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Neuroregulation of the Autophosphorylatable Regulatory Subunit of Adenosine Cyclic 3':5' Monophosphate-Dependent Protein Kinase Type II

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NEUROREGULATION OF THE AUTOPHOSPHORYLATABLE REGULATORY SUBUNIT OF ADENOSINE CYCLIC 3':5' MONOPHOSPHATE-DEPENDENT PROTEIN KINASE TYPE II

by

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VITA

The author, Stephen Paul Squinto, is the son of Leonard Squinto and Emily (Gorowski) Squinto. He was born September 19, 1956, in Berwyn, Illinois.

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The author received an Individual National Research Service Award from the National Institute of Health in 1984 which will be served under the direction of Dr. Richard Jungmann, a Professor in the Department of Molecular Biology at Northwestern University Medical School, Chicago, Illinois.

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I. INTRODUCTORY STATEMENT OF PURPOSE AND SIGNIFICANCE

The objectives of the research for my dissertation evolved from preliminary studies of cytosolic protein phosphorylation in denervated slow-twitch soleus muscle of the rat hindlimb (Squinto et al., 1980). These studies showed a denervation time-dependent increase in the *in vitro* phosphorylation of soluble protein in cytosolic muscle fractions. However, this cytosolic protein phosphorylation was delayed for 3 hr and reached a peak after 24 hr when phosphorylation of these proteins in the denervated muscle was approximately twice that found in contralateral, sham-operated muscles. To pursue the significance of this early denervation effect which occurred before denervation atrophy, but not immediately after nerve transection, I set forth the following specific aims: 1) to determine whether significant changes in the phosphorylation of soluble protein from rat soleus muscle are dependent upon neural influences other than the impulse-directed release of acetylcholine; 2) to characterize the denervation-dependent soluble cytosolic ATP:protein phosphotransferase reaction of rat soleus muscle; and 3) to identify the predominant soluble cytosolic phosphoprotein substrate for the denervation-dependent phosphorylation reaction.

Research literature relevant to these specific aims is reviewed under section II. I will emphasize the alterations in skeletal muscle which are known to occur early after

denervation and before the onset of denervation atrophy since these changes probably represent events more closely mediated by neurotrophic influences. However, other muscle parameters which are affected later and occur in conjunction with or following muscle atrophy will also be mentioned. The general topics which I will review in section II are sequenced logically in the outline presented below. A brief introduction to these general topics follows the outline.

A. Neuromuscular Communication

B. Effect of Alterations in Neuromuscular Communication upon the Integrity of Skeletal Muscle

C. Cyclic AMP in Neuroregulatory Functions

D. Protein Phosphorylation in Skeletal Muscle

E. Protein Dephosphorylation in Skeletal Muscle

F. Cyclic AMP-Dependent Protein Kinase Isozymes I and II

Cell biologists have classically defined"trophic influence" in terms of a long term maintenance requirement necessary for the survival of a cell and supplied by extracellular effectors (reviewed in Gospodarowicz, 1976). This concept of an extracellular influence on cell viability has been applied to neuromuscular communication in that motor neurons may exert regulatory influences upon muscle which are expressed as alterations in the physiology and biochemistry of the muscle cell. These modifications may, in turn, be essential in maintaining the structural and functional integrity of the target muscle cell. In this dissertation,

trophic influences exerted by the motor nerve upon muscle are defined as neural influences not related to impulse-dependent acetylcholine transmission.

The motor nerve transmits information to the contiguous muscle cell through impulse-directed and spontaneously released acetylcholine. Impulse-dependent transmission of acetylcholine occurs in response to the propagation of a nerve impulse which is generated in the form of an action potential. When the action potential arrives at the nerve terminal of a functional synapse, it results in the release of the neurotransmitter - acetylcholine. Thus, the electrical signal is transformed into a chemical signal. The chemical transmitter, in turn, crosses the synaptic cleft and binds to a specific postsynaptic receptor. Upon binding to the receptor, impulse-directed acetylcholine opens ion gates on the postsynaptic membrane and reconstitutes the electrical process through the development of endplate potentials which lead ultimately to muscle contraction. Spontaneous transmission of acetylcholine, which can occur in the absence of nerve impulse activity, does not result in muscle contraction. However, it may have a trophic role in regulating some properties of the muscle membrane.

Neuromuscular communication also occurs through the release of putative neurotrophic factors which may be synthesized in the neuronal perikarya. Much of the experimental evidence suggesting that trophic factors are involved in

neuromuscular communication has come from the observations that the motor neuron can influence the target muscle cell even in the absence of a functional innervation. Axoplasmic transport provides a viable mechanism by which these axonal components are delivered from their sites of synthesis to the neuromuscular junction.

The vital necessity of an intact and functional innervation for the maintenance of the structural and functional integrity of skeletal muscle is well documented. Denervation disrupts neural influences on the target muscle cell by eliminating communication between the motor nerve and the contiguous muscle cell which can be in the form of impulse-directed and spontaneous acetylcholinerelease or axonally-derived neurotrophic factors. Elimination of neuromuscular communication causes marked changes in morphological, physiological and biochemical properties of the muscle. These changes, in turn, result in the development of denervation atrophy.

Neurotrophic factors and the postsynaptic molecular events affected by putative neurotrophic factors are not clearly defined. Additionally, the mechanisms by which target muscle cells translate neurotrophic messages into molecular events, which may be essential for the maintenance of muscle structure and function, is an intriguingly important and undefined aspect of this mode of neuromuscular communication. Elucidation of these molecular events and the mechanisms by which they are neurotrophically-regulated

is significant in that it may yield information useful toward determining the initial site of action of unidentified, axonally transported, trophic factors.

Cyclic AMP has been implicated in neuromuscular communication, but its role is unclear. Skeletal muscle undergoes excitability changes upon impulse transmission and these changes may involve cyclic nucleotides and calcium ions. Denervated skeletal muscle exhibits marked changesin adenylate cyclase and cyclic nucleotide phosphodiesterase activities. These enzymes regulate cyclic AMP metabolism. Also, cyclic AMP and drugs which stimulate cyclic AMP synthesis by binding to beta-adrenergic receptor-coupled adenylate cyclase increase the number of acetylcholine receptors in skeletal muscle.

Cyclic AMP produces many, if not all, of its effects in eukaryotes by stimulating the phosphorylation of cell proteins. Various neurotransmitters,non-steriod hormones and other extracellular depolarizing agents are known to increase the phosphorylative state of muscle proteins by binding to receptors coupled to adenylate cyclase. This binding leads to the increased synthesis of cyclic AMP and the stimulation of the cyclic AMP-dependent protein kinase. Therefore, the reversible phosphorylation-dephosphorylation mechanism which mediates the intracellular regulation of many metabolic processes may be involved in the interrelationship between motor neuron and contiguous muscle cell.

II. RELEVANT LITERATURE REVIEW

A. Neuromuscular Communication

1. Acetylcholine

a. role of acetylcholine in synaptic transmission

Current knowledge suggests that the motornerve is limited in its mode of communication with the muscle cell. Presently, the most understood mode of neuromuscular communication centers around the neurotransmitter - acetylcholine. Electrical impulses propagate down the motor nerve axon as brief waves which consist of an excitation-induced depolarization of the neuronal membrane followed by a spontaneous repolarization to the resting membrane potential. These brief positive changes in the membrane potential are called action potentials and lead to the release of acetylcholine from the motor endplate in multimolecular packets or "quanta" (reviewed in Krnjevic, 1974; MacIntosh, 1981). Upon receiving an impulse, the nerve ending becomes depolarized and the frequency of acetylcholine release is greatly increased. At the neuromuscular junction, a few hundred quanta may be released almost synchronously from numerous sites on the motor axon's terminal region (reviewed in Krnjevic, 1974). During the nerve's resting state when the membrane potential is negative, quanta may be released singly. This is referred to as spontaneous quantal release. Acetylcholine may also be released by spontaneous leakage

from the nerve ending in a nonquantal form (reviewed in Drachman, 1974; Schmidt, 1978; Drachman et al, 1982). Thesleff (1983) recently reported another type of acetylcholine transmission which occurs as a result of a quantal spontaneous release of acetylcholine and which is not dependent upon a Ca^{2+}-mediated depolarization of the nerve ending.

Acetylcholine is formed from the reaction of choline andacetyl CoA catalyzed by the enzyme - choline acetyltransferase. Both acetylcholine and choline acetyltransferase are present throughout the length of the motor neuron and are concentrated in the axonal endings. Cell fractionation studies have shown that much of the acetylcholine at the nerve endings is contained in the synaptic vesicles, butsome is free in the cytosol. However, most of the choline acetyltransferase is free in the cytosol, but some of it may adhere to the outside of the vesicles (reviewed in Fonnum, 1973). Acetylcholine is hydrolyzed by the enzyme - acetylcholinesterase. This enzyme is characteristically membrane-bound. At the presynaptic region, most of its active sites face outward enabling the enzyme to hydrolyze acetylcholine that has just been released. Also, a small percentage of the enzyme faces inward and hydrolyzes surplus acetlycholine. Choline acetyltransferase and acetylcholinesterase are synthesized in the neuronal perikarya and are transported down the axon to the synaptic region by axoplasmic transport.

These enzymes are present both at the nerve terminal and throughout the whole length of the cholinergic neuron (MacIntosh, 1981). Their highest concentrations are in the axon terminal. However, acetylcholine functions only as a synaptic transmitter and does not have a role in the conduction of impulses along the axon or the delivery of axonal components by axoplasmic transport.

Acetylcholine that is released diffuses across the synaptic junction and binds to a highly specific postsynaptic receptor (reviewed in Heidmann and Changeux, 1978). The snake venom toxin, alpha-bungarotoxin, binds specifically and irreversibly to this acetylcholine receptor. These properties of the neurotoxin, therefore, have provided a useful tool for studying the biochemical properties of the receptor (reviewed in Devreotes and Fambrough, 1975; Fambrough and Devreotes, 1979; Pumplin and Fambrough, 1982). It is now known from these studies and studies with other cholinergic neurotoxins that the acetylcholine receptor is an integral membrane glycoprotein which can be split into 5 or 6 non-equivalent subunits by sodium dodecyl sulfate (SDS). These subunits range in molecular weight from 40,000 to 140,000 when the receptor is purified from rat skeletal muscle (Heidmann and Changeux, 1978; Fambrough, 1979; Conti-Tronconi and Raftery, 1982). The protein subunits of the receptor are synthesized on polyribosomes and are immediately incorporated into membranes which form vesicles on the Golgi

apparatus where posttranslational modification of the receptor occurs. The vesicles migrate to and fuse with the external membrane exposing the acetylcholine receptor on the external surface. Degradation of the receptor involves its internalization and subsequent proteolytic cleavage in the lysosomes. The acetylcholine receptor is found at the junctional region of the postsynaptic muscle cell membrane in the adult muscle and at both the junctional and extrajunctional regions in embryonic muscle. A chemical and physical denervation of adult muscle leads to the development of extrajunctional acetylcholine receptors. Extrajunctional receptors have a turnover time of 1 to 2 days (Burden, 1977; Edwards, 1979; Pumplin and Fambrough, 1982) while intact adult junctional receptors are more stable and have a turnover time of 7 to 10 days (Edwards, 1979; Linden and Fambrough, 1979; Loring and Salpeter, 1980; Stanley and Drachman, 1983). However, approximately 20% of the intact junctional acetylcholine receptors have a faster turnover time of 18 hr (Stanley and Drachman, 1983).

A difference in electrical potential, the membrane potential, usually exists between the inside of the muscle cell and the extracellular fluid surrounding it. This is referred to as the resting membrane potential. Binding of acetylcholine to its receptor induces a transient depolarization of the postsynaptic membrane as a result of an activation of ionic channels into conductive or open states (Katz and Miledi, 1971; reviewed in Fambrough, 1979). Opening of

ion channels permits Na^{+2}, K^{+} and Ca^{+2} to move down concentration gradients which depolarizes the muscle membrane and generates endplate potentials. Endplate potentials (epps) are the product of impulse-related acetylcholine transmission, while miniatureendplate potentials (mepps) are the result of spontaneous quantal acetylcholine transmission (reviewed in Drachman, 1974; Schmidt, 1978). Only epps, however, generate muscle contraction. Therefore, spontaneous quantal or non-quantal acetylcholine release may be related to a trophic influence of the motor nerve.

b. role of acetylcholine in skeletal muscle contraction

Contraction occurs in a muscle fiber as a result of acetylcholine-induced depolarization of the plasma membrane. The activation of the contractile elements (myosin, actin and the ATPase of myosin) which are distributed over the whole cross-section of the fiber depends on the spreading of the excitatory state from the plasma membrane transversally across the muscle fiber.

There is evidence that the transverse tubule system (T tube system) of the sarcomere is involved in the propagation of impulses inward from the plasma membrane (Landon, 1982). These tubules measure about 300 Angstroms in diameter and run in a transverse direction through the muscle fiber. They are called the T tubule system to distinguish them from the tubules of the sarcoplasmic reticulum which are mainly

longitudinal in orientation. On each side of a transverse tubule, but not continuous with it, are structures called terminal cisternae which form part of the sarcoplasmic reticulum and act as a calcium storage area.

It is now known that the effect of an action potential propagating along the fiber membrane is to produce some kind of action potential in the T-tubule system and that this in turn causes the release of calcium ions from the terminal cisternae into the sarcoplasmic reticulum (Landon, 1982). The released Ca^{2+} binds to the calcium binding subunit of the troponin protein (TN-C) and causes conformational changes that are transmitted to the tropomyosin protein and finally to the thin filament protein - actin. These structural changes enable actin to interact with the thick filament protein, myosin, resulting in muscle contraction and ATP hydrolysis. Muscle contraction and ATP hydrolysis will persist until Ca^{2+} is removed. The relaxation stage is accomplished by the pumping back of Ca^{2+} into the sarcoplasmic reticulum by an ATP-driven Ca^{2+} pump.

Different types of skeletal muscles are characterized according to their fiber types. Fiber types are distinguished, however, by the kind of innervation they receive. In some fibers, the membrane (sarcolemma) is electrically exicitable. In these fibers, an all-or-none action potential is generated at the neuromuscular junction and is propagated along the entire length of the fiber; this allows the whole muscle to contract. Fibers of this kind normally

have a single motor endplate and are described as having uniterminal innervation (Huddart, 1975). Included in this category are most of the twitch fibers in skeletal muscles. Other fibers have membranes that are electrically inexcitable; in these, the action potential at the neuromuscular junction only causes a localized stimulation of the contractile apparatus. For this reason, these fibers (tonic fibers) tend to have multiterminal innervation. A single stimulus has relatively little effect on this fiber type, but repetitive stimulation causes the gradual development of muscle tension.

Although almost all of the skeletal muscles in mammals are of the phasic type (uniterminal innervation), a considerable range of contractile activity is found. This is because not all fibers in a particular muscle are of the same type. For example, fibers can be distinguished by their energy supply. Those muscles whose energy supply is largely glycolytic are relatively fast in contractile response and generally appear white in color (white fast-twitch muscle). Those fibers that have an abundance of mitochondria are said to be oxidative. These fibers are relatively slow in contractile response and appear reddish in color because of the oxygen carrier protein - myoglobin. Also, there is a considerable overlap between the two energy sources in several fibers and these are referred to as intermediate fiber types. They are generally termed fast oxida-

tive/glycolytic fibers and are often red in color.

The slow and fast fibers are often referred to as Type I and Type II fibers. Type II (fast) fibers are further subdivided into Type IIA (fast oxidative/glycolytic) and Type IIB (fast glycolytic). These fiber types were originally distinguished most reproducibly by histochemical methods (Dubowitz and Brooke, 1973). Type I fibers have acid-resistant, alkali-sensitive myofibrillar ATPase properties, relatively high content of mitochondria, high succinic dehydrogenase activity, low glycogen content and phosphorylase activity, a relatively poorly developed sarcoplasmic reticulum, low contraction speeds and high resistance to fatigue (Lewis et al., 1974). Slow muscles undergo sustained rhythmic activity, respond to stimuli with prolonged contractures, constantly adjust to postural change, and operate during periods of prolonged anoxic stress. The slow muscle permits muscular contraction to proceed with lower utilization of ATP because it has a low amount of myosin ATPase activity. This facilitates the appropriate balance between chemical energy supply and mechanical energy utilization that must occur in a muscle that contracts steadily and without rest for long periods of time. White fast muscles (Type II fibers) have the converse properties and undergo only short bursts of activity and possess little myoglobin. It is noteworthy, however, that most muscles are a mixture of white, red and intermediate (Type IIA) fibers and have properties intermediate to the main two fiber types.

The diversity of properties makes it difficult at times not only to classify particular fibers but to compare results using these intermediate fiber type muscles . However, rat soleus muscle employed for all experiments reported in this dissertation is considered a classical example of the slow-twitch oxidative muscle in that its fiber composition is a homogeneous (approximately 95%) composition of Type I fibers (Squire, 1981).

2. Neurotrophic factors

It has been proposed that neuromuscular communication also involves the delivery of putative neurotrophic factors to the target muscle cell (reviewed in Guth, 1968 and 1969; Harris, 1974 and 1980; Gutmann, 1976; Varon and Bunge, 1978; Thesleff, 1983). If trophic factors do contribute to the neural regulation and maintenance of skeletal muscle structure and function, then the motor neuron must possess the biochemical machinery necessary for their synthesis and a mechanism for the transportation of such factors from their site of synthesis to their release site.

a. synthesis of transportable material

The nerve cell displays an extreme degree of regional specialization in that most of the machinery for synthesizing proteins and other complex macromolecules is confined to the cell body (reviewed in Grafstein and Forman,

1980). The perikaryon, therefore, is the primary source of assembly for most of the materials needed by the long cellular extensions that constitute the axons of neurons. These needed materials include membrane constituents, nucleotides, RNA and synaptic components. The nerve cell soma is also the primary site of protein synthesis in mammalian motor neurons and a high level of protein synthesis is continually occurring there (reviewed in Lajtha, 1964; McIlwain and Bachelard, 1971; Barondes and Dutton, 1972; Grafstein and Forman, 1980). Perhaps the first definitive demonstration of protein synthesis occurring in the nerve cell soma was reported in the classic experiments of Droz and LeBlond (1963). By means of high-resolution autoradiography, these workers demonstrated directly that after an amino acid (^{3}H-leucine) had been injected systemically into adult rats and had become incorporated into nerve cells, a wave of radioactive protein moved from the soma of anterior horn cells and sensory ganglion cells into the region of the axon hillock. The radiolabeled proteins then appeared further along the axon at succeeding intervals of time. These studies, therefore, also implied a movement or flow of material from the nerve soma to more distal regions of the axon. Other studies have also examined the possibility of the nerve cell soma as the primary site of protein synthesis and have confirmed the findings of Droz and LeBlond (Taylor and Weiss, 1965; Grafstein, 1967; Lasek, 1968). It is interesting to note that more recent studies have shown that protein synthesis can

also occur to a limited extent in the axon (reviewed in Peters et al., 1976) and possibly in the dendrites of central nervous system neurons as well (Steward, 1982).

b. axoplasmic transport: movement of material within the axon

The intricate and detailed morphological specializations of the neuron which includes axons, dendrites and synapses (and possibly the maintenance of target tissues) are thought to depend upon the specific and targeted transport of macromolecules from the neuronal perikaryon (reviewed in Grafstein and Forman, 1980). A model of the nerve as a dynamic structure conducting unique functions including the maintenance of nerve structure, excitation and conduction of the nerve impulse, neurotransmission, and the release of trophic substances affecting cells in contact with the nerve terminals has developed from the studies of axoplasmic transport (Ochs, 1982).

Transport of macromolecules from their site of synthesis in the neuronal perikarya to nerve endings was first inferred from the work of Waller (1850) who described a progressive degeneration of nerve fibers distal to the point of nerve transection. This process, which occurs within a few days after nerve transection, is now classically referred to as "Wallerian degeneration" or the "dying back phenomenon". In their classical studies on nerve compres-

sion, Weiss and Hiscoe (1948) postulated that the axon was continuously growing from the cell body. They based this interpretation on their experimental observations of a swelling of nerve fibers proximal to a constriction point, produced by nerve ligation, and a narrowing of fibers distal to the ligation. These results were interpreted as a demonstration of a continuous movement of axoplasm inside the nerve fiber. It has been possible to show with histofluorescence staining that norepinephrine and dopamine-beta-hydroxylase, which are both involved in noradrenergic transmission at noradrenergic junctions, accumulate at a nerve ligature (Dahlstrom, 1965). Accumulation at a constriction has also been demonstrated for acetylcholine (Haggendal, 1971) and acetylcholinesterase using an *in vitro* bioassay (O'Brien, 1978). Various enzymes accumulate at a nerve constriction and include lactate dehydrogenase, choline acetyltransferase, phosphoglucuronic dehydrogenase and catechol-O-methyltransferase (reviewed in Lubinska, 1964; Ochs, 1974, Lubinska, 1975; Pleasure, 1980; Ochs, 1982). Electron microscopic autoradiography has also been used to identify structures labeled during the axoplasmic transport of radioactive materials. The label which accumulates in the nerve endings is concentrated in synaptic vesicles, in the presynaptic membrane and to a lesser extent in the mitochondria (Lubinska, 1975; Ochs, 1982). The fraction retained in the axon appears preferentially in the axolemma and in the smooth endoplasmic reticulum. Much of the label in the axoplasm is

associated with microtubules and neurofilaments (reviewed in Lubinska, 1975; Ochs, 1982).

Direct microscopic observation of living nerve fibers show that small particles, including mitochondria and elements of the endoplasmic reticulum, can move within axons (Lubinska, 1975; Grafstein and Forman, 1980; Pleasure, 1980; Ochs, 1982). These observations have been made in neurites growing in tissue culture as well as in axon segments excised from an intact nerve.

From the studies on the components of protein movement within neurons, a dual transport hypothesis emerged. For example, a population of small particulate organelles were found to proceed down the axon at a relatively high velocity while the axoplasm itself advanced very slowly (Grafstein and Forman, 1980; Ochs, 1982).

The fast component appears to travel within the axon at a rate of 410 mm per day at 37°C (Ochs, 1974). To a large extent this fast moving fraction includes various types of vesicles derived from the Golgi system and endoplasmic reticulum (Ochs, 1982). Some of these vesicles may be responsible for the renewal of the plasma membrane, since it appears likely that in the nerve cell, as in other cells, new plasma membrane is formed as a continuous sheet by the addition to the membrane of vesicular elements that fuse to the membrane by exocytosis (reviewed in Lentz, 1983). The fast component also appears to contain elements related to

the transmitter storage vesicles that participate in synaptic transmission. These vesicles probably originate with the synthesis of a protein core in the rough endoplasmic reticulum of the cell body. Presumably, this core material consists of binding proteins for the transmitter and enzymes involved in transmitter metabolism. The material is then transferred to the Golgi apparatus, packaged into membrane-bound bodies and then conveyed along the axon by fast axoplasmic transport (reviewed in Ochs, 1982).

Fast transport is critically dependent upon oxidative metabolism. Transport may be blocked in a nerve _in vitro_ by applying metabolic blocking agents such as sodiun cyanide, dinitrophenol, azide or immersing it in an atmosphere of nitrogen (Ochs, 1974; Wilson and Stone, 1979; Ochs, 1982). The effectivness of dinitrophenol, which uncouples oxidative phosphorylation, shows that the transport requires a continuous supply of ATP. Ochs (1974 and 1982) has also shown that Ca^{2+}, intact microtubules and temperature are important in the functioning of fast transport. The rate of fast transport is not linked to the electrical activity of the axon. This has been demonstrated by showing that the rate of transport in an isolated inactive segment of nerve is the same as in an active intact nerve _in vivo_ and electrical stimulation of an isolated nerve preparation has no effect on the rate of transport (Ochs, 1982). Additionally, axoplasmic transport is maintained in isolated segments of peripheral nerve (Ochs and Ranish, 1969) and,

therefore, is independent of a continuity between nerve cell soma and axon. The mechanism of fast and slow transport is not known (Ochs, 1982).

The properties of slow transport have so far received much less attention. Slow transport reportedly has a rate of 1 to 5 mm per day (Droz and LeBlond, 1963; McEwen and Grafstein, 1968; Lasek and Hoffman, 1975; Lasek, 1980). Slow transport has recently been divided into categories based on distinct classes of neuronal constituents commonly associated with slow transport, including numerous cytoskeletalelements such as neurofilaments, tubulin, actin and clathrin. Black and Lasek (1980) have described a slow component of slow transport with an estimated rate of 1 to 2 mm per day (slow component a) and a slightly faster component of slow transport with an estimated rate of 4 to 5 mm per day (slow component b). The influence of temperature, electrical activity and metabolism on the rate of slow transport remains to be established.

Microscopy has revealed that neuronal constituents can move in either direction along the axon. Transport of materials in the disto-proximal direction is known as retrograde transport (Lubinska, 1975). The accumulation of material distal to a nerve ligation or cut has been shown to occur with organelles detectable with electron microscopy (Lubinska, 1964). Quantitative studies on the rates of accumulation of various enzymes on both sides of a lesion

have made possible some comparisons between the retrograde and orthograde transport. Acetylcholinesterase, for example, travels in the orthograde direction with the fast component of protein transport while a small pecentage of the axonal content of the enzyme participates in the retrograde transport (Edstrom and Hanson, 1973). Retrograde transport rate is also half the rate of the orthograde transport rate for this particular component (Edstrom and Hanson, 1973). These authors also showed that the two types of axoplasmic transport (retrograde and orthograde) had similar sensitivities to various transport blocking agents and, therefore, suggested that they are related.

The significance of retrograde axoplasmic transport remains to be established. It has been suggested that retrograde transport may provide a mechanism for feedback control of neurons by the target tissues which they innervate (reviewed in Price, 1974; Varon and Bunge, 1978). In fact, recent evidence suggests that an extract of rat skeletal muscle may provide protein-like motoneurotrophic factors which stimulate neurite sprouting and cholinergic activity of cultured spinal motor neurons (Smith and Appel, 1983). Retrograde transport may also have an important function during the regeneration of a cut axon. After an axon is cut, its cell body may undergo a series of complex metabolic and morphological changes that may be a prelude to axonal regeneration (reviewed in Grafstein, 1977). These changes are usually initiated before any axon outgrowth is

detectable. It is not clear how the cell body is informed of the axonal changes following nerve section. However, the arrival of some abnormal material conveyed by retrograde transport from the damaged area or the interruption of the retrograde transport of some substance originating in the nerve terminals might serve as a signal for the cell body response (Bisby, 1982). Another possibility is that some materials normally conveyed by orthograde transport to the axon terminals would be unable to reach their usual destination and might return prematurely to the cell body where they would alter the pattern of their own metabolism. This suggests that orthograde transport might play an important role in the neural control of metabolic activity in the neuron by determining the level of certain materials in the cell body.

Direct evidence for the transsynaptic transport of trophic material from the nerve to muscle is limited at present. Autoradiographs have suggested a translocation of radiolabeled protein from the neuron to the muscle and entry of labeled protein into the muscle (Korr et al., 1967; Musich and Hubbard, 1972; Grafstein, 1977). Additionally, Hines and Garwood (1977) reported a significant release of axonally transported proteins from frog sciatic nerve _in vitro_. However, it has been difficult to determine the mechanism of protein release and whether there is specific release or general leakage of this axonal material.

c. experimental analysis of trophic effects

(1) disuse vs. surgical denervation

In the motor system, the nerve impulse and the neurotrophic message can both travel in the proximo-distal direction from the neuronal perikaryon to the nerve terminal region. For this reason, it is often difficult to distinguish between the effects of these two kinds of neural influences on target muscle cells. Many early studies chose to operationally distinguish between neurotrophic and impulse-mediated influences of the nerve by comparing the effect of surgical denervation on muscular plasticity to that of muscle inactivation. In these early studies, inactivation (disuse) was produced by several experimental models including tenotomy, spinal cord isolation and limb immobilization (reviewed in Jolesz and Sreter, 1981; Hoyle, 1983). Perhaps the first to suggest that skeletal muscle is regulated by neural influences other than impulse-directed contractile activity alone was Tower (1941) who observed that the development of fibrillatory activity in denervated dog muscle appeared earlier and with a quantitatively greater response than that observed in disused but innervated muscle. Also, others have suggested that muscle atrophy is regulated by neural influences apart from impulse-directed contractile activity alone. Muscle atrophy can be experimentally produced by inactivating physically innervated muscles

(reviewed in Guth, 1968 and 1969; Jolesz and Sreter 1981; Hoyle, 1983). The morphological changes observed in the inactivated muscle included myofibrillar atrophy and loss of nuclear integrity (Guth, 1968) as well as changes in sarcomere length (Williams and Goldspink, 1978) and an increase in protein degradation (Goldspink et al., 1983). The onset of these changes, however, was delayed in disused muscles relative to surgically denervated muscles. Also, a late phase of increased protein synthesis observed in 7 day-denervated muscle did not occur with inactivation (Goldspink, 1978). Other early studies have shown that changes in endplate morphology were not as pronounced in disused muscle when compared with denervation-induced changes (Johns and Thesleff, 1961). With refined techniques, however, including long term integrated electromyography, the use of experimental models for muscle disuse has recently been questioned (Fournier et al., 1983). These studies showed that the degree of atrophy in rat soleus and gastrocnemius muscles was not closely related to the recorded electromyographic activity of these muscles.

(2) cross-reinnervation experiments

The experimental observations of Buller, Eccles and Eccles (1960 and 1960a) have provided evidence suggesting that the dynamic properties of skeletal muscle are regulated by a neural influence other than impulse-directed acetylcholine transmission (reviewed in Close, 1972). These authors reinnervated a fast-contracting muscle with a nerve that normally supplies a slow-contracting muscle and reinnervated a slow-contracting muscle with a nerve that normally innervates a fast-contracting muscle. They found that the isometric contraction time of the fast-contracting muscle became longer and that of the slow-contracting muscle became shorter. Subsequently, Close (1969) observed that the intrinsic speed of shortening of the muscle sarcomere is modified by these cross-reinnervation procedures and suggested, therefore, that the basic properties of the myosin molecule might be neurotrophically regulated. A close correlation has been reported between the contraction of different skeletal muscle types and the Ca^{2+}-activated myosin ATPase activity for these muscle types (Unsworth et al., 1982). It has been shown that different muscles types contain myosins with different ATPase properties and that the proportions of these myosin ATPase activities change as a result of cross-reinnervation (Guth and Samaha, 1969; Buller et al., 1969; Mommaerts et al., 1969; Barany and Close, 1971; Sreter et al., 1974; Hoh, 1975; discussed further in section

II B). It has also been shown that the relative proportions of polymorphic forms of other contractile proteins (troponin, actin and tropomyosin) also change as a result of cross-reinnervation (Amphlett et al., 1975).

The neural message responsible for eliciting changes in several skeletal muscle contractile proteins and myosin ATPase activity of cross-reinnervated muscles is unknown. The authors of early studies suggested that the motor nerve regulates the dynamic properties of fast twitch and slow twitch muscle through the release of some specific neurotrophic factor(s). Recently, however, evidence has been presented which suggests that the conversion of muscle properties observed in cross-reinnervated muscles might be due to a change in the frequency and pattern of impulse activity (Salmons and Sreter, 1976; Eisenberg and Salmons, 1980).

(3) experimental use of neurotoxins

Recently, various neurotoxins which block specific modes of neuromuscular communication have been used to differentiate a role for impulse-directed acetylcholine release, spontaneous acetylcholine release and axonally transported neurotrophic factors in the regulation of specific muscle properties. For example, tetrodotoxin (TTX) has been used to successfully block nerve impulse conduction by inhibiting axonal sodium conductance while leaving the muscle physically innervated. Application of TTX to

peripheral nerve results in a denervation-like increase in extrajunctional acetylcholine sensitivity (Pestronk et al., 1976; Bray et al., 1979) and a decline in the resting membrane potential (Drachman et al., 1982) of the innervated muscle without affecting axoplasmic transport or inducing nerve degeneration. The TTX-induced alterations in these muscle properties, however, were quantitatively less than the changes induced by surgical denervation. Chemical denervations producing quantitative changes in these muscle parameters similar to the effect of TTX have also been carried out with other neurotoxins (botulinum toxin and beta-bungarotoxin). These neurotoxins are capable of producing a pharmacological blockade of impulse-dependent acetylcholine transmission. The results of these studies and studies conducted with TTX have suggested that the neural regulation of muscle resting membrane potential and extrajunctional acetylcholine sensitivity involves additional influences of the motor nerve apart from impulse-directed muscle contraction.

In addition to these physiological properties of muscle, biochemical parameters of denervated and TTX-inactivated muscle have been compared. In a study of the oxidative processes in skeletal muscle, it was observed that TTX-inactivation of rabbit soleus muscle for 2 weeks did not affect the activities of several mitochondrial enzymes while denervation for 2 weeks led to a decrease in the metabolic

rate and in mitochondrial enzyme activity of the muscle (Nemeth et al., 1980). Additionally, a recent study has shown that the denervation-induced increase in cell proliferation occurring within 36 hours after denervation is not observed in muscle inactivated by TTX (Murray and Robbins, 1982). The decrease in acetylcholinesterase activity observed in denervated muscle, however, was found to be quantitatively equivalent to that observed in TTX-inactivated muscle (Butler et al., 1978).

If the neural regulation of some skeletal muscle properties depends on the delivery of putative neurotrophic factor(s) to the muscle, then blocking axoplasmic transport while leaving the muscle electrically active should produce changes in muscle properties quantitatively equivalent to those observed in surgically denervated muscle. Experiments to test this hypothesis have been done by applying the neurotoxins colchicine or vinblastine to the motor nerve (Albuquerque et al., 1972; Albuquerque et al., 1974). These toxins are said to interfere with fast axonal transport by disrupting microtubules without affecting the propagation of action potentials or acetylcholine transmission (Fernandez and Ramirez, 1974; Tiedt et al., 1977a). The techniques used to apply the neurotoxin to the peripheral nerve consisted of a single subepineural injection or a neurotoxin-impregnated cylindrical silastic cuff wrapped around the axon. However, the injection of colchicine or vinblastine beneath the perineurium of the motor neuron apparently causes denervation-

like alterations in membrane physiology in both the target tissue and the contralateral, control muscle (Albuquerque et al., 1974; Cangiano and Fried, 1977; Teidt, 1977a). This implies that application of neurotoxin by injection leads to a general systemic effect on muscle membrane physiology. However, it was reported that the dosage of neurotoxin injected was an important consideration in the design of these types of experiments (Guth and Albuquerque, 1979). Neurotoxin-impregnated cylindrical cuffs offered an advantage in that they affected only the motor nerve to which they were attached. Also, application of the neurotoxins by this method did not result in any morphological alteration of the neural membrane. Using this technique, blockage of axoplasmic transport with colchicine or vinblastine was achieved and also there was a decline in resting membrane potential, development of TTX-resistant action potentials, an appearance of extrajunctional acetylcholine sensitivity (Albuquerque et al., 1972 and 1974) and the development of muscle fibrillatory activity (Inestrosa and Fernandez, 1976). The quantitative change in these membrane physiological parameters was equivalent to the changes induced by surgical denervation, but their appearance was delayed with the chemical denervation. These results implied that axoplasmic transport might be directly involved in the supply of some neurotrophic factor(s) to the post-neural cell which, in turn, may have some role in the neural regulation of these electrophysio-

logical events. Inestrosa and Fernandez (1976) also showed that the activities of malic dehydrogenase, pyruvate kinase and phosphorylase b were decreased within 4 to 5 days after chemical denervation wih colchicine. The time course of the decline in enzyme activity after chemical denervation was the same as that observed with surgical denervation, but the quantitative effect was less. Therefore, these results suggested a partial role for the axoplasmic transport of some neurotrophic factor in the regulation of these enzyme activities.

Batrachotoxin, a toxin from frog (Phyllobates aurotaenic), has also been used as an experimental tool to study the role of axoplasmic transport in the regulation of various muscle parameters. This neurotoxin reportedly blocks impulse conduction (Albuquerque et al., 1971a) and axoplasmic transport, but does not affect muscle when applied directly to the tissue (Ochs and Worth, 1975). Studies comparing impulse blockade (using TTX) to a combined impulse conduction and axoplasmic transport blockade (using batrachotoxin) have demonstrated a role for axoplasmic transport in the neural regulation of the muscle resting membrane potential (Boegman et al., 1980), hydrolytic enzyme activity (Boegman and Scarth, 1981 and 1981a), Ca^{2+} uptake by the sarcoplasmic reticulum (Wan et al., 1982) and glucose-6-phosphate dehydrogenase activity (Max et al., 1982).

The role of spontaneously released acetylcholine has been studied recently by using the neurotoxin - alpha-

bungarotoxin. This toxin eliminates transmission of impulse-directed and spontaneously released acetylcholine through a complete pharmacological blockade of the postsynaptic acetylcholine receptor (Drachman et al., 1982). It was reported that treatment of rat soleus muscle with alpha-bungarotoxin produced a decline in muscle resting membrane potential and an increase in extrajunctional acetylcholine receptor number quantitatively equivalent to that induced by surgical denervation (Drachman et al., 1982). The quantitative change in these two muscle properties observed after blocking impulse conduction with TTX, however, was less than the change induced by surgical denervation. The authors interpreted their findings as evidence that acetylcholine transmission (impulse-dependent and spontaneous) is the neural factor responsible for the regulation of these two muscle properties. Whether other specific muscle properties affected by denervation are regulated entirely by acetylcholine has not been defined.

(4) comparative studies: embryonic vs. adult skeletal muscle

It has been suggested that trophic influences of the motor neuron apart from impulse-directed contractile activity might be of significance on an evolutionary scale by contributing to the development of the size, strength and endurance needed by skeletal muscles relative to the extent

to which they are used (Harris, 1980). From this it has been argued that the trophic actions of the nerve which are independent of impulse activity of the nerve may be of significance during the embryonic maturation of the muscle. Experimental support for the coexistence of trophic and activity-related mechanisms for maintaining adult skeletal muscle has come from studies of embryonic muscle. Results of these experiments indicate that the relative contributions of trophic actions of the nerve and of impulse-related muscular contractions to the development of embryonic and adult muscle are not strikingly different. For example, the motor nerve functioning independently of impulse-directed acetylcholine release regulates acetylcholine receptor number and contributes to the clustering of receptors on embryonic muscle (Burden, 1977). Muscle growth, however, seems to require impulse-directed contractile activity. Nerve stump length and neurotoxin experiments such as those described previously indicate that a trophic function of the motor nerve is responsible for regulating acetylcholine receptor number and position in adult skeletal muscle as well (Uchitel and Robbins, 1978; Drachman et al., 1982). Also, the embryonic nerve, independent of nerve impulse activity, contributes to the inhibition of extrajunctional receptor sensitivity as monitored by changes in acetylcholine receptor number (Braithwaite and Harris, 1979). This neurotrophic function is apparently important in adult skeletal muscle as well (Pestronk et al., 1976; Harborne and Smith, 1979; Olek

et al., 1981; Steinbach, 1981; Drachman et al., 1982).

(5) neurally-derived myotrophic factors

It is apparent from the studies described above that motor neurons may influence several properties of skeletal muscle through a trophic function of the nerve involving axonally transported neurotrophic factors. The isolation and characterization of neurally-derived myotrophic factors, however, is at present limited.

A protein purified from adult chicken sciatic nerve has been shown to have a trophic or "innervation-like" effect on embryonic muscle cells in culture (Markelonis and Oh, 1979). This bioactive protein was shown to enhance the rate of morphological maturation and protein synthesis of the cultured muscle cells (Markelonis and Oh, 1979; Markelonis et al., 1980) and maintain cross-striated myotubes in the absence of innervation in vitro for up to several months (Oh, 1976). The myotrophic protein, referred to as "sciatin" (Oh and Markelonis, 1980), has been recently reported to be structurally and functionally similar to the iron-carrying protein of chick serum - transferrin (Markelonis et al., 1982; Oh and Markelonis, 1982). The two proteins have similar amino acid compositions and are indistinguishable immunologically. In vitro and in vivo immunohistochemical studies have demonstrated that the myotrophic protein is present in the perikarya of spinal cord neurons and the

axoplasm of chicken sciatic nerves (Oh et al., 1981). Recently, it was demonstrated that the myotrophic transferrin (M_r = 84,000) is synthesized in spinal neurons and co-purifies with a protein of M_r = 52,000 which may represent the neuronal receptor for this trophic protein (Markelonis and Oh, 1983). The myotrophic transferrin has also been identified by others (Popiela et al., 1982). Interestingly, this protein is identical in structure to chicken transferrins isolated from embryo extract, serum and eggs, but distinguishable from human and horse transferrin as determined by peptide mapping (Beach et al., 1983). The myotrophic transferrin apparently exerts its trophic effects by altering the cell cycle (Popiela et al., 1983).

The 84,000-dalton myotrophic transferrin has been shown to cause a significant increase in acetylcholine receptor number and receptor clustering on aneural embryonic chicken skeletal muscle (Markelonis et al.,1982a; Oh and Markelonis, 1982). Additionally, it has been proposed that acetylcholine may be packaged in synaptic vesicles at the nerve ending together with proteins and ATP (Musich and Hubbard, 1972). These studies reported that ATP and acetylcholine-binding proteins were released, together with acetylcholine, upon nerve stimulation of mouse nerve-muscle preparations (phrenic nerve-diaphragm muscle) <u>in vitro</u>. Other diffusable neural factors from the motor neuron have also been described as having a trophic effect on acetylcholine receptor number and receptor aggregation (Cohen an

Fischbach, 1977; Podelski et al., 1978; Jessel et al., 1979; Kuromi et al., 1981; Kalcheim et al., 1982), but the biochemical isolation and characterization of these factors from nerve extracts has not been achieved.

Also of interest is a report by Davis and Kiernan (1980) demonstrating that sciatic nerve extract was able to reverse some of the muscle changes characteristic of denervation atrophy *in vivo* (i.e., weight and protein loss and reduction in diameter of type IIB myofibrils) observed in 7 day-denervated fast-twitch extensor digitorum longus muscle. Lentz et al. (1981) also described a myotrophic protein which they partially purified from rat brain. The protein, termed "neurotrophin", was characterized as a small (34,000-daltons) basic protein which was able to significantly increase the activity of 16.5 S acetylcholinesterase in cultured newt triceps muscle. However, the biochemical mechanism of action of neurotrophin is unknown.

B. Effect of Alterations of Neuromuscular Communication upon the Integrity of Skeletal Muscle

1. Effect of denervation upon muscle ultrastructure

When a skeletal muscle is deprived of its motor neuron supply (acetylcholineand axonally-transported neurotrophic factors), it undergoes certain anatomical and functional changes which collectively have been referred to as denervation atrophy (reviewed in Harris, 1974; Drachman,

1974; Gutmann, 1976; Harris, 1980). However, relatively few denervation-induced changes in skeletal muscle structure and function occurring before the onset of denervation atrophy have been described. These early changes are important in that they might be related to events closely regulated by a neurotrophic function of the motor nerve.

Degenerative changes at the neuromuscular junctions of rat diaphragm muscle have been described following cutting of the phrenic nerve which innervates the diaphragm (Pulliam and April, 1979 and 1979a). In these studies, particular attention was directed at unique changes in the various fiber types (red slow-twitch, white fast-twitch and intermediate muscle fibers) and also the effect of denervation period and nerve stump length on these changes. The axon terminals innervating white fast-twitch and intermediate muscle fibers were found to degenerate more rapidly than the axon terminals innervating red slow-twitch fibers. However, axon terminal degeneration preceded muscle fiber changes in all three fiber types and was visually apparent by 2 days after denervation. An increase in rough endoplasmic reticulum and Golgi membranes at subsarcolemmal regions were observed in all three fiber types at about 3 to 4 days after denervation. This in turn was followed by a disruption of sarcomere structure at 12 days post-denervation with white fast-twitch fibers affected greatest (Pulliam and April, 1979; Manalov, 1974). Interestingly, the morphological

changes described above appear later in a muscle denervated with a longer distal nerve stump length left attached to the muscle (Pulliam and April, 1979 and 1979a).

The relationship of denervation-induced morphological changes to the length of the distal nerve stump is significant in that it indicates that the alteration is due to an influence of the motor nerve apart from the impulse-directed release of acetylcholine. Impulse conduction in the distal nerve stump will cease immediately when the nerve is transected at any position along the length of the axon. However, neurotrophic mediators which are supplied by axoplasmic transport will be limited quantitatively and temporally by the length of the distal nerve stump attached to the muscle. Changes in muscle properties that are directly related to denervation period and nerve stump length, therefore, are interpreted to be neurotrophically regulated (Guth, 1968; Guth, 1974; Lubinska, 1975; Ochs, 1982).

An early onset in the loss of the post-junctional sarcolemmal infolding has been reported in soleus muscles of the mouse denervated for 5 days (Brown et al., 1982). These results were also reported by Tachikawa and Clementi (1979) who used the technique of freeze-fracture electron microscopy to show that soleus muscles denervated for 5 days also have an increased number of intramembrane particles in the postsynaptic infoldings as well as a significant increase in the number of pinocytotic vesicles. These findings are consistent with a denervation-induced alteration in the turnover of

membrane constituents (Tachikawa and Clementi, 1979) and the increase in the acetylcholine receptor population at denervated myoneural junctions (Levitt and Salpeter, 1981; Olek and Robbins, 1983). Additionally, histochemical analysis has indicated a loss of both endplate choline acetyltransferase (Davey and Younkin, 1978) and acetylcholinesterase (Davey and Younkin, 1978; Ranish et al., 1980) in muscle denervated for 3 days; these changes have also been related to denervation period and nerve stump length (Ranish and Dettbarn, 1978).

My studies have focused on denervation period and nerve stump length-dependent phosphorylative changes in the slow-twitch soleus muscle of the rat. Denervation atrophy (i.e., loss of muscle weight and protein) occurs in this homogeneous (Type I fiber) muscle of the rat hindlimb approximately three days after nerve section when a long 50 mm distal nerve stump is left attached to the muscle (Solandt and Magladery, 1940; Stonnington and Engel, 1973; Cullen and Pluskal, 1977). At this time mitochondrial swelling and an increase in rough endoplasmic reticulum are observed (Cullen and Pluskal, 1977). Within 2 weeks after the midthigh denervation, soleus muscle is reduced to one half its original weight (Solandt and Magledery, 1940) with myofibrillar proteins affected more than sarcoplasmic proteins (Goldberg, 1969). At 2 weeks post-denervation the cross-sectional myofibril area is also significantly decreased and by 4 to 8

weeks myofibrils are often completely lost when studied by light microscopy (Gutmann and Zelena, 1962). Early electron microscopy studies confirmed the finding of a decreased myofibril cross-sectional area and also revealed a proliferation of the sarcoplasmic reticulum and the transverse tubule system (Pellegrino and Franzini, 1963; Romanul et al., 1965; Engel and Stonnington, 1974). The decrease in the size of the myofibrils was interpreted to occur by a progressive lysis of the peripheral filaments. Interestingly, similar morphological changes were described in denervated muscle by Tower as early as 1939 and were also interpreted to represent a process of myofibrillar degeneration.

Sarcoplasmic changes that have been observed in denervated muscle include an increase in size and relative number of sarcolemmal nuclei (Sunderland and Ray, 1950; Guth, 1968). Also, the nuclei appear more rounded and migrate into the more central regions of the fibers (Guth, 1968). The increase in the number of sarcolemmal nuclei is apparently relative and this has been demonstrated biochemically by the finding of a constant total amount of DNA in muscles denervated for two weeks (Hogan et al., 1965). An increase in the relative amount of interfiber connective tissue has also been reported without a change in the total amount of collagen (Fischer and Ramsey, 1946; Stewart, 1955).

2. Effect of denervation upon muscle membrane electrophysiology

Denervation produces several changes in the electrophysiological properties of the muscle membrane. These electrophysiological changes in the muscle membrane are the earliest reported alterations observed in the denervated muscle. A reduction in the resting membrane potential occurs within 3 to 18 hours after denervation when a short nerve stump (2 mm or less) is left attached to the muscle. This response is delayed several hours when a long (30 to 40 mm) distal nerve stump was left attached (Albuquerque et al., 1971; Bray et al., 1976; Deshpande et al., 1980; Stanley and Drachman, 1980; Drachman et al., 1982). The denervation-induced decline in resting membrane potential occurs initially at the endplate region and then spreads to the extrajunctional region at later periods (Bray et al., 1976). The quantitative fall in potential is approximately 10 mV. The decline in resting membrane potential is thought to be related to the loss of some membrane component responsible for electrogenic hyperpolarization of the endplate (Bray et al., 1976) since the potential can be restored in denervated rat soleus muscle by increasing the activity of the Na^+/K^+ pump (Clausen and Flatman, 1977). Also, the quantitative fall in membrane potential is also obtained following a block of the pump with ouabain (Locke and Solomon, 1967). The rapidity of the denervation-induced partial depolarization of

the motor endplate (3 to 18 hr after denervation) suggested that it might be the initial and rate-limiting step in a chain of neurally-regulated membrane events (Deshpande et al., 1976).

Other changes in membrane physiology follow the decline in resting membrane potential. Resistance to the neurotoxin, tetrodotoxin (TTX), is observed at the endplate region within 2 days after denervation (Harris and Thesleff, 1972) and at any region of the muscle fiber after one week (Redfern and Thesleff, 1971). The denervation-dependent change in TTX resistance has also been related to the length of distal nerve stump left attached to the muscle since the effect appears earlier when the nerve is transected closer to the muscle (Thesleff, 1974). TTX is known to inhibit the propagation of the action potential by blocking Na^+ conductance.

Additionally, denervated muscle develops acetylcholine supersensitivity in the extrajunctional region as early as two days after denervation (Miledi and Potter, 1971; Harris and Thesleff, 1972; Uchitel and Robbins, 1978; Olek et al., 1981; Steinbach, 1981). The increase in extrajunctional acetylcholine supersensitivity has been described in terms of ionotophoretic changes involving changes in membrane permeability (Miledi and Zelena, 1966; Miledi and Potter, 1971; Kuffler and Yoshikawa, 1975). Also, an apparent increase in acetylcholine receptor number has been reported (Pestronk et al., 1976; Olek et al., 1981;

Steinbach, 1981; Drachman et al., 1982) which has been related to nerve stump length in denervated diaphragm muscle (Uchitel and Robbins, 1978). Denervation accelerates the turnover of junctional acetylcholine receptors from a half life of ten days to approximately two to three days (Loring and Salpeter, 1980; Levitt et al., 1980; Olek and Robbins, 1983; Stanley and Drachman, 1983) and also extrajunctional receptors from a half-life of 1 to 2 days to approximately 18 hours (reviewed in Fambrough, 1979; Pumplin and Fambrough, 1982).

Finally, fibrillatory potentials (which are observed no earlier than 42 hr after denervation when a short (5 mm) nerve stump is left attached to the soleus muscle and 48 hr when a longer (30 mm) nerve stump is left attached) usually appear near the endplate region first and then propagate from there along the entire length of the muscle fiber (Salafsky et al., 1968). A nerve stump length-related alteration in the fibrillatory activity of denervated muscle has also been reported by others (Luco and Eyzaguirre, 1955; Thesleff, 1974). Fibrillatory activity has been classified recently as an electrophysiological membrane parameter regulated by a trophic influence of the nerve possibly through the non-quantal release of acetylcholine (Thesleff, 1982).

3. Effect of denervation upon muscle biochemistry

The neuroregulation of the biochemical properties of skeletal muscle provides a molecular basis for neural maintenance of muscle structure and function. Early biochemical changes in muscle which have been related to denervation period and nerve stump length are summarized in Table 1 and include an increase in muscle proteolytic activity (McLaughlin et al., 1974), an increase in RNA synthesis (Politoff and Blitz, 1978), an increase in cyclic adenosine 3':5' monophosphate (Carlsen, 1975), an increase in the concentration of phosphatidyl ethanolamine (Fernandez et al., 1979), a decrease in glucose-6-phosphate dehydrogenase activity (Robbins and Carlson, 1979; Wagner and Max, 1979; Robbins, 1981) and a decrease in endplate acetylcholinesterase activity (Davey and Younkin, 1978; Ranish and Dettbarn, 1978; Ranish et al., 1980). The denervation period and nerve stump length-dependent change in glucose-6-phosphate dehydrogenase activity, however, was not observed when the slow-twitch rat soleus muscle was examined (Wagner and Max, 1979). Although the nerve stump length-dependent biochemical changes described occur within 12 to 72 hr after denervation, only those changes occurring before the onset of denervation atrophy are potentially important in defining the early muscle processes closely regulated by non-impulse mediated neural influences.

TABLE 1. BIOCHEMICAL PARAMETERS RELATED TO DENERVATION PERIOD AND NERVE STUMP LENGTH[a]

Parameter	muscle	nervestump length	lagperiod
proteolytic activity	rat extensor digitorum longus	2 to 5 mm	48 hr
"	"	30 to 35 mm	no increase
RNA synthesis	frog sartorius	0	3 days
"	"	2 cm	no increase
cyclic AMP concentration	rat gastrocnemius	5 mm	24 hr
"	"	1.5 cm	48 hr
phosphatidyl ethanolamine concentration	rat gastrocnemius	5 to 8 mm	24 hr
"	"	30 to 35 mm	3 days
glucose-6-phosphate dehydrogenase	rat diaphragm	0.5 cm	12 hr
"	"	3.5 cm	18 to 24 hr
endplate acetyl-cholinesterase	rat diaphragm	3 to 5 mm	24 hr
"	"	50 to 55 mm	36 hr

[a]Biochemical parameters which are altered after denervation and are temporally related to the denervation period and the length of the distal nerve stump left attached to the muscle. Relevant references are cited in the text.

Other biochemical changes occur in muscles denervated for a longer period of time and after the onset of denervation atrophy. The Ca^{+2}-activated adenosine triphosphatase (ATPase) activity, the actomyosin ATPase activity and the sarcoplasmic Ca^{+2} transport rate are altered in a muscle denervated for 1 week (Margreth et al., 1972; Palexas et al., 1981; Tate et al., 1983). Inhibition of Ca^{2+} uptake by the sarcoplasmic reticulum in muscles denervated for 9 days has also been confirmed by others (Wan et al., 1982). It is significant that the Ca^{+2}-transport process as well as the Ca^{+2}-dependent hydrolysis of ATP is affected after a 1 week denervation period. Ca^{2+} is known to regulate a Ca^{2+}-activated protease (Reddy et al., 1983) which may play a significant role in the development of muscle necrosis at the neuromuscular junction by catalyzing the dissolution of the Z-disks (Leonard and Salpeter, 1979). Furthermore, the denervation effect upon the functional properties of the sarcoplasmic reticulum may be important since these properties are closely correlated with the contractile properties of the muscle fibers (Heilmann and Pette, 1977; Wan et al., 1982).

The turnover of muscle membrane components mayalso be altered following denervation. A change in both ganglioside and lipid composition in skeletal muscle denervated for 7 to 9 days has been reported (Max et al., 1970; Kabara and Tweedle, 1981, respectively). These changes may be related

to the removal and insertion of membrane constituents by the process of endo- and exocytosis which also appear to be altered following denervation of skeletal muscle (Sellin et al., 1980). An elevated rate of endocytosis has been observed by following the increased uptake of extracellular markers (i.e., ^{3}H-inulin and horseradish peroxidase) into denervated fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus muscle (Libelius et al., 1981; Boegman and Scarth, 1981 and 1981a). This change along with a denervation-induced increase in lysosomal enzyme activity (Weinstock and Iodice, 1969) may be important in the turnover of membrane constituents in the denervated muscle. Interestingly, these biochemical changes are preceded by certain membrane physiological changes; i.e., the decline in resting membrane potential and the appearance of TTX-resistant action potentials (Sellin et al., 1980).

4. Neural regulation of skeletal muscle gene expression

It has been suggested that peripheral motorneurons exert a control over the skeletal muscles they innervate which may be expressed at the level of genome. This is based on the elimination of denervation-induced alterations in skeletal muscle with inhibitors of RNA and protein synthesis and also the effect of denervation upon the synthesis of specific muscle proteins. Blocking RNA synthesis with a single dosage of actinomycin D prevents the development of TTX-resistant action potentials, extrajunctional acetyl-

choline supersensitivity and the decline in the resting membrane potential for up to four days in a denervated muscle (Grampp et al., 1972). These parameters could also be blocked for longer periods with repeated injections of the antibiotic. Blocking transcription with a single dose of actinomycin D also delays the fibrillatory activity associated with denervated muscle for as long as three days (Muchnik et al., 1973). The increased number of extra-junctional acetylcholine receptors observed in denervated muscle as measured by ^{125}I-labeled alpha-bungarotoxin binding was blocked by actinomycin D and cycloheximide (Fambrough, 1979). This suggests that the increase in receptor number involved an acceleration of receptor biosynthesis (Fambrough, 1979). Actinomycin D was also shown to prevent the increase in glucose-6-phosphate dehydrogenase activity observed in rabbit muscle denervated for 18 hr (Ilyin et al., 1979).

The neural regulation of skeletal muscle gene expression has also been suggested from studies showing an effect of denervation, inactivity and cross-reinnervation upon the synthesis of specific proteins. In addition to the denervation-induced increase in the synthesis of the extra-junctional acetylcholine receptor protein described above (Fambrough, 1979), denervation also causes an increase in sarcoplasmic reticulum proteins (Pellegrino and Franzini, 1963) with a concomitant decrease in the synthesis of contractile proteins such as myosin (Goldberg, 1969). It

has also been shown that the myosin protein isolated from fast and slow-contracting muscle are qualitatively different and that these differences in the structure of the myosin molecule are reversed by cross-reinnervation of these muscles (Guth and Samaha, 1969; Buller et al., 1969; Mommaerts et al., 1969; Barany and Close, 1971; Sreter et al., 1974; Hoh, 1975). Myosins from slow and fast-contracting muscle can be distinguished on the basis of their light chain patterns resolved on polyacrylamide gels and also by the pattern of peptides generated by proteolytic digestion of the heavy chains (Unsworth et al., 1982; Pluskal and Sreter, 1983). Unsworth et al. (1982) also reported that immobilization (for 6 weeks) caused the slow-twitch soleus muscle to resemble a fast-twitch muscle in contractile properties, myosin-ATPase activity and the heavy chain pattern of myosin. Immobilization, however, did not affect these properties of fast-twitch muscle. Also, denervation caused a change in the isozyme patterns of glucose-6-phosphate dehydrogenase and lactate dehydrogenase which were restored upon reinnervation (Ilyin et al., 1979). The amount of a particular protein within the cell is controlled by its rate of synthesis and degradation and these processes, in turn, are controlled by a variety of biochemical mechanisms. The qualitative expression of a protein, such as that observed for myosin, glucose-6-phosphate dehydrogenase and lactate dehydrogenase, is determined by the activity and regulation of the genome. Therefore, it has been suggested that several biochemical

properties of skeletal muscle may be exerted through a regulation of gene expression in the muscle cell (reviewed in Guth, 1969).

The neural regulation of skeletal muscle transcriptional and translational events has been studied more specifically by showing an effect of denervation upon these processes. It has been reported that an increase in the activities of the nuclear RNA polymerases I and II in skeletal muscle occurs within 48 hr after midthigh denervation of the rat soleus (Held, 1978). Using a cell-free translational assay system and cDNA/RNA hybridization techniques, Metafora et al. (1980) reported a sequence-selective alteration in mRNA translation products at later times after denervation (8 days). It was suggested from these studies that the neural control of the types of muscle mRNA is at the level of transcriptional events. These results indicate the importance of defining early genetic changes associated with denervation. Alterations in genetic parameters which occur prior to denervation atrophy (but not immediately after the cessation of impulse-directed acetylcholine transmission) are of significance because they may suggest a neurotrophic regulation of muscle protein synthesis.

Several biochemical studies of denervated muscle have also focused on the neuroregulation of protein turnover in skeletal muscle. There is evidence that marked changes

in the average rate of protein degradation, as well as synthesis, contribute to muscle wasting during denervation atrophy and muscle growth during compensatory hypertrophy (Goldberg et al, 1974; Goldspink et al, 1983). Also, it is generally agreed that the appearance of denervation atrophy is related to an increased rate of protein degradation with little change or a decrease in protein synthesis (Goldberg, 1969; Goldspink, 1976, 1977 and 1978; Goldspink et al., 1983; Goldspink and Goldspink, 1977; Ramirez, 1980).

C. Cyclic AMP in Neurotrophic Functions

The molecular mechanism by which the muscle cell translates neurotrophic messages into biological responses is not known. Cyclic AMP has been implicated in the regulation of various intracellular processes, particularly in the translation of extracellular influences exerted by protein hormones, various neurotransmitters and depolarizing drugs into intracellular effects. These extracellular trophic effectors exert their effects in eukaryotic cells through the activation of receptor-coupled adenylate cyclase which generates cyclic AMP from ATP. The specific mode of action of cyclic AMP is to stimulate the inactive cytosolic cyclic AMP-dependent protein kinase holoenzyme which exists as a tetramer consisting of two catalytic subunits (C) and two cyclic AMP-binding regulatory subunits (R). Cyclic AMP activates the enzyme by binding to the R subunits. Binding leads to a rapid dissociation of the C and R subunits to

yield the free, active C subunit according to the following stoichiometry (Builder et al., 1980):

$$R_2C_2 + 4\ cAMP \rightleftharpoons R_2(cAMP)_4 + 2C$$

Activation of the cyclic AMP-dependent protein kinase by cyclic AMP leads to the subsequent phosphorylation of cell proteins (reviewed in Greengard, 1978; Cohen, 1982). Also, activation of the cyclic AMP-dependent protein kinase is the only known function of cyclic AMP in eukaryotic cells (Greengard, 1978).

Numerous properties of skeletal muscle are affected by the motor neuron through synaptic transmission and/or neurotrophic factors and some of these effects may be mediated by cyclic AMP. The evidence suggesting that cyclic AMP may be involved in neuromuscular communication centers around its role in pre- and post-synaptic events which occur in innervated tissue following nerve terminal depolarization (Greengard, 1978). One example involves synaptic transmission where neurotransmitters alter the endogenous cyclic AMP concentration of their target cells. Norepinephrine, epinephrine, dopamine, histamine, serotonin, glutamate, aspartate and octopamine all were shown to alter cyclic AMP concentration of the cerebral cortex, cerebellum and striatum in several species, including rat, rabbit, monkey and man (Kebabian, 1977).

It is thought that the membrane receptors for these neurotransmitters may be closely associated with the

regulatory subunit of adenylate cyclase - the membrane-bound enzyme which catalyzes the formation of cyclic AMP from adenosine triphosphate (Ross and Gilman, 1980). When the neurotransmitter or hormone binds with the regulatory subunit of the enzyme, the catalytic subunit (thought to be located on the inner surface of the cell membrane) is stimulated to form cyclic AMP which then mediates the intracellular effects of the extracellular effector (Ross and Gilman, 1980). Cyclic AMP is hydrolyzed in the cell by cyclic nucleotide phosphodiesterase to 5'-AMP (Appleman et al., 1973). Interestingly, it has been reported that the activities of both adenylate cyclase and phosphodiesterase are increased in skeletal muscle denervated for 1 to 2 days (McLane and Held, 1981). These results suggest that cyclic AMP metabolism in skeletal muscle is dependent upon a functional innervation. Also, the concentration of cyclic AMP in the target muscle cell is nerve stump length-dependent (Carlsen, 1975).

The intracellular effects of hormones and catecholamine neurotransmitters upon skeletal muscle which are mediated by the activation of membrane-bound adenylate cyclase are initiated at the cell membrane by binding to beta-adrenergic receptors coupled to adenylate cyclase. In homogenized muscle tissue, changes in beta-adrenergic receptor functions have been inferred from reported changes in membrane-bound adenylate cyclase activity and by using highly specific radiolabeled receptor binding analogs of beta-adrenergic agonists such as ^{3}H-dihydroalprenolol

(reviewed in Minneman et al., 1981; Chan-Palay et al., 1982). This radiolabeled receptor ligand has been used to obtain information regarding the neural regulation of beta-adrenergic receptors on skeletal muscle. Banerjee et al. (1977) reported a marked increase in the specific binding of ^{3}H-dihydroalprenolol to muscle particulate fractions as early as 4 days after denervation. However, Smith et al. (1978) reported that the population of beta-adrenergic receptors on skeletal muscle denervated for 5 days did not differ from unoperated control muscles. It has been proposed that neural factors other than acetylcholine released by the motor neuron may participate in the regulation of beta-adrenergic receptor density at post-synaptic sites (Banerjee et al., 1977). The neural factor has not been determined, but catecholamine synthesizing enzymes and peptides have been found at the neuromuscular junction (Chan-Palay et al., 1982; Adams and O'Shea, 1983).

Other evidence suggesting that cyclic AMP is a mediator of some neurotrophic message is less direct and involves the ability of cyclic AMP to affect the morphology of the motor endplate and to stimulate and maintain cellular differentiation in embryonic skeletal muscle . Cyclic AMP has an effect similar to peripheral nerve extracts in maintaining the endplate acetylcholinesterase activity in denervated muscle (Lentz, 1972 and 1972a). Also, its concentration in cultured embryonic chick muscle cells is

affected by a neurally-derived myotrophic protein and this myotrophic protein increases the acetylcholine receptor number of aneural embryonic chick skeletal muscle (Markelonis et al.,1982a; Oh and Markelonis, 1982). It was suggested from these studies that the myotrophic protein might induce acetylcholine receptor synthesis via regulation of muscle cyclic AMP concentration. This is an attractive hypothesis in view of the findings which indicate that cyclic AMP regulates acetylcholine receptor number directly (Blosser and Appel, 1980) and indirectly through beta-adrenergic receptor activation by isoproterenol (Blosser, 1983). When skeletal muscle treated with cyclic AMP is examined microscopically, the morphology of the endplate is maintained in a manner similar to muscle cells cultured in the presence of peripheral nerve extract (Lentz, 1972a). Cyclic AMP, therefore, appears to have some influence on the structure and biochemical properties of the endplate. However, this may not be a specific effect since cyclic AMP generally stimulates the growth, differentiation and shape of the neural cell (Prasad et al., 1972; Prasad and Kumar, 1975; Oey, 1975). Cyclic AMP also mimics some of the neural influence on muscle by stimulating the efflux of sodium ions in muscle fibers (Bittar et al., 1976).

D. Protein Phosphorylation in Skeletal Muscle

1. Types of protein kinases

The biochemical basis for the ability of mammalian skeletal muscle protein kinases to alter the activity and regulatory function of key cellular proteins lies in the ability of these proteins to preserve some of the high energy of the nucleoside triphosphate bond within their structure when phosphate is covalently bound to a specific residue (i.e., usually serine or threonine). Protein kinases (EC 2.7.1.37-ATP:protein phosphotransferases) are enzymes which catalyze the transfer of the terminal (gamma) phosphate of ATP to the hydroxyl containing amino acid (reviewed in Carlson et al., 1979). Initially, the classification of the various protein kinases from mammalian skeletal muscle was based upon the specificity of the kinase with respect to the protein substrate (Krebs et al., 1972; Krebs, 1983). However, the protein kinases activated by cyclic AMP have a broad substrate specificity and several of the substrates for cyclic AMP-dependent protein kinase are phosphorylated by other protein kinases not affected by cyclic AMP. Thus, the classical nomenclature becomes inoperable. Weller (1979) has organized a classification system for protein kinases based on both substrate specificity and the nucleotide dependency or independency of the phosphotransferase reaction. Type a_1 protein kinases are defined as catalyzing the phosphorylation of histones and other proteins and are stimulated by cyclic

AMP. Type a_2 kinases also catalyze the phosphorylation of histones, but are activated by cyclic GMP. Type b protein kinases catalyze the phosphorylation of phosvitin and are not affected by cyclic nucleotides. Type c kinases are generally considered to be nuclear kinases and phosphorylate histones, but are cyclic AMP-independent.

a. type a_1 protein kinases

The activation of cyclic AMP-dependent protein kinases occurs by a cyclic AMP-promoted dissociation of the holoenzyme to yield the catalytic species (C) and a complex consisting of regulatory subunit (R) and cyclic AMP as described earlier in this literature review (pg. 49). The concentration of cyclic AMP necessary to obtain half-maximal phosphotransferase activity (K_a) for pure preparations of holoenzyme was found to be in the range of 0.2 to 0.3 micromolar (Flockhart and Corbin, 1982). The maximum amount of cyclic AMP bound to skeletal muscle holoenzyme is 4 moles of cyclic AMP per mole of tetramer.

The effect of MgATP on the holoenzyme is to increase the apparent dissociation constant for cyclic AMP by a factor of approximately 10-fold (Hofmann et al., 1975). As a result, the activation of holoenzyme can be modulated by the range of cyclic AMP and ATP concentrations found in the tissue. In skeletal muscle the ATP concentration is in the millimolar range; therefore, the holoenzyme should contain

bound ATP under most conditions. The result of MgATP binding is to increase the K_d for cyclic AMP to coincide with the physiological concentrations of that effector. It has been suggested that any agent capable of altering ATP binding would offer an additional regulatory feature to the enzyme (Flockhart and Corbin, 1979).

The concentration of cyclic AMP in skeletal muscle isapproximately equivalent to the K_a (0.2 to 0.3 micromolar) of the holoenzyme for cyclic AMP (Beavo et al., 1974). As a result, a number of investigators have speculated that much ofthe cyclic AMP in the cell must be compartmentalized for otherwise protein kinase would always be completely activated by the basal level of cyclic AMP in the muscle cell (Walsh et al., 1972). However, another intracellular regulator known to affect the activity of cyclic AMP-dependent protein kinase is the heat-stable protein kinase inhibitor first described by Ashby and Walsh (1972). The inhibitor binds tightly to the free catalytic subunit, but not to the regulatory-catalytic subunit complex (Ashby and Walsh, 1972). These authors speculated that enough of this protein is present in most tissues to inhibit about 20% of the total protein kinase activity present in the tissue. As such, the inhibitor could indirectly alter the amount of cyclic AMP needed to allow expression of protein kinase activity. Kinetic studies have shown that the heat-stable protein is a noncompetitive inhibitor with respect to ATP and protein substrates (Demaille et al., 1978; Ashby and Walsh, 1972). However,

further studies based upon analyses of tightly bound substrates suggested that it is a competitive inhibitor with a K_i of 2×10^{-9} M (Demaille et al., 1978).

b. type a_2 protein kinases

Guanosine 3':5'-monophosphate-dependent protein kinase has been identified in various tissues of many species (reviewed in Glass and Krebs, 1980). However, the concentration of this enzyme is reportedly highest in mammalian brain (Aswad and Greengard, 1981) and lowest in mammalian skeletal muscle (Lincoln and Corbin, 1977). In analogy with the observations that cyclic AMP effects are mediated through cyclic AMP-dependent protein kinase, Kuo and Greengard (1970) have proposed that a specific cyclic GMP-dependent protein kinase mediates the biological effects of cyclic GMP. Cyclic GMP-dependent protein kinase resembles the cyclic AMP-dependent protein kinase in exhibiting high specificity and affinity for the cyclic nucleotide and in the ability of the enzyme to catalyze the transfer of ATP to protein, usually to serine residues (Glass and Krebs, 1980). Studies of the subunit structure of the holoenzyme have been carried out with the enzyme isolated from bovine lung and have shown that the enzyme is composed of two identical 75,000-dalton subunits (Gill et al., 1977). Unlike the cyclic AMP-dependent protein kinase, the subunits of the cyclic GMP-dependent protein kinase do not dissociate upon activation by cyclic

GMP (Gill et al., 1977). However, the cyclic GMP- and cyclic AMP-dependent protein kinases are similar with respect to substrate specificity, in particular histone, and both enzymes show a 70% similarity in amino acid composition. Some authors have suggested that these kinases evolved from a common ancestral protein (Gill, 1978; Lincoln and Corbin, 1977).

Cyclic GMP-dependent protein kinases are not affected by the heat-stable inhibitor which acts on the free catalytic subunit of the cyclic AMP-dependent protein kinase. However, crude preparations of the inhibitor protein were found to stimulate cyclic GMP-dependent protein kinase activity, but this was later found to be due to a modulator protein which co-purified with the inhibitor (Kuo et al., 1976). This modulator does not affect cyclic AMP-dependent protein kinase activity. It has also been shown that cyclic GMP-dependent protein kinase can undergo an autophosporylation reaction of unknown function (Foster et al., 1981; Takio et al., 1983).

c. cyclic nucleotide-independent protein kinases

The function and regulation of the cyclic nucleotide-independent protein kinases in skeletal muscle has not received as much attention as the cyclic nucleotide-dependent protein kinases and, therefore, much less is known about these enzymes. Cyclic nucleotide-independent protein kinases which are activated by Ca^{2+} and calmodulin (Cheung,

1980) and Ca^{2+} and phospholipid (Katoh et al., 1983) have been isolated from skeletal muscle and characterized. Various studies have established that these kinases play important roles in the regulation of muscle contraction through the phosphorylative modification of myofibrillar proteins (Adelstein et al., 1981; Walsh and Guilleux, 1981; Marston, 1982; Katoh et al., 1983). In contrast, the physiological significance of the cyclic nucleotide- and Ca^{2+}-independent protein kinases is unknown. This class of protein kinase, initially isolated as casein kinase, have been shown to phosphorylate glycogen synthetase (DePaoli et al., 1981), protein synthesis initiation factors (Hathaway et al. 1979), non-histone nuclear chromatin proteins (Christman an Dahmus, 1981) and troponin T (Villar-Palasi and Kumon, 1981). Although many of these phosphorylations were reported in tissues other than skeletal muscle, it is interesting that a protein kinase from rabbit skeletal muscle which functions independent of Ca^{2+} and cyclic nucleotides was found to phosphorylate glycogen synthetase, phosphorylase kinase, troponin, myosin light chain and myosin light chain kinase (Singh et al., 1982).

2. Physiological functions of protein kinases

The role of cyclic AMP-dependent protein phosphorylation as an important mechanism involved in the regulation of various cellular processes was first discovered

in skeletal muscle by studies of the neural and hormonal regulation of glycogen metabolism (reviewed in Krebs et al., 1972; Krebs and Beavo, 1979; Cohen, 1980; Adelstein et al., 1981; Cohen, 1983). Glycogen is a major source of energy for muscle contraction. Its breakdown and synthesis (glycogen metabolism) and utilization in the form of glucose (glycolysis) are regulated by neural influences related to muscle contraction and by hormones through changes in the phosphorylation of muscle proteins. This regulatory scheme is now known to involve several protein kinases, protein phosphatases and specific cytosolic and particulate muscle proteins. Some of these proteins are phosphorylated in response to the second messengers Ca^{2+} and cyclic AMP. Phosphorylation reactions in skeletal muscle may also be important for the regulation of the impulse-dependent contractile process. Calcium ions released from the sarcoplasmic reticulum in response to a nerve stimulus activate the interaction between the thick myosin filaments and the thin filaments of actin. The interaction between these two major contractile proteins, actin and myosin (and other proteins such as tropomyosin and troponin) contribute to the regulation of muscle contraction. This may occur through the phosphorylative modification of these proteins (Adelstein et al., 1981).

These systems (glycogen metabolism, glycolysis and muscle contraction) and associated enzymes and substrates provide a detailed examination of the phosphorylation-

dephosphorylation process in skeletal muscle. A review of these processes during the course of my research was pertinent toward characterization of the denervation period and nerve stump length-dependent phosphorylation reaction (specific aim 2) and identification of the substrate(s) for this neurally-regulated reaction (specific aim 3).

a. protein phosphorylation and the regulation of muscle glycogen metabolism

Although an important aspect of the regulation of glycogen metabolism in skeletal muscle involves the interaction of metabolites with key enzymes (i.e., allosteric or metabolic control), the major regulatory mechanism governing the rate of glycogenesis and glycogenolysis in this tissue is the metabolic interconversion of enzymes by phosphorylation and dephosphorylation. Studies of these pathways led to the initial identification of cyclic AMP (Rall et al., 1957) and the cyclic AMP-dependent protein kinase (Walsh et al., 1968). When skeletal muscle is stimulated electrochemically by the transsynaptic diffusion of acetylcholine, calcium ions from the sarcoplasmic reticulum initiate muscle contraction and also activate phosphorylase kinase. As a result, glycogen phosphorylase undergoes phosphorylative modification and glycogenolysis is increased to provide ATP required to sustain muscle contraction. Phosphorylase kinase, in turn, phosphorylates glycogen synthetase. This

results in a decrease in activity of glycogen synthetase. Thus, the two opposing pathways are regulated. Glycogen synthetase is the rate-limiting enzyme in the biosynthetic pathway and exists in a non-phosphorylated or "I" form and a phosphorylated or "D" form (Friedman and Larner, 1963). Glycogen synthetase I, the glucose-6-phosphate independent form of the enzyme, is considered to be more active physiologically than the glucose-6-phosphate dependent or "D" form which is strongly inhibited by metabolites. Recent evidence suggests that the conversion may be complex, potentially involving multiple phosphorylation sites on the 85,000-dalton glycogen synthetase subunit and several distinct protein kinases (reviewed in Soderling, 1979). For example, both cyclic AMP-dependent protein kinase and phosphorylase kinase can phosphorylate and inactivate glycogen synthetase (DePaoli-Roach et al., 1979a). It has also been established that another general class of protein kinase, unaffected by cyclic AMP, can phosphorylate glycogen synthetase (DePaoli-Roach et al., 1981).

Phosphorylase kinase is the key to both the neural and hormonal mechanisms for stimulating glycogenolysis. This enzyme is dependent on Ca^{+2} and can also be activated by cyclic AMP-dependent protein kinase. Phosphorylase kinase acts on glycogen phosphorylase b to catalyze a Ca^{+2}-dependent phosphorylation of phosphorylase b to yield the active "a" form (reviewed in Krebs and Beavo, 1979). Skeletal muscle

phosphorylase kinase is currently believed to be a hexadecamer with four different types of subunits designated alpha, beta, gamma and delta (Shenolikar et al., 1979). The alpha and beta subunits each contain a unique amino acid sequence with a serine residue as phosphate acceptor (Yeaman et al., 1977). Phosphorylation of these sites is catalyzed by cyclic AMP-dependent protein kinase. The beta subunits are phosphosphorylated most readily and this action has been correlated with activation of the enzyme (Cohen, 1973). Catalytic activity has been correlated with the beta and gamma subunit of the enzyme (Skuster et al., 1980).

The relationship of calcium to phosphorylase kinase is complex. For example, the recently detected small molecular weight gamma subunit has been shown to be identical to calmodulin - a calcium binding protein first identified in brain (Shenolikar et al., 1979; Grand et al., 1981). Calmodulin has been shown to activate cyclic nucleotide phosphodiesterase (Teo and Wang, 1973), myosin light chain kinase (Yagi et al., 1978) and Ca^{+2}-dependent adenosine triphosphatase(Gopinath and Vincenzi, 1977). Also, it is becoming increasingly evident that calmodulin is a primary intracellular calcium binding protein. The biochemistry of calmodulin has been recently reviewed (Cheung, 1980; Wolff and Brostrom, 1979; Klee et al., 1980; Means and Dedman, 1980). A second role of calcium in the regulation of phosphorylase kinase has recently been determined. This regulatory role is also due to the calcium/calmodulin

complex. In this case, calmodulin is not acting as the gamma subunit, but in a looser association with phosphorylase kinase. This exogenous calmodulin has been shown to activate phosphorylase kinase to a greater extent than that observed with calcium alone (DePaoli-Roach et al., 1979a). Apparently, phosphorylase kinase possesses two types of calmodulin binding sites. Picton et al. (1980) have presented evidence through the use of a cross-linking reagent that an association exists between the gamma and delta subunits and between exogenous calmodulin and both alpha and beta subunits. Finally, a calcium-dependent protease can irreversibly activate phosphorylase kinase by cleavage of a peptide from the protein (Huston and Krebs, 1968).

It has also been recently demonstrated that cyclic GMP-dependent protein kinase can phosphorylate the same two serine residues on phosphorylase kinase as cyclic AMP-dependent protein kinase (Cohen, 1980). The physiological significance of the cyclic GMP-dependent phosphorylation is questionable, however, in that it occurs at a very slow rate and the concentration of cyclic GMP in skeletal muscle is very low (Lincoln and Corbin, 1977).

Also, rabbit skeletal muscle phosphorylase kinase is activated through a cyclic nucleotide and calcium-independent phosphorylation reaction (Singh et al., 1982). The protein kinase involved in this phosphorylation reaction was also found to phosphorylate troponin, myosin light chain and

myosin light chain kinase *in vitro* (Singh et al., 1982). These results suggest that this protein kinase may resemble the cyclic AMP-dependent protein kinase in that it appears to have multiple cellular functions.

b. protein phosphorylation and the regulation of muscle glycolytic enzymes

Although the cyclic AMP-mediated regulation of glycolysis in liver has been well documented (reviewed in Exton and Harper, 1975), a role for this nucleotide in skeletal muscle glycolysis is not firmly established. Studies of the cyclic AMP-dependent phosphorylation of glycolytic enzymes in skeletal muscle have focused on two key regulatory enzymes in glycolysis - phosphofructokinase and pyruvate kinase.

Phosphofructokinase is subject to a complex allosteric regulation by a number of metabolites (reviewed in Uyeda, 1979). It has been shown that skeletal muscle phosphofructokinase exists in a partially phosphorylated state *in vivo* (Hussey et al., 1977; Hofer and Sorenson-Ziganke, 1979) and consists of 4 phosphates per tetramer when the muscle is at rest and 8 phosphates per tetramer when the muscle is stimulated to contract (Hofer and Sorenson-Ziganke, 1979). Furthermore, the *in vitro* phosphorylation of the enzyme by the catalytic subunit of cyclic AMP-dependent protein kinase has been demonstrated (Riquelme et al., 1978 and 1982). It has been reported that the phosphorylation of

phosphofructokinase by the cyclic AMP-dependent protein kinase leads to inactivation of the enzyme (Schaftingen et al., 1981). Also, it is now known that the *in vivo* site of phosphorylation by the cyclic AMP-dependent protein kinase occurs on an 80,000-dalton subunit of skeletal muscle phosphofructokinase (Schaftingen et al., 1981).

Other studies have attempted to demonstrate the cyclic AMP-dependent phosphorylation of skeletal muscle pyruvate kinase. Pyruvate kinase is also involved in a key irreversible reaction in glycolysis by catalyzing the conversion of phosphoenolpyruvate to pyruvate. To date, however, no known phosphorylation of skeletal muscle pyruvate kinase has been demonstrated (Humble et al., 1975; Cohen et al., 1975; Berglund et al., 1977; Kirschenlohr and Hofer, 1983). In fact, only phosphofructokinase has been demonstrated to be a physiologically important soluble phosphorylatable glycolytic enzyme in skeletal muscle (Kirschenlohr and Hofer, 1983).

c. protein phosphorylation and the regulation of muscle contraction

Among the myofibrillar proteins shown to be phosphorylated are the light chains of skeletal muscle myosin (Perrie et al., 1973; Adelstein and Eisenberg, 1980; Stull, 1980; Adelstein et al., 1981). The myosin light chains are phosphorylated bya myosin light chain kinase dependent on

Ca^{+2}/calmodulin (reviewed in Stull, 1980; Adelstein and Eisenberg, 1980) and also by a protease-activated kinase I independent of Ca^{+2}/calmodulin (Tuazon et al., 1982). It is currently thought that phosphorylation of the smooth muscle myosin light chains may play a major role in regulating the actin-activated adenosine triphosphatase activity of myosin. phosphorylation apparently reverses the inhibition of the 20,000-dalton light chain on the actin-activated adenosine triphosphatase activity (Seidel, 1980). The role of myosin light chain phosphorylation in skeletal muscle is currently unclear. Recent reports suggest that myosin light chain phosphorylation is correlated with a decrease in the myosin adenosine triphosphatase activity in the fast-twitch extensor digitorum longus muscle *in vivo*, but this was not observed in the slow-twitch soleus muscle (Crow and Kushmerik, 1982). Also, it was reported that myosin light chain phosphorylation in skeletal muscle was increased *in vivo* following stimulation of the muscle with a low frequency electrical probe (Klug et al., 1982; Stull et al., 1983). Phosphorylation was increased in tetanized fast and slow-twitch muscle also (Barany et al., 1983).

Another myofibrillar protein known to undergo enzymatic phosphorylation-dephosphorylation is troponin (Stull et al., 1972). This protein is necessary for the calcium sensitization of the actomyosin adenosine triphosphatase activity. Examination of the individual subunits of rat skeletal muscle troponin revealed that cyclic

AMP-dependent protein kinase phosphorylates the inhibitory subunit of troponin (troponin I) *in vitro* (Bailey and Villar-Palasi, 1971). However, troponin T (tropomyosin binding subunit) and troponin C (calcium binding subunit) did not undergo this cyclic AMP-dependent phosphorylative modification (Bailey and Villar-Palasi, 1971; Perry and Cole, 1973). It was also shown that phosphorylase kinase could phosphorylate troponin I (Stull, 1972) as well as purified troponin T (Perry and Cole, 1973). Currently, an association between the phosphorylation of troponin T or I and changes in the contractile properties of skeletal muscle has not been demonstrated.

Smooth muscle myosin light chain kinase can also serve as a substrate for the cyclic AMP-dependent protein kinase (reviewed in Adelstein et al., 1981). When the calmodulin-bound myosin light chain kinase is used as a substrate for cyclic AMP-dependent protein kinase, there is an inhibition of the phosphorylation of one of the two acceptor sites on the enzyme. Additionally, cyclic AMP-dependent phosphorylation of the myosin light chain kinase leads to a marked decrease in the binding affinity of the enzyme for calmodulin which, in turn, leads to a decrease in myosin light chain kinase activity (reviewed in Adelstein et al., 1981). The dynamic interactions between myosin light chain kinase, calmodulin and cyclic AMP-dependent protein kinase which lead to an alteration of myosin light chain

kinase activity have not been demonstrated for skeletal muscle.

E. Protein Dephosphorylation in Skeletal Muscle

The level of phosphorylation of any cellular protein is dependent on the balance of the activities of the protein kinases and protein phosphatases that act on it. It is becoming increasingly clear that protein phosphatases like protein kinases are the target of extracellular effectors of skeletal muscle function. Four distinct protein phosphatases which are termed protein phosphatase 1, 2A, 2B and 2C have been isolated and characterized from skeletal muscle (Ingebritsen and Cohen, 1983). These are capable of dephosphorylating the enzymes of glycogen metabolism (Cohen, 1981). Protein phosphatase 1 specifically dephosphorylates the beta subunit of phosphorylase kinase and is inhibited by protein phosphatase inhibitors 1 and 2. The type 2 phosphatases preferentially dephosphorylate the alpha subunit and are not affected by the inhibitor proteins (Cohen, 1981). The concentrations of phosphatases 2A and 2C are very low and only account for a small percentage of the phosphatase activity directed towards the enzymes of glycogen metabolism (Cohen, 1978).

Protein phosphatase 1 is probably the most important of the dephosphorylating enzymes in skeletal muscle since it is the major phosphatase for glycogen synthetase, glycogen phosphorylase and the beta subunit of phosphorylase kinase

(Cohen et al., 1982; Cohen, 1983). Also, protein phosphatase 1 is not only associated with glycogen (Ingebtitsen and Cohen, 1983), but also with microsomes. This suggests a role for protein phosphatase 1 in cholesterol synthesis and protein synthesis (Ingebritsen and Cohen, 1983). In liver, this phosphatase dephosphorylates acetyl-CoA carboxylase, hydroxy-methylglutaryl-CoA (HMG-CoA) reductase and HMG-CoA reductase kinase (Ingebritsen et al., 1981).

Protein phosphatases 2A and 2C have been isolated from muscle as myosin light chain phosphatases and may be involved in the regulation of muscle contraction (Ingebritsen and Cohen, 1983). However, protein phosphatase 1 and 2B are also myosin light chain phosphatases <u>in vitro</u> and are also the major phosphatases which dephosphorylate protein synthesis initiation factor eIF-2 (Ingebritsen and Cohen, 1983).

Protein phosphatase 2B is a calcium/calmodulin-dependent protein phosphatase and is present at high concentrations in skeletal muscle and brain (Ingebritsen and Cohen, 1983). It appears to be identical to calcineurin - a major calcium binding protein in brain (Stewart et al., 1982). Its immunohistochemical localization at postsynaptic densities and microtubules of postsynaptic dendrites has suggested a role for this enzyme in neurotransmitter action and microtubular function (Wallace et al., 1980). Although it may be the primary alpha-phosphorylase kinase phosphatase

(Cohen et al., 1982), the function of phosphatase 2B in skeletal muscle is still unclear.

F. Cyclic AMP-Dependent Protein Kinase Isozymes I and II

1. Holoenzymes

In mammalian systems there are two types of soluble cytosolic cyclic AMP-dependent protein kinases (I and II) which are distinguished by their elution from anion exchange resins (Beavo et al., 1975; Corbin et al., 1975; Corbin and Keely, 1977). Type I protein kinases are eluted with approximately 0.1 M NaCl, whereas type II kinases are eluted at higher NaCl concentrations of 0.15 to 0.2 M. In addition to electrostatic differences, the two types of cyclic AMP-dependent protein kinases differ immunologically (Fleischer et al., 1976; Hofmann et al., 1977; Weber et al., 1981) and genetically (Costa et al., 1976a). Differences are also observed in the kinetics of their subunit (regulatory and catalytic) dissociation/reassociation in the presence of cyclic AMP (Hofmann et al., 1975; Rosen and Erlichman, 1975; Rangel-Aldao and Rosen, 1977). Additionally, the inactive holoenzyme of type I protein kinase has a high affinity (dissociation constant = 50 nanomolar) binding site for ATP which serves as an important regulatory mechanism for this isozyme (Bechtel et al., 1975). Also, the regulatory and catalytic subunits of the two protein kinase isozymes from several diverse species can form heterologous holoenzymes

which indicates that these proteins contain highly conserved structures.

2. Catalytic subunits

The C subunits of types I and II cyclic AMP-dependent protein kinase appear to be identical proteins (reviewed in Flockhart and Corbin, 1982). Both proteins have a relative molecular weight on SDS polyacrylamide gels between 37,000 and 41,000 (Hofmann et al., 1975; Taylor and Stafford, 1978). The isoelectric profiles of the type I catalytic subunit indicates that this protein has two isoelectric points of 7.4 and 8.5 which are similar to those reported for the type II enzyme (Bechtel et al., 1977; Peters et al., 1977). These authors also showed that the two proteins have similar amino acid compositions and contain a high proportion of serine and glycine residues. The complete amino acid sequence of the 349-residue catalytic subunit of bovine cardiac muscle is known and the molecular weight of the protein predicted from the amino acid sequence is 40,580 (Shoji et al., 1981). The type I subunit has been shown to undergo an autophosphorylation reaction when incubated with ^{32}P-ATP *in vitro* (Chiu and Tao, 1978). This autophosphorylation reaction has not been demonstrated for the type II enzyme, but phosphate bound to serine and threonine residues has been found in the purified protein (Shoji et al., 1979; Shoji et al., 1981). Schwoch et al. (1980) has determined that the catalytic subunits from types

I and II protein kinases are immunologically indistinguishable. Also, the catalytic subunits have a similar K_m for ATP (approximately 2 millimolar) and phosphorylate the same protein substrates *in vitro* with similar reaction rates (reviewed in Flockhart and Corbin, 1982). They also have a similar ability to interact with either type I or type II regulatory subunits (reviewed in Flockhart and Corbin, 1982).

3. Regulatory subunits

The differences in the protein kinase isozymes appear to be due to differences in the regulatory subunits only (Hofmann et al., 1975). The regulatory subunit of the type I kinase (RI) has an estimated molecular weight of 47,000 to 49,000 as determined by SDS gel electrophoresis (Hofmann et al., 1975; Zoller et al., 1979). In contrast, the cyclic AMP-binding subunit of the type II cyclic AMP-dependent protein kinase (RII) has a molecular weight of 55,000 to 56,000 estimated by SDS polyacrylamide gel electrophoresis (Zoller et al., 1979) and exists as a dimer of two 56,000-dalton proteins (Erlichman et al., 1973; Corbin et al., 1978).

The RI protein can be phosphorylated by C in an intermolecular reaction (Huang et al., 1983). RII not only differs significantly from RI in its primary structure, but also in its ability to undergo an autophosphorylation

catalyzed in an intramolecular reaction by C (Erlichman et al., 1974; Hofmann et al., 1975; Rangel-Aldao and Rosen, 1977; Geahlen et al., 1982; Erlichman et al., 1983). The phosphorylation was determined to be an intramolecular reaction because the rate of the reaction was not affected by either serial dilution of the enzyme or by the presence of an inhibitor of the free cyclic AMP-dependent protein kinase catalytic subunit (Erlichman et al., 1983).

RI can also be phosphorylated by cyclic GMP-dependent protein kinase *in vitro* and *in vivo* (Geahlen and Krebs, 1980; Geahlen et al., 1982). The physiological significance of RI phosphorylation by cyclic GMP-dependent protein kinase in tissues like skeletal muscle which have low cyclic GMP concentrations is questionable (Lincoln and Corbin, 1977). Also, the reaction proceeds at a veryslow rate (Geahlen et al., 1982).

The cyclic AMP-binding sites on the regulatory subunits of types I and II cyclic AMP-dependent protein kinase have been shown to differ considerably with respect to cyclic nucleotide analog specificity (Yagura et al., 1980). Also, the effect of MgATP on the dissociation of cyclic AMP from the regulatory subunits differs in the two isozymes (Hoppe et al., 1977). The RI protein is known to have a high affinity binding site for MgATP which is not found on the RII protein. The effects of analogs on cyclic AMP dissociation from the regulatory subunits also indicates that these two proteins have different cyclic AMP binding sites. Using

intrinsic tryptophan fluorescence and a fluorescent analog of cyclic AMP, it was experimentally determined that the RI subunits appear to have only one class of cyclic AMP-binding site (LaPorte et al., 1980; Smith et al., 1981) because each of the two RI subunits bind 2 molecules of cyclic AMP with positive homotropic cooperativity (Smith et al., 1981). Two cyclic AMP-binding sites on RII (A and B) have been described. It has been reported that cyclic AMP will bind to site A at a faster rate than it will bind to site B *in vitro* under reaction conditions of 37°, pH 7.0 and 0.15 M KCl (Ogreid and Doskeland, 1981). These authors have also suggested that only site B can react with cyclic AMP *in vivo*. Binding of cyclic AMP to site B presumably alters the conformation of RII allowing the more rapid binding of cyclic AMP to site A under physiological conditions. This cooperativity of cyclic AMP binding to RII has also been recently described by Robinson-Steiner et al. (1983). These authors also reported that binding of cyclic AMP to both sites is necessary for dissociation of the R and C subunits and, therefore, enzyme activation. Additionally, the conformational change in the RII protein first suggested by Ogreid and Doskeland (1981a) is now known to occur via an intersubunit crosslinking reaction which is a consequence of the binding of cyclic AMP to RII at site B (Huang, 1982).

4. Physiological roles

It is currently thought that the structural and functional differences in the cyclic AMP-dependent protein kinases may be related to subcellular localization of the isozymes and, in turn, to specification of their physiological functions. It is known that the two isozymes differ in their tissue distribution and vary in their relative amounts during development (Corbin et al., 1975; Lee et al., 1976). Type I kinase accounts for about 70% of the total soluble cyclic AMP-dependent protein kinase activity of skeletal muscle and type II accounts for the remaining 30% (Corbin et al., 1975).

Although very little is currently known about the biological differences of these two isozymes, they apparently have differing effects on the regulation of normal cellular processes such as cell proliferation (Fleischer et al., 1976), differentiation (Lee et al., 1976; Russell, 1976; Haddox et al., 1979; Boynton et al., 1981; McClung and Kletzian, 1981; Kwost-Welfeld and Kaniuga, 1981), hormonal and mitogenic cellular stimulation (Harper et al., 1981; Koide et al., 1981; Mednieks and Jungmann, 1982) as well as abnormal processes such as tumor cell growth (Majumder, 1977; Walter et al., 1979; Evans et al., 1982; Mednieks et al., 1982; Cho-Chung, 1979).

Additionally, the RII subunit, unlike the RI protein, can be separated into a subclass that appears to be

neural tissue specific and another subclass of non-neural origin (Erlichman et al., 1980). The subclassification of these proteins was based on immunochemical analysis where the type II protein derived from neural tissues bound anti-RII antibodies raised against neural RII more tightly than RII proteins from non-neural tissues. A comprehensive analysis of the RII protein from a variety of tissues using type-specific antibody has demonstrated the presence of cyclic AMP-binding proteins of 52,000 and 50,000-daltons (Weber et al., 1981). These cyclic AMP-binding proteins have been found in kidney, HeLa cells, lymphocytes (Weber et al., 1981) and adrenal chromaffin secretory granule cells (Treiman et al., 1983). Although these proteins have been referred to as RII variants (Weber et al., 1981), there is some evidence that they may represent proteolytic cleavage of the 56,000-dalton RII protein (Rangel-Aldao et al., 1979). These authors also found the 52,000-dalton RII protein in heart muscle which also had an identical tryptic peptide mapping pattern as the 56,000-dalton RII protein. Variants of the RI protein have not been demonstrated.

It is generally believed that several normal and abnormal cellular functions are regulated by extracellular effectors which are capable of dissociating soluble cyclic AMP-dependent protein kinase by increasing cyclic AMP levels. This is followed by an alteration in nuclear protein phosphorylation as a result of a cyclic AMP-mediated translocation of regulatory and catalytic subunits of

cytoplasmic protein kinase to the nucleus (reviewed in Jungmann et al., 1975; Jungmann and Russell, 1977; Jungmann and Kranias, 1977; Rosenfeld and Barrieux, 1979; Sharma, 1982). This series of events provides a molecular mechanism for the transfer of regulatory information from the cell membrane to the nucleus via cyclic AMP-dependent protein kinase. In support of this hypothesis, several reports have appeared showing the regulatory role of cyclic AMP and translocated cyclic AMP-dependent protein kinase in the structural and functional modification of eukaryctic chromatin via phosphorylation of chromatin-associated histone and non-histone proteins (Costa et al., 1976; Spielvogel et al., 1977; Chuang et al., 1977; Cho-Chung et al., 1979; Murdoch et al., 1982). Also, cyclic AMP regulates the induction of specific mRNA's (Bachrach, 1978; Derda et al., 1980; Kumar et al., 1980; Miles et al., 1981; Murdoch et al., 1982; Cimbala et al., 1982). These changes in eukaryotic gene activity have been functionally linked with the selective phosphorylative modification of nuclear proteins by translocated cyclic AMP-dependent protein kinase isozymes (Harrison et al., 1980; Murdoch et al., 1982; Harrison et al., 1983).

II. MATERIALS AND METHODS

A. Description and Care of Animals

Male albino Sprague-Dawley rats (approximately 90 days of age) were obtained from King Animal Laboratories (Oregon, WI) and housed in groups of 3 to 5 in the Animal Care Facility of both Hines Veterans Administration Hospital, Hines, IL and Loyola University Stritch School of Medicine, Maywood, IL. The animals were maintained on a 12 hr light/dark cycle at ambient temperature and received food and water ad libitum. The average age of the animals used during this research project was 118 days with a range of 84 to 195 days.

B. Denervation Surgery

The various animal groups (usually 3 rats per group) used during this research project are described in Table 2. For all experimental groups an initial incision of 20 cm was made through the skin of the left hindlimb approximately at midthigh. Following this surgical procedure, the 20 cm cut was also performed on the right hindlimb at midthigh in order to carry out the sham-operation as described below. All surgical procedures were carried out while the animal was under light ether anesthesia.

In group one the soleus muscle in the left hindlimb was denervated by surgically removing a 2 mm segment of the

Table 2

EXPERIMENTAL ANIMAL GROUPS

Group #	Surgical Treatment of Nerve and Muscle*		Denervation Period
	left soleus	right soleus	
1	left sciatic nerve cut proximally (32-35 mm)	right sciatic nerve sham-operation	**
2	same as 1 + intermediate cut (17-20 mm)	right sciatic nerve sham-operation	***
3	same as 1 + low cut (2 mm)	right sciatic nerve sham-operation	****
4	no surgery	no surgery	0 hr unoperated
5	same as 3	right sciatic nerve cut proximally (32-35 mm)	24 hr

* Solei of the rat hindlimb (3 muscles pooled/ group) were treated as described in the Table and in detail in Method B.

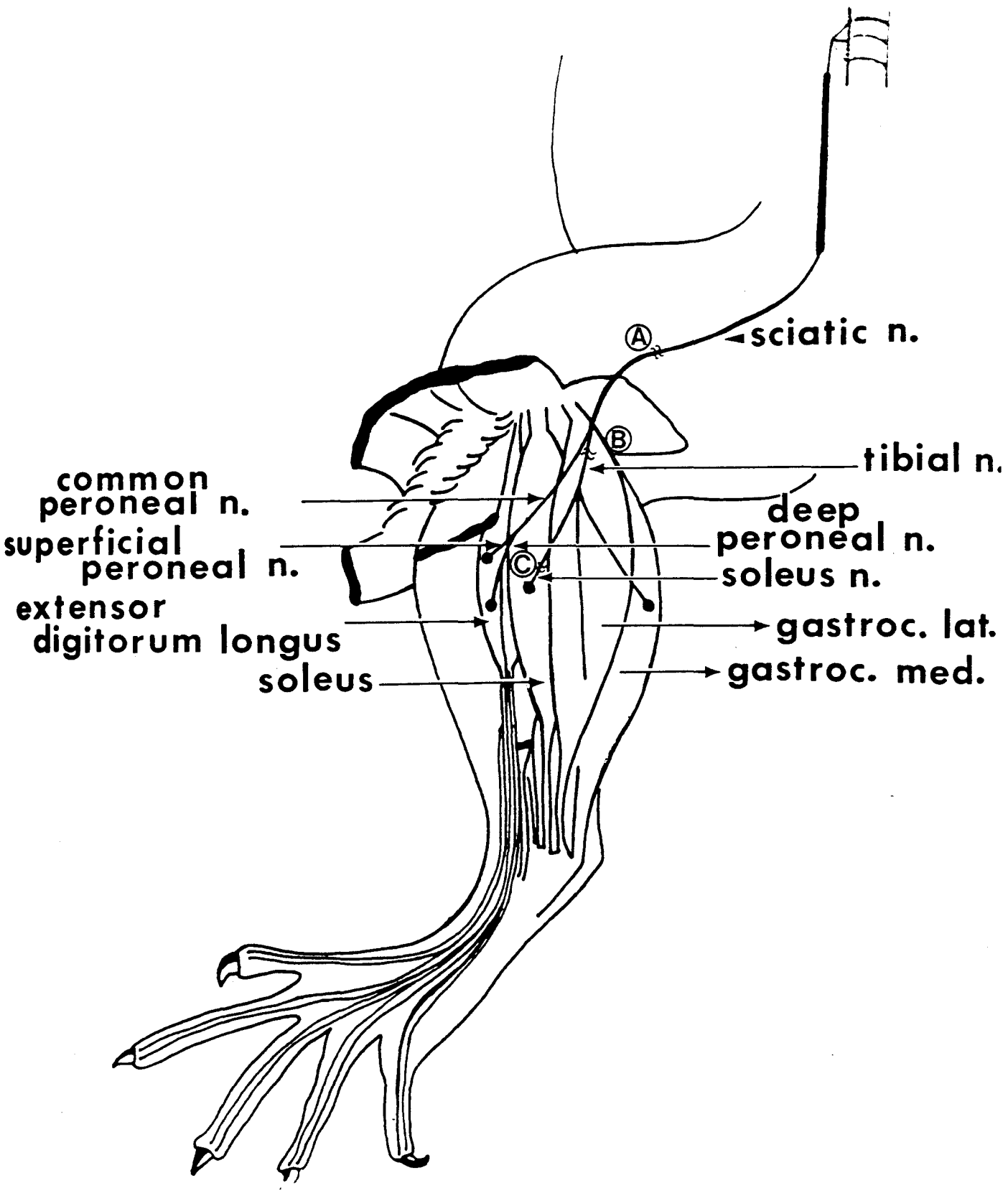
** Animals were sacrificed at 1, 3, 6, 12, 18, 24, 36, 48 and 72 hr after the surgical procedures described.

*** Animals were sacrificed at 3, 12, 24, 30, 36, 42, 48, 54, 60, 66 and 72 hr after the surgical procedures described.

**** Animals were sacrificed at 3, 12, 24, 36, 42, 48, 54, 60, 66, 72 and 78 hr after the surgical procedures described.

left sciatic nerve at midthigh level to leave a distal, long nerve stump of 32 to 35 mm (Figure 1A). In the second group of animals the left soleus was denervated by cutting the left tibial branch of the sciatic nerve at the level of the popliteal fossa to leave a distal, intermediate nerve stump of 17 to 20 mm (Figure 1B). In the third group of animals a lower skin incision of 10 cm was also carefully made with a scalpel to prevent excessive bleeding. The denervation surgery was then carried out by cutting the left soleus branch of the tibial nerve to leave a distal, short nerve stump of 2 mm attached to the muscle (Figure 1C). When the nerve was cut distally at either the intermediate (17 to 20 mm nerve stump) or low (2 mm nerve stump) location, first the proximal cut was also made. A "double denervation" protocol was carried out on a separate group of animals (group 5). That is, the left soleus was denervated by cutting the left sciatic nerve at both the proximal (32 to 35 mm) and distal (2 mm) locations. Then the right, contralateral soleus muscle was also denervated by cutting the right sciatic nerve at the proximal location only.

In groups 1, 2, and 3 the right soleus served as the control muscle in that a sham-operation was performed on this muscle. First a 20 cm incision was made through the skin at the midthigh region. Then the sham-operation was performed by gently pulling on the sciatic nerve at a distance of approximately 32 to 35 mm from its point of insertion into the muscle. This protocol allowed for a similar surgical


sciatic n.
tibial n.
common peroneal n.
deep peroneal n.
superficial peroneal n.
soleus n.
extensor digitorum longus
gastroc. lat.
soleus
gastroc. med.
A
B
C

trauma to the sciatic nerve on both the left (denervated) and right (sham-operated) side at an equal distance from insertion into the muscle. In animal group 3, a lower skin incision was also made on the right side of the animal. Additionally, unoperated solei (group 4) were obtained from either the left or right hindlimb.

All surgical preparations for animal groups 1 through 5 were carried out at 2:00 AM, 5:00 AM, 7:00 AM, 8:00 AM, 2:00 PM or 8:00 PM. Animals were consistently sacrificed between 8 and 9 AM. Animals were sacrificed by decapitation at 0 (unoperated), 1, 3, 6, and 6 hr intervals thereafter to 78 hr following the surgical procedures. Animals recovered from the surgical procedures and anesthesia within 2 to 5 min as monitored by their increased activity and alertness.

C. Preparation of Soluble Cytosolic Protein Fraction from Rat Soleus Muscle

Within 2 to 3 min following sacrifice of the animals by decapitation, the denervated and sham-operated solei were removed and placed in ice-cold homogenizing media consisting of 0.32 M sucrose (ribonuclease-free from Schwarz/Mann Co., Orangeburg, NY) and 3 mM $MgCl_2$ (Fisher Chemical Co., Fair Lawn, NJ), pH 6.5. Tendons were carefully removed with a scalpel while the muscles were on a chilled cutting surface (parafilm covered glass on ice) in a cold room. Muscles were then blot-dried with a disposable wiper (Kimwipes, Kimberly-Clark Corp., Neenah, WI), weighed on a top-loading Mettler balance (Mettler Instrument Corp., Highstown, NJ) and minced with scissors while in a cold room. Homogenization of three muscles (denervated or sham-operated solei pooled from 3 rats) was performed at one time with a Virtis blade-type homogenizer (Virtis Co., Inc., Gardiner, NY) in sufficient homogenizing media to give a final muscle concentration of 2.5 % (w/v). The Virtis homogenizer was set at 50 and homogenization was carried out for 5 consecutive 30 second intervals. Vacuum filtration of the homogenate was done consecutively through 50-, 100-, and 150-mesh stainless steel screens. The filtered homogenate was centrifuged first at 800 x g and 4°C for 20 min in an International centrifuge (International Instrument Corp., Needham Heights, MA). The supernatant was recovered by pouring off the aqueous layer.

Five milliliters of the supernatant was then ultracentrifuged (Beckman L2-65, Beckman Spinco, Palo Alto, CA) at 105,000 x g and 4°C for 45 min. The high speed supernatant fraction obtained after the ultracentrifugation step is referred to as the soluble cytosolic protein fraction. Only freshly prepared soluble cytosolic protein fractions were used in the protein phosphotransferase assays described in Method E. The protein content of these fractions was quantitated by the method of Lowry et al. (1951) as described below.

D. Protein Determination

The two distinct steps which lead to the color development in the Lowry method (1951) for the quantitation of protein are as follows: (1) a reaction of protein with copper in alkali; and (2) a reduction of the phosphomolybdic-phosphotungstic acid reagent by the copper treated protein. One milliliter of reagent "C" (described below) was placed in each of a series of 12 x 75 mm glass tubes. Two hundred microliters of the high speed supernatant fraction (soluble cytosolic protein fraction) from denervated and sham-operated solei (60 to 180 ug protein diluted 15 times) was added to appropriately marked tubes and 200 ul of glass-distilled water was added to a pair of tubes which served as "blank". The contents of the tubes were mixed and allowed to stand for ten min. One hundred microliters of 1 N Folin-Ciocalteu reagent (Fisher Scientific Co., Fair Lawn, NJ) was

added to each tube and the tubes were mixed thoroughly with a vortex mixer immediately after addition of reagent. The reaction mixtures were allowed to stand at least 30 min but no longer than 1 hr to allow for full color development. The absorbance of the sample solutions was determined with a Gilford model 250 spectrophotometer (Gilford Instrument Co., Oberlin, OH) at a wavelength of 750 nanometers (nm). The instrument was zeroed against glass-distilled water. A standard curve was prepared using bovine serum albumin as a reference protein each time this procedure was used for protein determinations. The concentration of the unknown soluble cytosolic protein sample was derived from the slope of the linear regression line for the standard curve (4 to 25 ug of protein). All calculations (linear regression analysis and protein concentrations) were carried out with a computer program and a Hewlett-Packard model 9830A computer.

Solutions

1. Reagent A

   20 g of Na_2CO_3, 4 g NaOH and 200 mg of KNa $C_4H_4O_6(4H_2O)$ (all from Fisher Chemical Co.) were dissolved in one liter of glass-distilled water.

2. Reagent B

   5 g of $CuSO_4$ $(5H_2O)$ (Fisher Chemical Co.) were dissolved in one liter of water.

3. Reagent C

   Fifty parts of Reagent A and one part Reagent B were combined and used within 1 hr.

E. *In Vitro* Cytosolic Protein Phosphorylation Assay

The final concentration of the components constituting the phosphorylation incubation medium in a total volume of 0.25 ml were as follows: 0.1 M NaCl, 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, and 6.7 x 10^{-9} M [gamma-^{32}P] ATP (3000 Ci/mmol) obtained from Amersham Corp., Des Plaines, IL. The phosphotransferase reaction was started by the addition of an aliquot of a freshly prepared fraction of cytosolic protein (15 to 90 ug) which served as the source for endogenous protein kinases and phosphorylatable substrates. In some experiments the final concentrations of additional components were as follows: 10^{-11} to 10^{-9} M and 1 uM adenosine 3':5' cyclic monophosphate (cyclic AMP), 1 uM guanosine 3':5' cyclic monophosphate (cyclic GMP), 2.0 x 10^{-5} to 10^{-3} M $CaCl_2$, 1 mM $ZnCl_2$, 65 mM (ethylenedinitrilo)tetra-acetic acid (EDTA), 1 mM ethyleneglycol-bis-(beta-aminoethyl ether)N,N'-tetra-acetic acid (EGTA) or 50 inhibitory units of cyclic AMP-dependent protein kinase inhibitor from bovine heart muscle (Sigma Chemical Co., St. Louis, MO) which was further purified prior to use in some experiments essentially according to Kantor and Brunton (1981) as described below (refer to Method F). After incubation of the phosphotransferase medium for 5 min, the assay tubes (12 x 75 mm) were transferred to ice and the reaction was terminated first by the addition of 25 ul of 5 mM adenosine 5' triphosphate (ATP) and then 100 ul of 5 % sodium pyrophosphate (NaPPi).

Aliquots of 50 ul from the reaction mixture were transferred in duplicate to Millipore filters (type PH from Millipore Corp., Bedford, MA) which were pre-treated by placing them in 5 mM ATP for 1 hr prior to use. The radioactive Millipore filters were subsequently treated overnight by washing them with constant stirring in 5 % trichloroacetic acid (TCA) and 1.5 % NaPPi in a cold room. The TCA-precipitable, ^{32}P-radioactivity on each filter was quantitated with a Packard model 3380 liquid scintillation spectrometer in a 7.5 ml toluene solution containing Permafluor I (Packard Instrument Co., Downers Grove, IL). Data obtained from liquid scintillation counting were converted from counts per minute to soluble cytosolic protein phosphorylation with the use of a computer program (Method 0). Soluble cytosolic protein phosphorylation for denervated and contralateral, sham-operated solei were reported as femtomoles ^{32}P-phosphate incorporated per milligram of soluble cytosolic protein per 5 min incubation period.

F. Partial Purification of Cyclic AMP-Dependent Protein Kinase Inhibitor

One hundred milligrams of crude inhibitor (lyophilized powder from bovine heart, Sigma Chemical Co., St. Louis, MO) were dissolved in a minimal volume (usually 10 ml) of 5 mM sodium acetate and 1 mM EDTA, pH 5.3. This solution of inhibitor was passed over a diethylaminoethyl (DEAE)-Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) anion

exchange column (0.9 x 9.0 cm) which was previously equilibrated with the 5 mM sodium acetate buffer, pH 5.3, described above. Protein was eluted from the column with a linear gradient of 5 to 300 mM sodium acetate and absorbance of the effluent was monitored at 280 nm with a Uvicord II model 8300 (LKB Instruments, Stockholm, Sweden). Consecutive fractions of 4.0 ml were collected immediately after the gradient was started and the relative conductance of each fraction was recorded immediately after collection with an Industrial Instruments conductivity meter (Cedar Grove, NJ) in order to avoid errors in measurement due to carbon dioxide contamination. The protein kinase inhibitor activity (refer to definition below) of each fraction was assayed *in vitro* in the buffered phosphorylation incubation medium described above (Method E) with the addition of 200 ul of each DEAE fraction and also 0.25 mg of hydrolyzed and partially dephosphorylated casein (Sigma Chemical Co., St. Louis, MO) and 1 uM cyclic AMP. The reaction was started by the addition of 12 units (refer to definition below) of cyclic AMP-dependent protein kinase type II isozyme (Sigma Chemical Co.) and was allowed to proceed for 5 min before the assay tubes were transferred to ice. At this time the reaction was terminated with ATP and NaPPi as described in Method E. Aliquots of 50 ul were transferred in duplicate to ATP-treated Millipore filters. The filters were treated overnight by washing them with constant stirring in 5 % TCA and 1.5 % NaPPi and the TCA-

precipitable, ^{32}P-radioactivity was quantitated by liquid scintillation spectrometry. The DEAE fractions corresponding to inhibitor activity were pooled, lyophilized and then resuspended in a minimal volume of 5 mM sodium acetate and 1 mM EDTA, pH 5.3. The chromatograph indicating relative protein absorbance, relative conductance and inhibitor activity is shown in Figure 47 of the Appendix.

Definitions

1. Unit of Inhibitor Activity

The amount of cyclic AMP-dependent protein kinase inhibitor needed to inhibit one unit of cyclic AMP-dependent protein kinase phosphorylating activity.

2. Unit of Cyclic AMP-Dependent Protein Kinase Phosphorylating Activity

At 37°C and pH 7.5, one unit will transfer 1.0 picomole of phosphate from [gamma-^{32}P]ATP to hydrolyzed and partially dephosphorylated casein per min.

G. Adenosine Triphosphatase (ATPase) Assay

Soluble cytosolic protein was prepared from rat solei and assayed for ATPase activity using the incubation medium described previously in Method E. The ATPase assay, which monitored the conversion of [gamma-^{32}P]ATP to ^{32}P-inorganic phosphate, was started by the addition of aliquots of soluble cytosolic protein (between 20 and 130 ug) and incubated for 1, 2, 5, 10 or 20 min. The reaction was

terminated with 25 ul of 5 mM ATP and 100 ul of 5% NaPPi. Five microliters of the reaction products were quantitatively transferred by disposable glass pipet (Clay Adams, Parsippany, NJ) to polyethyleneimine-impregnated cellulose thin layer chromatography paper (Brinkman Instuments, Inc., Westbury, NY) which was cut into strips (1 cm x 6.5 cm). The 5 ul of reaction product was spotted at a location 1 cm from the bottom of the paper strip designated the origin. After spotting, the thin layer chromatography paper strips were air-dried and then developed by ascending chromatography for 30 min in a glass chamber containing approximately 20 ml of 0.3 M LiCl (Fisher Chemical Co). The paper strips were then allowed to dry overnight and the reaction products ([gamma-^{32}P]ATP and ^{32}P-inorganic phosphate) were visualized under ultraviolet light, appropriately marked and then cut out. The ^{32}P-inorganic phosphate spot migrated from the origin while the [gamma-^{32}P]ATP remained at the origin. ^{32}P-radioactivity associated with the UV-sensitive spots was measured by liquid scintillation spectrometry in a 7.5 ml toluene solution containing Permafluor I (Method 0).

H. "Mixing" Experiments

The effect of endogenous protein kinase modulators upon the soluble cytosolic protein phosphorylation in denervated and sham-operated solei was evaluated by conducting "mixing" experiments. For these experiments, equal volumes (75 ul) of the cytosolic protein fractions from denervated

and sham-operated solei were added to the *in vitro* phosphorylation assay (Method E) as a combined sample. Cytosolic protein phosphorylation of the combined sample (150 ul) was compared quantitatively and statistically to that of each individual cytosolic protein fraction from denervated and sham-operated solei in a 150 ul aliquot. All samples of 150 ul (combined or individual) were pre-incubated for 0, 10, 20 or 30 min.

I. Determination of Alkali-Labile Phosphate

Protein-bound phosphate was determined as alkali-labile phosphate according to Weller (1979) with minor modifications. Soluble cytosolic protein phosphorylation was assayed *in vitro* in the standard phosphorylation incubation medium (Method E). After the 5 min incubation, the phosphorylation reaction was terminated by adding 1.0 ml of "precipitating solution" (10 % TCA and 0.05 M ATP) and 0.1 ml of bovine serum albumin (Miles Laboratories, Inc., Elkhart, IN) prepared as a 20 mg/ml solution in glass distilled water. The assay tubes were immediately transferred to ice and were allowed to stand for 30 min. Then the incubation medium was centrifuged at 2000 r.p.m. and 4°C for 20 min. The supernatant was discarded and the pellet was rinsed 8 to 10 times with 2 ml of 5 % TCA and 0.01 M NaPPi. The precipitate was pelleted each time by centrifugation at 2000 r.p.m. and 4°C for 10 min. ^{32}P-phospholipid was extracted by treating the

pellet once with 1.0 ml ethanol:diethyl ether (1:1, v/v) and once with 1.0 ml of chloroform:methanol (2:1, v/v). Again the precipitate was pelleted each time by centrifugation at 2000 r.p.m. and 4°C for 10 min. The supernatant fractions from these two steps were combined and allowed to evaporate overnight. ^{32}P-phospholipid was quantitated by liquid scintillation spectrometry after resuspending the dried phospholipid in 0.75 ml Soluene 350 (Packard Instrument Co., Downers Grove, IL) and 7.5 ml of a toluene solution containing Permafluor I. ^{32}P-phospholipid was reported as percent of the total TCA-precipitable ^{32}P-radioactivity (untreated sample).

The pellet remaining after phospholipid extraction was then treated to remove acid-labile phosphate by heating in 1.0 ml of 10 % TCA and 0.05 M ATP for 12 min at 100°C. The acid-treated protein was then centrifuged at 2000 r.p.m. and 4°C for 10 min. The supernatant was retained and ^{32}P-acid-labile phosphate was quantitated by liquid scintillation spectrometry in a 7.5 ml pre-mixed liquid scintillation cocktail (Phase Combining System, Amersham Corp., Des Plaines, IL). The ^{32}P-acid-labile phosphate was reported as percent of the total ^{32}P-radioactivity.

Free acid was removed from the remaining pellet by extracting in 1.0 ml ethanol:diethyl ether (1:1, v/v). Alkali-labile phosphate was determined by heating the lipid-free, acid-treated pellet in 1.0 ml 1 M NaOH for 12 min at 100°C. After treatment with base, protein was reprecipitated

with 1.0 ml of 30 % TCA and centrifuged at 2000 r.p.m. and 4°C for 20 min. The final resulting pellet was resuspended in 0.75 ml of Soluene 350 and the ^{32}P-radioactivity was quantitated by liquid scintillation spectrometry in a 7.5 ml toluene solution containing Permafluor I. Acid-stable, alkali-labile ^{32}P-phosphoprotein was reported as percent of the total ^{32}P-radioactivity of the untreated sample.

J. Chromatographic Methods

1. Gel filtration

A Sephadex G-150 Superfine (Pharmacia Fine Chemicals) gel filtration column (1.5 x 90 cm, Bio-Rad Laboratories, Richmond, CA) was used to resolve ^{32}P-phosphoproteins from the cytosols of both denervated and sham-operated solei. Soluble cytosolic protein fractions from denervated and sham-operated solei were prepared and aliquots phosphorylated as previously described (Methods C and E). ^{32}P-labeled soluble cytosolic protein from the *in vitro* assay (60 to 90 ug soluble cytosolic protein) was combined with unlabeled cytosolic protein (200 to 250 ug) in non-phosphorylated aliquots of the denervated and sham-operated cytosolic protein fractions. The unlabeled cytosolic protein served as cold carrier protein. The combined samples were quantitatively transferred to membrane tubing (Spectrapor, Spectrum Medical Industries, Los Angeles, CA) and then dialyzed overnight in a 500-fold volume of 0.1 M NaCl, 2 mM $MgCl_2$ and 50 mM Tris-HCl

buffer, pH 7.5, at 4°C. The dialysis buffer was changed three times during this period. The Sephadex G-150 columns were equilibrated with this same buffer. Care was taken to apply equal aliquots of the dialyzed denervated and sham-operated cytosolic protein to individual Sephadex G-150 columns. After the protein had entered the column, elution was carried out under non-denaturing conditions with the same buffer used during dialysis at an approximate flow rate of 0.1 ml/min. During the elution of protein, consecutive 1.5 ml fractions were collected. The ^{32}P-radioactivity in each fraction was quantitated in 200 ul aliquots by liquid scintillation spectrometry using a 7.5 ml pre-mixed scintillation cocktail (Phase Combining System, PCS). The protein profiles from both denervated and sham-operated solei were obtained by recording the relative absorbance (280 nm) of each fraction using a Gilford model 250 spectrophotometer. These profiles were compared quantitatively and qualitatively. The relative molecular weight range of various soluble cytosolic phosphoproteins was established after calibrating the Sephadex G-150 column. This was done by calculating the partition coefficient (defined below) of known molecular weight marker proteins (given in the legend to Figure 35 of the Appendix) and plotting this calculated value against the log molecular weight of these marker proteins. A linear relationship was established and then used to estimate the relative molecular weight of specific

soluble cytosolic phosphoproteins from denervated and sham-operated solei.

Definition: Partition Coefficient (K_{av})

$$K_{av} = \frac{\text{Elution Volume } (V_e) - \text{Void Volume } (V_o)}{\text{Bed Volume } (V_t) - \text{Void Volume } (V_o)}$$

2. High performance liquid chromatography

Soluble proteins from the cytosolic fractions of denervated and sham-operated solei were resolved by high performance liquid chromatography (HPLC). One advantage of HPLC over more conventional liquid chromatographic systems is that it employs a high pressure pumping system which allows for a rapid separation of components.

A Waters I-125 Protein Purification size exclusion column (30 cm, Waters Associates, Inc., Milford, MA) was used to separate soluble cytosolic proteins prepared from denervated and sham-operated solei (Method C). The soluble cytosolic protein fractions were phosphorylated *in vitro* as previously described (Method E). An aliquot of the cytosolic fraction (30 to 50 ug of protein) was combined with unlabeled cytosolic protein (70 to 90 ug) which served as cold carrier protein. The combined samples were quantitatively transferred to Spectrapor membrane tubing and then dialyzed overnight against a 500-fold volume of 0.05 M sodium phosphate buffer, pH 7.4, at 4°C. The dialysis buffer was changed three to four times during an 18 hr period. Care was taken to apply similar aliquots (approximately 80 ug) of both

denervated and sham-operated soluble cytosolic protein to individual HPLC columns. Immediately following application of the protein (via a syringe-type injection system of Waters Assoc.), elution was started under non-denaturing conditions using the 0.05 M sodium phosphate buffer described above. A constant flow rate of 2.0 ml/min was maintained using a single pump system (Waters Assoc.) and a constant pressure of 1500 pounds per square inch (psi). Consecutive fractions of 0.5 ml were collected immediately after elution was started. Protein was monitored continuously at 254 nm and recorded at a constant chart speed of 2.0 cm/min using a Houston Instruments (Houston, TX) chart recorder. ^{32}P-radicactivity in each fraction was quantitated by liquid scintillation spectrometry. This was done by combining each 0.5 ml fraction with 7.5 ml of a pre-mixed scintillation cocktail (Phase Combining System, PCS). The relative molecular weights of specific phosphoproteins from denervated and sham-operated soleus cytosols were obtained after calibrating the HPLC column using the calculated partition coefficients (K_{av}) of molecular weight marker proteins (described in the legend to Figure 36 of the Appendix).

3. Chromatofocusing

The approximate isoelectric points of soluble cytosolic proteins from denervated and sham-operated solei were obtained by column chromatography under non-denaturing

conditions. A relatively new technique was used - chromatofocusing (Sluyterman and Elgersma, 1978). Chromatofocusing is a technique which ideally combines the high resolution of isoelectric focusing with the high capacity of ion-exchange chromatography.

Relatively small columns (15 to 30 ml bed volumes) are used which have the capacity to rapidly separate proteins on a preparative scale. A 1.5 x 90 cm column (Bio-Rad) was packed with Polybuffer Exchanger (PBE 94, Pharmacia Fine Chemicals) and equipped with flow adaptors (Bio-Rad) to ensure even sample application. The bed volume of the column was 20 ml. PBE contains ion exchangers carrying basic buffering groups. The column was equilibrated with 0.025 M histidine-HCl buffer, pH 6.3. The pH of the eluting buffer, Polybuffer 74 (Pharmacia), was adjusted to pH 4.0. Polybuffer 74 contains a large number of differently charged ionic species the most acidic of which binds the basic anion exchanger (PBE 94) and elutes last. This automatically establishes an internal descending pH gradient which allows for the elution of proteins as sharply focused zones in the order of their isoelectric points.

To resolve soluble phosphoproteins from the cytosolic fraction of rat solei, an aliquot of soluble cytosolic protein (approximately 0.5 ug) ^{32}P-labeled *in vitro* was combined with 10 to 15 mg of unlabeled protein from the same cytosolic fraction. The combined samples were quantitatively transferred to Spectrapor membrane tubing and then dialyzed

overnight at 4°C against a 500-fold volume of 0.025 M histidine-HCl buffer, pH 6.3. The dialysis buffer was changed three to four times. The next day the protein was applied to the column and then eluted by passing 200 to 300 ml (10 to 14 bed volumes) of elution buffer (PB 74) over the column. A constant flow rate was maintained during elution (1.0 ml/min) by a Technicon Auto Analyzer hi-pressure micro pump (Technicon Instrument Corp., Chauncey, NY) operating at 50 % maximal output. Consecutive fractions of 2.0 ml were collected and protein was monitored at 280 nm with a Uvicord II 8300 (LKB Instruments, Stockholm, Sweden) and recorded with a LKB Chopper Bar Recorder model 6520. When protein elution with PB 74 was completed, the pH of each individual fraction was recorded immediately with a Markson pH meter and electrode (Markson Science Inc., Del Mar, CA) in order to avoid errors in measurement due to CO_2 contamination. ^{32}P-radioactivity was quantitated in 200 ul aliquots of each fraction by liquid scintillation spectrometry in a 7.5 ml pre-mixed scintillation cocktail (PCS).

4. Cyclic adenosine 3':5' monophosphate affinity chromatography

a. synthesis of N^6-(2-aminoethyl)-cyclic adenosine 3':5' monophosphate

The preparation of N^6-(2-aminoethyl)-cyclic AMP was carried out essentially according to Dills et al. (1975,

1979). The procedure is based on the conversion of 6-chloropurine-riboside-3':5'-monophosphate (6-Cl-cPRMP) to the desired cyclic AMP-affinity ligand. 6-Cl-cPRMP (25 mg or 0.067 mmoles) was added to 0.26 g (4.35 mmoles) of ethylenediamine free base in 5 ml of glass-distilled water. This solution was refluxed for 6 hr at 100°C. The incubation medium was allowed to cool to room temperature and was then applied to an anion exchange column (1.8 x 30 cm). The anion exchange resin used was AG 1-X2 (Bio-Rad Laboratories) of 100 to 200 mesh in the acetate form. The resin had been prewashed with glass-distilled water. After the sample was applied, the column was washed successively with 25 ml of glass-distilled water, 25 ml of 0.01 M NH_4Cl and 25 ml of glass-distilled water. N^6-(2-Aminoethyl) cyclic AMP was then eluted with a linear gradient of 0 to 0.5 N acetic acid (approximately 160 ml elution buffer or 4 column bed volumes). When the gradient was started, consecutive fractions of 4.0 ml were collected and absorbance was monitored at 265 nm by reading each fraction with a Gilford model 250 spectrophotometer. A 50 ul aliquot of each fraction was transferred to a sheet of Whatman #1 filter paper and the free amino group of the nucleotide was observed in a chromatic reaction after treating the spotted filter paper with ninhydrin (Spray-type reagent from Gelman Instument Co., Ann Arbor, MI). Ninhydrin is a strong oxidizing agent which is commonly used as a qualitative identification of an amino acid or a free amino group. Ninhydrin brings about an

oxidative deamination of N^6-(2-aminoethyl) cyclic AMP (Figure 2). The ammonia and hydrindantin formed react with a second molecule of ninhydrin to yield a purple pigment. A purple color observed in the ninhydrin reaction served as a positive test for the presence of the free amino group of N^6-(2-aminoethyl) cyclic AMP. Those fractions which absorbed at 265 nm and were ninhydrin positive were pooled, lyophilized overnight and then resuspended in a minimal volume (usually 4.0 ml) of glass-distilled water. This nucleotide solution was diluted 1000 times in glass-distilled water adjusted to pH 1.0 with 12 N HCl. Absorbance was recorded at 262 nm. Dills et al. (1975) reported that the nucleotide showed maximum absorbances at 262 nm and at 267 nm in solutions of pH 1.0 and 11.0, respectively. The molar extinction coefficients are 16,000 at pH 1.0 and 11.0. Using this molar extinction coefficient, a 71.12 % (17.78 mg) yield of N^6-(2-aminoethyl) cyclic AMP was obtained.

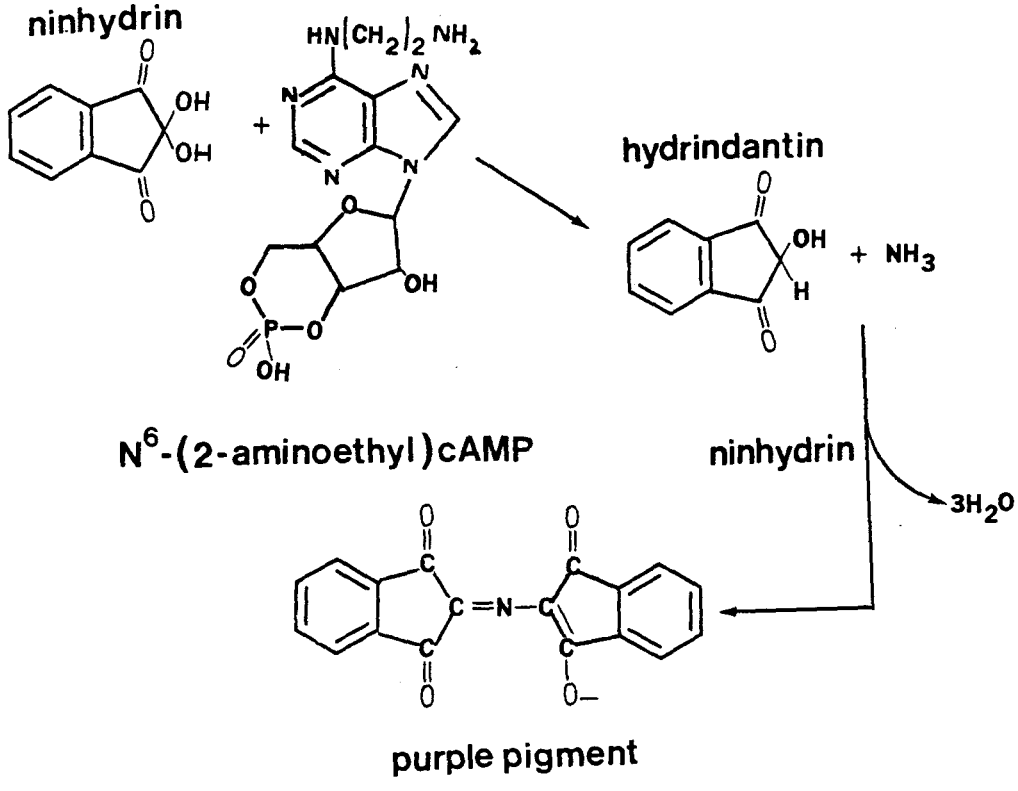


Figure 2. Chromatic reaction to detect N^6-(2-aminoethyl)cAMP with ninhydrin reagent.

b. coupling of N^6-(2-aminoethyl) cyclic AMP to Sepharose 4B

The protocol used to couple N^6-(2-aminoethyl) cyclic AMP to Sepharose 4B was essentially according to Dills et al. (1975 and 1979) and the steps are illustrated in Figure 3. To prepare approximately 9.0 ml of gel, 2.5 gm of Cyanogen bromide-activated Sepharose 4B (CNBr-activated Sepharose) obtained from Sigma Chemical Co. was washed with 5 ml of 1.0 mM HCl. The washed gel was then suspended in an equal volume of 100 mM sodium bicarbonate buffer, pH 9.5, in the presence of enough N^6-(2-aminoethyl) cyclic AMP to give a final concentration of 4 umoles of nucleotide per ml of washed gel. The coupling reaction was allowed to proceed for 2 hr at room temperature. At this time, an equal volume of 1 M ethanolamine hydrochloride, pH 9.5, was added and the reaction was allowed to proceed overnight for 16 hr at 4°C. The gel was then filtered by vacuum filtration using a Millipore type NCWP filter (14 micron pore size and 47 mm diameter) and washed with 5 to 10 volumes of the bicarbonate buffer (pH 9.5) followed by at least 5 volumes of glass-distilled water. The affinity gel was stored in 0.02 % sodium azide at 4°C.

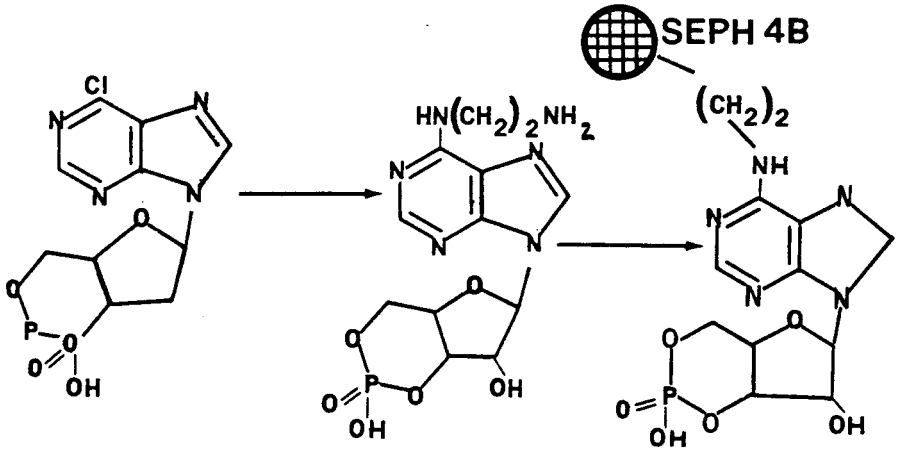


Figure 3. Coupling reaction of N^6-(2-aminoethyl) cyclic AMP to Sepharose 4B chromatography resin

c. adsorption of soluble cyclic AMP-binding proteins from cytosolic fractions of rat soleus muscle

N^6-(2-Aminoethyl) cyclic adenosine 3':5' monophosphate coupled to Sepharose 4B was used to adsorb cyclic AMP-binding proteins from the cytosolic fractions of denervated and sham-operated solei. Approximately 2.0 ml of affinity gel were poured into a disposable polypropylene column (0.7 x 4 cm) from Bio-Rad Laboratories. A small piece of rubber tubing (approximately 2.0 cm long) was attached to the outlet of the column and flow was regulated with a pinch clamp. The cyclic AMP affinity resin was equilibrated with 5 mM 2-(N-morpholino) ethanesulfonic acid (MES) containing 0.1 mM EDTA, 15 mM 2-mercaptoethanol and 100 mM NaCl, pH 6.8 (buffer A). ^{32}P-radiolabeled phosphoproteins from the cytosolic fractions of denervated and sham-operated solei were prepared as previously described (Method E). Approximately 200 ug of protein from both denervated and sham-operated solei were quantitatively transferred to Spectrapor membrane tubing and then dialyzed against a 500-fold volume of buffer A overnight at 4°C. The dialysis buffer was replaced three to four times during this period. ^{32}P-labeled soluble cytosolic protein from denervated and sham-operated solei were then passed over separate cyclic AMP affinity columns. The protein was applied to the column and was incubated with the resin for 2 hr at 4°C in order to allow for the interaction of cyclic AMP-binding proteins with the cyclic AMP ligand. After

incubation, the cyclic AMP affinity resin was washed with 10 ml of buffer A, then with 10 ml of buffer A containing 750 mM NaCl, and finally with 5 ml of buffer A. The washed column was then allowed to come to room temperature.

d. elution of soluble cytosolic cyclic AMP-binding phosphoproteins

Soluble cytosolic cyclic AMP-binding proteins from denervated and sham-operated solei were specifically eluted from the affinity gel essentially according to Dills et al. (1979). Proteins were eluted from the cyclic AMP affinity ligand by first washing the column with 5.0 ml of buffer A at room temperature and then two sequential 5.0 ml washes (1.5 hr elution time for each wash) with buffer A containing 1.0 mM cyclic AMP. The cyclic AMP displacement wash was retained and concentrated by lyophilization overnight. The lyophilized protein was resuspended in a minimal volume (usually 0.5 ml) of dialysis buffer containing 0.065 M Tris-HCl, pH 6.8, and 0.1 % sodium dodecyl sulfate (SDS). 2-mercaptoethanol was then added at a final concentration of 5% immediately before use. The suspension was then heated for 2 min at 100°C and dialyzed overnight at room temperature against a 500-fold volume of the dialysis buffer. The next day, 200 ul of the cyclic AMP-binding protein suspensions from denervated and sham-operated solei were electrophoretically resolved on SDS polyacrylamide gradient slab gels and ^{32}P-labeled soluble cytosolic cyclic AMP-binding proteins

were visualized by autoradiography of the dried slab gel. The gel electrophoresis and autoradiography protocol is described in detail in Method K.

K. SDS-Polyacrylamide Gel Electrophoresis

1. Rod gel protocol

a. casting the polyacrylamide rod gels

Soluble cytosolic proteins which had been phosphorylated *in vitro* (Method E) were electrophoretically resolved in SDS-10% polyacrylamide rod gels (SDS rod gels) essentially according to Weber and Osborn (1969). Rod gels were cast in cylindrical glass rods 0.5 cm in diameter and 7.5 cm in length. Usually 12 gels were cast at one time by setting the glass rods onto a plexiglass holder (Buchler Instruments, Fort Lee, NJ) equipped with a level to ensure even casting. A small piece of parafilm was used to seal the bottom opening of the glass cylinder. To make the gel casting solution, 20 ml of a 10 % acrylamide solution containing 0.5 % N,N'-methylene-bis-acrylamide (filtered through Whatman #1 paper before using) was mixed with 15 ul of N,N,N',N'-tetramethylethylenediamine and 60 ul of 10 % ammonium persulfate. This gel solution (1.0 ml) is transferred into the glass cylinders and a few drops of glass-distilled water are carefully added to the top of the gel solution to eliminate the meniscus. The gels are allowed

to polymerize by exposing them to fluorescent light for 30 min.

b. electrophoretic separation of protein

Twenty five microliters of 18 % SDS was added to the terminated in vitro phosphorylation incubation medium (refer to Method E) and the SDS-treated samples were heated for 2 min at 100°C. The SDS- and heat-treated ^{32}P-labeled cytosolic fractions from denervated and sham-operated solei were transferred to Spectrapor membrane tubing and then dialyzed overnight at room temperature against a 500-fold volume of electrophoresis buffer consisting of 0.01 M sodium phosphate buffer, pH 7.4, 0.1 % SDS and 0.14 M 2-mercaptoethanol. The dialysis buffer was changed three to four times during this period. In order to directly compare the ^{32}P-labeling and electrophoretic patterns of soluble cytosolic protein from denervated and sham-operated solei, equivalent aliquots of dialyzed, cytosolic protein (usually 30 ug protein) were always taken and quantitatively transferred to the SDS-10% polyacrylamide rod gels. Electrophoretic separation in these gels was conducted at a constant current of 8 mA/gel for approximately 2 hr. The buffer used at the anode (upper reservoir) and cathode (lower reservoir) of the electrophoresis cell (Buchler Instruments) was the same as that used for dialysis. When the tracking dye had migrated to within 2 to 5 mm from the bottom of the gels, electrophoresis was terminated.

c. staining of resolved proteins

Following electrophoresis, the gels were removed from the glass rods by carefully injecting water with a needle and syringe between the polyacrylamide rod gel and the wall of the glass tubes. The gels were then placed in a solution of methanol-acetic acid-water (5:1:5) for 1 hr. Each gel was then stained for 2 hr at room temperature in a solution of 1.25 g of Coomassie brilliant blue R (Sigma Chemical Co.), 454 ml of 50 % methanol and 46 ml of glacial acetic acid. The staining solution was filtered through Whatman #1 filter paper before using. After staining, the gels were rinsed with distilled water before being destained electrophoretically in 7 % acetic acid for approximately 2 hr using a Canalco gel destaining apparatus (Canalco, Rockville, MD).

d. evaluation of resolved proteins

The distribution of protein-stained bands was analyzed by scanning each gel at 600 nm using a Gilford model 250 spectrophotometer equipped with a model 2410-S linear transport and a model 6050 recorder.

(1) estimation of molecular weights

The relative molecular weight (M_r) of the resolved proteins was estimated after calibrating the electrophoresis system. This was done by plotting the relative mobility (R_f)

of the molecular weight marker proteins against the log of their molecular weights. SDS molecular weight marker proteins (bovine serum albumin, ovalbumin, trypsinogen and lysozyme from Sigma Chemical Co. and aldolase from Pharmacia Fine Chemicals) were heated at 100°C for 2 min in dialysis buffer (1 mg marker protein/ml buffer). The linear relationship established (example shown in Figure 13) was used to determine the relative molecular weights of specific soluble cytosolic phosphoproteins. Relative mobility was calculated as described by Weber and Osborn (1969) according to the following equation:

$$R_f = \frac{(\text{migration (mm) of protein})}{(\text{length of gel (mm) after destaining})} \quad \frac{(\text{length (mm) of gel})}{(\text{migration (mm) of dye})}$$

(2) quantitation of ^{32}P-labeling in gel slices

The protein-stained gels were frozen with dry-ice and then sectioned into consecutive 1 mm slices with a Brinkmann gel slicer (Brinkmann Instruments, Westbury, NY). The ^{32}P-radiolabeling of each consecutive 1.0 mm gel slice was quantitated in a 7.5 ml toluene solution of Permafluor I by liquid scintillation spectrometry. The gels were also sliced longitudinally, sealed in Saran wrap and placed flat side down on Kodak X-Omat film (Eastman Kodak Co., Rochester, NY) which was then kept in a lightproof box for 3 to 7 days. After this exposure time, the film was developed and the resulting autoradiograph was used to qualitatively

detect the distribution of ^{32}P-radiolabeling in the rod gels.

2. Slab gel protocol

a. casting the polyacrylamide slab gels

Soluble cytosolic proteins which were phosphorylated in vitro using the assay protocol described in Method E were electrophoretically resolved in SDS-polyacrylamide gradient slab gels (SDS gradient slab gels) essentially according to Laemmli (1970) with a 5 to 15% exponential acrylamide gradient. The 5% acrylamide gel was prepared with 3.5 ml of 1.5 M Tris-HCl buffer (pH 8.8) containing 0.4% SDS (buffer L), 2.5 ml of 29.9% acrylamide containing 0.8% N,N'-methylene-bis-acrylamide (solution N), 8.75 ml of glass-distilled water, 17 ul of 10% ammonium persulfate and 5 ul N,N,N',N'-tetramethylethylenediamine (TEMED). The 15% acrylamide gel was prepared with 5.0 ml of buffer L, 10.0 ml of acrylamide solution N, 3.0 ml glass-distilled water, 2.0 ml glycerol, 22 ul of 10% ammonium persulfate and 5 ul TEMED. The gel solutions were degassed by vacuum before being cast between two glass plates arranged in a plastic gel casting apparatus (Bio-Rad Laboratories) to give a polyacrylamide slab gel 1.5 mm thick with a 5 (top of the gel) to 15% (bottom of the gel) exponential acrylamide gradient. The 5 to 15% polyacrylamide gradient was formed from the bottom to the top by layering the 15% gel solution under the 5% gel

solution using a pumping device to ensure an even flow rate. After the gradient slab gel had polymerized (usually after 1 hr), the stacking gel was prepared. The stacking gel was a 4.5% acrylamide gel prepared with 2.5 ml of 0.5 M Tris-HCl buffer (pH 6.8) containing 0.4% SDS, 1.5 ml acrylamide solution N, 6.0 ml glass-distilled water, 30 ul of 10% ammonium persulfate and 10 ul of TEMED. A plastic well former was used during polymerization of the stacking gel to allow for application of 10 samples per gel.

b. electrophoretic separation of proteins

Fifty microliters of an "SDS-stop solution" (18 % SDS, 12.5 mM EDTA, 3.0 mM EGTA, 30 % 2-mercaptoethanol and 0.375 M Tris-HCl buffer, pH 6.8) was added to assay tubes containing terminated phosphotransferase incubation medium from the *in vitro* assay (Method E) and the covered tubes were placed in a boiling water bath for 2 min. The SDS-treated, ^{32}P-labeled cytosolic protein was quantitatively transferred to membrane tubing (Spectrapor) and dialyzed overnight at room temperature against a 500-fold volume of buffer consisting of 0.0625 M Tris-HCl, pH 6.8, 0.1% SDS and 5% 2-mercaptoethanol. The dialysis buffer was changed three to four times. The next day ^{32}P-labeled protein was removed from the membrane tubing and transferred to graduated conical glass cylinders so that the sample volume in the membrane tube could be measured. The protein concentration ofthe dialysate was calculated with corrections made for changes in

sample volume which occurred during dialysis. In order to directly compare the ^{32}P-labeling and electrophoretic patterns of soluble cytosolic proteins from denervated and sham-operated solei, equivalent aliquots of dialyzed, cytosolic protein (approximately 30 to 45 ug protein) were always taken and quantitatively transferred to individual sample wells of the gel. The maximum sample volume applied did not exceed 200 ul. Along with the protein applied, 10 ul of 66% sucrose and 5 ul of tracking dye (0.04% bromophenol blue solution from Eastman Kodak) was also added to each sample well. The electrophoresis buffer at the anode (upper reservoir) and the cathode (lower reservoir) of the double slab electrophoresis cell (Bio-Rad Laboratories) was 0.025 M Tris (free base) buffer, pH 8.3, with 0.192 M glycine and 0.1% SDS. A constant current of 20 mA/gel was used until the protein entered the stacking gel. It was then increased to 30 mA/gel and later to 40 mA/gel when the protein entered the gradient gel (running gel). The current was kept at 40 mA/gel until the tracking dye had migrated approximately 110 mm. Electrophoresis was then terminated and the slab gel was removed from between the glass plates and fixed in a solution of methanol-acetic acid-water (5:1:5) overnight.

c. staining of resolved proteins

Gels were stained for 30 min with a solution containing 1.25 g Coomassie brilliant blue R, 454 ml of 50%

methanol and 46 ml glacial acetic acid. The staining solution was filtered with Whatmann #1 filter paper before using. The gels were destained successively with methanol-acetic acid-water solutions of 5:1:5 and 1:1:9 for 1 hr each. Then the gels were placed in 7% acetic acid with 5% glycerol overnight. The protein-stained gels were dried onto filter paper by applying vacuum and heat for 2 hr with a gel dryer (Bio-Rad).

d. evaluation of resolved proteins

(1) estimation of molecular weights

The relative molecular weights of the resolved proteins were estimated after calibrating the electrophoresis system. This was done by plotting the relative mobility of the molecular weight marker proteins against the log of their molecular weights.

Molecular weight marker proteins (Sigma Chemical Co.) were boiled for 2 min in 0.0625 M Tris-HCl, pH 6.8, 0.1% SDS and 5% 2-mercaptoethanol (1 mg marker protein/ ml buffer). The linear relationship established was used to determine the relative molecular weights of specific soluble cytosolic phosphoproteins. The relative mobilities of the molecular weight marker proteins were calculated using the following equation:

$$\text{Relative Mobility } (R_f) = \frac{\text{migration (mm) of protein}}{\text{migration (mm) of tracking dye}}$$

(2) quantitation of ^{32}P-labeling in gel slices

The dried gel was placed on Kodak X-Omat AR film (Eastman Kodak) for 1 to 7 days. The film was placed in a lightproof film holder (Buck X-Ray Accessories, Cincinnati, OH) and stored at -20°C. The lightproof film carriage also housed a Cronex Intensifying Screen (Du Pont Chemical Co., Cleveland, OH) which was used to shorten the exposure time needed to obtain an autoradiograph. The distribution of ^{32}P-radiolabeling was determined qualitatively from the autoradiograph and quantitatively by slicing the dried gel into consecutive gel slices of 2 mm which were placed in a 7.5 ml toluene solution containing Permafluor I. The ^{32}P content of these slices was analyzed by liquid scintillation spectrometry.

L. Immunological Methods

1. Development of anti-RII (regulatory subunit of cyclic AMP-dependent protein kinase type II) antiserum

Anti-RII antiserum was a generous gift from the Sigma Chemical Co., St. Louis, MO. The antiserum was raised by Sigma in New Zealand white rabbits by the subcutaneous (s.c.) injection of 500 ug of purified cyclic AMP-dependent protein kinase regulatory subunit type II from bovine heart (Sigma Chemical Co.). Complete Freund's adjuvant accompanied the injection of protein kinase regulatory subunit. Booster injections (s.c.) of regulatory subunit (200 ug) were given

at 4, 8 and 10 weeks following the initial injection. The rabbits were bled through the ear vein 7 days following the final booster injection to harvest anti-RII antiserum. The rabbits continued to receive booster injections once every four weeks for three months and were bled three weeks after each of these injections. Antiserum was stored at 4°C in 0.1 % sodium azide.

2. Partial purification of RII antibody immunoglobulin G (IgG) fraction

The IgG fraction of the anti-RII antiserum was purified using sodium sulfate fractionation and DEAE-Sephadex anion exchange chromatography. Crude antiserum (2 ml) donated by Sigma Chemical Co. was diluted with 2 ml of 0.14 M NaCl, pH 7.2 (physiological saline). Solid sodium sulfate (Fisher Chemical Co., Fair Lawn, NJ) was added at a final concentration of 18 % (0.72 g) and the solution was allowed to incubate at room temperature for 30 min. The precipitate formed was pelleted by centrifugation at 2000 x g and 4°C for 10 min. The pellet obtained was resuspended in 1 ml of a 10 mM potassium phosphate buffer, pH 7.2, containing 15 mM NaCl and then dialyzed overnight against a 100 to 200-fold volume of this buffer at 4°C. The dialyzed antiserum was applied to a 5 ml DEAE-Sephadex (Pharmacia Fine Chemicals) column which had been previously equilibrated with the same buffer used for dialysis. The void volume was

collected and represented the partially purified IgG fraction of the anti-RII antiserum.

3. Antibody specificity

A double diffusion test was performed essentially according to Ouchterlony (1967) in 1 % agar gels to test the specificity of the purified IgG fraction of the anti-RII antibody. The double diffusion protocol was also used to test the anti-RII antibody against 15 and 20 ug of the cyclic AMP-dependent protein kinase type I holoenzyme (Sigma Chemical Co.), 20 ug of the purified catalytic subunit of this enzyme (Sigma Chemical Co.) and 20 ul of 0.15 M NaCl.

To prepare the double diffusion gel plates, 1 g of purified agar (Difco Laboratories, Detroit, MI) was dissolved in 100 ml of 0.05 M sodium borate, pH 7.2, by heating. After the solution cooled to room temperature, 10 ml was transferred to a 100 x 15 mm plastic petri dish (Falcon Plastics, Oxnard, CA) placed on a level surface to ensure even distribution of the agar gel. A 2 mm thick gel was formed. Four sample wells (4 mm diameter) were formed with a cork borer at an equal distance (5 mm) from the center antisera well (6 mm diameter) and equidistant from each other (10 mm). Purified IgG anti-RII antibody fraction (50 ul) was placed in the center well. Various aliquots of a 1 mg/ml solution of purified RII protein (20, 15, 10 and 5 ul) were placed in the four sample wells. Diffusion was carried out for 24 hr at room temperature.

Non-precipitated protein was washed from the gel with 0.15 M NaCl twice for 24 hr and once with glass-distilled water for 24 hr. Antibody-neutralized RII was visualized by staining the agar gel with a Coomassie brilliant blue R solution (1.25 g Coomassie brilliant blue R in 454 ml 50 % methanol and 46 ml glacial acetic acid) for 2 hr at room temperature and by destaining overnight in a solution of methanol:acetic acid: water (1:1:9) and then overnight again in 7 % acetic acid.

4. Immunoaffinity chromatography

a. preparation of anti-RII-IgG affinity column

Anti-RII antibody was coupled to Cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals). The activated Sepharose (2.9 g) was treated with 500 ml of 1 mM HCl (1 g is approximately equivalent to 3.5 ml of affinity gel). The treated gel was collected on a sintered glass filter and then washed with 15 ml of a coupling buffer consisting of 0.1 M $NaHCO_3$, pH 8.3, and 0.5 M NaCl. The purified IgG anti-RII antiserum fraction was diluted to 10 ml with 0.15 M NaCl and this solution was mixed with 30.0 ml of the coupling buffer. The gel suspension was added to the antibody/coupling buffer solution and allowed to incubate overnight at 4°C. The antibody affinity gel was then transferred to 10.0 ml of 0.2 M $NaHCO_3$ buffer, pH 8.0, with 0.1 M NaCl and 1 M ethanolamine-HCl and incubated with this buffer for 2 hr at

room temperature to block remaining active groups. The excess of uncoupled antibodies and blocking agent were removed by washing the gel alternately with 10 ml of low pH buffer (0.1 M sodium acetate buffer, pH 4.0, with 0.5 M NaCl) and 10 ml high pH buffer (coupling buffer, pH 8.3, with 0.5 M NaCl). This process was repeated four to five times.

b. adsorption of ^{32}P-labeled soluble cytosolic protein

Denervated and sham-operated soleus cytosolic fractions were prepared (Method C) and phosphorylated *in vitro* (Method E). Equal aliquots (approximately 200 ug protein) of ^{32}P-labeled denervated and sham-operated cytosolic protein were quantitatively transferred to membrane dialysis tubing (Spectrapor) and dialyzed overnight against a 500-fold volume of 50 mM sodium phosphate buffer, pH 7.0, with 1 mM EDTA at 4°C. The dialyzed ^{32}P-labeled cytosolic protein from denervated and sham-operated solei was applied to separate 1.0 ml anti-RII-IgG affinity columns and incubated with the resin for 1.0 hr at 4°C in a disposable 0.7 x 4.0 cm polypropylene column (Bio-Rad Laboratories). The antibody affinity columns were washed initially with 2.0 ml of 50 mM sodium phosphate buffer, pH 7.0. The eluate was saved and served as the unadsorbed fraction. Salts and non-specifically bound proteins were removed from the columns by washing with 10.0 ml of the sodium phosphate buffer containing 0.5 M NaCl and then with 2.0 ml of 10 mM Tris-HCl

buffer, pH 7.4 (Uno, 1980).

c. elution of specifically-bound ^{32}P-labeled protein

^{32}P-radiolabeled adsorbed antigens were eluted from the anti-RII IgG affinity columns with 0.5 ml of 0.2 M glycine buffer, pH 2.2, and the eluates were then neutralized with 1.0 M Tris base (Uno, 1980). Fifty microliters of SDS-stop solution (Method K) was added to each 200 ul aliquot of the eluates from the denervated and sham-operated solei. The SDS-treated eluates were heated for 2 min at 100°C, quantitatively transferred to Spectrapor membrane dialysis tubing, and then dialyzed overnight against a 500-fold volume of 0.0625 M Tris-HCl, pH 6.8, with 0.1% SDS and 5% 2-mercaptoethanol. Also, the unadsorbed fractions collected previously (b) were concentrated overnight by lyophilization and resuspended with boiling in 0.5 ml of 0.0625 M Tris-HCl buffer, pH 6.8, with 0.1 % SDS and 5 % 2-mercaptoethanol.

d. resolution of ^{32}P-labeled eluates

Aliquots (200 ul) of the unadsorbed protein fractions from denervated and sham-operated solei were electrophoresed on a SDS-polyacrylamide gradient slab gel (Method K). The dialyzed proteins eluted from the anti-RII affinity column were also electrophoretically separated in a SDS-polyacrylamide gradient slab gel (Method K). ^{32}P-labeled soluble proteins from the cytosolic fractions of denervated and sham-operated solei (adsorbed and unadsorbed fractions)

were analyzed qualitatively by autoradiography of the slab gel and quantitatively in 2 mm gel slices by liquid scintillation spectrometry (Method O). Relative molecular weights of specifically-bound soluble cytosolic phosphoproteins were established by calibrating the SDS-polyacrylamide gradient slab gel using SDS molecular weight marker proteins (Method K).

M. Peptide Mapping

Peptide maps of a specific 56,000-dalton phosphoprotein resolved from the cytosolic fraction of denervated and sham-operated solei were obtained essentially according to the method of Cleveland et al. (1977). This method allows for a rapid and convenient analysis of the enzymatic proteolytic fragments of specific proteins isolated in SDS gels.

Cytosolic fractions were prepared from denervated and sham-operated rat solei (Method C). These fractions were phosphorylated *in vitro* (Method E) and the ^{32}P-labeled 56,000-dalton phosphoprotein was resolved from these muscles by SDS gradient slab PAGE (Method K). After electrophoresis, the gel was removed from between the glass plates and placed in a solution of methanol: acetic acid: water (5:0.5:5) overnight. The next day the gel was transferred immediately to a staining solution consisting of 1.25 g Coomassie brilliant blue R in 454 ml 50% methanol and 46 ml glacial acetic acid. The gel was stained for no longer

than 15 to 20 min. The gel was destained briefly (45 min to 1 hr) in a methanol-acetic acid-water (5:0.5:5) solution and then in 5% acetic acid for 1 hr. The protein-stained gel was placed on a transparent plastic sheet and protein-staining bands were visualized with a light box. The 56,000-dalton phosphoproteins were sliced from each sample column of the slab gel. Each gel slice was incubated for 30 min in 10 ml of a 0.125 M Tris-HCl buffer, pH 6.8, with 0.1% SDS and 1 mM EDTA with occasional stirring.

While the gel slices were incubating, the polyacrylamide gel to be used for peptide mapping was cast. This gel (1.5 mm thick x 8.5 mm long) was cast between two glass plates as described for the SDS-polyacrylamide gradient slab gel (Method K). To make the 15% acrylamide gel casting solution, 20 ml of a 29.2% acrylamide solution containing 0.8% N,N-methylene-bisacrylamide was combined with 10.0 ml of 1.5 M Tris-HCl buffer, pH 8.8, with 0.4% SDS, 6.0 ml glass-distilled water, 4.0 ml glycerol, 44 ul of 10.0% ammonium persulfate and 10.0 ul of TEMED. The 8.5 cm peptide gel was also cast with a longer than usual stacking gel (5 cm). The stacking gel was cast by combining 3 ml of the acrylamide solution described above with 5.0 ml of 0.5 M Tris-HCl buffer, pH 6.8, with 0.4% SDS, 12.0 ml of glass-distilled water, 60.0 ul of 10.0% ammonium persulfate and 20.0 ul TEMED. A Teflon well former was used when casting the stacking gel to allow for application of 10 samples.

The specific gel slices for peptide mapping were removed from the 0.125 M Tris-HCl incubation buffer and trimmed to 2 x 5 mm. Trimming was necessary in order to place these gel slices in the formed sample wells of the 15% polyacrylamide peptide mapping gel. Approximately 88.3 ± 1.0% (mean ± S.E.M. with n=4) of the total ^{32}P-radioactivity in the whole gel slice (usually about 2 x 8 mm after incubation) was consistently and reproducibly recovered in the 2 x 5 mm trimmed gel slice. The trimmed gel slices were placed in the formed sample wells. The outer two wells of the slab gel contained a mixture of standard known molecular weight marker proteins prepared for electrophoresis as previously described (Method K). Sample wells containing the gel slices were filled with 200 ul of 0.125 M Tris-HCl buffer, pH 6.8, with 0.1% SDS and 1 mM EDTA, 10.0 ul of glycerol and 10.0 ul of a 0.04% Bromophenol blue tracking dye solution. The gel slices were pushed to the bottom of the sample wells with a spatula. Finally, 0.5, 1.0 or 10.0 ug of bovine pancreas trypsin (Sigma type III from Sigma Chemical Co.) was added to some sample wells. One microgram of trypsin was added to only one of the side wells containing the marker proteins. Electrophoresis was conducted as described for the SDS-polyacrylamide gradient slab gel system (Method K) except that the current was turned off for 30 min when the tracking dye was 5 to 10 mm from the bottom of the stacking gel. Electrophoresis was terminated when the tracking dye had migrated to within 2 to 5 mm from the bottom of the gel.

After electrophoresis, the peptide gel was stained and destained (Method K). The gel was dried with a Bio-Rad gel dryer and the dried gel was placed on Kodak X-Omat AR film for 1 to 7 days. An autoradiograph was obtained with the aid of Cronex Intensifying screens. The autoradiograph was used to qualitatively compare the ^{32}P-labeled tryptic peptides of the 56,000-dalton phosphoprotein from denervated and sham-operated solei. All molecular weights were determined after calibrating the gels as described in Method K. Quantitative analysis of the tryptic ^{32}P-labeled phosphopeptides generated from the ^{32}P-labeled 56,000-dalton phosphoprotein was carried out by sectioning the peptide gel into consecutive 2 mm gel slices and counting the radioactivity of each slice by liquid scintillation spectrometry. It was determined that 66.4 $\pm$ 7.6 % (mean $\pm$ S.E.M. with n=24) of the total ^{32}P-radioactivity applied to the peptide gel (total radioactivity associated with the 56,000-dalton phosphoprotein in the trimmed gel slice) was consistently and reproducibly recovered in the peptide mapping gel. Also, by paired t-test the percentage of ^{32}P-radioactivity recovered when the 56,000-dalton phosphoprotein from denervated muscle was applied was not significantly different from the percentage recovered when the 56,000-dalton phosphoprotein from sham-operated, contralateral muscle was applied.

N. Phosphorylation of the Regulatory Subunit of Cyclic AMP-Dependent Protein Kinase Type II

The regulatory subunit of the type II cyclic AMP-dependent protein kinase purified from bovine heart was obtained from Sigma Chemical Co. This protein was phosphorylated *in vitro* using the phosphorylation assay protocol described in Method E. Four micrograms of purified cyclic AMP-dependent protein kinase catalytic subunit from bovine heart (Sigma Chemical Co.) was added to the phosphorylation incubation medium along with 0.13 ml of 0.32 M sucrose with 3.0 mM $MgCl_2$, pH 6.5. The phosphorylation reaction was started with the addition of 4.0 ug of regulatory subunit type II and terminated after a 5 min incubation period at 37°C with 5 mM ATP and 1.5 % sodium pyrophosphate (Method E).

O. Liquid Scintillation Spectrometry

1. Instrument

A Packard Tri-Carb model 3380 liquid scintillation spectrometer (Packard Instruments, Downers Grove, IL) was used for analysis of all ^{32}P-labeled radioactive samples. The spectrometer was integrated with a teletype printer and a tape punch mechanism. All ^{32}P-labeled radioactive samples were analyzed with a factory set ^{32}P-channel which was optimized for counting of single-labeled ^{32}P-samples. The pre-set channel (X) had an amplification setting of 0.3% to

0.5% and a window of 20 divisions (lower limit discriminator, 0.03 MeV) to 1000 divisions (upper limit discriminator, 1.7 MeV).

2. Sample preparation

^{32}P-radioactivity of nitrocellulose Millipore filters, thin layer chromatography paper strips or SDS-polyacrylamide gel slices were analyzed by liquid scintillation spectrometry in a 7.5 ml liquid scintillant. A 1 liter stock solution of the liquid scintillant was prepared by combining 960 ml of scintillation grade toluene (Research Products International Corp., Elk Grove Village, IL) with 40 ml of Permafluor I (scintillation grade, Packard Instrument Co.). The resulting "scintillation cocktail" contained 5.0 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP). Samples (filters, gel slices or thin layer paper strips) were counted in 20 ml low-phosphate borosilicate glass vials fitted with plastic screw caps. Filters, gel slices and thin layer paper strips were always placed at the bottom of the glass vials.

^{32}P-labeled soluble cytosolic protein in fractions collected during chromatographic separations were analyzed by liquid scintillation spectrometry using a liquid scintillant (Phase Combining System, Amersham Corp., Des Plaines, IL) especially suitable for use with aqueous solutions. Aliquots of these fractions were introduced into the fluor by pipetting. Then the borosilicate glass vials were capped and

shaken vigorously to dissolve the test material.

3. Counting accuracy

The Packard model 3380 scintillation counter indicates the statistical counting accuracy for each sample counted as percent standard deviation. The percent standard deviation is the quotient of 100 and the square root of the gross counts in the ^{32}P channel. This is expressed to the nearest of 12 values ranging from 9.5 to 0.1. All ^{32}P-radioactive samples were counted for 10.0 min. Only those counts per minute that were within a percent standard deviation range of 0.1 to 2.5 were considered for analysis of results. Thus, for example, if a sample had 200 counts per minute with a 2% counting accuracy this would indicate a 2% counting error associated with those 200 counts per minute at a 68.27% confidence level.

4. Counting efficiency

The efficiency of counting the ^{32}P was determined by external standarization for the two liquid scintillants used. External standardization was carried out by counting a known amount (dpm) of [gamma-^{32}P]ATP in 7.5 ml of either of the two scintillants using varying concentrations of quencher (0 to 0.5 % chloroform). Chloroform is a chemical quencher which competes with the scintillator for the energy being transferred from the solvent. Counting efficiency correla-

tion curves were obtained by plotting efficiency ratios (dpm obtained/known dpm) versus the automatic external standard ratio. The Packard model 3380 automatic external standardization (AES) system uses a compound gamma source consisting of americium-241 and radium-226. These two sources are spaced apart so that americium-241 irradiates the sample near the top of the sample and radium-226 irradiates it near the bottom. Counts in the automatic external standard channel are accumulated during two automatic one-half min counting periods. The AES count ratio is obtained by division of the counts recorded in two fixed, internal AES counting channels. This ratio is calculated by the computer and is recorded for each sample counted. The correlation curves in Figure 48 I and II of the Appendix show that the counting efficiency was 97 to 99% for both scintillants. This factor was not included in calculations of counting data.

5. Conversion of ^{32}P-cpm to cytosolic protein phosphorylation

Counting data (^{32}P-counts per minute) from the *in vitro* phosphorylation assay (Method E) was converted to cytosolic protein phosphorylation by a computer program. The Packard liquid scintillation spectrometer was integrated with a digital computer (Hewlett Packard model 9830 with tape reader model 2748B and printer model 9866A) so that counting data (cpm) could be entered into the computer via punched paper tape. A teletype was used to punch tape automatically

after each sample count. A computer program which had been written in BASIC language was used to convert cpm to dpm. This program incorporated factors for ^{32}P-decay, background counts per minute and the specific activity of the [gamma-^{32}P]ATP which was the phosphoryl donor in the *in vitro* phosphorylation assay. The computer program was also able to convert dpm/ug soluble cytosolic protein to cytosolic protein phosphorylation reported as femtomoles (10^{-15} moles) of ^{32}P-phosphate incorporated per mg soluble cytosolic protein per 5 min incubation period.

P. Statisical Treatment of Data

The statistical significance of differences between experimental and control measurements was determined using a two-sided Student's t-test. A paired t-test was used when comparing paired experimental and control samples. A level of $p < 0.05$ was accepted as minimally significant.

III. RESULTS

A. Phosphorylation of Soluble Cytosolic Protein from Rat Soleus Muscle

1. Requirements and characterization of the *in vitro* phosphorylation reaction

The phosphorylation of soluble protein from cytosolic fractions of rat soleus muscle was assayed *in vitro* using an incubation medium consisting of 0.1 M NaCl, 50 mM Tris-HCl buffer, pH 7.5, 2 mM $MgCl_2$ and 6.7×10^{-9} M [gamma-^{32}P]ATP (3000 Ci/mM) as described in Method E. The soluble protein obtained after high speed centrifugation of the soleus muscle homogenate (Method C) included endogenous protein kinases and phosphorylatable protein substrates. Phosphorylation was determined as the femtomoles (10^{-15} moles) of ^{32}P-phosphate incorporated into trichloroacetic acid precipitable material per mg of soluble cytosolic protein per incubation period. The phosphorylation of soluble cytosolic protein was found to be heat-labile (Table 3). When Mg^{+2} was deleted from the phosphorylation assay, a slight (22%) but significant ($p < 0.05$) reduction in cytosolic protein phosphorylation was observed (Table 3). The lack of a larger reduction in phosphorylation might be due to endogenous Mg^{+2} or the small amount of Mg^{+2} derived during homogenization of the muscles with 0.32 M sucrose and 3 mM $MgCl_2$, pH 6.5, since addition of 6.5 mM EDTA, a chelator of

TABLE 3. REQUIREMENTS FOR THE PHOSPHORYLATION OF SOLUBLE CYTOSOLIC PROTEIN FROM RAT SOLEUS MUSCLE

Incubation Medium	Protein Phosphorylation[a]
complete[b]	9.1 ± 0.7
without Mg^{+2}	7.1 ± 0.1 *
with EDTA (6.5 mM)	0
with Ca^{+2} (0.2 mM)	9.0 ± 0.5
with Ca^{+2} (2.0 mM)	9.7 ± 0.3
with EGTA (1.0 mM)	9.9 ± 0.4
with cyclic AMP (10^{-11} to 10^{-9} M)	10.4 ± 1.2
with cyclic AMP (10^{-6} M)	5.7 ± 1.0 *
with cyclic GMP (10^{-6} M)	9.3 ± 0.2
with protein kinase inhibitor[c] (40 ug)	4.6 ± 0.4 **
boiled cytosolic protein fraction	0

[a] cytosolic protein phosphorylation (fmoles ^{32}P-PO_4/incubation period (5 min)/mg protein) are the means ± S.E.M. from 3 to 4 groups of 3 rats. * $p < 0.05$ and ** $p < 0.01$.

[b] The complete incubation medium (Method E) consists of 0.1 M NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$ and 5 uCi [gamma-^{32}P]ATP (3000 Ci/mmole). The phosphorylation reaction was carried out as described in Method E after the addition of 40 to 45 ug of soluble cytosolic protein.

[c] Purchased from Sigma Chemical Co., St. Louis, MO, Type II from bovine heart fractionated essentially as described by Ashby and Walsh (1971) for the cyclic AMP-dependent protein kinase inhibitor.

Mg^{+2}, completely inhibited the phosphorylation reaction. These results are consistent with protein kinase catalyzed phosphotransferase reactions (EC 2.7.1.37-ATP:protein phosphotransferases) which are dependent upon the presence of this divalent cation. Ca^{+2}, which serves as a modulator of other cytosolic protein kinases when combined in a ternary complex with calmodulin (Cheung, 1980), and EGTA, a strong chelator of Ca^{+2} ions, had no effect upon this phosphotransferase reaction. Also, no change in cytosolic protein phosphorylation was observed after the addition of 10^{-6} M cyclic GMP. This nucleotide stimulates a soluble cytosolic cyclic GMP-dependent protein kinase. A slight and statistically insignificant stimulation (14%) of the endogenous phosphorylation reaction was seen after the addition of exogenous concentrations of cyclic AMP in the range of 10^{-11} to 10^{-9} M, but a significant reduction (37%) occurs with micromolar additions. Cyclic AMP activates cytosolic cyclic AMP-dependent protein kinases by binding to the regulatory subunit of the holoenzyme. This results in a dissociation of the regulatory and catalytic subunits and activation of the free catalytic subunit (Builder et al., 1980). Addition of 40 ug of a specific inhibitor (commercial preparation) of the free catalytic subunit of cyclic AMP-dependent protein kinase, first identified by Ashby and Walsh (1972), significantly inhibited (49%) the cytosolic phosphorylation reaction.

Protein phosphorylation was also studied in cytosolic fractions from rat solei denervated for 24 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before insertion into the muscle. Protein phosphorylation in these cytosolic fractions was compared to cytosolic protein phosphorylation in the contralateral, sham-operated muscles. After incubation of 15 to 90 ug of soluble cytosolic protein from either 24 hr denervated short nerve stump-solei or contralateral, sham-operated solei, a direct relationship was observed between the concentration of soluble cytosolic protein added to the buffered (pH 7.5) phosphorylation incubation medium and the ^{32}P-radiolabeling of the trichloroacetic acid precipitable material (Figure 4). Within this range, therefore, it was valid to compare the cytosolic protein phosphorylation from denervated and sham-operated muscles. This protein range was used for all denervation studies.

An incubation time-course study of the <u>in vitro</u> phosphorylation of soluble cytosolic protein shows that when the phosphotransferase reaction is terminated at 1 or 2 min instead of after 5 min (the incubation time used throughout), the phosphorylation of soluble protein from the cytosolic fractions of 24 hr denervated short nerve stump-solei is still consistently increased more than twofold relative to the contralateral, sham-operated solei. Also, even though the rate of cytosolic protein phosphorylation declined after a 10 min incubation period, the difference in the

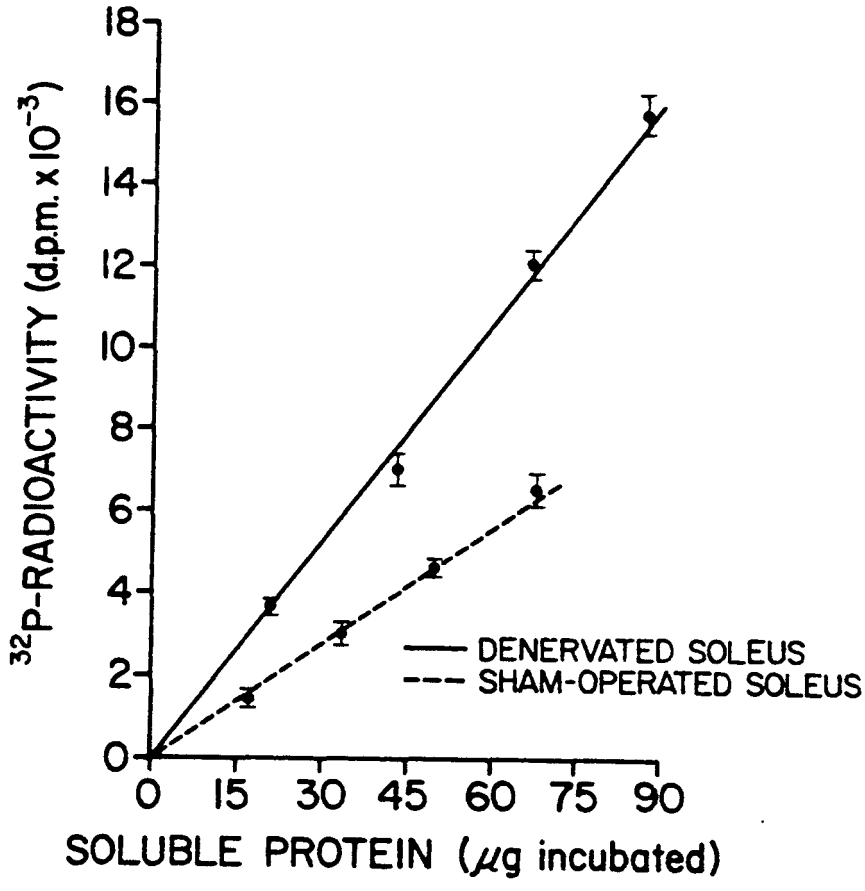


Figure 4. The relationship of phosphorylation of soluble cytosolic protein in denervated (———) and sham-operated (-----) solei of the rat to the concentration of soluble protein in the incubation medium. The left soleus was denervated 24 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before insertion into the muscle. Control samples were from sham-operated, contralateral muscles. Phosphorylation of soluble cytosolic protein was conducted in a phosphorylation reaction buffer containing 0.1 M NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$ and 6.7×10^{-9} M [gamma-^{32}P]ATP (3000 Ci/mmole) in a total volume of 0.25 ml (Method E), but with several concentrations of soluble cytosolic protein. Values are reported as means and ranges of duplicate assays for each level of protein.

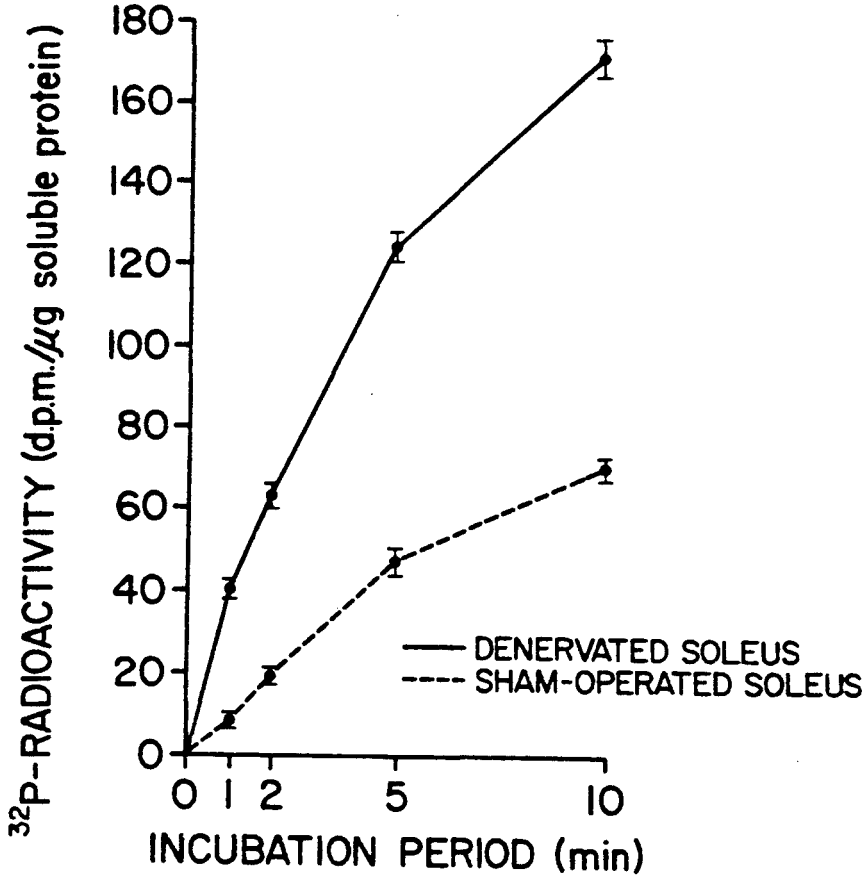


Figure 5. Time course of phosphorylation of soluble cytosolic protein in denervated (———) and sham-operated (----) solei of the rat. The left soleus was denervated 24 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before insertion into the muscle. Control samples were obtained from sham-operated, contralateral muscles. Phosphorylation of soluble cytosolic protein was conducted in a phosphorylation reaction buffer containing 0.1 M NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$ and 6.7×10^{-9} M [gamma-^{32}P]ATP (3000 Ci/mmole) in a total volume of 0.25 ml (Method E), but after incubation periods of 1, 2, 5 and 10 min. Values are reported as means and ranges of duplicate assays at each time period.

phosphorylation of cytosolic protein is again approximately twofold greater in denervated solei relative to the contralateral, sham-operated solei (Figure 5).

Incorporation of the *in vitro* ^{32}P-radiolabel into trichloroacetic acid precipitable soluble cytosolic material was determined as alkali-labile, acid-stabile protein-bound phosphate (Method I). Table 4 shows that most of the ^{32}P-label associated with the soluble cytosolic substrates from both 24 hr denervated short nerve stump-solei (85.6 ± 1.7%, mean ± S.E.M. with n=3) and contralateral, sham-operated solei (84.1 ± 2.0%, mean ± S.E.M with n=3) is incorporated into protein as alkali-labile, acid-stable phosphate probably linked to serine or threonine residues. Also, the percentage of the total ^{32}P-label incorporated into protein asalkali-labile phosphate is not statistically different in denervated solei (by paired t-test) relative to contralateral, sham-operated solei.

2. Conversion of [gamma-^{32}P]ATP to ^{32}P-inorganic phosphate by endogenous adenosine triphosphatase (ATPase)

The conversion of [gamma-^{32}P]ATP to ^{32}P-inorganic phosphate by endogenous ATPase was assessed for varying concentrations of soluble cytosolic protein under the *in vitro* phosphorylation conditions described previously (Method E). The separation of these two ^{32}P-radiolabeled components was carried out by one-dimensional thin layer chromatography

TABLE 4. PERCENTAGE OF ^{32}P-PHOSPHATE AS PHOSPHOLIPID, ACID-LABILE AND ALKALI-LABILE PHOSPHATE IN CYTOSOLIC FRACTIONS OF DENERVATED AND CONTRALATERAL, SHAM-OPERATED RAT SOLEI AFTER *IN VITRO* PHOSPHORYLATION[a]

Extract	% of Total ^{32}P-Radioactivity[b]	
	Denervated	Sham-operated
Phospholipid	2.4 ±0.5	1.8 ±0.3
Acid-labile Phosphate	10.2 ±0.8	12.7 ±0.6
Alkali-labile Phosphate	85.6 ±1.7	84.1 ±2.0

[a] Phosphorylation of soluble cytosolic protein from denervated and sham-operated solei was determined as described in Method I. Cytosolic fractions were prepared from rat solei denervated with a short (< 2 mm) distal nerve stump for 24 hr prior to sacrifice and from contralateral, sham-operated muscles.

[b] Percentages were calculated from the ratios of ^{32}P-radioactivity in the form of phospholipid, acid-labile phosphate and alkali-labile phosphate to the total ^{32}P-radioactivity determined in TCA-precipitable soluble cytosolic protein essentially according to Weller (1979) as described in Method I. Percentages are reported as means ± S.E.M with n=3.

B. Effect of Denervation and Nerve Stump Length Upon the Phosphorylation of Soluble Cytosolic Protein from Rat Soleus Muscle

A comparison of the phosphorylation of soluble protein from the cytosolic fractions of denervated and contralateral, sham-operated rat solei is shown in Figure 8. Cytosolic protein phosphorylation was determined as the femtomoles of ^{32}P-phosphate incorporated into trichloroacetic acid-precipitable material per mg soluble cytosolic protein in the buffered phosphorylation incubation medium (pH 7.5) per incubation period (Method E).

Rat solei were denervated for 1, 3, and 6 hr and also at 6 hr intervals to a maximum of 78 hr by cutting the left sciatic nerve or one of its branches so that either a short nerve stump of 2 mm or less, an intermediate nerve stump of 17 to 20 mm, or a long nerve stump of 32 to 35 mm was left attached to the muscle (illustrated in Figure 1). With distal nerve stumps of short and intermediate lengths, the sciatic nerve was cut in the midthigh region leaving a 32 to 35 mm distal nerve stump. The midthigh cut denervates all the muscles of the lower hindlimb eliminating the possibility of passive stretching of the soleus by contraction of neighboring muscles.

The level of cytosolic protein phosphorylation of denervated muscles was compared to that of the corresponding sham-operated muscle from the contralateral hindlimb. A

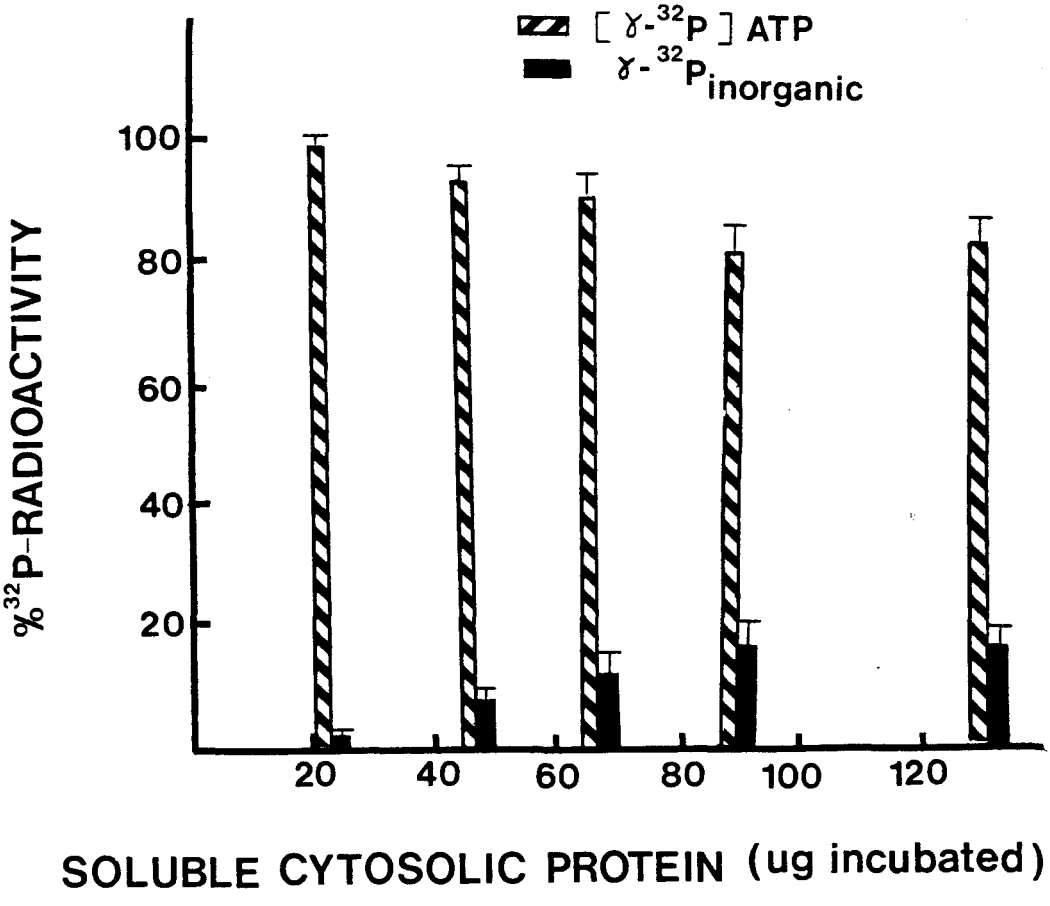
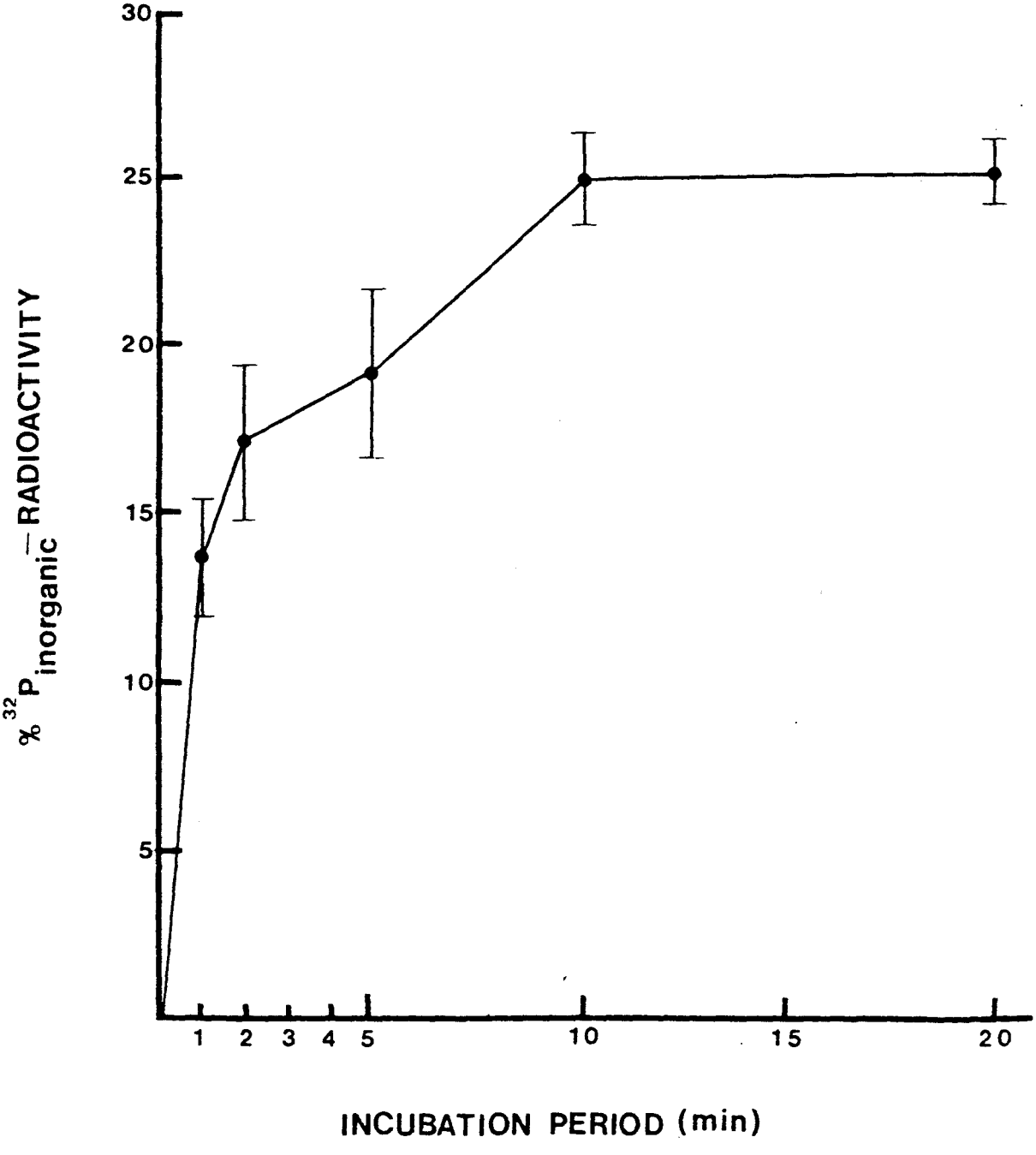


Figure 6. Relationship of ATPase activity in the soluble cytosolic fraction from unoperated rat soleus muscle to the concentration of soluble cytosolic protein in the incubation medium. The ^{32}P-radioactivity associated with ATP and inorganic phosphate was determined in cytosolic fractions pooled from three muscles by thin layer chromatography and liquid scintillation spectrometry as described in Method G with aliquots of the 100,000 x g supernatant fraction containing 20 to 130 ug of protein. Values are reported as means ± S.E.M. of triplicate assays for each level of protein.

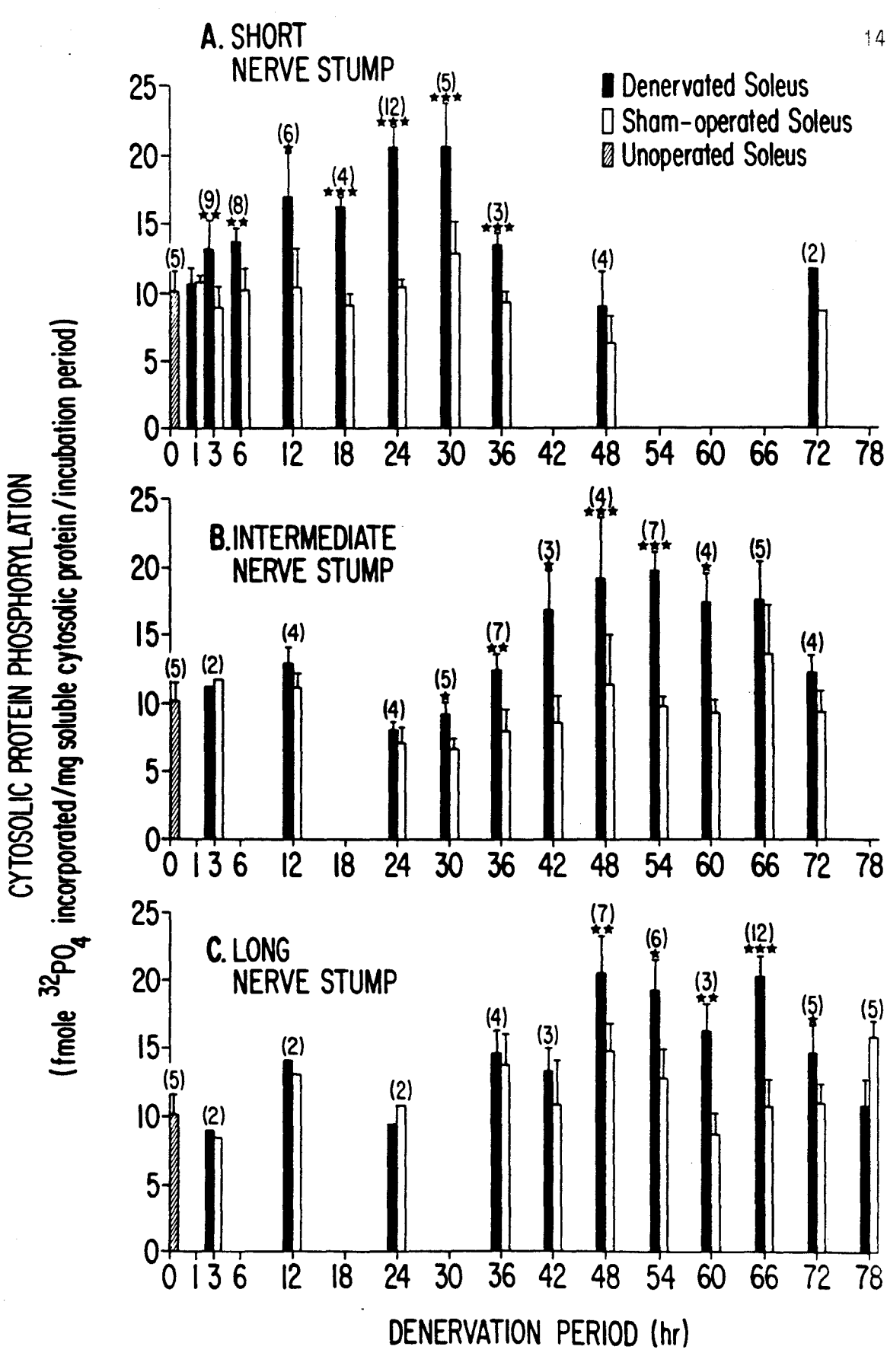
Figure 7. Time course of the appearance of ^{32}P-inorganic phosphate in the soluble cytosolic protein fraction from three pooled unoperated rat solei. The percentage of ^{32}P-radioactivity in the form of inorganic phosphate was calculated by determining the ratio of ^{32}P-inorganic phosphate to ^{32}P-ATP. Separation of radiolabeled components from the incubation medium was carried out by thin layer chromatography as described in Method G. Incubation of protein (87.5 ug) was for 1, 2, 5, 10, 15 or 20 minutes. Values are reported as means ± S.E.M. of triplicate assays at each incubation period.


30
25
20
15
10
5
% $^{32}P_{inorganic}$ – RADIOACTIVITY
1
2
3
4
5
10
15
20
INCUBATION PERIOD (min)

paired Student's t-test was used to determine statistical significance. With this statistical evaluation, therefore, each animal served as its own control.

Cytosolic protein phosphorylation was significantly increased approximately twofold in the denervated muscles whether a short, intermediate or long nerve stump was left attached to the soleus muscle (Figure 8). Additionally, the elapsed time during which cytosolic protein phosphorylation was significantly stimulated in the denervated muscles (24 to 33 hr) was also the same for each of the three nerve stump lengths. The lag period, however, which was observed after denervation of the soleus muscle before the phosphorylation of cytosolic protein was significantly increased, was different for each of the three distal nerve stumps. Specifically, with the short 2 mm nerve stump a significant increase in cytosolic protein phosphorylation was observed as early as 3 hr ($p < 0.02$) after denervation. However, a significant increase was not observed until 30 hr ($p < 0.05$) and 48 hr ($p < 0.02$) for the intermediate (17 to 20 mm) and long (32 to 35 mm) nerve stumps, respectively. At the early periods of 3, 12 and 24 hr, a significant increase in cytosolic protein phosphorylation was observed with the short 2 mm nerve stump. However, no significant change in phosphorylation occurs at these times or at 24 hr when an intermediate (17 to 20 mm) nerve stump is left attached to the muscle. Also, no change is observed in cytosolic protein

Figure 8. Temporal dependency of cytosolic protein phosphorylation of denervated rat solei upon the length of distal nerve stump remaining attached to the muscle. The left soleus muscle of the rat hindlimb was denervated for the given periods prior to sacrifice by transecting the sciatic nerve or its branches so that the distal nerve stumps were short < 2 mm (A), intermediate 17-20 mm (B) or long 32-35 mm (C). A sham-operation was performed on the contralateral hindlimb. Unoperated muscles were obtained from separate groups of animals. Phosphorylation of soluble cytosolic protein was conducted as described in Method E in a phosphorylation reaction buffer containing 0.1 M NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$ and 6.7 x 10^{-9} M [gamma-^{32}P]ATP (3000 Ci/mmole) in a total volume of 0.25 ml. The results are means $\pm$ S.E.M. for the number of groups of 3 rats indicated in the parentheses. * $p < 0.05$, ** $p < 0.02$ and *** $p < 0.01$ based upon paired t-tests.


A. SHORT NERVE STUMP
B. INTERMEDIATE NERVE STUMP
C. LONG NERVE STUMP
Denervated Soleus
Sham-operated Soleus
Unoperated Soleus
CYTOSOLIC PROTEIN PHOSPHORYLATION
(fmole 32PO4 incorporated/mg soluble cytosolic protein/incubation period)
DENERVATION PERIOD (hr)

phosphorylation at 3, 12, 24, 36 and 42 hr when the long (32 to 35 mm) nerve stump is left attached to the muscle. However, a significant increase in phosphorylation is observed at 3, 12 and 24 hr and at 36 and 42 hr for the short and intermediate nerve stump-solei, respectively.

The denervation period corresponding to the maximum increase in cytosolic protein phosphorylation was observed was also different for the three distal nerve stump lengths. For example, with the short nerve stump-solei the maximal increase was observed at 24 hr. However, with the intermediate and long nerve stump solei the maximal increase in cytosolic protein phosphorylation was observed at 54 and 66 hr, respectively.

Cytosolic protein phosphorylation was not significantly increased in denervated muscles relative to contralateral sham-operated muscles at longer denervation periods. This was observed at 48 and 72 hr with the short 2 mm nerve stump muscles, 66 and 72 hr with the 17 to 20 mm intermediate nerve stump muscles and 78 hr with the long 32 to 35 mm nerve stump muscles.

Protein phosphorylation in the cytosolic fractions of muscles from unoperated animals did not differ significantly from sham-operated muscles. Also, cytosolic protein phosphorylation in sham-operated muscles did not differ significantly from each other.

In a separate group of animals the left soleus muscle was denervated by cutting the soleus branch of the

sciatic nerve to leave a 2 mm nerve stump while the right soleus muscle was also denervated by cutting the sciatic nerve at a distance of 32 to 35 mm from its insertion into the muscle. On the left side the proximal cut (32 to 35 mm from insertion) was also made and preceded the distal denervation. Twenty four hours after these surgical procedures soluble cytosolic protein phosphorylation in the right versus left solei was compared. For this "double denervation" protocol, therefore, cytosolic protein phosphorylation was compared in two denervated muscles (right and left solei) that differed only in the length of the distal nerve stump left attached to these muscles. With previous animal groups, denervated solei was always compared to contralateral, sham-operated solei.

When both the left and right solei were electrically silenced for 24 hr as described above, a significant increase (162.8 ± 17.3%, mean ± S.E.M. with n=4 and $p < 0.02$ by paired t-test) in cytosolic protein phosphorylation was observed in the left denervated short nerve stump-solei when compared to the contralateral, denervated long nerve stump-solei (Table 5). Also, this increase (162.8 ± 17.3%) in cytosolic protein phosphorylation in the "double denervation" group was not significantly different (by Student's t-test) from the increase in cytosolic protein phosphorylation in 24 hr short nerve stump solei versus contralateral, sham-operated solei (199.8 ± 3.4, mean ± S.E.M., n=12, Figure 8).

TABLE 5. COMPARISON OF THE EFFECT OF "DOUBLE DENERVATION" AND DENERVATION UPON THE PHOSPHORYLATION OF SOLUBLE CYTOSOLIC PROTEIN FROM RAT SOLEUS MUSCLE

Denervation Protocol	% Change[c] (Left/Right x 100)
Double Denervation[a] (4)	162.8 ± 17.3 **
Denervation[b] (12)	199.8 ± 3.4 ***

[a] The left soleus was denervated for 24 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before its insertion into the muscle. This surgical procedure was immediately preceded by cutting the left sciatic nerve 32 to 35 mm before insertion into the muscle. The right contralateral soleus was also denervated by cutting the right sciatic nerve 32 to 35 mm before insertion into the muscle. However, the more distal cut (< 2 mm distal nerve stump) was not made on the right side.

[b] In this group of rats the left soleus muscle was denervated for 24 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before insertion into the muscle (Figure 1C). The sciatic nerve was also cut at a distance of 32 to 35 mm from insertion into the left soleus (Figure 1A). The right contralateral soleus muscle was sham-operated.

[c] The percent changes are determined by dividing the cytosolic protein phosphorylation in the left soleus by the cytosolic protein phosphorylation in the right soleus muscle and are reported in the Table as means ± S.E.M. ** $p < 0.02$ and *** $p < 0.01$. Statisitical differences were evaluated by paired t-tests. The number of groups of three rats at the 24 hr denervation period is given in the parentheses. Cytosolic protein phosphorylation (femtomoles $^{32}PO_4$ incorporated/ mg soluble cytosolic protein/ incubation period) was determined as described in Method E.

A direct relationship between the earliest denervation period with a significant increase in cytosolic protein phosphorylation (onset period) and the length of the distal nerve stump left attached to the muscle was found based upon these nerve stump lengths (Figure 9). The onset periods from Figure 8 are 3 hr ($p < 0.02$) for the short (2 mm) nerve stump, 30 hr ($p < 0.05$) for the intermediate (17 to 20 mm) nerve stump and 48 hr ($p < 0.02$) for the long (32 to 35 mm) nerve stump. The slope of the solid linear regression line in Figure 9 indicates that the onset period is delayed 1.4 hr for every millimeter of distal nerve stump remaining attached to the muscle.

The dashed linear regression line in Figure 9 which is based upon the denervation period corresponding to the maximum increase in cytosolic protein phosphorylation relative to sham-operated muscles also indicates that this change is directly related to nerve stump length. The lag period derived from the slope of the line is 1.3 hr for every millimeter of distal nerve stump remaining attached to the muscle and is similar to the delay (1.4 hr/mm nerve) prior to onset. The periods corresponding to the maximal increase in cytosolic protein phosphorylation (see Figure 8) are 24, 54 and 66 hr for muscles with short, intermediate and long nerve stumps, respectively.

The development of muscle atrophy was assessed for each denervation period with each nerve stump length indicated in Figure 8 by comparing the wet weights of

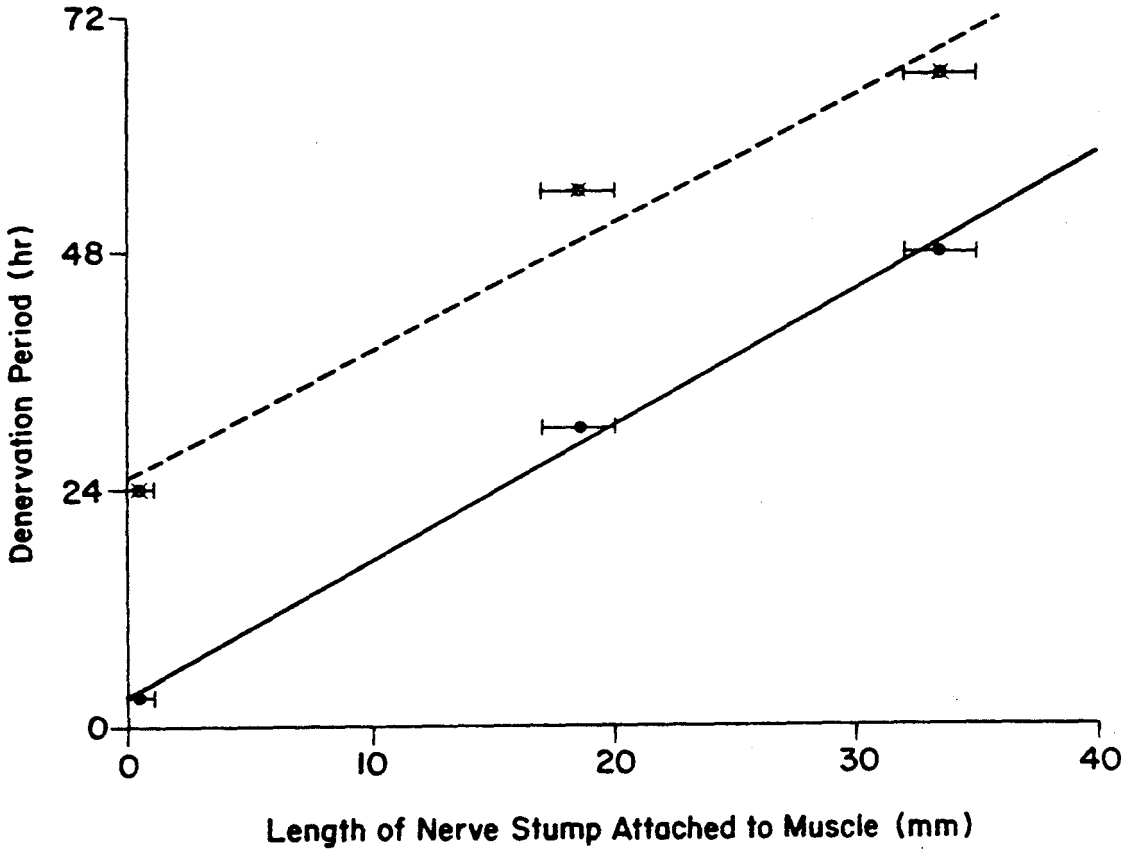


Figure 9. The relationship of the times of onset and of maximal increase of cytosolic protein phosphorylation in denervated solei to the length of the nerve stump remaining attached to the muscle. Time of onset was defined as the period of the first statistically significant increase in cytosolic protein phosphorylation ($p < 0.05$). As shown in Figure 8, this occurred at 3, 30 and 48 hr for solei with distal nerve stumps of < 2 mm, 17-20 mm and 32-35 mm, respectively. The slope of the solid linear regression line derived from these points indicates that the onset of increased protein phosphorylation is delayed 1.4 hr for each mm of nerve left attached to the muscle. The time of maximal increase in cytosolic protein phosphorylation as shown in Figure 8 occurred at 24, 54 and 66 hr for muscles with distal nerve stumps of < 2 mm, 17-20 mm and 32-35 mm, respectively. The dotted linear regression line derived from these points indicates that the maximal increase is delayed 1.3 hr for each mm of distal nerve remaining attached to the muscle.

denervated versus contralateral, sham-operated solei. During the denervation periods of 3, 6, 12, 18, 24, 30 and 36 hr when a significant increase in cytosolic protein phosphorylation in short nerve stump muscles is observed (Figure 8), muscle weight is not affected by denervation (Table 6). Also, no muscle weight change is observed during denervation periods corresponding to a significant increase in cytosolic protein phosphorylation (30, 36, 42, 48, 54 and 60 hr) with the intermediate nerve stump length and most of the periods corresponding to a significant increase in cytosolic protein phosphorylation (48, 54 and 60 hr) with the long nerve stump (Figure 8 and # in Table 6). In addition, although there is significant weight loss ($p < 0.01$) observed during the 66 hr denervation time period for the intermediate nerve stump muscles and 60 and 66 hr with long nerve stump muscles, still cytosolic protein phosphorylation is significantly increased for these groups.

C. Resolution of Denervation Period and Nerve Stump Length-Dependent Soluble Cytosolic Phosphoproteins from Rat Soleus Muscle

1. Resolution of proteins under non-denaturing conditions

a. gel filtration chromatography

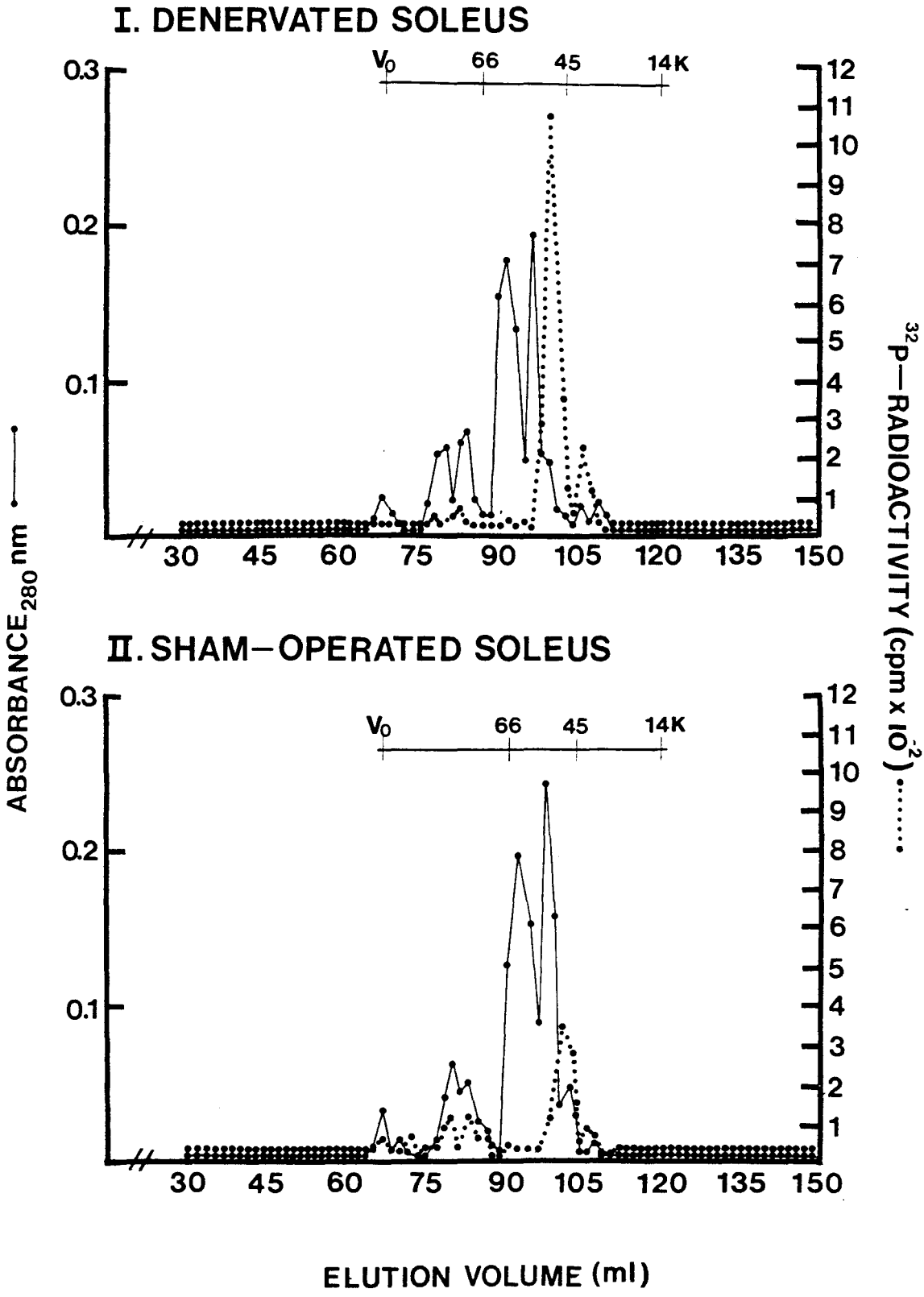
Gel filtration chromatography was chosen as a first step in resolving soluble protein substrates for the denerva-

TABLE 6. COMPARISON OF DENERVATED AND CONTRALATERAL, SHAM-OPERATED SOLEUS MUSCLE WEIGHTS[a]

Period (hr) After Surgery	Short Nerve Stump		Intermediate Nerve Stump		Long Nerve Stump	
Denervated(+) or Sham (-)	+	-	+	-	+	-
1	0.45	0.45				
3	0.40	0.39#	0.42	0.43	0.45	0.44
6	0.43	0.42#				
12	0.49	0.49#	0.48	0.47	0.41	0.42
18	0.37	0.40#				
24	0.49	0.49#	0.42	0.43	0.40	0.46
30	0.50	0.51#	0.44	0.41		
36	0.39	0.42#	0.46	0.45#	0.44	0.45
42			0.45	0.44#	0.46	0.40
48	0.39	0.40	0.43	0.44#	0.47	0.47#
54			0.42	0.42#	0.39	0.42#
60			0.38	0.39#	0.36	0.41#*
66			0.38	0.40*	0.40	0.43#*
72	0.44	0.45	0.40	0.41	0.46	0.49#
78					0.38	0.45*

tion period and nerve stump length-dependent cytosolic phosphorylation reaction due to its reliability and simplicity. Separation of components by gel filtration chromatography is related to the capacities of the various components to enter pores of the stationary phase. Smaller molecules which can enter the gel pores move much slower than large molecules which cannot enter the pores. Components are, therefore, eluted in order of decreasing molecular size.

Samples of ^{32}P-labeled soluble cytosolic protein fractions from 24 hr denervated short nerve stump-solei and contralateral, sham-operated solei were dialyzed separately overnight in ice-cold buffer (50 mM Tris-HCl, pH 7.5, 0.1 M NaCl and 2 mM $MgCl_2$) and then quantitatively applied to a Sephadex G-150 gel filtration column (1.5 x 90 cm) which was equilibrated prior to use with the same buffer (Method J). A similar amount of dialyzed protein from denervated (275 ug) and sham-operated (286 ug) cytosols was applied to individual columns. Protein was eluted under non-denaturing conditions using the dialysis buffer. The relative absorbance profile at 280 nm generated during elution of soluble protein from the cytosolic fractions of denervated and sham-operated muscles is shown in Figure 10-I and II, respectively (refer to solid lines). No striking quantitative or qualitative differences were observed when comparing 24 hr denervated short nerve stump-solei to contralateral, sham-operated solei. Along with the absorbance profiles, the distribution of ^{32}P-radioactivity quantitated in consecutive 1.5 ml


I. DENERVATED SOLEUS
II. SHAM–OPERATED SOLEUS
V_0
66
45
14K
0.3
0.2
0.1
12
11
10
9
8
7
6
5
4
3
2
1
30
45
60
75
90
105
120
135
150
ABSORBANCE$_{280}$ nm
^{32}P—RADIOACTIVITY (cpm x 10^{-2})
ELUTION VOLUME (ml)

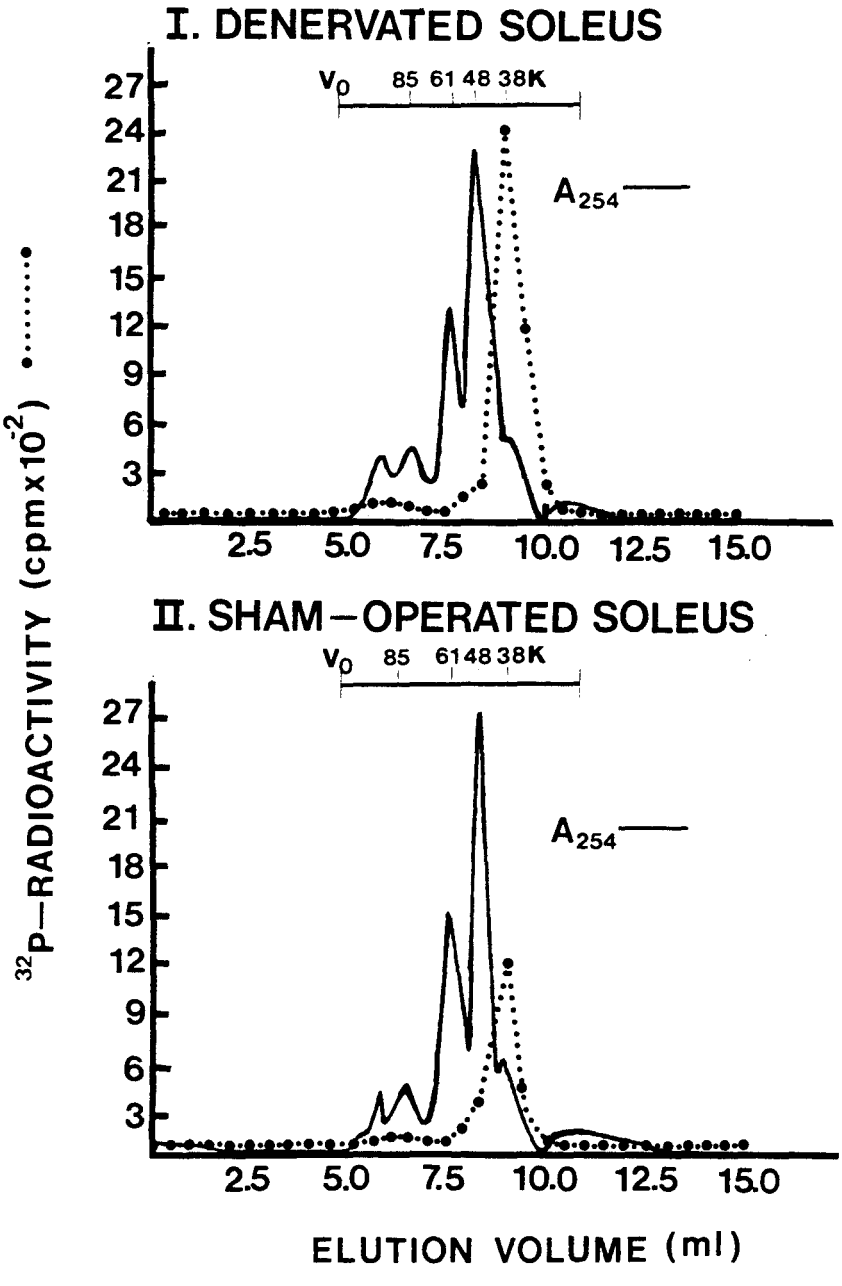
fractions is shown in Figure 10 (dotted line). Approximately 60 to 75% of the total ^{32}P-radioactivity eluted from columns to which denervated (Figure 10-I) or sham-operated (Figure 10-II) cytosolic fractions were applied was localized in four consecutive 1.5 ml fractions corresponding to a total elution volume of 96.0 to 102.0 ml. The level of ^{32}P-radioactivity recovered in these four consecutive fractions, however, is 2.5 fold greater in the cytosolic fraction from denervated solei relative to a similar sample from the cytosolic fraction of the contralateral, sham-operated solei. Also, the four consecutive fractions from both muscle cytosols correspond to a region of very low protein absorbance in a relative molecular weight range of M_r = 58,000 to 45,000. The approximate molecular weight range under this four fraction peak was determined from the partition coefficients of the first and last 1.5 ml fraction of the peak and the molecular weight calibration curve shown in Figure 35 of the Appendix. The calibration curve is based upon the partition coefficients of known molecular weight marker proteins. Additionally, when the four consecutive major ^{32}P-labeled fractions were pooled, resolved by SDS gradient slab PAGE and then ^{32}P-labeled proteins were visualized by autoradiography, only a 56,000-dalton radiolabeled phosphoprotein was apparent (data not shown).

b. high pressure liquid chromatography (HPLC)

HPLC was chosen as an alternate method to conventional gel filtration chromatography to shorten the time needed for separation of soluble cytosolic proteins and to achieve greater resolution of components. Conventional Sephadex gel filtration was found to be extremely time consuming, often taking 18 hr.

^{32}P-labeled soluble phosphoproteins from the cytosolic fractions of 24 hr denervated short nerve stump-solei and contralateral, sham-operated solei were resolved under non-denaturing conditions by HPLC using a 0.05 M sodium phosphate buffer, pH 7.4, and a Waters I-125 protein purification size exclusion HPLC column. Cytosolic protein applied to the column from denervated (76.0 ug) and sham-operated muscles (81.5 ug) were eluted under non-denaturing conditions with the sodium phosphate buffer. The relative absorbance profiles (254 nm) for denervated and contralateral, sham-operated muscles are essentially identical (compare continuous solid lines in Figure 11-I and II).

The distribution of ^{32}P-radioactivity in consecutive 0.5 ml fractions from denervated and sham-operated solei indicated that the majority (85 to 90%) of the total ^{32}P-radioactivity eluted is localized in five consecutive fractions corresponding to a total elution volume of 7.5 to 9.5 ml. The level of ^{32}P-radioactivity of this region was approximately twofold higher for the denervated cytosol


I. DENERVATED SOLEUS
V_0 85 61 48 38K
A_{254}
II. SHAM–OPERATED SOLEUS
V_0 85 61 48 38K
A_{254}
^{32}P–RADIOACTIVITY (cpm x 10^{-2}) ••••••••
27
24
21
18
15
12
9
6
3
2.5 5.0 7.5 10.0 12.5 15.0
ELUTION VOLUME (ml)

relative to a similar sample from the contralateral, sham-operated muscle (Figure 11-I vs. 11-II). Also, the five consecutive fractions from both muscles corresponded to a region of low protein absorbance in a broad molecular weight range of M_r = 58,000 to 35,000. The approximate molecular weight range of this peak was determined from the partition coefficients of the first and last 0.5 ml fraction of the peak and the molecular weight calibration curve shown in Figure 36 of the Appendix. The calibration curve is based on the partition coefficients of known molecular weight marker proteins. The approximate molecular weight of each peak was calculated and is indicated in the molecular weight scale presented at the top of Figure 11-I and II. The HPLC chromatographic procedure resolved cytosolic proteins more rapidly (approximately 9 min for separation of cytosol proteins) than the conventional Sephadex gel filtration chromatography (approximately 18 hr). However, neither method of protein separation yielded a good resolution of soluble cytosolic proteins.

2. Resolution of proteins under denaturing conditions

a. rod gel electrophoresis

The soluble proteins in cytosolic fractions from 24 hr denervated short nerve stump-solei and contralateral, sham-operated solei of the rat were phosphorylated *in vitro* in a buffered incubation medium containing [gamma-^{32}P]ATP as

the phosphoryl donor (Method E). Aliquots of these phosphorylated cytosolic fractions were heat- and SDS-treated, dialyzed and then subjected to SDS-10% polyacrylamide rod gel electrophoresis (SDS rod PAGE) in a phosphate buffered (pH 7.4) system (Method L). The absorbance profiles of Coomassie blue protein-stained gels at 600 nm obtained with equivalent amounts of soluble cytosolic protein (30 ug) from denervated and sham-operated solei were qualitatively comparable as shown in Figure 12A and B, respectively. Also, only minor quantitative differences between denervated and sham-operated muscles were observed. When the distribution of the ^{32}P-radiolabel on the Coomassie blue protein-stained gels was analyzed by autoradiography of longitudinally sliced gels, the obviously overexposed film was darkened only in the region of lightly stained protein bands (refer to double arrows in Figure 12A and B). Four consecutive 1 mm gel slices from the ^{32}P-labeled region of the gel contained 70 $\pm$ 2% (mean $\pm$ S.E.M. with n=8) of the ^{32}P-radioactivity recovered after electrophoresis (i.e., 61 $\pm$ 6% of applied radioactivity; mean $\pm$ S.E.M. with n=4). This distribution of the ^{32}P-labeling was found for cytosolic protein from both denervated and sham-operated muscles. The ^{32}P-radioactivity associated with the relatively minor polypeptide band(s) was 186 $\pm$ 18% (mean $\pm$ S.E.M with n=5) higher when resolved from *in vitro* phosphorylated soluble protein in cytosolic fractions of solei which were denervated for 24 hr leaving a

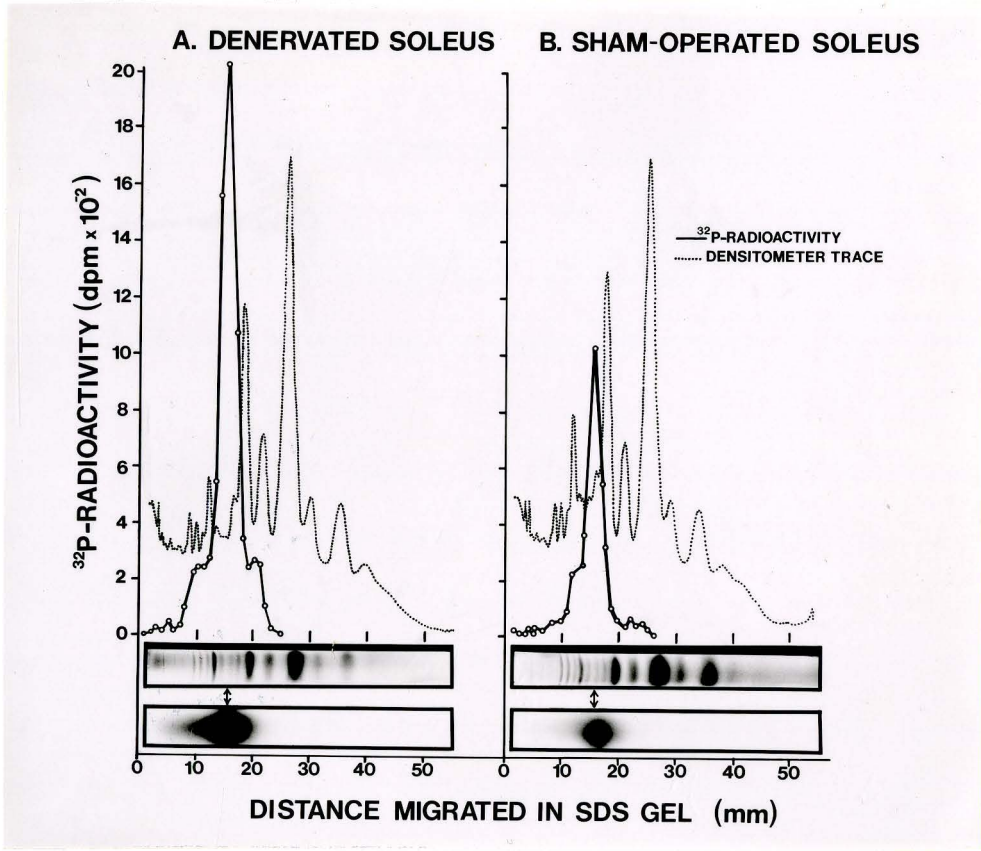


Figure 12. Resolution of a phosphorylated polypeptide from the cytosolic fractions of denervated and sham-operated solei of the rat in SDS-10% polyacrylamide rod gels. The left soleus was denervated 24 hr prior to sacrifice by cuttingthe soleus branch of the sciatic nerve 2 mm before insertion into the muscle. Control samples were obtained from sham-operated, contralateral muscles. Cytosolic fractions were incubated in the phosphorylation medium (Method E) and then prepared for SDS rod gel electrophoresis (Method K). Densitometric tracings recorded at 600 nm (.....) of Coomassie blue-stained rod gels are shown along with ^{32}P-radioactivity (———) quantitated in consecutive 1 mm gel slices by liquid scintillation spectrometry. An equivalent amount of soluble cytosolic protein (30 ug) was applied in (A) and (B). Photographs of these protein-stained gels and autoradiographs of longitudinally sliced gels are shown at the bottom of the Figure.

short (< 2 mm) nerve stump (Figure 12A) than when resolved from contralateral, sham-operated muscles (Figure 12B).

Based upon the relative electrophoretic mobilities of molecular weight marker proteins (i.e., albumin-66,000; ovalbumin-45,000; pepsin-34,700; trypsinogen-24,000 and beta-lactoglobulin-18,400) in SDS-10% polyacrylamide rod gels (Figure 13), the relative molecular weight of the denervation-dependent phosphopolypeptide is 40,400 ± 800 (mean ± S.E.M. with n=8). The distance (in mm) migrated by the polypeptide was taken as the distance of the gel slice from the origin which was the most radioactive (e.g., the peak at 15 mm in Figure 12A and B). All measurements were corrected for differences and changes in the lengths of the rod gels due to the staining and destaining procedures according to the equation given by Weber and Osborn (1969) (Method K).

The relationship of the 40,400-dalton denervation-dependent soluble cytosolic phosphoprotein to the catalytic subunit of the cyclic AMP-dependent protein kinase was also examined using the SDS rod PAGE system. This comparison was significant in that the catalytic subunit of cyclic AMP-dependent protein kinase has reported molcular weights of M_r = 37,000 to 41,300 (Kinzel and Kubler, 1976; Bechtel et al., 1977) and undergoes autocatalytic self-phosphorylation (Chiu and Tao, 1978) under reaction conditions similar to those employed in the *in vitro* cytosolic phosphorylation assay (Method E). The relative molecular weight of the cyclic AMP-

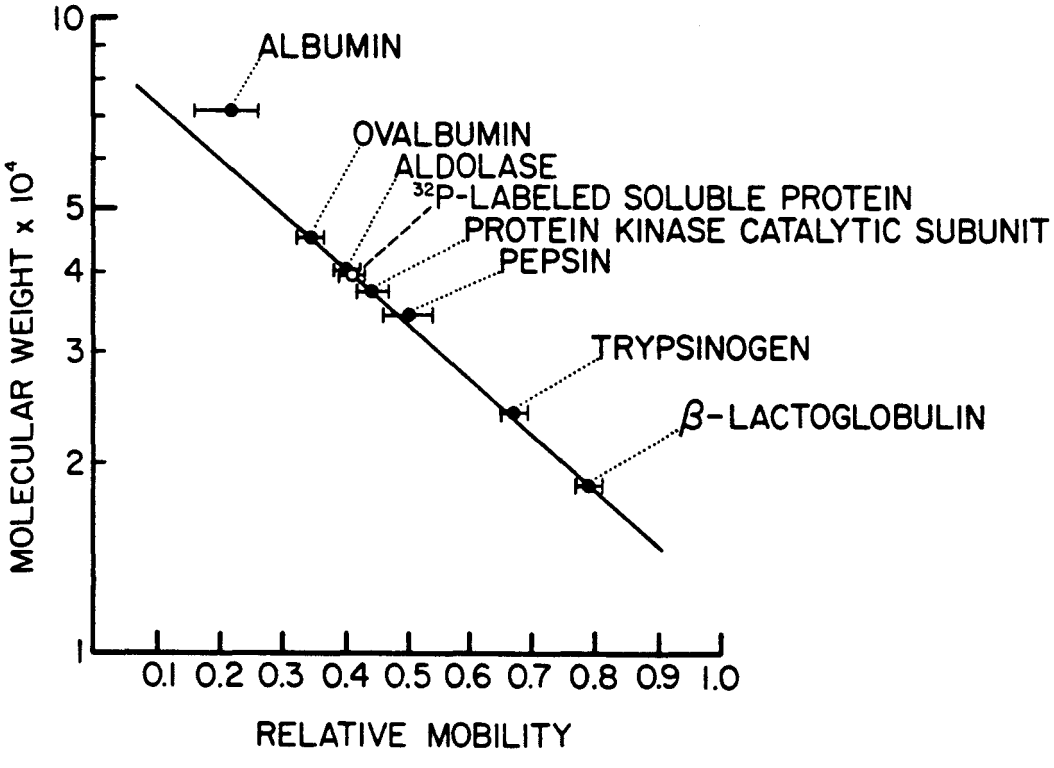


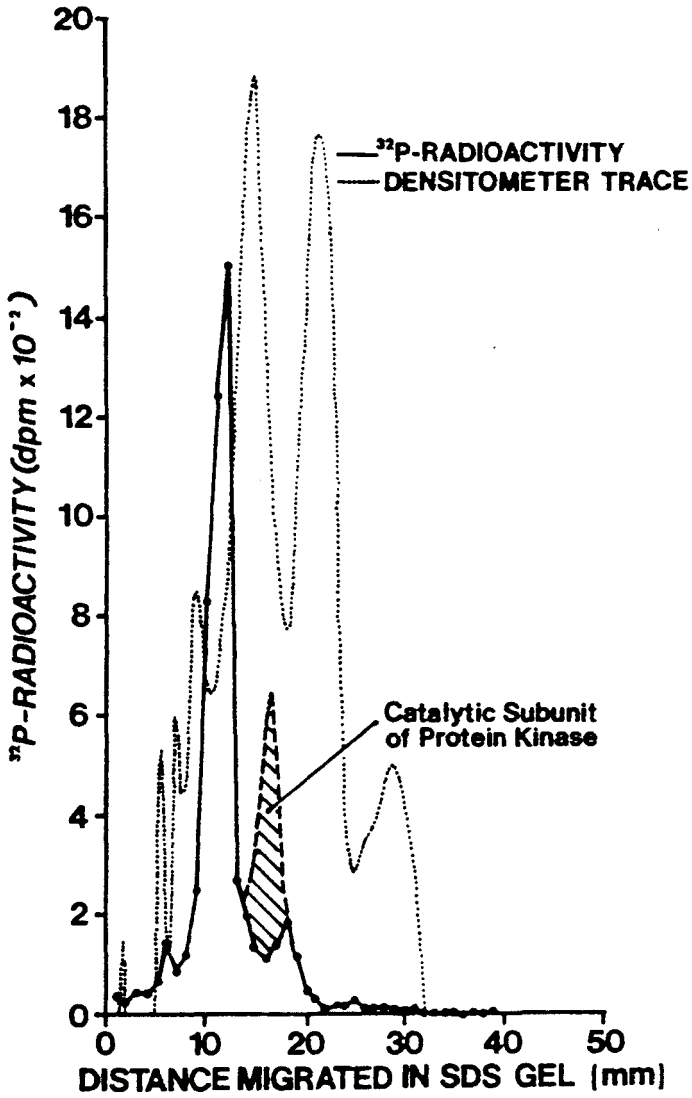
Figure 13. Estimation of the relative molecular weight of a ^{32}P-labeled soluble cytosolic phosphoprotein from rat soleus muscle and the protein kinase catalytic subunit by SDS-10% polyacrylamide rod gel electrophoresis. The protein standards of known molecular weight (i.e., albumin-66,000; ovalbumin-45,000; pepsin-34,700, trypsinogen-24,000 and beta-lactoglobulin-18,400) used for the calibration and the catalytic subunit (from bovine heart muscle) were obtained from Sigma Chemical Co., St. Louis, MO. The molecular weight marker proteins were prepared for electrophoresis as described in Method K. Relative mobilities were determined according to Weber and Osborn (1969) and are shown as means ± S.E.M. with n=8 for the ^{32}P-labeled soluble protein and n=3 for all other proteins.

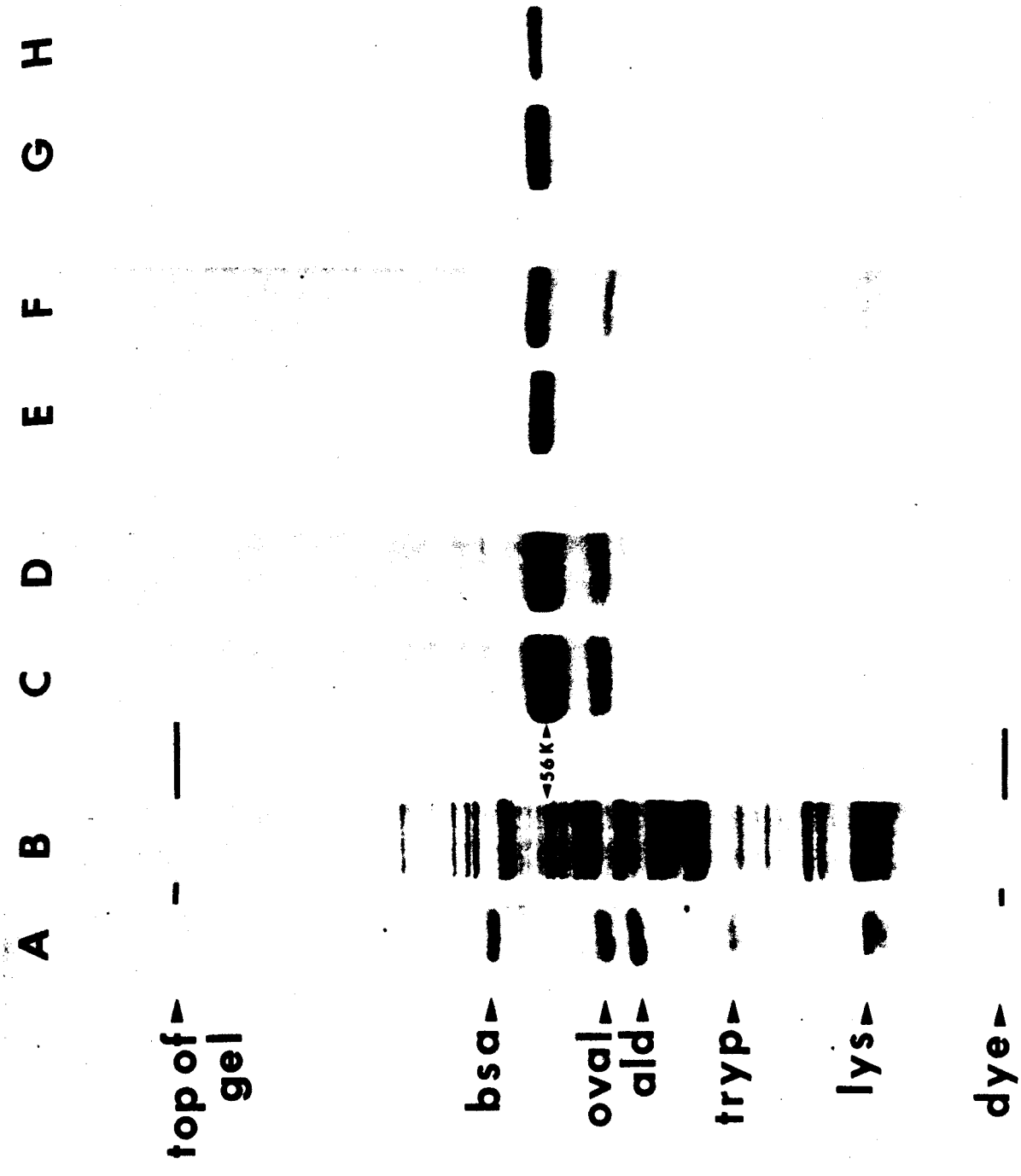
dependent protein kinase catalytic subunit from bovine heart (commercial preparation) was determined to be 38,000 ± 580 (mean ± S.E.M. with n=3) as demonstrated in Figure 13. Additionally, after coelectrophoresis with ^{32}P-labeled soluble cytosolic protein from rat soleus muscle, this commercially purified catalytic subunit was distinctly separated from the 40,400-dalton major soluble phosphoprotein resolved from the cytosol of rat soleus muscle (Figure 14).

b. slab gel electrophoresis

Aliquotsof phosphorylated soluble proteins which were denatured (heat- and SDS-treated) and dialyzed were also subjected to SDS polyacrylamide gradient slab gel electrophoresis (SDS slab PAGE) using a 5 to 15% exponential gradient according to Laemmli (1970) (Method K). This gel system was used to achieve a better resolution of cytosolic proteins from rat soleus muscle. With this gel system, approximately 45 to 50 Coomassie blue protein-staining bands were reproducibly resolved (Figure 15B).

Autoradiography of the dried slab gels indicates that 8 to 11 soluble cytosolic proteins were phosphorylated during the *in vitro* assay (Figure 15C-H). Based upon liquid scintillation counting of consecutive 2 mm gel slices, 76.5 ± 2.8 % (mean ± S.E.M. with n=6) of the total ^{32}P-radioactivity applied to the gel was recovered after electrophoresis when soluble cytosolic proteins were resolved from


20
18
16
14
12
10
8
6
4
2
0
^{32}P-RADIOACTIVITY (dpm x 10^{-2})
—^{32}P-RADIOACTIVITY
—DENSITOMETER TRACE
Catalytic Subunit
of Protein Kinase
0
10
20
30
40
50
DISTANCE MIGRATED IN SDS GEL (mm)


A B C D E F G H
top of gel
bsa
ovalbd
tryp
lys
dye
56 K

denervated solei. When soluble proteins were resolved from contralateral, sham-operated solei, 77.8 ± 2.8 % (mean ± S.E.M. with n=6) of the total ^{32}P-radioactivity applied to the gel was recovered after electrophoresis. However, 67.5 ± 5.1 % (mean ± S.E.M. with n=6) of the ^{32}P-radioactivity recovered after electrophoresis of soluble cytosolic proteins from denervated solei was associated with two consecutive 2 mm gel slices corresponding to a lightly stained protein band (Figure 15C, E and G). Similarly, 69.9 ± 4.3 % (mean ± S.E.M. with n=6) of the ^{32}P-radioactivity recovered after electrophoresis of soluble cytosolic proteins from contralateral, sham-operated solei was also associated with two consecutive 2 mm gel slices corresponding to a lightly stained protein band (Figure 15D, F and H). The low levels of ^{32}P-radioactivity associated with the other phosphoproteins (100 cpm or less) detected by autoradiography prevented their reliable quantitative assessment by liquid scintillation spectrometry.

The predominant soluble cytosolic phosphoprotein from the cytosolic fractions of both denervated and sham-operated solei has a relative molecular weight of M_r = 56,000 ± 400 (mean ± S.E.M. with n=12). The distance migrated by the predominant soluble phosphoprotein was taken as the most radioactive gel slice from the origin (top) of the SDS gradient slab gel. The relative molecular weight of this predominant phosphoprotein was estimated from the molecular weight calibration curve shown in Figure 16. This calibra-

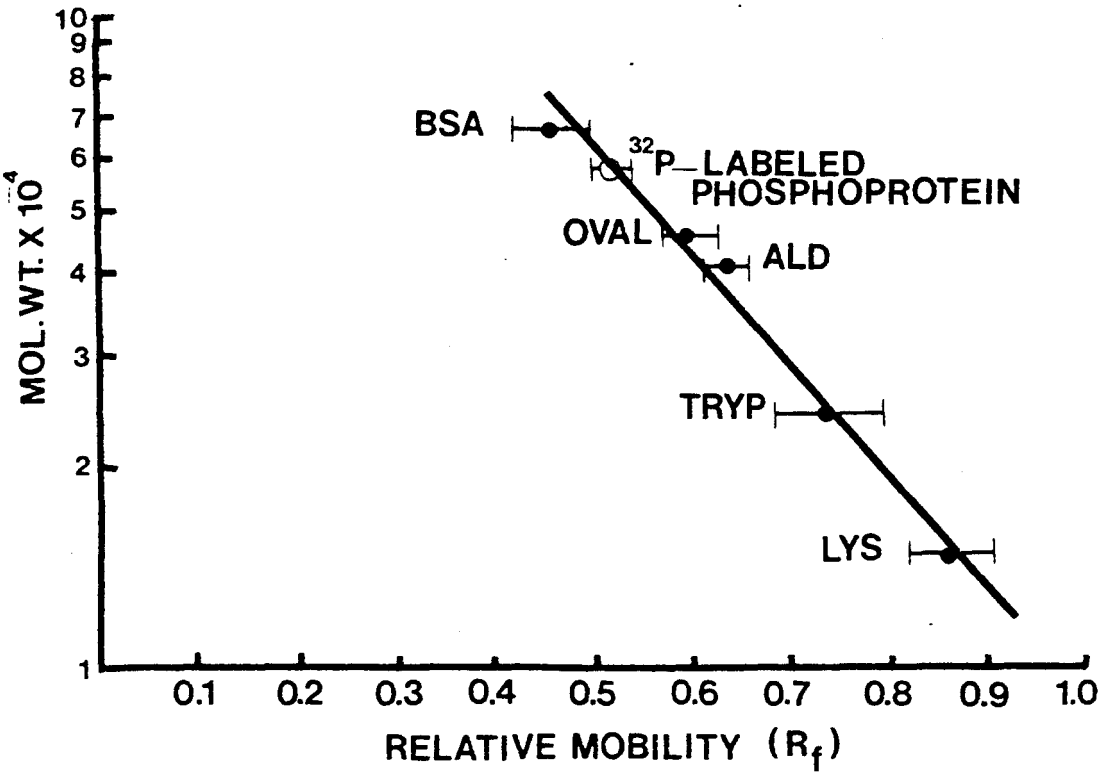


Figure 16. Estimation of the relative molecular weight of the denervation period and nerve stump length-dependent soluble cytosolic phosphoprotein from rat soleus muscle by SDS gradient slab PAGE. The protein standards of known molecular weight described in Figure 15 were purchased from Sigma Chemical Co. with the exception of aldolase which was purchased from Pharmacia Chemical Co. The standard proteins were prepared as solutions of 1 mg protein/ml of a 0.0625 M Tris-HCl buffer, pH 6.8, with 0.1% SDS and 5% 2-mercapto-ethanol. The protein solutions were boiled for 2 min prior to electrophoresis to denature the proteins. An equal aliquot (10 ug) of each protein was applied to the gradient slab gel. Relative mobilities were calculated according to the equation given in Method K and are shown as means ± S.E.M. for 12 electrophoretic separations.

tion curve was derived by calculating the relative electrophoretic mobilities of the known molecular weight standard marker proteins shown in Figure 15A.

An increased phosphorylation of the predominant 56,000-dalton phosphoprotein was observed when ^{32}P-labeled soluble proteins were resolved from the cytosolic fractions of rat solei denervated for 24, 54 and 66 hr with the three nerve stump lengths of 2, 17-20 and 32-35 mm, respectively. These are the denervation periods at which a maximal increase in cytosolic protein phosphorylation was found (Figure 8). The marked increase in the ^{32}P-labeling of the major 56,000-dalton soluble cytosolic phosphoprotein from denervated muscles can be observed in Figure 15 by comparing the autoradiographic density of this protein in short nerve stump (C), intermediate nerve stump (E) and long nerve stump (G) solei relative to their respective, sham-operated (D, F and H) muscles. Similar amounts of soluble protein from the cytosolic fractions of denervated and contralateral, sham-operated muscles were applied to the gel. Denervation had no effect on synthesis of soluble cytosolic proteins as judged from the Coomassie staining pattern. It is pertinent to note that the density of the autoradiographs of one pair of samples cannot be compared with another pair (e.g., C-D vs E-F or G-H) since the half-life of ^{32}P is relatively short (14.2 days) and the samples for the three pairs of denervated and sham-operated muscles were not available for electro-

phoresis simultaneously.

Due to the discrepancy in apparent molecular weight of the predominant soluble phosphoprotein observed when ^{32}P-labeled cytosolic proteins were resolved by SDS rod PAGE and SDS gradient slab PAGE, an experiment was conducted to directly compare the relative molecular weight of this protein by SDS rod PAGE and SDS gradient slab PAGE. ^{32}P-labeled soluble cytosolic proteins were resolved initially by SDS rod PAGE. After electrophoresis the rod gels were sliced into five consecutive 10 mm segments and the radiolabeled segment was detected with a Geiger counter (ElScint Ltd., Israel). The radiolabeled segment was prepared for re-electrophoresis by hand homogenizing the 10 mm segment and resuspending the radiolabeled protein in the electrophoresis buffer used during SDS gradient slab PAGE. The buffer consisted of 0.0625 M Tris-HCl, pH 6.8, 0.1% SDS and 5% 2-mercaptoethanol. It can be seen from the autoradiograph shown in Figure 17B that the major radiolabeled protein resolved initially by SDS rod PAGE and re-electrophoresed on SDS gradient slab gels had a molecular weight similar to the major ^{32}P-labeled protein of M_r = 56,000-daltons also resolved by SDS gradient slab PAGE (17A).

During the denervation periods corresponding to a significant increase in cytosolic protein phosphorylation with a short nerve stump (3, 6, 12, 18 and 24 hr), a similar percentage increase in the ^{32}P-radiolabeling of the 56,000-dalton soluble cytosolic phosphoprotein was observed in

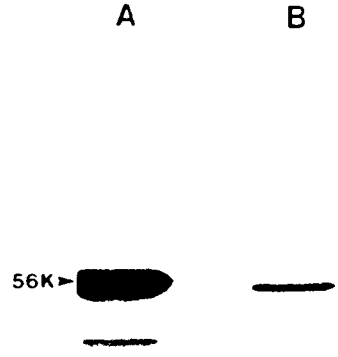
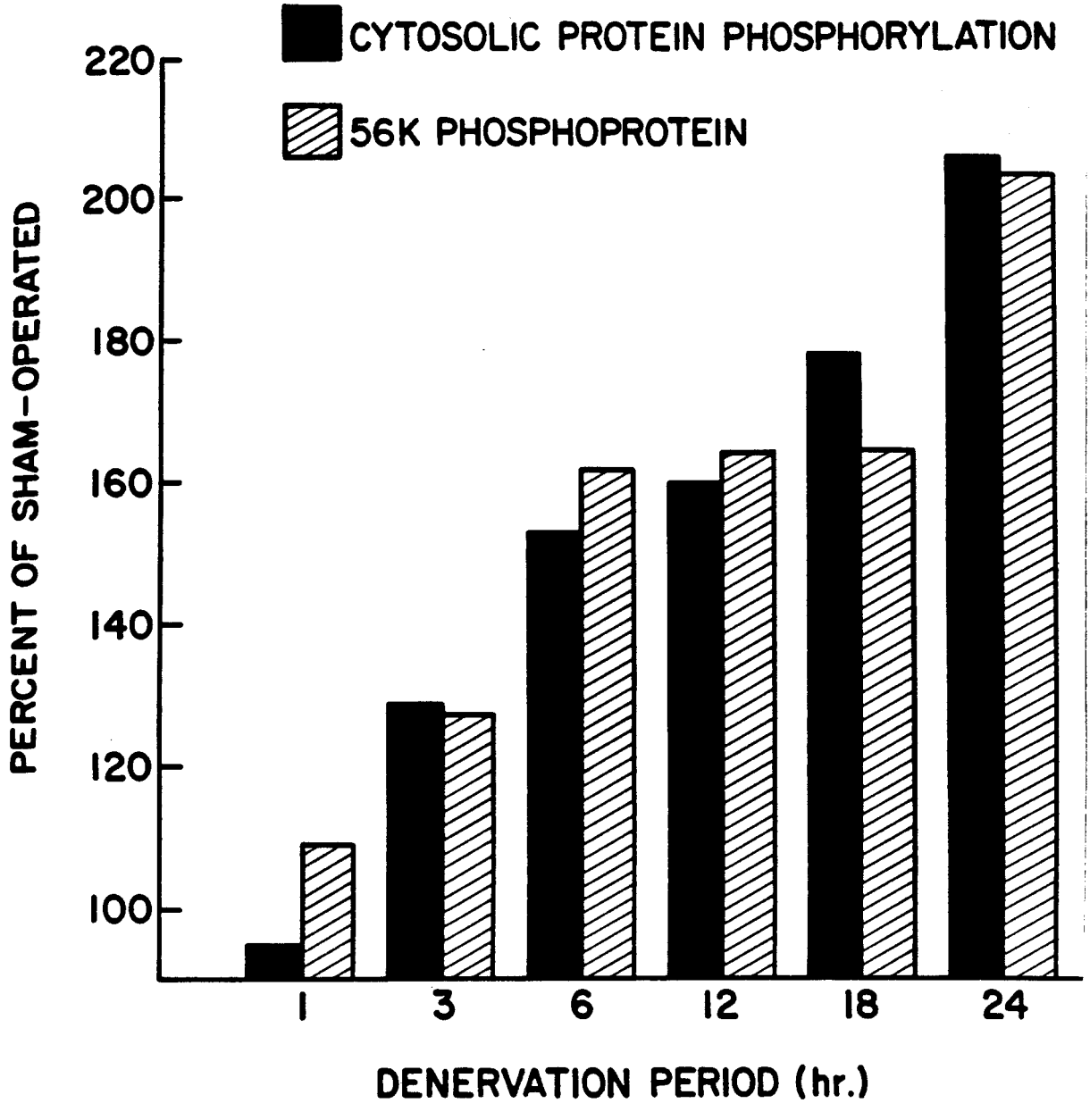


Figure 17. Molecular weight determination of the denervation period and nerve stump length-dependent soluble cytosolic phosphoprotein from rat soleus muscle by SDS rod PAGE and SDS gradient slab PAGE. ^{32}P-labeled cytosol samples of 3 pooled unoperated rat solei were prepared for electrophoresis in SDS rod gels and SDS gradient slab gels as described in Method K. Autoradiographs of the ^{32}P-labeled cytosolic samples resolved by SDS gradient slab PAGE (A) and of the major ^{32}P-radiolabeled soluble cytosolic phosphoprotein (40,000-daltons) which was sliced from an SDS rod gel and then re-electrophoresed in an SDS gradient slab gel (B) are shown. Molecular weight of the major radiolabeled protein band was determined relative to the electrophoretic mobilities of proteins of known molecular weight and the calibration curve shown in Figure 16.

denervated solei relative to sham-operated solei (Figure 18). Where no significant change in cytosolic protein phosphorylation was observed at 1 hr, no significant increase in phosphorylation of the 56,000-dalton protein was observed. Relative phosphorylation of the 56,000-dalton phosphoprotein was determined by dividing the total ^{32}P-radioactivity (counts per minute in two consecutive 2 mm gel slices) associated with this protein band by the total amount of soluble cytosolic protein (usually between 30 and 50 ug) from denervated or sham-operated samples applied to corresponding individual sample wells of the gel. Histograms showing the quantitative distribution of ^{32}P-radioactivity in SDS gradient slab gels for 1, 3, 6, 12, 18 and 24 hr denervated and sham-operated solei can be found in Figures 41 to 46 of the Appendix.


CYTOSOLIC PROTEIN PHOSPHORYLATION
56K PHOSPHOPROTEIN
PERCENT OF SHAM-OPERATED
220
200
180
160
140
120
100
1
3
6
12
18
24
DENERVATION PERIOD (hr.)

D. Modulators of the Denervation Period and Nerve Stump Length-Dependent Phosphorylation of Cytosolic Protein(s)

1. Effect of exogenous modulators and protein substrates

a. cyclic AMP

The Coomassie blue protein-staining pattern (Figure 19-I) indicates that the addition of cyclic AMP and also other exogenous modulators to the *in vitro* assay had no qualitative effect upon the soluble cytosolic protein pattern of rat soleus muscle. However, the autoradiograph of the dried slab gel shows that the level of ^{32}P-labeling of the 56,000-dalton protein band from both denervated and sham-operated cytosolic fractions is markedly inhibited by the addition of 1 uM cyclic AMP to the *in vitro* phosphorylation assay (Figure 19-II). Quantitatively, the ^{32}P-radiolabeling of the two consecutive gel slices (2 mm) corresponding to the 56,000-dalton protein band was significantly reduced ($p < 0.01$ by paired t-test) 77.93 $\pm$ 3.37% (mean $\pm$ S.E.M. with n=5) in the denervated sample and 76.12 $\pm$ 3.77% (mean $\pm$ S.E.M. with n=5) in the contralateral, sham-operated sample (Table 7). These results were obtained after determining the relative phosphorylation of the 56,000-dalton cytosolic phosphoprotein. This was calculated as the ^{32}P-radiolabeling of the 56,000-dalton protein relative to the total amount of soluble protein in cytosolic fractions of denervated or sham-operated solei applied to corresponding individual sample

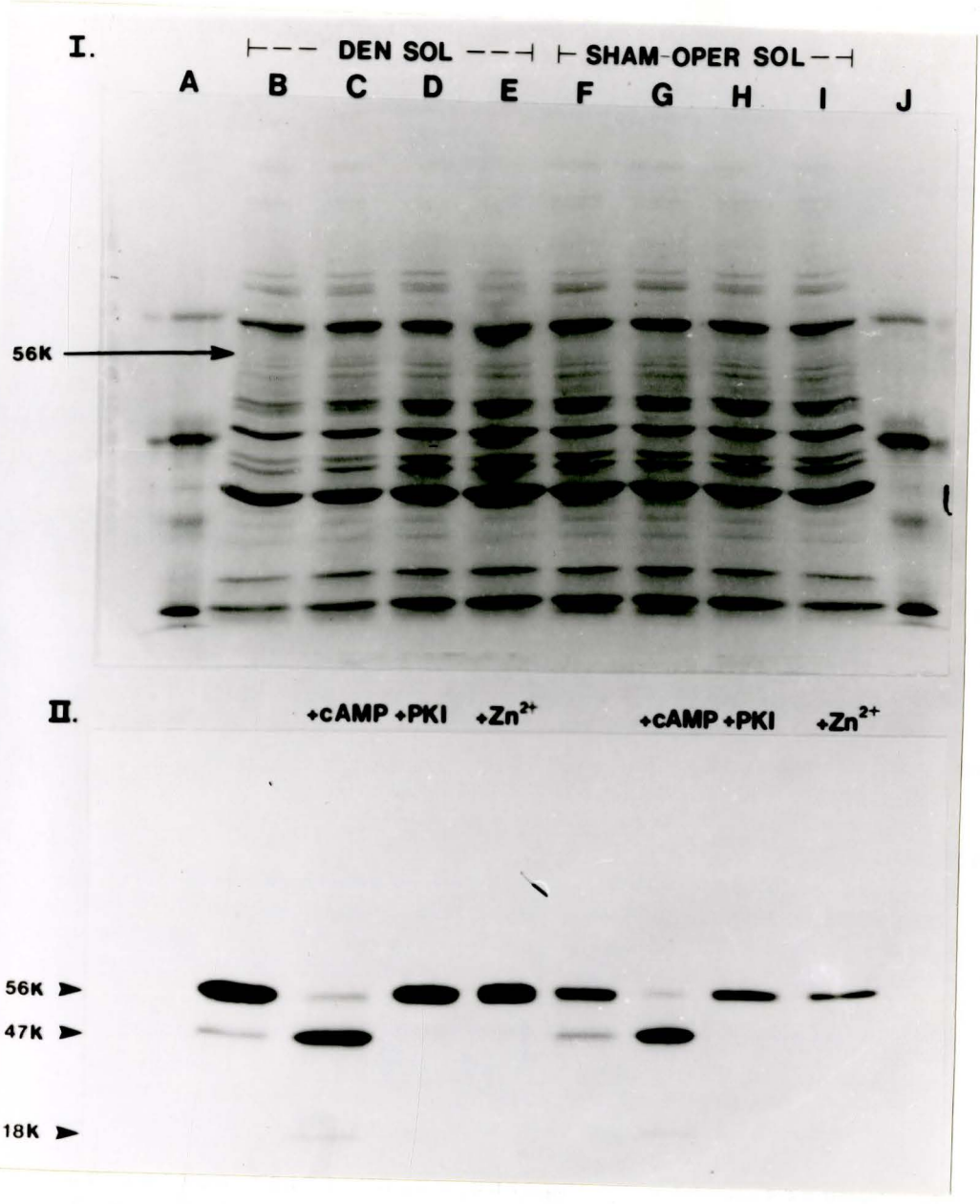

I.
DEN SOL
SHAM-OPER SOL
A
B
C
D
E
F
G
H
I
J
56K
II.
+cAMP +PKI +Zn2+
+cAMP +PKI +Zn2+
56K
47K
18K

TABLE 7. EFFECT OF CERTAIN MODULATORS UPON THE PHOSPHORYLATION OF THE SOLUBLE 56,000-DALTON PHOSPHOPROTEIN FROM THE CYTOSOLIC FRACTION OF DENERVATED AND SHAM-OPERATED RAT SOLEUS MUSCLE

Modulator[a]	% Change in Phosphorylation of the 56,000-Dalton Phosphoprotein[b]	
	Denervated[c] Soleus	Sham-operated Soleus
1 uM cyclic AMP	(5) -77.93 ±3.37***	(5) -76.12 ±3.77***
1 uM cyclic GMP	(3) +1.46 ±6.99	(3) -0.07 ±9.37
2.0 mM Ca^{2+}	(3) -2.32 ±5.27	(3) -6.43 ±2.80
1 mM Zn^{2+}	(5) -7.34 ±3.98	(5) -4.90 ±4.12
50 units PKI[d]	(5) -5.74 ±4.59	(5) -7.70 ±5.23

[a]Soluble cytosolic protein in cytosolic fractions of denervated and contralateral, sham-operated rat solei. was phosphorylated *in vitro* (Method E) with the addition of 1 mM Zn^{2+} (replacing Mg^{2+}), 1 uM cyclic AMP, 1 uM cyclic GMP, 2.0 mM Ca^{2+} and 50 units of the protein kinase inhibitor (see d below). The 56,000-dalton phosphoprotein was resolved from these fractions by SDS gradient slab PAGE (Method K).

[b]Phosphorylation of the 56,000-dalton phosphoprotein resolved by SDS gradient slab gel electrophoresis was calculated by dividing the total cpm in 2 consecutive gel slices corresponding to the region of the gel containing the 56,000-dalton protein by the amount of soluble protein (ug) applied to individual sample wells of the slab gel. Percent changes in phosphorylation with the addition of certain modulators to the *in vitro* phosphorylation assay are presented as means ± S.E.M. for the number of groups of three rats indicated in the parentheses. *** $p < 0.01$ by paired t-test. + indicates an increase in phosphorylation and - indicates a decrease in phosphorylation relative to phosphorylation without the addition of modulators.

[c]Experimental muscles were denervated unilaterally to leave a short (< 2 mm) distal nerve stump 24 hr prior to sacrifice. Sham-operated, contralateral muscles served as controls.

[d]Protein kinase inhibitor purchased from Sigma Chemical Co., St. Louis, MO was fractionated from bovine heart. The inhibitor was purified further (Method F).

wells of the SDS polyacrylamide gradient slab gel. Also, note in Figure 19-II the increase in the phosphorylation of a 47,000-dalton and an 18,000-dalton protein when cyclic AMP was added to the *in vitro* assay system. Quantitative changes in the phosphorylation of these soluble proteins (47,000 and 18,000-dalton proteins) could not be accurately assessed, however, due to the low level of counts (< 100 cpm) associated with these bands when cytosolic protein phosphorylation is assayed without cyclic AMP. Molecular weights indicated in Figure 19-I and II were calculated relative to the electrophoretic mobilities of known molecular weight marker proteins (Figure 37 of the Appendix).

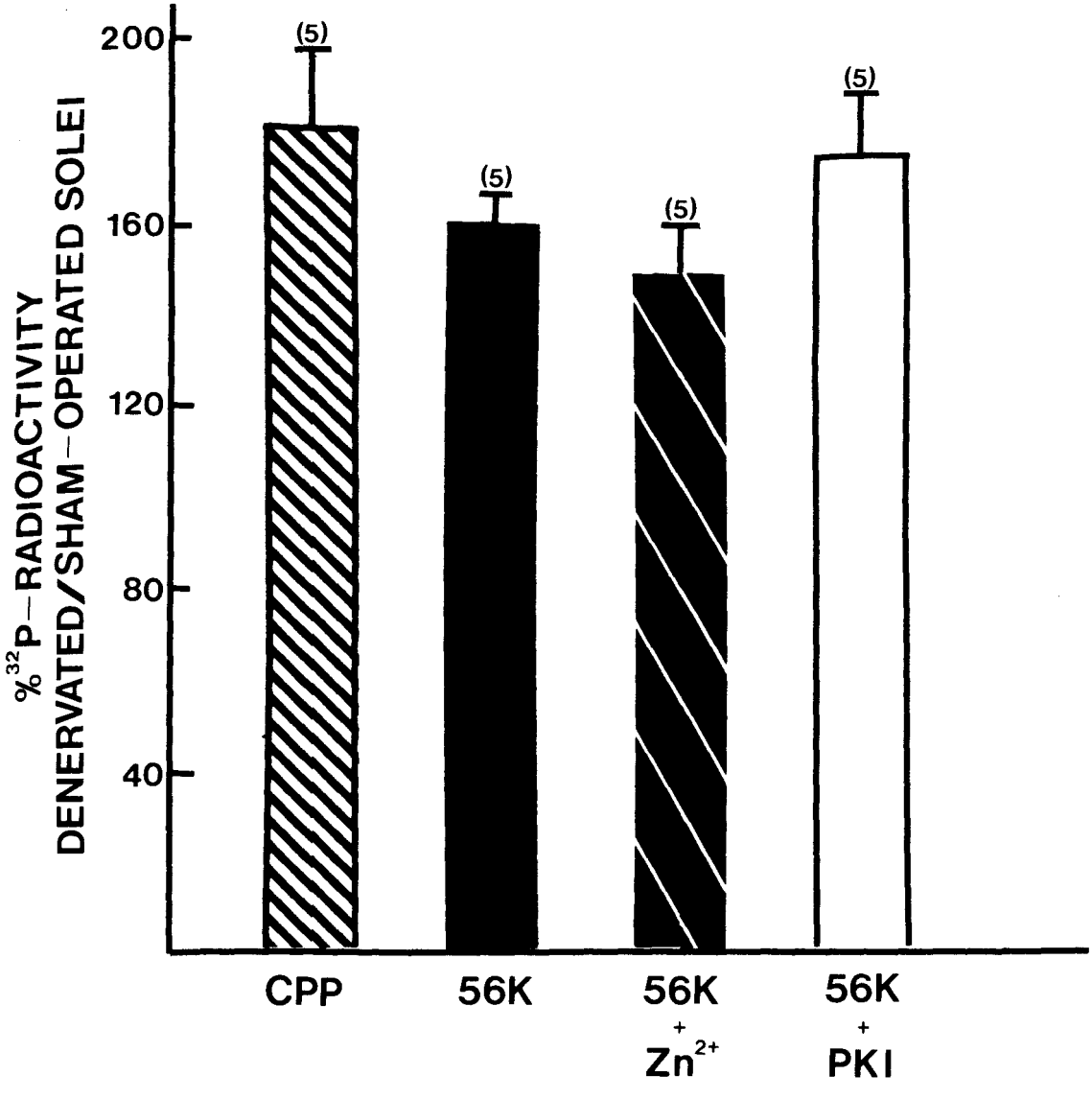
b. protein kinase inhibitor

The specific inhibitor of the free catalytic subunit of cyclic AMP-dependent protein kinase had only a slight (statistically insignificant) effect upon the phosphorylation of the predominant 56,000-dalton phosphoprotein resolved from denervated (5.74 ± 4.59% inhibition, mean ± S.E.M. with n=5) and contralateral, sham-operated solei (7.70 ± 5.23% inhibition, mean ± S.E.M. with n=5) (Table 7). However, addition of 50 units of the specific inhibitor reduced the phosphorylation of the cyclic AMP-stimulated 47,000-dalton phosphoprotein in both denervated and sham-operated solei (lanes D and H in Figure 19-II).

c. Zn^{2+}

When 1 mM Zn^{2+} was used in the *in vitro* phosphorylation assay instead of 2 mM Mg^{2+}, only a slight (statistically insignificant) inhibitory effect upon the phosphorylation of the predominant 56,000-dalton phosphoprotein resolved by SDS gradient slab PAGE from denervated solei (7.34 ± 3.98% inhibition, mean ± S.E.M with n=5) and contralateral, sham-operated solei (4.90 ± 4.12% inhibition, mean ± S.E.M. with n=5) was observed (Table 7). However, Zn^{2+} had an effect similar to the protein kinase inhibitor in reducing the phosphorylation of the cyclic AMP-stimulated 47,000-dalton phosphoprotein in both denervated and sham-operated solei (lanes E and I in Figure 19-II).

Also, Figure 20 shows that the increase in the phosphorylative modification of the 56,000-dalton cytosolic protein from 24 hr denervated short nerve stump-solei in the presence of either the specific cyclic AMP-dependent protein kinase inhibitor or Zn^{2+} is comparable (not statistically different by paired t-test) to the overall increase in total cytosolic protein phosphorylation in these muscles; i.e. the percentage change in the phosphorylation of the 56,000-dalton protein in denervated solei under the two conditions shown in Figure 20 (abscissa) relative to their corresponding, sham-operated solei was similar.


200
160
120
80
40
% ^{32}P–RADIOACTIVITY
DENERVATED/SHAM–OPERATED SOLEI
(5)
(5)
(5)
(5)
CPP
56K
56K + Zn^{2+}
56K + PKI

d. Ca^{2+}

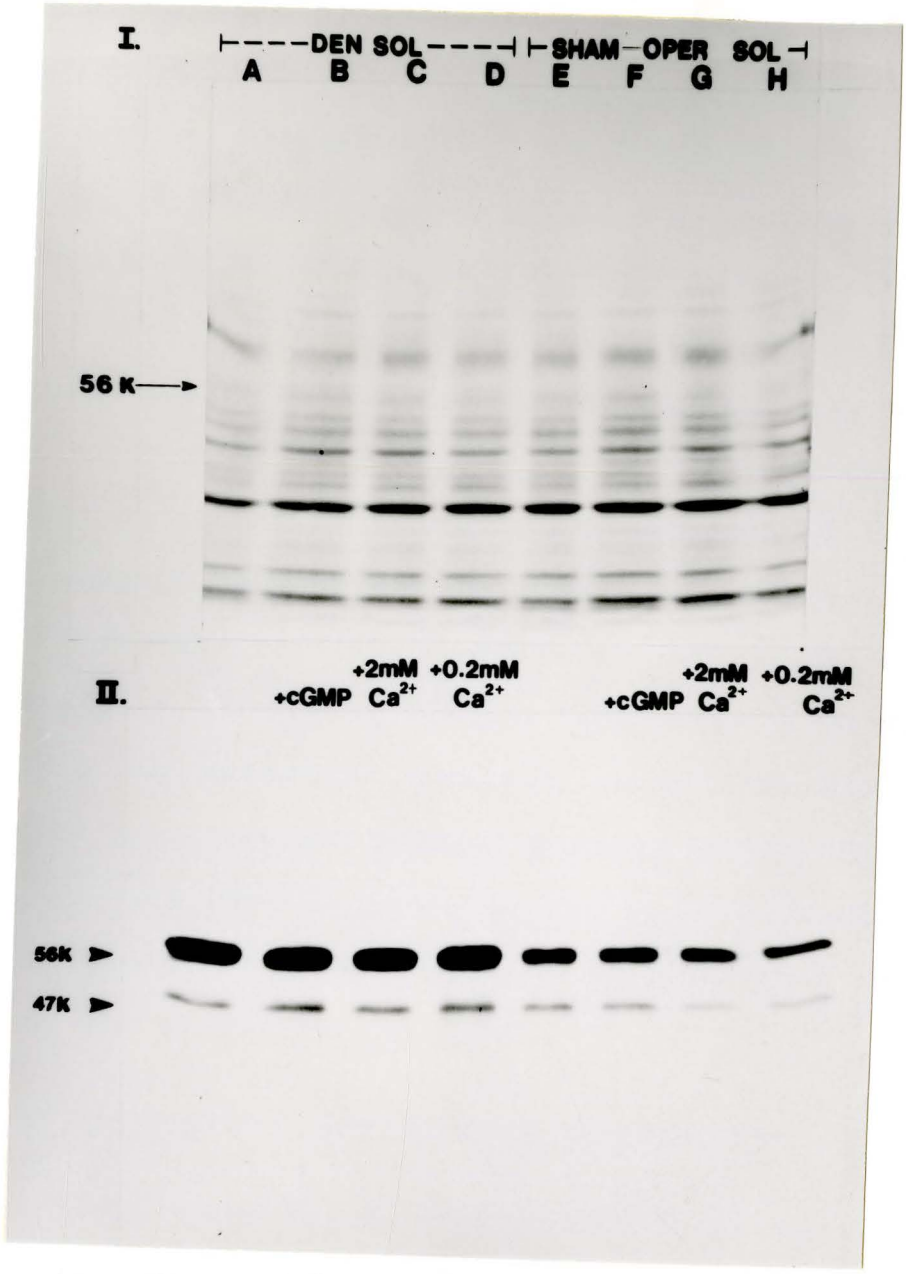
In Figure 21-I, the photograph of the Coomassie blue stained proteins from 24 hr denervated short nerve stump-solei and contralateral, sham-operated solei shows that neither 0.2 nor 2.0 mM Ca^{2+} had any qualitative effect upon soluble proteins resolved from the cytosolic fractions of these muscles. Ca^{2+} (0.2 and 2.0 mM) also had no qualitative (Figure 21-II) or quantitative (Table 7) effect upon the phosphorylation of the predominant 56,000-dalton phosphoprotein.

e. cyclic GMP

Like Ca^{2+}, 1 uM cyclic GMP had no qualitative (Figure 21-II) or quantitative (Table 7) effect upon the phosphorylation of the predominant 56,000-dalton phosphoprotein resolved from the cytosolic fractions of 24 hr denervated short nerve stump solei and their sham-operated, contralateral muscles. Molecular weights indicated in Figure 21-II were determined relative to the electrophoretic mobilities of standard known molecular weight marker proteins (Figure 38 of the Appendix).

f. histone

Cytosolic protein phosphorylation was determined in 24 hr denervated short nerve stump-solei relative to sham-operated solei with or without histone added to the <u>in vitro</u>


I.
DEN SOL
SHAM OPER SOL
A B C D E F G H
56 K
II.
+cGMP
+2mM Ca2+
+0.2mM Ca2+
+cGMP
+2mM Ca2+
+0.2mM Ca2+
56K
47K

phosphorylation assay. Histone is a natural substrate for the cytosolic cyclic AMP-dependent protein kinase (Weller, 1979). Without histone added to the incubation medium, cytosolic protein phosphorylation was increased in the denervated muscle 172.5 ± 3.2% (mean ± S.E.M. with n=5). With 50 ug of a commercial preparation of calf thymus histone added to the *in vitro* assay, cytosolic protein phosphorylation (determined as femtomoles $^{32}PO_4$ incorporated/ mg soluble cytosolic protein/ incubation time period) increased 369.3 ± 5.9% (mean ± S.E.M. with n=5) in the denervated sample relative to cytosolic protein phosphorylation in denervated solei assayed without histone (Table 8). However, cytosolic protein phosphorylation in sham-operated solei assayed with histone was increased 884.7 ± 7.3% (mean ± S.E.M. with n=5) relative to cytosolic protein phosphorylation in sham-operated solei assayed without histone (Table 8). Also, when exogenous histone was added to the phosphorylation reaction, cytosolic protein phosphorylation in denervated solei was 72% that of the sham-operated solei.

2. Effect of endogenous modulators

The relationship between the stimulation of the cytosolic protein phosphorylation in denervated rat solei and a change in the concentration of an endogenous modulator of protein phosphorylation was studied in "mixing" experiments in which protein phosphorylation in a sample containing equal

TABLE 8. ENDOGENOUS VS. EXOGENOUS PROTEIN PHOSPHORYLATION BY CYTOSOLIC FRACTIONS FROM DENERVATED AND CONTRALATERAL, SHAM-OPERATED RAT SOLEI

Substrate[a]	DEN SOL[b]		SHAM-OPER SOL		DEN/SHAM x 100
	CPP[c]	%increase	CPP	%increase	
Soluble Cytosolic Protein	19.3 ±2.0	---	11.2 ±1.7	---	172.5**
Histone	71.1 ±5.1	369**	98.8 ±5.5	884**	72.0*

[a]The phosphorylation of cytosolic protein (40 to 45 ug) was assayed *in vitro* with the phosphorylation medium described in Method E. Histone (50 ug, from Sigma Chemical Co., St. Louis, MO) was also added to some assays as exogenous substrate.

[b]The left soleus muscle was denervated 24 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before insertion into the muscle. Sham-operated, contralateral muscles served as controls.

[c]CPP - Cytosolic protein phosphorylation (fmoles $^{32}PO_4$ incorporated/mg soluble protein/incubation period (5 min)) are the means ± S.E.M. from 5 groups of 3 rats. * $p < 0.05$ and ** $p < 0.01$ by Student's t-test.

aliquots of cytosolic fractions from both 24 hr denervated short nerve stump-solei and contralateral, sham-operated solei was compared to protein phosphorylation in these individual fractions alone. If the concentration of an endogenous modulator of the cytosolic protein phosphorylation reaction in either denervated or sham-operated cytosolic fractions is altered, then protein phosphorylation of the combined aliquots of these samples will deviate from the expected additive value derived from the results of assays of these samples separately. When equal aliquots of cytosolic fractions from 24 hr denervated short nerve stump-solei and sham-operated solei were combined and assayed *in vitro*, cytosolic protein phosphorylation was significantly ($p < 0.01$ by paired t-test) decreased by 9.4 ± 2.1% (mean ± S.E.M., n=8) from the expected value (Figure 22, refer to 0 min pre-incubation period). It is significant to note that phosphorylation of cytosolic protein in denervated solei is consistently increased (1.6 to 2.0 fold) compared to that of the contralateral, sham-operated solei over the entire incubation time course. Pre-incubation, however, results in the formation of less phosphorylated product than that formed without pre-incubation. Also, longer pre-incubation of the combined sample resulted in an even greater and significant decrease in protein phosphorylation from the expected value. For example, a 10.4 ± 1.7% (mean ± S.E.M. with n=8; $p < 0.02$) decrease from the expected value was observed with the 10 min pre-incubation period. An 11.9 ± 2.2% (mean ± S.E.M.

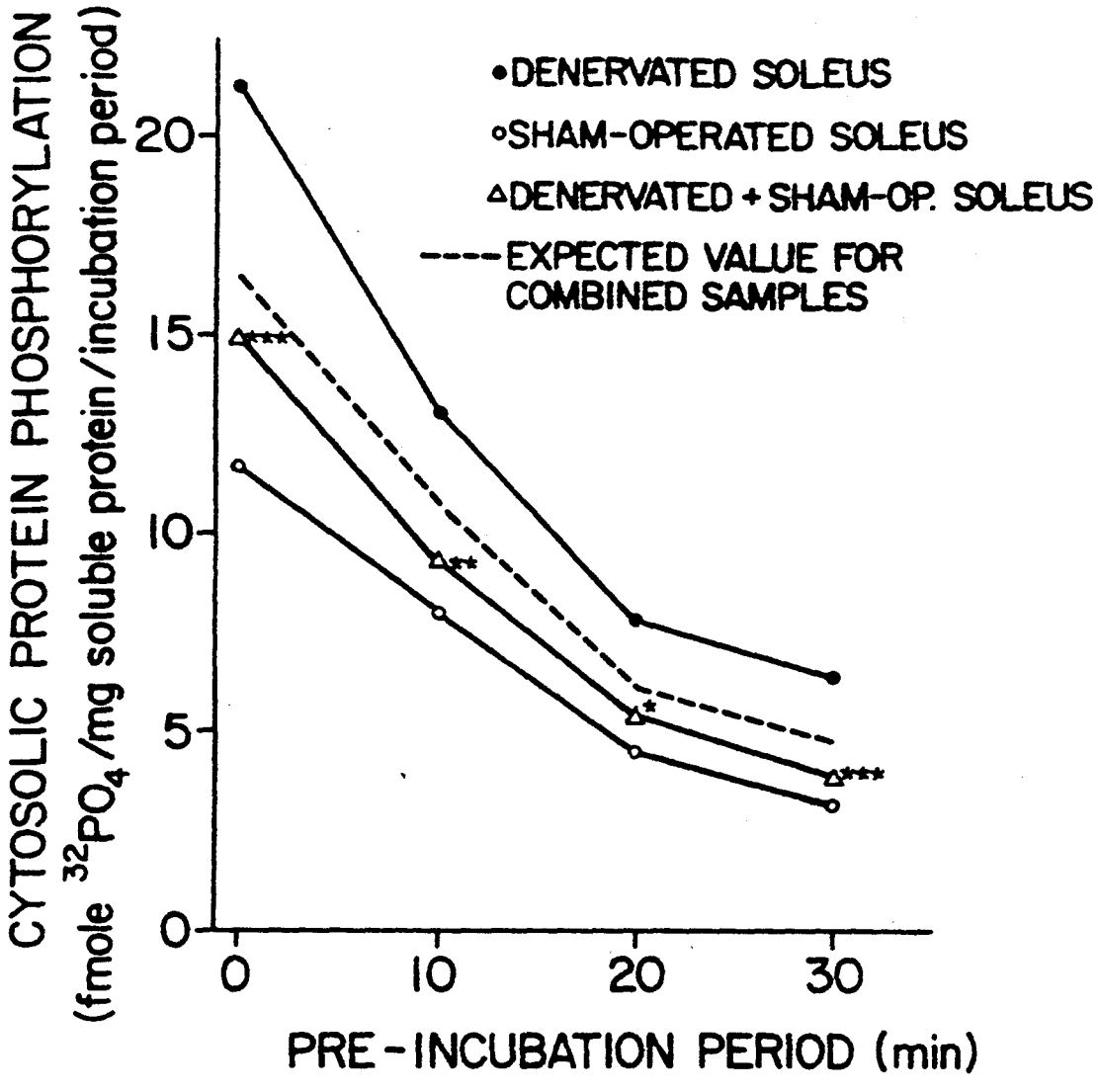


Figure 22. Effect of endogenous soluble modulators upon cytosolic protein phosphorylation in rat solei. Combined aliquots from the cytosolic fractions of 24 hr denervated short (< 2 mm) nerve stump-solei and contralateral, sham-operated solei were assayed and cytosolic protein phosphorylation (fmoles ^{32}P-phosphosphate incorporated/ mg soluble cytosolic protein/ incubation period) was determined in these combined samples as described in Method E varying only the time of pre-incubation. These results were compared to those obtained from assays of each sample separately. Each point represents the mean of eight determinations obtained from eight separate experiments. Statistical analyses were performed by paired t-test to examine differences between experimentally determined cytosolic protein phosphorylation (———) and expected values (-----) for combined samples. * $p \leq 0.05$, ** $p \leq 0.02$ and *** $p \leq 0.01$.

with n=8; $p < 0.05$) decrease from the expected value was observed with the 20 min pre-incubation period and an 18.7 $\pm$ 1.6% (mean $\pm$ S.E.M. with n=8; $p < 0.01$) decrease was observed with the 30 min pre-incubation period.

3. Effect of endogenous divalent cation-dependent and independent protein phosphatase activity

Soluble proteins from the cytosolic fractions of denervated and sham-operated solei were phosphorylated _in vitro_ in a buffered (pH 7.5) phosphorylation medium containing Mg^{2+} and [gamma-^{32}P]ATP (Method E). The phosphorylation reaction was terminated after 1, 2, 5, 10, 20 and 30 min. Under these reaction conditions, cation-dependent dephosphorylation of ^{32}P-labeled soluble proteins was examined. ^{32}P-labeled soluble proteins were resolved by SDS gradient slab PAGE (Figure 23-I) and visualized by autoradiography (Figure 23-II). Similar amounts of ^{32}P-labeled soluble protein from the cytosolic fractions of denervated (30.77 ug) and sham-operated solei (30.44 ug) were applied to the SDS gradient slab gel. The Coomassie blue protein staining patterns for denervated samples were essentially identical to the protein pattern obtained with sham-operated solei (Figure 23-I). Autoradiography of the dried slab gel shows an increase in the ^{32}P-labeling associated with the major 56,000-dalton phosphoprotein with longer incubation time periods. The increase in phosphorylative modification

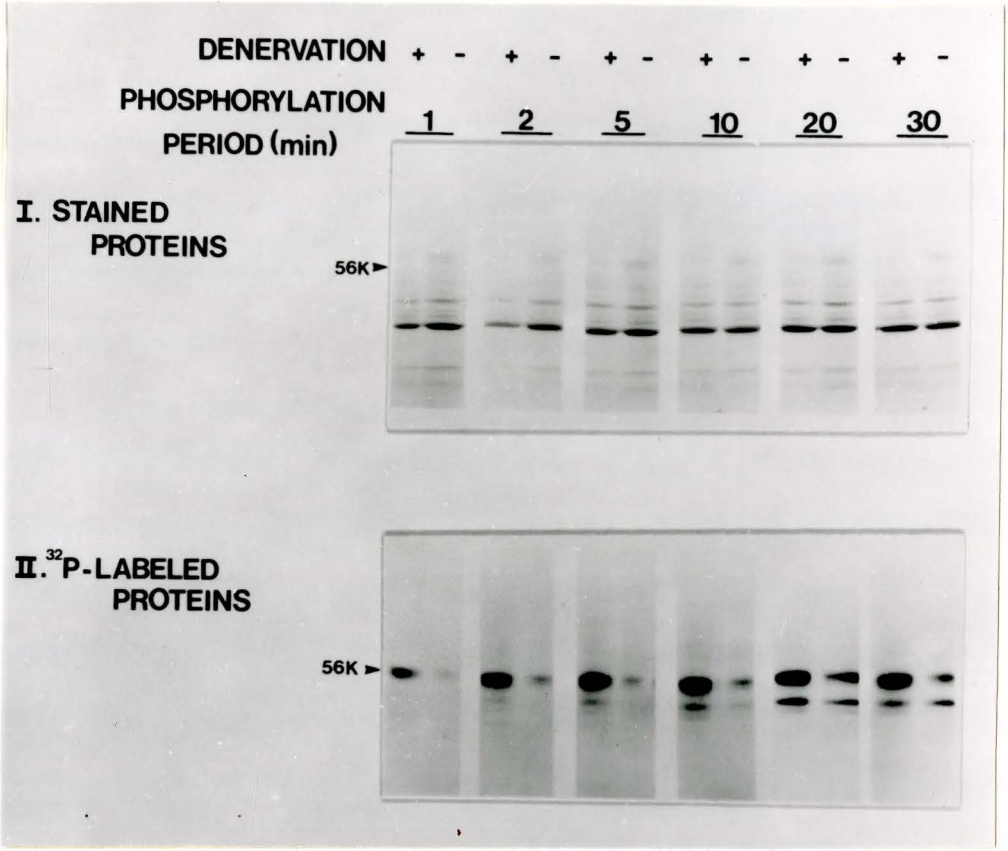


Figure 23. The effect of incubation time on the phosphorylation and cation-dependent dephosphorylation of the major 56,000-dalton soluble phosphoprotein in denervated and sham-operated cytosolic fractions of rat solei. Cytosol samples from solei denervated for 24 hr with a short (< 2 mm) distal nerve stump (+) and the contralateral, sham-operated muscles (-) were radiolabeled *in vitro* as in Method E except that times of incubation were 1, 2, 5, 10, 20 and 30 min. ^{32}P-radiolabeled reaction products were resolved by SDS gradient slab PAGE. A photograph of the Coomassie blue protein staining pattern from the cytosolic fractions of denervated and sham-operated muscles is shown in I. The autoradiograph of the dried slab gel is also shown (II). Molecular weights indicated were determined relative to the electrophoretic mobilities of known molecular weight marker proteins. These proteins were used to derive the calibration curve shown in Figure 39 of the Appendix.

of this protein occurs in both denervated and sham-operated samples. Also, a marked increase in the phosphorylation of the 56,000-dalton phosphoprotein in denervated compared to sham-operated muscles was observed for all incubation periods. However, a cation-dependent dephosphorylation of the 56,000-dalton phosphoprotein in either denervated or sham-operated solei was not observed over the incubation time course studied.

The effect of a cation-independent dephosphorylation of the predominant 56,000-dalton soluble phosphoprotein was examined in the cytosolic fractions of 24 hr denervated short nerve stump-solei and contralateral, sham-operated solei. To specifically study the cation-independent dephosphorylation of the major 56,000-dalton phosphoprotein, soluble cytosolic protein from denervated and sham-operated solei were incubated for 5 min in the buffered phosphorylation reaction system (Method E). After the 5 min incubation period, 6.5 mM EDTA was added to the assay to terminate phosphorylation and to inhibit any cation-dependent phosphatase activity. The dephosphorylation reaction catalyzed by the cation-independent protein phosphatase was allowed to proceed for another 0, 5, 15, and 25 min. The reaction products were resolved by SDS gradient PAGE (Figure 24-I) and visualized by autoradiography of the dried slab gel (Figure 24-II). Similar amounts of soluble protein from denervated (30.77 ug) and sham-operated (30.44 ug) soleus cytosolic fractions were applied to individual sample wells of the SDS gel. An increased

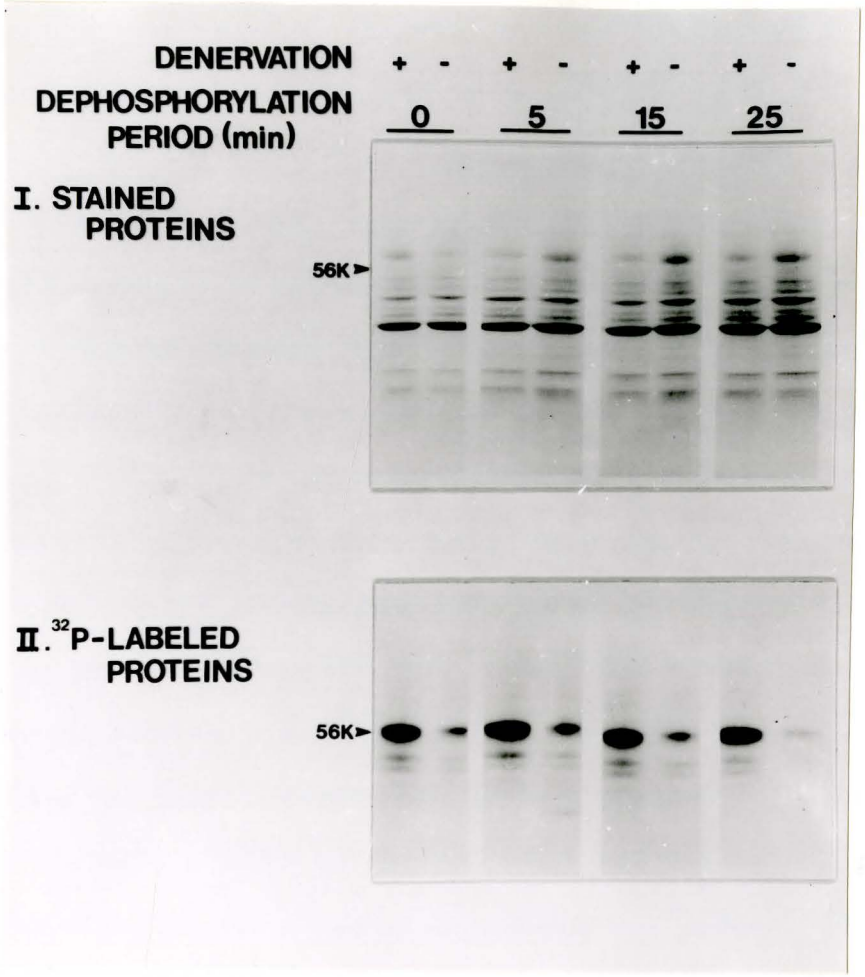


Figure 24. Effect of cation-independent protein phosphatase activity upon the ^{32}P-labeling of the major soluble 56,000-dalton phosphoprotein in the cytosolic fractions of denervated and sham-operated solei. Cytosol samples from rat solei denervated for 24 hr with a short (< 2 mm) distal nerve stump (+) and contralateral, sham-operated solei (-) were ^{32}P-labeled *in vitro* with a 5 min incubation period (Method E). After the 5 min incubation period, 6.5 mM EDTA was added to the incubation medium and the dephosphorylation reaction catalyzed by a cation-independent phosphoprotein phosphatase was allowed to proceed for another 0, 5, 15 or 25 min. ^{32}P-radiolabeled reaction products were resolved by SDS gradient slab PAGE. A photograph of the Coomassie blue protein staining pattern from the cytosolic fractions of denervated and sham-operated muscles is shown in I. The autoradiograph of the dried slab gel is also shown (II). Molecular weights indicated were determined relative to the electrophoretic mobilities of known molecular weight marker proteins. These proteins were used to derive the calibration curve shown in Figure 39 of the Appendix.

phosphorylation of the 56,000-dalton phosphoprotein was observed in denervated samples compared to sham-operated samples and appears to be consistent for each incubation period. Little change in the ^{32}P-radiolabeling of the 56,000-dalton protein from either denervated or sham-operated solei occurred at either the 5 or 15 min incubation pericds. A slight dephosphorylation of the 56,000-dalton protein from the cytosolic fraction of sham-operated solei occurs after 25 min. These results indicate that both denervated and sham-operated solei have a similar low level of cation-independent protein phosphatase activity. The molecular weights of proteins indicated in Figures 23 and 24 were calculated relative to the electrophoretic mobilities of standard known molecular weight marker proteins. The molecular weight calibration curve for these gels is shown in Figure 39 of the Appendix.

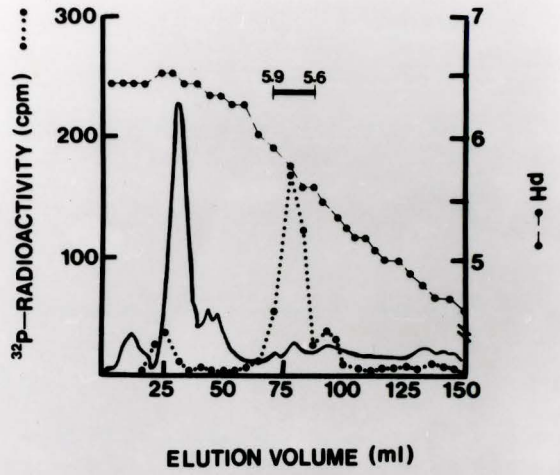
E. Identification of the 56,000-Dalton Denervation Period and Nerve Stump Length-Dependent Cytosolic Phosphoprotein from Rat Soleus Muscle

1. Chromatofocusing

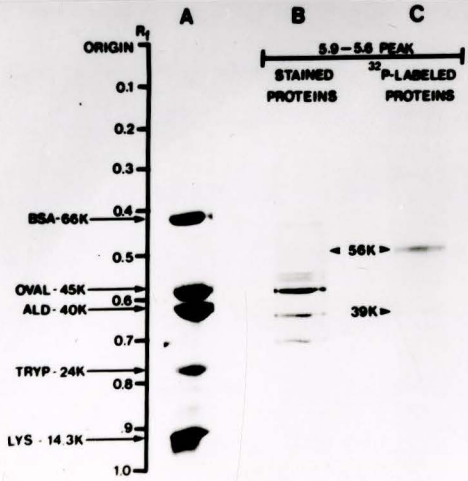
Soluble proteins from the cytosolic fraction of unoperated rat solei were phosphorylated <u>in vitro</u> (Method E) and were prepared for chromatofocusing by dialyzing aliquots overnight against a buffer of 0.025 M histidine-HCl, pH 6.4, at 4°C (Method J). An aliquot of unlabeled soluble cytosolic

protein (12.75 mg) was combined with an another aliquot of soluble protein (43.0 ug) which was ^{32}P-labeled *in vitro* and the combined sample was applied to a pH-equilibrated (pH 6.4) chromatofocusing column. Unlabeled soluble cytosolic protein was used as cold carrier protein. The majority of the soluble cytosolic protein elutes in the void volume corresponding to a pH of 6.4 or higher (Figure 25-I). The ^{32}P-radioactivity determined in 250 ul aliquots of consecutive 5.0 ml fractions by liquid scintillation spectrometry shows that the majority (55%) of the recovered ^{32}P-radioactivity (i.e., 68.3% of the radioactivity applied to the column) is localized in four consecutive fractions eluting within a pH range of 5.9 to 5.6. When these four fractions were pooled, concentrated by lyophilization, resuspended in a buffer containing SDS, dialyzed overnight in the buffer containing SDS and then resolved by SDS gradient slab PAGE, the major cytosolic ^{32}P-labeled phosphoprotein observed in this pH range (5.9 to 5.6) was a 56,000-dalton phosphoprotein (lane C in Figure 25-II). The molecular weight of this protein was determined relative to the molecular weight marker proteins shown in Figure 25. Therefore, this protein has the same electrophoretic mobility on the SDS slab gel system as the 56,000-dalton denervation period and nerve stump length-dependent phosphoprotein (Figure 15). Also, the less intense ^{32}P-labeled 39,000-dalton band probably represents a proteclytic fragment of the 56,000-dalton phosphoprotein because it

I. CHROMATOFOCUSING



II. SDS PAGE

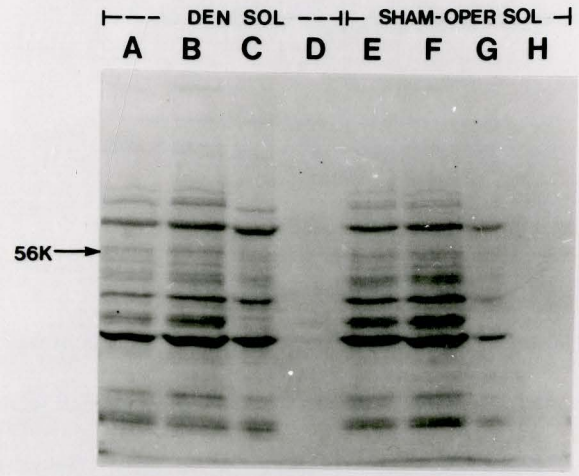


is not observed when total phosphorylated soluble cytosolic proteins are resolved by SDS gradient slab PAGE and visualized by autoradiography of the dried slab gel (Figure 15).

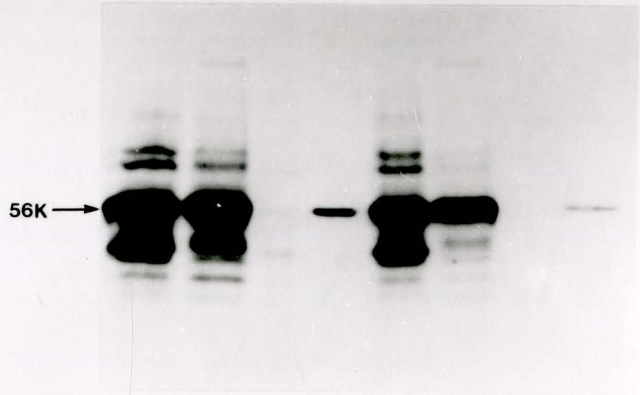
2. Cyclic AMP-affinity chromatography

The possible identity of the 56,000-dalton denervation period and nerve stump length-dependent phosphoprotein from rat soleus muscle as the type II regulatory subunit of cyclic AMP-dependent protein kinase (RII) was studied by examining the specific binding of ^{32}P-labeled soluble cytosolic phosphoproteins from 24 hr denervated short stump-solei and contralateral, sham-operated solei onto N^6-(2-aminoethyl)-cyclic AMP-Sepharose. Binding and non-binding phosphoproteins were resolved by SDS gradient slab PAGE and visualized by autoradiography (Figure 26). The Coomassie blue protein-staining patterns obtained when soluble proteins from the cytosolic fractions of denervated (176 ug) and sham-operated solei (188 ug) were applied to the affinity column are shown in lanes A and E of Figure 26-I. Most of the soluble cytosolic proteins from denervated and sham-operated solei applied to the cyclic AMP affinity column did not bind to the resin and appeared in the void volume (lanes B and F of Figure 26-I). A small amount of non-specifically bound protein was eluted from the cyclic AMP affinity resin with 750 mM NaCl when soluble cytosolic protein from either denervated or sham-operated solei was applied to the column

I. STAINED PROTEINS



II. ^{32}P-LABELED PROTEINS



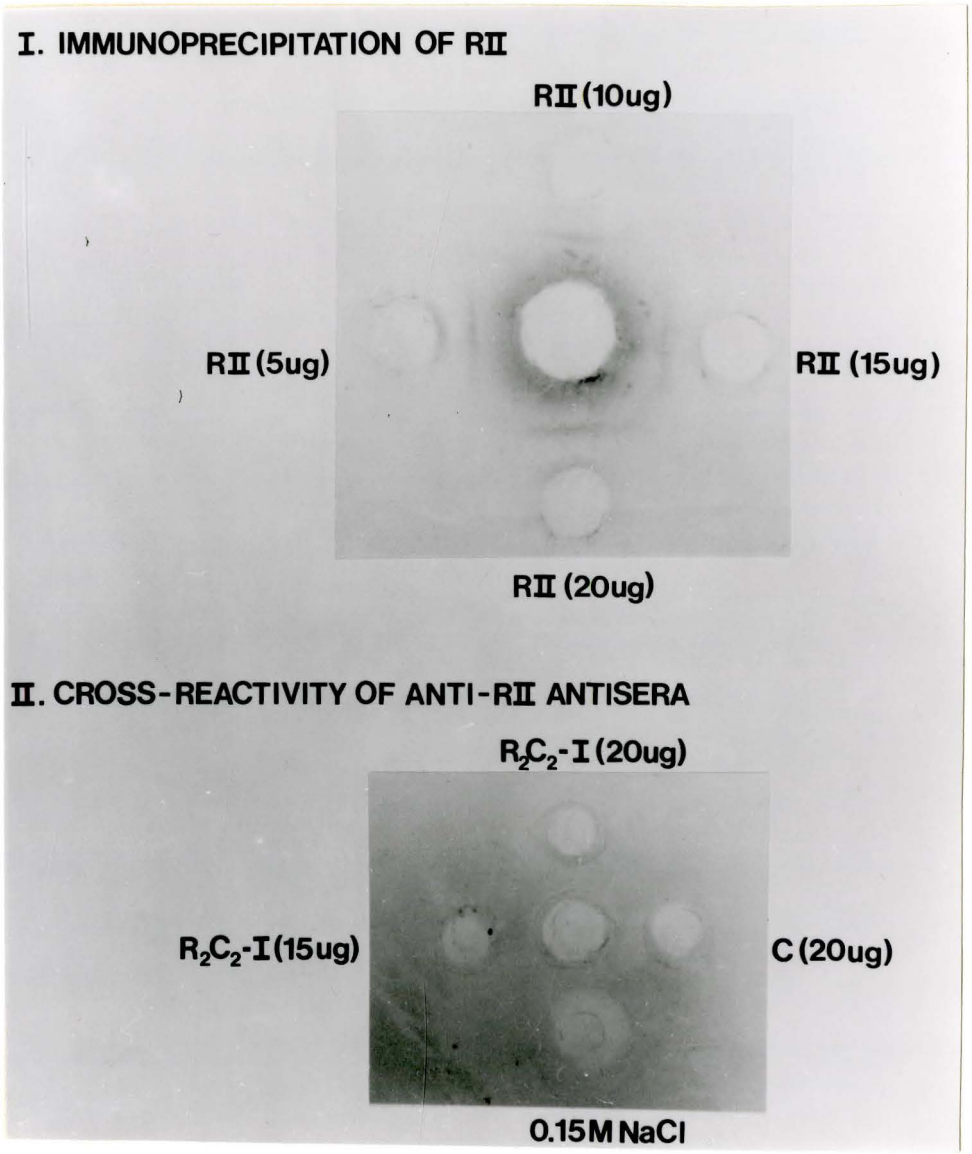
(lanes C and G of Figure 26-I). Some protein from denervated and sham-operated solei appeared to be specifically bound to the cyclic AMP affinity resin and was displaced from these columns with high concentrations (1 mM) of cyclic AMP (lanes D and H of Figure 26-I). This protein(s) was not visualized with the Coomassie stain, but was detected by autoradiography (lanes D and H of Figure 26-II).

The specificity of the N^6-(2-aminoethyl)-cyclic AMP-Sepharose in retaining ^{32}P-labeled soluble cytosolic phosphoproteins from 24 hr denervated short stump-solei and sham-operated solei is illustrated in the autoradiograph (Figure 26-II) derived from the dried slab gel shown in Figure 26-I. A single ^{32}P-labeled soluble cytosolic phosphoprotein was specifically adsorbed by the cyclic AMP affinity resin when soluble protein from either the cytosolic fractions of denervated solei (Figure 26 II-lane D) or contralateral, sham-operated solei (Figure 26 II-lane H) was applied to separate cyclic AMP affinity columns. This ^{32}P-radiolabeled phosphoprotein was eluted from these columns with 1 mM cyclic AMP. The relative molecular weight of the cyclic AMP-binding phosphoprotein resolved from the cytosolic fractions of denervated and sham-operated rat solei was determined to be 56,000. This relative molecular weight was determined by comparing its relative electrophoretic mobility to the electrophoretic mobilities of known molecular weight marker proteins which were used to establish the molecular weight

calibration curve shown in Figure 40 of the Appendix. The ^{32}P-labeling of the 56,000-dalton cyclic AMP-binding protein resolved from denervated solei is greater relative to sham-operated, contralateral solei. Also, the electrophoretic mobility determined on SDS gradient slab gels of the 56,000-dalton cyclic AMP-binding phosphoprotein from rat solei is the same as the electrophoretic mobility of the 56,000-dalton denervation period and nerve stump length-dependent phosphoprotein from rat solei.

3. Anti-RII (regulatory subunit of cyclic AMP-dependent protein kinase type II) immunoaffinity chromatography

Antiserum was raised by Sigma Chemical Company (St. Louis, MO) as described in Method L against a commercial preparation of the cyclic AMP-dependent protein kinase type II regulatory subunit (RII) derived from bovine heart muscle. The immunoglobulin G (IgG) component of the crude antiserum was partially purified by sodium sulfate fractionation and ion exchange chromatography and then tested for its specificity by the Ouchterlony double diffusion method (Ouchterlony, 1967). The partially purified IgG fraction of the anti-RII antiserum immunoprecipitates commercially purified bovine heart RII (Figure 27-I). The antigenic response was weak (faint immunoprecipitin line) when the reaction was carried out against 5, 10, 15 and 20 ug of RII. However, only one immunoprecipitin line was observed which is characteristic of a monospecific, antibody/antigen conjugation. Also the IgG


I. IMMUNOPRECIPITATION OF RII
RII (10ug)
RII (5ug)
RII (15ug)
RII (20ug)
II. CROSS-REACTIVITY OF ANTI-RII ANTISERA
R_2C_2-I (20ug)
R_2C_2-I (15ug)
C (20ug)
0.15M NaCl

fraction of the anti-RII antiserum did not cross react with a commercial preparation of cyclic AMP-dependent protein kinase type I holoenzyme (15 and 20 ug), a commercial preparation of cyclic AMP-dependent protein kinase catalytic subunit (20 ug), or saline (Figure 27-II).

An anti-RII immunoaffinity column was prepared by conjugating the partially purified IgG fraction of the anti-RII antiserum to CNBr-activated Sepharose 4B (Method L). Soluble proteins from the cytosolic fractions of 24 hr denervated short nerve stump-solei and contralateral, sham-operated solei were ^{32}P-labeled <u>in vitro</u> (Method E) and then prepared for immunoaffinity chromatography (Method L). Similar amounts of soluble cytosolic protein from denervated and sham-operated solei (approximately 200 ug) were applied to separate immunoaffinity chromatography columns. Non-adsorbing proteins collected in the column void volume, non-specifically bound proteins eluting with NaCl, and those proteins eluting with low pH (2.2) glycine buffer were then concentrated by lyophilization, resuspended in 200 ul of "SDS stop solution" and resolved by SDS gradient slab PAGE. Also, a separate 200 ul aliquot of soluble cytosolic protein from denervated and sham-operated solei was not subjected to immunoaffinity chromatography, but was concentrated by lyophilization, resuspended in an "SDS stop solution" and resolved by SDS gradient slab PAGE. This procedure made it possible to quantitate and statistically compare the

adsorption of specific soluble phosphoproteins from both denervated and sham-operated cytosols on an anti-RII immunoaffinity resin. The 56,000-dalton phosphoprotein in each of these fractions resolved on SDS gradient slab gels was located by staining the gel with Coomassie brilliant blue. The Commassie blue stained band corresponding to the 56,000-dalton protein was cut from the gel (2 mm slice) and ^{32}P-radioactivity in this 2 mm gel slice was quantitated by liquid scintillation spectrometry.

Quantitatively, 80.4 ± 2.8% (mean ± S.E.M. with n=3) of the ^{32}P-radioactivity specifically associated with the 56,000-dalton phosphoprotein from the cytosolic fraction of denervated solei (total cpm in 2 mm gel slice) does not bind to the affinity resin and can be accounted for in the void fraction (Table 9). Similarly, 75.9 ± 5.6% (mean ± S.E.M. with n=3) of the ^{32}P-radioactivity associated with the 56,000-dalton phosphoprotein from the cytosolic fraction of contralateral, sham-operated soleidoesnotbind to the affinity resin and appears in the void fraction (Table 9). Only 2.1 ± 0.7% (mean ± S.E.M. with n=3) of the total ^{32}P-radioactivity associated with the 56,000-dalton protein from the cytosolic fraction of denervated solei was eluted from the affinity column with 0.5 M NaCl followed by 10 mM Tris-HCl, pH 7.4 (Table 9). Also, only 1.5 ± 1.0% (mean ± S.E.M. with n=3) was eluted from immunoaffinity columns to which cytosolic fractions of contralateral, sham-operated solei were applied (Table 9). However, 12.0 ± 1.7% (mean ±

TABLE 9. ADSORPTION OF THE 56,000-DALTON PHOSPHOPROTEIN FROM THE CYTOSOLIC FRACTIONS OF DENERVATED AND SHAM-OPERATED SOLEI BY ANTI-RII IMMUNOAFFINITY CHROMATOGRAPHY

	Denervated[b]	Sham-operated
Source[a] of 56K	%Total CPM	%Total CPM
Cytosol	100	100
Void Vol.	80.4 ± 2.8	75.9 ± 5.6
NaCl Elution	2.1 ± 0.7	1.5 ± 1.0
Low pH (2.2) Elution	12.0 ± 1.7	16.2 ± 3.9

[a] Soluble protein from the cytosolic fractions of denervated and contralateral, sham-oprated rat solei was phosphorylated *in vitro* (Method E). ^{32}P-labeled reaction products (200 ul aliquot of the incubation medium) were resolved by SDS gradient slab PAGE (Method K). The 200 ul aliquot from the cytosolic fractions of denervated cytosol contained 188.7 ug of protein and the 200 ul aliquot from sham-operated cytosol contained 195.6 ug of protein. The region of the gel (2 mm gel slice) containing the the ^{32}P-labeled 56,000-protein was visualized after Coomassie staining and located by examining consecutive 2 mm gel slices with a Geiger counter. Another aliquot from the phosphorylation assay containing a similar amount of soluble ^{32}P-labeled protein from denervated cytosols (188.7 ug) and sham-operated cytosols (195.6) were applied to separate immunoaffinity columns. Non-binding proteins eluting in the void volume, non-specifically bound proteins eluting with NaCl and those eluted specifically with low pH (2.2) buffer were concentrated by lyophilization and applied to the SDS gel. The ^{32}P-labeling of the 56,000-dalton protein was quantitated by liquid scintillation spectrometry of 2 mm gel slices. Percentages are reported in the Table as means ± S.E.M for three groups of three animals.

[b] The left soleus was denervated 24 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve leaving a 2 mm distal nerve stump left attached to the muscle. Sham-operated, contralateral muscles served as controls.

S.E.M. with n=3) of the ^{32}P-radioactivity associated with the 56,000-dalton protein from the cytosolic fractions of denervated solei and 16.2 ± 3.9% (mean ± S.E.M. with n=3) from sham-operated solei bound to anti-RII immunoaffinity columns and was displaced only after treating these columns with low pH (2.2) 0.2 M glycine buffer (Table 9). Also, the ^{32}P-radiolabeling associated with the 56,000-dalton soluble cytosolic phosphoprotein resolved by anti-RII immunoaffinity chromatography from denervated solei was significantly ($p < 0.05$) greater (160.6 ± 27.5%; mean ± S.E.M. with n=3) than the ^{32}P-labeling associated with the 56,000-dalton protein resolved from contralateral, sham-operated solei.

Since the percentage of 56,000-dalton phosphoprotein bound to the anti-RII immunoaffinity resin was low when either denervated (12.0%) or sham-operated (16.2%) sample was applied to individual columns, the ability of the immunoaffinity column to bind a commercial preparation of RII, phosphorylated *in vitro* (Method N), was examined. Anti-RII immunoaffinity columns were prepared by conjugating either a commercial preparation of anti-RII to Sepharose 4B or by conjugating purified anti-RII (gift from the laboratory of Dr. Richard Jungmann, Department of Molecular Biology, Northwestern University Medical School, Chicago, Il.) to the Sepharose resin (Method L). Binding and non-binding ^{32}P-labeled RII protein were identified by SDS gradient slab PAGE. It is important to point out that purified 56,000-

dalton RII protein obtained from commercial sources shows several contaminating proteins when resolved by SDS gradient slab PAGE (not shown). When equivalent amounts of phosphorylated bovine heart RII (16 ug) were passed over these two immunoaffinity columns, only 23.4% of the total ^{32}P-radioactivity associated with the 56,000-dalton RII protein was retained by the immunoaffinity column prepared with commercially produced anti-RII antiserum. Also, only 17.6% was bound to the immunoaffinity column prepared with purified anti-RII antiserum (Table 10).

4. ^{32}P-Phosphopeptide mapping

Limited tryptic digestion of proteins carried out in SDS polacrylamide slab gels (15%) according to Cleveland et al. (1977) provides a convenient method for analyzing peptide maps generated from the proteolytic fragmentation of specific proteins purified by SDS gradient slab PAGE. Cytosolic fractions from rat solei were incubated in the phosphorylation medium containing 2 mM $MgCl_2$ and [gamma-^{32}P]ATP (Method E). The ^{32}P-radiolabeled 56,000-dalton soluble phosphoprotein was resolved from the cytosolic fractions of these muscles by SDS gradient slab PAGE, sliced from the slab gel (2 x 5 mm gel slice) and prepared for tryptic peptide mapping (Method M) . Phosphopeptides generated by proteolysis of the 56,000-dalton phosphoprotein with bovine pancreas trypsin for 30 min were resolved by SDS slab PAGE (15% polyacrylamide

TABLE 10. ADSORPTION OF RII BY ANTI-RII IMMUNOAFFINITY CHROMATOGRAPHY AFTER *IN VITRO* PHOSPHORYLATION

Antibody Source	Source[c] of RII Protein	CPM	%Total
a	Assay	94712	100
	Void Vol.	66541	70.3
	NaCl Elution	3080	3.3
	Low pH (2.2) Elution	22142	23.4
b	Assay	100113	100
	Void Vol.	71292	71.2
	NaCl Elution	803	0.8
	Low pH (2.2) Elution	17538	17.5

[a]Antiserum against commercially prepared RII subunit of cyclic AMP-dependent protein kinase type II was supplied by Sigma Chemical Co., St. Louis, MO. The IgG fraction from this antiserum was partially purified and coupled to CNBr-activated Sepharose 4B (Method L).

[b]Antiserum raised against RII protein was a gift from the laboratory of Dr. Richard Jungmann. This antiserum was also prepared for use in immunoaffinty chromatography by the protocol described in Method L.

[c]Commercially available RII protein (Sigma Chemical Co.) was phosphorylated *in vitro* (Method N) and prepared for immunoaffinity chromatography (Method L). An equivalent amount of ^{32}P-labeled RII protein (16 ug) was applied to individual immunoaffinity chromatography columns prepared with either antisera a or b. Non-binding ^{32}P-labeled RII protein eluting in the void volume, non-specifically bound RII protein eluting with NaCl and those eluting with 0.2 M glycine (pH 2.2) were concentrated by lyophilization and applied to the SDS gel. The region of the gel containing the 56,000-dalton RII protein (2 mm gel slice) was detected by cutting the gel into consecutive 2 mm gel slices and examing each slice with a Geiger counter. The ^{32}P-labeling of the 56,000-dalton RII protein was quantitated by liquid scintillation spectrometry.

gel) and visualized by autoradiography (Figure 28). Re-electrophoresis of the ^{32}P-labeled 56,000-dalton phosphoprotein by SDS slab PAGE resolved the 56,000-dalton phosphoprotein, another major 52,000-dalton phosphopeptide and a minor 39,000-dalton phosphopeptide (Figure 28A). These phosphopeptides probably represent proteolytic fragments generated from the 56,000-dalton protein during the preparation of the protein for re-electrophoresis (30 min incubation). Proteolytic fragments of 52,000 and 39,000-daltons have been previously reported for the 56,000-dalton regulatory subunit of cyclic AMP-dependent protein kinase type II (Rangel-Aldao et al., 1979). When the ^{32}P-labeled 56,000-dalton phosphoprotein is treated with 0.5 ug of trypsin, much of the protein is digested to minor ^{32}P-labeled phosphopeptides of M_r=52,000, 31,000, 24,000 and 12,000 and major phosphopeptides of 39,000 and 16,500 (Figure 28B). Increasing the amount of trypsin to 1.0 ug results in an apparent increase in the ^{32}P-labeled 16,500-dalton and 12,000-dalton phosphopeptides and a slight decrease in the ^{32}P-labeled 39,000-dalton phosphopeptide (Figure 28C). Complete digestion of the 56,000-dalton phosphoprotein with 10.0 ug of trypsin yields phosphopeptides of 16,500-daltons and 12,000-daltons (Figure 28D). Molecular weights were estimated after establishing a molecular weight calibration curve for this SDS slab gel using known molecular weight marker proteins (Figure 28).

In order to determine whether the low molecular

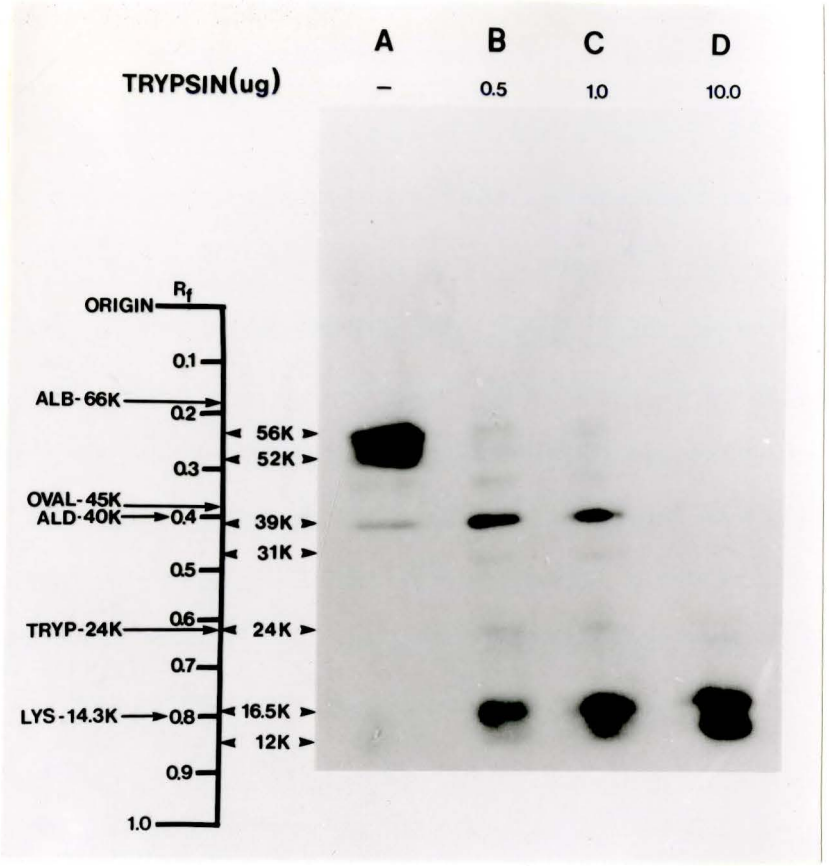
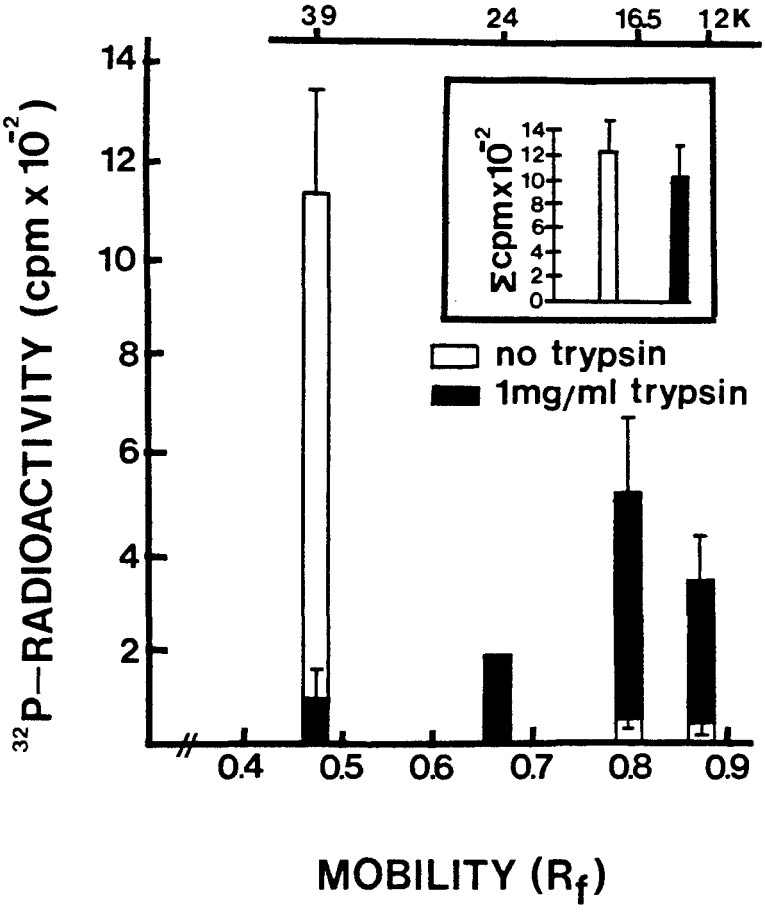


Figure 28. ^{32}P-phosphopeptide map obtained after tryptic digestion of the 56,000-dalton phosphoprotein from unoperated rat soleus muscle. The 56,000-dalton soluble phosphoprotein was ^{32}P-radiolabeled *in vitro* using a phosphorylation incubation media containing 2 mM $MgCl_2$ and [gamma-^{32}P]ATP. The phosphorylated 56,000-dalton protein was resolved by SDS gradient slab PAGE. The Coomassie blue stained band containing the 56,000-dalton phosphoprotein was sliced from the gel and prepared for peptide mapping (Method M). The 56,000-dalton phosphoprotein was re-electrophoresed on an SDS 15% polyacrylamide slab gel. The autoradiograph of the peptide mapping gel is shown. The 56,000-dalton phosphoprotein was treated as follows: (A) no trypsin; (B) + 0.5 ug trypsin; (C) + 1.0 ug trypsin; (D) + 10 ug trypsin. The molecular weights of radiolabeled phosphopeptides are indicated and were established relative to the electrophoretic mobilities of standard known molecular weight marker proteins. The protein standards (i.e., bovine serum albumin (BSA)-66,000; ovalbumin (OVAL)-45,000; aldolase (ALD)-40,000; trypsinogen (TRYP)-24,000; lysozyme (LYS)-14,300) were obtained from commercial sources. Standard proteins were prepared as solutions of 1 mg protein/ml of buffer (0.0625 M Tris-HCl, pH 6.8, 0.1% SDS and 5% 2-mercaptoethanol) and were denatured by boiling for 2 min prior to electrophoresis. Ten micrograms of each standard protein were applied to the peptide mapping gel. Relative mobilities (R_f) are the values obtained from a single electrophoretic separation.

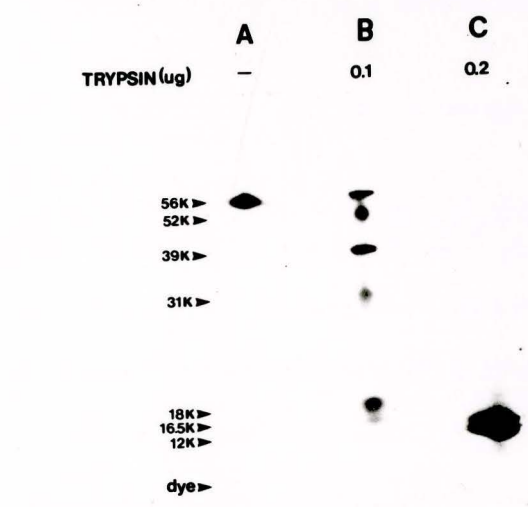
weight phosphopeptides observed in Figure 28 (16,500 and 12,000-daltons) represented further proteolytic digestion of the 39,000-dalton phosphopeptide, the ^{32}P-labeled 39,000-dalton phosphopeptide obtained from the limited tryptic digestion of the 56,000-dalton phosphoprotein (lane B of Figure 28) was subjected to further proteolysis with 10.0 ug trypsin. The generated ^{32}P-labeled peptides were resolved by SDS slab PAGE (15% gel). The ^{32}P-cpm of consecutive 2 mm gel slices was determined by liquid scintillation spectrometry. Tryptic proteolysis of the 39,000-dalton phosphopeptide generated only 24,000, 16,500 and 12,000-dalton phosphopeptides (Figure 29). Also, the total ^{32}P-cpm associated with these tryptic products (1069 ± 262 cpm; mean ± S.E.M. with n=3 sum of solid bars in the inset of Figure 29) is not statistically different by paired t-test to the total ^{32}P-cpm associated with the 39,000, 16,500 and 12,000-dalton phosphopeptides observed before tryptic digestion (1208 ± 228 cpm; mean ± S.E.M. with n=3 sum of open bars in the inset of Figure 29). The relative molecular weights of these peptides were estimated after establishing a calibration curve using known molecular weight marker proteins as described for Figure 28.

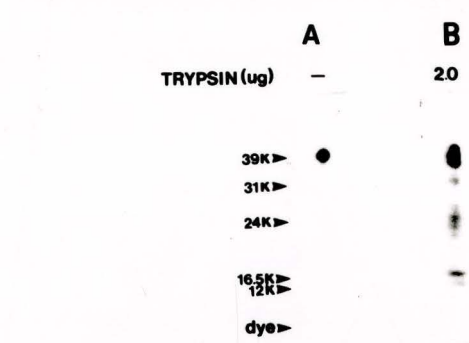
The phosphopeptide map of the ^{32}P-labeled 56,000-dalton phosphoprotein from rat soleus muscle was compared to the ^{32}P-phosphopeptide map of a commercial preparation of bovine heart cyclic AMP-dependent protein kinase type II


39
24
16.5
12K
14
12
10
8
6
4
2
^{32}P–RADIOACTIVITY (cpm x 10^{-2})
Σcpm x 10^{-2}
no trypsin
1mg/ml trypsin
0.4
0.5
0.6
0.7
0.8
0.9
MOBILITY (R_f)

regulatory subunit (RII). The 56,000-dalton RII protein was autophosphorylated in vitro with the protein kinase catalytic subunit using [gamma-^{32}P]ATP as the phosphoryl donor (Method N). The reaction products were resolved by SDS gradient slab PAGE. The ^{32}P-labeled 56,000-dalton RII protein was sliced from the gel and digested with trypsin. Then the generated ^{32}P-phosphopeptides were resolved by SDS slab PAGE (Figure 30-I). Like the 56,000-dalton phosphoprotein from rat soleus muscle, limited tryptic proteolysis of the 56,000-dalton RII protein with 0.1 ug trypsin generated major phosphopeptides of 52,000, 39,000, 31,000, 18,000 and 16,500-daltons (lane B of Figure 30-I). Also, like the 56,000-dalton soleus phosphoprotein, the 56,000-dalton RII protein was completely digested to phosphopeptides of 16,500 and 12,000-daltons (lane C of Figure 30-I).

One of the tryptic phosphopeptides of the 56,000-dalton RII protein, the 39,000-dalton phosphopeptide, has been reported to contain the autophosphorylation site (Potter and Taylor, 1979; Rangel-Aldao et al., 1979; Weber and Hilz, 1979; Takio et al., 1980). This 39,000-dalton autophosphorylation peptide generated from the limited tryptic digestion of the 56,000-dalton RII protein (lane B of Figure 30-I) was digested further with 2.0 ug trypsin and its proteolytic products were also resolved by SDS slab PAGE. Only the 39,000-dalton phosphopeptide was observed following re-electrophoresis of this phosphopeptide by SDS slab PAGE without trypsin present (lane A of Figure 30-II). However,


I. DIGEST OF 56K RII
A
B
C
TRYPSIN (ug)
–
0.1
0.2
56K
52K
39K
31K
18K
16.5K
12K
dye


II. DIGEST OF 39K RII PEPTIDE
A
B
TRYPSIN (ug)
–
2.0
39K
31K
24K
16.5K
12K
dye

the 39,000-dalton autophosphorylation peptide generated the same low molecular weight proteolytic products as the 56,000-dalton RII protein and the 56,000-dalton soleus phosphoprotein. These include the 31,000, 24,000, 16,500 and 12,000-dalton tryptic fragments (lane B of Figure 30-II).

Cytosolic fractions from 24 hr denervated short (< 2 mm) nerve stump-solei and their contralateral, sham-operated solei were ^{32}P-labeled *in vitro* and the radiolabeled 56,000-dalton phosphoprotein was resolved from these fractions by SDS gradient slab PAGE. Then phosphopeptides generated by proteolysis (0.5 ug trypsin) of the 56,000-dalton phosphoproteins from both denervated and sham-operated muscles were resolved by SDS slab PAGE and visualized by autoradiography (Figure 31). Re-electrophoresis of the 56,000-dalton phosphoprotein from both denervated (Figure 31A) and sham-operated solei (Figure 31B) by SDS slab PAGE generated the 56,000-dalton phosphoprotein and a 52,000-dalton phosphopeptide. Tryptic proteolysis of the ^{32}P-labeled 56,000-dalton phosphoprotein from either denervated (Figure 31C) or sham-operated muscles (Figure 31D) generated major 39,000-dalton phosphopeptide and a minor phosphopeptide of M_r =16,500. The phosphorylation of the 56,000-dalton phosphoprotein and also the 39,000-dalton fragment is increased in the denervated compared to the sham-operated muscle. Molecular weights were estimated after establishing a calibration curve using known molecular weight marker proteins as

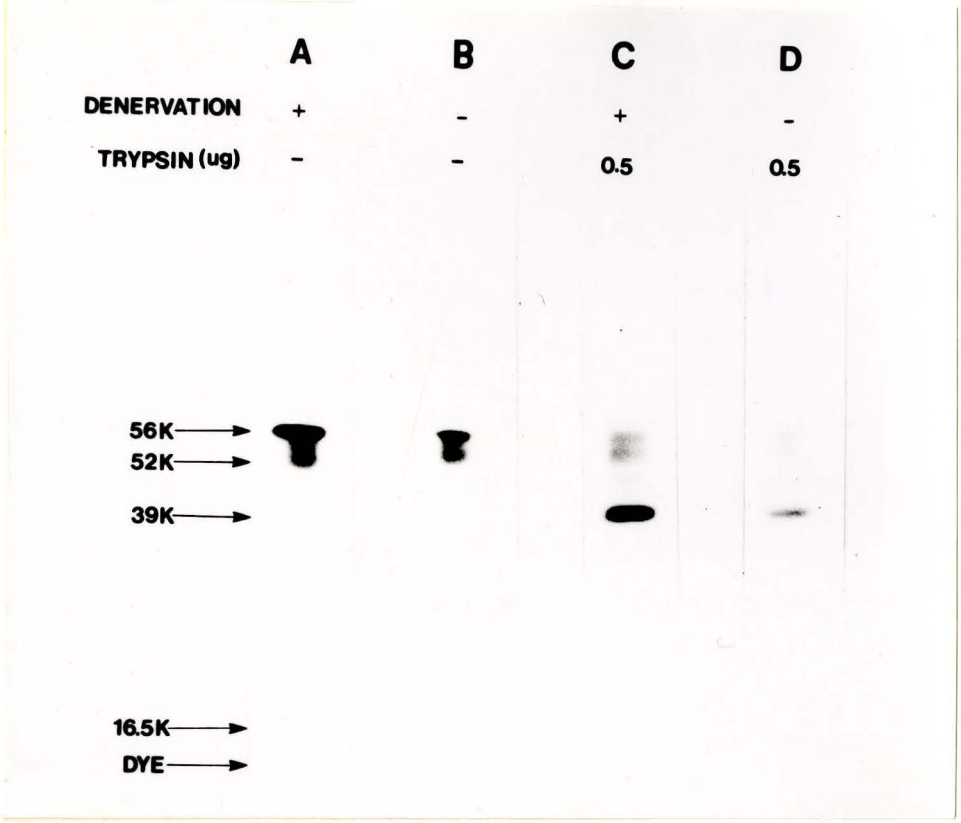


Figure 31. ^{32}P-phosphopeptides generated from the tryptic proteolysis of the ^{32}P-labeled 56,000-dalton phosphoprotein from the cytosolic fractions of denervated and sham-operated rat solei. The 56,000-dalton soluble phosphoproteins from the cytosolic fractions of solei denervated for 24 hr with a short (< 2 mm) distal nerve stump and their contralateral, sham-operated muscles were phosphorylated in vitro and resolved by SDS gradient slab PAGE. Equal aliquots of denervated and sham-operated soluble cytosolic protein (35 ug) were applied to individual sample wells of the slab gel. The 56,000-dalton ^{32}P-labeled phosphoproteins were sliced from the gel (2 mm gel slice) and prepared for peptide mapping. An autoradiograph of the resolved ^{32}P-labeled phosphopeptides derived from the 56,000-dalton phosphoproteins from denervated and sham-operated solei and resolved in an SDS 15% polyacrylamide slab gel is shown. The 56,000-dalton protein from the cytosolic fractions of denervated solei was treated as follows: (A) no trypsin; (C) + 0.5 ug trypsin. The 56,000-dalton proteins from the cytosolic fractions of sham-operated solei were treated as follows: (B) no trypsin; (D) + 0.5 ug trypsin. Relative molecular weights of the tryptic phosphopeptides are shown and were established relative to the known standard molecular weight marker proteins shown in Figure 28.

described for Figure 28.

The ^{32}P-cpm of each phosphoprotein and phosphopeptide shown in Figure 31 (A-D) was determined in consecutive 2 mm gel slices by liquid scintillation spectrometry. The data shown in Figure 32 were derived from three individual gels of which a representative example is shown in Figure 31. The quantitative result shown in Figure 32 supports the qualitative result shown in Figure 31. The phosphorylation of the 56,000-dalton protein and also the 39,000-dalton fragment is increased in denervated (Figure 32 lanes A and C) compared to sham-operated muscles (Figure 32 lanes B and D).

Cytosolic fractions from 66 hr denervated long (32 to 35 mm) nerve stump-solei and their contralateral, sham-operated muscles were ^{32}P-labeled <u>in vitro</u> and the radiolabeled 56,000-dalton phosphoprotein was resolved from these fractions by SDS gradient PAGE. Then phosphopeptides generated by proteolysis (0.5 ug trypsin) of the 56,000-dalton phosphoproteins from both denervated and sham-operated muscles were resolved by SDS slab PAGE and visualized by autoradiography (Figure 33). Tryptic proteolysis of the 56,000-dalton phosphoprotein from both denervated and sham-operated solei generated major phosphopeptides of 50,000, 39,000 and 16,500-daltons. Minor phosphopeptides of 31,000, 24,000 and 12,000 were also resolved. The ^{32}P-cpm associated with each of the major phosphopeptides was determined by slicing the gel into consecutive 2 mm slices and counting

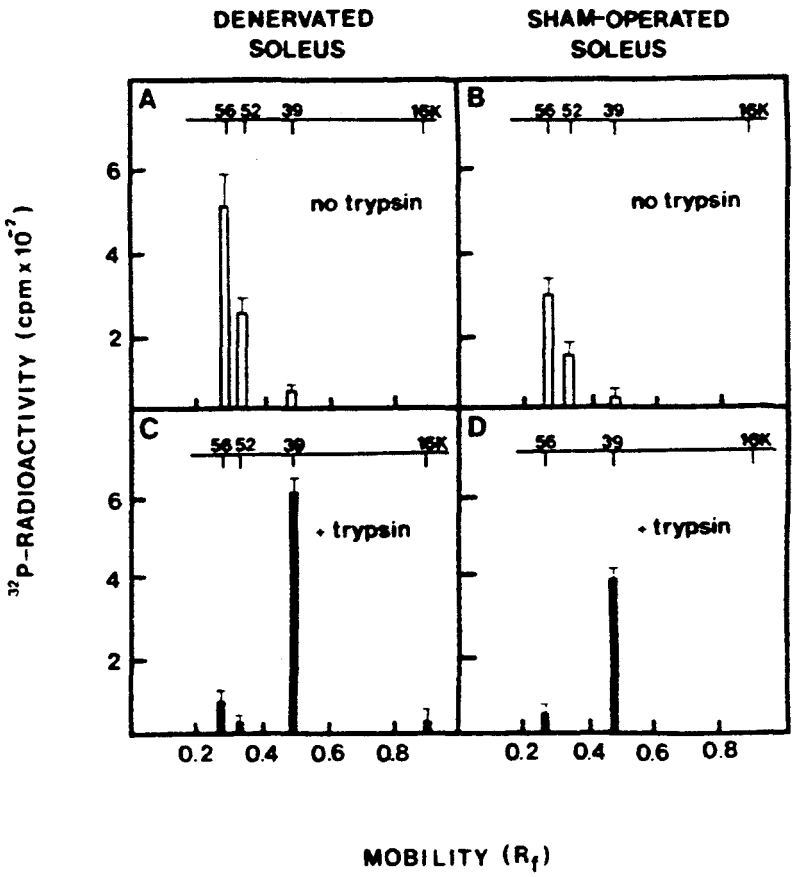


Figure 32. The distribution of ^{32}P-radioactivity associated with phosphopeptides derived from the ^{32}P-labeled 56,000-dalton soluble protein resolved from the cytosolic fractions of denervated and sham-operated solei. The left soleus was denervated 24 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before its insertion into the muscle. Control samples were obtained from sham-operated, contralateral solei. Cytosolic fractions were incubated in a phosphorylation media containing [gamma-^{32}P]ATP and the soluble ^{32}P-labeled 56,000-dalton protein was resolved by SDS gradient slab PAGE, sliced from the gel and prepared for tryptic proteolysis and peptide mapping in an SDS 15% polyacrylamide slab gel. ^{32}P-radioactivity was quantitated in consecutive 2 mm gel slices by liquid scintillation spectrometry. The source of the 56,000-dalton protein in these samples was as follows: A) 24 hr denervated short (< 2 mm) distal nerve stump-solei; B) contralateral, sham-operated solei; C) denervated solei + 0.5 ug trypsin; D) sham-operated solei + 0.5 ug trypsin. Values for each bar represent the means ± S.E.M. with n=3. Molecular weights indicated at the top of each quadrant (A-D) were estimated relative to the electrophoretic mobilities of known molecular weight marker proteins shown in Figure 28.

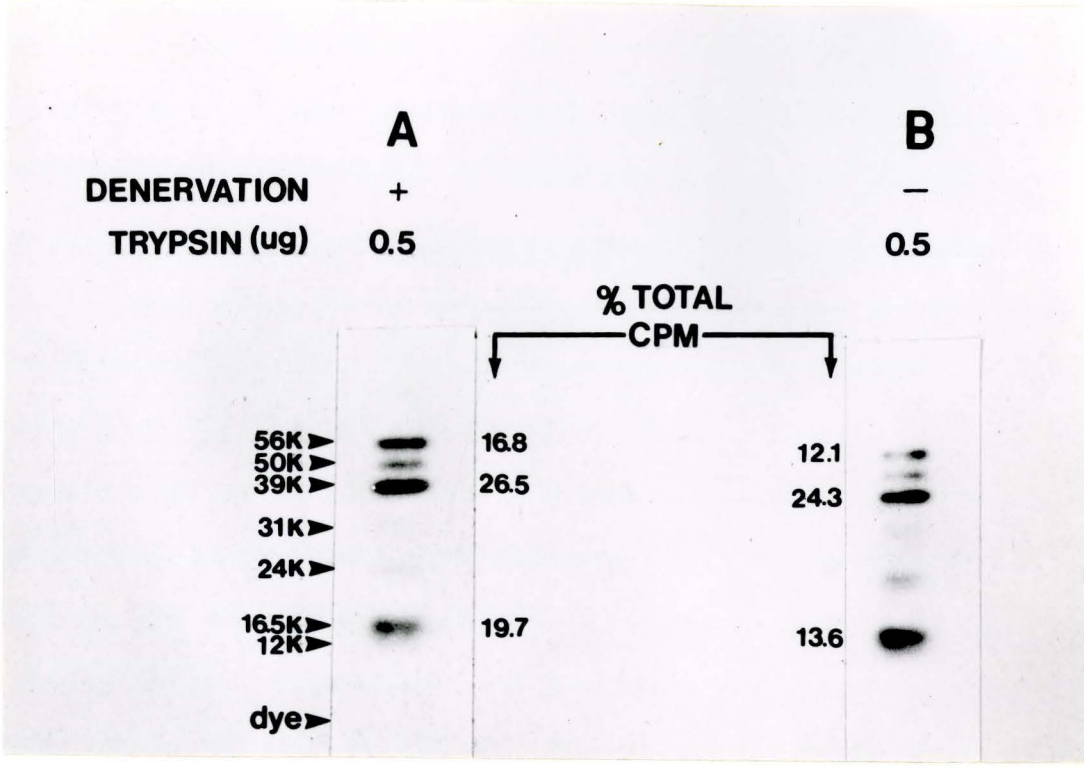
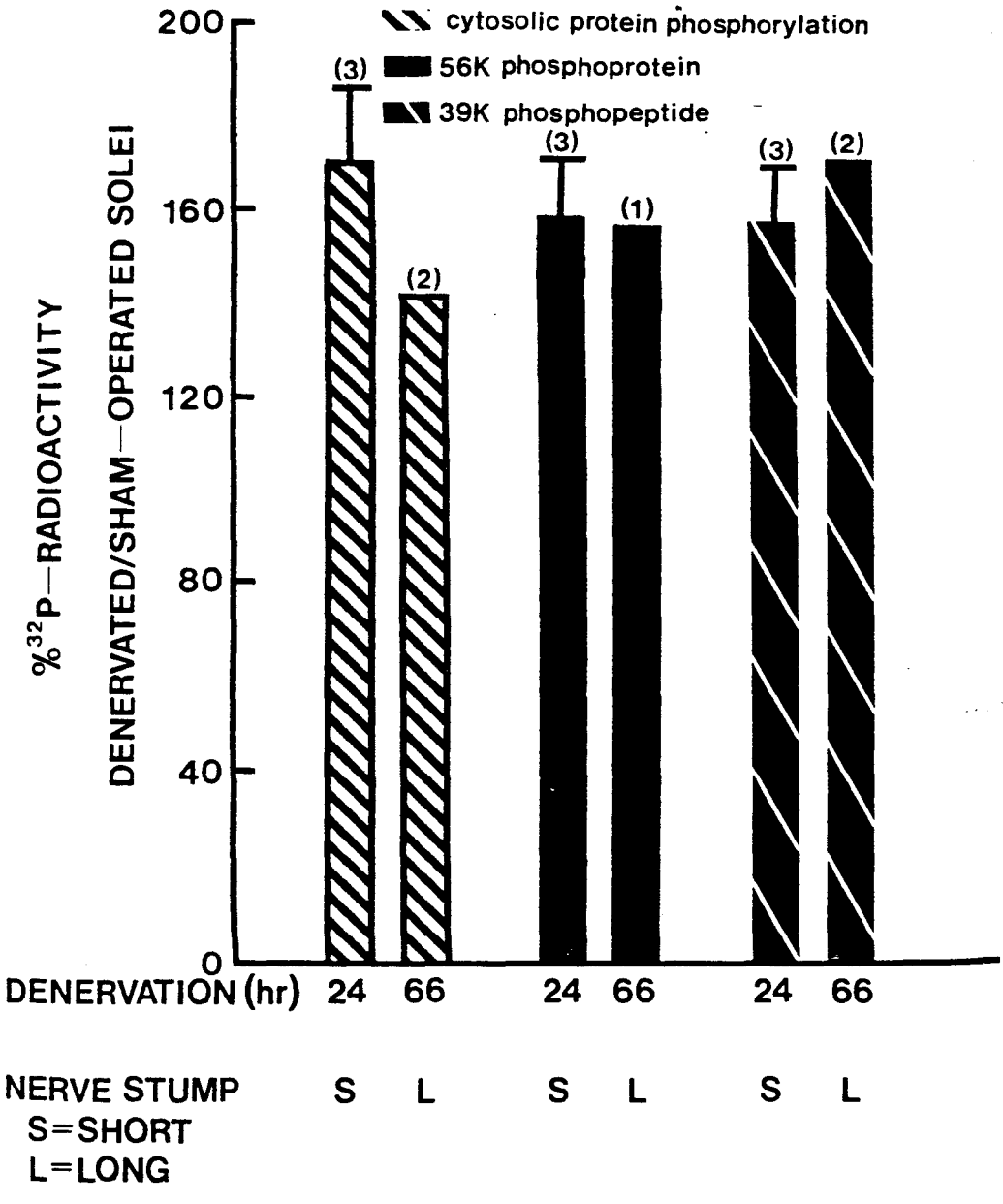


Figure 33. ^{32}P-phosphopeptides generated from the tryptic proteolysis of the ^{32}P-labeled 56,000-dalton phosphoprotein resolved from the cytosolic fractions of denervated (long nerve stump) and sham-operated solei. The left soleus was denervated 66 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 32 to 35 mm before its insertion into the muscle. Control samples were obtained from sham-operated, contralateral solei. Cytosolic fractions were incubated in the phosphorylation medium containing [gamma-^{32}P]ATP. A similar aliquot of radiolabeled cytosol protein from denervated (30.2 ug) and sham-operated (29.6 ug) solei was applied to an SDS gradient slab gel. The soluble radiolabeled 56,000-dalton protein was resolved by SDS gradient slab PAGE, sliced from the gel and prepared for tryptic proteolysis and peptide mapping in an SDS 15% polyacrylamide slab gel. An autoradiograph of the resolved ^{32}P-labeled phosphopeptides derived from the tryptic digest (0.5 ug trypsin) of the radiolabeled 56,000-dalton protein from denervated and sham-operated solei is shown. The distribution of ^{32}P-radioactivity was quantitated in consecutive gel slices of 2 mm by liquid scintillation spectrometry. The percentage of the total radioactivity recovered in the gel (cpm) repesented by each phosphopeptide is shown. Relative molecular weights of the tryptic phosphopeptides are also shown and were established relative to the electrophoretic mobilities of the known molecular weight marker proteins shown in Figure 28.

each slice by liquid scintillation spectrometry. By these procedures, it was determined that the percentage of the total ^{32}P-cpm recovered in each of the major phosphopeptides was similar when the 56,000-dalton ^{32}P-labeled phosphoprotein was derived from 66 hr denervated long nerve stump solei or the contralateral, sham-operated solei. Also, with the exception of the 50,000-dalton phosphopeptide, the phosphopeptides generated are the same as those generated from the 56,000-dalton RII protein and the 56,000-dalton protein from 24 hr denervated short nerve stump-solei and their contralateral, sham-operated muscles. Finally, the phosphorylation of the 56,000-dalton phosphoprotein and also the 39,000-dalton fragment is increased in denervated (Figure 33A) compared to the sham-operated muscle (Figure 33B).

The quantitative increase in cytosolic protein phosphorylation was determined in denervated solei (24 hr short nerve stump and 66 hr long nerve stump) relative to contralateral, sham-operated solei as described for Figure 8 and is shown as the percentage of ^{32}P-radioactivity in the denervated versus sham-operated solei (Figure 34). The quantitative increase in the phosphorylation of the radiolabeled 56,000-dalton phosphoprotein was also determined in these samples using the gel slice data from Figure 32 (for 24 hr denervated muscles) and Figure 33 (for 66 hr denervated muscles). The quantitative increase in the phosphorylation of the 39,000-dalton phosphopeptide generated from the


cytosolic protein phosphorylation
56K phosphoprotein
39K phosphopeptide
200
160
120
80
40
0
(3)
(2)
(3)
(1)
(3)
(2)
%32P—RADIOACTIVITY
DENERVATED/SHAM—OPERATED SOLEI
DENERVATION (hr) 24 66 24 66 24 66
NERVE STUMP S L S L S L
S=SHORT
L=LONG

56,000-dalton phosphoprotein was determined in the same manner. When cytosolic protein phosphorylation is increased in 24 hr denervated short nerve stump-solei compared to sham-operated solei (171.0 ± 13.4%, mean ± S.E.M. with n=3), a comparable increase is observed in the phosphorylation of the 56,000-dalton phosphoprotein resolved from these muscles (160.6 ± 10.1%, mean ± S.E.M. with n=3) and also in the 39,000-dalton tryptic phosphopeptide derived from the 56,000-dalton phosphoprotein (157.1 ± 7.3%, mean ± with n=3) (Figure 34). Additionally, when cytosolic protein phosphorylation is increased in 66 hr denervated long nerve stump-solei compared to sham-operated solei (148.9%), a comparable increase is observed in the phosphorylation of the 56,000-dalton phosphoprotein resolved from these muscles (157.7%) and also in the 39,000-dalton tryptic phosphopeptide derived from the 56,000-dalton phosphoprotein (168.9%).

IV. DISCUSSION OF RESULTS

A denervation-induced alteration of cytosolic protein phosphorylation in rat soleus muscle was found to be directly related to the denervation period and the length of the distal nerve stump. The phosphorylative changes observed in denervated solei were reported relative to contralateral, sham-operated solei. When the branch of the sciatic nerve innervating the soleus muscle was cut leaving a distal, short nerve stump of 2 mm or less, a significant increase in the phosphorylation of soluble cytosolic protein was first observed as early as 3 hr after denervation. The maximal increase of cytosolic protein phosphorylation in this short nerve stump-muscle occurred at 24 hr and a significant increase was still observed at 30 and 36 hr after denervation. A significant increase in cytosolic protein phosphorylation was not observed, however, at the early denervation periods (i.e., 3, 12 or 24 hr) when an intermediate (17 to 20 mm) or long (32 to 35 mm) nerve stump was left attached to the muscle. Additionally, a significant increase in the phosphorylation of soluble cytosolic protein was not observed until 30 hr after denervation when a distal, intermediate nerve stump was left attached to the soleus muscle and 48 hr after denervation when a distal, long nerve stump stump was left attached to the muscle. A significant increase in the phosphorylation of cytosolic protein in the "double denervation" group (short nerve stump-solei dener-

vated for 24 hr relative to contralateral, solei denervated for 24 hr with a distal, long nerve stump) was also found. All these denervation period and nerve stump length-dependent changes suggest that the neuroregulation of cytosolic protein phosphorylation is related to a trophic influence of the motoneuron and is not simply a consequence of muscle disuse. Additionally, the "double denervation" results suggest that cytosolic protein phosphorylation is not related to an alteration in the supply of blood-borne humoral factors due to a denervation-induced change in blood flow.

Additionally, when a significant loss in muscle weight was observed in long nerve stump solei at 66 hr after denervation, the phosphorylation of soluble cytosolic protein was still significantly increased relative to contralateral, sham-operated muscles. This indicates that denervation atrophy (as evidenced by a significant decrease in muscle wet weight) also does not contribute to the alteration in protein phosphorylation within the denervation periods of 1 to 66 hr.

The delay in the times of onset and also of maximal increase in cytosolic protein phosphorylation were found to be directly related to the three lengths of distal nerve stumps left attached to denervated solei. The delay in the onset of increased cytosolic protein phosphorylation of 1.4 hr/mm distal nerve stump and the comparable delay of 1.3 hr/mm distal nerve stump based upon the denervation periods at which a maximal increase was observed indicate that the

rate of change in the phosphorylation event during the 21 hr interval from onset to peak is the same for the three nerve stump-muscles. Also, the comparable rates suggest that the three nerve stump-muscles are undergoing similar changes which lead to the appearance and development of the phosphorylative modification of soluble cytosolic protein. However, the return of cytosolic protein phosphorylation to the levels of the contralateral, sham-operated solei which was observed after long periods of denervation with short, intermediate and long nerve stump-muscles may not be related to neurotrophic effects, but rather to the many metabolic changes occurring as the muscle becomes increasingly atrophic.

The onset of biochemical and physiological changes in muscle which can be related to the length of the distal nerve stump remaining attached to the muscle have been interpreted as neurotrophic events regulated by a function of the nerve independent of the impulse-directed release of acetylcholine (reviewed in Lubinska, 1975; Grafstein and Forman, 1980). The impulse-directed release of acetylcholine at the neuromuscular junction will cease immediately in the distal nerve stump of any length as a consequence of nerve transection. Axoplasmic transport, however, has been shown to continue in isolated segments of peripheral nerve (Ochs and Ranish, 1969). The supply of axonally transported trophic substances to the neuromuscular junction <u>in vivo</u> will be limited quantitatively and temporally by the length of distal

nerve stump left attached to the muscle. Therefore, denervation period and nerve stump length-dependent alterations in cytosolic protein phosphorylation may be related to a gradual depletion in the supply of an axonally transported neurotrophic factor(s).

Elimination of the impulse-directed contractile activity of soleus muscle may also affect the utilization and supply of energy in the tissue. Speculatively, therefore, energy changes in muscle may contribute to the altered phosphorylative modification of soluble cytosolic protein observed in the denervated muscle. However, early denervation effects are apparently not energy related. A decrease in high energy phosphate has been observed in denervated muscle and is apparently related to the reduction in metabolic rate observed in denervated slow-twitch and fast-twitch skeletal muscle (Hogan et al., 1965; Kauffman and Albuquerque, 1970). These changes may be related to the reported denervation-induced decrease in the activities of key metabolic enzymes such as glycogen phosphorylase and glycogen synthetase (Pichey and Smith, 1979; Moruzi and Bergamini, 1983) and the decreased levels of lactate, phosphocreatine, inorganic phosphate and ATP (Kauffman and Albuquerque, 1970). Changes in these energy-related parameters were studied in skeletal muscle denervated for two to nine days. However, alterations in these parameters reportedly do not appear until five to nine days after denervation of the

slow-twitch soleus muscle. Additionally, it is unlikely that the twofold increase in cytosolic protein phosphorylation observed in denervated solei compared to sham-operated solei is related to a change in endogenous ATPase since my results show that the conversion of ^{32}P-ATP to ^{32}P-inorganic phosphate during the 5 min incubation pericd was relatively limited (i.e., ~ 10 tc 15%).

As mitochondria play a major role in energy production and utilization in mammalian muscle cells, it seems likely that alterations in their functioning might also be observed in denervated muscles. In fact, an impairment in the first two energy coupling regions of the respiratory chain have been observed in denervated rat hindlimb muscle, but not before 28 days after denervation (Joffe et al., 1981). DuBois and Max (1983) studied the oxidation of 6-^{14}C-glucose to $^{14}CO_2$ in fast-twitch extensor digitorum longus muscles denervated for 1 to 60 days with a distal, short nerve stump. They found that the oxidation of glucose was not affected by denervation until 6 days after denervation. A decreased oxidation of specific substrates of the Krebs cycle (Kark et al., 1974; Nemeth et al., 1980) as well as a decrease in respiratory control and mitochondrial ATPase activity (Joffe et al., 1981) have also been observed in 28 day-denervated muscle, but not in 14 day-denervated muscle. The denervation-induced decrease in respiratory control and mitochondrial ATPase activity are especially interesting in that they are apparently not affected during disuse atrophy

(Max, 1972). In the disuse atrophy model the muscle is physically innervated, but mechanical contraction is experimentally prevented. This suggests that energy-related alterations, while not contributing to the early denervation effect on cytosolic protein phosphorylation, may be affected by some neural message independent of impulse-related muscle activity.

Although the nerve stump length protocol has been used to demonstrate a trophic function of the motor nerve, some investigators have argued that products of nerve terminal degeneration may contribute to the denervation-induced effects observed in muscle. Nerve stump length-related degeneration of distal nerve stumps has been reported for transected peripheral nerves (Miledi and Slater, 1970; Card, 1977; Pulliam and April, 1979a and b). It has been suggested that the products of nerve terminal degeneration may have a direct effect on muscle by exacerbating or accelerating the effects of inactivity on changes in some nerve stump length-related electrophysiological parameters such as the muscle resting membrane potential and extrajunctional acetylcholine sensitivity. Evidence for this hypothesis is supported by the findings that partial denervation of the fast-twitch extensor digitorum longus muscle of the rat with tetrodotoxin (TTX) produced denervation changes in the TTX sensitivity of both denervated fibers and adjacent innervated fibers (Cangiano and Lutzemberger, 1980).

However, a more comprehensive study suggested that degenerating nerve fibers did not affect the muscle resting membrane potential, extrajunctional acetylcholine sensitivity or TTX sensitivity of the adjacent innervated fibers (Teidt et al., 1977). These authors (Teidt et al., 1977) also suggested that the conclusions of Cangiano and Lutzemberger (1977) may have been related to the inability of these investigators to accurately record changes in the electrophysiological parameters of the innervated adjacent fibers. The role of degenerating nerve products has also been supported by the findings that silastic cuffs impregated with various substances such as nerve extract and trypsin caused the development of extrajunctional acetylcholine sensitivity when placed on the surface of the extensor digitorum longus muscle (Jones and Vrbova, 1974; Jones and Vyskocil, 1975; Vyskocil and Syrovy, 1975). A similar effect was observed when a graft of peripheral nerve or a piece of suture material was placed on the muscle surface (Jones and Vrbova, 1974). When these studies were repeated, marked histopathological abnormalities were observed on the muscle surface along with a decline in the resting membrane potential and development of extrajunctional acetylcholine sensitivity. These studies do not support the hypothesis that products of the degenerating nerve are important in the effect of denervation upon acetylcholine sensitivity and the resting membrane potential since this histopathology was not found concomitantly with these electrophysiological changes in a denervated muscle

(Bray et al., 1979; Guth and Albuquerque, 1979; Guth et al., 1980; Oh et al., 1980). Also, extracts of degenerating peripheral nerve stumps are able to sustain the development of embryonic muscle cells in culture presumably through the release of some viable trophic substance (Oh et al., 1980).

Endplate potentials and muscle contraction which are dependent upon impulse-dependent acetylcholine transmission are immediately eliminated after transection of the peripheral nerve. However, muscle fibrillation has been reported to occur at 42 and 48 hr after the sciatic nerve is sectioned 5 mm or 30 mm, respectively, from its insertion into the soleus muscle of the rat (Salafsky et al., 1968). The early onset of increased phosphorylation of cytosolic protein occurring at 3 hr and 30 hr for the short and intermediate nerve stump solei, if not at 48 hr for the long nerve stump muscle, therefore, precedes the development of fibrillatory potentials.

It has been reported recently that spontaneous transmission of acetylcholine may be the neural element involved in the denervation period and nerve stump length-dependent neurotrophic regulation of both the resting membrane potential and extrajunctional acetylcholine receptor sensitivity of muscle (Drachman et al., 1982; reviewed in section II). This conclusion was based upon the finding that alpha-bungarotoxin blockade of acetylcholine transmission (both quantal and nonquantal) and denervation have a

quantitatively similar effect on the decline in resting membrane potential and the increase in extrajunctional acetylcholine receptor number of muscle. Alpha-bungarotoxin binds specifically and irreversibly to the postsynaptic acetylcholine receptor. Blockage of impulse-dependent quantal acetylcholine release alone with TTX did not have as great an effect on changes in these muscle parameters. This result strongly supports a role for the acetylcholine receptor in the neural regulation of these parameters and implies a role for spontaneous quantal or nonquantal acetylcholine transmission. Also, since much more acetylcholine is released from the nerve terminal by nonquantal leakage than by spontaneous quantal release (Katz and Miledi, 1977), Drachman et al. (1982) also suggested that nonquantal acetylcholine release is most important in the neuroregulation of the resting membrane potential. However, whether other specific muscle properties are regulated entirely by an alteration in the spontaneous quantal or nonquantal transmission of acetylcholine has not been determined. It has been reported that the spontaneous, quantal release of acetylcholine will continue for no longer than 12 to 14 hr after denervation of solei when a short nerve stump is left attached to the muscle. These findings are based on the recording of miniature endplate potentials in vivo in these denervated muscles for up to 14 hr after nerve transection (Deshpande et al., 1976; Stanley and Drachman, 1980). Nonquantal spontaneous acetylcholine transmission is reportedly

eliminated within a few hr in a short nerve stump muscle (Stanley and Drachman, 1980). A significant increase of cytosolic protein phosphorylation in the short nerve stump-solei occurred as early as 3 hr, reached a peak at 24 hr, and remained significantly increased for another 12 hr. Whether this temporal pattern would correlate with a nerve stump-related cessation of quantal or nonquantal spontaneously released acetylcholine is not clear and would provide an interesting topic for future research.

The specific role of spontaneously released acetylcholine in the neuroregulation of skeletal muscle parameters has been a controversial issue. The controversy has evolved from apparently conflicting results for the time course of membrane depolarization (decline in resting membrane potential) and the disappearance of miniature end plate potentials (mepps) which are the product of the spontaneous quantal release of acetylcholine. Alterations in both of these electrophysiological parameters have been found to be related to the length of distal nerve stump left attached to the muscle (reviewed in section II). Deshpande et al. (1976 and 1980) reported that the decline in resting membrane potential of rat soleus muscle was related to nerve stump length and that the change in potential, which occurred within 6 hr after denervation with a distal, short (2 mm) nerve stump, preceded the disappearance of mepps measured <u>in vitro</u>. These results suggested that the denervation period and nerve stump

length dependent change in the resting membrane potential was not regulated by the spontaneous quantal release of acetylcholine and, therefore, might be regulated by an axonally transported trophic factor(s). However, Stanley and Drachman (1980) confirmed the nerve stump length effect on membrane depolarization, but reported that the change was not observed in vivo until 18 hr after denervation of rat soleus muscle with a distal, short (< 2 mm) nerve stump and followed the elimination of mepps. These results are in close agreement with the findings of Linden et al. (1983) who assessed nerve stump length-dependent changes in resting membrane potential and mepps (recorded in vivo) in rat extensor digitorum longus muscle. Although it is not clear which changes occur first, the decline in resting membrane potential, elimination of mepps and the increase in cytosolic protein phosphorylation reportedly represent the earliest changes associated with denervated muscle and each of these are related to denervation period and nerve stump length. Speculatively, therefore, these events may represent those muscle changes most closely regulated by non-impulse mediated communication between nerve and muscle.

Additionally, another mode of regulation has been suggestedfor the resting membrane potential in that it has been recently reported that the nerve stump length decline in this parameter is dependent upon protein synthesis in the target muscle cell (Komatsu et al., 1983). Earlier studies also showed that blocking RNA synthesis prevented the

denervation-dependent decline in the resting membrane potential (Grampp et al., 1972). Also of interest is the finding that newly synthesized extrajunctional acetylcholine receptors appear very early (within hours) after denervation (Olek and Robbins, 1983). However, it is not yet clear whether the neural regulation of either mepps, the resting membrane potential or cytosolic protein phosphorylation are related to the induction of acetylcholine receptor synthesis.

Relatively few biochemical parameters have been reported to be related to the length of the distal nerve stump left attached to the muscle. Those reported are listed in Table 1 (Section II). It can be seen from this Table that before changes in these events occurred a latent period of 12 to 72 hr was required. This is much longer than the 3 hr required before a significant increase in cytosolic protein phosphorylation is observed in short nerve stump-solei. To my knowledge, a denervation period and nerve stump length-dependent increase in cytosolic protein phosphorylation has not been reported by other investigators.

The requirements for the phosphorylation of cytosolic protein were determined in the cytosolic fractions of unoperated rat soleus muscle. The phosphotransferase reaction was found to be heat-labile, dependent upon Mg^{2+} and cyclic AMP, but not Ca^{2+} or cyclic GMP, and directly related to the amount of cytosolic protein which provides the endogenous source of the protein kinase enzyme (EC 2.7.1.37-

ATP:protein phosphotransferases), ATP, cyclic AMP and phosphorylatable protein substrate. Also, cytosolic protein phosphorylation in unoperated solei did not differ from cytosolic protein phosphorylation in sham-operated solei.

The results of these studies in unoperated muscle suggested that the *in vitro* phosphotransferase reaction was dependent upon endogenous cyclic AMP in the cytosol (Squinto et al., 1980). This is based upon the findings of a slight increase in cytosolic protein phosphorylation with additions of exogenous cyclic AMP in the range of 10^{-11} to 10^{-9} M, but a significant reduction in phosphorylation with micromolar additions of cyclic AMP. Other investigators have found that while low concentrations of cyclic AMP are stimulatory to some purified protein kinases, higher levels were inhibitory (Kuo and Greengard, 1970). Also, although exogenous cyclic AMP only slightly stimulates the phosphorylation of cytosolic protein, the specific inhibitor of Ashby and Walsh (1972) for cyclic AMP-dependent protein kinase markedly reduces cytosolic protein phosphorylation. However, these results were obtained with a crude commercial preparation of the inhibitor which reportedly may contain other enzymes that affect protein phosphorylation (Kantor and Brunton, 1981).

An evaluation of the substrates for the neurally regulated cytosolic phosphorylation reaction initially involved the biochemical resolution of ^{32}P-labeled soluble cytosolic proteins from rat soleus muscle by gel filtration chromatography and HPLC size exclusion chromatography.

Radiolabeled phosphate was incorporated predominantly into a minor protein or group of proteins within a molecular weight range of M_r = 58,000 to 35,000. In the denervated solei, the ^{32}P-radiolabeling of the minor protein(s) was increased approximately 2.5-fold when resolved by gel filtration chromatography and 2.0-fold when resolved by HPLC. Therefore, the increase in cytosolic protein phosphorylation during the period of maximal stimulation for the short nerve stump muscles (24 hr) was comparable to the increased ^{32}P-labeling of a minor protein or group of proteins between M_r=58,000 and 35,000.

Evaluation of the substrates of the denervation period and nerve stump length-dependent cytosolic phosphorylation reaction also involved the resolution of *in vitro* ^{32}P-labeled soluble cytosolic proteins by SDS gradient slab PAGE. A minor protein with a relative molecular weight of M_r=56,000 was immediately apparent as the predominant substrate and quantitatively contained approximately 70% of the ^{32}P-label incorporated *in vitro* into soluble cytosolic protein. Also, ~85% of the *in vitro* label was incorporated into alkali-labile phosphate probably linked, therefore, to serine or threonine residues. Mastri et al. (1982) reported the *in vitro* phosphorylation of a 54,000-dalton soluble protein resolved from the cytosolic fractions of both the slow-twitch soleus and fast-twitch extensor digitorum longus muscles by SDS slab PAGE. These authors showed that the phosphorylative

modification of this protein was increased after chronic low frequency stimulation of the fast-twitch muscle but not the slow-twitch soleus muscle. These results suggest a neural regulation of this phosphorylation. However, unlike the 56,000-dalton phosphoprotein reported here, the 54,000-dalton protein was a minor cytosolic phosphoprotein and phosphorylation of this protein was stimulated by cyclic AMP.

Neuroregulation of the phosphorylative modification of the 56,000-dalton protein was established by two criteria. First, phosphorylation of the major soluble 56,000-dalton phosphoprotein was consistently and predominantly increased in the three nerve stump length-solei relative to sham-operated solei at each of the denervation periods corresponding to a maximal increase in cytosolic protein phosphorylation. Secondly, denervation period and nerve stump length-dependent changes in both cytosolic protein phosphorylation and phosphorylation of the 56,000-dalton protein were similar during the course of development of these changes in a short nerve stump-muscle. The quantitative assessment of the denervation period and nerve stump length-dependent alteration in the phosphorylation of soluble cytosolic proteins was only possible for the 56,000-dalton protein due to the low level of counts associated with the other minor phosphoproteins. The 56,000-dalton protein was also identified as having a molecular weight of M_r=40,000 in a SDS rod PAGE system (Squinto et al., 1981). The apparent discrepancy in molecular weight of the neurally regulated

phosphoprotein was resolved by determining that the ^{32}P-labeled, rod gel slice corresponding to a protein of M_r = 40,000 was 56,000 after re-electrophoresis and improved resolution in the SDS gradient slab PAGE system.

The 56,000-dalton predominant substrate for the neurally regulated cytosolic phosphorylation reaction is probably not a subunit tightly associated with a larger protein complex. This is based upon the findings of a similar relative size of the protein when resolved under both non-denaturing (gel filtration chromatography and HPLC) and denaturing conditions of SDS gradient slab PAGE.

Several phosphorylatable cytosolic phosphoproteins have been identified in skeletal muscle and are listed in Table 11. The phosphorylative modification of these proteins has been described in section II. However, the molecular weight of the denervation pericd and nerve stump length-dependent phosphoprotein (56,000) and its localization in the soluble cytosolic fraction rule out a number of these substrates which are found associated with mitochondria, myofibrils or glycogen particles. Even if trace amounts of certain particulate phosphoproteins should be present, the molecular weights of their subunits would be either considerably lower or higher than 56,000. For example, the molecular weights of the phosphorylated light chains of myosin are less than 29,000 while the subunits of myosin light chain kinase, glycogen phosphorylase, phosphorylase

TABLE 11. PHOSPHORYLATABLE CYTOSOLIC SKELETAL MUSCLE PROTEINS

Phosphorylatable Substrate	Phosphorylation Site	Molecular Wt.
glycogen phosphorylase	4 beta subunits	95,000 each
phosphorylase kinase	alpha and beta subunits	80,000 each
glycogen synthetase	4 subunits	85,000 each
phosphofructokinase	subunit	80,000
cyclic GMP-dependent protein kinase	2 subunits	75,000 each
vimentin	?	57,000
cyclic AMP-dependent protein kinase Type II	regulatory subunit	56,000
cyclic AMP-dependent protein kinase Type I	regulatory subunit	49,000
cyclic AMP-dependent protein kinase Type I and II	catalytic subunit	40,000
RNA-binding protein of embryonic skeletal muscle	polypeptide	38,000
myosin light chain red muscle	2 of 3 types in red muscle	29,000 27,000
phosphorylase phosphatase inhibitor I		26,000
myosin light chain white muscle	1 of 4 types in white muscle	25,000
myosin light chain kinase		18,000

A listing of reported phosphorylatable cytosolic proteins from skeletal muscle, their site(s) of phosphorylation and their approximate molecular weights.

kinase, glycogen synthetase and phosphofructokinase all have molecular weights reportedly greater than 75,000 (Weller, 1979; Stull, 1980). The molecular weight of the muscle phosphoprotein vimentin (57,000) is close to that of the 56,000-dalton neurally regulated phosphoprotein from the cytosolic fraction of soleus muscle. However, vimentin, unlike the 56,000-dalton soleus phosphoprotein, can only be extracted from muscle with high concentration of nonionic detergent and salt and is solubilized with urea (Browning and Sanders, 1981).

The relative size (M_r = 56,000) and approximate isoelectric point (pI of 5.6 to 5.9 as determined by chromatofocusing) of the denervation period and nerve stump length-dependent soluble cytosolic phosphoprotein indicated that it might be the regulatory subunit of the soluble cyclic AMP-dependent protein kinase type II (RII). The relative molecular weight of RII by SDS gradient slab PAGE is also 56,000 (Table 11 and Zoller et al., 1979; Rangel-Aldao et al., 1979) and the phosphorylated RII protein has an isoelectric point between 5.3 and 5.4 determined by isoelectric focusing on polyacrylamide gels (Rangel-Aldao et al., 1979). The reported pI of phospho-RII indicated that it is slightly more acidic than the minor 56,000-dalton phosphoprotein (5.6 to 5.9). However, the slightly higher isoelectric point reported here is not surprising because with the chromatofocusing method an internal descending pH gradient is established in the chromatography column; therefore, proteins

will elute in a pH range slightly more basic than their true pI.

RII is reported to be a cyclic AMP binding protein and accounts for approximately 30% of the total cyclic AMP binding activity of skeletal muscle cytosol (Corbin et al., 1975; Hofmann et al., 1975; Taylor and Stafford, 1978). The cyclic AMP-binding property has aided in the isolation, purification and identification of the types I and II regulatory subunits of cyclic AMP-dependent protein kinase by cyclic AMP-affinity chromatography (Ramseyer et al., 1974; Dills et al., 1975; Dills et al., 1976 and 1976a; Corbin et al., 1978; Dills et al., 1979). My preliminary approach toward identification of the 56,000-dalton denervation and nerve stump length-dependent phosphoprotein as RII was based upon the biospecific interaction of the ^{32}P-labeled 56,000-dalton protein from 24 hr denervated short nerve stump-solei and contralateral, sham-operated solei with N^6-(2-aminoethyl)-cyclic AMP coupled to Sepharose 4B. The type II regulatory subunit isolated by this affinity chromatography protocol has a relative molecular weight of M_r=56,000 when resolved by SDS gradient slab PAGE (Liu et al., 1981).

The 56,000-dalton phosphoprotein was the only ^{32}P-labeled phosphoprotein bound to the cyclic AMP-affinity resin that was eluted specifically witha highconcentration of cyclic AMP. However, since only a small amount of this protein bound to the affinity resin, it cannot be

unequivocally established from this result that the 56,000-dalton phosphoprotein is a soluble cyclic AMP-binding protein in the cytosolic fraction of denervated and sham-operated solei. The low level of specific binding can be explained if most of the ^{32}P-labeled 56,000-dalton protein is already bound to endogenous cyclic AMP which would interfere with specific binding to the immobilized cyclic AMP affinity ligand.

The identification and purification of cyclic AMP-dependent protein kinase regulatory subunits (types I and II) using type specific antibodies and immunoaffinity chromatography has been employed less frequently than cyclic AMP affinity chromatography. For the type II regulatory subunit, this may be due to its poor antigenic properties (Weber et al., 1981). Antiserum raised against the type II regulatory subunit of cyclic AMP-dependent protein kinase from bovine heart (RII antigen and antiserum were commercial preparations) and coupled to CNBr-activated Sepharose 4B was used to identify the neurally regulated 56,000-dalton phosphoprotein as RII. But less than 20% of the total phosphorylated 56,000-dalton protein recovered from the cytosolic fraction of either denervated or contralateral, sham-operated solei appeared in the fraction corresponding to specifically bound RII antigen. However, the anti-RII immunoaffinity column was approximately as efficient in adsorbing (< 20% specifically bound) purified and *in vitro* phosphorylated RII antigen. The specific reason for the poor quantitative adsorption of

either the soluble cytosolic 56,000-dalton phosphoprotein or purified RII protein with the anti-RII immunoaffinity resin is not clear, but may be attributable to several factors: 1) a poor preparation of RII antigen leading to development of non-specific antibodies exhibiting a low titer; 2) poor antigenic properties of the RII protein; 3) the possibility of proteolytic activity associated with the purified antibody (personal communication from Bio-Rad Laboratories, Richmond, CA); 4) an inefficient coupling of antibody to the resin; or 5) a lack of cross-reactivity between bovine anti-RII antiserum and rat muscle RII.

The RII subunit of cyclic AMP-dependent protein kinase can be phosphorylated *in vitro* and *in vivo* by an autophosphorylation reaction in which the catalytic subunit of the soluble holoenzyme catalyzes the intramolecular transfer of the gamma phosphate of ATP to serine residue 95 (Erlichman et al., 1974; Hofmann et al., 1975; Rangel-Aldao and Rosen, 1977; Geahlen et al., 1982). An inhibitory effect of cyclic AMP is consistent with the intramolecular autophosphorylation of the RII protein because cyclic AMP dissociates the regulatory and catalytic subunits and thus prevents the intramolecular autophosphorylation reaction. This property (autophosphorylation) has been used by several investigators as a means of identifying 56,000-dalton phosphoproteins from various tissues as RII (DeLorenzo and Greengard, 1973; Maeno et al., 1974; Liu et al., 1981). Therefore, the inhibitory

effect of cyclic AMP upon the phosphorylation of the 56,000-dalton protein when added to the *in vitro* assay with either cytosolic fractions from 24 hr denervated short nerve stump-solei or contralateral, sham-operated solei can be taken as evidence for an autophosphorylation phenomenon. Also, the lack of effect upon the phosphorylation of the 56,000-dalton protein when a specific inhibitor of the free catalytic subunit of cyclic AMP-dependent protein kinase (Ashby and Walsh, 1972) was added to the assay is also consistent with an intramolecular autophosphorylation of RII.

Addition of cyclic AMP to the assay, however, markedly increased the phosphorylation of a 47,000-dalton phosphoprotein. The phosphorylation of this protein was markedly inhibited by the addition of the specific inhibitor of the free catalytic subunit of cyclic AMP-dependent protein kinase to the *in vitro* assay. Also, the phosphorylation of other minor proteins was markedly increased by cyclic AMP and reduced by the addition of the protein kinase inhibitor. The identity of this 47,000 cyclic AMP-dependent cytosolic phosphoprotein is currently unknown. It is possible, however, that this protein is the cyclic AMP-dependent protein kinase regulatory subunit type I. This regulatory subunit has a relative molecular weight of approximately 47,000 to 49,000 when resolved by SDS slab PAGE (Hofmann et al., 1975) and can be phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase in an intermolecular autophosphorylation reaction (Huang et al., 1983).

Other subunits of cyclic nucleotide-dependent protein kinases are also involved in autophosphorylation reactions and are dissociated from the holoenzyme upon binding cyclic AMP or cyclic GMP. However, it is not likely that they represent the 56,000-dalton substrate of the neurally regulated phosphotransferase reaction due to the dissimilarity in their molecular weights and their phosphorylation properties. The autophosphorylatable catalytic subunits of cyclic AMP-dependent protein kinases type I and II have molecular weights of approximately 40,000 (Sharma, 1982 and Table 11). They were also experimentally ruled out by comparing the electrophoretic mobilities of the major soluble cytosolic phosphoprotein to that of a commercial preparation of the protein kinase catalytic subunit by SDS rod PAGE. The molecular weights of the autophosphorylatable regulatory subunits of cyclic GMP-dependent protein kinases are reported to be approximately 75,000 (Geahlen and Krebs, 1980 and Table 11). Also, unlike the 56,000-dalton phosphoprotein, the phosphorylation of the cyclic GMP-dependent protein kinase regulatory subunits are not affected by cyclic AMP.

Previous studies have indicated that high concentrations of Zn^{2+} (1 to 5 mM) inhibit both protein phosphatase activity and cyclic AMP-dependent protein kinase activity (DeLorenzo and Greengard, 1973). However, these high concentrations of Zn^{2+} have little effect upon the autophos-

phorylation of RII (Maeno et al., 1974; Liu and Greengard, 1976; Liu et al., 1981). When Zn^{2+} was added to the *in vitro* phosphorylation reaction, it inhibited the phosphorylation of several minor phosphoproteins without affecting the ^{32}P-labeling of the predominant 56,000-dalton phosphoprotein. This lack of effect upon the phosphorylation of the 56,000-dalton protein in the presence of Zn^{2+} was also observed with the cytosolic fractions from 24 hr denervated short nerve stump-solei and sham-operated, contralateral solei. Also, the cyclic AMP-dependent protein kinase inhibitor did not affect the phosphorylation of the 56,000-dalton phosphoprotein. Interestingly, the increase of cytosolic protein phosphorylation in 24 hr denervated short nerve stump-solei relative to sham-operated solei evaluated *in vitro* in the presence of Zn^{+2} or cyclic AMP-dependent protein kinase inhibitor was not statistically different from the increase in the phosphorylation of the 56,000-dalton protein resolved in an aliquot of these samples by SDS gradient slab PAGE. This indicates that the denervation-induced increase in the phosphorylative modification of a 56,000-dalton soluble cytosolic protein may involve predominantly the autophosphorylation of RII.

In order to substantiate the evidence suggesting that the 56,000-dalton phosphoprotein is the autophosphorylatable RII protein, the phosphopeptide map of a purified RII (commercial preparation resolved by SDS gradient slab PAGE) was compared to the phosphopeptide map of the predominant

soluble cytosolic 56,000-dalton phosphoprotein from rat soleus muscle which was also resolved by SDS gradient slab PAGE. Similar to the purified type II regulatory subunit from bovine heart, the 56,000-dalton phosphoprotein yielded phosphopeptides of M_r = 52,000, 39,000, 31,000, 24,000, 16,500 and 12,000 after partial tryptic proteolysis. Also, both the 56,000-dalton soleus phosphoprotein and purified RII could be completely digested to phosphopeptides of 16,500 and 12,000-daltons. These phosphopeptides have also been previously described by others for a partial tryptic digest of purified RII (Rangel-Aldao et al., 1979, Rannels and Corbin, 1979; Potter and Taylor, 1979; Takio et al., 1980; Corbin and Rannels, 1981; Hemmings et al, 1982).

The 39,000-dalton tryptic phosphopeptide common to both the 56,000-dalton phosphoprotein and purified RII is the autophosphorylation peptide (Potter and Taylor, 1979; Rangel-Aldao et al., 1979; Flockhart and Corbin, 1982). Amino acid sequencing and phosphoamino acid analysis of the 39,000-dalton tryptic fragment of the 56,000-dalton RII protein, autophosphorylated <u>in vitro</u> with the free catalytic subunit of cyclic AMP-dependent protein kinase, has shown that serine 95 is the autophosphorylation site (Weber and Hilz, 1979; Takio et al., 1980).

When cytosolic protein phosphorylation was increased in a denervated muscle (24 hr short nerve stump and 66 hr long nerve stump) relative to its sham-operated, contralateral muscle, a comparable increase was observed in the phosphorylative modification of the 56,000-dalton protein resolved from the cytosolic fractions of these muscles and the 39,000-dalton phosphopeptide derived from the tryptic proteolysis of the 56,000-dalton phosphoprotein. Other lower molecular weight phosphopeptides (M_r = 16,500 and 12,000) also appeared to show a denervation-induced increased phosphorylation. However, these low molecular weight tryptic fragments were shown to be derived from the major 39,000-dalton peptide. These results rule out the possibility that the increased phosphorylation of the low molecular weight phosphopeptides (16,500 and 12,000) derived from the 39,000-dalton phosphopeptide are related to a denervation-induced phosphorylation of the 56,000-dalton protein by glycogen synthetase kinases. The phosphorylation of RII catalyzed by the glycogen synthetase kinases involving serines 44, 47, 74 and 76 has been found to occur exclusively on a 17,000-dalton phosphopeptide. This 17,000-dalton peptide, however, is not a tryptic product of the 39,000-dalton autophosphorylation peptide (Carmichael et al., 1982; Hemmings et al., 1982). Therefore, it can be concluded that the neurally regulated phosphorylation of soluble cytosolic protein occurs at the serine 95 autophosphorylation site on the 56,000-dalton RII

protein.

The neurally regulated increase in the *in vitro* autophosphorylation of RII from the denervated muscle indicates an increase in the number of available autophosphorylation sites *in vivo*. An increase in the number of RII autophosphorylation sites in the denervated muscle may be due to an increased type II holoenzyme concentration *in vivo* or an increased association of the type II cyclic AMP-dependent protein kinase subunits (R and C) *in vivo* without a change in the concentration of holoenzyme. These interpretations are consistent with an increase in the capacity of RII to incorporate ^{32}P from [gamma-^{32}P]ATP when assayed *in vitro* and, therefore, suggest a decreased autophosphorylation of RII *in vivo*. Demonstration of a neural regulation of RII autophosphorylation in denervated muscle *in vivo* might provide insight into the validity of these interpretations.

An increased level of cyclic AMP-dependent protein kinase holoenzyme in denervated muscle might be accounted for by an increase in the cyclic AMP-stimulated RII protein phosphatase activity of the denervated muscle. It has been reported that the 56,000-dalton type II regulatory subunit of cyclic AMP-dependent protein kinase is dephosphorylated by a 35,000-dalton protein phosphatase which, unlike most protein phosphatases, does not require divalent cations for the expression of enzyme activity (Chou et al., 1977). This cation-independent phosphatase catalyzes the dephosphorylation of the free RII protein which allows for the reassocia-

tion of the catalytic and regulatory subunits. This occurs due to the increased binding affinity of the dephospho-RII for the catalytic subunit relative to phospho-RII (Hofmann et al., 1975; Rangel-Aldao and Rosen, 1977). However, when the dephosphorylation of the 56,000-dalton phosphoprotein was specifically studied in 24 hr denervated short nerve stump-solei and contralateral, sham-operated solei using EDTA to terminate phosphorylation and inhibit cation-dependent phosphatase activity, very little cation-independent RII phosphatase activity was observed and differences in phosphatase activity between the two muscle samples were not apparent. Additionally, very little cation-dependent protein phosphatase activity was found in either muscle as well. Therefore, it is unlikely that the two-fold increase in RII autophosphorylation observed in denervated muscle could be accounted for by an increase of either the RII protein phosphatase or protein phosphatase activity.

An apparent increase in the state of association and inactivation of the type II cyclic AMP-dependent protein kinase holoenzyme in denervated muscle might also be explained by a decrease in the concentration of cyclic AMP in that tissue relative to sham-operated muscles. The marked increase in the phosphorylation of exogenous histone in sham-operated solei relative to 24 hr denervated short nerve stump-solei, presumably by the endogenous catalytic subunit of cyclic AMP-dependent protein kinase, suggests that the

concentration of free catalytic subunit is greater in sham-operated muscles relative to denervated muscles. Since the concentration of free catalytic subunit may be related to the concentration of cyclic AMP, these results in turn suggest that the concentration of cyclic AMP is greater in sham-operated muscles relative to denervated muscles. This would also be consistent with an increased state of association of the holoenzyme in denervated muscles. However, these results must be interpreted with caution since histone reportedly can increase the dissociation of cyclic AMP-dependent protein kinase. Also, histone can be phosphorylated by kinases other than cyclic AMP-dependent protein kinase (reviewed in Weller, 1979).

The results of combining equal aliquots of cytosolic fractions from denervated and sham-operated solei indicate that the concentration of an endogenous inhibitor of the denervation period and nerve stump length-dependent cytosolic phosphorylation reaction is greater in sham-operated muscles relative to 24 hr denervated short nerve stump-solei. Cyclic AMP dissociates the holoenzyme and inhibits the autophoshorylation reaction. If the concentration of cyclic AMP is greater in sham-operated muscles than denervated muscles, then the state of dissociation of the holoenzyme might be relatively greater in sham-operated muscles. Therefore, it seems likely that cyclic AMP is the endogenous inhibitor because the evidence presented with the "mixing" experiments is consistent with the inhibitory action of cyclic AMP upon

the autophosphorylation of 56,000-dalton RII.

It is now well established that the physiological actions of some neurotransmitters and non-steriod hormones are mediated by cyclic AMP-dependent protein kinases (Greengard, 1978). Although autophosphorylation of RII apparently alters the activity of cyclic AMP-dependent protein kinase, the physiological significance of this reaction is not known. The functional significance of phospho and dephospho forms of cyclic AMP-dependent protein kinase type II has been studied in bovine heart (Hofmann et al., 1975; Rangel-Aldao and Rosen, 1977). An alteration in the kinetics of association/dissociation of the cyclic AMP-dependent protein kinase has been observed with the phosphorylated form of RII. This alteration is reportedly due to an increase in the binding affinity of phosphorylated RII for cyclic AMP (Hofmann et al., 1975; Rangel-Aldao and Rosen, 1977). These results suggest that the increased intramolecular autophosphorylation of RII alters the conformation of this subunit. This, in turn, increases its sensitivity to subtle changes in cyclic AMP flux. However, a conformational change in the RII protein corresponding to a change in cyclic AMP binding has not been shown experimentally. Also of interest is the recent finding that steroid hormones alter the autophosphorylation of RII *in vitro* (Liu et al., 1981). Since steroids are not known to act at membrane receptors via an adenylate cyclase/cyclic AMP mechanism, the link between steroids and

cyclic AMP-mediated events involving RII is not clear. It was suggested from these studies that autophosphorylation may link steroid-induced cellular processes with cyclic AMP-mediated events.

Interestingly, an autophosphorylation has been described for several protein kinases including the catalytic subunit of cyclic AMP-dependent protein kinase (Chiu and Tao, 1978; Shoji et al., 1979), cyclic GMP-dependent protein kinase (deJonge and Rosen, 1977), phosphorylase kinase (Walsh et al., 1971), myosin light chain kinase (Wolff and Hoffman, 1980) and virus-induced transformation-related phosphotyrosine kinases (Erikson et al., 1979). A function has been proposed for the latter of these autophosphorylations because the self-catalyzed incorporation of phosphate has been shown to be related to a change in their enzyme activities.

Functions of the RII protein independent of the catalytic subunit and associated with changes in nuclear events have been described (Jungmann et al., 1974; Cho-Chung, 1979; Boynton et al., 1981; Nesterova et al., 1981). Presumably, these RII-dependent nuclear changes come about after the nuclear translocation of the protein (reviewed in Jungmann et al, 1975; Jungmann and Russell, 1977; Jungmann and Kranias, 1977; Rosenfeld and Barrieux, 1979; Sharma, 1982). However, a specific function of RII in the genome of eukaryotic cells has not been established. Interesting speculations concerning the nuclear function of RII can be made in that a recent report has shown that eukaryotic RII

and the E. coli cyclic AMP receptor protein (commonly referred to as the catabolite gene activator protein) have significant primary and secondary sequence homology (Weber et al., 1982). The authors suggested from these studies that the eukaryotic RII protein may have a nuclear function similar to the E. coli protein in activating specific gene expression. This suggestion is supported experimentally by the work of Severin and Nesterova (1981) who reported that RII functions in eukaryotic gene transcription by increasing the number of RNA polymerase binding sites on chromatin. These authors also showed that RII binds to eukaryotic chromatin with a high affinity (Kd = 10^{-10} M). This indicates a highly specific interaction between RII and eukaryotic chromatin. The high affinity binding of RII for chromatin has also been recently reported by others (Tang and Catapano, 1983).

Other recent studies have shown that RII is not translocated in transformed cells (SV40 virally-transformed 3T3 cell line) which exhibit a decreased cyclic AMP concentration relative to the normal cell (Nesterova et al., 1981). Also, addition of cyclic AMP to the system restored RII translocation. Using this transformed cell line, these authors recently reported (Nesterova et al., 1982) that addition of a cyclic AMP-RII complex results in the induced synthesis of a small protein of M_r = 15,000 (P-15 protein). Synthesis of this protein was not observed with normal 3T3 cells and cyclic AMP alone could not induce synthesis of P-15

in the virally-transformed cells. These results imply a close relationship between the induction of P-15, the nuclear translocation of the RII-cyclic AMP complex, binding of this complex to chromatin and altered gene activity. It was also reported that the phosphorylated RII protein is not translocated and does not affect *in vitro* transcription (Severin and Nesterova, 1981). Additionally, it was observed in porcine ovary (Hunzicker-Dunn, 1982) and a mammalian tumor cell line (Cho-Chung et al., 1979) that both steroids and cyclic AMP stimulate the specific translocation of RII. Also, an increase in nuclear RII derived from the cytoplasm is related to the development of the ovary (Hunzicker-Dunn, 1982). Interestingly, both cyclic AMP and steroids (Liu et al., 1981) inhibit the autophosphorylation of RII *in vitro*.

It is not known whether the non-impulse related neural regulation of RII autophosphorylation in rat soleus muscle is related to the alterations in nuclear events (i.e., phosphorylation of nuclear proteins and increased activity of RNA polymerases I and II) which also occur early after denervation of the rat soleus muscle, but not immediately after nerve transection (Held, 1978; Held, 1983). However, before these relationships can be established, the temporal changes in the nuclear events must be re-examined with particular attention to denervation period and nerve stump length-dependency. Whether the neurotrophic regulation of RII autophosphorylation in rat soleus muscle is also related to a change in the nuclear translocation of the

protein and an alteration in gene activity is not known and would provide a stimulating and potentially important project for future investigation.

V. SUMMARY

The non-impulse related neural regulation of the autophosphorylation of RII has been established. I have found that the *in vitro* phosphorylation of soluble cytosolic protein in the slow-twitch soleus muscle of the rat is heat-labile, dependent upon Mg^{2+} and cyclic AMP, but independent of Ca^{2+} or cyclic GMP. The predominant substrate for this cyclic AMP-dependent phosphorylation is a 56,000-dalton soluble cytosolic protein. The 56,000-dalton soluble cytosolic phosphoprotein has been identified as the regulatory subunit of type II cyclic AMP-dependent protein kinase (RII) based upon several criteria: 1) RII and the 56,000-dalton protein from soleus muscle have a similar molecular weight as determined by SDS gradient slab PAGE; 2) RII and the 56,000-dalton protein have a similar isoelectric point; 3) RII and the 56,000-dalton protein are cyclic AMP-binding proteins; 4) RII and the 56,000-dalton protein are able to tightly bind to anti-RII immunoaffinity resin; 5) based on the effects observed with Zn^{2+}, cyclic AMP and the specific inhibitor of the free catalytic subunit of cyclic AMP-dependent protein kinase, RII and the 56,000-dalton protein are autophosphorylatable when associated with the catalytic subunit of protein kinase; 6) RII and the 56,000-dalton protein yield a similar tryptic phosphopeptide map, including the 39,000-dalton autophosphorylation peptide.

A nerve stump length paradigm was employed to demonstrate that the autophosphorylation of the 56,000-dalton

RII protein from rat soleus muscle is regulated by non-impulse related neural influences. The *in vitro* phosphorylation of the 56,000-dalton RII protein is directly correlated with the denervation period and the distal nerve stump length. Also, the denervation-induced increase in cytosolic protein phosphorylation observed with 24 hr denervated short nerve stump-solei and 66 hr denervated long nerve stump-solei is quantitatively comparable to the increase in phosphorylation of the 56,000-dalton phosphoprotein resolved from these muscles and in the increased autophosphorylation of the 39,000-dalton phosphopeptide generated from the tryptic proteolysis of the 56,000-dalton protein. Based upon these findings, I suggest that some neurotrophic message(s) communicates with skeletal muscle through the autophosphorylation of the regulatory subunit of the type II cyclic AMP-dependent protein kinase. The denervation period and nerve stump length-dependent alteration of the autophosphorylation of the 56,000-dalton RII protein also suggests that the phosphorylative modification of this protein may be related to a depletion of axonally transported trophic factors. Other non-impulse related neural factors, however, have not been ruled out experimentally. These results, therefore, provide new insight into the neurotrophic communication between motor nerve and adult skeletal muscle by identifying a physiologically significant reaction which is the earliest reported biochemical change directly related to

denervation period and nerve stump length.

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VII. APPENDIX

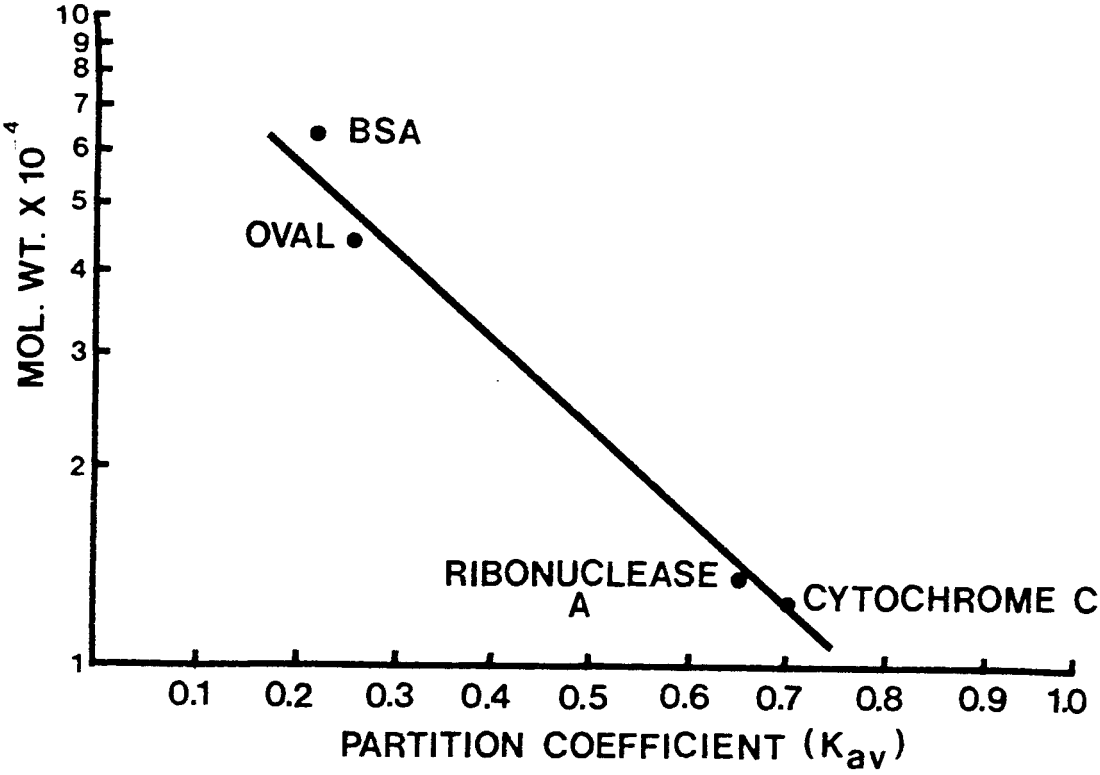


Figure 35. Calibration curve for the estimation of relative molecular weights of ^{32}P-radiolabeled soluble phosphoproteins in Figure 10 resolved from the cytosolic fractions of denervated and sham-operated rat solei by Sephadex gel filtration chromatography. The protein standards of known molecular weight (i.e., bovine serum albumin (BSA)-66,000; ovalbumin (OVAL)-45,000; ribonuclease A-13,700 and cytochrome c-12,270) were obtained from Pharmacia Chemical Co., Piscataway, NJ. Standard proteins were prepared as solutions of 1 mg protein/ ml of buffer (50 mM Tris HCl, pH 7.5, 0.1 M NaCl and 2 mM $MgCl_2$). The individual solutions were pooled and an aliquot of the combined solution containing 500 ug of protein was applied to the gel filtration column. Partition coefficients were determined according to the equation given in Method J and are shown as the values obtained from a single chromatographic separation.

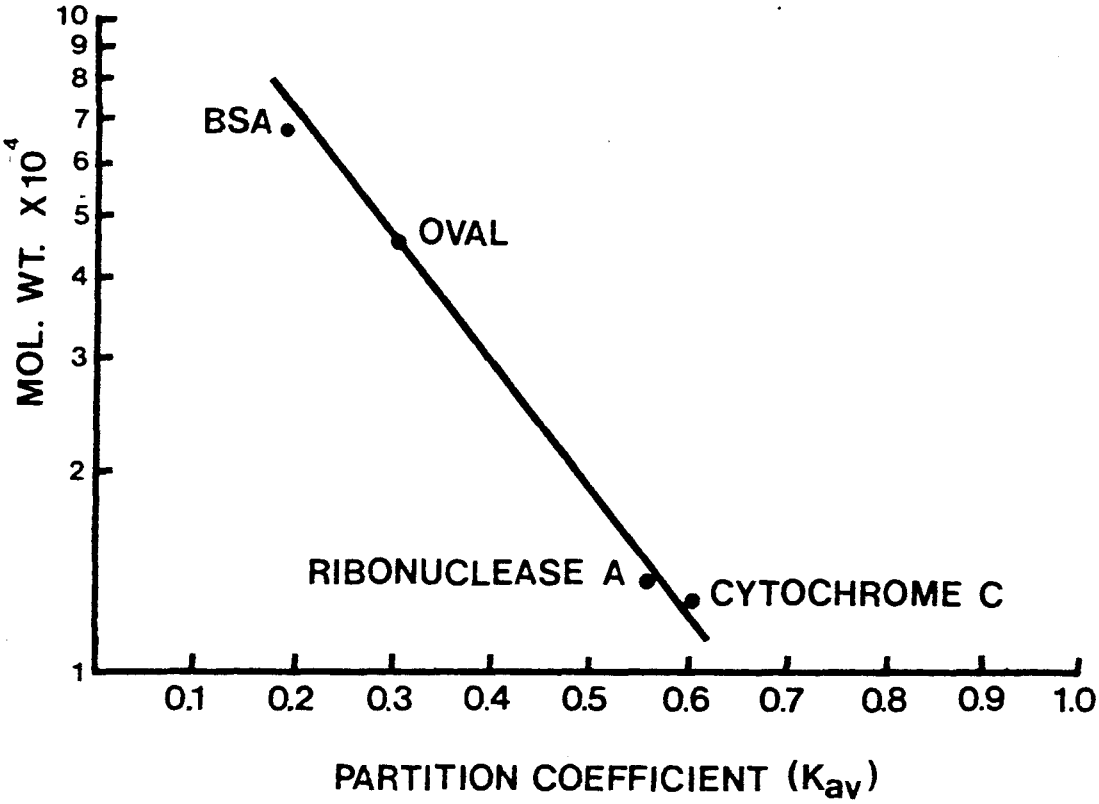


Figure 36. Calibration curve for the estimation of relative molecular weights of ^{32}P-radiolabeled soluble phosphoproteins (Figure 11) resolved from the cytosolic fractions of denervated and sham-operated solei by HPLC. The protein standards of known molecular weight (i.e. bovine serum albumin (BSA)-66,000; ovalbumin (OVAL)-45,000; ribonuclease A-13,700 and cytochrome c-12,270) were obtained from Pharmacia Chemical Co., Piscataway, NJ. The molecular weight marker proteins were prepared as solutions of 1 mg protein/ml of buffer (0.05 M sodium phosphate, pH 7.4). The solutions were pooled and an aliquot of the combined solution containing 100 ug of each protein was injected onto the HPLC column. Partition coefficients were determined according to the equation given in Method J and are shown as the means of two HPLC determinations.

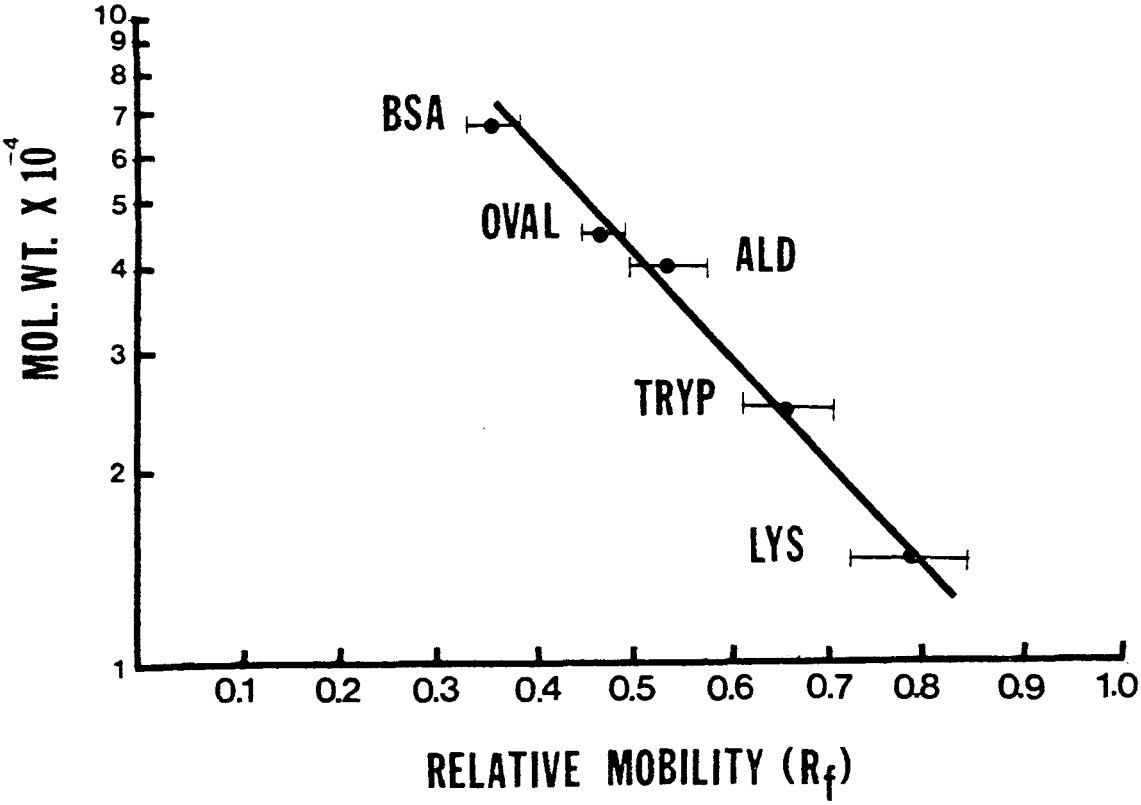


Figure 37. Calibration curve for the estimation of the relative molecular weights of ^{32}P-labeled soluble cytosolic proteins phosphorylated in the presence of the protein kinase modulators, cyclic AMP, Zn^{2+} and protein kinase inhibitor. The protein standards of known molecular weight (i.e., bovine serum albumin (BSA)-66,000; ovalbumin (OVAL)-45,000; aldolase (ALD)-40,000; trypsinogen (TRYP)-24,000 and lysozyme (LYS)-14,300) were purchased from commercial sources (described in the legend of Figure 16). Standard proteins were prepared as solutions of 1 mg protein/ml of buffer (0.0625 M Tris-HCl, pH 6.8, 0.1% SDS and 5% 2-mercaptoethanol). The protein solutions were boiled for 2 min prior to electrophoresis to denature the proteins. Ten micrograms of each protein was applied to the slab gel. Relative mobilities were calculated using the equation given in Method K and are shown as the means ± S.E.M. for 5 electrophoretic separations.

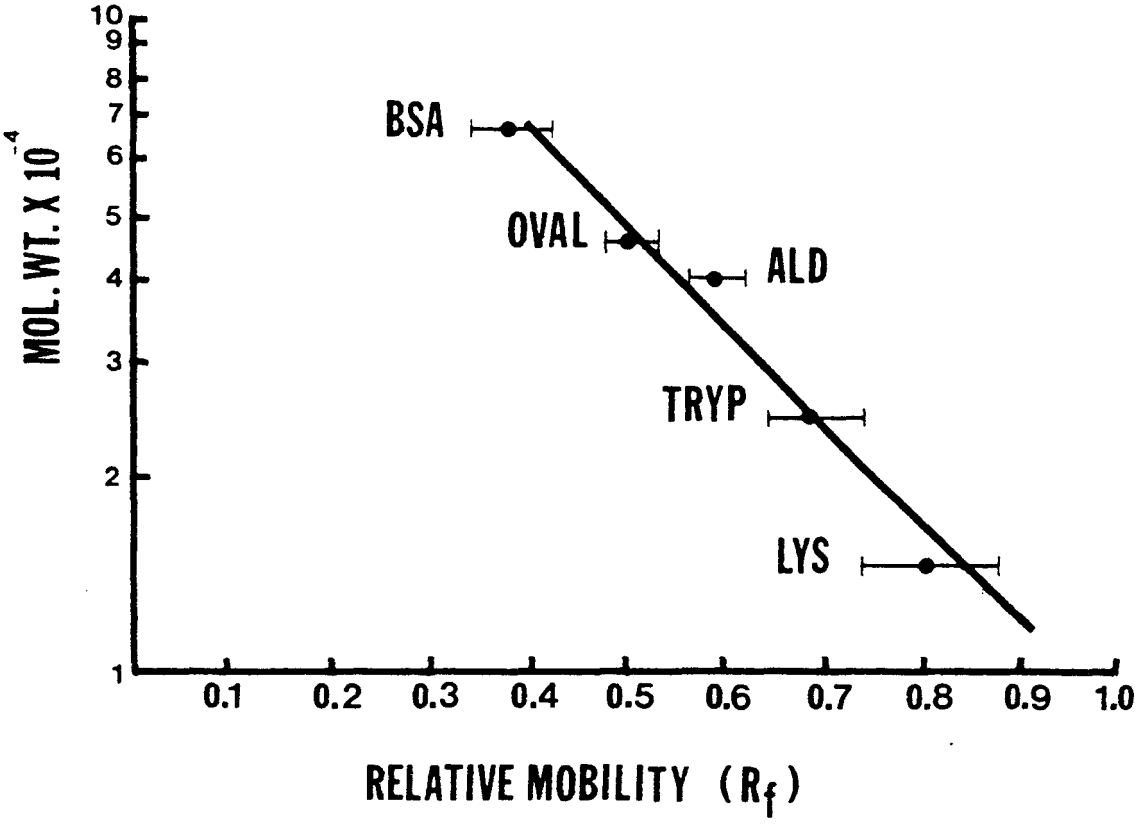


Figure 38. Calibration curve for the estimation of relative molecular weights of ^{32}P-labeled soluble cytosolic proteins phosphorylated in the presence of the specific modulators, Ca^{2+} and cyclic GMP. The protein standards of known molecular weight (i.e. bovine serum albumin (BSA)-66,000; ovalbumin (OVAL)-45,000; aldolase (ALD)-40,000; trypsinogen (TRYP)-24,000 and lysozyme (LYS)-14,300) were obtained from commercial sources (described in the legend of Figure 16). Standard proteins were prepared as solutions of 1 mg protein/ml buffer (0.0625 M Tris-HCL, pH 6.8, 0.1% SDS and 5% 2-mercaptoethanol). Proteins were boiled for 2 min prior to electrophoresis to denature the proteins and 10 ug of each protein were applied to the gel. Relative mobilities were calculated according to the equation given in Method K and are shown as the means ± S.E.M. for 3 electrophoretic separations.

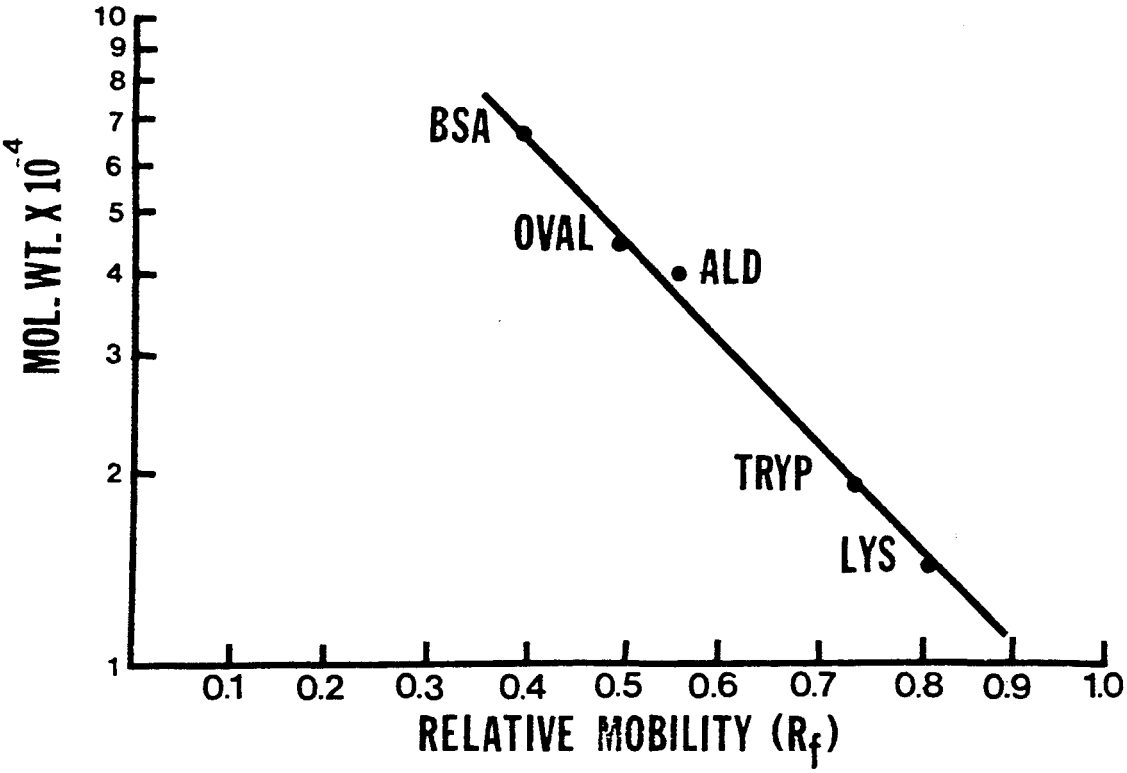


Figure 39. Calibration curve for the estimation of the relative molecular weight of ^{32}P-labeled phosphoproteins shown in Figures 23 and 24 resolved by SDS gradient slab PAGE. The protein standards of known molecular weight (i.e. bovine serum albumin (BSA)-66,000; ovalbumin (OVAL)-45,000; aldolase (ALD)-40,000; trypsinogen (TRYP)-24,000 and lysozyme (LYS)-14,300) were obtained from commercial sources (described in the legend of Figure 16). Molecular weight marker proteins (1 mg protein/ml of 0.0625 M Tris-HCl, pH 6.8, 0.1% SDS and 5% 2-mercaptoethanol) were boiled for 2 min to denature the protein and 10 ug of each protein were applied to the gel. Relative mobilities were calculated according to the equation given in Method K and are the values obtained from a single electrophoretic separation.

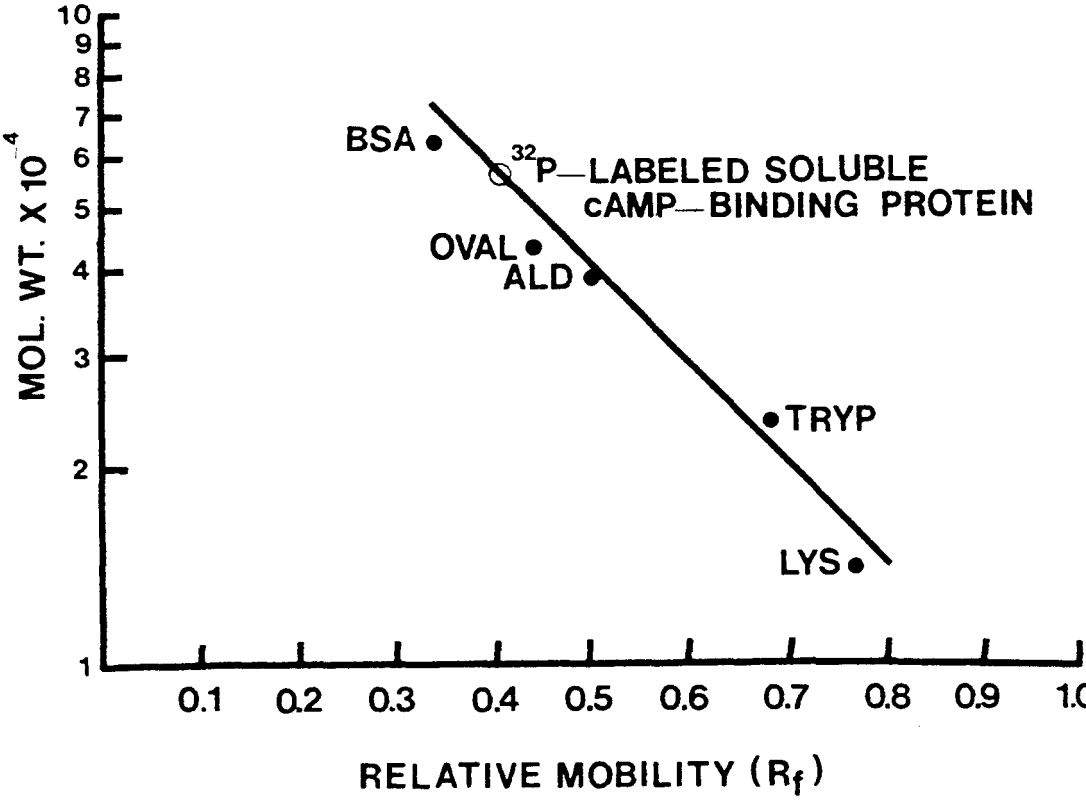


Figure 40. Calibration curve for the estimation of the relative molecular weight of the ^{32}P-labeled soluble cyclic AMP-binding protein resolved from the cytosolic fraction of denervated and sham-operated rat solei by cyclic AMP affinity chromatography and SDS gradient slab gel electrophoresis. The protein standards of known molecular weight were obtained from commercial sources (described in the legend of Figure 16). The molecular weight marker proteins were prepared as solutions of 1 mg protein/ml buffer (0.0625 were boiled for 2 min prior to electrophoresis to denature the proteins. Ten micrograms of each protein were applied to the gel. Relative mobilities were calculated according to the equation given in Method K and are the values obtained from a single electrophoretic separation.

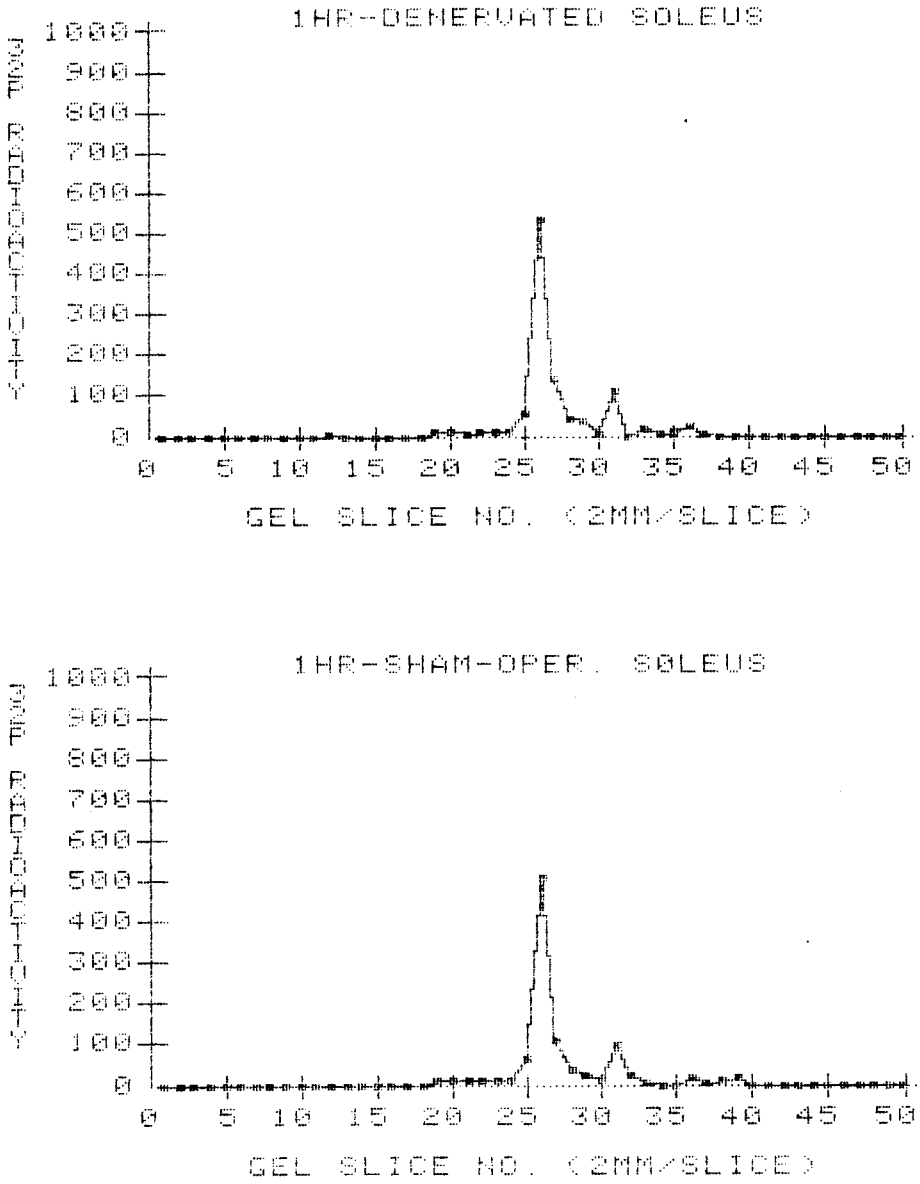


Figure 41. The distibution of ^{32}P-radioactivity associated with soluble proteins resolved from the cytosolic fractions of denervated and sham-operated rat soleus muscle by SDS gradient slab PAGE. The left soleus was denervated 1 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before insertion into the muscle. Control samoles were obtained from sham-operated, contralateral solei. Cytosolic fractions were incubaed in a phosphorylation medium containing [gamma-^{32}P]ATP and soluble cytosolic proteins were resolved by SDS gradient slab PAGE. ^{32}P-radioactivity was quantitated in consecutive 2 mm gel slices by liquid scintillation spectrometry. An equivalent amount of protein (~40 ug) from denervated (top figure) and sham-operated (bottom figure) samples was applied to individual sample wells of the gel.

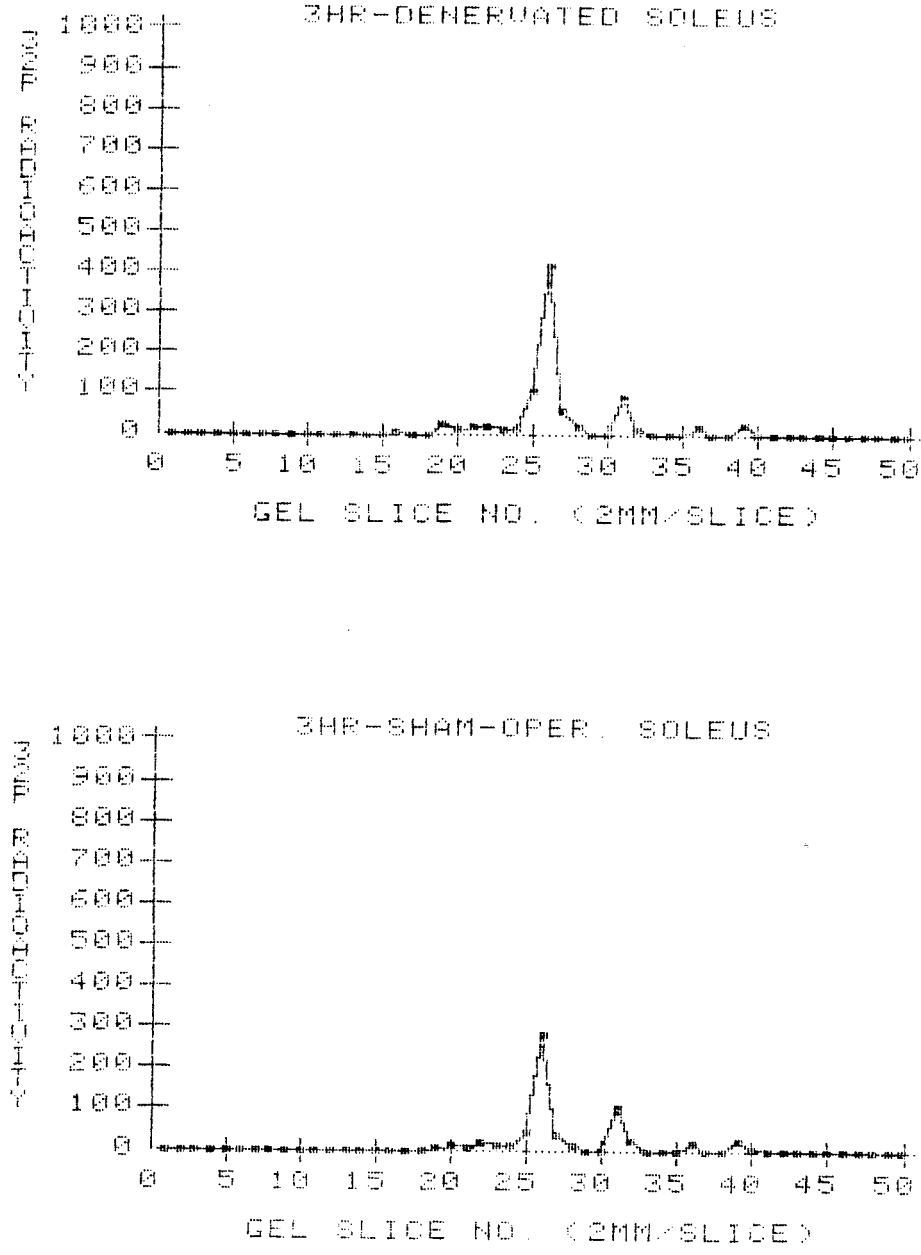


Figure 42. The distribution of ^{32}P-radioactivity associated with soluble proteins resolved from the cytosolic fractions of denervated and sham-operated rat solei by SDS gradient slab PAGE. The left soleus was denervated 3 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before insertion into the muscle. Control samples were obtained from sham-operated, contralateral solei. Cytosolic fractions were incubated in a phosphorylation medium containg [gamma-^{32}P]ATP and soluble cytosolic proteins were resolved by SDS gradient slab PAGE. ^{32}P-radioactivity was quantitated in consecutive 2 mm gel slices by liquid scintillation spectrometry. An equivalent amount of protein (~40 ug) from denervated (top figure) and sham-operated (bottom figure) samples were applied to individual sample wells of the gel.

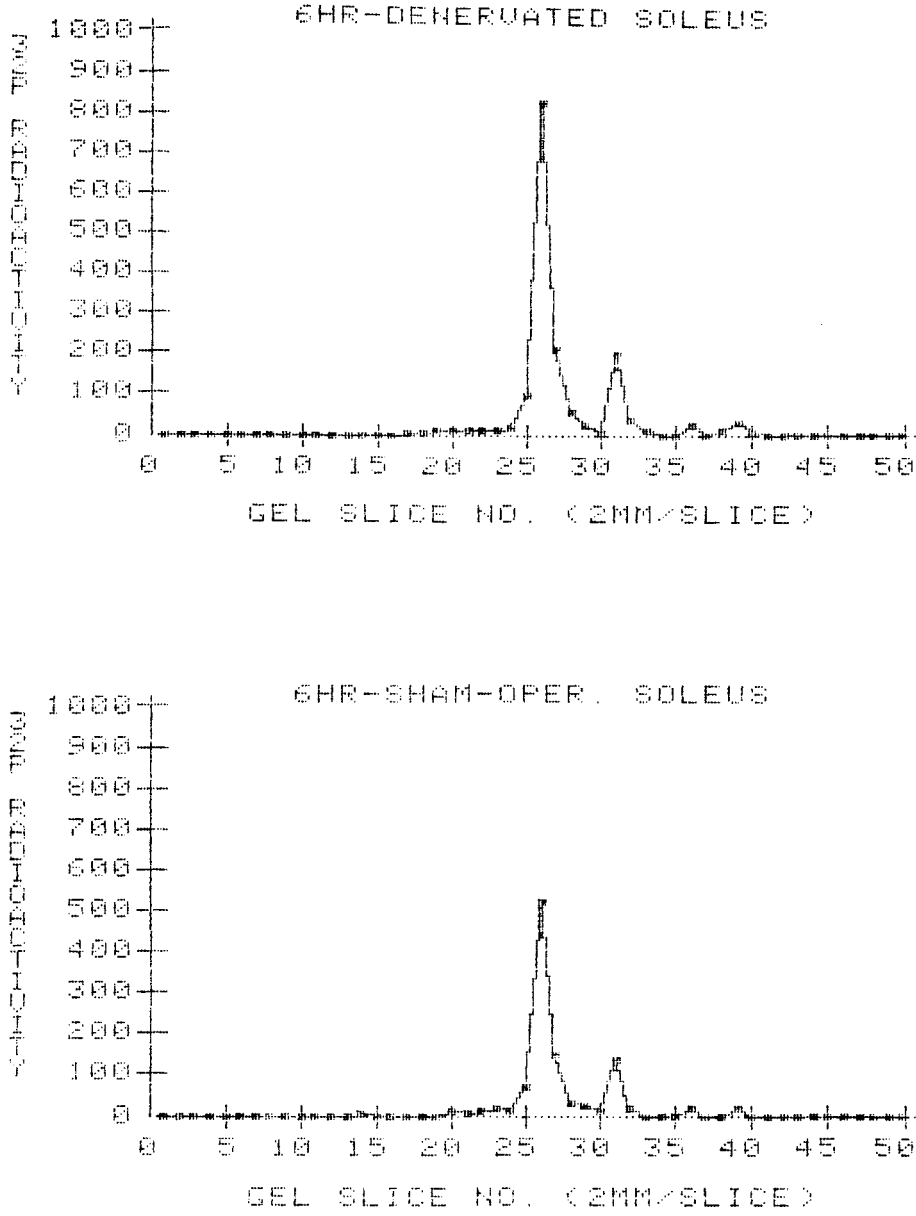


Figure 43. The distribution of ^{32}P-radioactivity associated with soluble proteins resolved from the cytosolic fractions of denervated and sham-operated rat solei by SDS gradient slab PAGE. The left soleus was denervated 6 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before its insertion into the muscle. Control samples were obtained from sham-operated, contralateral solei. Cytosolic fractions were incubated in a phosphorylation medium containing [gamma-^{32}]ATP and soluble cytosolic proteins were resolved by SDS gradient slab PAGE. ^{32}P-radioactivity was quantitated in consecutive 2 mm gel slices by liquid scintillation spectrometry. An equivalent amount of protein (~40 ug) from denervated (top figure) and sham-operated (bottom figure) samples were applied to individual sample wells of the gel.

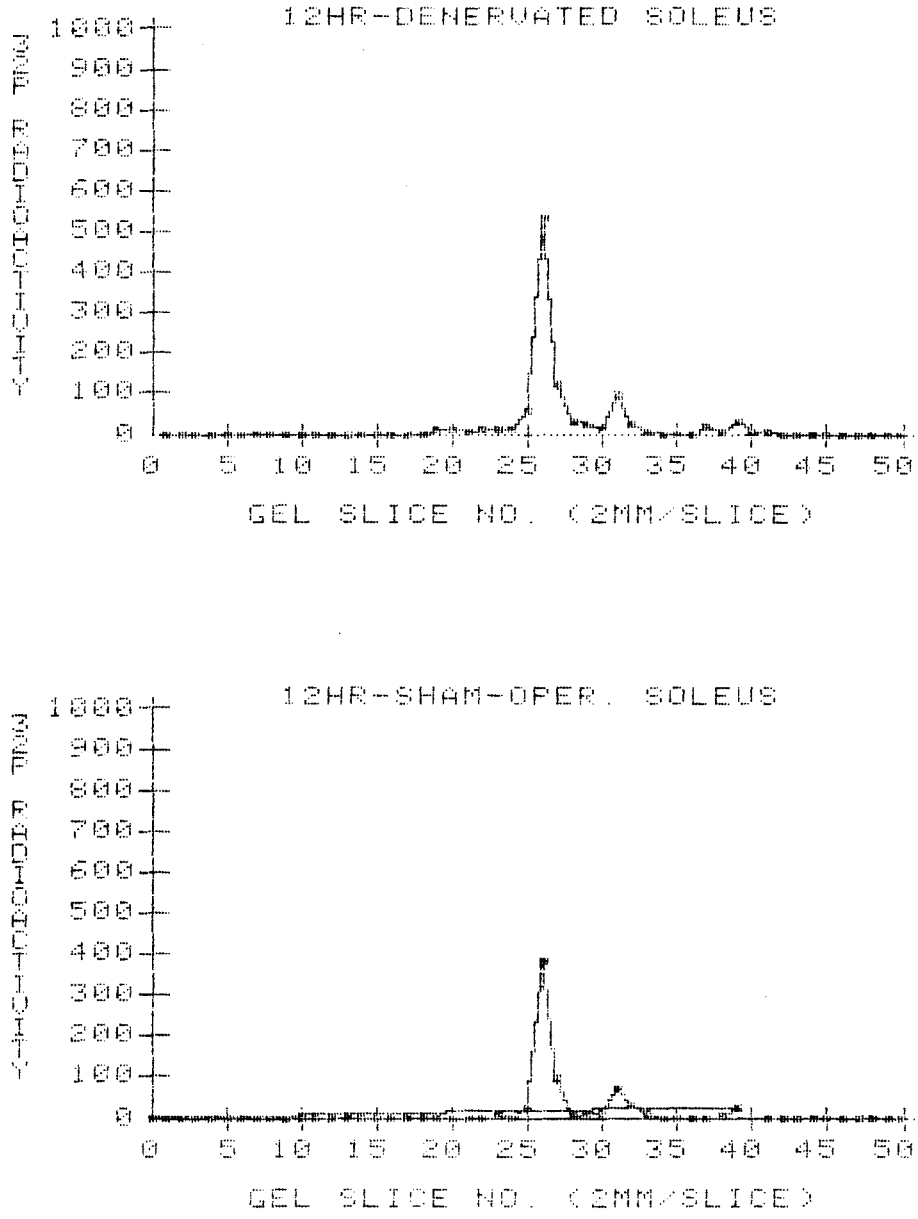


Figure 44. The distribution of ^{32}P-radioactivity associated with soluble proteins resolved from the cytosolic fractions of denervated and sham-operated rat solei by SDS gradient slab PAGE. The left soleus was denervated 12 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before insertion into the muscle. Control samples were obtained from sham-operated, contralateral solei. Cytosolic fractions were incubated in a phosphorylation medium containing [gamma-^{32}]ATP and soluble cytosolic proteins were resolved by SDS gradient slab PAGE. ^{32}P-radioactivity was quantitated in consecutive 2 mm gel slices by liquid scintillation spectrometry. An equivalent amount of protein (~40 ug) from denervated (top figure) and sham-operated (bottom figure) samples was applied to individual sample wells of the gel.

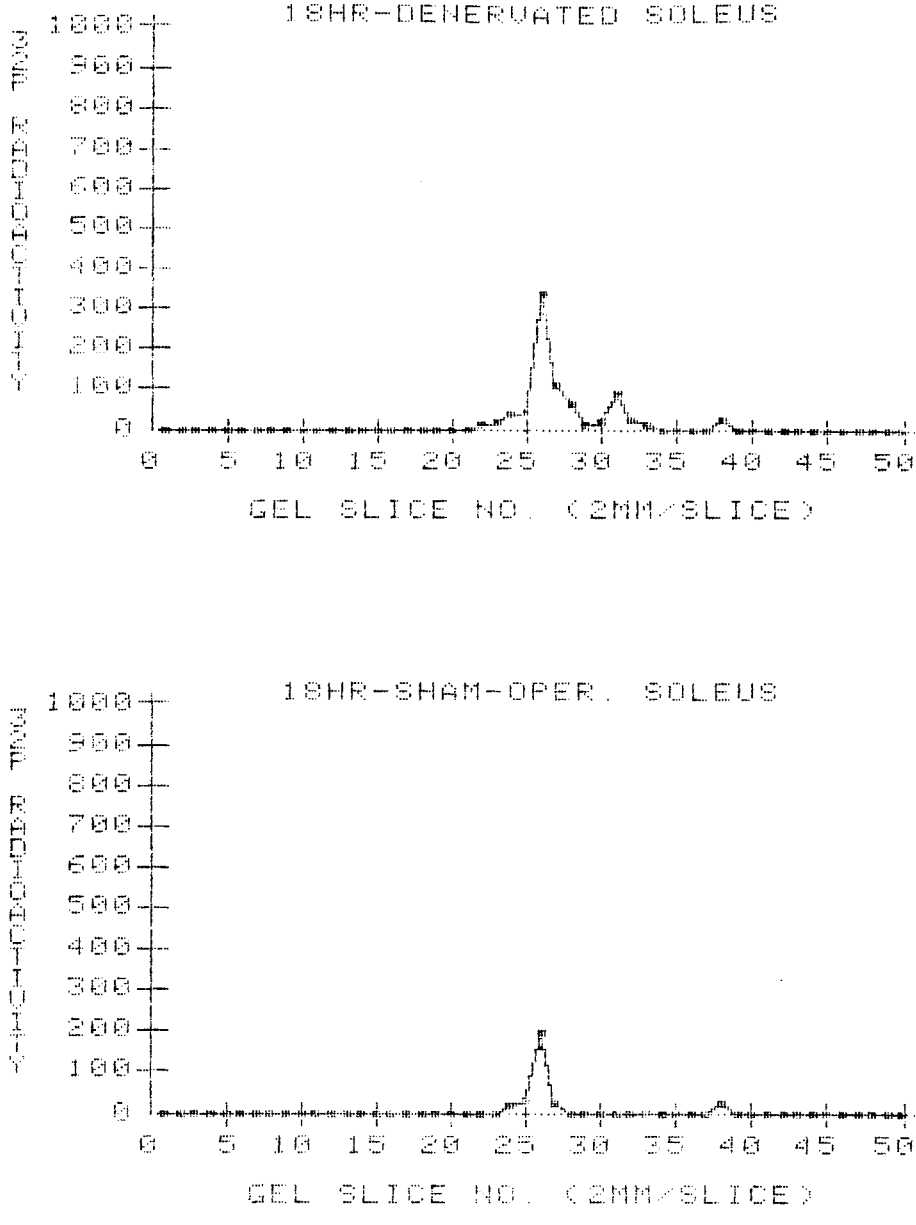
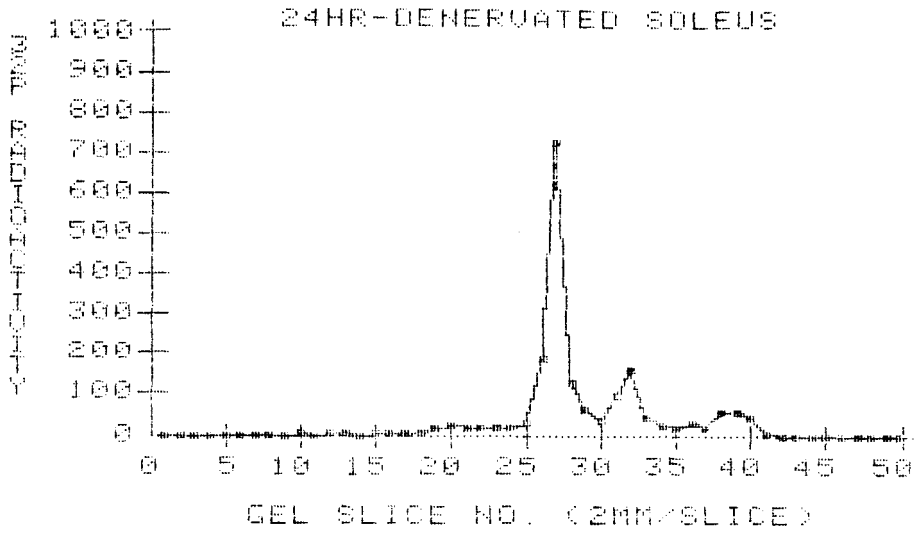


Figure 45. The distribution of ^{32}P-radioactivity associated with soluble proteins resolved from the cytosolic fractions of denervated and sham-operated rat solei by SDS gradient slab PAGE. The left soleus was denervated 18 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before insertion into the muscle. Control samples were obtained from sham-operated, contralateral solei. Cytosolic fractions were incubated in a phosphorylation medium containg [gamma-^{32}P]ATP and soluble cytosolic proteins were resolved by SDS gradient slab PAGE. ^{32}P-radioactivity was quantitated in consecutive 2 mm gel slices by liquid scintillation spectrometry. An equivalent amount of protein (~40 ug) from denervated (top figure) and sham-operated (bottom figure) samples was applied to individual sample wells of the gel.



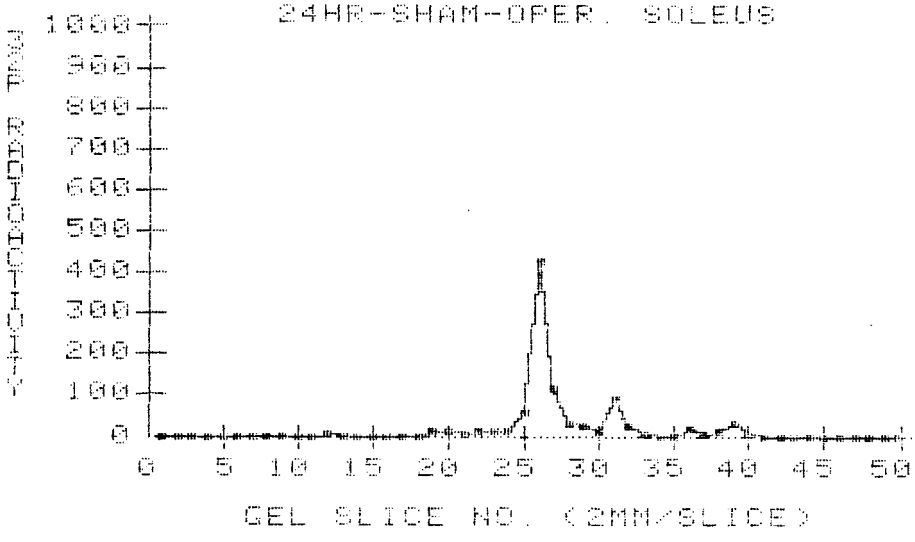


Figure 46. The distribution of ^{32}P-radioactivity associated with soluble proteins resolved from the cytosolic fractions of denervated and sham-operated rat solei by SDS gradient slab PAGE. The left soleus was denervated 24 hr prior to sacrifice by cutting the soleus branch of the sciatic nerve 2 mm before insertion into the muscle. Control samples were obtained from sham-operated, contralateral solei. Cytosolic fractions were incubated in a phosphorylation medium containing [gamma-^{32}]ATP and soluble cytosolic proteins were resolved by SDS gradient slab PAGE. ^{32}P-radioactivity was quantitated in consecutive 2 mm gel slices by liquid scintillation spectrometry. An equivalent amount of protein (~40 ug) from denervated (top figure) and sham-operated (bottom figure) samples was apllied to individual sample wells of the gel.

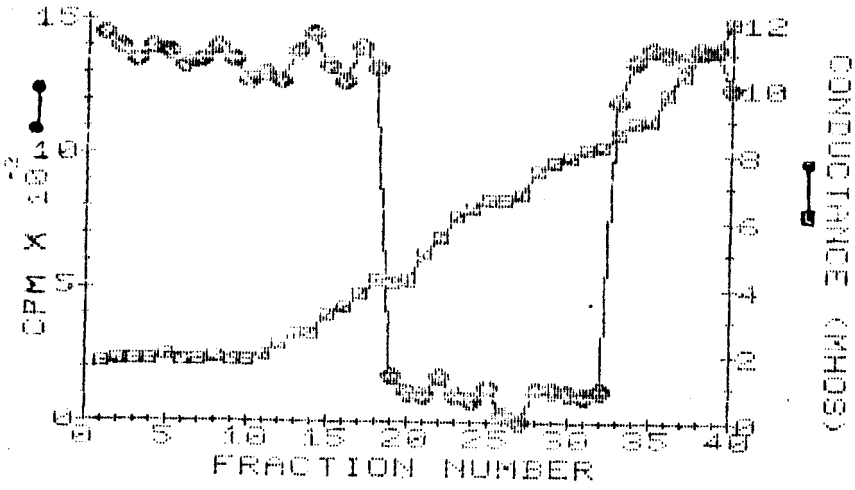


Figure 47. Elution of the partially purified inhibitor of cyclic AMP-dependent protein kinase from DEAE Sephadex. The protocol is described in detail in Method F. Conductance (●——●) is shown along with ^{32}P-radioactivity (CPM) (●——●).

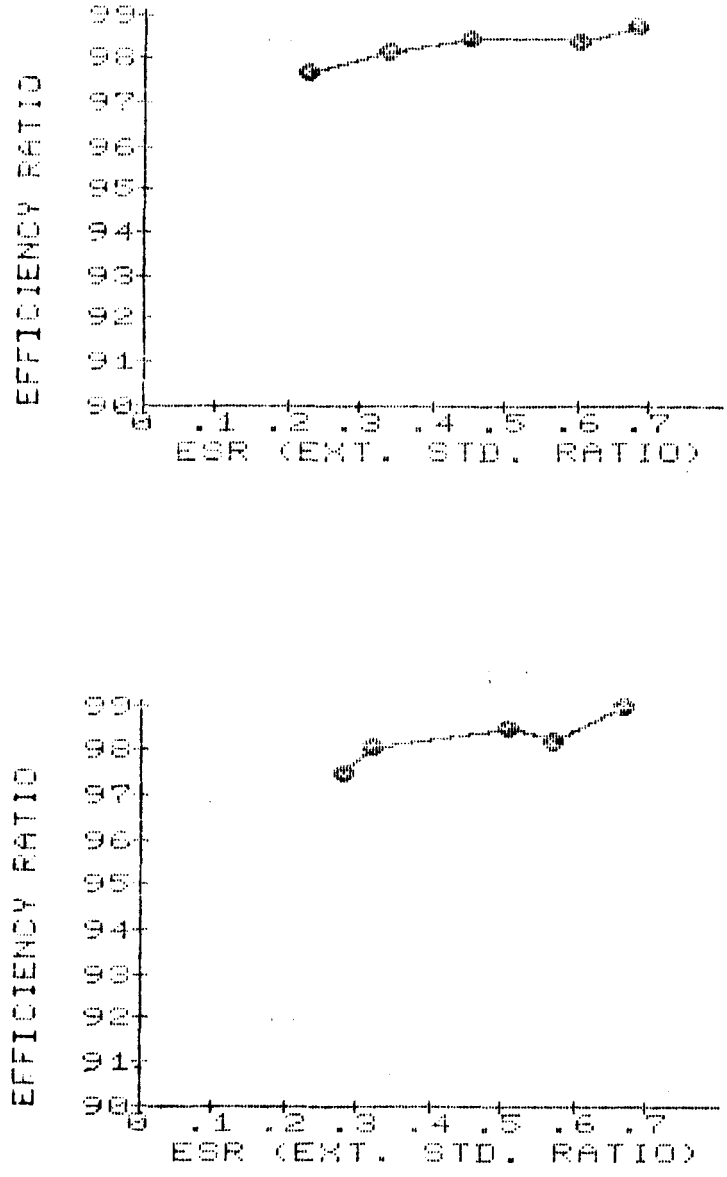


Figure 48. Counting efficiency correlation curves for ^{32}P counted with the toluene-based fluor (top figure) and the phase combing system (PCS) (bottom figure) in the presence of varying concentrations of chloroform. The protocol is described in detail in Method O.

APPROVAL SHEET

The dissertation submitted by Stephen P. Squinto has been read and approved by the following committee:

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Dr. Allen Frankfater, Ph.D., Associate Professor, Biochemistry, Loyola.

Dr. Mary Manteuffel, Ph.D., Associate Professor, Biochemistry, Loyola.

Dr. Jerry McLane, Ph.D., Assistant Professor, Pharmacology, Loyola.

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Dr. Warren Wells, Associate Professor, Molecular Biology, Northwestern University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April 4, 1984
Date

Irene R. Held
Director's Signature