POSSIBLE ROLE OF CYCLIC AMP IN THE NEUROTROPHIC REGULATION OF SKELETAL MUSCLE

by

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Historical Survey

"Trophic" is defined in Webster's New World Dictionary as "having to do with the processes of nutrition". This is not a particularly precise definition, and fails to convey a feeling for the full extent of the phenomena currently considered to be "neurotrophic". The phenomenological nature of the available information concerning "neurotrophism" makes a precise definition all but impossible. However, in order to provide a working level of understanding of what is meant by "neurotrophism" attempts have been made to at least give the term a conceptual definition. Two such conceptual definitions are as follows:

A) NEUROTROPHIC RELATIONS: nervous non-impulse mechanisms maintaining (and recovering) metabolism and structure of tissues (74).

B) TROPHIC EFFECTS: interactions between nerves and other cells which initiate or control molecular modifications in the other cell (66).

The term "neurotrophism", then, presupposes that, in addition to the transmission of impulses for the initiation of short-term events, nerves are capable of supplying long-term regulatory input to their end-organs. The mechanisms by which nerves provide neurotrophic

regulation have not been clearly identified, but such regulation has been shown to be necessary for the development, maintenance, and restoration of end-organ structure and function. The evidence for neurotrophic regulation is primarily indirect, and has been derived from a variety of studies, including those involving a) morphogenesis (80; 198, 93), b) denervation (66, 67, 75, 76), c) nerve cross union (30, 45, 66, 67), and d) regeneration (66, 67, 74).

Clinical observations of neurotrophic phenomena are quite old, even predating Galvani's observations of bioelectricity. Prochaska, in 1784 (79), speculated as to whether nerves "participated in the substitutuion of lost particles, and, thus, the building up and renewal of our organism". Several of his contemporaries, Sylvius and Willis among them, had already made the assumption that two kinds of fluid flowed in neural tubes; one a "dense protein fluid which serves nutritional processes", and the other, "vaporizing, more liquid, diffused in the former and destined for eliciting motion and sensation".

The early clinical observations of neurotrophic phenomena involved primarily the morphological changes which accompanied peripheral nerve damage. Such morphological disturbances were called "trophoneuroses" and included tissue dystrophy, muscular atrophy, and neuroarthropathy (a marked destruction of joint and bone following peripheral denervation) (Mitchell, 1831; Charcot, 1868 from 5; and Romberg, 1846; Mitchell, 1864 from 79). The

initial experimental studies concerning neurotrophism focused on the experimental production of "trophoneuroses". Magendie (1824 from 79) was able to demonstrate the development of keratitis following section of the trigeminal nerve, and Brown-Sequard (1849 from 79) demonstrated muscular atrophy after section of the sciatic nerve.

Although no neurotrophic mechanisms had been isolated, clinical and experimental observations favored a hypothesis involving the direct nervous regulation of tissue metabolism (or nutrition, which apparently was used interchangeably with metabolism). Claude Bernard's concept of the constancy of the "milieu interieur" (74) no doubt played a role in the orientation of early observers. Sechenov in particular (1866 from 79) stated that "these processes which ensure the anatomical and functional integrity of tissues during life are generally designated as nutritional processes. These functions enable muscles, nerves, bones and other tissues in the organism to remain unchanged as far as their physiological properties are concerned". Sechenov then speculated as to whether "these [nutritional] processes are not also dependent on nervous influences". A phenomenon demonstrated earlier by Bernard led to an attempt by Samuel (1860 from 79) to prove the existence of anatomically distinct "trophic nerves". Bernard had shown that sectioning the superior cervical ganglion in a rabbit resulted in both a vasodilation and an increase in temperature in the rabbit's ear. Samuel attempted to show that <u>vasomotor</u>

nerves did not directly influence tissue metabolism, and hence, would not increase temperature. In a monograph entitled "The Trophic Nerves", he defined the trophic influence of a nerve fiber in direct relation to its effect on tissue metabolism. Then on the basis of Bernard's observation, and his own evidence showing the lack of a direct metabolic influence mediated by vasomotor nerves, he postulated the existence of anatomically distinct "trophic nerves". Both Bernard and Sechenov remained skeptical of Samuel's hypothesis. Bernard in particular (1871 from 79), pointed out that no one had been able to demonstrate the anatomical existence of such nerves, and, in fact, that there was no reason why nerve fibers regulating functional activity could not also regulate metabolic activity.

The idea of a "trophic nerve" as an anatomical entity was not, however, laid to rest by Bernard. Heidenhein (1866 from 79 assumed the existence of trophic nerves supplying the salivary gland. He differentiated salivary nerves as secretory nerves, which stimulated the production of saliva, and trophic nerves, which regulated the production of the organic constituents of saliva. Pavlov, as late as 1920 (79), accepted the existence of trophic nerves. He, in fact, postulated the existence of a "triple control" of organs, i. e.

> every organ is under the influence of triple nervous control, i. e. functional nerves which either evoke or interrupt functional activity (muscle contraction, secretion in glands, etc.), vasomotor nerves regulating roughly the supply of chemical material (and removal of metabolic products) by increasing

> or decreasing blood circulation through the organ, and finally trophic nerves, which control in the interests of the organism the exact amount of final utilization of this material in each organ.

Although trophic nerves per se probably do not exist, nerves can influence distant metabolic events, as was dramatically illustrated by Waller (1850, 1856 from 74). He found that after nerve section the portion of the axon distal to the cut, i. e. separated from the cell body, rapidly degenerated. The nerve cell, or "trophic center" was absolutely necessary to the maintenance of the axon, and could be said to have a "trophic" relationship with the axon. The independence of some neurotrophic relationships from nerve-impulse generation was first pointed out by Bouchard (1866 from 79) who declared that "as the distal portion of a sensory nerve degenerates after section, transmission of impulses cannot constitute the trophic function of the neuron."

Early anatomical observations also indicated that nerves might play an important role in the embryonic differentiation and development of innervated structures, particularly skeletal muscle. Alessandrini (1824, 1834 from 198) described cases of partial amyelia in the dorsal and lumbar segments of domestic animals, which were accompanied by the absence of appropriate hind limb muscles. Only connective tissue was present, and extensive fibrous changes had caused fixation and ankylosis of joints. Neumann (1846 from 198)

dissected a malformed human fetus which had hemicephaly and partial amyelia, but found both well-developed spinal roots and nerves, together with well-developed muscles. This observation was accepted as positive evidence supporting the role of nerves in muscle development. Subsequent observations by Weber (1851 from 198) seemed to confirm the influence of nerves on muscle development. He reported a case of amyelia of the distal half of the spinal cord in which he found the corresponding body region to have well-developed skin, a normal skeleton, and well differentiated tendons, but a complete absence of muscle. Weber concluded that muscles could not be formed independently of the nervous system.

A critical point not included in these early reports was the age of the animal observed. Sherrington (162) reported an amyelous human fetus of 7 to 8 months which lacked any trace of brain or spinal cord, but in which "the striated muscles and their fibers revealed no obvious departures from normal". Sherrington accepted the idea that a neurotrophic influence was necessary for the maintenance of muscle structure, but concluded that "the trophic influence must therefore be still in abeyance during the period of fetal life".

The extent to which vertebrate muscle fibers can differentiate and develop when deprived of their nerve supply has been extensively studied, using both a variety of animal

species and a variety of experimental techniques. The initial studies were conducted by Schaper (1898 from 93) on Anuran embryos and larva. His work was continued and extended by a number of people, in particular by Harrison (84), who removed parts of the spinal cord of tadpoles, thus producing experimental amyelia. Harrison also placed barriers in the paths of growing nerve fibers, thereby preventing them from reaching the peripheral musculature. Under both conditions, Harrison found that peripheral muscle fibers differentiated and remained present and clearly defined. If, however, he followed the course of development up to metamorphosis, he noted that the muscle fibers became atrophic.

The neuroembryological literature is filled with similar studies using a variety of rather clever techniques, i. e. the production and transplantation of aneurogenic limbs in Anurans (93, 141, 181), the grafting of limbs to non-innervated body regions in Anurans and birds (81, 93, 198), denervation at late developmental stages in mammals (47, 93, 198) and lately, the use of tissue culture techniques (93, 195). The consensus of opinion appears to favor the idea that muscles can differentiate and develop in the absence of innervation, but that such aneurogenic development may be slower than normal (47, 198). The animal also apparently reaches a point where innervation becomes necessary for maintenance and growth. If innervation continues to be suppressed beyond this time the muscles will cease growing and

begin to atrophy. There remains some confusion over this point, however, since limb regeneration studies in Anurans, which supposedly mimic developmental phenomena, show an absolute requirement for the presence of nerves (166). The situation becomes even cloudier when it is pointed out that the nervous requirement for limb regeneration in Urodeles involves only the presence of a minimum quantity of axoplasm. Both motor and sensory nerves can induce limb regeneration if they are present in a quantity sufficient to exceed the minimum axoplasmic requirement. After regeneration is completed motor nerves must innervate limb musculature in order to prevent atrophy (93, 166, 167). It should also be noted that in tissue culture studies, cloned myoblasts have shown a normal ability to grow and differentiate in the complete absence of nerves (195). Such cultures will apparently never reach the point of nerve dependence noted in vivo. It may be concluded that some organizing process takes place during development which makes the continued growth and maintenance of muscle dependent on some factor supplied by its innervation. This organizing process is apparently not inherent in the muscle fibers themselves, but requires some interaction with surrounding tissues, including nerves. It represents, perhaps, an additional example of "embryonic induction" between tissues (67, 73, 74, 181).

Further difficulty in establishing a unified concept of the role

of nerves in tissue differentiation and development arises when one discovers that some structures are absolutely dependent on innervation for their development. Such dependence has been demonstrated in several types of receptor organs, e.g. taste buds, muscle spindles, lateral-line organs and skin receptors (67, 197, 198). In some of these receptors the nervous effect on differentiation probably precedes the onset of function (197). In fact, in some cases, sensory innervation is necessary before the organ will differentiate from its component tissue. The specificity may be so pronounced as to require a sensory nerve of the correct functional relationship (e.g. gustatory nerves are apparently the only ones capable of inducing taste bud differentiation) (67, 197).

The denervation of structures in the adult animal produces a progressive series of changes which may culminate in the complete atrophy and disappearance of the structure. Early observations noted the morphological deterioration accompanying denervation (74, 79, 175). As attention became focused on the neurohumoral relationships between nerve and end-organ, a large number of investigators noted that denervated structures became increasingly sensitive to certain chemical agents, in particular to acetylcholine and catecholamines (21, 28, 157). This phenomenon has been termed "denervation hypersensitivity" or "supersensitivity" by Cannon (28), and it represents perhaps the most extensively reported neurotrophic

phenomenon. Finally, as both the techniques for investigating and the understanding of metabolic mechanisms have improved, it has been possible to determine some of the specific metabolic relationships which are altered by manipulating the nerve-end-organ relationship (66, 67, 73, 74, 78). Thus the old postulates concerning the involvement of the nerve in end-organ "nutrition" have taken on a firmer, experimentally verified, foundation.

Since this project is aimed at using skeletal muscle to investigate possible neurotrophic mechanisms, it might be advantageous to point out specific neurotrophic relationships using the neuromuscular system as a model.

Neurotrophic Influences in Skeletal Muscle

Neurotrophic relationships in the neuromuscular system of adult animals have been studied using several approaches, i.e. the denervation of muscle fibers, reinnervation after denervation, and the cross-union of nerves supplying different muscles. Nerves have been shown to be necessary for the maintenance of the structural, chemical, and functional integrity of muscle fibers, and for the determination of certain qualitative distinctions in their histochemical profiles and contractile characteristics (30, 66, 67, 74, 76).

The complete denervation of a muscle leads to a complex series of events which occur both simultaneously and progressively over an

extended period of time. Synaptic transmission is lost within a few hours after nerve section, while progressive atrophic events may continue for several months. Certain changes occurring after denervation are specific and cannot be reproduced by disuse alone (67, 74). These factors must be assumed to be under the control of some "trophic" influence of the nervous system. Further verification of this assumption has been supplied by studies in which changes in the length of the nerve fiber remaining distal to the point of section have led to changes in the time to onset of the muscle alterations associated with denervation. This phenomenon has been viewed as evidence for the transport of a trophic substance in the nerve fiber; i. e. the greater the remaining length of fiber, the larger the amount of residual trophic material available, the longer the time period before the residual substance is exhausted (46, 74, 117, 128).

Specifically, muscle denervation first produces a decline in resting membrane potential, followed by a loss of junctional transmission, due probably to the early deterioration of fine nerve terminals (128, 137). Spontaneous miniature end-plate potentials disappear at the same time nerve terminals degenerate (15, 126). Following a period of from 2 days in rodents, to 18 days in humans, spontaneous, rhythmical contractions of the denervated muscle fibers appear. This phenomenon, termed denervation fibrillation, may be observed visually, after removal of the overlying skin and fascia, or it may be

recorded electrophysiologically. While fibrillation has been extensively investigated (12, 36, 111, 117, 158, 182), there is currently little agreement as to the underlying mechanisms producing the spontaneous activity. It is presently believed, however, that fibrillation potentials originate at the denervated end-plate, and are then propagated along the muscle fiber (12, 101, 110, 111).

Changes in the electrical and chemical excitability of the muscle fiber after denervation have also been extensively reported (28, 66, 67, 74, 75, 76, 182). Changes in the properties of the muscle membrane may be directly responsible for such alterations in excitability. Membrane property changes include: a) a decrease in the resting membrane potential (3, 92), b) an increase in transmembrane resistance (3, 136), c) a decrease in potassium conductance (40, 82, 92), and d) a prolongation of membrane accommodation and chronaxie (38).

The increase in chemical excitability is perhaps the single most extensively investigated phenomenon associated with denervation. The denervated muscle fiber's extrajunctional sensitivity to neurohumorals, particularly acetylcholine (ACh), is dramatically increased (3, 21, 28, 46, 101, 126, 157, 179). Normal, innervated muscle fibers are sensitive to ACh only at their end-plates and within a distance of 100-200 μ from each end-plate. The peak of sensitivity is centered at the end-plate and drops off rapidly as one moves away from it. Within a few days after denervation, the area

of muscle fiber membrane responsive to ACh has enlarged. If denervation is maintained, the entire muscle membrane will eventually become sensitive to ACh. Interestingly, the denervated end-plate does not become more sensitive to ACh, but the membrane regions outside of the end-plate tend toward the same level of sensitivity as that normally found at the end plate (126). Miledi (125, 126) postulates that the removal of some influence mediated by the nerve allows the spatial spread of membrane located ACh-receptor units, and that this leads to an increase in ACh-receptor density in membrane regions other than that of the end-plate. Recent evidence now indicates, moreover, that the spread of extrajunctional ACh sensitivity depends on de novo protein synthesis (50, 64). Inhibitors of protein synthesis, applied to rat muscle either in vitro or in vivo during the first two days following denervation, delayed the appearance of extrajunctional ACh sensitivity. Once an increase in sensitivity had been established, however, the addition of the inhibitors would not reduce it. These experiments at least tentatively indicate that the increase in extrajunctional ACh sensitivity depends on a mechanism possibly involving gene induction. Furthermore, the new receptors, once established, apparently have a relatively long half-life. Reinnervation, however, does produce a retraction of the ACh-sensitive region, until the normal, innervated situation is resumed (127). Of additional interest is the observation that prior

to innervation, developing skeletal muscle is sensitive to ACh along its entire length. The ensuing development of neuromuscular connections then produces a retraction in the ACh-sensitive region until the normal adult relationship is reached (39).

There are also specific intracellular changes occurring in a denervated muscle. While both denervated and disused muscles atrophy, there are basic differences in the biochemical alterations which occur in each case (67, 73, 74, 76). Neurotrophic mechanisms appear to involve the regulation of protein synthesis and protein degradation (67, 74, 75, 76). Gutmann (75, 76) has divided the metabolic changes following muscle denervation into three phases: a) initial denervation hypertrophy, apparently due to acute immobilization, b) changes due to the loss of neurotrophic influences, and c) metabolic adaptation to muscle inactivity. The initial hypertrophy phase includes transient increases in muscle protein content, muscle glycogen content and end-plate cholinesterase (ChE) activity. This phase can be duplicated by disuse experiments, in which transmission across the synapse is blocked while innervation remains intact. Denervation hypertrophy may be pronounced in certain muscles (e.g. rat diaphragm), but completely absent in others (e.g. rat gastrocnemius). The trophic phase starts, according to Gutmann (7 5, 76), approximately 3 days after nerve section. Using rabbit muscle, he demonstrated a large (200%) increase in muscle DNA

content, and a smaller (15%), but significant, decrease in protein content. These changes could not be duplicated by a nerve block of 3 days duration produced by novocaine. Gutmann (75, 76) has also reported a dissociation, shortly after denervation, between the resynthesis of RNA and protein synthesis. In the initial stages of denervation phase 2, there is relatively little change in RNA content, but a considerable loss of protein. This appears to be due to an increase in proteolytic activity without any accompanying change in proteosynthesis (74). It is also possible to stimulate RNA resynthesis in denervated muscles without stimulating an accompanying increase in proteosynthesis (75). Goldberg (59) in fact, found proteosynthesis to be decreased after denervation. Gutmann raised the question of whether "this dissociation between nucleic acid and protein synthesis may not be the characteristic feature of the (trophically induced) metabolic disturbance in denervated muscle".

The regulation of muscle protein metabolism by nerve is more specifically demonstrated by studies involving specific muscle enzymes, located in particular intracellular regions. The sarcoplasmic constituents of denervated frog muscle show an increase in the incorporation of labeled amino acids into protein, while myoplasmic constituents show a progressive decrease in proteosynthesis (132). The demonstrated increase in proteolysis after denervation may be correlated with an increase in the activity of particular

lyosomal enzymes (e.g. B-glucuronidase and acid phosphatase) (67). The most extensively investigated enzyme system associated with neurotrophic influences, however, is cholinesterase (ChE). Morphogenetic evidence has firmly established the cause and effect relationship between the onset of innervation and the appearance of muscle ChE (67, 93). Additional evidence strongly supports the concept that the control of muscle ChE in the adult is mediated by neural influences not associated with impulse activity (67). Most of the adult muscle ChE is accumulated at the end-plates (35) and is extremely dependent on the presence of the nerve. Three days following denervation, 50-70% of the ChE in the rat sternomastoid muscle had disappeared. While tenotomy produced a similar amount of atrophy, it did not produce a loss of muscle ChE (71). The loss of muscle ChE has also been shown to be proportional to the percent of innervating fibers which are lost (67). Reinnervation, or the collateral sprouting of nerve fibers from intact neurones to denervated end plates, is capable of restoring ChE activity to denervated muscle fibers (67, 70, 72). As additional evidence, DFP (diisopropylfluorophosphate), an irreversible inhibitor of ChE, was injected into young chickens, producing a disappearance of end-plate ChE.
ChE would gradually reaccumulate in the end-plates of the affected muscles only if the muscles were innervated. If the muscle was denervated at the time of application of DFP, ChE would not reappear

(53). Recently, an in vitro study has shown that explanted sensory ganglia, separated from cultured segments of muscle by a filter, and added filtered nerve homogenates, are able to maintain ChE activity in cultured denervated muscle (109). Thus providing additional evidence for the hypothesis that some neurotrophic influences are mediated by chemical factor(s).

Mammalian skeletal muscles have been differentiated into two types, slow and fast, on the basis of their contractile velocities (e.g. time to peak tension for a twitch response) (24, 25, 32, 45, 55, 135, 185). It has also been possible to make distinctions between muscle types on the basis of other parameters. Besides having slower contraction velocities, slow muscle fibers also receive nerve fibers of a smaller average diameter, which discharge at a lower average frequency, and have slower conduction velocities than do the nerve fibers innervating fast muscle (45, 67, 86, 123, 194). The tetanic frequency necessary for fusion of the muscle response is always lower in slow muscles (45, 67). On a functional basis, slow muscles are generally thought to be tonic in nature, subserving functions requiring the maintenance of long-term sustained contractions. Fast muscles, on the other hand, are considered to be phasic in nature, subserving functions requiring brief, rapid, forceful contractions (45, 67, 86).

Similar distinctions may be made on the basis of the

histochemical and enzymatic activity profiles of the two types of muscle (23, 66, 156). While histochemical studies have shown that skeletal muscles are made up of a variety of fiber types, certain patterns are evident. Slow muscle has a more uniform, or homogeneous, population of histochemical fiber types; in some studies it appears as if all the fibers of a slow muscle (e.g. soleus) are the same histochemical type (85). Fast muscles (e.g. gastrocnemius), however, have a more heterogeneous population of histochemical fiber types (85). Enzymatic activity profile studies have also demonstrated that there exists a continuum of fiber types in both types of muscle. However, the majority of the evidence favors skewed distributions, with most slow muscle fibers showing higher oxidative enzymatic activity, as opposed to the higher glycolytic enzymatic activity found in most fast muscles (66, 67, 155). The two muscle types differ in visual appearance as well; hence, slow, oxidative muscles are designated "red" muscles, and fast, glycolytic muscles are designated pale or "white" muscles (10, 66, 67, 155).

While this brief summary hardly does justice to the extensive current literature involving the physiological and biochemical characteristics of muscle, the important point to be made is that these distinctions between muscle types apparently depend, in part, on neurotrophic influences (66, 67, 73). Cross-innervating slow and fast muscles produces changes in the contractile patterns (7, 23, 24,

25, 30, 32, 45), and in the histochemical and enzymatic activity profiles of the affected muscles (7, 131, 156). The muscles are altered so as to become compatible with their innervation. However, the conversion is usually not complete; all cross-innervated slow muscle fibers do not attain completely, the set of characteristics normally associated with fast muscle fibers. The reverse of this is also true for cross-innervated fast muscle fibers (22, 23, 24, 25, 45, 67). The end result of cross-innervation is usually an intermediate muscle type, with the original parameters altered in the direction of the new innervation.

Unfortunately, therefore, cross-innervation studies apparently do not represent clearly defined examples of chemically mediated neurotrophism, and other neural influences must be considered. One of the distinctions made between the two muscle types concerns the pattern of impulse activity in the nerves supplying them. Slow muscles receive rather continuous (tonic) low-frequency discharge, while fast muscles receive bursts of high-frequency input. A large number of studies have attempted to produce changes in muscle parameters by altering the activity patterns in the nerves supplying the muscles. Most of these studies have involved the production of muscular disuse by a reduction in nerve activity. Several techniques have been used, i. e. tenotomy, immobilization, spinal cord isolation, and pharmacological block (24, 25, 43, 55, 135, 185). Only rarely

have studies involving chronic increases in muscular activity been attempted (66, 115, 184). The net result of the disuse studies, thus far, has been the production of partial changes in the contractile characteristics and biochemical profiles of slow muscles in particular. Fast muscles appear to be less responsive to decreases in neural activity (135).

The final conclusion resulting from the cross-innervation-disuse-denervation approach seems to be that the control of muscle properties requires two superimposed regulatory mechanisms: 1) the impulse activity and pattern in the nerve supplying the muscle, and 2) nerve-supplied trophic substances which act independently of impulse-activity. The informational content of the nerve impulse activity could be an impulse-mediated release of trophic material from the nerve, or could be directly related to its influence on muscle activity (55, 135).

Summary and the Consideration of a Possible Mechanism for Neurotrophism

It is evident that nerves are capable of supplying information to end organs through mechanisms other than the transmission of impulses. It is also apparent that the effects produced by non-impulse mechanisms are of long-duration, as opposed to short-duration impulse-mediated effects. In particular, nerves seem

able to exert long-term regulatory influences on the metabolic patterns of their end-organs. It is difficult to generalize across the large number of phenomenological observations summarized in the previous sections. However, there do appear to be some general conclusions which can be extracted from the various pieces of evidence.

First, neurotrophic influences may have a stimulatory effect on end-organs. Nerves are necessary for the stimulation of growth in the regenerating salamander limb (166). Lebowitz and Singer (108) have recently shown that the infusion of nerve homogenates into the regenerating blastema stimulates protein synthesis. Nerves are also necessary for stimulating the growth and differentiation of sensory organs, such as taste buds and muscle spindles (68, 196, 197, 198). Nerves are required for the production and maintenance of a number of enzymes, in particular cholinesterase and myosin ATPase (22, 67, 69, 131). Denervation of muscle produces atrophic changes which are qualitatively different than the atrophy produced by disuse (67, 74, 75). Finally, reinnervation of denervated muscles leads to an increase in RNA synthesis, an increase in protein synthesis, and a decrease in proteolysis (75).

However, there is also evidence that neurotrophic influences may be inhibitory. Gutmann (74) has described the metabolism of a denervated muscle as "exhibiting the characteristics of an

uninhibited system". There is an increase in DNA, an increase in proteolysis, a change in membrane properties which leads to oscillations of the resting membrane potential and spontaneous fibrillation, and the development of a supersensitivity to ACh (21, 28, 67, 74, 110, 111, 136, 182). Reinnervation stabilizes the membrane, halts fibrillation and returns ACh sensitivity to normal.

Apparently there must be more than one mechanism available for neurotrophic control. The first question then, becomes one of determining how the nerve supplies the necessary information to the end-organ; the second is one of specifying the molecular changes occurring in the end-organ in response to the nerve-supplied information (66). There is ample evidence, particularly from studies with sensory nerves showing neurotrophic influences moving in a direction opposite to the direction of impulse transmission, and from the recent in-vitro work of Lentz (109), to support the concept of chemical factors as the suppliers of neurotrophic information (66, 67, 73, 165). Considering the diversity of neurotrophic effects, it seems unlikely that only one chemical factor would be able to supply the necessary information. Thus far, however, no such neurotrophic factors have been isolated.

Given the hypothesis that neurotrophic influences are mediated by chemical substances released by the nerve, is there evidence that nerves are capable of functioning in this way? The axoplasmic

transport of material originally located in the perikaryon, first experimentally verified by Weiss and Hiscoe (190), is now a firmly established concept (8, 165). A large assortment of material, e.g. amino acids, protein, phospholipids, neurohumorals, has been demonstrated to move from the perikaryon into the axon and down the axon to the nerve terminals (8). There is evidence for variations in flow rates, which depended on axon size, the type of material and, in fact, on the region of the individual axon being traversed. To summarize from Barondes (8);

> It is generally believed that axoplasm is transported for four major purposes: 1) to replace axoplasmic constituents which are degraded during "turnover", 2) for secretion at nerve endings, as in the classic case of the migration of hormone containing granules from hypothalamic cells to the posterior pituitary, 3) for the growth of the axon in maturation and regeneration, and 4) for the transport of trophic substances to innervated cells.

Recent evidence (17, 63, 99) has demonstrated the ability of nerve terminals to release small proteins. The majority of studies have been concerned with the release of protein from sympathetic nerves in association with norepinephrine (17). The proteins are released in extremely small quantities and have not been associated with any function, but it was felt that they could interact with the external surface of either the pre or postsynaptic cell (17). On the other hand, Korr (8, 99) labeled hypoglossal neurons with P^{32}-inorganic phosphate or C^{14} amino acids; then observed the movement of activity down the

nerves, and eventually, its appearance in the muscle cells of the tongue. The labeled molecules apparently crossed the neuromuscular junction and entered the muscle cells. Korr suggests that this mechanism may represent the means by which neurotrophic factors are introduced into the end-organ.

Can neurotrophic factors be considered analogous to hormones? Huxley's (1935) definition of a hormone, i. e. "hormones may be regarded as agents which transfer information from one set of cells to another for the good of the cell population as a whole", would allow the inclusion of neurotrophic factors. Some hormones (in the classical sense) are neurosecretory products, e. g. the posterior pituitary hormones in mammals, and several insect hormones which also originate from neurosecretory cells in the central nervous system (191). The insect hormones represent a particularly good comparison since many of them are directly involved in target cell metabolism and protein synthesis (191). Several mammalian hormones have also been shown to produce significant changes in target cell enzyme activity, protein synthesis and nucleic acid metabolism. While a precise biochemical description of the mechanism of action for any hormone is still not available, the DNA → RNA → protein dogma seems to be implicated in many cases (e. g. thryoxin, aldosterone, estrogen, testosterone, ACTH, growth hormone, insulin). It seems probable that many hormones express their effects through

some interaction with the genetic constitution of the target cell (97, 177, 178).

Samaha et al. (159) have demonstrated the appearance of a qualitatively different myosin ATPase in cross-innervated slow muscle. Since protein synthesis is qualitatively specified by gene action it is implied that neurotrophic influences influence gene expression in the cell. Fambrough (50), using inhibitors of RNA and protein synthesis, prevented the increase in ACh sensitivity following the denervation of skeletal muscle. He concluded that "the neuronal restriction of ACh sensitivity in muscle fibers involves regulation of gene activity in muscle fibers."

If both neurotrophic factors and hormones express their effects through influences on the genetic constitution of the target cell, is it possible that they might have other mechanisms in common? Specifically, is it possible that adenosine 3', 5'-monophosphate (cyclic AMP), which has been correlated with the actions of a number of hormones (153, 174, 176), may also play a role in the mediation of neurotrophic effects? The widespread distribution of cAMP, and its demonstrated ability to influence the rate of various ongoing cellular processes makes it particularly attractive as a mechanism for the expression of neurotrophic actions.

The role of the adenyl cyclase-cAMP system in regulating cell processes will be reviewed in the next section. In particular cAMP's

relationship to hormonal actions will be considered, and the capability for specificity inherent in this system pointed out. Hopefully this will make what appears to be a strong potential correlation between cAMP and neurotrophism more apparent.

INTRODUCTION: CYCLIC AMP

Historical Survey

Cyclic 3', 5' Adenosine monophosphate (cAMP) is a mononucleotide of adenylic acid with the phosphate group diesterified at carbons 3' and 5' of the ribose molecule (see the figure diagraming the second messenger hypothesis). Since its discovery by Sutherland (176), the number of studies implicating cAMP as an intermediate in the mechanisms by which a variety of hormones produce their effects has grown at an exponential rate. There are numerous reviews (65, 152, 153, 174), covering hundreds of studies, which summarize the enormous number of hormonal responses with which cAMP has been associated. The concern of this brief survey is the proposed mechanisms of action by which cAMP may be stimulated (or inhibited), and by which it may produce its effects. Clearly, the intention is to show how cAMP could conceivably mediate the production of some of the effects associated with neurotrophism.

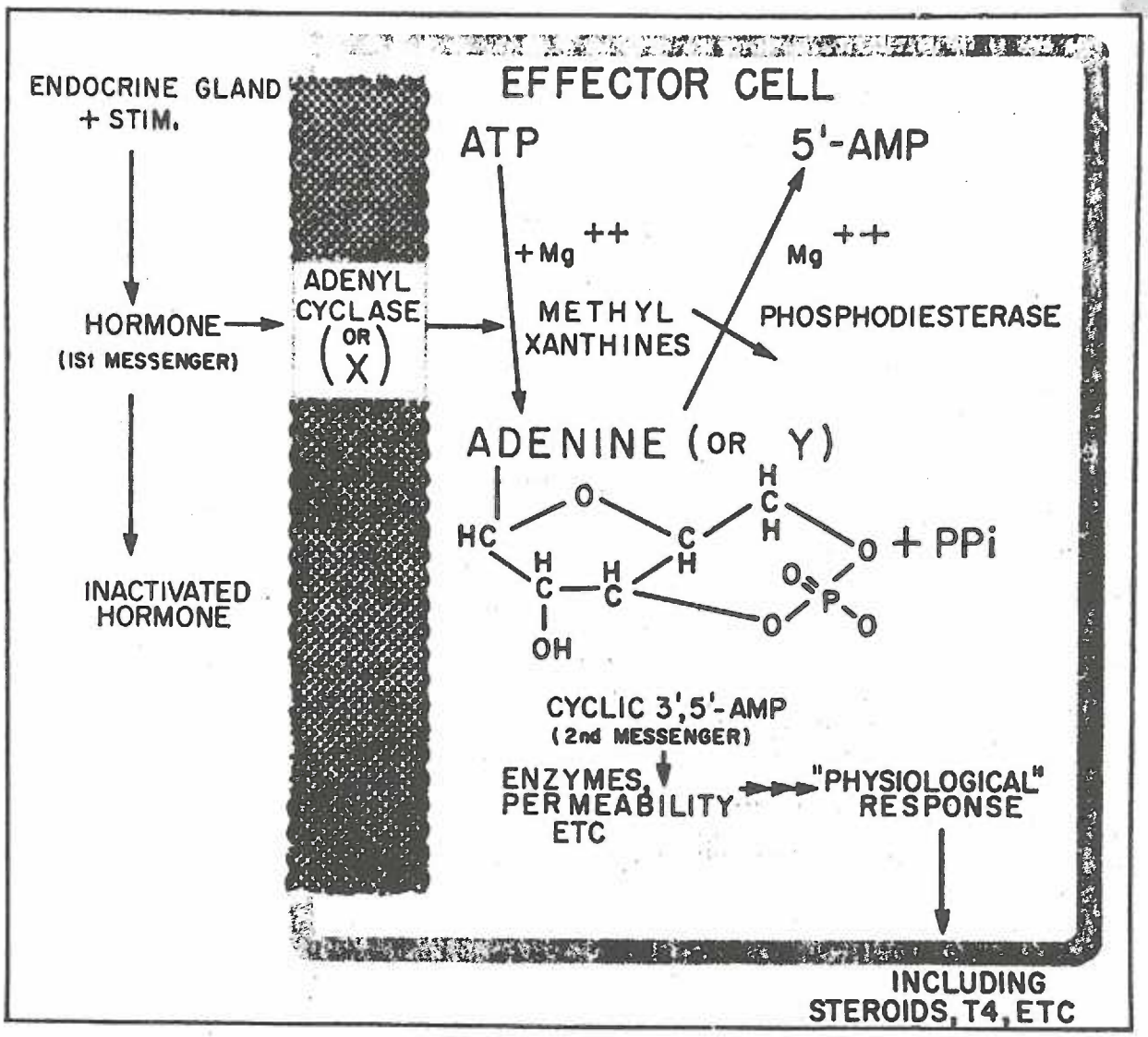
A brief synopsis of Sutherland's initial studies may provide a firmer foundation for the introduction of the second messenger concept; the postulated mechanism by which hormones or neurohumorals activate cyclic AMP. Sutherland was interested in determining the mechanism of action by which epinephrine and glucagon produced glycogenolysis in the liver. He first determined that hormone

stimulation of glycogenolysis was effected in part by the glycogen phosphorylase system (176). Phosphorylase was found to exist in active and inactive forms (i. e. the a and b form). Sutherland found enzymes capable of converting the active form to the inactive form (a to b by phosphorylase phosphatase), and the inactive form to the active form (b to a by dephosphorylase kinase). He further established that a system which contained all of these components was essentially unresponsive to epinephrine and/or glucagon unless a heat-stable factor isolated from liver homogenate, was added. This heat stable factor was ultimately isolated and identified as cyclic 3', 5'-AMP (175). Krebs (100) also confirmed the action of cAMP in the activation of phosphorylase kinase in rabbit skeletal muscle in response to epinephrine. Subsequently cAMP was shown to affect enzymes in other systems not associated with glycogenolysis (87, 138, 174). It therefore became important to attempt to provide some unifying scheme by which a diverse number of agents might stimulate (or inhibit) cAMP, and by which cAMP might aid in the production of the specific effects associated with hormone function. The first problem, that of cAMP activation, led to Sutherland's second messenger hypothesis (174).

The Second Messenger Hypothesis

Essentially, the hypothesis provides a means of incorporating hormone-target cell specificity into what seems to be a more

Figure 1. Second messenger hypothesis as proposed by Sutherland (174).



Second messenger system involving adenyl cyclase.

generalized phenomenon, i.e. the activation of cAMP. A summary of the hypothesis is shown in the figure on the previous page (Figure 1). The hormone or neurohumor (the first messenger) is released from the cell producing it. It is transported to its target cell where it interacts via its receptor, with the membrane-bound adenyl cyclase system. The process then becomes one of increasing cyclic AMP concentration, stimulation of the specific hormonally-initialled event, and the subsequent hydrolysis of cyclic AMP.

Adenyl cyclase is an enzyme, first determined by Sutherland (145, 175), which in the presence of Mg^{++}, catalyzes the production of cAMP from ATP. Greengard (65) has determined that the reaction $ATP \xrightarrow[\text{adenyl cyclase}]{Mg^{++}}$ cAMP is endergonic and reversible. Once produced, cAMP (the second messenger) proceeds to activate the cell systems necessary for the production of the "physiological response". A second enzyme, cyclic nucleotide phosphodiesterase (26), inactivates cAMP by hydrolyzing the 3' bond and converting 3', 5'-cAMP to 5'-AMP.

Adenyl cyclase has been found in every species examined, and in essentially all types of tissue. In mammals it has been found in every nucleated cell studied (152, 174, 187). Such a ubiquitous distribution would seem to defeat the necessity for specificity in the hormone-target organ relationship. The specificity of hormonal action is maintained, however, since the adenyl cyclase of each

particular tissue will respond only to the appropriate hormonal and neurohumoral agents (152, 187), which in turn, may bind only to specific receptor sites. Furthermore, for adenyl cyclase to function in the manner proposed by the second messenger hypothesis it must be readily available to the transmitted hormone or neurohumoral. The most accessible location would be the plasma membrane, and it is, therefore, of interest to note that recent studies have confirmed the location of adenyl cyclase in the membranes of skeletal muscle (143), brain (37, 188), pineal gland (188), kidney (29), fat cells (154) and avian erythrocytes (174). In order to serve as a mediator for neurotrophic factors it is necessary for adenyl cyclase to be associated with postsynaptic sites, and this has been demonstrated in the synaptosome fraction of rat brain cortex (37), in the pineal gland (189), in cerebellar Purkinje cells (164), and in the superior cervical ganglion (122). There has been some discussion as to whether adenyl cyclase itself might serve as the hormone receptor (174). Recent evidence, however, seems to indicate that adenyl cyclase is not the receptor, but that it exists in close relationship with it (16, 152).

Phosphodiesterase is more widely distributed throughout the cell than is adenyl cyclase (174). It has been demonstrated to be associated with cellular particulate material and also with the soluble fraction (26, 37). Several drugs, particularly the methylxanthines, have been shown to inhibit phosphodiesterase (26). This may form a basis for the action of these drugs, and has been used as a criterion

to establish the involvement of cAMP in the production of a particular hormone action (174). However, no hormone or neurohumoral has been shown to produce its effect on cAMP levels via the inhibition or stimulation of phosphodiesterase (152, 153, 187).

Possible Mechanisms of Action of Cyclic AMP

For an appropriate perspective in considering the postulated mechanisms by which cAMP acts, it is first necessary to briefly mention some of the end results of cAMP action. Basically this means listing some of the hormonal actions with which cAMP is associated. As previously mentioned, the number of hormonal and neurohumoral actions which have been shown to be mediated by cAMP is extensive, now approaching fifty in number (152, 174). Since, however, neurotrophic factors seem to preferentially affect membrane-related mechanisms, possibly via the control of intracellular nucleic acid and protein synthesis, those cAMP mediated-hormonal actions which involve membrane alterations and/or protein and nucleic acid synthesis will be emphasized here.

Hormones affect membrane associated phenomena such as ion permeability, electron transport, oxidative phosphorylation and lipid synthesis. It is also probable that some hormones play a major role in the maintenance of membrane integrity, possibly by their demonstrated ability to influence RNA and protein synthesis (97,

177, 178). Hormones having such effects have been shown to stimulate adenyl cyclase and produce increased intracellular concentrations of cAMP. Exogenously administered cAMP (or a more permeable analog, dibutyryl c AMP) is capable of mimicing the effects produced by the hormone in certain cases (152, 153, 174). In particular, the vasopressin-stimulated movement of Na^{+} and water in both the toad bladder and mammalian renal medulla has been correlated with a vasopressin-induced activation of cAMP (29, 138). cAMP has been postulated to act in this case, by altering the properties of the membrane or permeability barrier. Parathyroid hormone also stimulates the production of cAMP, and this fact has been correlated with PTH-induced changes in Ca^{++} and Mg^{++} permeability in the renal cortex (29), and across mitochondrial membranes (148).

A number of other hormones also induce permeability changes in their target cells, which may be related to their stimulation of cAMP synthesis. MSH, ACTH, and angiotensin for example, alter Ca^{++} movement, while glucagon increases the efflux of both Ca^{++} and K^{+} from liver cells (146, 153). There have also been attempts to correlate insulin-produced changes in muscle membrane function with the observation that insulin <u>decreases</u> intracellular cAMP levels. This explanation, however, is probably not sufficient to account for all of the effects of insulin on membrane function (153, 160). Finally, a growing accumulation of evidence now seems to have clearly

established the involvement of cAMP in the catecholamine-induced positive inotropic effect in cardiac muscle (106, 133, 168). This effect is undoubtedly at least partially related to the cAMP-mediated increase in the accumulation of Ca^{++} ions by cardiac muscle sarcoplasmic reticulum (134).

At least two studies have related hormone or neurohormonal induced changes in cAMP concentration with changes in resting membrane potentials. Cyclic AMP was shown to mediate potassium-dependent beta-adrenergic hyperpolarization in vascular smooth muscle (170). Cyclic AMP has also been demonstrated to mimic glucagon-produced increases in potassium and calcium efflux and membrane hyperpolarization in liver cells (56).

Hormones induce the synthesis of a variety of enzymes in a number of target organs. cAMP has been linked to hormone-induced enzyme synthesis in some cases, e.g. the induction of tyrosine aminotransferase by hydrocortisone in rat liver, and the induction of serine dehydratase by epinephrine and glucagon, also in rat liver. Similarly, the stimulation of steroidogenesis by ACTH in the adrenals and by gonadotrophins in the ovary require cAMP mediation (153). It has been suggested that ACTH stimulation of steroidogenesis might be mediated through an activation of protein synthesis by cyclic AMP (57). Puromycin was also shown to antagonize the steroidogenic effects of LH and cyclic AMP, but not the effects of LH on cAMP

levels, thus indicating an involvement of cAMP in the control of protein synthesis (120). TSH requires the activation of cAMP in order to stimulate the production of thyroid hormone, and this, too, has been correlated with the stimulation of protein and RNA synthesis in the thyroid gland (153).

More direct evidence for the role of cAMP in the hormonal induction of RNA and protein synthesis is available. Langan (104) has shown that hormonal-induction of enzyme synthesis is associated with an increase in histone phosphorylation. The phosphorylation of histones has, in turn, been associated with a cAMP activated histone kinase. The two events appear to be directly related; the phosphorylation of histones may be one of the primary responses which results from the increased intracellular cAMP levels produced by hormones or neurohumors (104, 105). Langan (144) has formulated a theoretical scheme for hormone action which is based on cAMP-mediated histone phosphorylation, the ensuing derepression of the DNA template, and the subsequent increase in RNA and protein synthesis. His supporting evidence includes both the observations of hormonal enzyme induction mediated by cAMP, and the extensive evidence relating histones to DNA regulation (4). Studies using both bacterial and mammalian systems have correlated cAMP with increases in DNA synthesis (118), the stimulation of polyribosomes (113, 139) and an involvement in the transcription process (48). On the basis of observations

relating cAMP activity to both pre- and postsynaptic events during impulse transmission (19, 60, 122, 164), Langan has postulated a role for cAMP in the improvement of synaptic connections (by the induction of particular proteins) and the phenomenon of memory. If one considers certain neurotrophic studies, especially those by Gutmann (75, 76), Samaha et al. (159) and Fambrough (50), which demonstrated trophically-produced changes in muscle RNA and protein synthesis, there seems good reason to postulate a role for cAMP in the mediation of neurotrophic effects.

Molecular Mechanisms of cAMP Action and the Possible Relationship of cAMP to Neurotrophic Factors

The specificity inherent in the hormone-target cell relationship may be adequately explained by postulating the existence of distinctive membrane receptors. However, the specificity of intracellular hormonal actions, especially if mediated by a common molecule (cAMP), requires additional explanation. There have been essentially two different mechanisms proposed to account for the molecular basis of cAMP action. One which has already been mentioned briefly, involves the activation of specific protein kinases by cAMP (65). Cyclic AMP has been demonstrated to cause widely different effects in different tissues, and even multiple effects in the same tissue (e. g. glycogenolysis and protein synthesis in liver) (65). The protein

kinase activation hypothesis postulates that cAMP stimulates a specific protein kinase, which in turn catalyzes the phosphorylation of a specific macromolecule. Phosphorylation of the macromolecule leads to a change in its biochemical activity and results in a specific functional change in the cell. For example, the phosphorylation of histone may lead to the possible derepression of the DNA template, and result in the synthesis of either more or qualitatively different proteins. This hypothesis allows specificity to be achieved by any or all of three possible mechanisms. First, Kuo et al. (103) demonstrated that protein kinases obtained from different tissues differ from each other in terms of their reactive properties, e.g. the concentration of cAMP required to give a half-maximal increase in kinase activity was found to vary between tissues. Second, Kuo and Greengard (104) provided evidence for the existence of multiple cAMP-dependent protein kinases within a given tissue. Each kinase showed a specificity for a particular macromolecular substrate. Third, it seems possible that there may be qualitative and quantitative substrate differences existing in each tissue. This would allow alterations in the activity of a particular kinase, depending on the availability of its substrates in a given tissue. This potential mechanism is still undergoing investigation (65).

An alternate mechanism proposed for the molecular basis of cAMP action involves the relationship of cAMP to Ca^{++} ions (146,147).

Rasmussen has noted that many effects mediated by cAMP also involve changes in the distribution of Ca^{++} ions. This is particularly true for the cAMP-mediated hormonal effects produced by polypeptide hormones, e.g. vasopressin-induced permeability changes and PTH-induced gluconeogenesis in the renal cortex (146). Rasmussen has proposed several potential mechanisms relating cAMP and Ca^{++}, but currently seems to favor one involving an initial hormone-induced activation of cAMP, followed by a cAMP-mediated change in membrane permeability, and a subsequent redistribution of Ca^{++} ions. The resulting change in Ca^{++} concentration in one or more intracellular compartments is viewed as a potential regulatory phenomenon, providing a basis for the ensuing changes in cellular function (146, 147).

While the protein-kinase mechanism is currently more widely accepted than is the Ca^{++} hypothesis, either could conceivably support the hypothesis that cAMP plays a role in the mediation of neurotrophic effects. Gutmann (73, 74) has stated that after denervation, "a muscle becomes an unregulated system" or a "system released from inhibition". The large increase in DNA, the alterations in membrane properties which lead to fibrillation, the induction of specific RNA and protein synthesis leading to ACh hypersensitivity, and the progressive decrease in glycogen and increase in proteolysis, seem to present a picture of a system which has lost a controlling

influence. On the other hand, the activities occurring in denervated muscle are similar to hormonally-induced activities which occur in normal tissue, and which have been shown to be mediated by cAMP. This similarity between the two situations provides the possibility that one function of the neurotrophic substance is the inhibition of adenyl cyclase and the reduction of cAMP concentration. Denervation would remove the inhibition and allow an increase in cAMP, leading to the phenomena associated with denervation. Two additional related observations are of interest. In the only denervation study involving measurements of either adenyl cyclase activity or cAMP concentration currently found in the literature, Weiss (187) showed that superior cervical ganglionectomy (which produces denervation of the pineal gland) enhanced adenyl cyclase activity in the pineal gland. Additionally, although the identity of the trophic substance is unknown, and the subject of some controversy (66), one hypothesis proposes that the spontaneous quantal release of ACh may function as the neurotrophic transmitter (42). It is interesting, in this regard, to note that ACh has been shown to inhibit adenyl cyclase and reduce cAMP concentration in the heart (196). Prostaglandins, which may be released from nerve endings during impulse activity, have also been shown to inhibit adenyl cyclase (144). Finally, a possible requirement for the Ca^{++} hypothesis is also fulfilled in denervated muscle. Brody

(20) has demonstrated an increased accumulation of Ca^{++} ions by the sarcoplasmic reticulum of denervated skeletal muscle. A correlated increase in cAMP levels has not been shown, but a specific increase in sarcoplasmic protein synthesis after denervation has been reported (73).

STATEMENT OF THE PROBLEM

It is proposed that neurotrophic influences, directed from motor neurons to skeletal muscles, are transmitted by chemical substances, which are similar to neurohormones in the nature of their synthesis and release. These neurotrophic substances are assumed to be released from motor nerve terminals, to cross the synaptic cleft, and to combine with receptors located on the membrane of the muscle fiber. The combination of neurotrophic factors with muscle membrane receptors supposedly leads to the regulation of some aspects of muscle metabolism. It is further proposed that this neurotrophically-induced regulation of muscle metabolism is mediated via the control of an adenyl cyclase-cyclic AMP system. Neurotrophic factors, through their interaction with muscle membrane receptors, are postulated to alter the activity of either adenyl cyclase or phosphodiesterase, and subsequently, to alter the concentration of cyclic AMP in the muscle.

It is possible to test the involvement of cyclic AMP in neurotrophically-induced phenomena. Such a test requires the measurement of cyclic AMP during the production of events defined as being neurotrophically-mediated. The problem must be approached through a series of experimental stages. First it must be demonstrated that the removal of neurotrophic influences from skeletal muscle results

in significant changes in the intracellular concentration of cAMP in the muscle. This will be accomplished by denervating a selected skeletal muscle, and assaying for cAMP at various times after denervation. The use of this technique requires that changes in the concentration of cAMP associated with denervation be distinguished from those produced by muscular disuse alone. A comparison between the concentration of cAMP after denervation versus the concentration after a similar period of disuse comprises the second experimental stage.

The third stage is designed to more carefully establish a correlation between postulated neurotrophic factors and changes in the intracellular concentration of cAMP. This will be accomplished by sectioning the nerve at varying distances from the muscle; then following the temporal pattern of changes in cAMP concentration. In the fourth stage, the changes in cAMP concentration produced by denervation and associated with neurotrophic events must be demonstrated to be reversed by reinnervation of the muscle.

METHODS AND MATERIALS

Animal Selection and Maintenance

Female, Sprague-Dawley rats, weighing between 200 and 250 grams were used in all experiments. The animals were between 4 and 6 months of age at the time they were used. From the time of arrival the rats were housed in large wire cages, at a density of no more than 4 per cage. They were fed a standard rat chow and given water ad libitum. The animals were briefly removed from the colony, surgical procedures accomplished, and the animals returned to their cage until the time they were removed for measurement. Despite the use of wire cages none of the animals showed any evidence of trophic ulceration following denervation.

Rats were chosen for a number of reasons. Since the measurements reported here are to be temporally correlated with other measurements reported in the literature, an effort has been made to reduce cross species variability by selecting the animal used in a majority of the literature reports. Rats also possess other desirable attributes which make them the logical choice for experiments of this type. They have a high resistance to infection, adapt quickly to functional losses produced by denervation, and show a capability for the relatively rapid regeneration of damaged nerve fibers, thus leading to the early reinnervation of denervated structures.

Animal Preparation

The animals were prepared for either denervation or disuse studies by performing an initial surgical procedure. For denervation studies a specific nerve was exposed at a specific point along its length, and the nerve destroyed by cutting or crushing it at that point. Alternatively, for disuse studies, "pins" (hypodermic needles) were inserted into a joint in such a way as to prevent rotation about that joint. All surgical procedures were performed on animals anesthetized with ether. In all of the denervation preparations, with the exception of the reinnervation series, the contralateral side was sham-operated, i.e. an incision was made, the nerve exposed, and then the wound closed with skin sutures. The experimental and sham-operated sides were alternated in different animals. No specific attempt was made to maintain aseptic surgical conditions, with the exception of using sterile hypodermic needles as pins. However, none of the animals showed any apparent sign of infection.

For the series of experiments involving the distal denervation of the gastrocnemius muscle, the sciatic nerve was exposed at the point of its division into the common peroneal, tibial and sural branches. The tibial nerve was then sectioned just distal (1-2 mm) to the point of sciatic branching. Care was taken to avoid damaging either the common peroneal or sural branches. The wound was

closed with skin sutures.

Proximal denervation of the gastrocnemius muscle was accomplished by exposing the sciatic nerve at the level of the trochanter and sectioning the nerve at that point. The wound was closed with skin sutures. Proximal denervation of the gastrocnemius, as opposed to the more distal denervation produced by tibial nerve section, increased the length of nerve remaining attached to the muscle by an average of 1.5 cm. In other words, the supposed reservoir of trophic material remaining available to the denervated muscle was increased by an additional 1.5 cm length of nerve.

An additional series of experiments was designed to focus on this same point, i.e., the possible change in the temporal pattern of a postdenervation response produced by altering the length of the residual nerve stump. Both tibialis anterior muscles in an animal were denervated, one distally, the other proximally. Distal denervation was produced by exposing the common peroneal nerve at the point of its insertion into the tibialis anterior, and sectioning the nerve just proximal to that point. Proximal denervation was accomplished on the opposite side, by exposing the sciatic nerve just anterior to the trochanter, and sectioning the nerve at that point. Wounds were again closed with skin sutures. The average length of residual nerve stump added by the more proximal nerve section was 3.5 cm. A proximal and distal denervation produced in the same animal had the

advantage of allowing a direct comparison of cyclic AMP concentration changes between the two denervated muscles. In the earlier experiments of this type, using the gastrocnemius, comparisons were made between muscles from different animals, thus increasing the number of potential variables.

In the series of experiments concerned with reinnervation, the gastrocnemius muscle--tibial nerve combination was again used. The tibial nerve was exposed as mentioned previously, but, rather than sectioning, the nerve was crushed between the blades of a smooth-bladed pair of hemostats. The blade width of the hemostats was approximately 1 mm, and the crush force was maintained for at least 30 seconds. This procedure leaves the nerve sheath intact to guide regenerating nerve fibers, and therefore decreases the period between denervation and reinnervation. Since at least 14 days elapsed between nerve crush and the removal of the gastrocnemius muscle for measurement, no sham operation was performed.

Muscle disuse was produced using the method of joint fixation (55, 169). The knee and ankle joints of one limb were immobilized by locking them in a fixed position, using hypodermic needles as pins. The foot was fixed at a right angle to the lower leg by driving a 25 gauge hypodermic needle through the calcaneous into the periosteum of the anterior aspect of the lower tibia. The knee joint was fixed with the tibia at right angles to the femur, by driving a 21 gauge

hypodermic needle through the distal femur into the head of the tibia. The pins were cut off at skin level, leaving a small segment of the needle hub to prevent their becoming dislodged. The small incision opened in the knee to aid in pin placement was closed with skin sutures. The fixed joints were examined periodically to insure that the pins were retained. X-ray photographs were taken of twelve of the fixed joints in order to insure the correct positioning of the pins (see Appendix A). Fixed joints showed some inflammation and swelling over the first 2-3 days, but usually the reaction was considerably reduced by the fourth day. The animals generally held the limb elevated, but moved freely about their cage, and would bear weight on the limb almost from the moment they recovered from anesthesia.

Electrophysiological Methods

Electrophysiological measurements have been made in order to monitor the functional status of affected muscles following denervation, and during the earliest period of reinnervation. Two parameters were measured; the capability of the residual nerve stump to transmit electrical activity to the muscle, and the extent of spontaneous, muscle-initiated electrical activity. Initially, it had been planned to measure the electrophysiological status of a denervated muscle just prior to measuring its cyclic AMP concentration. However, the procedures necessary for electrical recording introduced

excessive variability into the cyclic AMP measurements. Therefore, a separate series of experiments was performed to obtain the electrophysiological data.

All electrical recordings have been obtained from gastrocnemius muscles. The denervation series of measurements was obtained from gastrocnemii denervated by sectioning the tibial nerve. For the reinnervation series, the tibial nerve was crushed rather than sectioned.

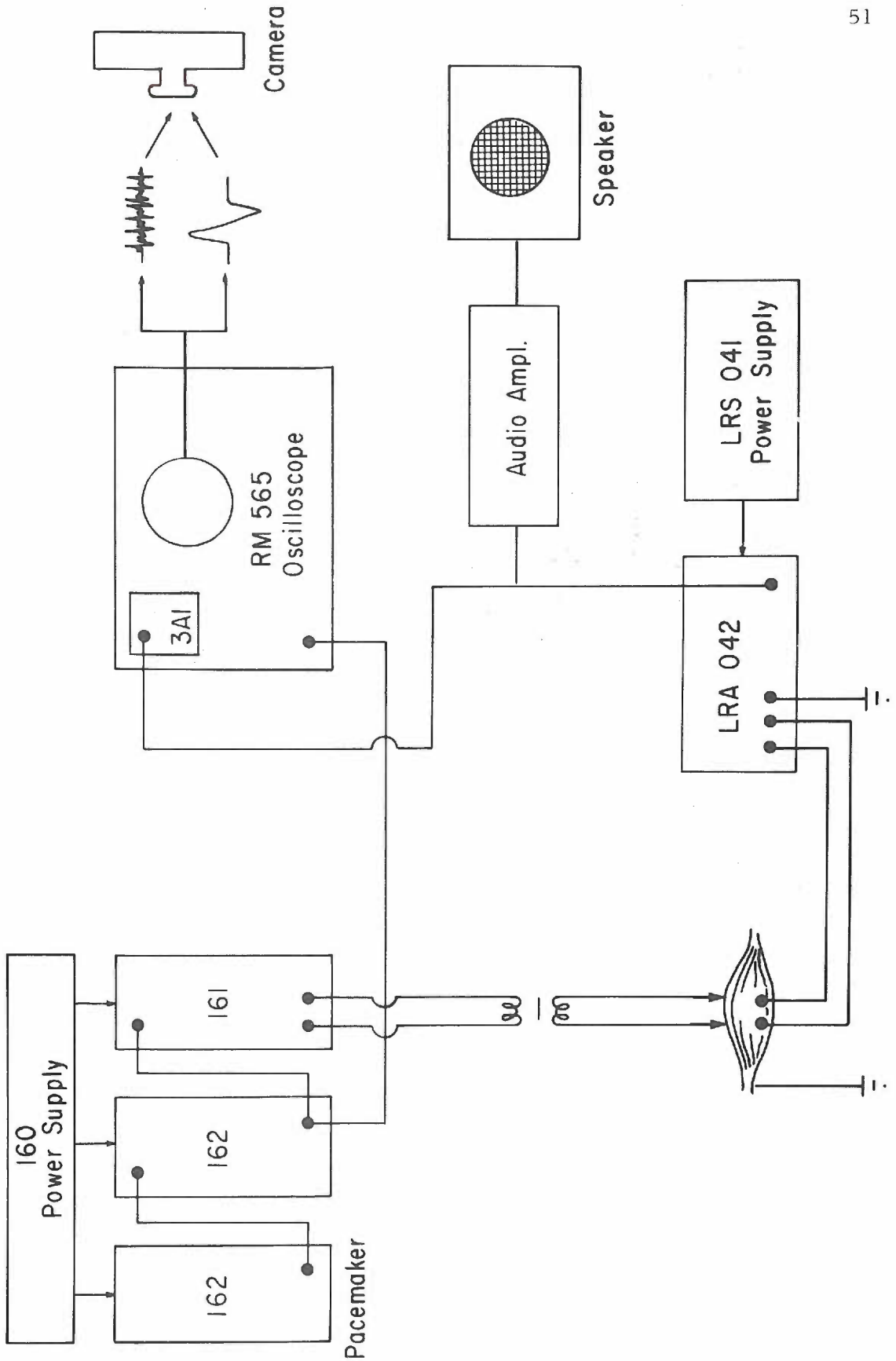
Preparation for recording consisted of first anesthetizing the animal with sodium pentobarbital (50 mgm/kgm, I. P.). The anesthetized animal was fastened to a surgical board, and the gastrocnemius muscle and its nerve supply exposed in both hind limbs. The exposed muscles and nerves were covered by a pool of warm mineral oil (38° C). The animal preparation was connected to the necessary instrumentation by gently placing the tibial nerve on the stimulating electrodes, and inserting the recording electrodes into the muscle.

The instrumentation used for measuring electrical activity is shown as a block diagram in Figure 2. Starting at the point of initiation of a stimulus pulse, a description of the equipment used and its purpose is as follows:

Stimulation

A Tektronix 162 Wave Form Generator is used as a pacemaker to set the frequency and duration of the output of a second 162. The

Figure 2. Schematic of Electrophysiological Instrumentation.


Camera
Speaker
Audio Ampl.
LRS 041
Power Supply
RM 565
Oscilloscope
3A1
LRA 042
160
Power Supply
161
162
162
Pacemaker

second Tek 162 is used to trigger both the output of a Tektronix 161 Pulse Generator and the sweep of the Tektronix RM565 Oscilloscope. The stimulus pulse from the 161 is delivered, via an Argonaut LIT 069 Isolation Transformer, to the stimulating electrodes. The stimulus pulse is a supramaximal rectangular pulse with a pulse width of between .05 and .5 msec. Pulses are delivered to the stimulating electrodes at a frequency of 1/sec. The stimulating electrodes themselves are made of .01 inch platinum wire, insulated, except for a 2 mm segment at the tip, with epoxylite 6001-M (Epoxylite Corp.). The two electrodes are stabilized in a silastic cuff, and the interelectrode distance is fixed at 3 mm. The entire electrode arrangement is clamped in a plastic block at the end of a micromanipulator, and lowered into the vicinity of the nerve. The tibial nerve is gently laid over the slightly curved tips of the stimulating electrode.

Recording

Electrical activity, either spontaneous or evoked, is recorded from a bipolar recording electrode inserted into the gastrocnemius muscle. The electrode is made of two size 000 stainless steel insect pins, insulated, except for 1 mm at the tips, with epoxylite 6001-M. The pins are arranged in parallel and fixed on a small piece of cardboard. The interelectrode distance is stabilized at 3 mm. A

fine copper wire lead, .0025 inches in diameter, is soldered to each pin. These small wire leads are connected to two terminals on a three terminal plexiglas block. The third terminal is connected to a ground lead which is clamped to an exposed Achilles tendon. The output from the terminal block is connected to the Argonaut LRA 042 Differential Preamplifier via the preamp's shielded input cable. The bandpass of the preamp is set at 10 hertz on the low side, and 10 K hertz on the high side. Gain is set at 1000. The shielded output cable from the LRA 042 is connected to both the input of a Tektronix 3A1 Dual Trace Amplifier and to the input of a Grass Audio Amplifier. This arrangement allows both visual and auditory monitoring of the recorded signal.

The recorded activity was displayed on the oscilloscope screen, and permanent records made by photographing the displayed signal. A Tektronix C27 Oscilloscope Camera with a polaroid film back was used for photography. The photographs have been used to provide both qualitative and quantitative information about the electrophysiological status of the muscle.

Biochemical Methods

Measurement of Cyclic AMP

Tissue levels of cyclic AMP are extremely labile and may undergo change within seconds after hormonal stimulation (61, 152).

Therefore, in order to successfully measure "resting" concentrations of cyclic AMP in a tissue, one must be able to "fix" the tissue as rapidly as possible. Once "fixed", the tissue must be removed and cyclic AMP extracted, without allowing cyclic AMP concentration to be altered. Once extracted and removed from the influences of the enzymes responsible for its synthesis and degradation, cyclic AMP is very stable and may be stored indefinitely at -70° C (186). In general, the techniques used for fixation and extraction are those specified by Goldberg and O'Toole (61), with slight modifications.

Animal Preparation

The animal was anesthetized with sodium pentobarbital (60 mgm/kgm, I. P.). At least 30 minutes was allowed to elapse before any of the subsequent procedures were performed. This time period is considered to be sufficient to allow muscle cyclic AMP concentration, which may be increased by the stress-induced release of epinephrine, to return to resting levels (44). For the first two experimental series (distal denervation and joint fixation) an anesthetic dosage of 50 mgm/kgm, I. P., and a waiting period of 20 minutes were used. Some animals, however, showed both twitch responses and shivering during subsequent dissection and exposure to the cold room. The higher anesthetic dosage and longer waiting period were used to insure deep anesthesia, and to reduce the degree of variability

in cyclic AMP measurements. While variability was reduced, as indicated by a smaller standard error of the mean, a lower value for the control concentration of cyclic AMP was also obtained (332 picomoles/gram wet weight in the first two series vs. 265 picomoles/gram wet weight in the final two series). Recent evidence now indicates that pentobarbitol depresses the concentration of cyclic AMP in skeletal muscle (98). However, since all animals within a given experimental series were treated alike, and since statistical comparisons were made on the basis of pairing the control and experimental muscles from the same animal (See Statistical Methods), the variability in control values obtained for the different series was not of major concern. For statistical comparisons made between series, normalized data (i. e. the percent change from control), which are independent of the differences in control concentration, were used.

Muscle Fixation and Removal

All dissection was performed in the deep cold room (-20° C). The anesthetized animal was fixed on a surgical board. At the end of the waiting period the animal was immediately taken into the cold room. The gastrocnemius or tibialis anterior muscle from each side was quickly dissected free and immediately frozen, using Wollenberger clamps (192). Briefly, the clamps consist of two large aluminum blocks (3 cm x 3 cm x 8 cm) fixed on each jaw of a large pair of pliers. The blocks are first cooled in liquid nitrogen, and

then the tissue to be frozen is clamped between the two chilled blocks. Since the aluminum blocks both conduct heat rapidly, and compress the tissue to a thickness of approximately 3 mm, the sample is rapidly and uniformly frozen. The blood-supply to the muscle was not severed until after the muscle was frozen. Complete dissection and freeqing of the two muscles required approximately 90 seconds, measured from the time the animal was brought into the cold room. The order of dissection and freezing was randomized, i. e. the experimental and control muscles were dissected out and frozen in random order. A control series of experiments (see Appendix B) was performed in order to show that there was no systematic error introduced when a muscle was dissected out and frozen either first or last.

After both muscles were frozen, aliquots from each were taken and placed in separate mortars, previously cooled with liquid nitrogen. The muscle aliquots were then pulverized under liquid nitrogen. The powdered muscle aliquot was transferred to a tared glass homogenizer tube (Duall 22, Kontes Glass), which had been chilled in a dry ice and 95% ethanol bath (-20° C). The muscle aliquot plus tared tube was quickly weighed on a Mettler Balance, and then returned to the dry ice and ethanol bath.

Cyclic AMP Extraction

The homogenizer tubes containing the muscle aliquots were transferred, still in the dry ice-ethanol bath, to a 4° C cold room.

Two mls of ice-cold 10% trichloroacetic acid (TCA) was added to each homogenizer tube, and the powdered aliquot quickly homogenized, using a ground-glass pestle. The 2 mls of homogenate were transferred to a centrifuge tube. Since the TCA-precipitate was to be used to measure the non-collagenous protein concentration of the aliquot, the homogenizer tube was washed with an additional 1 ml of 10% TCA, which was also transferred to the centrifuge tube.

In order to measure the percent recovery of extracted cyclic AMP, 2 picomoles of tritium-labeled cyclic AMP (New England Nuclear, specific activity 25 Ci/mM) were added to each centrifuge tube. This added cyclic AMP-H^3 represents a concentration of 2×10^{-9} M in the final extract volume. Since the average concentration of cyclic AMP extracted from muscle aliquots is approximately 1×10^{-7} M in the final volume, the added cAMP-H^3 represents approximately 1% of the extract concentration. This is unlikely to interfere with the assay of cold cyclic AMP.

The homogenate is centrifuged at 10,000 x g for 20 minutes. The supernatant is decanted into a 25 ml boiling flask, and the TCA removed by extracting 5 times with 5 volumes of water-saturated diethylether. The residual ether is blown off under a stream of nitrogen.

The extract was taken to dryness in one of two ways. During the first two experimental series a Rinco Rotary Evaporator was

available, and the extract was dried on this apparatus using a bath temperature of 60° C. During the later two experimental series, the rotary evaporator was not available. Therefore, the extracts were dried under a stream of nitrogen, in a water bath maintained at 60° C.

Once extracted, cyclic AMP is very stable and may be stored in the cold (-70° C) indefinitely (186). It was usual to store the samples prepared during these experiments for no longer than a week prior to assay. Each of the dried samples was taken up in 1 ml of 50 mM sodium acetate buffer (pH 4.0) just prior to assay.

Cyclic AMP Assay

The method used to assay cyclic AMP was that devised by Gilman (58), with minor modifications. The Gilman assay depends on a competition between tritium labeled and cold cyclic AMP for the binding sites on a specific protein. The cyclic AMP-protein complex is isolated on cellulose filters, and the radioactivity counted in a liquid scintillation spectrometer.

Preparation of the Binding Protein

The specific binding protein was prepared from fresh bovine heart muscle, using the routine method suggested by Gilman. The tissue was obtained from a local slaughter house, placed on ice, and

brought to the laboratory. A portion (250 grams) of the tissue was taken through the first three separation steps devised by Miyamoto et al. (130). These include homogenization, acid precipitation, ammonium sulfate precipitation and a final dialysis against 5 mm potassium phosphate buffer (pH 7.0). The supernatant obtained from the final dialysis step was applied to a column of DEAE-cellulose (Whatman DE 52, 1 mEq/gram on a column 32 cm x 2.5 cm) which had been previously equilibrated with 5 mM potassium phosphate (pH 7.0). The column was pumped, using a sigma motor pump, to obtain a flow rate of 3-4 ml/minute. Fractions were taken off in 20 ml aliquots. The first 37 such fractions were eluted with 100 mM potassium phosphate (pH 7.0). Fractions 38 through 52 were eluted with 300 mM potassium phosphate (pH 7.0). These later fractions were pooled, and dialyzed against 5 mM potassium phosphate (pH 7.0) for 24 hours. The resulting dialysate was made up in 2-3 ml aliquots and stored in a Revco Freezer at -70° C. The prepared binding protein may be stored for at least 18 months at this temperature (58).

The concentration of protein in the dialysate was assayed using the Lowry technique (116) with bovine serum albumin as a standard. Finally, a cAMP-H^3-binding protein saturation curve was obtained (see Appendix C).

Assay of Tissue Extracts for Cyclic AMP

Tissue extracts were assayed for cyclic AMP in the following manner:

1. In an incubation tube were mixed: 2×10^{-12} moles of cAMP-H^3 (New England Nuclear, specific activity 25 Ci/mM), 1.8% bovine serum albumin (Calbiochem, L.A., Calif.), either a known concentration of cold cAMP (Schwarz-Mann, Orangeburg, N.Y.) or an aliquot of tissue extract. The reaction was initiated by the addition of 2.5 μgm of binding protein. Final incubation volume was made up to 0.3 ml with 50 mM sodium acetate buffer (pH 4.0).

2. Incubation was carried out in a cold room at 4° C for a time period greater than 60 minutes.

3. The reaction was stopped by adding 2 ml of cold 20 mM potassium phosphate buffer (pH 6.0) to the incubation tube.

4. Approximately, 4-5 minutes after stopping the reaction, the tube contents were filtered through 24 mm Millipore cellulose filters (0.45 μM, Millipore Corp., Bedford, Mass.). The filters had been presoaked in 20 mM potassium phosphate buffer (pH 6.0). The tube was washed with an additional 7-8 mls of phosphate buffer and this added to the filter as well.

5. After filtering, the cellulose filters were placed directly into scintillation vials containing 10 mls of a scintillation cocktail

composed of toluene plus ethylene glycol monoethyl ether (cellosolve) in a ratio of 1:1, with 6 grams of PPO/liter and 75 mgms of POPOP/ liter.

6. Radioactivity was counted in a Model 3320 Packard TriCarb Liquid Scintillation Spectrometer. It required approximately 6-9 hours for the filters to dissolve completely, and the counting rate to stabilize. Three successive counting periods following count stabilization were used to obtain a mean count rate. Counting efficiency, measured by the addition of an internal standard (tritium standard, New England Nuclear, Boston, Mass.), was between 25 and 30%.

Assay Results

Each tissue assay was accompanied by a standard curve, produced by adding known concentrations of cold cyclic AMP to the incubation tubes (see Appendix D for a sample standard curve). The standard curve was plotted on log-log paper as suggested by Gilman. This produces an approximately linear curve.

Values for the concentration of cyclic AMP in the tissue extract were read from the standard curve, corrected for the percent recovery of cyclic AMP, and the dilution factor (10 fold). This value was divided by the wet weight of the tissue aliquot to give a tissue cyclic AMP concentration in moles x 10^{-12} per gram wet weight of tissue. This value in turn, was divided by the concentration of noncollagenous

protein (NCP), in mgms NCP per gram wet weight of tissue, to give a value for cyclic AMP concentration in moles x 10^{-12} per mgm NCP.

Assay Validation

The assay procedure was tested for possible erroneous measurements of cyclic AMP concentration, which might result from the competitive nonspecific binding of materials other than cyclic AMP. Incubation of aliquots of muscle extract were carried out in the presence and absence of bovine heart 3':5' cyclic nucleotide phosphodiesterase (Sigma Chem. Co., St. Louis, Mo.). The incubation media contained; tissue extract, 100 µgm phosphodiesterase, or buffer without phosphodiesterase, and 100 mM sodium acetate buffer (pH 8.0) containing 10 mM magnesium acetate and 30 mM imidazole (102), in a final volume of 0.3 ml. Incubation was carried out at 30° C for 1 hour. The incubation tube was placed in boiling water for 5 minutes to stop the reaction. Aliquots of the incubation media were assayed for cyclic AMP as usual. The results of one assay are shown in Appendix E. The phosphodiesterase experiments show that the cyclic AMP assay is specific for cyclic AMP, and other compounds do not contribute significantly to the binding reaction.

Protein Measurement

Perhaps the clearest phenomenon associated with muscle disuse or denervation is atrophy. The rapidity with which a muscle must be "fixed" in order to measure cyclic AMP, makes it very difficult to also measure changes in either the wet or dry weight of an affected muscle. Therefore, since muscle protein is the major constituent changed during atrophy, it was decided to use muscle protein measurements as an indication of muscle atrophy.

Muscle protein may be separated into three fractions: a) connective tissue protein which comprises 5% of normal muscle protein. b) Sarcoplasmic protein comprising 30% and c) contractile protein comprising 65% (171). Following denervation there is a period of at least 15 days when both connective and sarcoplasmic protein increase (increasing to levels of 9 and 37% respectively), and contractile protein decreases (reaching a level of 54% at 15 days). If the muscle is not reinnervated, both sarcoplasmic and contractile protein will eventually be lost, and the muscle will be replaced by connective tissue and fat. Since there is little if any cyclic AMP associated with connective tissue (153), it is more useful to associate cyclic AMP concentration only with sarcoplasmic and contractile protein. This may be accomplished by fractionating the TCA-precipitated protein into two classes: connective tissue protein and sarcoplasmic plus

contractile protein. Fractionation is obtained using the method of Lilienthal et al. (112) as modified by Beatty et al. (11). There would be little point to further fractionating the sarcoplasmic-contractile protein pool since the distribution of cyclic AMP between the two fractions is unknown.

Fractionation of the TCA-Precipitate and Assay

The TCA-precipitate obtained after centrifuging the muscle extract may be fractioned by taking advantage of the fact that connective protein is insoluble in dilute hydroxide. The precipitate is first washed with 20-30 mls of distilled water. The precipitate is then made up as a 1% solution (weight/volume) in .05N NaOH. The mixture is agitated in a vortex mixer and left to stand from 18-20 hours at room temperature. The remaining connective tissue protein precipitate is removed by centrifugation (10, 000 x g for 20 minutes). The supernatant is assayed for protein using the Lowry technique (116), with bovine serum albumin as a standard.

Since the Lowry technique provides a standard curve which is approximately linear up to a concentration of 500 μgm/ml, an aliquot of the supernatant is diluted to contain a concentration in this linear range. If one knows the weight of the tissue aliquot being assayed, and one assumes a protein concentration of approximately 20% of wet weight, the proper dilution may be easily calculated.

The Lowry or Folin-Ciocalteau method is a standard technique, the details of which are available in numerous sources (33, 116). The assay was performed exactly as specified, using a commercially prepared Folin-Ciocalteau Reagent (Harleco, Phil., Penn.). Color was read at 750 mm.

Statistical Methods

The choice of how best to statistically compare data obtained in these experiments is, as usual, based on the assumptions one must make when using a particular statistical test. While parametric tests are the most powerful statistically, they also require that a number of specific assumptions be met. Student's t-test for example, requires that the observations be independent, that they come from a normally distributed population, that they have the same variance, and that they are measured on at least an interval scale (163). Distribution-free tests, on the other hand, require fewer assumptions, and for small samples, are almost as powerful as parametric tests (18, 163). Since in these experiments, each animal served as its own control, it seemed logical to both avoid the assumptions inherent in parametric tests, and to make use of data which provided a direct comparison between experimental and control muscles. Therefore, the Wilcoxon Matched Pairs Signed-Ranks Test has been used to test the significance of the postdenervation or postfixation changes

observed in the concentration of cyclic AMP after a specific time interval. This statistical test assumes only that the observations are independent, and that the measured variable has underlying continuity.

Statistical comparisons between the different experimental series were made using a similiar nonparametric test, the Mann-Whitney U Test. This test handles data taken from independent groups, and compares it in a fashion much the same as the Wilcoxon test. For the Mann-Whitney U Test, the data from one experimental series taken at a particular time interval are compared with the data from a different experimental series taken at the same time interval. To allow for the differences in control concentration obtained for the different experimental series, the data compared were expressed as the calculated percentage of control.

In each case, statistical significance was tested with the .05 probability level accepted as criterion for rejecting the null hypothesis of no difference between the two populations.

RESULTS

Electrophysiological Analysis of Denervated Muscle

Neuromuscular Transmission

In general, neuromuscular transmission fails abruptly at some time following denervation. Miniature end plate potentials (m.e.p.p.s.) usually disappear at the same time, although they may reappear several days later (15). While the length of the postdenervation time interval prior to transmission failure differs according to species, the pattern of failure is quite similar across species. Failure occurs within hours after the first noticeable signs of the morphological disruption of nerve terminals (128). Transmission failure, therefore, may be taken as an indirect indication of nerve terminal disruption. Although each neuromuscular terminal fails abruptly, without showing any progressive decline in the end plate potential (e.p.p.), not every junction fails at the same time. One therefore sees a progressive decline in the amplitude of the evoked response, recorded extracellularly, and an increase in the asynchrony of the EMG response (e.g. an increase in polyphasic potentials) (49).

Terminal disruption and the failure of neuromuscular transmission apparently depend on the length of the residual nerve stump. Failure occurs in a proximal to distal progression within the muscle,

i. e. terminals close to the point of nerve entry into the muscle will fail before those located more distally (128). In the rat, however, terminal failure spreads very rapidly, and the addition of one centimeter of residual nerve stump postpones terminal failure by no more than 45 minutes. Therefore, while the measurements reported here are based only on experiments in which the tibial nerve was sectioned, one would expect the temporal pattern of failure resulting from sciatic section to be very similar. The results of measurements of nerve transmission after denervation are tabulated in Table 1A , and shown pictorially in Figure 3. Twenty-four hours following tibial nerve section, EMG recordings from both the proximal and distal gastrocnemius show a severe decrease in the amplitude of the evoked potential (a decline of approximately 10 fold), and a decrease in the duration of activity. Postdenervation recordings from the proximal gastrocnemius show a lower amplitude and duration of activity than do those from the distal segment. Figure 3 shows a polyphasic muscle potential recorded from the proximal gastrocnemius at 24 hours, while the distal recording still shows a biphasic potential. Thirty-six hours after tibial nerve section, no evoked activity can be recorded from the gastrocnemius, either proximally or distally. These results are in accord with measurements of the time course, and proximal to distal progression of failure reported by other investigators (128).

Table 1. Electrophysiological analysis of denervated muscle.

A) Measurement of the parameters associated with the evoked muscle potentials produced by a single, supra-maximal stimulus applied to the tibial nerve.

B) Measurements of the frequency of fibrillatory potentials at 3 and 4 days postdenervation.

Electrophysiological analysis of denervated muscle

A) Neuromuscular transmission

Time Postdenervation (days)	Latency (msec)		Amplitude (mv)		Duration (msec)	
	Prox.	Distal	Prox.	Distal	Prox.	Distal
0 (Control)	1.0	1.0	0.93	1.5	4.3	5.0
1	1.0	1.0	0.093	0.15	2.8	3.6
1.5	Neuromuscular transmission fails					

B) Fibrillatory Activity

Time Postdenervation (days)	Frequency (impulses/sec) (Potentials > 10 μV)	
	Prox.	Distal
3	100	100
4	80	50

	Frequency (impulses/sec) (Potentials > 20 μV)	
	Prox.	Distal
3	40	30
4	27	10

Figure 3. Electrophysiological recordings from the rat gastrocnemius muscle following section of the tibial nerve. Recordings were obtained from both the proximal and distal segments of the muscle. Time and voltage scales refer to the scales presented at the bottom of each column of recordings.

A) Control, innervated muscle. Muscle potentials evoked by tibial nerve stimulation. Time scale: 5 msec. Voltage scale: 0.5 mV.

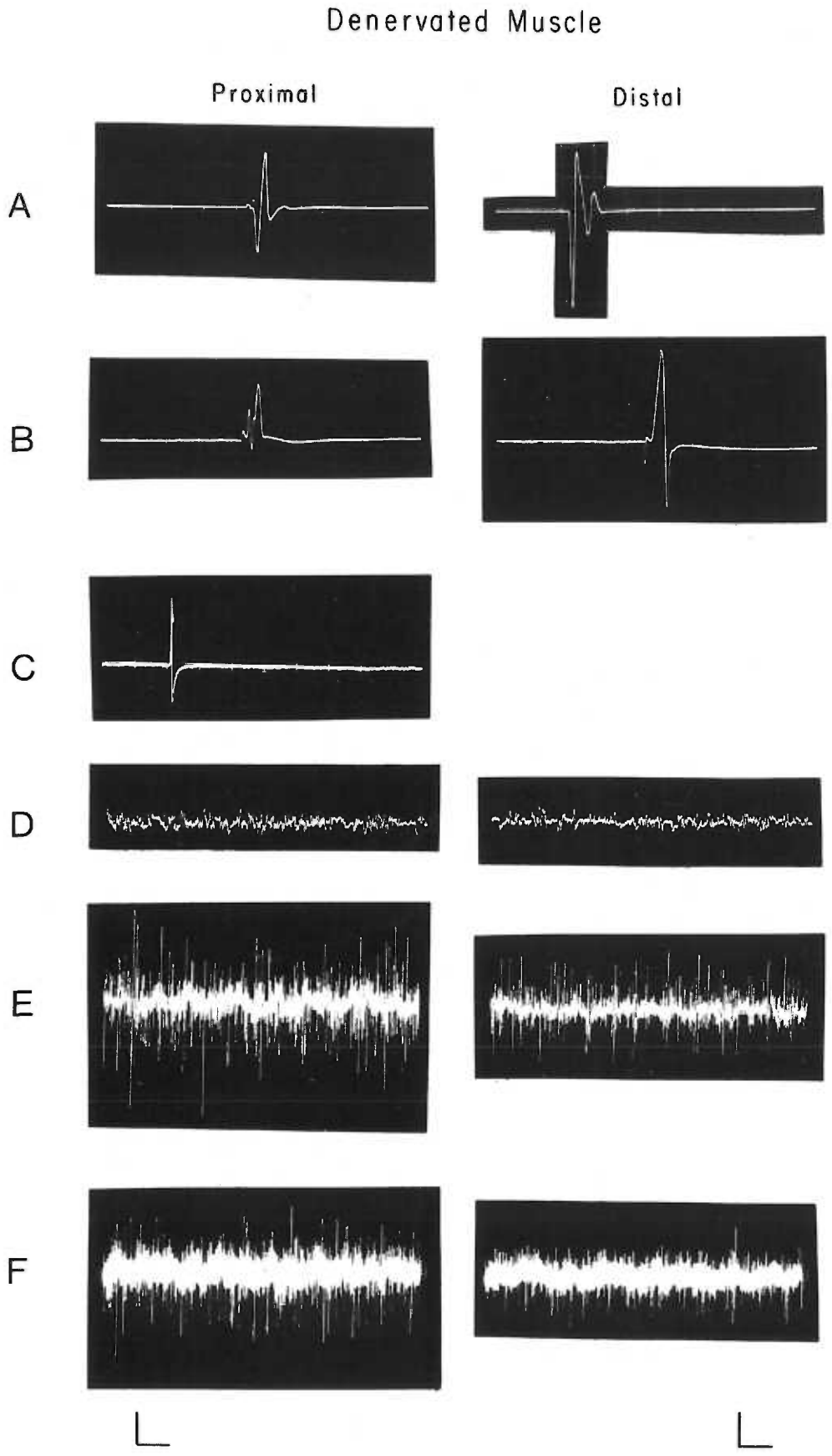
B) One day postdenervation. Muscle potentials evoked by tibial nerve stimulation. Time scale: 5 msec. Voltage scale: 0.05 mV.

C) 36 hours postdenervation. No muscle potential can be evoked by tibial nerve stimulation. Potential recorded is a stimulus artifact. Time scale: 10 msec. Voltage scale: 20 μV.

D) 2 days postdenervation. No muscle potential can be evoked. Fibrillatory potentials of very low amplitude now present. Time scale: 10 msec. Voltage scale: 20 μV.

E) 3 days postdenervation. Fibrillatory potentials are of considerably greater amplitude. Time scale: 100 msec. Voltage scale: 20 μV.

F) 4 days postdenervation. Fibrillatory potentials do not appear to be significantly larger in amplitude. Time scale: 200 msec. Voltage scale: 20 μV.


Denervated Muscle
Proximal
Distal
A
B
C
D
E
F

Fibrillation

Fibrillatory activity in a muscle is a characteristic indication of denervation. The onset of fibrillation also apparently depends on the length of the residual nerve stump (117). While the mechanisms responsible for fibrillation are not known, it is probable that some change in the intrinsic properties of the muscle cell membrane provides its foundation (12, 111). Therefore, fibrillatory activity offers an indirect indication of alterations occurring in some muscle membrane properties (38). Fibrillation activity itself is difficult to quantify. The extent of recorded activity depends on various factors such as: the postdenervation time interval, the region of the muscle from which recordings are made, the temperature of the muscle, the type of muscle fiber recorded from, the endocrine activity of the animal, etc. (52). Activity is, however, apparently unaffected by barbiturate anesthesia. In general, it appears as if the early increase in fibrillatory activity depends on a progressive increase in the number of larger amplitude potentials (158). It also appears that high amplitude activity spreads in a proximal to distal manner. However, in the rat, the spread of activity occurs quite rapidly, with the difference between the proximal and distal appearance of large potentials being no greater than 6 hours (158).

Fibrillatory activity in this preparation was first recorded at 2

days postdenervation (see Figure 3). The potentials, however, were very small, less than 10 μV in amplitude, and difficult to distinguish from the background noise. By 3 days postdenervation, fibrillatory potentials were easily distinguishable. Figure 3 shows examples of activity recorded from both the proximal and distal segments of the gastrocnemius. Table IB tabulates the frequencies of the recorded potentials. Included are measurements of activity involving all potentials of 10 μV and above, and of activity involving only potentials of 20 μV and above. The largest potentials recorded in this preparation were between 50 and 60 μV. Recordings made from the proximal segment show a larger number of higher amplitude potentials than do recordings from the distal segment. The degree of variability inherent in the extracellular measurement of fibrillatory activity is indicated by the recordings made at 4 days. While the frequencies recorded from the proximal segment are essentially the same as those recorded at 3 days, the recorded distal frequencies are actually less than the comparable 3 day values. It is this variability, which depends on a multitude of factors as mentioned, that makes more quantitative measurements of little value. Attempts, for instance, in these experiments to integrate the spontaneous EMG activity, provided little additional information concerning the functional status of the denervated muscle. One may assume, therefore, that fibrillatory activity is fully developed by day 3 in this preparation, and

that the activity will continue as long as the muscle remains denervated, and contractile tissue survives (90, 182).

Atrophy in Denervated and Disused Muscles

A comparison of the effects of denervation and disuse on the cyclic AMP concentration of skeletal muscle, requires at least an indirect indication that the two procedures produce an approximately equivalent amount of disuse in the muscle. Denervation clearly produces absolute disuse of the muscle. Disuse, produced by joint fixation, however, allows some degree of muscle activity. This residual activity can only be measured directly by the use of chronic EMG recording techniques, as explained in the Discussion section. However, an indirect indication of the degree of muscle disuse may be provided by measuring muscle atrophy. This has been accomplished in these experiments by measuring the change in the concentration of noncollagenous protein per gram wet weight of tissue. A comparison of the values obtained at various time periods after denervation, or joint fixation, is provided in Table 2. Figure 4 provides the same information graphically. It is clear that there is no significant difference in either the degree or time course of protein concentration change following denervation or fixation. The values for the concentration of noncollagenous protein reported here are equivalent to the values reported in the literature for similar

Table 2. Concentration of noncollagenous protein (NCP) following denervation or joint fixation.

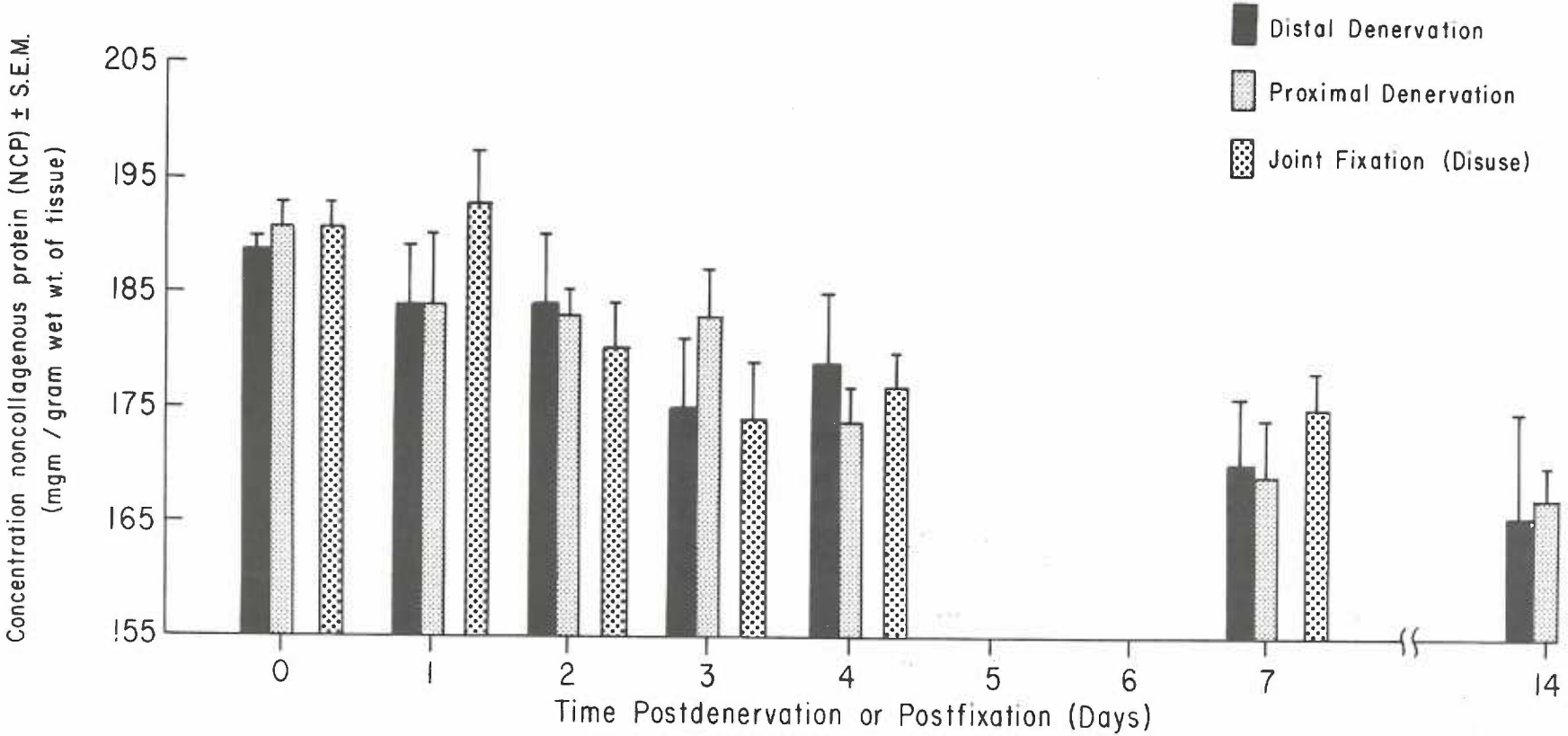
	Time	Postdenervation (Days)					
	0	1	2	3	4	7	14
Series 1 Distal Denervation	189 ±1	184 ±5	182 ±6	175 ±6	179 ±6	170 ±6	166 ±10

	Time	Postfixation (Days)					
	0	1	2	3	4	7	14
Series 2 Joint Fixation	191 ±2	193 ±4	180 ±4	174 ±5	177 ±3	175 ±3	167 ±3

	Time	Postdenervation (Days)				
	0	1	2	3	4	7
Series 3 Proximal Denervation	191 ±2	184 ±6	183 ±2	183 ±4	174 ±3	169 ±5

NOTE: Concentration of noncollagenous protein (NCP) given as mgm NCP per gram wet weight of tissue ±standard error of the mean.

Figure 4. Concentration of noncollagenous protein (NCP) following denervation or joint fixation. NCP concentration given on the ordinate as mgm NCP per gram wet weight of tissue, $\pm$ standard error of the mean. Abscissa: time postdenervation or post-fixation in days.


Distal Denervation
Proximal Denervation
Joint Fixation (Disuse)
Concentration noncollagenous protein (NCP) ± S.E.M.
(mgm / gram wet wt. of tissue)
205
195
185
175
165
155
0
1
2
3
4
5
6
7
14
Time Postdenervation or Postfixation (Days)

time periods (171).

Cyclic AMP Concentration in Denervated and Disused Muscle

The problem has been approached through a series of experimental stages. The first stage, involving the distal denervation of the gastrocnemius, was designed to determine whether the removal of neurotrophic influences would affect the concentration of cyclic AMP in a muscle. The second stage, the production of muscle disuse by joint fixation, was designed to distinguish between changes in cyclic AMP concentration resulting from the removal of neurotrophic influences, and those which might result from muscle disuse alone. The third stage, involving the proximal denervation of the gastrocnemius, was an attempt to more firmly establish the possible neurotrophic control of cyclic AMP, by demonstrating a temporal relationship between the length of the residual nerve stump, and the onset of the change in cyclic AMP concentration. The results obtained from the latter two experimental stages, made the establishment of a neurotrophic contol of cyclic AMP somewhat equivocal. Therefore, an additional series of experiments, involving the proximal and distal denervation of both tibialis anterior muscles in the same animal, was designed. This section is organized to first present the data from each of the first three series individually, then to compare

these three series, and finally to report the results of the tibialis anterior experiments.

Cyclic AMP Concentration Changes Following Distal Denervation

Section of the tibial nerve produced an increase in the concentration of cyclic AMP in the denervated gastrocnemius. The increase began very early after denervation; the concentration in the denervated muscle was significantly elevated over control by one day postdenervation. The increase reached a peak at two days, and then the concentration slowly declined. Cyclic AMP in the denervated muscle was still significantly elevated at four days, but returned to the control level at 7 days. Expressing the concentration of cyclic AMP in relation to the concentration of noncollagenous protein (NCP), did not change either the temporal pattern or significance of the increase. The results of this series are presented in Tables 3A and 4A, expressed as the mean concentration of cyclic AMP either per gram wet weight (Table 3A) or per mgm NCP (Table 4A). The results are also displayed graphically in Figure 5.

Cyclic AMP Concentration Changes Following Joint Fixation

The concentration of cyclic AMP in the disused muscle, following joint fixation, was never significantly different from the

Table 3. Concentration of cyclic AMP in the gastrocnemius muscle following denervation or joint fixation. Concentration given as moles x 10^{-12} per gram wet weight of tissue ± standard error of the mean. * indicates that the difference in concentration between the experimentally treated and contralateral control muscles is statistically significant ($p < .05$) at the specified time period. N. S. indicates that the difference is not statistically significant ($p > .05$).

Table 3. Concentration of cyclic AMP in the gastrocnemius muscle following denervation or joint fixation (disuse).

A) Series 1: Distal Denervation

	Time Postdenervation (days)						
	1	1.5	2	3	4	7	14
Number of Animals	8	8	7	7	8	8	6
Mean Denervated ±SEM	389±44	497±77	526±80	508±38	464±47	388±43	423±55
Mean Control ±SEM	295±32	268±40	328±29	338±25	329±32	374±55	340±56
Denervated/Control x 100	132	154	161	150	141	104	125
	*	*	*	*	*	N. S.	N. S.

B) Series 2: Joint Fixation (Disuse)

	Time Postfixation (days)					
	1	2	3	4	7	14
Number of Animals	7	8	6	12	5	8
Mean Disused ±SEM	375±48	290±40	337±28	569±28	441±69	341±37
Mean Control ±SEM	359±49	306±26	367±64	507±52	501±19	313±45
Disused/Control x 100	105	95	92	112	87	109
	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.

C) Series 3: Proximal Denervation

	Time Postdenervation (days)				
	1	2	3	4	7
Number of Animals	6	6	6	6	6
Mean Denervated ±SEM	275±21	354±32	329±29	313±12	306±16
Mean Control ±SEM	234±13	266±15	249±12	283±13	281±4
Denervated/Control x 100	117	134	133	111	109
	*	*	*	*	N. S.

Table 4. Concentration of cyclic AMP in the gastrocnemius muscle following denervation or joint fixation. Concentration given as moles x 10^{-12} per mgm noncollagenous protein ± standard error of the mean. * indicates that the difference in concentration between the experimentally treated and contralateral control muscles is statistically significant ($p < .05$) at the specified time period. N. S. indicates that the difference is not statistically significant ($p > .05$).

Table 4. Concentration of cyclic AMP in the gastrocnemius muscle following denervation or joint fixation (disuse).

A) Series 1: Distal Denervation

	Time Postdenervation (days)						
	1	1.5	2	3	4	7	14
Number of Animals	8	8	7	7	8	8	6
Mean Denervated Muscles ±SEM	2.1±.2	2.7±.4	2.9±.4	2.9±.3	2.6±.3	2.3±.3	2.7±.6
Mean Control Muscles ±SEM	1.6±.2	1.7±.2	1.7±.2	1.8±.1	1.7±.2	1.9±.3	1.8±.3
Denervated/Control x 100	132	157	174	162	156	122	153
	*	*	*	*	*	N. S.	N. S.

B) Series 2: Joint Fixation (Disuse)

	Time Postfixation (days)					
	1	2	3	4	7	14
Number of Animals	7	8	6	12	5	8
Mean Disused Muscles ± SEM	1.9±.3	1.7±.3	1.8±.1	3.4±.2	2.5±.4	2.1±.2
Mean Control Muscles ± SEM	1.8±.3	1.7±.2	1.7±.3	2.8±.4	2.4±.3	1.7±.2
Disused/Control x 100	107	101	107	121	102	123
	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.

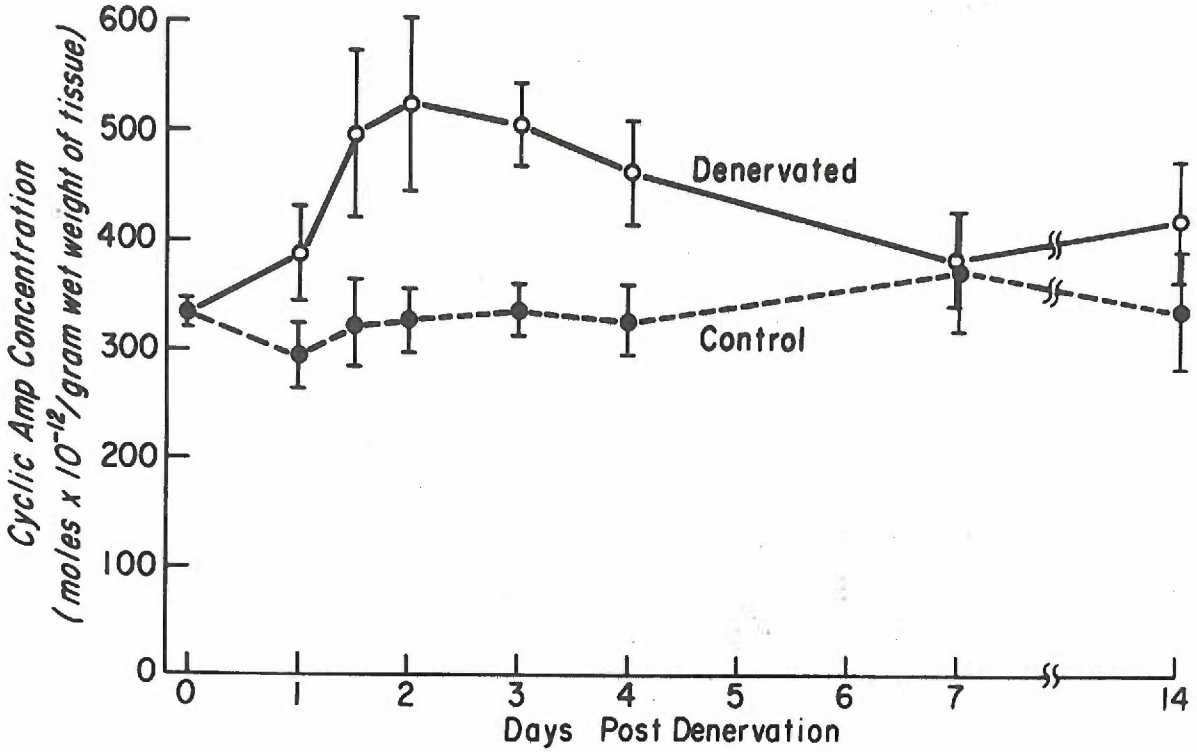
C) Series 3: Proximal Denervation

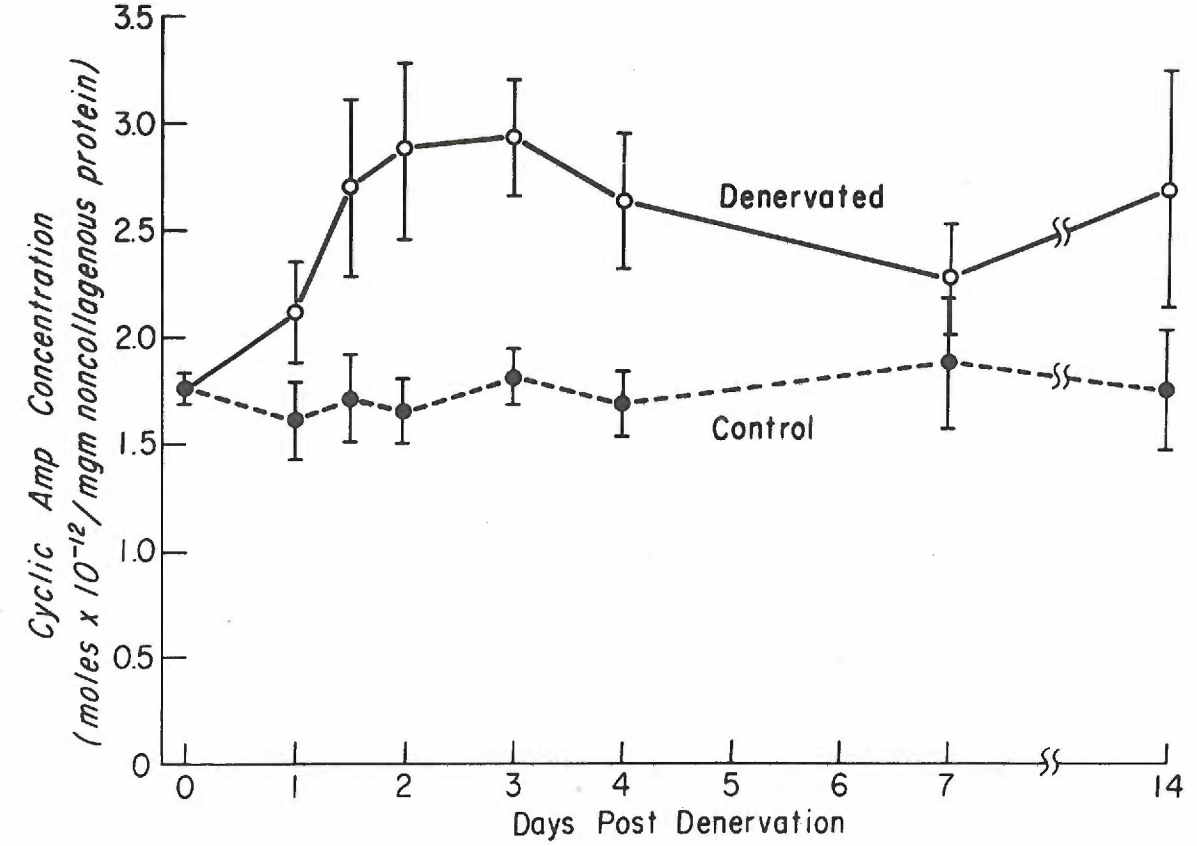
	Time Postdenervation (days)				
	1	2	3	4	7
Number of Animals	6	6	6	6	6
Mean Denervated Muscles ±SEM	1.5±.1	2.0±.2	1.8±.2	1.8±.1	1.8±.1
Mean Control Muscles ± SEM	1.3±.1	1.4±.1	1.3±.1	1.5±.1	1.5±.1
Denervated/Control x 100	119	142	137	122	122
	*	*	*	*	*

Figure 5. Concentration of cyclic AMP in the gastrocnemius muscle following section of the tibial nerve (denervated) and in the contralateral innervated gastrocnemius muscle (control).

Top graph: concentration of cyclic AMP given in moles x 10^{-12} per gram wet weight of tissue ± standard error of the mean.

Bottom graph: concentration of cyclic AMP given in moles x 10^{-12} per mgm noncollagenous protein ± standard error of the mean.

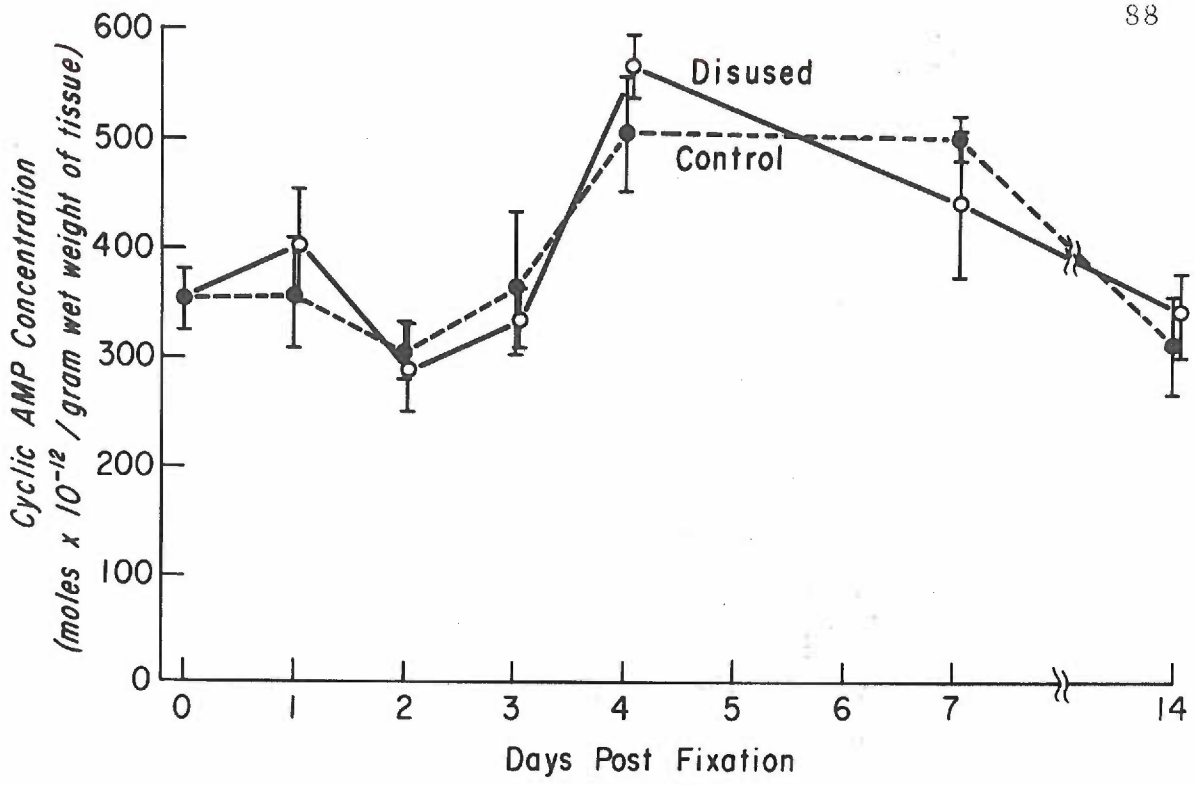

Cyclic Amp Concentration
(moles x 10⁻¹²/gram wet weight of tissue)
600
500
400
300
200
100
0
Denervated
Control
0
1
2
3
4
5
6
7
14
Days Post Denervation


Cyclic Amp Concentration
(moles x 10⁻¹²/mgm noncollagenous protein)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Denervated
Control
0
1
2
3
4
5
6
7
14
Days Post Denervation

concentration in the control muscle. The results are given in Tables 3B and 4B, and are shown graphically in Figure 6. While there appeared to be no change in cyclic AMP concentration for the first three days following fixation, an abrupt increase occurred in both the disused and control muscle at day four. The increased concentration was maintained at day seven, but returned to normal levels in both muscles by day 14. This sudden increase was both surprising, and difficult to explain. As may be noticed from Tables 3B and 4B, twelve animals were used to verify the results obtained at day four. In every one of these preparations, the concentration of cyclic AMP in both the control and disused muscle was elevated. It would appear, therefore, that the increase is real, rather than due to experimental error. Various possibilities have been considered in order to explain the phenomenon. One of these has been tested experimentally.

The first potential explanation considered, was the possibility that the activity level of the animal changed between day three and four. Perhaps the pain and trauma associated with pin insertion reduced the animal's activity for the first few days. The animals, therefore, were carefully observed at various periods after joint fixation. They were observed especially at night, when rats are most active. There seemed to be no noticeable difference in the activity of the "fixed" rats compared with normal rats. From the time they were returned to their cage following fixation the animals

Figure 6. Concentration of cyclic AMP in the gastrocnemius muscle following joint fixation (disuse) and in the contralateral gastrocnemius muscle (control). Top graph: concentration of cyclic AMP given in moles x 10^{-12} per gram wet weight of tissue ± standard error of the mean. Bottom graph: concentration of cyclic AMP given in moles x 10^{-12} per mgm noncollagenous protein ± standard error of the mean.


Cyclic AMP Concentration
(moles x 10⁻¹² / gram wet weight of tissue)
600
500
400
300
200
100
0
Disused
Control
0
1
2
3
4
5
6
7
14
Days Post Fixation

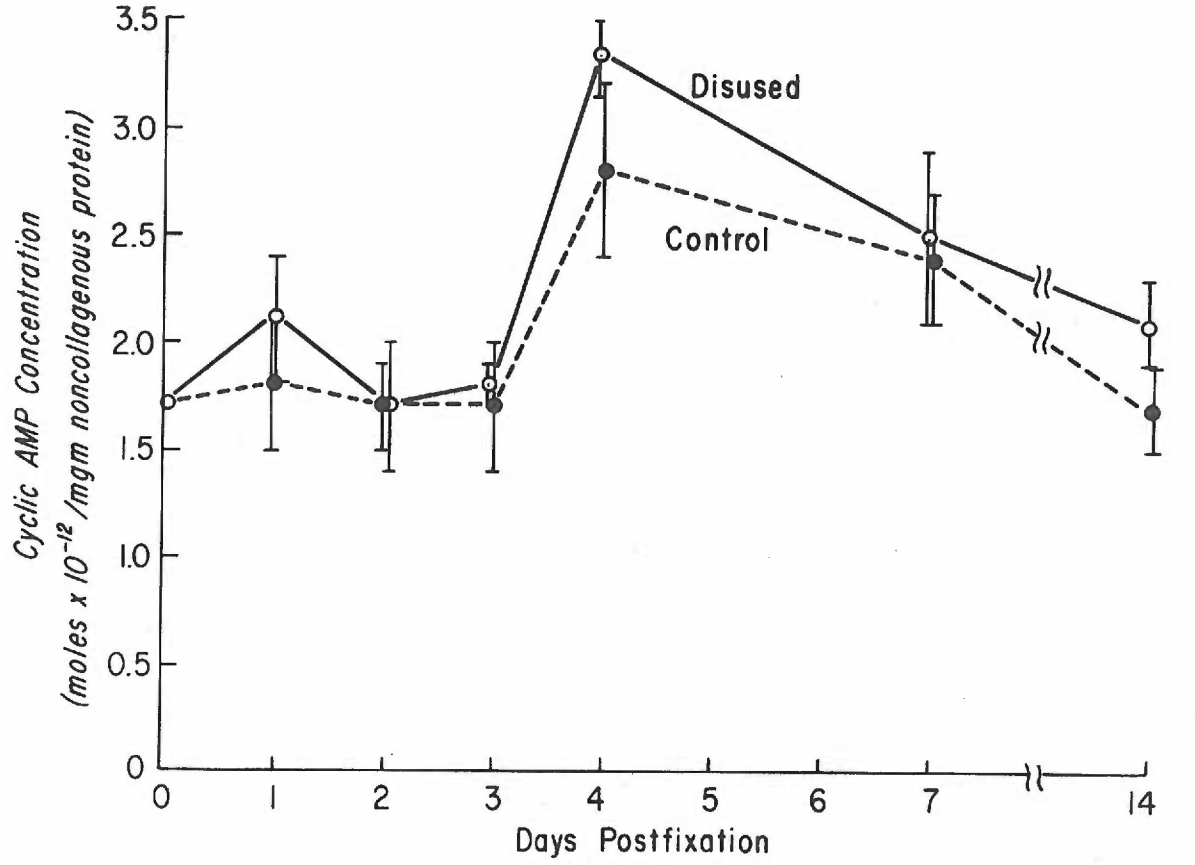
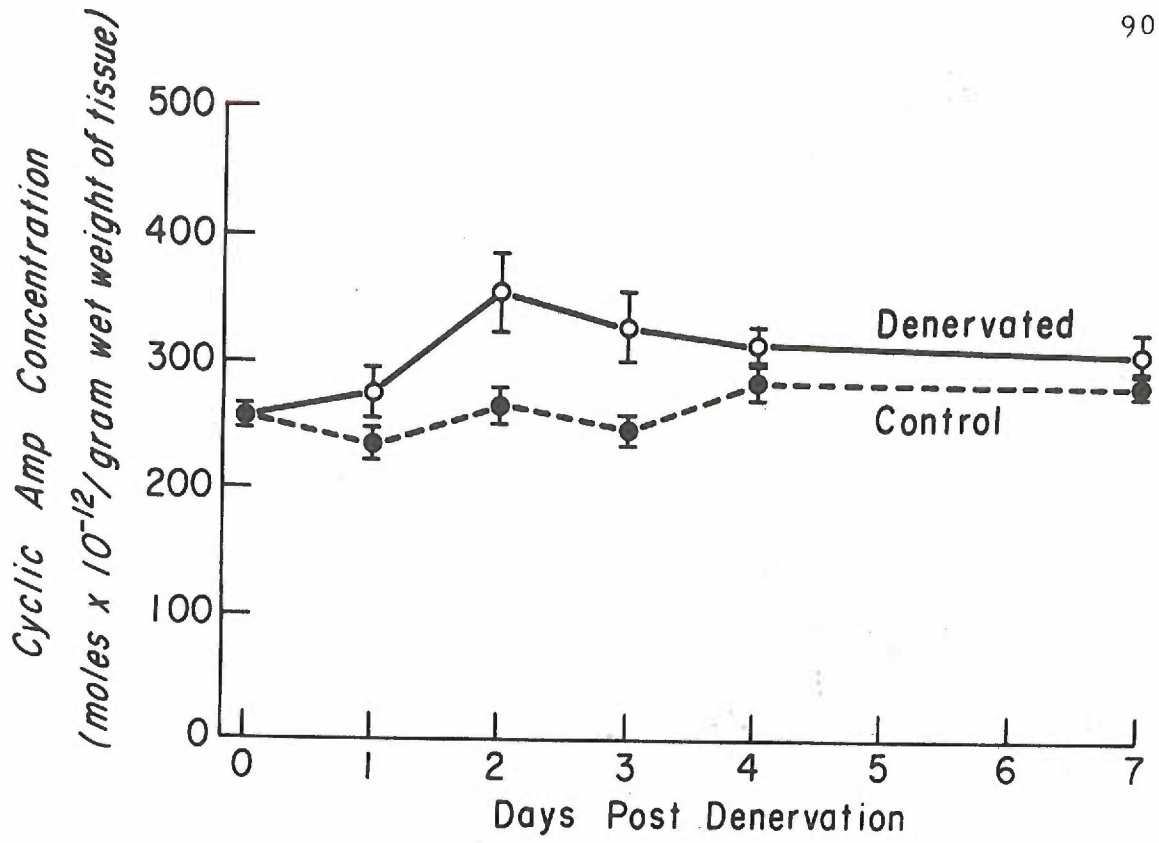
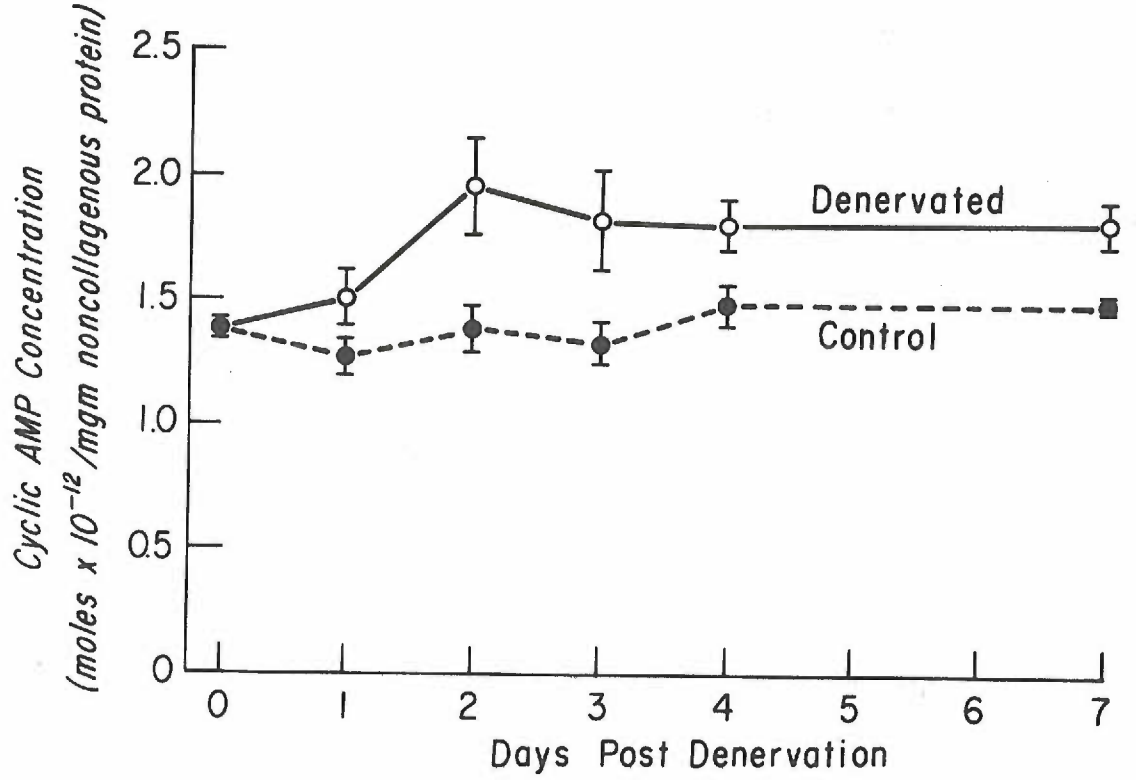

Cyclic AMP Concentration
(moles x 10⁻¹² /mgm noncollagenous protein)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Disused
Control
0
1
2
3
4
5
6
7
14
Days Postfixation

Figure 7. Concentration of cyclic AMP in the gastrocnemius muscle following section of the sciatic nerve at the level of the trochanter (denervated), and in the innervated contralateral gastrocnemius muscle (control). Top graph: concentration of cyclic AMP given in moles x 10^{-12} per gram wet weight of tissue ± standard error of the mean. Bottom graph: concentration of cyclic AMP given in moles x 10^{-12} per mgm noncollagenous protein ± standard error of the mean.


Cyclic Amp Concentration
(moles x 10⁻¹²/gram wet weight of tissue)
500
400
300
200
100
0
Denervated
Control
0
1
2
3
4
5
6
7
Days Post Denervation


Cyclic AMP Concentration
(moles x 10⁻¹²/mgm noncollagenous protein)
2.5
2.0
1.5
1.0
0.5
0
Denervated
Control
0
1
2
3
4
5
6
7
Days Post Denervation

moved freely about the cage, even bearing weight on the fixed limb. While no quantitative measurements of activity were made, it would seem unlikely that activity per se, or lack of it, might explain the sudden elevation in cyclic AMP concentration.

As an alternative to the first possible explanation, it might be possible that muscle disuse patterns change with time after fixation. The animal may attempt to use the fixed muscle less initially, perhaps because of pain, and then increase his attempts as the pain and inflammation recede. Alternatively, he may use the fixed muscle more at first, until he determines he cannot extend his knee and ankle and adapts his attempted usage to the situation. While it is not possible to answer this question directly without chronic EMG recordings, alterations in the disuse pattern of the fixed muscle would not explain the elevation of cyclic AMP found in the control muscle.

Finally, given the fact that the degree of disuse produced at each time interval is still uncertain, a third possible explanation may involve the complex series of reactions known as the inflammatory response. Inflammation is subdivided into acute and chronic phases; the latter may last several weeks. Therefore, while the immediate, visually evident inflammation resulting from pin insertion usually appears to be significantly reduced by day 4, other, more biochemically evident reactions may still be occurring. In order to test the possibility that the elevation in cyclic AMP concentration seen in

both the control and fixed muscles might be part of a general systemic response to joint damage, an additional series of experiments was performed. The results are given in Table 5. First a control series, composed of three normal animals, was prepared. The gastrocnemius muscles from both sides were frozen and removed as usual. Immediately after removing the muscles, the liver was exposed and a small aliquot frozen and removed. Both muscle and liver extracts were assayed for cyclic AMP as usual. The concentration obtained for the liver aliquot was considered to be the control concentration for liver tissue obtained in this manner. Four additional types of experiments were performed. Disuse was produced in eight animals by unilateral joint fixation. Three of these animals were sacrificed at four days postfixation, the other five at seven days. Liver extracts were obtained, along with the muscle extracts, from each preparation. Six additional animals were sham-operated. Sham-operation involved the insertion of the pins into the joints, and then the immediate removal of the pins. The sham-operated joints did develop a degree of ankylosis, and were less mobile than normal joints, but were certainly more mobile than fixed joints. Three sham-operated animals were sacrificed at four days, the remaining three at seven days. From the results shown in Table 5, it is evident that four and seven days after fixation, both control and disused muscles show elevated cyclic AMP concentrations. However, the

Table 5. A comparison of disused and sham-operated muscles, including aliquots of liver. Cyclic AMP concentration given in moles x 10^{-12} per gram wet weight of tissue ± standard error of the mean. Disused muscles were produced by joint fixation. Sham-operated muscles were produced by inserting pins in the knee and ankle joints and then immediately removing the pins. Aliquots of liver were obtained after muscles were removed.

Table 5. Comparison of disused and sham-operated muscles including liver aliquots.

	Number of Animals	Cyclic AMP Concentration
Control Muscle	3	355±43
Control Liver	3	889±85
4 Days Postfixation		
Disused Muscle	3	461±46
Control Muscle	3	455±49
Liver	3	1101±25
4 Days Post Sham		
Sham-operated Muscle	3	474±43
Control Muscle	3	436±31
Liver	3	991±36
7 Days Postfixation		
Disused Muscle	5	441±69
Control Muscle	5	501±19
Liver	5	1200±162
7 Days Postsham		
Sham-operated Muscle	3	362±29
Control Muscle	3	446±70
Liver	3	1112±188

sham-operated animals also show a similar elevation. Additionally, it appears as if the concentration of cyclic AMP in the livers of both sham and fixed animals is slightly elevated. The small sample number makes it difficult to determine unequivocally whether a systemic factor is operating, or whether this factor is related to some aspect of the tissue disruption following pin insertion. The evidence, however, does not rule out this possibility. Whatever the cause, the fact that both control and disused muscles show elevated cyclic AMP concentrations following joint fixation indicates that the process producing the increase is probably different from that which produces the increase in the denervated muscle. It was decided, therefore, not to continue to explore what may essentially be a side issue to the problem of neurotrophic control of cyclic AMP.

Cyclic AMP Concentration Changes Following Proximal Denervation

Sectioning the sciatic nerve at the level of the trochanter produced an increase in the concentration of cyclic AMP in the denervated muscle, which showed the same temporal pattern as the increase following distal denervation. Tables 3C and 4C give the results of these experiments, and the results are presented graphically in Figure 7. The dose level of anesthetic was increased in this series, and that probably accounts for both the reduction in control

concentration, and the lower peak increase in the denervated muscle (98). However, the temporal pattern of the increase was similar to that which occurred following distal denervation, and the time intervals at which the increase is significant are also the same.

Comparison of Series 1, 2 and 3

Table 6 presents a comparison of the results obtained from the two denervation experimental series, and from the experiments involving joint fixation. Figures 8 and 9 present the comparisons graphically. The results are expressed as percent of control concentration. The data have been analyzed using the Mann Whitney U Test, which compares data from two independent groups. The statistical test shows that, at any time interval, there is no significant difference between the percent increase in the concentration obtained after either proximal or distal nerve section. On the other hand, a comparison between the distal denervation and disuse experiments shows that the percent increase in the concentration of cyclic AMP in the denervated muscle is significantly greater at days two and three, when the results are expressed on a per gram wet weight basis. If the results are expressed on a per mgm NCP basis, the percent increase in the denervated muscle's cyclic AMP concentration is significantly elevated only at day two.

The percent increase in the cyclic AMP concentration of the

Table 6. Comparison of Series 1, 2, and 3

A) Procedure	Time Postdenervation or Postfixation (days)				
	1	2	3	4	7
Distal Denervation (Series 1)	132±15	161±31	150±12	141±17	104±14
Proximal Denervation (Series 3)	117±6	134±7	133±11	111±5	109±7
Joint Fixation (Series 2)	105±6	95±11	92±20	112±14	87±11

B) Procedure	Time Postdenervation or Postfixation (days)				
	1	2	3	4	7
Distal Denervation (Series 1)	132±11	174±34	162±15	156±22	122±20
Proximal Denervation (Series 3)	119±5	142±6	137±14	122±6	122±9
Joint Fixation (Series 2)	107±8	101±14	107±24	121±20	102±10

NOTE: Table A: Comparison of the results of series 1, 2 and 3. Concentration of cyclic AMP measured as moles x 10^{-12} per gram wet weight of tissue and expressed in the table as the concentration in the experimental muscle/concentration in the control muscle x 100, ± standard error of the mean.

Table B: Same data as in Table A except that cyclic AMP concentration was measured as moles x 10^{-12} per mgm NCP.

Figure 8. Comparison of series 1, 2 and 3. Concentration of cyclic AMP measured as moles x 10^{-12} per gram wet weight and expressed as the percent of control concentration $\pm$ standard error of the mean for each time interval.

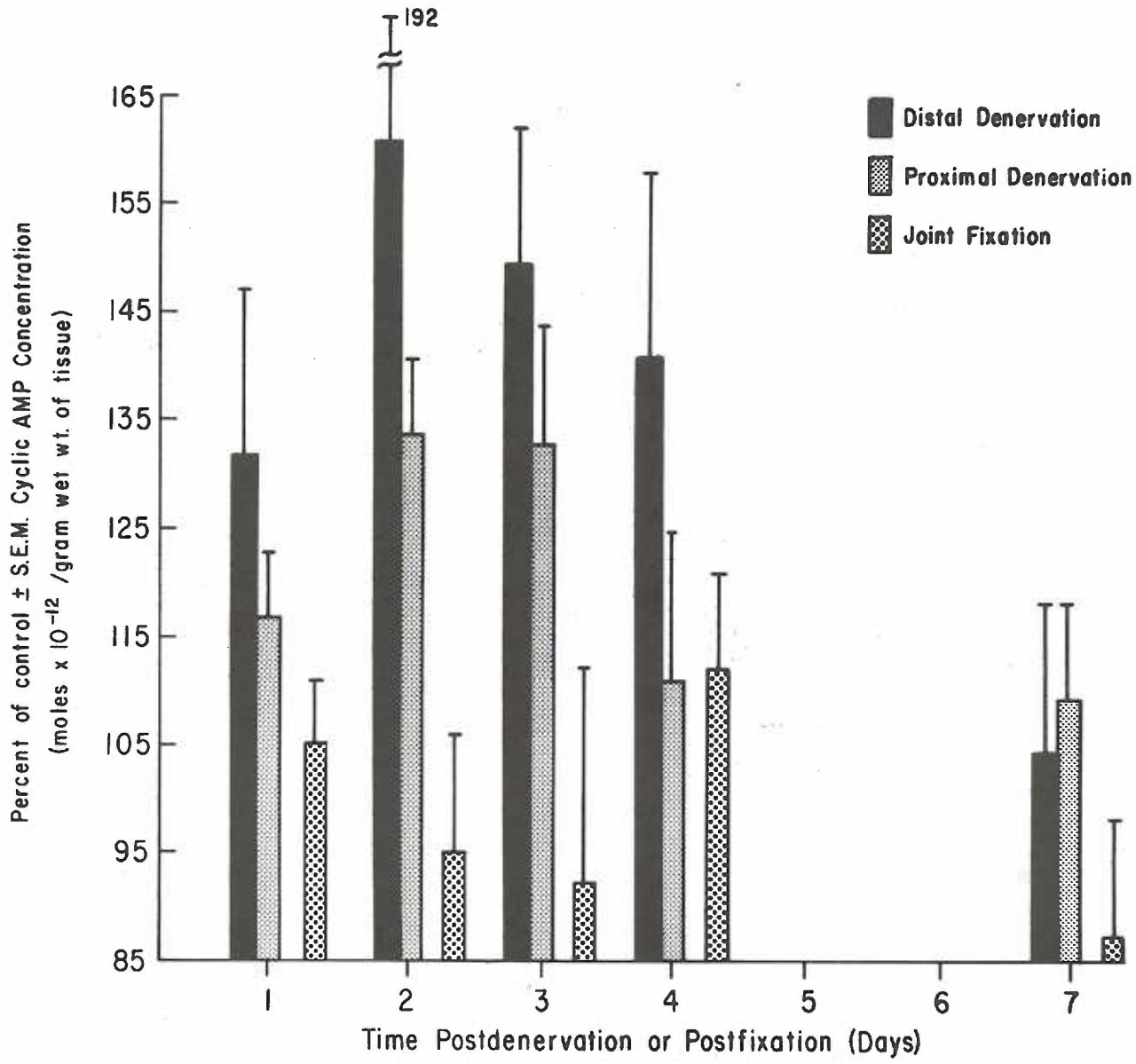
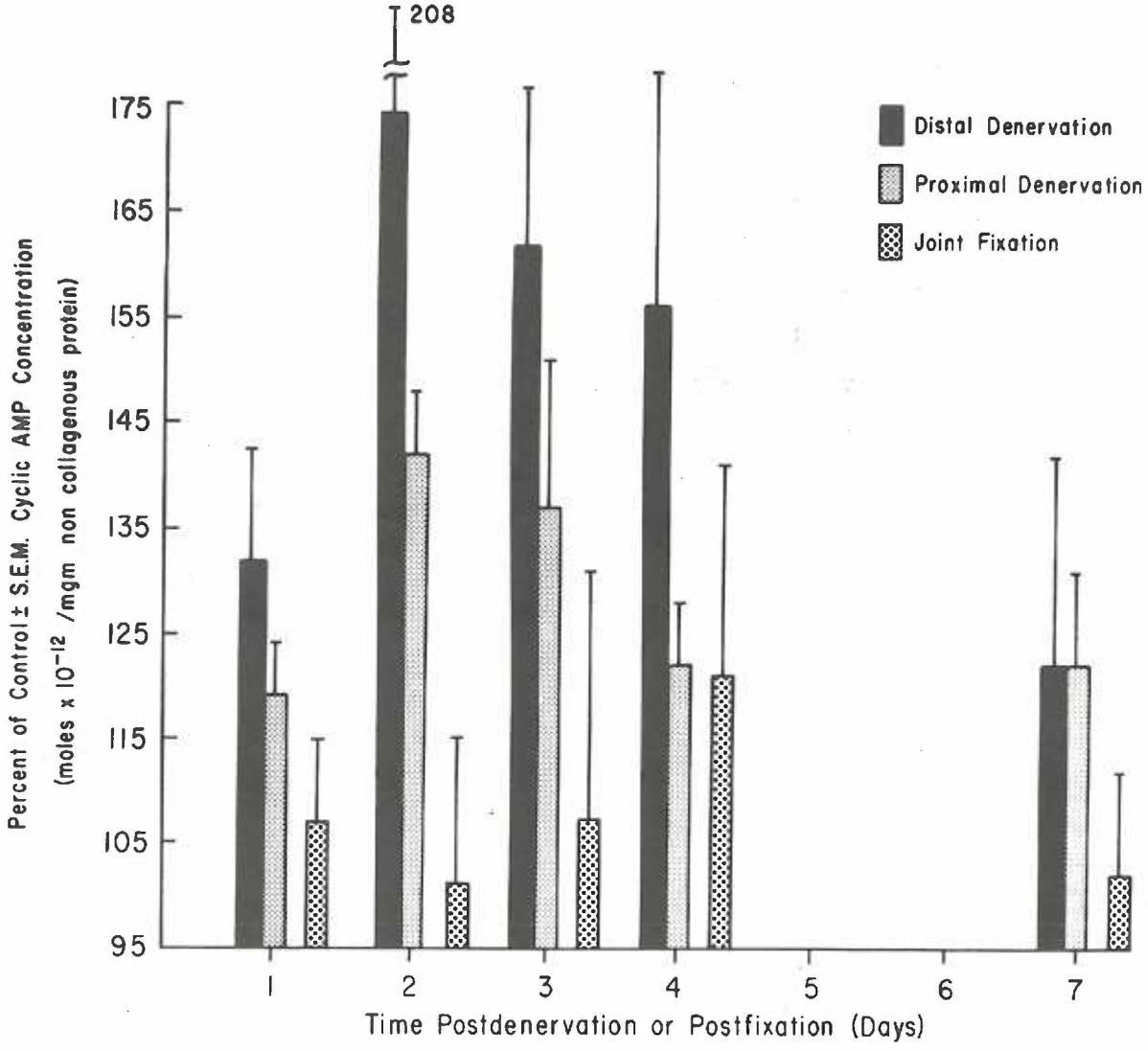

192
165
155
145
135
125
115
105
95
85
Distal Denervation
Proximal Denervation
Joint Fixation
Percent of control ± S.E.M. Cyclic AMP Concentration
(moles x 10^{-12} /gram wet wt. of tissue)
1
2
3
4
5
6
7
Time Postdenervation or Postfixation (Days)

Figure 9. Comparison of series 1, 2 and 3. Concentration of cyclic AMP measured as moles x 10^{-12} per mgm non-collagenous protein and expressed as the percent of control concentration ± standard error of the mean for each time interval.


208
175
165
155
145
135
125
115
105
95
Distal Denervation
Proximal Denervation
Joint Fixation
Percent of Control ± S.E.M. Cyclic AMP Concentration
(moles x 10⁻¹² /mgm non collagenous protein)
1
2
3
4
5
6
7
Time Postdenervation or Postfixation (Days)

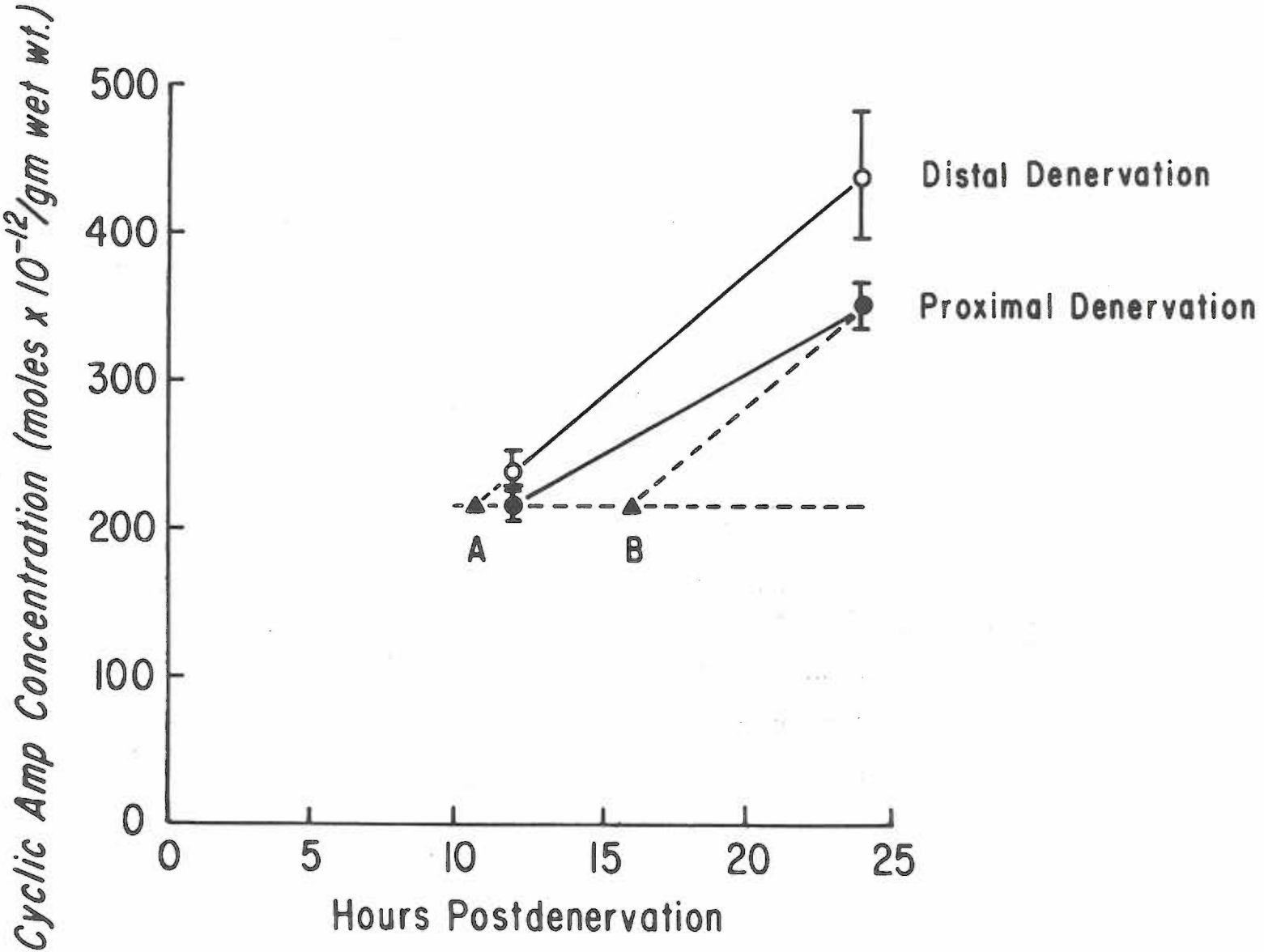
denervated muscle was significantly elevated over the percent increase in the concentration found after joint fixation only at day two, regardless of how the results are expressed.

The increase in cyclic AMP concentration occurred earlier after denervation than after disuse. However, the small number of time intervals which show a significant difference between the two makes it difficult to state unequivocally that the change in cyclic AMP concentration following denervation is not due simply to muscle disuse. Therefore, another series of experiments was designed specifically to answer this question.

Changes in the Concentration of Cyclic AMP Following the Proximal or Distal Denervation of the Tibialis Anterior

Both tibialis anterior muscles were denervated, one proximally, the other distally. Since both muscles were denervated at the same time, both would be equally disused. Therefore, any difference in the concentration of cyclic AMP which occurs between the two muscles, cannot be due to unequal use, but rather, must be due to the difference in the length of the residual nerve stumps. The results of these experiments are presented graphically in Figure 10. Each experimental point represents a mean of the concentration measurements made on seven muscles. At twelve hours postdenervation, there was no significant difference in the cyclic AMP concentration

Figure 10. Concentration of cyclic AMP in one tibialis anterior muscle after section of the peroneal nerve (distal denervation) and in the contralateral tibialis anterior after section of the sciatic nerve at the level of the trochanter (proximal denervation). Cyclic AMP concentration expressed as moles x 10^{-12} per gram wet weight ± standard error of the mean. Point A represents the extrapolation of the change in cyclic AMP concentration after distal denervation back to control concentration. Point B represents the same extrapolation for the proximally denervated muscle, assuming the change in concentration occurs at a rate equal to that in the distally denervated muscle. The difference between points A and B represents the time delay produced by an additional 3.5 cm of residual nerve stump (see Discussion).


Cyclic Amp Concentration (moles x 10⁻¹²/gm wet wt.)
500
400
300
200
100
0
0
5
10
15
20
25
Hours Postdenervation
Distal Denervation
Proximal Denervation
A
B

obtained from either muscle. At twenty-four hours postdenervation, the cyclic AMP concentration in the distally denervated muscle was significantly greater than the concentration found in the proximally denervated muscle. However, both muscles had cyclic AMP concentrations which were greater than that found in control muscles. Therefore, while it appears that the concentration of cyclic AMP was increasing in both muscles, in accord with the previous experiments, it also appears that the time of onset of the increase was different for the two muscles. The increase apparently began earlier in the distally denervated muscle.

Electrophysiological Analysis of Reinnervated Muscle

Fibrillatory Activity

Fibrillatory activity in a denervated muscle fiber ceases at the time the fiber becomes reinnervated. The disappearance of fibrillatory potentials, and the reappearance of miniature end plate potentials actually occurs before the re-establishment of neuromuscular transmission (127). However, not all muscle fibers are reinnervated at the same time. Therefore, one sees a gradual decline in the frequency of fibrillatory potentials recorded from different segments of the whole muscle. Once again there is a proximal to distal progression of activity. In this case, the more proximal portion of the

muscle was reinnervated first, as the nerve fibers regrew into the muscle. One would expect then, a proximal to distal difference in fibrillation frequency which is the reverse of that which occurs after denervation. This was the situation in these experiments, as may be seen from Tables 7A and Figure 11. It is apparent that the overall fibrillation frequency (potentials > 10 μV), and the frequency of large amplitude potentials (>20 μV), was greater in the recordings from the distal muscle at both day 12 and day 14. The largest amplitude potentials (50-60 μV), however, were present in both the proximal and distal recordings. The progression of reinnervation may also be noticed by the decline in fibrillation frequency recorded from either muscle segment between day 12 and day 14. No evoked activity could be produced in the muscle by stimulating the tibial nerve at either day 12 or day 14. At day 15, however, no fibrillatory potentials could be recorded, and evoked activity could be produced by nerve stimulation.

Neuromuscular Transmission

The most apparent phenomena associated with the return of neuromuscular transmission is the small amplitude and asynchrony of the evoked muscle potentials. As may be seen from Table 7B or Figure 11, the activity evoked at postdenervation day 15 was characterized by a series of low amplitude potentials which persisted for up to 15 msec following a single nerve stimulus. These separate

Table 7. Electrophysiological analysis of reinnervated muscle.
A) Measurements of the frequency of fibrillatory potentials at days 12 and 14 postdenervation.
B) Measurement of the parameters associated with the evoked muscle potentials produced by a single, supra-maximal stimulus applied to the tibial nerve.

A) Fibrillatory Activity

Time Postdenervation (days)	Frequency (impulses/sec) (Potentials > 10 V)	
	Prox.	Distal
12	40	65
14	25	30

	Frequency (impulses/sec) (Potentials > 20 V)	
	Prox.	Distal
12	10	20
14	3	10

B) Neuromuscular Transmission

Time Postdenervation (days)	Latency (msec)		Amplitude (mV)		Duration (msec)	
	Prox.	Distal	Prox.	Distal	Prox.	Distal
0 (Control)	1.0	1.0	0.93	1.5	4.3	5.0
15	4.0	7.0	0.04	0.02	11.0	15.0
17	2.5	4.5	0.21	0.22	12.0	14.0
21	2.5	3.5	0.13	0.21	8.0	11.0
28	2.0	2.0	0.30	0.14	8.0	11.0

Figure 11. Electrophysiological recordings from the denervated rat gastrocnemius during the course of reinnervation. Recordings were obtained from both the proximal and distal segments of the muscle. Time and voltage scales refer to the scales presented at the bottom of each column of recordings.

A) 12 days postdenervation. Fibrillatory potentials show a decrease in frequency although large amplitude potentials are still present. Time scale: 20 msec. Voltage scale: 20 μV.

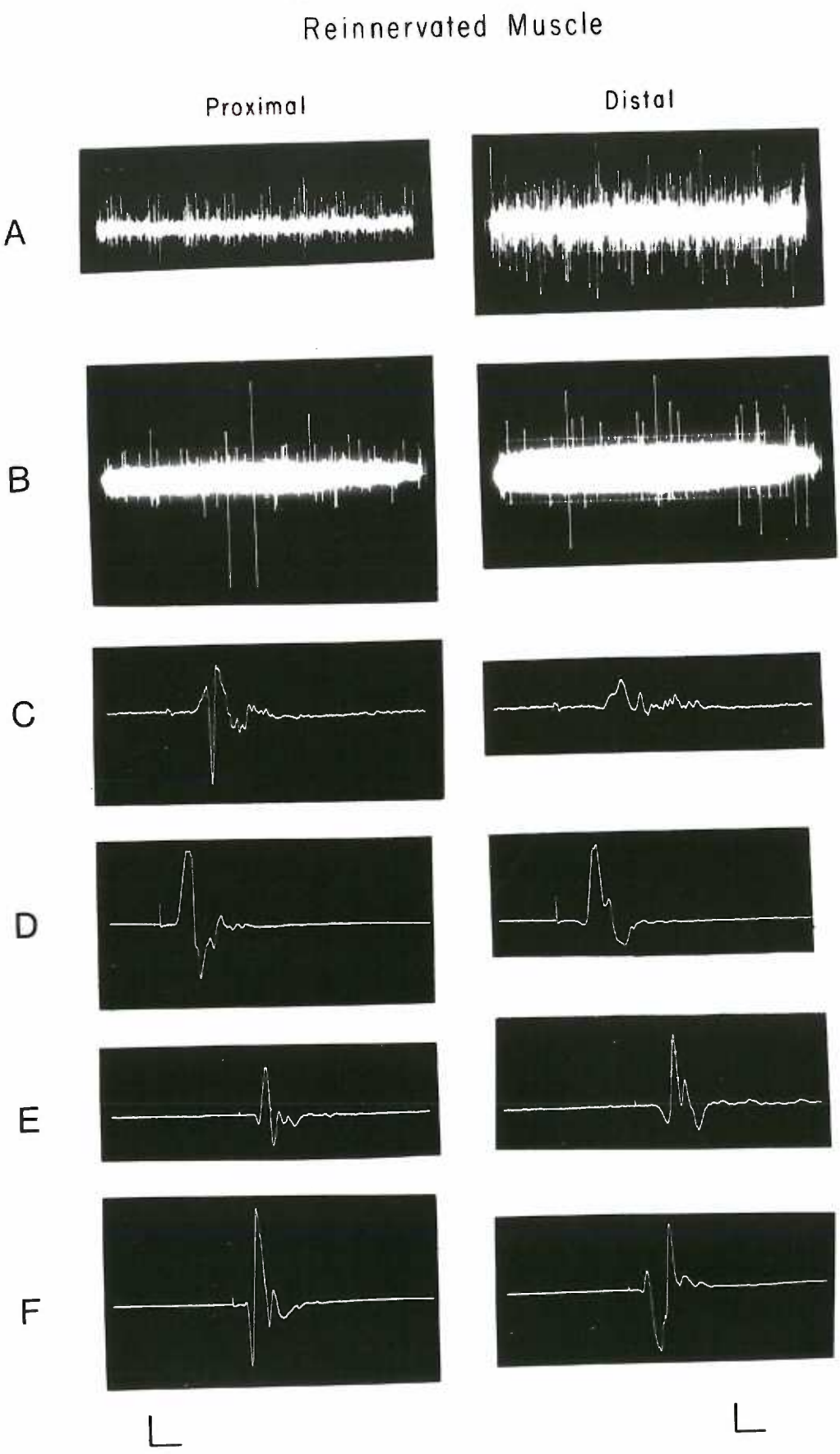
B) 14 days postdenervation. Fibrillatory potentials are further reduced in amplitude, but no muscle potentials may be evoked by nerve stimulation. Time scale: 200 msec. Voltage scale: 20 μV.

C) 15 days postdenervation. Fibrillatory potentials are no longer present. Muscle potentials may be evoked by stimulation of the tibial nerve. Time scale: 5 msec. Voltage scale: 20 μV.

D) 17 days postdenervation. Evoked potentials are increased in amplitude and latency is decreased. Time scale: 5 msec. Voltage scale: 0.1 mV.

E) 21 days postdenervation. Evoked potential shows a further reduction in latency but little change in amplitude. Time scale: 5 msec. Voltage scale: 0.1 mV.

F) 28 days postdenervation. Latency is further reduced, amplitude slightly increased in proximal recording. Time scale: 5 msec. Voltage scale: 0.1 mV.


Reinnervated Muscle
Proximal
Distal
A
B
C
D
E
F

potentials are probably the result of individual motor units which may be discharging at widely separate times. A further indication of the basis for the asynchronous pattern of the evoked muscle activity is provided by the latency measurements. The latency from nerve stimulation to muscle discharge is clearly prolonged in the reinnervated muscle. This phenomenon has been attributed to the very small size, and hence low conduction velocity, of the regenerating nerve fibers (14). It is easy to imagine how motor units, which are innervated by nerve fibers of different lengths, might discharge at different times when the conduction velocity of the nerve fibers may be as low as 1 meter/sec. The progressive increase in the amplitude and decline in the latency and duration of the muscle potential during reinnervation are a reflection of both an increase in the number of muscle fibers innervated, and the growth and maturation of the innervating nerve fibers. Ideally, as in the control muscle, intramuscular conduction is essentially instantaneous, and, after a supramaximal stimulus, all muscle fibers will contract synchronously. The synchronous depolarization of all muscle fibers leads to the characteristic bi or triphasic muscle potential seen in recordings from the control muscle. Clearly the control situation has not been reached by day 28, but it is probable that all muscle fibers have been reinnervated. Studies in which the development of tension during muscle contraction was measured have demonstrated approximately the same time

course for the return of function to the reinnervated rat gastrocnemius (180). Tension development had also not returned to control values by day 28, but complete return required a much longer period (i. e. 83 percent return by day 84). Since these experiments are concerned with the initial response of the muscle to reinnervation, no experiments covering a longer time course were conducted.

Cyclic AMP Concentration Changes in the Reinnervated Muscle

Cyclic AMP concentration measurements made on muscles during the process of reinnervation are presented in Table 8 and Figure 12. It is interesting to note that cyclic AMP concentration was elevated in the denervated muscle, but as reinnervation progressed the concentration returned to the control level. It is also interesting to note that the concentration of cyclic AMP in the denervated muscle was significantly higher than control at days 14 and 15. In the denervation experiments, where no reinnervation took place, there was no significant difference in concentration between denervated and control muscles at day 14. This point will be considered further in the Discussion section.

Table 8. Concentration of cyclic AMP in thegastrocnemius muscle during the period of reinnervation (experimental muscle) compared with contralateral gastrocnemius muscle (control).

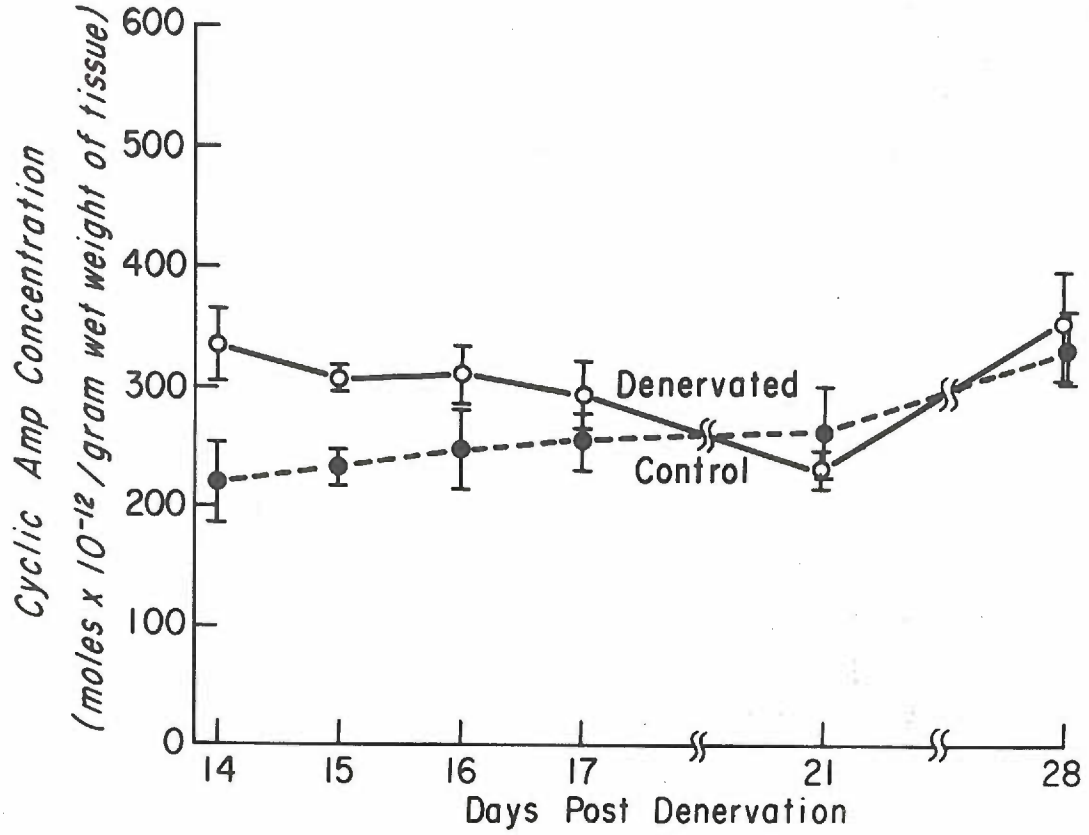
A) Concentration of cyclic AMP expressed as moles x 10^{-12} per gram wet weight.

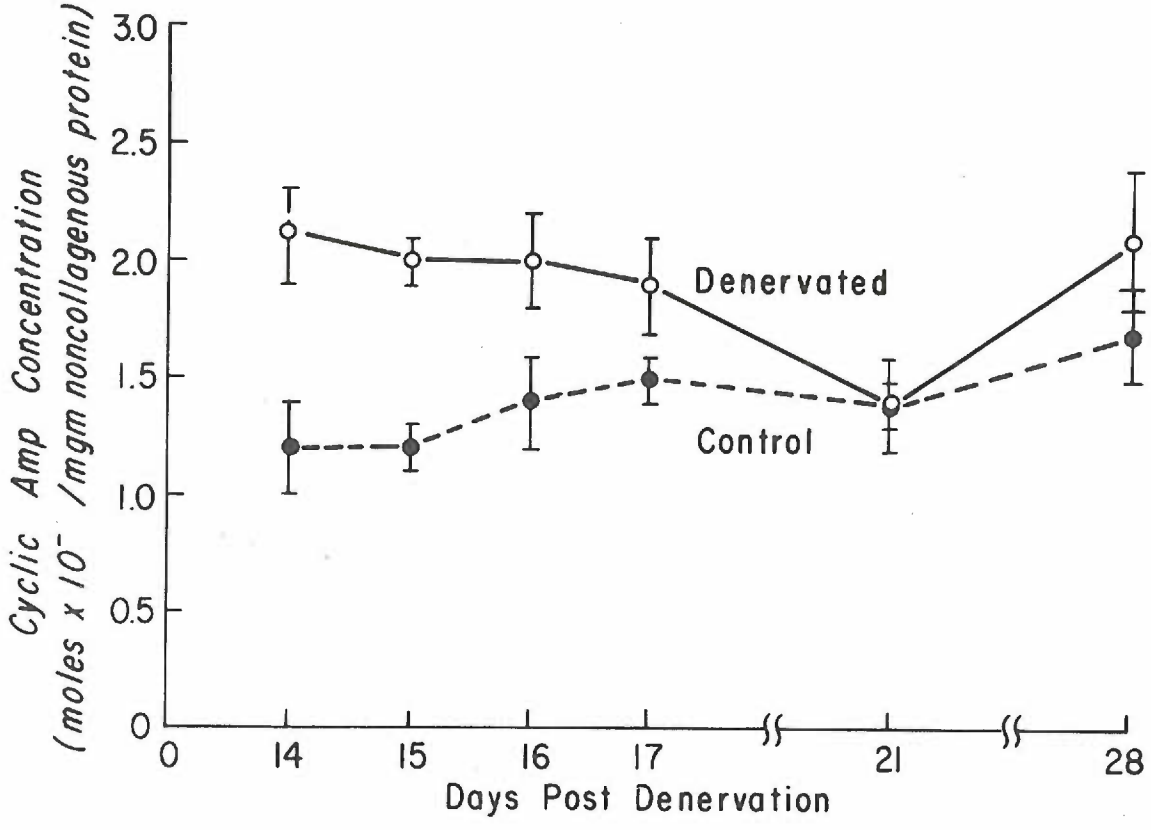
B) Concentration of cyclic AMP expressed as moles x 10^{-12} per mgm NCP.

A)	Time Postdenervation (days)					
	14	15	16	17	21	28
Number of Animals	6	6	6	6	6	5
Mean Experimental Muscles ±SEM	335±32	309±11	311±23	294±29	233±15	354±46
Mean Control Muscles ±SEM	220±34	233±14	249±33	255±24	263±35	330±30
Experimental/Control x 100	152	133	125	115	89	107
	*	*	N. S.	N. S.	N. S.	N. S.

B)	Time Postdenervation (days)					
	14	15	16	17	21	28
Number of Animals	6	6	6	6	6	5
Mean Experimental Muscles ±SEM	2.1±.2	2.0±.1	2.0±.2	1.9±.2	1.4±.1	2.1±.3
Mean Control Muscles ±SEM	1.2±.2	1.2±.1	1.4±.2	1.5±.1	1.4±.2	1.7±.2
Experimental/Control x 100	169	164	151	131	100	123
	*	*	*	N. S.	N. S.	N. S.

Figure 12. Concentration of cyclic AMP in the gastrocnemius muscle during the period of reinnervation (denervated) and in the contralateral gastrocnemius muscle (control). Top graph: Cyclic AMP concentration expressed as moles x 10^{-12} per gram wet weight of tissue ± standard error of the mean. Bottom graph: Cyclic AMP concentration expressed as moles x 10^{-12} per mgm NCP ± standard error of the mean.


Cyclic Amp Concentration
(moles x 10⁻¹² /gram wet weight of tissue)
600
500
400
300
200
100
0
Denervated
Control
14
15
16
17
21
28
Days Post Denervation


Cyclic Amp Concentration
(moles x 10⁻ /mgm noncollagenous protein)
3.0
2.5
2.0
1.5
1.0
0.5
0
Denervated
Control
0
14
15
16
17
21
28
Days Post Denervation

DISCUSSION

Introduction

The major question being asked in this study is whether the concentration of cyclic AMP in skeletal muscle is neurotrophically controlled. The phrase "neurotrophic control" implies, in this case, that some aspect of nerve-muscle interaction controls the activity of the adenylate cyclase-cyclic AMP system. The first half of the discussion, therefore, considers the currently accepted methods and criteria necessary to demonstrate neurotrophic regulation. The discussion then considers how well the results reported in this study fit the specific criteria. Clearly, it is necessary to determine whether cyclic AMP concentration is neurotrophically controlled before attempting to establish a possible role for cyclic AMP in the mechanisms involved in neurotrophic regulation. The experiments reported here do not provide data sufficient to show cause and effect relationships between changes in cyclic AMP and changes in other trophically-controlled phenomena. However, it is possible to show correlations between the temporal pattern of the postdenervation change in cyclic AMP, and the temporal pattern of other postdenervation changes in the muscle. Although temporal correlations do not demonstrate causality, they at least make a causal relationship possible. Therefore, the final part of the discussion presents a

speculative overview of a number of possibilities which could form the basis for future experiments.

Is the Concentration of Cyclic AMP in Skeletal Muscle Neurotrophically Regulated?

Once again, "neurotrophic regulation" in this case is meant to imply that the nerve provides some mechanism for controlling the activity of the adenylate cyclase-cyclic AMP system.

Disuse vs. Denervation

It has been assumed that any phenomenon which could be demonstrated after denervation, but not after muscle disuse, must be due to some trophic effect of the nerve (182). The degree of disuse produced, however, by most of the techniques currently used, is extremely variable. A complete paralysis, like that produced by denervation, has been obtained by only one method used to produce disuse in an innervated muscle. This procedure, however, which involves the placement of a silastic cuff filled with local anesthetic around the muscle nerve, has yielded conflicting results, and is complicated by possible drug effects (115, 151).

There are also obvious limitations associated with the technique used to produce muscle disuse in this study. The object of fixing the knee and ankle joints in a flexed position is to hold the gastrocnemius,

an extensor muscle, at its rest length. It is assumed that this degree of immobilization will lead to a reduction in the primary afferent input to gastrocnemius motoneurons. A reduction in afferent input will presumably lead to a subsequent reduction in motoneuron activity, and, therefore, to a reduction in muscle use. Unfortunately, the actual degree of disuse produced is uncertain, since no direct measurements of the electrical activity in a fixed gastrocnemius muscle have been made. However, Fischbach and Robbins (55) have chronically recorded the electrical activity in a soleus muscle which was immobilized by joint fixation. Soleus activity was found to be profoundly reduced following immobilization. Moreover, the soleus is also an extensor muscle whose insertions about the knee and ankle are essentially the same as those of the gastrocnemius. Therefore, it may be assumed that joint fixation reduces activity in the gastrocnemius to a similar extent. One point of caution should be mentioned, however, when comparing the gastrocnemius and soleus muscles. The soleus is an essentially homogeneous tonic muscle which normally shows continuous low frequency electrical activity. The gastrocnemius, on the other hand, is a mixed muscle containing phasic motor units which normally show electrical activity patterns consisting of quiescent periods coupled with periods of high frequency activity, and tonic motor units which

show characteristic tonic activity patterns. Whether both tonic and phasic muscles are equally disused after joint fixation can only be determined by direct measurements of the electrical activity in the muscle following fixation.

Despite these limitations, however, it is generally felt that this method of producing muscle disuse is superior to other techniques, most of which produce more profound rearrangements of muscle environment (66, 67). Techniques such as tenotomy (severing the distal tendon of the muscle) (185) and casting (34), may produce severe disruptions in muscle blood flow. An older technique, which involves isolating the motoneuron pool of a specific muscle by transecting the spinal cord above the pool and cutting all dorsal root inputs to the pool, disrupts both the reflex arc, and any suprasegmental input to the motoneurons (182). A surgical manipulation of this complexity also produces a high degree of stress, and is not suitable for experiments designed to measure early postdisuse changes in the concentration of cyclic AMP.

It is clear that, regardless of the technique used, the degree of disuse is variable and difficult to quantify. Few attempts have been made to measure the extent of disuse directly (55, 185). Most reports have attempted to indirectly measure the equivalence of muscle disuse and denervation by comparing the degree of muscle atrophy accompanying both. This comparison assumes that if the

extent and time course of muscle atrophy are equivalent following both procedures, the degree of muscle disuse must be equivalent. Atrophy, however, is a complex phenomenon which may be only indirectly related to the electrical activity of a muscle. Gutmann (77) for example, has emphasized that "disuse atrophy" is a heterogeneous event which is dependent on a number of factors. Disuse atrophy may not be directly comparable to denervation atrophy. Therefore, while the temporal pattern and degree of atrophy produced by either denervation or joint fixation were equivalent in the experiments reported here, one cannot state, on this basis, that the degree of disuse produced by the two techniques is equivalent.

A comparison of the change in cyclic AMP concentration following denervation with the change following joint fixation has been used in this study, to test the hypothesis that the concentration of cyclic AMP in skeletal muscle is neurotrophically regulated. It is clear from the previous discussion that the evidence provided by disuse experiments has certain limitations. Despite these limitations however, disuse experiments can provide an indication as to whether the phenomenon being studied is likely to be neurotrophically controlled. If there is, for instance, a clear distinction between the changes produced by denervation, and those produced by disuse, there would be reason to believe that a neurotrophic factor may be involved. The evidence provided by disuse experiments, then, when taken together

with evidence provided by other types of experiments, may strengthen the arguments supporting or rejecting a neurotrophic hypothesis.

It is evident that muscle cyclic AMP concentration following denervation is elevated over the concentration present following joint fixation, for the first 3 days. The temporal pattern of cyclic AMP change is also quite different in the two preparations. The concentration of cyclic AMP in the denervated muscle rises much more rapidly than occurs after joint fixation. Cyclic AMP concentration also declines more rapidly in the denervated muscle. Moreover, the elevation of cyclic AMP concentration seen in the control muscle four days after joint fixation is a phenomenon distinct from anything which occurs after denervation. Therefore, it seems fair to assume that the process producing the increase in cyclic AMP concentration after denervation is different from that producing an increase after fixation. The evidence obtained from the sham-operated, "fixed" muscles substantiates this, since cyclic AMP concentration is also elevated in this preparation. However, although the difference between the denervation and disuse experiments provides a necessary condition to support the hypothesis, it does not provide a sufficient condition. Therefore, a different approach was used to provide additional support relevant to the neurotrophic hypothesis.

Comparison of the Temporal Pattern of Postdenervation Change after Sectioning the Nerve at Different Levels

Sectioning a nerve produces an immediate paralysis of the innervated structure. Disuse is immediate and absolute regardless of the level of nerve section. Therefore, if the temporal pattern of the postdenervation change in a characteristic is altered by changing the level of nerve section, the normal maintenance of the characteristic may be assumed to depend on some factor associated with the nerve. This assumption is based on the evidence provided by studies concerned with the axoplasmic transport of material in nerve fibers (107).

Briefly, the transport studies have shown that material necessary for the maintenance of the nerve terminals is synthesized in the perikaryon, and transported down the axon. Therefore, the axon may be considered to be a reservoir of material which is in transit to the nerve terminals. An additional hypothesis assumes that trophic factors are part of the material contained in the axon. If the concentration of the various substances in axon may be assumed to be uniformly distributed along its length, the quantity of material available to the nerve terminals following nerve section will depend on the length of the residual nerve stump. Any difference then, in the temporal pattern of change in a characteristic following section of the nerve at varying levels, may be assumed to be due to the difference in the quantity of available trophic material.

Comparing the effect of transecting a nerve close to and more distant from the muscle, is a technique which has been used to demonstrate the dependence of several muscle characteristics on nerve-related factors (66, 67). Sectioning the nerve close to the muscle (more distally) versus sectioning the nerve more proximally produces an earlier disappearance of miniature end plate potentials, and an earlier failure of neuromuscular transmission (128). Distal denervation also leads to an earlier appearance of fibrillatory potentials (117), an earlier breakdown of the neuromuscular end plate (79), an earlier appearance of an increased extrajunctional sensitivity to acetylcholine (46), a more rapid disappearance of glycogen (79), and an earlier increase in proteolytic activity (79).

While the comparison of muscles denervated at different levels provides one of the clearest methods of demonstrating the trophic control of a muscle characteristic, one limitation should be mentioned. The ability to distinguish differences in the temporal pattern of change of a characteristic will depend on the rapidity with which the change occurs. For instance, the disappearance of miniature end plate potentials at the rat neuromuscular junction after nerve section is delayed by only 45 minutes per additional one centimeter of residual nerve fiber (128). The temporal resolution provided by the sampling schedule will determine, therefore, if any potential differences in the temporal pattern can be detected.

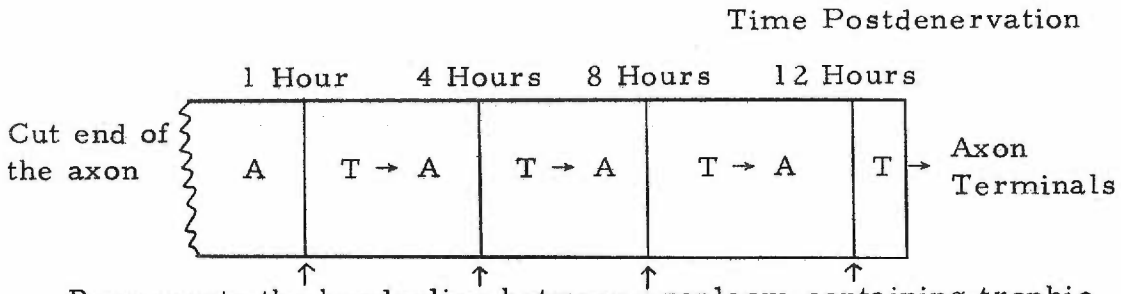
Temporal resolution presented a problem in this study. The experiments were designed to sample denervated muscles at 24 hour intervals. Proximal section of the sciatic nerve added approximately 1.5 centimeters of residual nerve stump to the denervated gastrocnemius when compared to the nerve stump remaining after tibial nerve section. A comparison of the two denervation experiments (Series 1 and 3, Figures 5 and 7) indicates that the peak increase in cyclic AMP concentration occurred at day 2 in both experiments. The peak increase is lower for the proximally denervated muscle, but this can probably be explained by the increased dose of pentobarbital used for anesthesia in series 3 (98). It is not immediately apparent from the figure, that an alteration in the temporal pattern of cyclic AMP concentration change is produced by the additional 1.5 cm of nerve stump. The temporal resolution provided by sampling at 24 hour intervals is not sufficient to clearly indicate a temporal change. However, the slope of the increase in concentration appears to be greater in the distally denervated muscle. One may, therefore, be able to discern a difference in the temporal pattern by comparing the rate of change of cyclic AMP concentration after proximal and distal nerve section. The rates of change may be compared by determining the time to half maximum cyclic AMP concentration for each experiment. These calculations indicate that the concentration of cyclic AMP in the distally denervated muscle

reaches half maximum concentration approximately 4 hours earlier than the concentration in the proximally denervated muscle. This then, represents the temporal delay provided by 1.5 cm of additional nerve stump, which supports the hypothesis that cyclic AMP in skeletal muscle is trophically regulated.

A stronger case could be made in support of trophic regulation, if the temporal resolution provided by the experimental protocol could be made finer by shortening the interval between samples. This was the purpose of the experimental series involving the denervation of both tibialis anterior muscles in the same animal. The experimental procedure provided an increased difference between the level of distal and the level of proximal nerve section (3.5 cm), and a shorter sampling interval (12 hours). Figure 10 provides clear evidence that an increase in the residual nerve stump alters the temporal pattern of the change in cyclic AMP concentration. This provides additional support for the hypothesis that the concentration of cyclic AMP in skeletal muscle is trophically regulated.

The information supplied by the denervation experiments also allows the calculation of a hypothetical velocity for the axoplasmic transport of the factor(s) responsible for regulating cyclic AMP. One might consider at least two alternative methods for the delivery of trophic material by the nerve to the muscle. The first alternative assumes that the concentration and rate of release of trophic factors

from the nerve terminals are constant because the materials move down the axon at a constant rate and concentration. In other words, there is a sharp division between the axoplasm containing trophic material after nerve section, and the axoplasm which has been drained of trophic material. This assumption is diagrammed below:



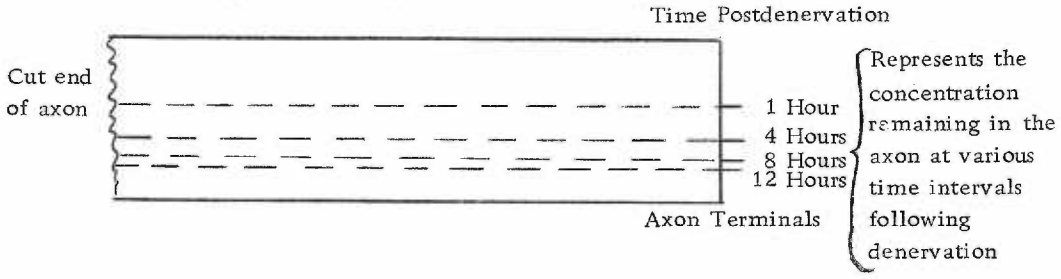
Represents the borderline between axoplasm containing trophic material (T), and that without (A) at various postdenervation time intervals.

Clearly, from this assumption, there will be no decrease in the supply of trophic material to the muscle until the axon reservoir empties, and the supply of trophic material ceases abruptly. This hypothesis is considered graphically in Figure 10. Point A represents the time when the trophic material remaining in the distally sectioned peroneal nerve is exhausted, and the trophic regulation of cyclic AMP in the muscle ends. Cyclic AMP concentration then proceeds to increase at a rate dependent on the intracellular mechanisms responsible for its synthesis and degradation. Point B represents the time when the trophic material in the longer, proximally sectioned nerve stump is exhausted. Cyclic AMP will now begin to

increase in the proximally denervated muscle. The rate of increase, however, will be the same as that for the distally denervated muscle, since it depends on the same factors. The dotted lines represent the extrapolations based on these assumptions. Clearly, then, the difference between point A and point B represents the time it takes for the residual concentration of trophic material in the proximally sectioned nerve to be exhausted. If one measures the difference in the lengths of the residual nerve stumps, approximately 3.5 cm, and divides this distance by the time interval, approximately 5.7 hours, then multiplies by 24 hours/day, one obtains a hypothetical velocity for the transport of the trophic material responsible for the control of cyclic AMP in skeletal muscle, approximately 147 mm/day.

A second, and equally likely alternative to explain the decline in the concentration of trophic material available to the denervated muscle assumes that the rate of transport of trophic material is directly related to the concentration of material contained in the axon. In other words, the concentration in the axon is uniform, and both the delivery and rate of release are dependent on the axon concentration. Once the axon is separated from its supply source, the perikaryon, the concentration of trophic material in the whole axon will uniformly diminish with time. The delivery of material to the nerve terminals will decrease exponentially. Since the release of trophic material depends on the concentration available at the nerve

terminals, the amount of trophic material supplied to the muscle will also decrease exponentially. This assumption is diagrammed below:



In this case, cyclic AMP concentration would increase at a rate dependent on both the diminishing concentration of trophic material, and the intramuscular mechanisms responsible for cyclic AMP synthesis and degradation. Increasing the length of the residual nerve stump would increase the reservoir of trophic material, and increase the period over which an effective concentration of trophic material is made available to the muscle. Using this assumption, the initial slope of the increase in cyclic AMP concentration would be reduced by increasing the length of the residual nerve stump Eventually, however, when all of the trophic material contained in the nerve was exhausted, the rate of change of cyclic AMP concentration would be the same in both the proximally and distally denervated muscles. Applying these assumptions to the comparison of the time to half maximum concentration increase from series 1 and

series 3, one obtains an axoplasmic velocity of approximately 91 mm/day for the transport of the trophic factor(s) regulating skeletal muscle cyclic AMP concentration.

Both of the values for axoplasmic transport velocity calculated are within the range of axoplasmic velocities representing fast axoplasmic transport (107). The values themselves probably have little meaning; the important point to be made is that whatever the actual velocity or velocities, the trophic factor(s) responsible for the control of cyclic AMP in skeletal muscle travel with the fast components of axoplasmic transport. The slow components travel at velocities in the range of 1-2 mm/day (107).

The Demonstration of Neurotrophic Regulation by Replacement after Denervation: Reinnervation

Trophically-mediated changes in muscle characteristics develop over a long time course. It has been difficult to devise a system which could be maintained long enough to allow the reversal of denervation changes by the replacement of missing trophic factors. Until recently, only tissue-culture techniques offered the capability of long-term tissue maintenance. Explants of muscle and spinal cord, maintained together in culture, have demonstrated the capability of nervous tissue to induce the normal differentiation and development of muscle fibers (140). This is a nerve-mediated effect normally

associated with neurotrophic regulation, and it would seem that tissue-culture might offer an excellent technique for investigating trophic mechanisms. However, it would also be appealing to be able to investigate trophic relationships occurring in whole organs, either in vitro or in vivo. Two recent experiments may provide the necessary techniques. Both experiments were performed using an amphibian system. Lebowitz and Singer (108) were able to stimulate protein synthesis in the regenerating limb of the newt by infusing homogenates of nerve directly into the regenerating blastema. Lentz (109) has developed an organ culture of muscle, also obtained from a newt. He has been able to modify the postdenervation disappearance of acetylcholinesterase from the muscle end plate (71). Applying homogenates of nerve and of sensory ganglia to the cultured muscle prevented the loss of cholinesterase activity; homogenates from non-nervous tissue had no effect. Thus far, however, these experiments represent the only successful attempts to replace the trophic regulation eliminated by denervation.

A rather non-specific attempt at restoring trophic regulation has been made in this study. The crushed tibial nerve was allowed to regenerate and reinnervate the denervated gastrocnemius muscle. Clearly, this procedure will not be able to distinguish between the effects produced by a return of the neuromuscular transmission of impulses, and effects produced by a return of trophic factors. As a

later portion of the discussion will show, the electrophysiological evidence suggests that muscle activity may play some role in the regulation of cyclic AMP. However, previous experiments demonstrated that cyclic AMP concentation in the muscle is also dependent on trophic factors Trophic regulation and muscle activity may, therefore, provide a joint mechanism for controlling the concentration of cyclic AMP in skeletal muscle. Reinnervation restores both systems. "Replacement" of trophic control, in this case, does not discriminate between the possible influences of the two systems in the regulation of cyclic AMP. Figure 12 shows that as reinnervation progresses in the muscle, the concentration of cyclic AMP declines. The concentration of cyclic AMP in the reinnervated muscle is not significantly different from control at day 16 Therefore, while this "replacement" evidence is subject to qualification, it is consistent with the previous evidence, and indicates a nerve-mediated regulation of cyclic AMP in skeletal muscle.

Summary

Three techniques have been used to test the hypothesis that cyclic AMP in skeletal muscle is trophically regulated. Denervation of the gastrocnemius produced a transient increase in the concentration of cyclic AMP in the muscle. Comparing the results of denervation with the results due to joint fixation showed that the change in

concentration of cyclic AMP produced by each technique was distinctly different. Changing the level of nerve section altered the temporal pattern of the change in cyclic AMP concentration, indicating that the regulation of cyclic AMP depended on some factor(s) associated with the residual nerve stump Finally, reinnervation of the denervated muscle produced a decline in the concentration of cyclic AMP. Taken togehter, the evidence strongly supports the hypothesis that the concentration of cyclic AMP in skeletal muscle is under trophic control, i. e., that the activity of the adenylate cyclase-cyclic AMP system is controlled by some mechanism provided by the nerve.

A Possible Role for Cyclic AMP in the Neurotrophic Regulation of Skeletal Muscle: Temporal Correlations

As was evident from the brief treatment in the Introduction section, cyclic AMP has been shown to mediate a variety of hormonally-induced intracellular responses. However, the only response in skeletal muscle known to be mediated by cyclic AMP is the catecholamine-induced increase in glycogenolysis. Therefore, in order to speculate on the potential role of cyclic AMP in the mediation of the postdenervation changes in several muscle characteristics, one must extrapolate from proven cyclic AMP relationships in other tissues to skeletal muscle. Clearly, the data presented here does not allow the

establishment of any causal relationships. However, there are a number of suggestive temporal correlations between changes in cyclic AMP concentration, and postdenervation changes in other muscle characteristics. Such temporal correlations may provide the first step toward elucidating a causal relationship.

Correlations with Electrophysiological Measurements

A comparison of the postdenervation electrophysiological measurements, with the temporal pattern of the postdenervation increase in cyclic AMP indicates that cyclic AMP concentration begins to increase while the nerve still retains the capability for neuromuscular transmission. This comparison may be a bit misleading since the sectioned nerve is not transmitting impulses. However, the fact that transmission is still possible proves that nerve terminals are still intact, and that miniature end plate potentials (m. e. p p. s) are still present (128). M. e. p. p. s are small local depolarizations which may be recorded from the muscle end plate. They are thought to be the result of the spontaneous release of small packets of acetylcholine from presynaptic nerve terminals (51). M. e. p. p. s are not of suffucient amplitude to reach the threshold for muscle action potential generation, and therefore, are not propagated beyond the end plate.

At one day postdenervation, the evoked muscle response is

reduced in amplitude, and the electrical pattern appears polyphasic. This is an indication that the various motor units are no longer responding synchronously to supramaximal nerve stimulation; this probably means that at least some of the nerve terminals are no longer transmitting (49), because they have degenerated. M. e. p. p. activity also ceases at the time transmission fails (128). Since cyclic AMP concentration is already significantly elevated, it is possible that the absence of m. e. p. p s produces some alteration in the trophic control of the muscle as has been suggested (43). In order to test this hypothesis directly, one would have to be able to measure the change in cyclic AMP concentration which occurs in individual muscle fibers as m. e. p. p. activity ceases. This would obviously be very difficult. However, the indirect evidence provided by the data from these experiments makes it appear unlikely that spontaneously released acetylcholine regulates cyclic AMP concentration. Although the time course over which m. e. p. p s disappear, and cyclic AMP concentration increases is quite similar (1, 2), the decline in cyclic AMP concentration, which begins on the 2nd day, is probably not due to a reappearance of m. e. p p. s. Although there is some evidence, obtained from frog muscle, that m. e. p. p s may reappear at a frequency much lower than normal (15), there is no evidence showing a reappearance of m. e. p p activity in mammalian muscles following denervation.

Additional evidence against the m. e. p. p. hypothesis is provided by the reinnervation experiments. Measurements made on muscles during the course of reinnervation show that cyclic AMP concentration is significantly increased over control muscles at day 14. In the previous experiments, in which the muscle was not reinnervated, cyclic AMP concentration in the denervated muscle was not significantly increased at day 14. One would assume that as reinnervation progressed and new nerve terminals re-established contact with the muscle, m. e. p p. activity would be restored (127). An increase in m. e. p. p activity should not lead to an increase in the concentration of cyclic AMP if it is the spontaneous release of acetylcholine which normally provides the trophic suppression of cyclic AMP concentration.

It is possible, however, that some factor associated with muscle activity (i. e. muscle depolarization, excitation-contraction coupling, energy utilization, etc.) provides a mechanism for the trophic control of cyclic AMP. It would be possible for example, for acetylcholine, in the amounts released by a nerve impulse, to act as a trophic regulator by causing muscle depolarization as has also been suggested (42). The muscle, except for m. e. p. p. activity, is quiescent following denervation, until the time fibrillatory potentials appear. Cyclic AMP concentration begins to increase, and in fact reaches its peak value during the quiescent period. The concentration begins

to decline, however, as fibrillatory activity increases. Moreover, the decline in cyclic AMP concentration closely parallels the increase in the frequency of fibrillatory activity (158). Once the concentration of cyclic AMP is returned to control level, it apparently remains there as long as the muscle remains denervated and fibrillatory activity remains high. When reinnervation is allowed to occur there is a period during which fibrillatory activity is decreasing, but impulse transmission has not been re-established. This period occurs between 12 and 15 days in in this preparation (88), and coincides with the period at 14 days when the cyclic AMP concentration in the muscle being reinnervated is again significantly elevated. However, it is not possible, from these data, to determine whether cyclic AMP increases in proportion to the decline in fibrillatory activity.

It is also possible that the apparent correlation between cyclic AMP concentration and muscle activity may be due to a third factor which influences them both independently. There are, nevertheless, a number of studies which seem to indicate a correlation between trophic regulation and muscle activity. Thesleff (179), and later Drachman (42), have used botulinum toxin to block the release of acetylcholine from the presynaptic nerve terminals, and hence to block impulse transmission. Muscles treated with the toxin show many of the characteristics associated with denervation (e. g. fibrillation and an increase in the extrajunctional sensitivity to

acetylcholine), although the nerve terminals are still present and undamaged. There remain a number of questions concerning the technique (e.g. what other effects does the toxin have?, does it block the release of substances other than acetylcholine?), but the authors suggest that either acetylcholine, or the effect it produces (muscle depolarization) provides some form of trophic regulation.

The evidence provided by cross-innervation experiments also suggests that nerve impulse activity and/or the pattern of muscle activity it produces may play a role in determining the contractile characteristics of a muscle (45). The small changes which occur in some muscle characteristics (e.g. the increase in extrajunctional acetylcholine sensitivity) after muscle disuse, may also indicate the importance of muscle activity to the control of muscle characteristics (54, 115). As has been mentioned previously, it is unlikely that any of the techniques used to produce disuse will cause complete muscle silence. Therefore, residual muscle activity may determine the extent to which trophic changes develop (55). Finally, experiments in which the denervated muscle has been chronically stimulated have shown that the spread of acetylcholine sensitivity, which normally occurs following denervation, is prevented by evoked muscle activity (41, 115). It is possible therefore, that muscle activity provides a necessary ingredient to the trophic regulation of skeletal muscle. It is also possible that one of the characteristics influenced by muscle

activity is the concentration of cyclic AMP in the muscle. An additional point which bears on this suggestion is the fact that either direct or indirect electrical stimulation of skeletal muscle produces no change in the resting concentration of cyclic AMP in the muscle (44, 142). This would be expected if muscle activity were providing a mechanism for the trophic suppression of cyclic AMP concentration.

Correlations with Changes in the Properties of the Muscle Membrane

If muscle activity per se, along with postulated trophic factors, provide a joint mechanism for trophic regulation, the first available point for interaction would be at the muscle membrane. Membrane properties clearly change following denervation. The most obvious example is provided by experiments showing that the denervated muscle is able to accept innervation from foreign nerves (13, 119, 125). However, other membrane properties change as well, and when taken together, suggest that the sarcolemma is structurally altered following denervation.

Post-denervation changes in muscle membrane properties may be grouped into three categories; changes in the passive properties of the membrane, changes in the active properties, and changes in membrane receptor properties. Structural alterations which may underlie the changes in these various properties may take the form

of allosteric changes in the properties of specific membrane-bound enzymes. The enzymes responsible for cyclic AMP synthesis, and, to a certain extent, degradation are membrane-bound. Adenyl cyclase is clearly associated with both the sarcolemma and the sarcoplasmic reticulum (143, 161). For the most part, phosphodiesterase has been considered to be concentrated in the cytoplasm, but recent evidence now indicates that some form of the enzyme may also be bound to the sarcolemma (193).

Changes in passive and active properties may also represent changes in the active or passive conductance of specific ions. The earliest change in a passive membrane property is a decrease in the resting membrane potential (1, 3, 92). The initial decrease may occur as early as two hours following the section of a nerve very close to the muscle (1). A change in the extrajunctional sensitivity to acetylcholine is not apparent earlier than 24 hours after denervation in the same preparation. While changes in other passive membrane properties occur following denervation, the time course of these changes make it unlikely that the alterations are related to the postdenervation increase in cyclic AMP concentration. For example, transverse membrane resistance, membrane capacitance and the membrane time constant all increase following denervation (2, 92, 136). However, no increase in any of these parameters occurs earlier than the 3rd day following denervation.

The decrease in resting membrane potential, on the other hand, appears to correlate quite closely with the increase in cyclic AMP concentration. The decrease occurs quite rapidly; the potential falls approximately 20 mV in the first 48 hours following denervation, then plateaus and declines only an additional 3 mV over the next 13 days (1). The reason for the decrease is unknown. Most authors have attempted to explain it on the basis of a demonstrated postdenervation depression in the activity of Na^+-K^+ pump (114), or by suggesting that denervation produces a change in the intracellular distribution of K^+ (40). Intracellular potassium concentration is apparently related to innervation, since there is a difference in the potassium concentration found in slow muscles as opposed to that found in fast muscles (172). The difference can be reversed by cross-innervation (91). There is also evidence which indicates that cyclic AMP may play a role in Na^+-K^+ transport, at least in the toad bladder (138). However, whether the postdenervation changes in resting membrane potential and cyclic AMP concentration are causally related remains to be seen. The temporal pattern of the respective changes would at least allow a causal relationship.

The active membrane properties of the muscle involve the mechanisms which generate muscle action potentials. The most apparent response of the active membrane to denervation is the development of spontaneous activity, i. e. fibrillatory potentials.

In this study, fibrillation first appears on the 2nd day following denervation, at a time when the concentration of cyclic AMP has reached its peak increase. The possibility that some aspect of muscle activity provides a mechanism for trophic regulation has already been discussed. The mechanism which produces membrane instability following denervation, however, is unknown. An oscillation of the resting membrane potential has been suggested as the source of the spontaneous activity (111), but some alteration in the properties of the active membrane could also provide a mechanism. Redfern and Thesleff (149) reported that the rate of rise of the muscle action potenital is decreased starting about 30 to 40 hours after denervation. Their data indicate that the change in the properties of the action potential is distinct from a change in the resting membrane potential. Apparently some property of the action-potential-generating mechanism is altered by denervation. Additional evidence supporting this point has been provided by Redfern and Thesleff (150) and Harris and Thesleff (83). Tetrodotoxin (TTX) is a drug which is considered to selectively block the membrane channels which conduct Na^+ during the generation of the action potential. At a dose (10^{-6} M) which completely abolishes the generation of action potentials in normal muscle, TTX does not abolish all action potentials in the denervated muscle (150). TTX-resistant action potentials were first noticed on the 2nd day following denervation. Moreover, the onset and time course of development of

the capability for the generation of TTX-resistant action potentials is remarkably similar to the onset and development of the extrajunctional sensitivity to acetylcholine (83). However, since it was possible to selectively block either the generation of TTX-resistant action potentials, or the cholinergic receptor, it is unlikely that TTX-resistant action potentials are generated by new acetylcholine receptors.

In summary, the postdenervation changes in muscle properties which lead to a decrease in resting membrane potential, a decrease in the rate of rise of the action potential, the development of TTX-resistant action potentials, and an increase in the sensitivity of the extrajunctional membrane to acetylcholine follow a very similar time course. The temporal pattern of the postdenervation increase in the concentration of cyclic AMP also correlates quite closely with this time course. Casual relationships, however, if they exist, remain to be determined. Recent evidence though, now indicates that the decrease in resting membrane potential, the development of TTX-resistant action potentials, and the increase in extrajunctional acetylcholine sensitivity all depend on de novo protein synthesis (50, 64). This information clearly provides a possible framework which may indicate an active role for cyclic AMP in the mechanisms responsible for trophic regulation.

Cyclic AMP Suppression and Trophic Mechanisms

The time course of the postdenervation development of extrajunctional acetylcholine sensitivity has been described many times (2, 46, 126). Fambrough (50), however, has recently found that he could prevent the spread of sensitivity by incubating a denervated rat diaphragm with either of two inhibitors of protein synthesis, cycloheximide or actinomycin D. There was also an apparent critical period during which the inhibitors had to be present to prevent the spread of sensitivity. Once an increase in sensitivity developed, adding an inhibitor would not reverse it. Therefore, the turnover rate of the new receptors is quite slow. Fambrough speculated that the trophic regulation of extrajunctional sensitivity involved the regulation of gene activity in muscle fibers. Grampp and his colleagues (64) have confirmed Fambrough's results using inhibitors applied in vivo. They have also added the development of TTX-resistant action potentials, and the decrease in resting membrane potential to the list of phenomena which depend on de novo protein synthesis. Both studies recognize that the blocking agents are toxic drugs. Grampp in fact, considered cycloheximide too toxic to use, and added another inhibiting agent, chloramphenicol, instead. The results of the two reports, however, if accepted as presented, indicate that the absence of some trophic agent following

denervation is releasing a normally suppressed protein synthetic pathway. Moreover, the new protein being synthesized is probably related to membrane function. A critical period during which the inhibiting agents had to be present to be effective apparently existed for the first two days following denervation. Since actinomycin D presumably prevents the synthesis of new messenger RNA, some form of genetic induction may be assumed to be responsible for the denervation-produced alterations.

How does cyclic AMP potentially fit into this process? The temporal relationship of the change in cyclic AMP to the change in the other parameters has already been pointed out. It would be rather nice to be able to present evidence showing an involvement of cyclic AMP in the pathways responsible for protein synthesis in skeletal muscle. Unfortunately, no such evidence is available. However, there is a great deal of suggestive information concerning the role of cyclic AMP in nucleic acid and protein metabolism in other systems. For example, cyclic AMP is able to induce the synthesis of a number of enzymes (174). Cyclic AMP is able to stimulate the phosphorylation of histones, which may lead to the derepression of DNA (105). Information obtained from bacterial systems also indicates that cyclic AMP has the capability of acting at the level of the gene to induce the synthesis of a specific enzyme (139). In general then, cyclic AMP does possess the capability for inducing

protein synthesis, and cyclic AMP is available in an increasing concentration at the appropriate time after denervation. Further investigation, however, is required before a causal relationship may be established.

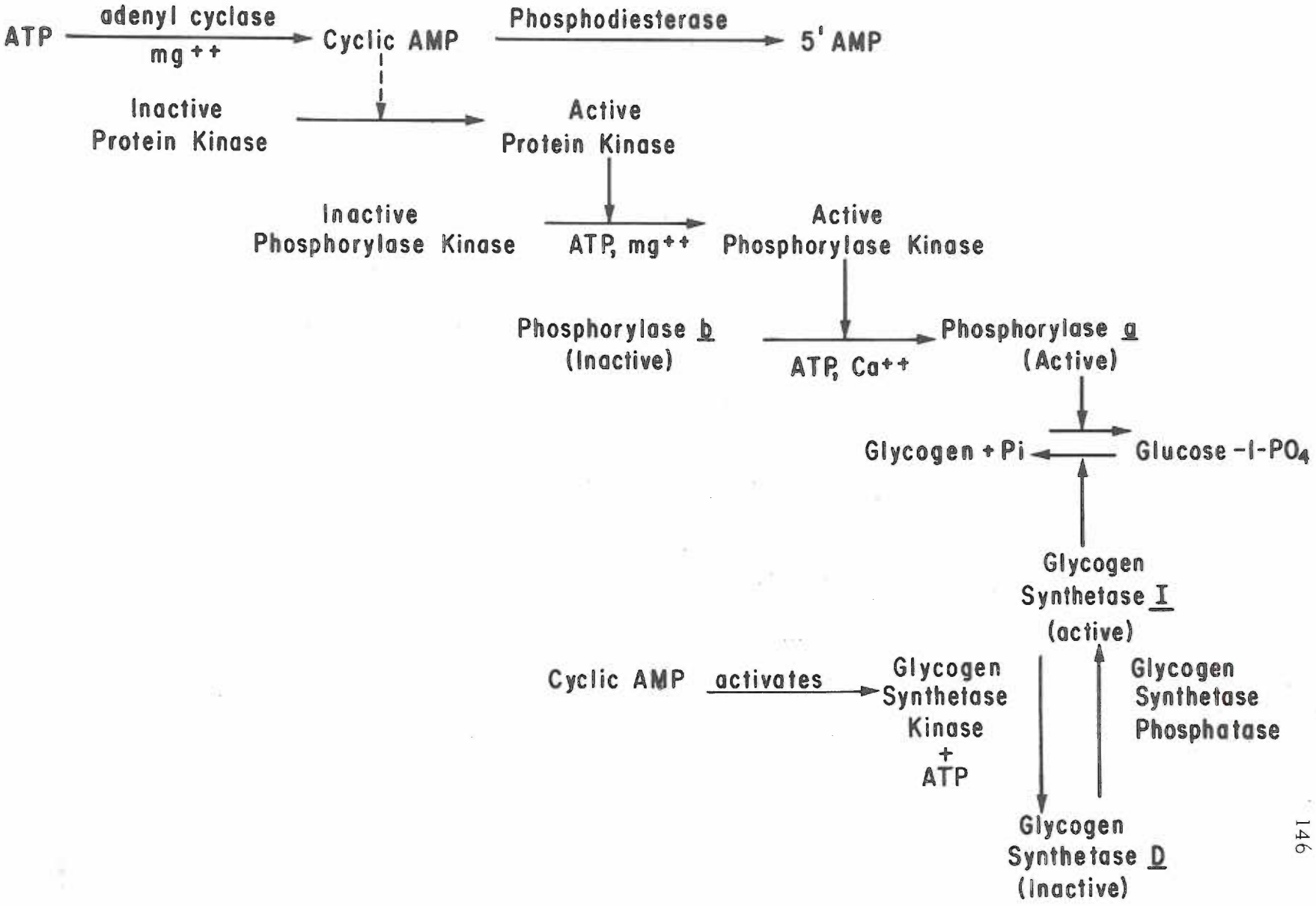
Correlations with Metabolic Changes

Probably the best understood cyclic AMP-mediated process is the epinephrine-induced breakdown of glycogen in skeletal muscle. Glycogen synthesis and degradation are central to the metabolic machinery of skeletal muscle. While the relationship of cyclic AMP to the development of phenomenon requiring the induction of protein synthesis must be speculative, the involvement of cyclic AMP in muscle glycogen metabolism is a clearly established relationship. Therefore, it will be of interest to discuss the changes which occur in the glycogen metabolic pathway following denervation, and to observe how these changes correlate temporally with the postdenervation increase in cyclic AMP.

A summary of the apparent reaction sequence which involves cyclic AMP in the synthesis and degradation of glycogen is presented in Figure 13 (153).

Briefly, muscle glycogen exists in a dynamic equilibrium with glucose-1-PO_4. The enzyme responsible for the breakdown of glycogen to glucose-1-PO_4, glycogen phosphorylase, and the enzyme

Figure 13. Proposed sequence of reactions for the synthesis and degradation of glycogen in skeletal muscle. Sequence includes proposed mechanisms involving cyclic AMP. (Robison et al., 1968).


ATP
adenyl cyclase
mg++
Cyclic AMP
Phosphodiesterase
5' AMP
Inactive
Protein Kinase
Active
Protein Kinase
Inactive
Phosphorylase Kinase
ATP, mg++
Active
Phosphorylase Kinase
Phosphorylase b
(Inactive)
ATP, Ca++
Phosphorylase a
(Active)
Glycogen + Pi
Glucose-1-PO4
Glycogen
Synthetase I
(active)
Cyclic AMP
activates
Glycogen
Synthetase
Kinase
+
ATP
Glycogen
Synthetase
Phosphatase
Glycogen
Synthetase D
(Inactive)

responsible for the synthesis of glycogen from glucose-1-PO_4, glycogen synthetase, may both be regulated by cyclic AMP. Glycogen phosphorylase exists in two forms, designated phosphorylase a and phosphorylase b. Phosphorylase a is the most active form of the enzyme, under normal conditions. It is produced from phosphorylase b the inactive form, by a phosphorylation reaction which is catalyzed by the enzyme phosphorylase kinase. Phosphorylase kinase, in turn, also exists in an active and inactive form. Activation of the kinase again requires a phosphorylation reaction, which is catalyzed by a third enzyme, phosphorylase kinase kinase, or protein kinase. It is the activity of protein kinase which is dependent on cyclic AMP. An increase in the concentration of cyclic AMP leads to the activation of protein kinase, and subsequently to the reactions which ultimately lead to the breakdown of muscle glycogen.

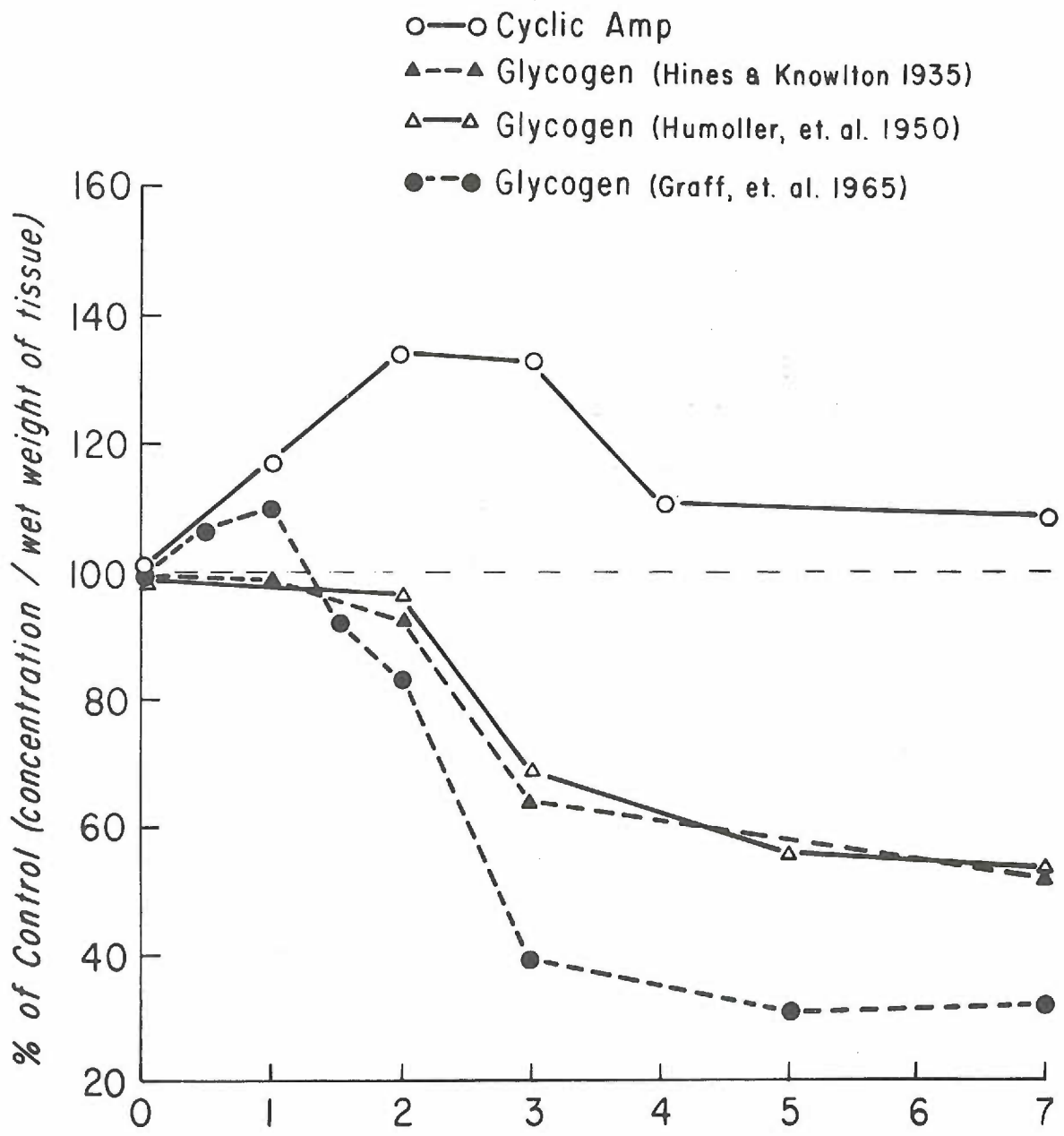
The synthesis of glycogen from glucose-1-PO_4 is catalyzed by glycogen synthetase. This enzyme also exists in an active and inactive form, glycogen synthetase I and D, respectively. In this case the activation reaction is a dephosphorylation, catalyzed by glycogen synthetase phosphatase. The inactivation reaction is a phosphorylation, catalyzed by glycogen synthetase kinase; it is this enzyme which is stimulated by cyclic AMP. The net result of an increase in cyclic AMP is a decrease in the activity of glycogen synthetase, and a decrease in glycogen synthesis.

Cyclic AMP, therefore, can regulate the glycogen concentration in skeletal muscle via two mechanisms; increasing glycogenolysis by stimulating glycogen phosphorylase activity and inhibiting glycogen synthesis by inactivating glycogen synthetase.

After denervation the glycogen concentration in skeletal muscle decreases. There is a period of approximately two days in which little change in glycogen concentration occurs. Between the 2nd and 3rd day, however, glycogen concentration declines abruptly. A temporal comparison between the postdenervation changes in cyclic AMP, and the postdenervation changes in muscle glycogen is presented in Figure 14. The three glycogen concentration curves represent data presented by three separate studies (62, 89, 95). It is apparent from the figure that the temporal comparison between cyclic AMP and glycogen concentration change is quite striking. This does not, however, demonstrate a causal relationship. There is additional evidence though, which strongly suggests that such a relationship may exist.

Bass (9) has demonstrated a reduction in the incorporation of glucose-C^{14} into muscle glycogen following denervation. Since the rate-limiting enzyme for glycogen synthesis is glycogen synthetase, which is in turn regulated by cyclic AMP, one would be interested in knowing the activity of the enzyme following muscle denervation. Canal and Frattola (27) measured synthetase activity in the rat

Figure 14. Comparison of the change in cyclic AMP concentration following denervation of the rat gastrocnemius with reported postdenervation changes in glycogen concentration. Concentration of cyclic AMP and glycogen given as the percent of control concentration. Days postdenervation given on the abscissa.


Cyclic Amp
Glycogen (Hines & Knowlton 1935)
Glycogen (Humoller, et. al. 1950)
Glycogen (Graff, et. al. 1965)
% of Control (concentration / wet weight of tissue)
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7

gastrocnemius at various times after denervation. Briefly, they found that the enzyme activity decreased precipitously almost immediately after nerve section. Activity had declined to 77 percent of control within two hours. The decrease became less abrupt with time, reaching a level of 50 percent of control at 3 days, and then essentially remained stable. Correlating the early abrupt decrease in synthetase activity with the postdenervation change in cyclic AMP conventration is tenuous. However, the more gradual decrease which occurs between two hours and three days correlates quite well with the cyclic AMP measurements.

A depression of glycogen synthesis is not the only reason glycogen concentration falls following denervation. An increase in glycogenolysis also follows nerve section Bass (9) has described the time course of the changes occurring in both synthesis and degradation. He finds that glycogen synthesis falls abruptly after denervation, reaching a level of about 37 percent of normal at 24 hours, which agrees with Canal and Frattola (27). Bass, however, also finds that glycogenolysis decreases immediately after denervation, the decrease essentially paralleling the decrease in synthesis. Hence, there should be little change in intracellular glycogen for the first 24 hours following denervation, as is found to be the case. Between 24 and 36 hours the rate of glycogen breakdown begins to increase, while the rate of synthesis remains depressed. By 72

hours postdenervation, the rate of glycogenolysis is approximately 20 percent greater than the rate of synthesis, and the concentration of glycogen falls quite rapidly. Again the temporal correlation between the increase in cyclic AMP concentration and the increase in glycogenolysis is striking. However, if the change in cyclic AMP could be correlated with an increase in glycogen phosphorylase activity, the possibility of a causal relationship between cyclic AMP and the decrease in glycogen concentration following denervation would be strengthened.

Measurements of glycogen phosphorylase activity in denervated muscle present a rather uncertain picture. While most studies (94, 95, 121, 129, 183) agree that total phosphorylase activity (phosphorylase a + phosphorylase b) declines from approximately day 6 onward, there remains some uncertainty as to what the individual components of the total activity are contributing. However, while the older studies must be suspect because of improper fixation of the enzyme, it appears that phosphorylase a activity increases up to day 5 following denervation. Most measurements have concentrated on time periods starting at least 7 days after denervation, and a careful analysis of enzyme activity immediately after denervation is lacking. Future experiments involving the measurement of cyclic AMP concentration, phosphorylase a activity and phosphorylase kinase activity should provide greater

insight into the relationship between the postdenervation changes in cyclic AMP and glycogen concentration in skeletal muscle.

SUMMARY AND CONCLUSIONS

Experiments were designed to first determine whether the adenylate cyclase-cyclic AMP system in skeletal muscle is neurotrophically controlled. The term "neurotrophic control", as used in this study, is meant to imply that some regulatory mechanism, supplied by the nerve, controls the activity of the cyclic AMP system in skeletal muscle. The hypothesis was tested by comparing the change in cyclic AMP concentration produced by denervation with the change produced by muscle disuse. Additionally, the change in the temporal pattern of the increase in cyclic AMP concentration following denervation was compared after sectioning the nerve at different distances from the muscle. In a final series of experiments, I measured the change in cyclic AMP concentration in a denervated muscle during the course of reinnervation.

Briefly, denervation produces a transient increase in muscle cyclic AMP concentration. The change produced by denervation is distinctly different from the change produced by muscle disuse. The increase in cyclic AMP concentration produced by denervation is also delayed by sectioning the nerve at a distance farther from the muscle. Reinnervation produces a decrease in the concentration of cyclic AMP, reducing it to control level within 3 days after the re-establishment of neuromuscular transmission. Taken together, the

evidence is consistent with the hypothesis that motoneurons are capable of suppressing the activity of the adenylate cyclase-cyclic AMP system in skeletal muscle.

The data obtained in these experiments do not allow a causal relationship to be established between the trophic control of cyclic AMP and the trophic control of other muscle characteristics. However, several temporal correlations may be made which allow further speculation on the possible role of the cyclic AMP system in the mechanisms responsible for trophic regulation. There is, for instance, a close temporal correlation between the increase in cyclic AMP concentration, and the onset of several postdenervation changes which apparently depend on de novo protein synthesis, e. g. the decrease in resting membrane potential, the increase in extrajunctional sensivity to acetylcholine, and the appearance of tetrodotoxin-resistant action potentials. There is also a close temporal correlation between the increase in cyclic AMP concentration and the postdenervation decrease in muscle glycogen.

It is apparent from these experiments that a nerve-mediated influence is capable of suppressing the activity of the cyclic AMP system in muscle. How this suppressive influence is manifested, i. e. via the inhibition of adenylate cyclase or the stimulation of phosphodiesterase, and what it means in terms of normal muscle development and function remain to be determined.

BIBLIOGRAPHY

1. Albuquerque, E. X., Schuh, F. T., and Kauffman, F. C. Early membrane depolarization of the fast mammalian muscle after denervation. Pflug. Arch. 328:36-50 (1971)

2. Albuquerque, E. X. and McIsaac, R. S. Fast and slow slow mammalian muscles after denervation. Exp. Neurol. 26:183-202 (1970)

3. Albuquerque, E. X. and Thesleff, S. A comparative study of membrane properties of innervated and chronically denervated fast and slow skeletal muscles of the rat. Acta Physiol. Scand. 73:471-480 (1968)

4. Allfrey, V. G. Some observations on histone acetylation and its temporal relationship to gene activation. In: Regulatory Mechanisms for Protein Synthesis in Mammalian Cells. A. San Pietro, M. P. Lamborg and F. T. Kenney (Eds.) New York: Academic Press. 1968 (pages 65-100)

5. Appenzeller, O. The Autonomic Nervous System. New York. American Elsevier 1970 (pages 72-79)

6. Axelsson, J. and Thesleff, S. A study of supersensitivity in denervated mammalian skeletal muscle. J. Physiol. 147: 178-193 (1959)

7. Barany, M. and Close, R. I. The transformation of myosin in cross-innervated rat muscles. J. Physiol. 213:455-474 (1971)

8. Barondes, S. H. and Samson, F. E. Axoplasmic transport. Neurosci Res. Prog. Bull. 5:191-299 (1967)

9. Bass, A. Energy metabolism in denervated muscle. In: The Denervated Muscle. E. Gutmann (Ed.). Prague: Czech Acad. Sci. 1962 (pages 203-273)

10. Beatty, C. H., Bassinger, G. M., Dully, C. C. and Bocek, R. M. Comparison of red and white voluntary skeletal muscles of several species of primates. J. Histochem. Cytochem. 14: 590-600 (1966)

11. Beatty, C. H., Peterson, R. D., Bocek, R. M., Craig, N. C. and Weleber, R. Effect of glucagon on incorporation of glycine-C^{14} into protein of voluntary skeletal muscle. Endo. 73:721-726 (1963)

12. Belmar, J. and Eyzaguirre, C. Pacemaker site of fibrillation potentials in denervated mammalian muscle. J. Neurophysiol. 29:425-441 (1966)

13. Bernstein, J. J. and Guth, L. Nonselectivity in establishment of neuromuscular connections following nerve regeneration in the rat. Exp. Neurol. 4:262-275 (1961)

14. Berry, C. M., Grundfest, H. and Hinsey, J. The electrical activity of regenerating nerves in a cat. J. Neurophysiol. 7: 103-116 (1944)

15. Birks, R., Katz, B. and Miledi, R. Physiological and structural changes at the amphibian myoneural junction, in the course of nerve degeneration. J. Physiol. 150:145-168 (1960)

16. Birnbaumer, L. and Rodbell, M. Adenyl cyclase in fat cells. II. Hormone Receptors. J. Biol. Chem.24 :3477-3482 (1969)

17. Bloom, F. E., Iversen, L. L. and Schmitt, F. O. Macromolecules in synaptic function. Neurosci Res. Prog. Bull. 8:325-455 (1970)

18. Bradley, J. V. Distribution-Free Statistical Tests. Englewood Cliffs, N. J.: Prentice-Hall, 1968 (pages 55-65)

19. Breckenridge, B. M., Buen, J. H. and Matschinsky, F. M. Theophylline, epinephrine and neostimine facilitation of neuromuscular transmission. Proc. Natl. Acad. Sci. 57:1893-1897 (1967)

20. Brody, I. A. Relaxing factor in denervated muscle: a possible explanation for fibrillations. Amer. J. Physiol. 211:1277-1280 (1966)

21. Brown, G. L. The action of acetylcholine on denervated mammalian and frog muscle J. Physiol. 89:438-461 (1937)

22. Buller, A. J., Mommaerts, W. F. H. M. and Seraydarian, K. Biochemical properties relevant to contractile function in slow and fast twitch muscles of the cat, as modified by nerve crossing. J. Physiol. 205:581-597 (1970)

23. Buller, A. J. and Lewis, D. M. Further observations on mammalian cross-innervated skeletal muscle. J. Physiol. 178:343-358 (1965)

24. Buller, A. J., Eccles, J. C. and Eccles, R. M. Differentiation of fast and slow muscles in the cat hind limb. J. Physiol. 150:399-416 (1960)

25. Buller, A. J., Eccles, J. C. and Eccles, R. M. Interactions between motoneurones and muscles in respect of the characteristic speeds of their responses. J. Physiol. 150:417-439 (1960)

26. Butcher, R. W. and Sutherland, E. W. Adenosine 3', 5'-phosphate in biological materials. I. Purification and properties of cyclic 3', 5' -nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3', 5'-phosphate in human urine. J. Biol. Chem. 237:124-1250 (1962)

27. Canal, N. and Frattola, L. Changes in muscular glycogen synthetase activity following denervation. Nature 211:416-417 (1966)

28. Cannon, W. C. and Rosenblueth, A. The Supersensitivity of Denervated Structures: A Law of Denervation. New York: Macmillan Co. 1949.

29. Chase, L. R. and Aurbach, G. D. Renal adenyl cyclase: anatomically separate sites for parathyroid hormone and vasopressin Science 159:545-547 (1968)

30. Close, R. I. Dynamic properties of mammalian skeletal muscles. Physiol. Rev. 52:129-197 (1972)

31. Close, R. Dynamic properties of fast and slow skeletal muscles of the rat after nerve cross-union. J. Physiol. 204: 331-346 (1969)

32. Close, R. Dynamic properties of fast and slow skeletal muscles of the rat during development. J. Physiol. 173:74-95 (1964)

33. Colowick, S. P. and Kaplan, N. O. Methods in Enzymology. Vol. III. New York: Academic Press 1955 (pages 448-450)

34. Cooper, R. R. Alterations during immobilization and regeneration of skeletal muscle in cats. J. Bone and Joint Surg. 54A:919-953 (1972)

35. Couteaux, R. The differentiation of synaptic areas. Proc. Royal Soc. (Ser. B) 158:451-480 (1963)

36. Denny-Brown, D. E. and Pennybacker, J. B. Fibrillation and fasciculation in voluntary muscle. Brain 61:311-334 (1938)

37. De Robertis, E., Rodriguez De Lores Arnaiz, G. and Alberici, M. Subcellular distribution of adenyl cyclase and cyclic phosphodiesterase in rat brain cortex. J. Biol. Chem. 242:3487-3493 (1967)

38. Desmedt, J. E. The physio-pathology of neuromuscular transmission and the trophic influence of motor innervation. Amer. J. Phys. Med. 38:248-259 (1959)

39. Diamond, S. and Miledi, R. A study of foetal and newborn rat muscle fibers. J. Physiol. 172:393-408 (1962)

40. Dockry, M., Kernan, R. P. and Tangney, A. Active transport of sodium and potassium in mammalian skeletal muscle and its modification by nerve and by cholinergie and adrenergic agents. J. Physiol. 186:187-200 (1966)

41. Drachman, D. B. The role of acetylcholine as a trophic neuromuscular transmitter. In: CIBA Foundation Symposium on Growth of the Nervous System. Wolstenholme, G. E. W. and O'Connor, M. (Eds.) London: Churchill. 1968 (pp. 251-273)

42. Drachman, D. B. Is acetylcholine the trophic neuromuscular transmitter? Arch. Neurol. 17:206-218 (1967)

43. Drachman, D. B. and Witzke, F. Trophic regulation of acetylcholine sensitivity of muscle: effect of electrical stimulation. Science 176:514-516 (1972)

44. Drummond, G. I., Harwood, J. P. and Powell, C. A. Studies on the activation of phosphorylase in skeletal muscle by contraction and by epinephrine. J. Biol. Chem. 244:4235-4240 (1969)

45. Eccles, J. C. Specificity of neural influence on speed of muscle contraction. In: The Effect of Use and Disuse on Neuromuscular Functions. E. Gutmann and D. Hnik (Eds.) Amsterdam: Elsevier. 1963 (pp. 19-28)

46. Emmelin, N. and Malm, L. Development of supersensitivity as dependent on the length of degenerating nerve fibers. Quart. J. Exp. Physiol. 50:142-145 (1965)

47. Engel, W. K. and Karpati, G. Impaired skeletal muscle maturation following neonatal neurectomy. Devel. Biol. 17: 713-723 (1968)

48. Eron, L., Arditti, R. R., Zubai, G., Connaway, S. and Beckwith, J. R. An adenosine 3':5' cyclic monophosphate-binding protein that acts on the transcription process. Proc. Nat. Acad. Sci. 68:215-219 (1971)

49. Eyzaguirre, C., Espildora, J. and Luco, J. V. Alterations in neuromuscular synapsis during Wallerian degeneration. Acta Physiol. Lat. Amer. 2:213-227 (1952)

50. Fambrough, D. M. Acetylcholine sensitivity of muscle fiber membranes: mechanisms of regulation by motoneurons. Science 168:372 (1970)

51. Fatt, P. and Katz, B. Spontaneous subthreshold activity at motor nerve endings. J. Physiol. 117:109-128 (1952)

52. Feinstein, B., Pattle, R. E. and Weddell, G. Metabolic factors affecting fibrillation in denervated muscle. J. Neurol. Neurosurg. Psychiat 8:1-11 (1945)

53. Filogamo, G. and Gabella, G. Cholinesterase behavior in the denervated and reinnervated muscles. Acta Anat. 63:199-214 (1966)

54. Fischbach, G. D. and Robbins, N. Effect of chronic disuse of rat soleus neuromuscular junctions on postsynaptic membrane. J. Neurophysiol.34:562-569 (1971)

55. Fischbach, G. D. and N. Robbins. Changes in contractile properties of disused solues muscles. J. Physiol. 201:305-320 (1969)

56. Friedmann, N., Somlyo, A.V.and Somlyo, A.P. Cyclic adenosine and guanosine monophosphates and glucagon: effect on liver membrane potentials. Science 171:400-402 (1971)

57. Garren, L. D., Davis, W. W. and Crocco, R. M. Puromycin analogs: action of adrenocorticotropic hormone and the role of glycogen. Science 152:1386-1388 (1966)

58. Gilman, A. G. A protein finding assay for adenosine 3'-5'-cyclic monophosphate. Proc. Nat. Acad. Sci. 67:305-312 (1970)

59. Goldberg, A. L. Protein turnover in skeletal muscle. Effects of denervation and cortisone on protein catabolism in skeletal muscle. J. Biol. Chem. 244:3223-3229 (1969)

60. Goldberg, A. L. and Singer, J. J. Evidence for a role of cyclic AMP in neuromuscular transmission. Proc. Nat. Acad. Sci. (USA) 64:134-141 (1969)

61. Goldberg, N. D. and O'Toole, A. G. Analysis of cyclic 3', 5'-adenosine monophosphate and cyclic 3', 5'-guanosine monophosphate. In: Methods of Biochemical Analysis. D. Glick (Ed.) New York: Wiley 1971 (pages 1-41)

62. Graff, G. L. A., Hudson, A. J. and Strickland, K. P. Changes in the relative amounts of ascorbic acid and glycogen in denervated rat gastrocnemius muscle. Cand. Biochem. 43:705-710 (1965)

63. Grafstein, B. Transneuronal transfer of radioactivity in the central nervous system. Science 172:177-179 (1971)

64. Grampp, W., Harris, J. B. and Thesleff, S. Inhibition of denervation changes in skeletal muscle by blockers of protein synthesis. J. Physiol. 221:743-754 (1972)

65. Greengard, P. and Kuo, J. F. On the mechanism of action of cyclic AMP. In: Advances in Biochemical Psychopharmacology Vol. 3 P. Greengard and E. Costa (Eds.). New York: Raven Press 1970 (pages 287-306)

66. Guth, L. "Trophic" effects of vertebrate neurons. Neurosci. Res. Prog. Bull. 7:1-73 (1969)

67. Guth, L. "Trophic" influences of nerve on muscle. Physiol. Rev. 48:645-687 (1968)

68. Guth, L. The effects of glossopharyngeal nerve transection on the circumvallate papilla of the rat. Anat. Rec. 128:715-731 (1957)

69. Guth, L. and Samaha, F. J. Qualitative differences between actomyosin ATPase of slow and fast mammalian muscle. Exp. Neurol. 25:138-163 (1969)

70. Guth, L. and Brown, W. C. The sequence of changes in cholinesterase activity during reinnervation of muscle. Exp. Neurol. 12:329-336 (1965)

71. Guth, L., Albers, R. W. and Brown, W. C. Quantitative changes in cholinesterase activity of denervated muscle fibers and sole plates. Exp. Neurol. 10:236-250 1964.

72. Guth, L. and Zalewski, A. A. Disposition of cholinesterase following implantation of nerve into innervated and denervated muscle. Exp. Neurol. 7:316-326 (1963)

73. Gutmann, E. Development and maintenance of neurotrophic relations between nerve and muscle. In: CIBA Foundation Symposium on Growth of the Nervous System. Wolstenholme, G. E. W. and O'Connor, M. Eds. London. Churchill 1968 (pp. 233-243)

74. Gutmann, E. Neurotrophic relations in the regeneration process. In: Progress in Brain Research Vol. 13 Mechanisms of Neural Regeneration. M. Singer and J. P. Schade (Eds.) Amsterdam: Elsevier. 1964 (pages 72-114)

75. Gutmann, E. Evidence for the trophic function of the nerve cell in neuromuscular relations. In: The Effect of Use and Disuse on Neuromuscular Functions. E. Gutmann and P. Hnik (Eds.) Amsterdam: Elsevier. 1963 (pages 29-34)

76. Gutmann, E. Metabolic reactability of the denervated muscle. In: The Denervated Muscle. E. Gutmann (Ed.) Prague: Czech Acad. Sci. 1962 (pages 337-432)

77. Gutmann, E. Denervation and disuse atrophy in cross-striated muscle. Rev. Canad. Biol. 21:353-365 (1962)

78. Gutmann, E. Metabolic reactions of denervated and reinnervated muscle. Am. J. Phys. Med. 38:104-117 (1959)

79. Gutmann, E. and Hnik, P. Denervation studies in research of neurotrophic relationships. In: The Denervated Muscle. E. Gutmann (Ed.) Prague: Czech. Acad. Sci. 1962 (pp. 13-56)

80. Hamburger, V. Trends in Neuroembryology. In: Biochemistry of the Developing Nervous System. New York: Academic Press. 1954 (pp. 52-73)

81. Hamburger, V. The development and innervation of transplanted limb primordia of chick embryos. J. Exp. Zool. 80: 347-389 (1939)

82. Harris, E. J. and Nicholls, J. G. The effect of denervation on the rate of entry of potassium into frog muscle. J. Physiol. 131:473-476 (1956)

83. Harris, J. B. and Thesleff, S. Studies on tetrodotoxin resistant action potentials in denervated skeletal muscle. Acta Physiol. Scand. 83:382-388 (1971)

84. Harrison, R. G. An experimental study of the relation of the nervous system to the developing musculature in the embryo of the frog. Amer. J. Anat. 3:197-220 (1904)

85. Henneman, E. and Olson, C. B. Relations between structure and function in the design of skeletal muscles. J. Neurophysiol. 28:581-598 (1965)

86. Henneman, E., Somjen, G., Carpenter, D. O. Functional significance of cell size in spinal motoneurones. J. Neurophysiol. 28:560-580 (1965)

87. Hilf, R. The mechanism of action of ACTH. New Eng. J. Med. 273:798-811 (1965)

88. Hines, H. M., Thomson, J. D. and Lazere, B. Quantitative studies on muscle and nerve regeneration in the rat. Am. J. Physiol. 137:527-532 (1942)

89. Hines, H. M. and Knowlton, G. C. The role of the nervous system in the regulation of the glycogen metabolism of skeletal muscle. Am. J. Physiol. 111:243-249 (1935)

90. Hines, H. M. and Knowlton, G. C. Changes in the skeletal muscle of the rat following denervation. Am. J. Physiol. 104:379-391 (1933)

91. Hoh, J. F. Y., and Salafsky, B. Effects of nerve cross-union on rat intracellular potassium in fast-twitch and slow-twitch rat muscles. J. Physiol. 216:171-180 (1971)

92. Hubbard, S. J. The electrical constants and the component conductances of frog skeletal muscle after denervation. J. Physiol. 165:443-456 (1963)

93. Hughes, A. F. W. Aspects of neural ontogeny. New York: Academic Press 1968 (pages 143-159)

94. Huls, H. N. and Leonard, S. L. Phosphorylase activity in denervated skeletal muscle. Proc. Soc. Exp. Biol. Med. 108:224-228 (1961)

95. Humoller, F. L., Hatch, D. and McIntyre, A. R. Effect of neurotomy on hexokinase and phosphorylase activities of rat muscle. Am. J. Physiol. 167:656-664 (1951)

96. Humoller, F. L., Griswold, B. and McIntyre, A. R. Comparative chemical studies in skeletal muscle following neurotomy and tenotomy. Am. J. Physiol. 161:406-412 (1950)

97. Karlson, P. New concepts on the mode of action of hormones. Pers. Biol. Med. 6:203-214 1963

98. Kimura, H., Thomas, E. and Murad, F. Effects of decapitation, ether and pentobarbitol on cyclic AMP and cyclic GMP levels in rat tissues. Fed. Proc. 32:680 Abs (1973)

99. Korr, I. M., Wilkinson, P. N. and Chornock, F. W. Axonal delivery of neuroplasmic components to muscle cells. Science 155:343-345 (1967)

100. Krebs, E. G., DeLange, R. J., Kemp, P. G. and Riley, W. D. Activation of skeletal muscle phosphorylase Pharm. Rev. 18:163-171 (1966)

101. Kuffler, S. W. Specific excitability of the end plate region in normal and denervated muscle. J. Neurophysiol. 6:99-110 (1943)

102. Kuo, J. F. and Greengard, P. Cyclic nucleotide-dependent protein kinases. VIII An assay method for the measurement of adenosine 3', 5'-monophosphate in various tissues and a study of agents influencing its level in adipose cells. J. Biol. Chem. 245:4067-4073 (1970)

103. Kuo, J. F., Krueger, B. K., Sanes, J. R. and Greengard, P. Cyclic nucleotide-dependent protein kinases. V. Preparation and properties of adenosine 3', 5'-monophosphate-dependent protein kinase from various bovine tissues. Biochim. et Biophys. Acta 212:79-91. 1970.

104. Langan, T. A. Action of adenosine 3', 5'-monophosphate-dependent histone kinase in vivo. J. Biol. Chem. 244:5763-5765 (1969)

105. Langan, T. A. Histone phosphorylation: stimulation by adenosine 3', 5'-monophosphate. Science 162:579-580 (1968)

106. La Raia, P. J. and Sonnenblick, E. H. Autonomic control of cardiac cyclic AMP system. Circ. Res. 28:377-384. 1971.

107. Lasek, R. J. Protein transport in neurons. Int. Rev. Neurobiol. 13:289-324 (1970)

108. Lebowitz, P. and Singer, M. Neurotrophic control of protein synthesis in the regenerating limb of the newt, Triturus. Nature 225:824-827 (1970)

109. Lentz, T. L. Nerve trophic function: In vitro assay of effects of nerve tissue on muscle cholinesterase activity. Science 171:187-189 (1971)

110. Li, C. H. Mechanism of fibrillation potentials in denervated mammalian skeletal muscle, Science 132:1889-1890 (1960)

111. Li, C. L., Shy, G. M., Weus, J. Some properties of mammalian skeletal muscle fibers with particular reference to fibrillation potentials. J. Physiol. 135:522-535 (1957)

112. Lilienthal, J. L., Jr., Zierler, K. L., Folk, B. P., Buka, R. and Riley, M. J. A reference base and system for analysis of muscle constituents. J. Biol. Chem. 182:501-508 (1950)

113. Lissitsky, S., Mantz, S., Attali, J. C. and Cartouzou, G. Action of 3', 5' -cyclic adenosine monophosphate in the protein synthesizing capacity of thyroid polyribosomes in vitro. Biochem. Biophys. Res. Comm. 35:437-443. 1969.

114. Locke, S., and Solomon, H.C. Relation of resting potenital of rat gastrocnemius and soleus muscles to innervation, activity, and the Na-K pump J. Exp. Zool. 166:377-386 (1967)

115. Lomo, T. and Rosenthal, J. Control of Ach-sensitivity by muscle activity in the rat. J. Physiol. 221:493-513 (1972)

116. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275 (1951)

117. Luco, J. V. and Eyzaguirre, C. Fibrillation and hypersensitivity to Ach in denervated muscle: effect of length of degenerating nerve fibers. J. Neurophysiol. 18:65-73 (1955)

118. Malamud, D. Adenyl cyclase: Relationship to stimulated DNA synthesis in parotid glands. Biochem. Biophys. Res. Comm. 35:754-758 (1969)

119. Mark, R. F. Matching muscles and motoneurones. A review of some experiments on motor nerve regeneration. Brain Res. 14:245-254 (1969)

120. Marsh, J. M., Butcher, R. W., Sanard, K. and Sutherland, E. W. Stimulatory effect of luteinizing hormone on adenosine 3', 5'-monophosphate accumulation in corpus luteum slices. J. Biol. Chem. 241:5436-5440 (1966)

121. Matsui, T. and Kuriaki, K. Effect of denervation and cocainization on activity of creatine synthesizing enzyme and phosphorylase in skeletal muscle. Am. J. Physiol. 196:461-464 (1959)

122. McAfee, D. A., Schorderet, M., and Greengard, P. Adenosine 3', 5'-monophosphate in nervous tissue: increase associated with synaptic transmission. Science 171:1156-1158 (1971)

123. McPhedran, A. M., Wuerker, R. B. and Henneman, E. Properties of motor units in a homogeneous red muscle (soleus) of the cat. J. Neurophysiol. 28:71-84 (1965)

124. Miledi, R. An influence of nerve not mediated by impulses. In: The Effect of Use and Disuse on Neuromuscular Functions. E. Gutmann and P. Hnik (Eds.) Amsterdam: Elsevier 1963 (pp. 35-40)

125. Miledi, R. Formation of extra nerve-muscle junction in innervated muscle. Nature 199:1191-1192 (1963)

126. Miledi, R. The Ach sensitivity of frog muscle fibers after complete or partial denervation. J. Physiol. 151:1-23 (1960)

127. Miledi, R. Properties of degenerating neuromuscular synapses in the frog. J. Physiol. 154:190-205 (1960)

128. Miledi, R. and Slater, C. R. On the degeneration of rat neuromuscular junctions after nerve section. J. Physiol. 207:507-529 (1970)

129. Mirski, A. and Wirtheimer, E. Muscle action and glycogen phosphorylysis. Biochem. J. 36:221-231. (1942.)

130. Miyamoto, E., Kuo, J. F. and Greengard, P. Cyclic nucleotide-dependent protein kinases. III. Purification and properties of adenosine 3', 5'-monophosphate-dependent protein kinase from bovine brain. J. Biol. Chem. 244:6395-6402 (1969)

131. Mommaerts, W. F. H. M., Buller, A. J., and Seraydarian, K. The modification of some biochemical properties of muscle by cross innervation. Proc. Natl. Acad. Sci. 64:128-133 (1969)

132. Muscatello, U., Margreth, A. and Aloisi, M. On the differential response of sarcoplasm and myoplasm to denervation in frog muscle. J. Cell. Biol. 27:1-24. 1965.

133. Namm, D. H., Mayer, S. E. and Maltbie, M. The role of K^+ and Ca^{++} ions in the effect of epinephrine on cardiac cyclic adenosine 3'-5'-monophosphate, phosphorylase kinase and phosphorylase. Mol. Pharmacol. 4:522-530 (1968)

134. Nayler, W. G. Calcium exchange in cardiac muscle: A basic mechanism of drug action. Am. Heart J. 73:379-391 (1967)

135. Nelson, P. G. Functional consequences of tenotomy in hind limb muscles of the cat. J. Physiol. 201:321-333 (1969)

136. Nicholls, J. G. The electrical properties of denervated skeletal muscle. J. Physiol. 131:1-12 (1956)

137. Okamoto, M. and Riker, W. F. Jr. Motor nerve terminals as the site of initial, functional changes after denervation. J. Gen. Physiol. 53:70-80. (1969)

138. Orloff, J. and Handler, J. Role of adenosine 3', 5'-phosphate in the action of antidiuretic hormone. Am. J. Med. 42:757-768 (1967)

139. Pastan, I. and Periman, R. L. Stimulation of tryptophanase synthesis in Escherichia coli by cyclic 3', 5'-adenosine monophosphate. J. Biol. Chem. 244:2226-2232 (1969)

140. Peterson, E. R. and Crain, S. M. Regeneration and innervation in cultures of adult mammalian skeletal muscle coupled with fetal rodent spinal cord. Exp. Neurol. 36:136-159 (1972)

141. Piatt, J. Transplantation of aneurogenic forelimbs in place of hindlimb in Amblystoma. J. Exp. Zool. 120:247-285 (1952)

142. Posner, J. B., Stern, R. and Krebs, E. G. Effects of electrical stimulation and epinephrine on muscle phosphorylase, phosphorylase b kinase and adenosine 3', 5'-phosphate. J. Biol. Chem. 240:982-985 (1965)

143. Rabinowitz, M., Desalles, L. and Meisler, J. Distribution of adenyl-cyclase activity in rabbit skeletal muscle fractions. Biochim. Biophys. Acta 97:29-36 (1965)

144. Rall, T. W. and Gilman, A. G. The role of cyclic AMP in the nervous system. Neurosci. Res. Prog. Bull. 8:221-323 (1970)

145. Rall, T. W. and Sutherland, E. W. Adenyl cyclase. II. The enzymatically catalyzed formation of adenosine 3', 5'-phosphate and inorganic pyrophosphate from adenosine triphosphate. J. Biol. Chem. 237:1228-1232 (1962)

146. Rasmussen, H. Cell communication, calcium ion, and cyclic adenosine monophsophate. Science 170:404-413 (1970)

147. Rasmussen, H. and Tenenhouse, A. Cyclic adenosine monophosphate, Ca^{++} and membranes. Proc. Nat. Acad. Sci. 59: 1364-1370 (1968)

148. Rasmussen, H. and Ogata, E. Parathyroid hormone and the reactions of mitochondria to cations. Biochem. 5:733-745 (1966)

149. Redfern, P. and Thesleff, S. Action potential generation in denervated rat skeletal muscle. I. Quantitative aspects. Acta Physiol. Scand. 81:557=564 (1971)

150. Redfern, P. and Thesleff, S. Action potential generation in denervated rat skeletal muscle. II. The action of tetrodotoxin. Acta Physiol. Scand. 82:70-78 (1971)

151. Robert, E. D. and Oester, Y. T. Absence of supersensitivity to Ach in innervated muscle subjected to a prolonged pharmacologic nerve block. J. Pharmacol. Exp. Therap 174:133-140 (1970)

152. Robison, G. A., Butcher, R. W., and Sutherland, E. W. Cyclic AMP. New York: Academic Press, 1971 (pages 85-125)

153. Robison, G. A., Butcher, R. W., and Sutherland, E. W. Cyclic AMP. Ann. Rev. Biochem. 37:149-174 (1968)

154. Rodbell, M. Metabolism of isolated fat cells. V. Preparation of "Ghosts" and their properties; adenyl cyclase and other enzymes. J. Biol. Chem. 242:5744-5750 (1967)

155. Romanul, F. C. A. Enzymes in muscles. I. Histochemical studies of enzymes in individual muscle fibers. Arch. Neurol. 11:355-368 (1964)

156. Romanul, F. C. A. and Van Der Meulen, J. P. Slow and fast muscle after cross innervation. Enzymatic and physiological changes. Archs. Neurol. Psych., Chicago 17:387-402 (1967)

157. Rosenblueth, A. and Luco, J. V. A study of denervated mammalian skeletal muscle. Am. J. Physiol. 120:781-797 (1937)

158. Salafsky, B., Bell, J. and Prewitt, M. A. Development of fibrillation potentials in denervated fast and slow skeletal muscle. Am. J. Physiol. 215:637-643 (1968)

159. Samaha, F. J., Guth, L., Albers, R. W. Phenotypic differences between the actomyosin ATPase of the three fiber types of mammalian skeletal muscle. Exp. Neurol. 26:120-125 (1970)

160. Schwartz, I. L. and Hechter, O. Insulin structure and function. Reflections on the present state of the problem. Am. J. Med. 40:765-772 (1966)

161. Severson, D. L., Drummond, G. I. and Sulakhe, P. V. Adenylate cyclase in skeletal muscle. Kinetic properties and hormonal stimulation. J. Biol. Chem. 247:2949-2958 (1972)

162. Sherrington, J. S. On the anatomical constitution of nerves of skeletal muscles; with remarks on recurrent fibers in the ventral spinal nerve-root. J. Physiol. 17:211-258 (1894)

163. Siegel, S. Nonparametric statistics for the behavioral sciences. New York: McGraw-Hill, 1956 (pages 40-55)

164. Siggins, G. R., Hoffer, B. J., and Bloom, F. E. Studies on norepinephrine-containing afferents to Purkinje cells of rat cerebellum. III. Evidence for mediation of norepinephrine effects by cyclic 3', 5'-adenosine monophosphate. Brain Res. 25:535-553 (1971)

165. Singer, M. The trophic quality of the neuron: Some theoretical considerations. In: Progress in Brain Research Vol. 13. Mechanisms of Neural Regeneration. M. Singer and J. P. Schade (Eds.) Amsterdam: Elsevier. 1964 (pages 228-232)

166. Singer, M. Nervous control of the regrowth of body parts in vertebrates. In: The Effect of Use and Disuse on Neuromuscular Functions Gutmann, E. and Hnik, P. (Eds.) Amsterdam: Elsevier 1963 (pp. 83-94)

167. Singer, M. The influence of the nerve in regeneration of the amphibian extremity. Quart. Rev. Biol. 27:169-200 (1952)

168. Skelton, C. L., Levey, G. S. and Epstein, S. E. Positive inotropic effects of dibutyryl cyclic adenosine 3', 5'-monophosphate. Circ. Res. 26:35-43 (1970)

169. Solandt, D. Y., Partridge, R. C. and Hunter, J. The effect of skeletal fixation on skeletal muscle. J. Neurophysiol. 6: 17-22 (1943)

170. Somlyo, A. V., Haeusler, G. and Somlyo, A. P. Cyclic adenosine monophosphate: Potassium dependent action on vascular smooth muscle membrane potential. Science 169: 490-491 (1970)

171. Stewart, D. M. Protein composition of denervated muscle. Am. J. Physiol. 202:281-284 (1962)

172. Streter, F. A. and Woo, G. Cell water, sodium and potassium in red and white mammalian muscles. Am. J. Physiol. 205: 1290-1294 (1963)

173. Sunderland, S. and L. J. Ray. Denervation changes in mammalian striated muscle. J. Neurol. Neurosurg. Psychiat. 13: 159-177 (1950)

174. Sutherland, E. W., Robison, G. A. and Butcher, R. W. Some aspects of the biological role of adenosine 3', 5'-monophosphate (cyclic AMP) Circ. 37:279-306 (1968)

175. Sutherland, E. W., Rall, T. W. and Menon, T. Adenyl cyclase. I. Distribution, preparation and properties. J. Biol. Chem. 237:1220-1227 (1962)

176. Sutherland, E. W. and Rall, T. W. Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. J. Biol. Chem. 232:1077-1091 (1958)

177. Tepperman, J. Metabolic and Endocrine Physiology. Chicago: Year Book. 1968. (pages 224-242)

178. Tepperman, J. and H. M. Tepperman. Some effects of hormones on cells and cell constituents. Pharm. Rev. 12:301-353 (1960)

179. Thesleff, S. Supersensitivity of skeletal muscle produced by botulinum toxin. J. Physiol. 151:598-607 (1960)

180. Thomson, J. D. Morgan, J. A. and Hines, H. M. Physiological characteristics of regenerating mammalian nerve and muscle. Am. J. Physiol. 161:142-150 (1950)

181. Thorton, C. S. Amphibian limb regeneration. Adv. Morphogen. 7:205-249. (1968)

182. Tower, S. S. The reaction of muscle to denervation. Physiol. Rev. 19:1-48 (1939)

183. Turner, L. V. and Manchester, K. L. Effects of denervation on the glycogen content and on the activities of enzymes of glucose and glycogen metabolism in rat diaphragm muscle. Biochem. J. 128:789-802 (1972)

184. Vrbova, G. The effect of motoneurone activity on the speed of contraction of striated muscle. J. Physiol. 169:513-526 (1963)

185. Vrbova, G. Changes in the motor reflexes produced by tenotomy. J. Physiol. 166:241-250 (1963)

186. Walton, G. M. and Garren, L. D. An assay for adenosine 3',5'-cyclic monophosphate based on the association of the nucleotide with a partially purified binding protein. Biochem. 9:4223-4229 (1970)

187. Weiss, B. Factors affecting adenyl cyclase activity and its sensitivity to biogenic amines. In: Biogenic Amines as Physiological Regulators. Blum. J. J. (Ed.) New Jersey: Prentice-Hall 1970. (pp. 35-73)

188. Weiss, B. and Costa, E. Regional and subcellular distribution of adenyl cyclase and 3', 5'-nucleotide phosphodiesterase in brain and pineal gland. Biochem. Pharmacol. 17:2107-2116 (1968)

189. Weiss, B. and E. Costa. Adenyl cyclase activity in rat pineal gland: Effects of chronic denervation and norepinephrine. Science 156:1750-1750-1752 (1967)

190. Weiss, P. and Hiscoe, H. B. Experiments on the mechanism of nerve growth. J. Exptl. Zool. 197:315-396 (1948)

191. Wigglesworth, V. B. Insect hormones. San Francisco: Freeman. 1970 (pages 104-133)

192. Wollenberger, A., Ristau, O. and Schoffa, G. Eine einfache Technik der extrem schrellen Abkuhlung groberer Gewebestucke Pflug. Arch. 270:399-412 (1960)

193. Woo, Y. T. and Manery, J. F. Cyclic AMP phosphodiesterase activity at the external surface of intact skeletal muscles and stimulation of the enzyme by insulin. Arch. Biochem. Biophys. 154:510-519 (1973)

194. Wuerker, R. B., McPhedran, A. M. and Henneman, E. Properties of motor units in a heterogeneous pale muscle (m. gastrocnemius) of the cat. J. Neurophysiol. 28:85-99 (1965)

195. Yaffe, D. Cellular aspects of muscle differentiation in vitro. In: Current Topics in Developmental Biology. A. A. Moscona (Ed.) New York: Academic Press. 1969. (pp. 37-78)

196. Zalewski, A. A. Combined effects of testosterone and motor, sensory, or gustatory nerve reinnervation on the regeneration of taste buds. Exp. Neurol. 24:285-297. (1969)

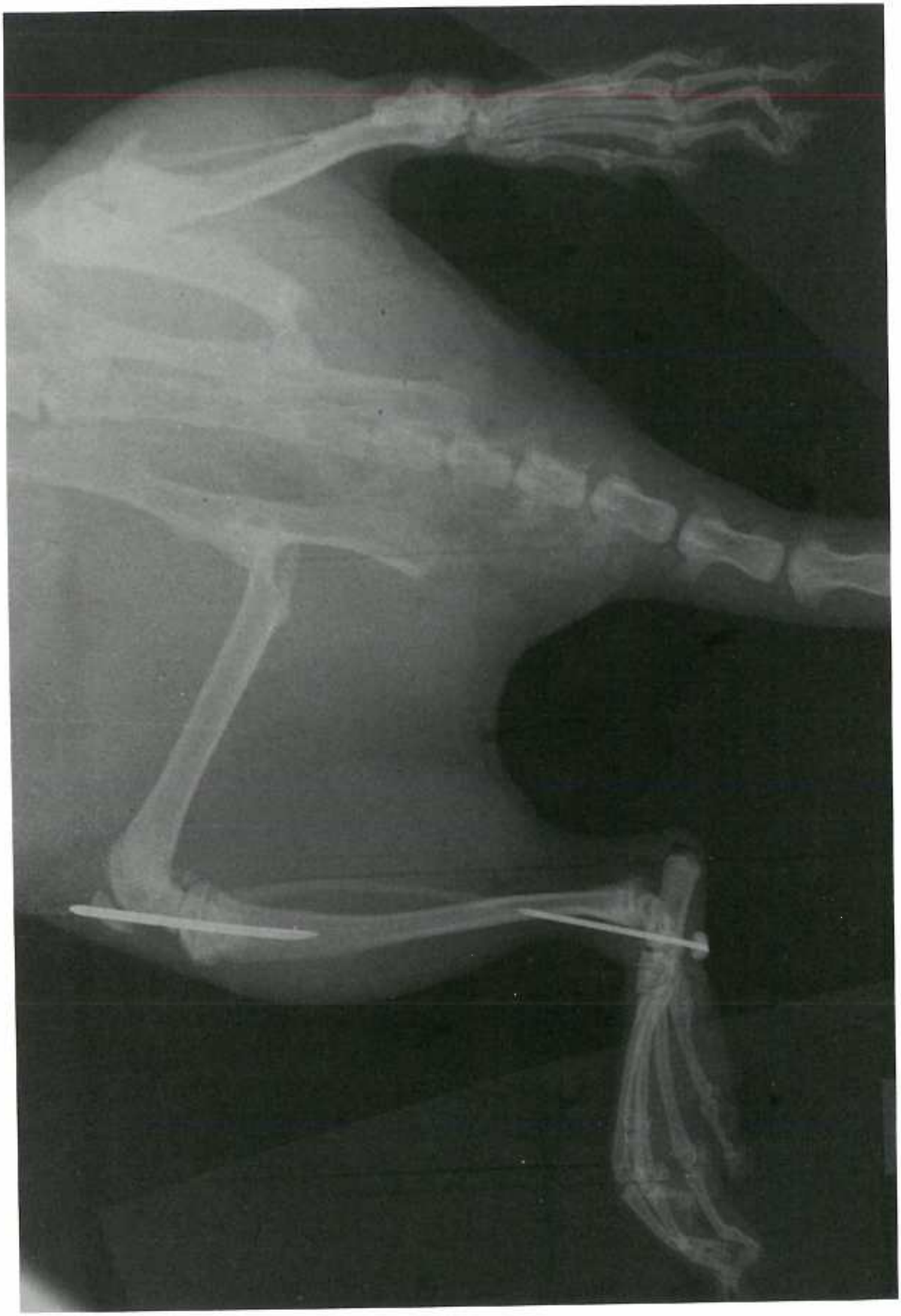
197. Zelena, J. Development, degeneration and regeneration of receptor organs. In: Progress in Brain Research Vol. 13. Mechanisms of Neural Regeneration. M. Singer and J. P. Schade (Eds.). Amsterdam: Elsevier 1964 (pages 175-213)

198. Zelena, J. The effect of denervation on muscle development. In: The Denervated Muscle. E. Gutmann (Ed.) Prague. Czechoslovak Academy of Sciences 1962 (pp 103-127)

APPENDICES

APPENDIX A

X-ray photograph of a fixed limb with the pins in place.



APPENDIX B

Control Assay and Statistical Comparison

Untreated, normal muscles were assayed for cyclic AMP and noncollagenous protein. Both gastrocnemius muscles from a normal animal were dissected free, frozen, removed, and the cyclic AMP extracted as described in the Methods section. The concentration of cyclic AMP was assayed, and the concentrations compared statistically using the Wilcoxon Signed-Rank Test. The concentration in the left gastrocnemius was compared with the concentration in the right gastrocnemius from the same animal. Muscles which had been frozen first were compared to those frozen last. No significant difference between the two groups was noted in either case. This indicates that no systematic error was introduced by the selection of either the right or left gastrocnemius as the experimental muscle, or by freezing either muscle first or last.

Appendix B: Control Assay

Right	Left	Sign	Difference	Rank	R^+	R^-
304	851	-	547	8		8
347	189	+	158	4	4	
288	428	-	140	3		3
209	323	-	114	2		2
283	230	+	53	1	1	
438	201	+	237	6	6	
633	198	+	435	7	7	
508	281	+	227	5	5	
					23	13
$\bar{X}$ 376	338			not significant ($p > .05$)		
SD 131	208					
SEM 50	79					

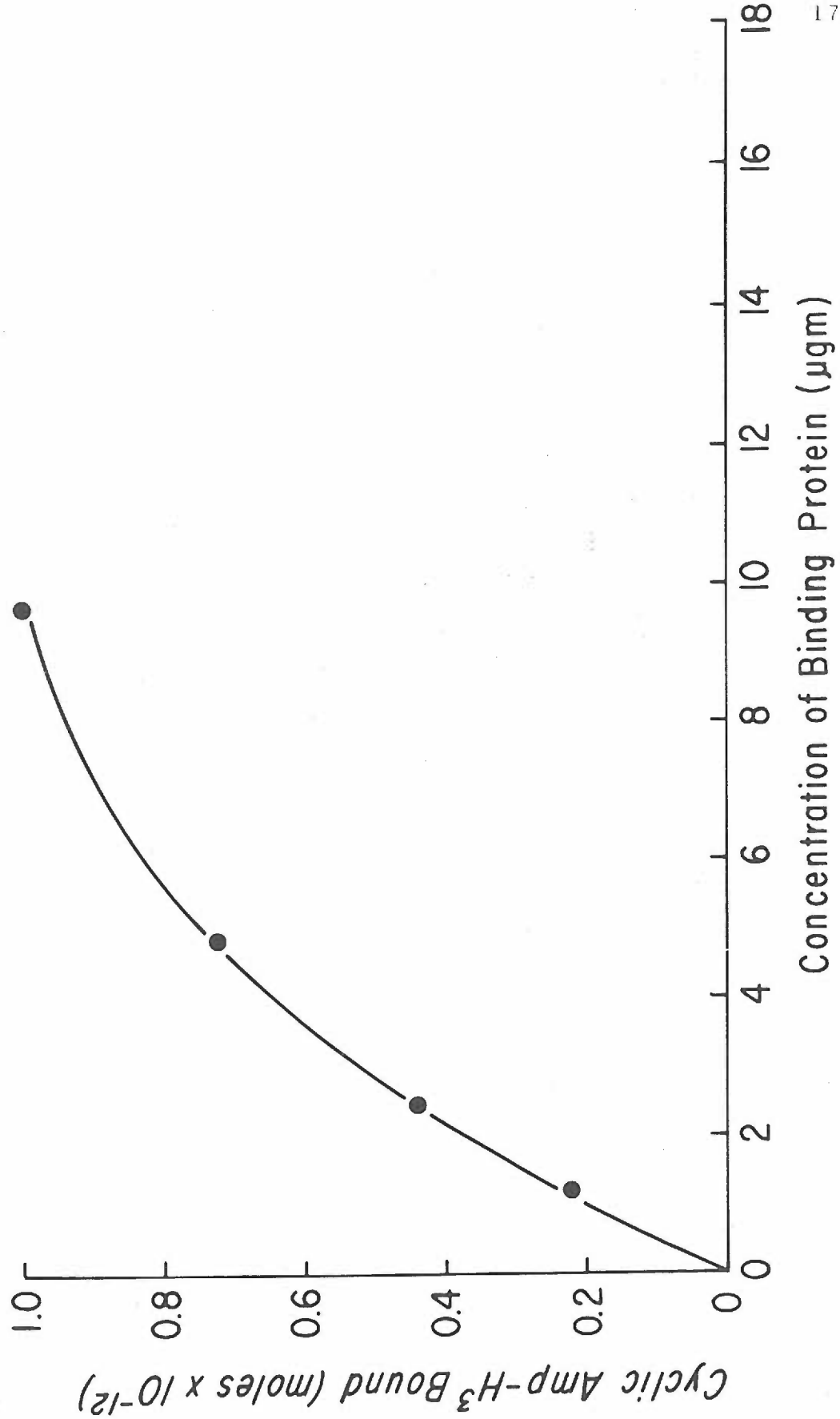
First	Last	Sign	Difference	Rank	R^+	R^-
304	851	-	547	8		8
347	189	+	158	4	4	
288	428	-	140	3		3
323	209	+	114	2	2	
283	230	+	53	1	1	
201	438	-	237	6		6
633	198	+	435	7	7	
508	281	+	227	5	5	
					19	17
$\bar{X}$ 361	353			not significant ($p > .05$)		
SD 131	210					
SEM 50	79					

Concentration of cyclic AMP expressed as moles x 10^{-12} per gram wet weight of tissue.

APPENDIX C

Binding Protein - Cyclic AMP - H^3 Saturation Curve

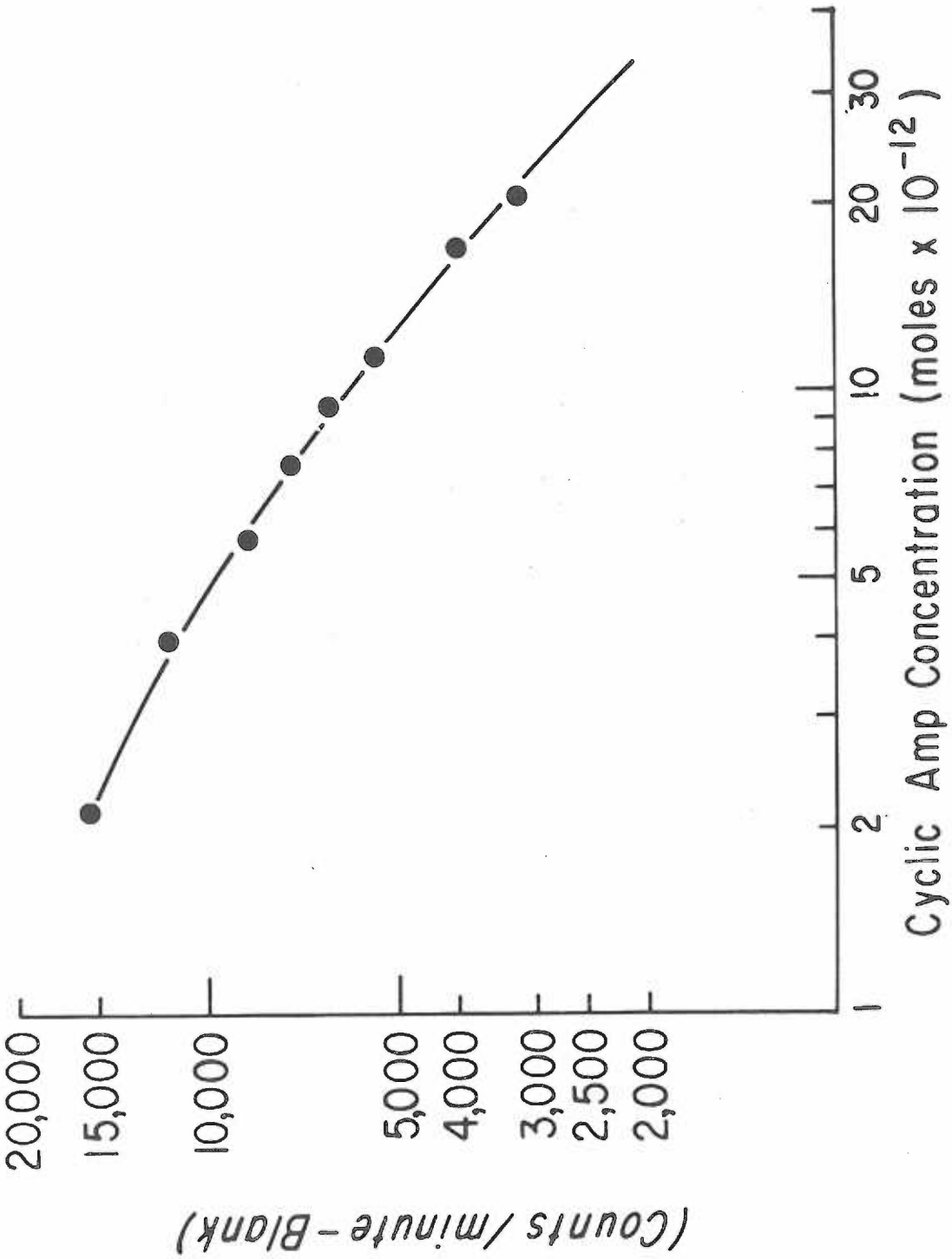
Binding activity of the isolated protein was assayed by incubating 1.0×10^{-12} moles of cyclic AMP-H^3 with varying amounts of protein. The protein is saturated by cyclic AMP-H^3 up to a concentration of 9.5 μgm, at which point the addition of more protein does not lead to an increase in bound activity. The protein isolated from bovine heart muscle therefore, binds 0.11 picomoles of cyclic AMP per μgm of protein. This is less than the binding activity reported by Gilman (1969), which was 0.19 picomoles per μgm of protein. The cyclic AMP assay procedure (Gilman, 1969) requires the addition of only enough protein to bind less than 30% of the added cyclic AMP-H^3. Adding 2 picomoles of cyclic AMP-H^3 to 2 μgm binding protein, as was the normal procedure for these experiments, fulfills the Gilman requirement.


Cyclic AMP-H³ Bound (moles x 10⁻¹²)
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
18
Concentration of Binding Protein (µgm)

APPENDIX D

Cyclic AMP Assay Standard Curve

The standard curve was produced by adding known concentrations of cold cyclic AMP to an incubation tube containing 2 picomoles of cyclic AMP-H^3 and 2 μgm of binding protein. The curve when plotted on a log-log scale is not precisely linear as reported by Gilman (1969), but is quite close. A similar standard curve was produced for each cyclic AMP assay.


Cyclic Amp Assay—Standard Curve
(Counts /minute - Blank)
20,000
15,000
10,000
5,000
4,000
3,000
2,500
2,000
1
2
5
10
20
30
Cyclic Amp Concentration (moles x 10^{-12})

APPENDIX E

The assay for cyclic AMP depends on the specific binding of cyclic AMP-H^3 to a receptor protein. The dilution of bound activity is assumed to be due to the competitive binding of cold cyclic AMP to the same receptor protein. If there is any nonspecific binding of material other than cyclic AMP to the receptor protein the assay will provide erroneous results. It is necessary therefore, to insure that the extracts from muscle containing cyclic AMP do not also contain other materials which would interfere with the assay. The extracted cyclic AMP, therefore, was hydrolyzed with cyclic nucleotide phosphodiesterase (Sigma Co., St Louis, Mo.) and the effect of hydrolysis on the assay for cyclic AMP determined.

The hydrolysis technique is modified from Kuo and Greengard (1970). The incubation tube contained 100 μgm of phosphodiesterase, or buffer, and either cold cyclic AMP or an aliquot of muscle extract. The incubation volume was made up to 0.3 ml using 0.1 M sodium acetate buffer (pH 8.0) which contained 10 mM Mg acetate and 30 mM imidazole. Incubation was carried out at 30° C for one hour. The reaction was stopped by boiling for 5 minutes. An aliquot of the incubation fluid was assayed for cyclic AMP as usual. Verification of the assay specificity was repeated for each experimental series. The results of one hydrolysis experiment are provided in Table 9.

The results show that the addition of cyclic nucleotide phosphodiesterase to the tissue extracts containing cyclic AMP eliminates any dilution of bound cyclic AMP-H^3 activity. Therefore, it may be assumed that the cyclic AMP extracts isolated from muscle in these experiments contained no material in sufficient concentration to interfere with the assay.

Appendix E

Table 9. The hydrolysis of cyclic AMP in tissue extracts with cyclic nucleotide phosphodiesterase.

Additions	Cyclic AMP-H^3 Bound (counts per minute)	
	+ Phosphodiesterase	- Phosphodiesterase
Buffer	12660	12162
Standard cyclic AMP (30×10^{-12} moles)	12434	7577
Muscle extract A	12132	8013
Muscle extract B	13101	6829
Muscle extract C	12256	5036