation in the tissue regeneration of muscle has occurred while the myogenic cells are still largely

isolated from one another by the basement membranes and endomysial connective tissue of the previous muscle.

In a normally functioning extremity most of the regenerating muscle fibers eventually become arranged in a fashion similar to that of the original intact muscle. If, as was indicated in the previous paragraph, the early organization of regenerating muscle is largely determined by that of the damaged muscle fibers, what factors bring about the restoration of near-normal internal architecture in severely damaged or disorganized muscle regenerating by the tissue mode?

Allbrook ('51) rotated 1-cm^2 pieces of muscle in the rabbit so that their muscle fibers were perpendicular to the long axis of the muscle. Regeneration ensued, and as the elongating myotubes extended past the basement membranes of the rotated implant, their orientation became sharply altered. Instead of continuing to extend in line with their original perpendicular orientation, they turned sharply to become parallel with the long axis of the muscle. From this experiment Allbrook concluded that the lines of tension within a muscle probably determine the orientation of regenerating muscle fibers.

Because it involves such extreme internal disorganization, the minced muscle system has proven to be a more favorable model for analysis. In a minced muscle, the internal architecture during the early phases of regeneration is totally chaotic, but in mature regenerates most muscle fibers are roughly parallel with one another. It was found that mechanical tension is the primary factor leading to the internal reorganization of minced muscle regenerates. If the minced gastrocnemius muscle of rats is allowed to regenerate under relatively tension-free circumstances (subcutaneous abdominal implantation or amputation of the lower segment of a limb containing a minced muscle), internal reorganization of the muscle fibers does not occur, and architectural disorder persists even in mature regenerates. When continuous directed tension is applied to these tension-free systems from the beginning, an ordering of the internal organization of the muscle fibers within these regenerates occurs, and they become aligned parallel to the lines of applied tension (Carlson, '71a, '72a).

There is considerable inferential evidence that tension is applied to the regenerating muscle fibers via the interstitial connective tissue which is formed early in the regenerative process. This is supported by an observation made by Gallucci et al. ('66), who found that in lathyritic rats the number of muscle fibers regenerating from the minced gastrocnemius was greater than normal but their orientation was poor in comparison with muscle fibers in control regenerates.

Identification of factors controlling the gross morphogenesis and molding of muscle regenerating by the tissue mode has also been facilitated by studies of the minced muscle system. In a regenerating minced muscle, a generalized model of a typical limb muscle is formed. The proximal portion is muscular, and the distal end tapers down to a tendon of dense connective tissue. The first clue to the nature of the morphogenetic forces was provided by Rumyantseva ('59, '60), who noticed that a regenerating mass of minced muscle, if released from tension, forms a rounded mass rather than an elongated structure. A tension-free environment is possible as long as tendon connections are not made with the regenerating muscle. Normally connections between a minced muscle and the proximal and distal tendon stumps are made within four to five days, and the mass of muscle soon acquires an elongated configuration. In contrast, a mass of minced muscle implanted beneath the abdominal skin quickly rounds up (Rumyantseva, '59). When a mass of minced muscle implanted beneath the abdominal skin was allowed to form connections with pieces of tendons which were, in turn, subjected to continuous tension, a pronounced elongation of the mass of regenerating muscle occurred (Carlson, '71a, '72a). It can be concluded that continuous tension is an important factor in the elongation of regenerating muscle.

Another striking example of the elongation of regenerating muscle is seen in the experiments of Litver et al. ('61), who reported a remarkable restoration of form as well as elongation of the stumps of amputated muscles in rats. I have repeated

this experiment on a small number of rats (Carlson, '72a). If an anatomical connection is made between a small proximal stump (4–5 mm) of the rat gastrocnemius muscle and the collagenous tissue regenerating proximad from the distal stump of the Achilles tendon, a two to three fold elongation of the muscle stump can occur. In contrast, if such a connection is not made, the muscle stump becomes attached to the underlying tissues and either remains the original size or disappears.

The molding of external shape in regenerating muscle appears to be due primarily to two factors. (1) The area of regeneration following many types of mechanical injury usually decreases in mass because of a quantitative deficiency of regenerating muscle fibers as well as the consolidation of scar tissue. (2) In areas of massive trauma, such as a minced muscle, overall gross molding of the regenerate seems to be accomplished almost entirely by means of pressures exerted by the surrounding tissues. This was demonstrated by a recent experiment in which pieces of Gelfoam were minced and soaked in saline or in supernates of muscle homogenates. The Gelfoam was then implanted in place of the gastrocnemius muscle of the rat (Carlson, '72a). Although no muscle fibers were ever seen, proximal and distal tendon connections were established with the implanted mass of Gelfoam, and the latter became molded into a structure grossly identical to an early minced muscle regenerate. The regenerating muscle fibers play no determining role in the gross morphogenesis of regenerating mammalian muscles.

One can summarize the morphogenetic forces known to be operating in the tissue mode of muscle regeneration by saying that most of these are not directly associated with the regenerating muscle fibers themselves. Mechanical tension appears to be the main force directing the internal organization of regenerating muscle whereas tendon connections and pressures of the surrounding tissues are largely responsible for gross molding. There is no evidence for the existence of any "morphogenetic field forces" which might play a role in the development of form in skeletal muscle regenerating by the tissue mode.

The epimorphic mode of regeneration

In any consideration of the epimorphic mode of muscle regeneration it is essential to recognize that muscle is not regenerating alone, but as part of a process which includes all components of the limb. Epimorphic regeneration is most commonly studied in amputated extremities of urodele amphibians, but it also occurs naturally in larval anurans, in certain extremities of fishes and in the tails of some lizards. Epimorphic regenerative processes have been stimulated experimentally in adult anurans (Polezhaev, '46; Rose, '44; Singer, '54; Schotté and Wilber, '58), in the limbs of lizards (Simpson, '61; Singer, '61; Kudokotsev, '62) and in the newborn opossum (Mizell, '68; Mizell and Isaacs, '70). The usual stimulus for epimorphic regeneration is the amputation of a limb, but a wide variety of other procedures can stimulate the formation of supernumerary limbs, another example of an epimorphic process (Carlson, '71b; in press). Following an initial period of epidermal wound healing and elimination of debris resulting from amputation, the limb stump enters the phase of dedifferentiation. In adult urodeles this period is relatively long and variable. In my laboratory it normally extends throughout the second and well into the third postamputational week. Despite the controversy concerning the cellular events which occur during this phase, it is generally agreed that dedifferentiation represents a phase during which the adult structure of the tissues near the amputation surface is lost and during which the population of cells which will form the regenerate is recruited. Although direct observation (Hay, '62) and some marking studies (Steen, '68, '70) have been employed, little is yet known about the origins and patterns of migration of the dedifferentiated cells except that they make their way apically to form a regeneration blastema beneath the thickened wound epidermis (Thornton, '68). The cells comprising the regeneration blastema are so similar in appearance that cells which may have originated from muscle or which will become muscle cannot be distinguished. Attempts to trace muscle cells by immunological methods (DeHaan, '56; Laufer, '59) have failed because detectable cellu-

lar immunological markers do not persist into the blastemal phase. At the present time it is not known which cells, if any, in the blastema will form muscle. Thus the extremely important question of whether cells in the blastema will form muscle because of their genetic background or because of their position within the developing blastema remains unanswered. There is also no information on possible unmasking of morphogenetic information within myogenic cells or transmission of morphogenetic information from one cell to another.

Some basic morphological features of muscle development within an epimorphic system are known. Differentiation of skeletal elements precedes that of muscles, and there is a strong proximodistal and a weaker preaxial to postaxial gradient of differentiation of both skeletal and muscular elements. In general, the differentiation of muscles within a regenerating limb follows a morphological pattern similar to that seen in the embryonic limb bud (Grim and Carlson, unpublished). The major morphological events are the formation of common muscle blastemas on the flexor and extensor sides of the newly differentiating skeleton. The muscle blastemas are composed of mononucleated myoblasts. The muscle blastemas then split into anlagen representing the individual muscles. Before the splitting becomes obvious, it can be seen that the spindle-shaped myoblasts are oriented parallel with the long axis of the future muscle. By the time the muscle anlagen are established, fusion of the myoblasts has occurred and both gross and internal morphogenesis of the muscles has been largely established. Then only the growth phase and minor remodelling remain.

There have been few studies of morphogenetic mechanisms operating on muscles in the regenerating limb. Much of the early work involved the transplantation of foreign muscles into limb stumps and observations on the effect on morphogenesis of the entire regenerate (reviewed by Vorontsova, '49; Vorontsova and Liosner, '60; Goss, '61). The effects of these muscle implants upon the muscular pattern within the regenerates was ignored.

There are no known systemic or environmental influences which have any specific effects upon the morphogenesis of epimorphically regenerating muscles. In a recent experiment axolotl larvae were kept continuously anesthetized with MS 222 (Sandoz) during the blastemal and all subsequent phases of the limb regenerative process. The formation of muscles within the regenerates was normal (Carlson, '71a).

Whether or not the musculature in the limb stump influences the morphogenesis of muscle in the regenerate is one of the few questions which has received experimental attention. It has recently been established in the axolotl that regenerates arising from limbs in which over 99% of the muscle fibers were surgically removed possess an anatomically normal muscular pattern and nearly normal numbers of muscle fibers (Carlson, '72b). This demstrates that an anatomically normal stump musculature is not required for the normal development of muscles within a limb regenerate. At first glance, these results appear to be contradictory to those of Pietsch ('61b, '62), who studied the regeneration of limbs transplanted to the orbit in *Ambystoma* larvae. After they healed in their new locations, the proximal portions of the transplanted limbs contained a mixture of limb muscles and extra-ocular muscles which had grown into the transplant. Following amputation of the transplanted limbs, the muscular pattern in the regenerates was abnormal. This indicates that the presence of foreign muscle in the limb stump exerts a morphological effect upon the muscles in the regenerating limb. On the basis of these two experiments, one can say that a regenerating limb can compensate for a morphologically abnormal and quantitatively deficient stump musculature, but that it cannot integrate the information provided by an extra supply of foreign muscle.

Because there are few known means of affecting specifically the musculature of regenerating extremities without altering other tissue components of the regenerate, mention should be made of several experimental manipulations which are reported to produce regenerates possessing normal gross form and normal skeletal structures, but which do not contain muscle. These

experimental models would be useful starting points for studies on muscle morphogenesis if it could be demonstrated that muscle is, indeed, totally absent from all regenerates. Then one could add foreign or experimentally altered muscle to the system and observe if and how these transplants contribute muscle to the otherwise muscleless regenerates.

In several papers Umansky ('46; et al., '51) mentioned that in the axolotl, regenerates which form from heavily x-irradiated limbs covered with unirradiated skin do not contain muscle. Examination of his original report (Umansky, '37), however, reveals that in certain of his experiments muscle did form in regenerates. These experiments and conclusions should be confirmed before this model could be used for experiments on muscle morphogenesis.

S. Holtzer ('56) found that surgical removal of masses of tail musculature caused a reduction or absence of muscle in the corresponding portions of the regenerates. Complete surgical removal of limb musculature is extremely difficult (Carlson, '72b), and even if it is accomplished, it would be difficult to exclude the possibility of migration of cells from damaged muscle in more proximal areas of the limb stump.

Following implantation into the caudal fin of early limb blastemas isolated from stump tissues in *Ambystoma*, Pietsch ('61c) reported that the subsequent regenerates possessed a well-formed skeleton but no muscle. In view of the formation of muscle in Stocum's ('68) experiments which involved essentially the same surgical technique, repetition of this work would be needed to determine whether or not there is a critical period of blastemal development before which muscle consistently fails to appear in regenerates arising from isolated blastemas.

Comparison of morphogenetic mechanisms in regenerating muscle

In summary, although the cytodifferentiation of muscle fibers is similar in all systems of regeneration studied to date, there appear to be two sets of conditions in which groups of muscle fibers regenerate (Carlson, '70a). One, the tissue mode of regeneration, is largely a local reparative process involving the rapid destruction of damaged muscle and the differentiation of new muscle fibers. It is not associated with a regeneration blastema, and the main morphogenetic forces acting upon the regenerating muscle cells are physical relationships with the surrounding tissues or with remaining structures, such as the basement membranes (or endomysial tubes) of the original muscle. Often the morphogenetic forces do not operate until the regenerating muscle cells are in the late myotube stage. The morphology of muscles or portions of muscles regenerated by this mode is imperfect.

In contrast, the epimorphic mode of muscle regeneration is a much more leisurely process, involving the phases of dedifferentiation and blastema formation before the redifferentiation of muscle fibers occurs. A regeneration blastema is always associated with the process, and the muscles resulting from an epimorphic process are usually exact duplicates of those lost by amputation. The morphology of the process essentially repeats that of embryonic development, and the external shape and internal architecture of the muscles is determined while the myogenic cells are still in the mononucleated myoblastic stage. The blastema appears to be the key factor in morphogenetic control, but the nature of the blastemal influence is unknown. Among the possibilities are the unmasking of morphogenetic information intrinsic to myogenic cells, intercellular transfers of morphogenetic instructions or positional influences.

It is important to recognize that cells of the same muscle may be capable of participating in either type of regenerative process, at least if the animal is able to regenerate amputated extremities. The pubo-ischio-tibialis muscle of the axolotl can regenerate either by the tissue mode following mincing or by the epimorphic mode if the hind limb is amputated (Carlson, '70b). In the frog, limb amputation is normally followed by cicatrization and the rapid tissue regeneration of muscle (Schotté and Harland, '43; Rose, 44; Thornton, '56), whereas after certain experimental manipulations, the damaged muscle, as well as the other tissues in an amputated limb, is drawn into an epi-

morphic regenerative response. Even in the epimorphic regeneration which normally follows limb amputation in urodele amphibia, there is evidence that both tissue and epimorphic regeneration may be occurring in the same muscle. If amputation is performed through the upper arm, for example, the distal musculature of the regenerated upper arm segment is usually anatomically normal whereas there is often an area between that and the proximal undamaged musculature which is anatomically imperfect, particularly with respect to the internal architecture of the muscles. This is interpreted as representing an area where the dedifferentiative reaction has been carried far enough proximad to remove some of the damaged muscle from the influence of the regeneration blastema. In this narrow zone muscle would be regenerating by the tissue mode. Differentiation of muscle fibers in this area is known to occur early, well before differentiation of any structures from the blastema. Thus the regeneration which occurs here fulfills the criteria set for regeneration by the tissue mode, and the reaction of the muscle is paralleled by a similar reaction of the skeletal tissues in this area. The latter response results in a cuff of periosteally-derived cartilage which surrounds the distal skeleton of the limb stump.

One of the major questions related to morphogenesis of regenerating muscle is whether myogenic cells possess intrinsic information which enables them to form perfect muscles in an epimorphic regenerative response or whether this information must be acquired from external sources. A related question of considerable practical importance is whether or not mammalian muscle can express or receive such information. This problem has received almost no attention.

NERVOUS INFLUENCE UPON REGENERATING SKELETAL MUSCLE

Mammalian muscle (tissue regeneration)

Normal relationships between nerves and regenerating muscle. The relationship between damaged muscle fibers and their nerve supply varies considerably in accordance with the experimental model which is used. In localized lesions produced by mechanical or thermal trauma there is often no direct damage to the nerve supplying the muscle or to the neuromuscular junction. In these cases the "continuous" regeneration which occurs at the damaged ends of the muscle could potentially be influenced by the continuously functioning nerve through the mediation of the undamaged segment of the muscle fiber. The work of Peterson and Crain ('72), who demonstrated in vitro a stimulatory effect of nerves upon the outgrowth of regenerating muscle indicates that this may be the case. Further support for the transmission of a nervous influence through both normal and regenerating muscle has come from the work of Hall-Craggs ('71). He found that segments of muscle fibers, separated from their motor end plates by crush lesions developed the characteristic signs of denervation atrophy. After several weeks, however, the patterns of metabolic activity of the isolated muscle segments returned to normal. He concluded that regenerating muscle restored connections between the two isolated segments of intact muscle fibers and that the nervous influence was transmitted through the regenerated portions of the muscle fibers to the temporarily denervated distal segment. The formation of new motor end plates was not seen in the formerly isolated segments of the muscle fibers.

The normal reinnervation of regenerating skeletal muscle is best studied in systems involving the complete functional and structural severance of neuromuscular connections, such as mincing, prolonged ischemia or free transplantation. In mammals, regeneration occurs entirely in the absence of nerve fibers during the first week (Allbrook and Aitken, '51; Zhenevskaya, '54, '58; Mong, unpublished). Approximations of ingrowing nerves with regenerating muscle fibers do not normally appear until the latter have formed late myotubes or early cross striated fibers. Typical motor end plates do not appear until considerably later. In the regenerating minced gastrocnemius muscle of the rat, Zhenevskaya ('54) describes "motor end plate like" formations by 21 days, and typical end plates were seen at 35 days

by the Bielschowsky-Gross technique. Working on the same minced muscle system, Mong (unpublished) has found a diffuse cholinesterase reaction along the muscle fibers throughout the third week of regeneration and localization of the reaction during the fourth and fifth weeks. This confirms the earlier studies of Betz and Reznik ('64), who demonstrated the progressive localization of cholinesterase activity in muscle fibers regenerating after ischemia. Allbrook and Aitken ('51) found a similar time-table of return of nerve-muscle relationships in the regenerating post-ischemic tibialis anterior muscle of the rabbit. The return of reinnervation in the regenerating gastrocnemius muscle of the frog is the same as in the mammal although the time course is slower (Hsu, '71).

Electron microscopic confirmation of the time of establishment of motor end plates as well as the morphological aspects of motor end plate formation has yet to be reported in regenerating mammalian muscle. This has been followed in the regenerating limb of the newt by Lentz ('69b), and in this case the essential morphological aspects of motor end plate formation appear to be quite similar to those occurring during embryological development of muscle (Hirano, '67; Teräväinen, '68).

There is little definitive information on the return of sensory nerve endings to regenerating muscle. Silver stained preparations by Zhenevskaya and Umnova ('65) have been interpreted as representing certain sensory terminations. Although Studitsky ('59, fig. 62) illustrated a structure which was interpreted to represent a muscle spindle in a regenerated muscle, Zelená and Sobotkova ('71) were recently unable to find any muscle spindles in their preparations of regenerated muscles.

The effects of denervation upon the course of muscle regeneration. One of the most effective means of determining the influences of the peripheral nerves upon muscle regeneration is to study the effects of denervation. Denervation can be performed at various stages in the regenerative process, and it is convenient to categorize the denervation experiments as those in which denervation was performed (1) at the time of muscle trauma, (2) at periods following muscle trauma and (3) prior to the time of muscle trauma.

Denervation at the Time of Muscle Trauma. The results of almost all experiments of this type have been remarkably similar although the interpretations have not been entirely uniform. There is agreement that the establishment of a population of myoblasts appears to occur normally in the absence of a functional nerve supply (Allbrook and Aitken, '51; Zhenevskaya, '54; Hsu, '71 and others). In fact, denervation may facilitate the formation of myoblasts. Lee ('65), Aloisi ('70) and Hess and Rosner ('70) described an increase in subsarcolemmal mononuclear cells following the simple denervation of muscle. These cells have been assumed to be myogenic. These findings suggest that functionally intact neuromuscular relations may, indeed, exert a morphological stabilizing influence upon the intact muscle fiber. It is true that in many experimental systems of muscle regeneration anatomical disruption of the motor nerve supply occurs, and it is likely that at least in the area of damage, there may be temporary interference with the propagation of the nervous impulse over the surface of the muscle fiber. Since almost nothing is presently known about the mechanisms controlling the recruitment and activation of myoblasts, an investigation into a possible inhibitory effect of a functional nerve supply to the muscle fiber may be rewarding.

The proliferation and subsequent fusion of myogenic cells, likewise, occurs in the absence of a nerve supply (Zhenevskaya, '62; Betz and Reznik, '64; Hsu, '71; Jirmanová and Thesleff, '72). On this point, however, Studitsky's interpretation of the experimental data of denervation experiments differs from the majority viewpoint. He maintains (Studitsky, '63; Studitsky et al., '63) that the presence of nerves is required for the fusion of myoblasts into myosymplants (myotubes). This viewpoint is contradicted by most experimental studies as well as by the ability of embryonic myoblasts to fuse in vitro.

Once the regenerating muscle has reached the myotube stage, the effects of denervation become apparent, at first as a retardation in the rate of maturation of the

myotubes (Allbrook and Aitken, '51; Zhenevskaya, '62; Hsu, '71). That a nervous influence is, indeed, transmitted to the early myotubes was recently shown by Jirmanová and Thesleff ('72), who demonstrated miniature end plate potentials in regenerating muscle fibers as early as three days following injury. The nature of the nervous influence during the early stages of regeneration remains wholly obscure. As late as two weeks in the regeneration of denervated mammalian muscle, the muscle fibers remain immature. The central position of the nuclei is often retained, and the alignment of the newly-formed myofilaments is imperfect. In the frog, differentiation of regenerating muscle fibers often appears to progress farther in the absence of a nerve supply than it does in mammals (Hsu, '71).

In the late periods following denervation, the regenerating muscle fibers begin to degenerate. This process is characterized by irregular central clumping and pycnosis of the nuclei and vacuolization of the cytoplasm. The relative proportion of connective tissue to muscle fibers increases, and long term denervated regenerates are usually represented by fields of dense connective tissue.

By selective denervation experiments, Zhenevskaya has carefully examined the respective roles of the functional components of the peripheral nervous system in regeneration of the minced gastrocnemius muscle of the rat. When spinal ganglia of the sensory nerves leading to the leg were severed, regeneration proceeded normally, with the exception of a slight retardation in rate (Zhenevskaya, '61). In contrast, selective destruction of the motor roots produced an effect similar to that seen following total denervation of the muscle (Zhenevskaya, '60). Destruction of sympathetic nerves to the limb had little effect upon the regenerative process (Zhenevskaya, '62). In the light of these experiments, there is little doubt that with respect to the nervous system, muscle regeneration is almost entirely dependent upon the motor component.

Denervation Performed During the Course of Muscle Regeneration. Almost no experiments of this type have been performed. In one suggestive experiment, Zhenevskaya ('58) denervated regenerating minced muscles ten days after the initial trauma. Twelve days later, the regenerates still contained numerous muscle fibers at a relatively advanced state of differentiation whereas muscle denervated for a similar period of time, but from the beginning of regeneration contained few regenerating fibers. This experiment was interpreted to indicate that the nerve fibers, during their early period of contact with the regenerating muscle, supply the muscle fibers (myotubes?) with a trophic agent, which allows them to continue their differentiation, at least for a while, in the absence of nerves. More recently Novoselova ('71), studying the effects of repeated denervation and reinnervation of the non-regenerating rat gastrocnemius muscle, concluded that during the period of primary contact the regenerating axons exert a trophic ("inductive") effect on the muscle fibers before functional activity of the neuromuscular junction is restored. In view of the important implications of this experiment, confirmation on other systems of regenerating muscle would be desirable.

Denervation Prior to the Time of Muscle Injury. Most of the early reports in this area were based upon clinical experience or experimental models designed to reproduce the effects of long-term neuromuscular disturbances (Adams et al., '62). They indicated that muscle which has been denervated for long periods of time (from several weeks to several months, depending upon the species) loses, to a great extent, its capacity to regenerate after injury.

During the late 1950's and the 1960's, the Russian school, in its studies on conditions facilitating the regeneration of skeletal muscle, stated that denervation for periods from two to four weeks brought muscle to a "plastic condition." The following parameters are included in their definition of the plastic state: a shift to an anerobic metabolic state, a proliferation of muscle fiber nuclei, an increase of sarcoplasmic mass, an increase in the number of myoblasts and a higher RNA content than in normal muscle (Studitsky et al., '63).

In recent years there have been several investigations on the effect of prior denervation (for 2–4 weeks) upon the course of

muscle regeneration. In mammalian muscle it has generally been concluded that the primary effect of denervation prior to the time of trauma is an acceleration of the early phases of the regenerative process, particularly the establishment of a population of myoblasts (Studitsky, '63; Barisoni, '69; Yeasting, '69; Carlson and Gutmann, '73). In contrast, Hsu ('71) could detect no difference between the regeneration of normal frog gastrocnemius muscles and those which had been denervated for two weeks prior to mincing. There have been no reports of changes from normal during the later stages of regeneration of previously denervated muscle.

A possible explanation for the acceleration of early stages of regeneration in previously denervated muscles may be embodied in the morphological studies of Lee ('65), Aloisi ('70) and Hess and Rosner ('70), who described the appearance of increased numbers of mononuclear cells beneath the basement membranes of denervated muscles fibers. If these observations can be confirmed, an explanation for the acceleration of regeneration of previously denervated muscles could be that a population of myoblastic cells has already been established. Thus in contrast to normal muscle, which must first build up a population of myoblasts, the first day or two following trauma to denervated muscle could be devoted to proliferation of a preexisting population of myoblasts and their fusion into myotubes. The absence of an acceleration of regeneration in two-week denervated frog muscle is probably due to the extreme slowness in the appearance of denervation changes in the frog (Muscatello et al., '65).

The regenerating limb

Throughout the regeneration of a limb it is difficult to attribute the various reactions of the muscles to a direct nervous influence because the entire regenerative process is nerve-dependent. As a result, any effects of denervation could be due to a direct effect of nerve either upon muscle or upon the entire complex of events involved at any given stage of regeneration. Particularly in the preblastemic phases, the evidence favors the latter alternative.

There is no information to suggest that the presence or absence of nerves materially affects muscle during the early stages of wound healing or demolition. The dedifferentiation of muscle is the stage first affected by the lack of a nerve supply. In larval urodeles, denervation results in an exaggerated phase of dedifferentiation; not only muscle but the entire limb stump often regresses to the shoulder (Thornton and Kraemer, '51). In contrast, denervation prevents the dedifferentiative process in adult newts (Rose, '48). Thus in larvae denervation produces a state of morphological instability whereas in adults the opposite effect is noted. This paradox is discussed in greater detail in another review (Carlson, in press). Lentz ('70) has noted a converse relationship during muscle dedifferentiation in the adult newt, namely that motor end plates associated with dedifferentiating muscle fibers undergo a corresponding loss of specialization.

Nerves are vital for the formation of the regeneration blastema. In a detailed series of experiments Singer ('52) demonstrated that blastema formation was not dependent upon the functional type of the nerve fibers, but rather their quantity.

Relatively little work has been done on the effects of denervation upon the later, postblastemal stages of regeneration (Singer and Craven, '48; Powell, '69). The major effect is an overall retardation of growth of the regenerating limb as a whole, and atrophic changes are marked in the muscular component of the limb. It is not known which stages of muscle differentiation are affected by late denervation of the regenerating limb, but one would expect the developing muscles to be affected in a way similar to that which occurs in denervated mammalian muscle.

THE DEGREE OF FUNCTIONAL RETURN IN REGENERATING MUSCLE

Until recently, little was known about the functional state of regenerated muscle beyond the fact that upon direct stimulation or stimulation of the motor nerve, regenerated muscles contract (Allbrook and Aitken, '51; Zhenevskaya, '54; Rumyantseva, '54; Studitsky, '59; Yeasting, '69).

Two recent reports (Salafsky, '71; Carlson and Gutmann, '72) have added considerable information to the earlier studies. These studies were both conducted on regenerating minced muscles.

Carlson and Gutmann ('72) followed the development of contractile function in a regenerating limb muscle of the rat. The gastrocnemius, soleus and plantaris muscles were removed. The soleus was discarded, and the gastrocnemius and plantaris muscles (both fast mixed muscles) were minced and replaced into the leg. A single muscle regenerated from the implanted minced fragments. Regenerating muscles first contracted in response to direct stimulation eight days following mincing. This is the time when cross striations are first noted in early muscle fibers at the periphery of the regenerate. Early regenerates possessed the contractile properties of very slow muscles. However, all parameters studied quickly speeded up until by 30–40 days after mincing, most were near normal values for a fast muscle. The latency period remained somewhat longer than normal (possibly due to imperfect restoration of the sarcoplasmic reticulum), and the half relaxation time continued to be longer than normal for at least 100 days. The latter is undoubtedly due to the increased amounts of connective tissue in minced muscle regenerates. The period when the contractile times came within the normal range (30–40 days) corresponds to the time when Snow ('71) was first able to distinguish different fiber types in regenerated gastrocnemius muscles of the rat on the basis of histochemical staining for succinic dehydrogenase. Both the twitch tension and tetanic tension were far weaker (10%) in regenerated muscles than in normal muscles. This is likely a reflection of both the small diameter and imperfect internal organization of regenerated minced muscles.

Salafsky ('71) studied regeneration of the minced tibialis anterior muscle in both normal and dystrophic mice. Twitch characteristics of 75-day regenerates taken from healthy animals were close to normal, and these regenerates generated a twitch tension almost 75% the strength of control muscles. In further studies, Salafsky found that transplantation of minced dystrophic muscle into normal animals resulted in regenerates possessing nearly normal contractile properties. In contrast, functional regenerates did not result when minced muscle from healthy mice was implanted into dystrophic hosts. In addition, Salafsky (personal communication) has implanted a minced red muscle (soleus) into the bed of a white muscle (tibialis anterior) in the rat. The converse operation was performed on the minced tibialis anterior. Seventy-five days later, the regenerated soleus muscle had the contractile properties of a white muscle, and the cross-transplanted tibialis anterior regenerate possessed the properties of a red muscle. Carlson and Gutmann (unpublished observations) have found a similar reversal of contractile and histochemical properties in muscle regenerates arising from the free transplantation of the intact soleus in place of the extensor digitorum longus muscle and vice versa in the rat.

In an interesting in vitro study on regenerating rat muscle, Peterson and Crain ('72) noted spontaneous contractions of early myotubes, but muscle fibers at the same stage of regeneration did not respond to the direct application of electric stimuli. A few days later, responses to direct stimulation did occur, and by the second week of culture, they demonstrated neuromuscular transmission. They also produced coordinated contraction of regenerated human muscle fibers cultured with mouse spinal cord explants.

The general impression arising from the functional studies performed to date is that there is a close correlation between the histological structure of regenerated muscle tissue and its functional state.

EXPERIMENTAL MODELS AVAILABLE FOR THE STUDY OF SKELETAL MUSCLE REGENERATION

Over the years numerous experimental models have been used for the study of muscle regeneration. Some have been chosen because they closely resemble traumatic situations to which muscle may be exposed and others because of their potential analytical value. Several of these latter models possess distinct advantages for studying certain aspects of regeneration and will be discussed below. Most,

however, are also not without their disadvantages.

Two of the most commonly studied lesions, transverse cuts and localized crushes, are difficult to analyze, particularly for quantitative studies, because of the small area of regeneration and the gradient between normal muscle and regenerating muscle on either side of the lesion. This type of lesion, however, has been studied to great advantage in the web muscles of the African fruit bat (Church, '70a,b; Church et al., '66). Because of the anatomical simplicity and the small number of fibers in these muscles, accurate studies of differences between normal and regenerating portions of the same muscle fiber can be made (Church, '70a). Since web muscles are found in most vespertilionid bats, greater exploitation of this system would likely be rewarding.

Localized lesions in muscle fibers should be quite useful means of investigating the hitherto little studied relationships between an intact segment of a muscle fiber and a terminal regenerating portion of the same fiber. A major, still unsolved problem, is the origin of the nuclei of a muscle bud, regenerating in continuity with an intact portion of a muscle fiber. Do myonuclei migrate down the injured fiber toward the damaged end or do mononucleated free myogenic cells fuse to and extend from the damaged end of the muscle fiber in a manner similar to that which seems to occur in the normal elongation of muscle fibers (Kitiyakara and Angevine, '63; Williams and Goldspink, '71)? Hall-Craggs ('71, '72) has used localized crush lesions in rats to demonstrate that non-selective reunion of muscle fibers isolated by crush lesions occurs and that the histochemical characteristics of the innervated fiber segments were imposed upon the non-innervated segments which united with them. Localized lesions could be used to advantage in studying effects of an intact, fully innervated segment of muscle upon structural and functional development of a regenerating muscle fiber. Would muscle fibers regenerating in continuity with old muscle fibers possess a different pattern of acetylcholine receptors from muscle fibers regenerating independently? Also could they form motor end plates, thus giving rise to doubly innervated muscle fibers?

Among the other experimental models, mincing has proven to be quite useful because at the onset almost all of the normal tissue and functional relationships of the muscle are severed (Studitsky, '59; Carlson, '68; '72a). A minced muscle is simultaneously denervated, devascularized and tenotomized. In addition its internal histological and cytological architecture is severely disrupted. A major advantage of mincing is that no intact muscle fibers remain as a "contaminant" to the regenerating system. Whereas this model has proven to be extremely useful in studies involving tissue interactions and morphogenesis of regenerating muscle as well as the pattern of functional return, it is sometimes difficult to analyze because so many different factors are simultaneously at work. The large amount of connective tissue which sometimes forms in these regenerates can interfere with both functional and morphological analysis.

Massive amounts of muscle regeneration in the absence of architectural disruption can be produced by a temporary disruption of the vascular supply to the muscle (Le Gros Clark, '46; Le Gros Clark and Wajda, '47). In the affected area, degeneration of the motor nerve supply accompanies necrosis of the muscle fibers (Allbrook and Aitken, '51; Betz and Reznik, '64), and this model has proven to be a particularly effective one for studying the reinnervation of regenerating muscle. Post-ischemic regeneration has also been used as a model to study changes in isoenzyme patterns within regenerating muscles (Kaspar et al., '69; Wiesman et al., '69). In most experiments reported to date, devascularization did not affect areas of fibers at the ends of the muscles. Thus a homogeneously regenerating muscle is normally not produced (Le Gros Clark and Blomfield, '45).

Recently the topical application of local anesthetics, such as bupivacaine (Marcaine), has been used to produce a selective destruction of muscle fibers within their basement membranes (Benoit and Belt, '70; Libelius et al., '70). A major advantage of this system is that the vascular

supply is not interrupted, and the motor nerve supply remains functionally intact (Sokoll et al., '68). The rate of regeneration following myonecrosis inflicted by methyl-bupivacaine is very rapid and complete (Jirmanová and Theslaff, '72). A disadvantage of this model with respect to functional studies is that destruction of muscle fibers is not complete throughout the muscle following topical application of the drug. It is possible that uniform regeneration might be accomplished by means of local perfusion of the drug to the muscle. Because of the intact nerve and blood supply following the administration of these drugs, a close comparison of muscle regeneration after bupivacaine treatment with regeneration following devascularization could represent a good means of determining early influences of nerves or the vasculature upon the regenerating muscle fiber.

Local application of another anesthetic, novocaine, has also been found to produce regenerative changes in skeletal muscle (Klishov, '71). An unusual feature of the regenerative process in this system is the apparent stimulatory effect upon nuclear proliferation (an almost 10-fold increase in nuclear number by seven days in affected rat muscle) and a pronounced peripheral clumping of nuclei (in groups of 2–6) in 17-day regenerated muscle fibers (Klishov, op. cit., fig. 19). This phenomenon is of interest because it may represent an effective means of studying early proliferative events in muscle regeneration.

Massive amounts of regeneration can also be produced by the free grafting of intact muscles. If a normal mammalian muscle weighing over a gram or two is freely autografted, it undergoes a characteristic necrosis (Volkmann, 1893; Studitsky and Zhenevskaya, '67; Carlson, '72a) and is replaced by a dense connective tissue scar. However, if a muscle is small (Zhenevskaya et al., '65; Laird-Rolston, '70; Carlson and Gutmann, '73) or if it has been previously denervated (reviewed by Studitsky and Zhenevskaya, '67; Zhenevskaya, '68; Bosova, '71; Thompson, '71), the degeneration subsequent to free grafting is followed by a massive regenerative response which is fairly uniform throughout the entire muscle. This experimental model has proved to be a good one for investigations of functional properties of regenerating muscles as well as for cross innervation studies (Carlson and Gutmann, '73, and unpublished observations). Transplanted muscles are superior to regenerated minced muscles, for functional studies of late stages of regeneration, but analysis of early stages is complicated by the functional survival of small numbers of denervated muscle fibers throughout the early post-transplantation days.

One model of muscle regeneration which deserves greater attention because of its potential theoretical and practical importance is regeneration from the wound surface of bisected or amputated muscles. In 1949 Studitsky removed approximately the middle two thirds of the biceps muscle in chickens, leaving stumps containing muscle fibers at both the origin and insertion. In many cases the space between the two stumps was restored by tissue containing muscle fibers. Then Dimitrova ('58, '59) and Litver et al. ('61) amputated the distal halves of a number of muscles in rats and reported that regeneration of muscle occurred from the cut surface. This regeneration apparently occurs by budding from the transected muscle fibers without the formation of a blastema of undifferentiated cells. The outwardly regenerating muscle stump forms connections with the distal tendon stump, and a remarkable degree of modeling of the regenerating muscle into a typical shape was reported. This does not occur in the amputated gastrocnemius muscle of the frog (Carlson, '68). Confirmatory experiments have been performed on about 30 amputated gastrocnemius muscles of the rat (Carlson, '72a, unpublished). The muscle stumps were 3–5 mm in length. If the ends of the muscle stumps became bound down to the underlying tissues, little elongation occurred. If, however, the end of a muscle stump established connections with the regenerating Achilles tendon, considerable elongation (up to 5-fold) and an increase in mass occurred.

In his studies of inductive phenomena in tissue regeneration of adult mammals, Levander ('64) claimed that pieces of

rabbit skeletal muscle, soaked in trypan blue and then implanted into the loose connective tissue of the host, induce undifferentiated cells to form new skeletal muscle fibers. Polezhaev ('70) repeated these experiments in rabbits and rats and has reported the formation of new muscle fibers around pieces of muscle which had been soaked in 1% trypan blue (Gurr) for 48 hours. Critics of these experiments have suggested the possible survival of portions of the implanted muscle (Romanova, '72; see discussion of Polezhaev, '70) and have questioned the interpretation of the results, but both Levander ('64) and Polezhaev ('70) have maintained that the muscle soaked in trypan blue undergoes total necrosis. These experiments remain highly controversial.

The use of in vitro methods to study cellular aspects of muscle regeneration has been highly successful in the past (reviewed by Murray, '60; Půža and Gayer, '66). The many recent developments in culture techniques for muscle are making possible some clear-cut studies on tissue interactions, particularly nerve-muscle, during muscle regeneration in culture (Lentz, '71; Peterson and Crain, '72). Studies on the degeneration (Hoja and Janik, '64) and regeneration (Bischoff, '72) of single muscle fibers in vitro have recently been begun.

The regeneration of muscles within a regenerating limb is an extremely important experimental system because it represents the only means by which virtually perfect morphological and functional restoration of mechanically damaged or totally missing muscles is known to occur. In view of the recent demonstration that the histologically recognizable phases of muscle formation in a regenerating limb repeat almost exactly those occurring in the embryonic limb bud (Grim and Carlson, unpublished observations), further emphasis should be placed upon determining whether there are corresponding similarities in morphogenetic control mechanisms. Much of the relevant literature relating to both early and late phases of epimorphic muscle regeneration has been reviewed by me elsewhere (Carlson, '72c, in press).

THE REGENERATION OF SKELETAL MUSCLE IN HUMANS — WHAT HAVE WE LEARNED FROM ANIMAL STUDIES?

It has been established without a doubt that injured or diseased human skeletal muscle is capable of regenerating. The early histological descriptions of muscle regeneration following disease (Zenker, 1864; Waldeyer, 1865) or trauma (Volkmann, 1893) have been amply confirmed by modern histological and electron microscopic studies (Gilbert and Hazard, '65; Allbrook et al., '66; Shafiq et al., '67; Mastaglia and Kakulas, '70; Mastaglia et al., '70). The morphological descriptions of human muscle regeneration reveal no major differences from regeneration in other mammals, and there is little reason to suspect that the mechanisms underlying the process are different. Since much of the classical handling of traumatized muscle has been predicated upon the once prevalent notion that skeletal muscle does not regenerate, it might be well to reconsider current procedures in the light of recent experimental findings.

Severely damaged muscle is commonly treated by surgical debridement of the directly injured tissues and wide excision of surrounding muscle of questionable viability. The major reasons for this procedure have been to eliminate a possible medium for the growth of anerobic bacteria and to attempt to reduce excessive scar tissue formation. The affected muscle is then usually immobilized and later subjected to limited exercise. This regimen is designed to produce the quickest healing of the damage without the expectation of any functional regeneration of new muscle fibers.

The following factors, learned primarily from experimental work conducted during the past two decades, should be kept in mind in planning a course of treatment which would maximize the natural regenerative potentialities of damaged skeletal muscle:

1. There must be a source of myoblastic cells. Quantitatively, this source is considerably reduced by the massive debridement which is currently practiced. The experience with minced muscles or with the transplantation of small pieces (1–2 cm^2) of intact muscle has shown that these can

provide an additional source of cells with myogenic potential.

2. Early muscle regeneration proceeds most satisfactorily if there are present basement membranes of old muscle fibers or other materials which can be used as a substrate upon which myoblasts can proliferate and fuse.

3. A good local blood supply is required once a population of myoblasts is established. A major limiting factor in the amount of muscle which regenerates is the spatial and temporal separation of the source of myoblasts from the vascular supply. Application of some of the newer arterial implantation techniques (Letts and Sorbie, '70) is one means of attacking this problem.

4. A motor nerve supply is needed to maintain the integrity of the regenerating muscle fibers. Continuity of regenerating muscle fibers with segments of already innervated fibers seems to obviate the need for direct innervation of the regenerating fibers themselves.

5. Muscles fibers that do regenerate, become morphologically and functionally normal. Therefore any regeneration which can be stimulated should be of direct functional benefit to the patient.

6. Continuing tension is of help in restoring the internal architecture of regenerating muscle, and there is some evidence that it may also be a positive factor in the differentiation of new muscle fibers (Denny-Brown, '51; Rumyantseva, '59, '60).

It has been demonstrated several times that muscle repair can be accomplished by the addition of pieces of previously denervated muscle or by the replacement of entire damaged muscles by free grafts of denervated muscles (Zhenevskaya, '68; Studitsky and Zhenevskaya, '67; Carlson and Gutmann, '73). Thompson ('71) has already employed this technique in the correction of disorders of the human facial musculature. One of the basic mechanisms in the success of these transplantation procedures is the massive regeneration which occurs in the free grafts. Since the homografting of muscle tissue is likely to receive more attention in the future, it is noteworthy that the early steps of muscle regeneration occur almost normally and that full cytodifferentiation of a small number of muscle fibers is possible in homografted muscle which is eventually strongly invaded by lymphocytes (Studitsky, '64; Studitsky and Rumyantseva, '64; Carlson, '70c).

Small foci of muscle degeneration followed by regeneration likely occur much more commonly during normal life than has been hitherto suspected. Biopsies of muscles from individuals, such as athletes engaged in contact sports, who have sustained moderate blows to the soft tissues would probably reveal a high incidence of areas of local regenerative activity. I have seen surprising numbers of regenerating muscle fibers in sections from the grossly normal diaphragm and psoas muscles of an individual who died from cardiac arrest 12 days after a relatively minor automobile accident.

At this time there is little reason to expect that methods for eliciting an epimorphic regenerative response in human limbs and muscles will soon be forthcoming. Nevertheless, the remarkable progress made in some facets of regeneration research make one unwilling to dismiss the possibility as a completely unattainable goal.

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