A UBIQUITOUS CALCIUM BINDING PROTEIN (CALMODULIN) AND ITS POSSIBLE PHYSIOLOGICAL FUNCTION

BY

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"Science began in the distant past, long before human history was being recorded. Its mother was tribal magic. The same mother gave birth to religion and probably even earlier, to art. Thus science, religion, and art have always been blood brothers. Their methods differ, but their aim is the same: to understand and interpret the universe and its workings and, from this, to promote the material and spiritual welfare of man where possible".

Paul B. Weisz, The Science of Biology, McGraw-Hill, 1963.

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ABSTRACT

This study has centered on the following aspects of the protein modulator: (1) the phylogenetic distribution of the protein modulator, (2) a comparison of the physical-chemical properties of protein modulator from a variety of crude animal extracts,(3) comparison of the physical, Ca^{2+} binding, and chemical properties of a purified primitive protein modulator (earthworm), with the purified protein modulator from bovine heart, (4) analysis of the physiological significance of protein modulator in rabbit skeletal muscle.

An examination of crude extracts from animals representative of the major phyla has revealed that the protein modulator has a ubiquitous phylogenetic distribution. This initial observation has been extended to the vascular plants. Since the protein modulator from crude plant and animal extracts was capable of the activation of mammalian cyclic nucleotide phosphodiesterase, and because polyacrylamide gel electrophoretic analysis of the crude animal extracts has revealed a single band of protein modulator activity of similar relative mobility, it is proposed that the functional as well as the physical-chemical properties of the protein modulator have been highly conserved during evolution.

A comparison between purified protein modulator from the earthworm Lumbricus terrestris and from bovine heart has revealed many similar properties. Both proteins have molecular weights of approximately 18,000 daltons, isoelectric points of about pH 4, similar and characteristic ultraviolet spectra, and similar amino acid compositions. Both proteins bind calcium ions with high affinity. However, the protein modulator from Lumbricus terrestris binds 2 mol of Ca^{2+} ions with equal affinity K_{diss} 6 μM whereas the protein modulator from bovine heart binds 4 mol of Ca^{2+} ions with differing affinities. Tryptic peptide maps of both protein modulators show identity in six of twenty peptides therefore suggesting homology, but clearly demonstrating non identity. The similarity of the protein modulator from Lumbricus terrestris and bovine heart has further supported the postulate that the protein modulator represents an universal and highly conserved protein.

The possible physiological function of protein modulator in rabbit skeletal muscle, has been investigated. The demonstration of the regulation of phosphorylase kinase, myosin light chain kinase, and an uncharacterized glycogen synthase kinase by Ca^{2+} and the protein modulator has suggested that in rabbit skeletal muscle the protein modulator serves to regulate glycogen metabolism and myosin light chain phosphorylation.

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EXPERIMENTAL RESULTS

LIST OF ABBREVIATIONS

CD	Circular dichroism
DEAE	Diethylaminoethyl
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediamine-N, N'-tetraacetic acid
EGTA	Ethylene glycol bis(β-aminaethyl ether)-N, N'-tetraacetic acid
ELC	Essential light chains
ICBP	Intestinal calcium binding protein
MDPK	Modulator dependent protein kinase
PDE	Cyclic nucleotide phosphodiesterase
PM	Protein modulator
RLC	Regulatory light chains
SDS	Sodium dodecyl sulfate
TRIS	Tris (hydroxymethyl) aminomethane
TN-C	Troponin-C
TN-I	Troponin-I
TN-T	Troponin-T

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INTRODUCTION

Ca^{2+} has been implicated in a great diversity of cellular functions including stimulus-secretion coupling, glycogenolysis, gluconeogenesis in liver and kidney cells, and stimulus-contraction coupling in all forms of muscle. Based on these observations Ca^{2+} has been proposed to be a universal second messenger and suggested not only to function within a variety of tissues within a single organism, but also in many different organisms (Rasmussen et al, 1972).

Since the principal if not exclusive mediators of the intracellular actions of the second messengers c-AMP and c-GMP have been identified as protein kinases much attention has been directed toward the elucidation of the intracellular mediators of the actions of Ca^{2+}. One mediator has recently been identified as the protein modulator, and the mechanism by which enzyme activity can be regulated by the protein modulator and Ca^{2+}, is as follows (Wang et al, 1975).

$$Ca^{2+} + PM \rightleftharpoons Ca^{2+} - PM \rightleftharpoons Ca^{2+} - PM^*$$

$$PM^* - Ca^{2+} + E \rightleftharpoons E - PM^* - Ca^{2+} \rightleftharpoons E^+ - PM^* - Ca^{2+}$$

where the symbols denote protein modulator (PM), regulated enzyme (E), and conformational changes (* +).

The model proposes a central role for the protein modulator as a Ca^{2+} receptor. In the absence of Ca^{2+} the protein modulator is inactive, however after binding Ca^{2+} the protein modulator is capable of stoichiometric interaction with the regulated enzyme. The importance of protein modulator mediated Ca^{2+} regulation of enzyme activity is best illustrated by the impressive number of enzymes regulated by this mechanism. These enzymes include cyclic nucleotide phosphodiesterase, brain adenylate cyclase, erythrocyte (Ca^{2+} - Mg^{2+}) ATPase, myosin light chain kinase, phosphorylase kinase, and synaptosomal membrane protein kinase. While Ca^{2+} has been implicated as a second messenger in many different organisms, the possibility that the protein modulator may function as a Ca^{2+} receptor in these organisms has not been investigated.

This thesis is dedicated in part to the elucidation of the phylogenetic significance of protein mediated Ca^{2+}-linked regulation of enzyme activity, and in part to the elucidation of the physiological functions of protein modulator in rabbit skeletal muscle.

Three experimental approaches were used to investigate the phylogenetic significance of protein modulator mediated Ca^{2+} regulation. The first approach involved the examination of crude plant and animal extracts for the

presence of protein modulator. The Ca^{2+}-dependent activation of bovine heart phosphodiesterase by extracts was used to determine protein modulator activity. For the second approach the relative mobility of protein modulator activity of the extracts was examined by polyacrylamide gel electrophoresis, which provided a means of examining possible evolutionary changes in the physical-chemical properties of the extract modulator. Third, an in depth analysis of the physical, chemical and Ca^{2+}-binding properties of a purified protein modulator from an invertebrate was undertaken, and a comparison between purified protein modulator from this invertebrate and from bovine heart allowed a more detailed evaluation of any evolutionary changes of the protein modulator.

After investigation of the phylogenetic significance of protein modulator mediated Ca^{2+} regulation it was decided to investigate the physiological function of protein modulator mediated Ca^{2+} regulation in rabbit skeletal muscle. Rabbit skeletal muscle was chosen because of the well established regulatory role of Ca^{2+} in that tissue. While Ca^{2+} has been demonstrated to coordinate muscle contraction, myosin light chain phosphorylation, and glycogenolysis in this tissue, a possible role for the protein modulator has not been investigated. To this end, the possible regulation of myosin light chain phosphorylation and glycogen metabolism by Ca^{2+} and the protein modulator has been studied.

The results of this study have allowed construction of a model outlining the possible role of protein modulator in rabbit skeletal muscle.

I. ISOLATION AND CHARACTERIZATION OF THE PROTEIN MODULATOR

1. Discovery of the Protein Modulator

The discovery of the protein modulator was actually as a result of investigations into the properties of cyclic nucleotide phosphodiesterase (EC 3.1.4.17). Cheung (1967) reported that during purification of phosphodiesterase from bovine brain cerebrum a substantial loss of phosphodiesterase activity occurred. Cheung suggested as a possible explanation for the inactivation of phosphodiesterase during purification that "a stimulatory factor present with the enzyme must have been dissociated during the course of its purification". This suggestion was based on two observations, the presence of a non-dialyzable substance obtained from the brain extract capable of activating the otherwise inactive partially purified phosphodiesterase, and second, the calculation that the activity of a mixture of crude and partially purified enzyme was greater than the sum of the activities of the two enzymes assayed separately. Further evidence was provided when it was discovered that chromatography of crude phosphodiesterase on DEAE-cellulose resulted in a loss of enzyme activity which could be reconstituted by addition of fractions eluted after the peak activity of phosphodiesterase (Cheung, 1969; Cheung, 1970). The phosphodiesterase activating factor was characterized as a

protein possessing neither proteolytic nor phosphodiesterase activity. The activating factor was reportedly stable to heat, acid pH, and 8M urea. Stimulation of the purified enzyme by the activator appeared specific, as several other proteins of various molecular weights were unable to mimic its stimulatory effect. Stimulation was independent of the time of preliminary incubation with the protein activating factor, but dependent on its concentration in the reaction mixture, suggesting a stoichiometric interaction between the protein activating factor and phosphodiesterase (Cheung, 1971).

A similar heat stable non-dialyzable protein activating factor was reported in bovine heart by Goren and Rosen (1971). The bovine heart phosphodiesterase and the protein activating factor were separated by DEAE-cellulose chromatography.

Kakiuchi and coworkers showed that phosphodiesterase activity in the 100,000 x g supernatant of a rat brain homogenate was stimulated by micromolar concentrations of Ca^{2+} in the presence of Mg^{2+} (Kakiuchi and Yamazaki, 1970a). Further results suggested that the stimulatory effect of Ca^{2+} on the crude enzyme was enhanced by the addition of a non-dialyzable and thermostable factor present in the brain extract (Kakiuchi and Yamazaki, 1970b). Gel filtration of rat cerebral supernatant fluid (EGTA was not added

to buffers) resolved two peaks of phosphodiesterase activity. The high molecular weight fraction (150,000 daltons) was named Ca^{2+}/Mg^{2+}-dependent cyclic nucleotide phosphodiesterase because of the stimulatory effect of Ca^{2+} and the protein activating factor (Kakiuchi et al, 1971). The stimulation of enzyme activity by Ca^{2+} was suggested to be completely dependent on the presence of the protein activating factor (Kakiuchi et al, 1973). The effect of the activating factor on enzyme activity was observed when the concentration of Ca^{2+} in the medium was above a threshold value (2 μM).

2. Species and Tissue Distribution

The protein modulator has been demonstrated in all mammalian tissues examined, which include bovine brain (Cheung, 1970), cardiac muscle (Goren and Rosen, 1971) rat brain (Kakiuchi and Yamazaki, 1970b) rat adrenal, kidney, epididymal fat pad, bone marrow, liver, testes, thymus, human blood platelet, brain (Smoake et al, 1974) uterus (Smoake et al, 1974; Kroeger et al, 1976), rat anterior pituitary (Russel and Thorn, 1977) and porcine coronary arteries (Wells et al, 1975). Since protein modulator from human, porcine, and rat brain and bovine heart cross-activated partially purified phosphodiesterase from the same tissues, Cheung (1971) has suggested that the protein

modulator lacks species specificity. This suggestion has been confirmed and extended by Waisman et al, (1975) who have examined a number of invertebrates and higher plants for protein modulator activity; in all species examined protein modulator activity (assayed with bovine heart phosphodiesterase) was demonstrated.

The distribution of the protein modulator has been quantitated in eight different rat tissues by Smoake et al, (1974). The rat testes contained the highest specific activity. Phosphodiesterase activity did not appear to correlate with modulator activity, for example rat testes was the richest source of protein modulator but one of the poorest sources of phosphodiesterase. Waisman et al, (1975) have also suggested a non parallel distribution of protein modulator and phosphodiesterase activities. Crude extracts prepared from a number of invertebrates were found to be rich sources of protein modulator but to contain little if any Ca^{2+}-activated phosphodiesterase activity. Similar results were documented for the higher plants. The results (Waisman et al, 1975) have suggested that unlike the protein modulator, Ca^{2+}-activated phosphodiesterase may have a limited phylogenetic distribution. It therefore appears that protein modulator and phosphodiesterase activities have non parallel tissue and phylogenetic distribution.

3. Subcellular Distribution

The subcellular distribution of the protein modulator has been determined in human blood platelets and liver parenchymal cells (Smoake et al, 1974), and bovine brain cortex (Cheung et al, 1975b). The majority of the protein modulator activity was found in the cytosol although small but significant modulator activity was reported in nuclear and microsomal preparations. Drabikowski et al, (1977b) have analyzed several tissues for the subcellular distribution of protein modulator, and found protein modulator activity to be present in both the 100,000 x g supernatant and pellet of skeletal muscle (rabbit), cardiac muscle (bovine), smooth muscle (rabbit uterus and chicken gizzard), adrenal medulla (bovine), brain (bovine), and platelets (bovine). Gnegy et al (1976) have demonstrated the presence of protein modulator in the washed 105,000 x g pellet of rat brain and adrenal medulla. In the subcellular particulate fractions of rat brain the concentration of modulator was highest in the microsomal fraction followed by the mitochondrial and nuclear fractions. Gradient centrifugation of the main mitochondria subfraction revealed that the modulator was concentrated in those fractions containing mainly synaptic membranes (Gnegy et al, 1977). The presence of protein modulator has also been reported by Schulman

and Greengard, (1978) in synaptosomal preparations.

These studies suggest a partly soluble, partly particulate subcellular distribution for the protein modulator.

4. Ontogenetic Development

Smoake et al, (1974) and Strada et al (1974) have examined the ontogenetic development of protein modulator in a variety of rat tissues. The ontogenetic development was found to be characteristic of the particular tissue examined. In general, it was observed that only minor changes in protein modulator activity occurred during ontogenetic development in many of the tissues examined (rat brain, thymus, and liver). In contrast, about a three fold increase in protein modulator activity was reported in testes. In none of the tissues examined was the development of protein modulator and phosphodiesterase activity parallel. For example, the Ca^{2+}-activated phosphodiesterase activity in rat cerebrum was found to increase twenty fold, whereas the protein modulator activity remained about the same (Strada et al, 1974).

5. Physical Properties

The protein modulator has been purified to homogeneity from bovine heart (Teo et al, 1973), bovine brain (Lin et al, 1974), porcine brain (Teshima and Kakiuchi, 1974;

Wolff and Brostrom, 1974; Klee, 1977a) rat testes (Beale et al, 1977) porcine and adrenal medulla (Egrie and Siegel, 1975) blood platelets (Muszbek et al, 1977) chicken gizzard (Dabrowski et al, 1977) electroplax of Electrophorus electricus (Childers and Siegel, 1975), and the earthworm (Waisman et al,1978a). Wolff and Siegel, (1972) purified a Ca^{2+}-binding phosphoprotein from porcine brain and from bovine adrenal medulla and testes (Brooks and Siegel, 1973a, b). This protein was later identified as the protein modulator (Wolff and Brostrom, 1974).

The protein modulator from a variety of sources has been extensively characterized by several groups of researchers. In general, all modulators examined exhibit very similar physical and chemical properties. Molecular weight determination by hydrodynamic methods have suggested a range of molecular weight from 15,000 to 19,000 daltons; these discrepancies appear to depend more on the method of determination than the source of the modulator. Watterson et al, (1976) have shown that determination of modulator molecular weight by sedimentation equilibrium method in low ionic solution results in low values. Overestimation of the molecular weight of modulator on gel filtration columns has suggested anomalous behavior of the protein modulator on gel filtration columns (molecular weight estimated by this method has been determined as 28,000 daltons, (Teo et al,

1973). The molecular weight of the bovine brain protein modulator has been determined from the amino acid sequence to be 16,723 daltons (Vanaman et al, 1977).

The protein modulator is very acidic, the isoelectric point has been determined to be about 4.0. The modulator has an atypical ultraviolet spectrum. Instead of having an absorption maximum at 280 nm as do most globular proteins, considerable vibrational structure in the region of 250 - 280 nm, and absorption peaks at 253, 259, 265, 258, and 276 nm have been reported for the modulator by Wang et al, (1975), Stevens et al, (1976), Watterson et al, (1976), Liu and Cheung, (1976), Kuo and Coffee, (1976a), Klee (1977a), and Dabrowski et al, (1977a). The unique optical properties result from the presence of a high phenylalanine/tyrosine ratio (8:2) and an absence of tryptophan. Furthermore, the protein modulator has an unusually low absorptive index of about 2.0 at 275 nm (1% solution; Watterson et al, 1976; Stevens et al, 1976).

6. Amino Acid Composition

The amino acid compositions of protein modulator purified from bovine heart (Wang et al, 1975) bovine brain (Lin et al, 1974; Watterson et al, 1976), porcine brain (Wolff and Siegel, 1972; Klee, 1977a), bovine adrenal medulla (Kuo and Coffee, 1976), rat testes (Beale et al,

1977), electroplax of Electrophorus electricus (Childers and Siegel, 1975) and Lumbricus terrestris (Waisman et al, 1978a) are remarkably similar. The protein modulator has a high content of acidic residues and a relatively low content of basic residues, consistent with the observed isoelectric point of 4.0. Liu and Cheung, (1976) and Walsh and Stevens (1977) have demonstrated that more than 70% of the total number of acidic amino acids in the acid hydrolyzate are represented by glutamic and aspartic acid, less than 30% are represented by glutamine and asparagine. Cysteine is commonly reported absent, although one residue of cysteine has been reported for the earthworm (Waisman et al, 1978a) and bovine adrenal medulla (Kuo and Coffee, 1976a) protein modulator. Tryptophan is consistantly reported as absent.

The presence of one mol of unidentified ninhydrin-positive basic compound in the acid hydrolyzate of bovine brain protein modulator was first reported by Watterson et al (1976). This unusual amino acid has been identified as ε- N - trimethyllysine, and demonstrated in the hydrolysates of all species tested including bovine, porcine, rabbit, and chicken brain protein modulator (Vanaman et al, 1977; Miyake and Kakiuchi, 1977), and rat testes (Jackson et al, 1977).

7. Amino Acid Sequence

Determination of the amino terminal of the protein modulator has yielded conflicting results. The dansyl chloride procedure has been used to identify valine (Lin et al, 1974) or isoleucine (Kuo and Coffee, 1976) as the amino terminal. Watterson et al, (1976) failed to detect an amino terminal amino acid, using a sequenator and suggested that the protein modulator contained a blocked amino terminal. Vanaman et al, (1977) have sequenced the bovine brain protein modulator and determined that the amino terminus of the modulator was acetylated and consisted of Ac-met-asp-asp. The amino terminus of the rat testes protein modulator was identified by Dedman et al, (1977) as Ac-ala-asp-glu.

Sequence studies have confirmed that the protein modulator is a simple protein (Vanaman et al,1977). Earlier claims that the bovine heart modulator was a glycoprotein (Teo et al, 1973) and that the porcine brain protein modulator was a phosphoprotein may have been due to contaminants (Wang et al, 1975).

Stevens et al (1976)have demonstrated that tryptic peptide maps of bovine heart and bovine brain protein modulator are indistinguishable,and have suggested that these are the same protein. Earlier comparisons by

Brooks and Siegel (1973a, b) demonstrated that the protein modulator was essentially identical in all bovine tissues examined (brain, heart, adrenal medulla, and testes), according to gel electrophoretic comparisons. Furthermore, Brooks and Siegel (1973a) demonstrated that tryptic peptide maps of bovine brain and adrenal medulla were identical. The results suggest that within the bovine tissue the protein modulator might exist as a single protein entity.

The complete sequence of the modulator from bovine brain (Vanaman et al, 1977) and rat testes (Dedman et al, 1978) has been determined, and a comparison of these sequences has established that the two modulators are remarkably similar.

II. MECHANISM OF ACTIVATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE BY PROTEIN MODULATOR

1. Overview

The characteristics of enzyme activation by protein modulator have been studied in many laboratories, using partially purified Ca^{2+}-activatable phosphodiesterase largely free of protein modulator. One of the most important observations was that enzyme activation depends on the simultaneous presence of Ca^{2+} and the protein modulator (Teo and Wang, 1973; Kakiuchi et al, 1973; Lin et al, 1974; Wolff and Brostrom, 1974; Wickson et al,

1975) i.e. the enzyme is not activated by Ca^{2+} in the absence of protein modulator nor is the enzyme activated by protein modulator in the absence of Ca^{2+}. Two mechanisms could account for this observation; enzyme activation could be due to binding of Ca^{2+} and protein modulator to phosphodiesterase, or the binding of Ca^{2+} by the protein modulator could constitute the active complex. The demonstration that protein modulator is a Ca^{2+}-binding protein has suggested that the binding of Ca^{2+} by modulator is necessary for enzyme activation. However, the two mechanisms do not have to be mutually exclusive and a possible contribution by the former mechanism can only be evaluated by examination of phosphodiesterase for Ca^{2+} binding properties. Wang et al, (1975) have shown that no significant Ca^{2+} binding was detected (gel filtration method of Hummel and Dreyer, 1962) with an enzyme preparation approximately 20% pure, at a concentration as high as 2 mg/ml. This data support the hypothesis that Ca^{2+} activates phosphodiesterase by binding to protein modulator.

Since enzyme activation depends on the simultaneous presence of Ca^{2+} and protein modulator the apparent Ka for Ca^{2+} (concentration of Ca^{2+} required for 50% enzyme activation) depends on the amount of protein modulator

present in the reaction mixture. At saturating amounts of protein modulator an apparent Ka of 2.3 μM has been reported for the bovine heart enzyme and modulator (Teo and Wang, 1973). Other investigators using protein modulator from mammalian brain and from the earthworm have obtained similar Ka values of 2 - 8 μM (Kakiuchi et al, 1973; Lin et al, 1974; Wolff and Brostrom, 1974; Wickson et al, 1975; Waisman et al, 1978).

Mammalian phosphodiesterase depends on Mg^{2+} for activity, although this cation requirement can also be satisfied by Mn^{2+} (Butcher and Sutherland, 1962). The Ca^{2+}-activated phosphodiesterase has been shown to require millimolar concentrations of Mg^{2+} (Kakiuchi and Yamazaki, 1970a) for catalytic activity, and micromolar concentrations of Ca^{2+} for activation. Although at least one order of magnitude less potent, other ions such as Mn^{2+}, Sr^{2+}, Ba^{2+} and Co^{2+} may replace Ca^{2+} as an activating ion (Kakiuchi et al, 1972; Teo and Wang, 1973; Lin et al, 1974). Mn^{2+} is unique in that it can fulfill the metal requirements for both activity and activation.

Ca^{2+} has also been shown to be required for the association between the enzyme and protein modulator. Teshima and Kakiuchi (1974) demonstrated in a series of gel filtration experiments that an active PDE-PM complex

was formed in the presence of Ca^{2+}, but in the absence of Ca^{2+} this complex dissociates into two separate components, PDE and PM. The results suggest that the active enzyme-modulator complex is formed only in the presence of Ca^{2+}. Similar results have been reported by Lin et al, (1975) and Wickson et al, (1975).

Lin et al, (1975) suggested that the effect of Ca^{2+} on the formation of a PDE-MP complex was instantaneous. Cheung (1971) showed that enzyme activation was dependent on the modulator concentration but independent of pre-incubation of enzyme and modulator. Teo et al (1973) demonstrated that activation of phosphodiesterase by protein modulator could be decreased by dilution of the enzyme. Activation of phosphodiesterase by Ca^{2+} has been shown to be readily reversible (Lin et al, 1974; Wolff and Brostrom, 1974; Waisman et al, 1975). The modulator concentration required to activate phosphodiesterase has been demonstrated to depend on the c-AMP concentration (Wang et al, 1972; Teo et al, 1973). These results suggest a reversible interaction between the enzyme and protein modulator.

The results of studies of the interaction of protein modulator and phosphodiesterase has allowed formulation of a model of the mechanisms of activation of phosphodiesterase by Ca^{2+} and protein modulator (Teshima

and Kakiuchi, 1974; Liu et al, 1974; Wang et al, 1975). Essentially these models propose that activation of phosphodiesterase is a stepwise procedure. As a consequence of Ca^{2+} binding, the protein modulator undergoes a conformational change to an active conformation. This complex (Ca^{2+}-PM) is capable of association with phosphodiesterase to form a ternary complex (Ca^{2+}-PM-PDE). As a result of the formation of the ternary complex, phosphodiesterase undergoes a conformational change and an enhancement of enzyme activity occurs.

The individual steps of this mechanism are considered in detail in the following sections.

2. Calcium Binding Properties

Discrepancies exist in the literature regarding the number of classes of Ca^{2+} binding sites on the protein modulator and their relative affinities and capacities. Wolff and Siegel (1972) using ultrafiltration technique found that the bovine brain protein modulator bound one mol of Ca^{2+}/mol of protein with K_d 25 μM. Similarly Brooks and Siegel (1973b) found that the adrenal medulla modulator had a single site K_d 17 μM. Teo and Wang (1973), using a gel filtration technique found one high affinity site (K_d 2.9 μM) and three lower affinity sites (K_d 11.9 μM) for the bovine heart modulator. Lin et al (1974) using

equilibrium dialysis reported four Ca^{2+} binding sites, three of high affinity (K_d 3.5 μM) and a single low affinity site (K_d 18 μM) for the bovine brain protein modulator. Kuo and Coffee, (1976a) reported two Ca^{2+} binding sites of K_d 20 μM, using equilibrium dialysis. Watterson et al, (1976) reported that bovine brain modulator bound two mol of Ca^{2+} with high affinity (K_d 1 μM) and two mol with very low affinity (K_d 860 μM). Micro-scale equilibrium dialysis was used in this study. Waisman et al (1978a) using gel filtration technique reported that the earthworm modulator bound two mol of Ca^{2+} with K_d 6 μM. Dedman et al, (1977b) using equilibrium dialysis demonstrated four equivalent Ca^{2+} binding sites K_d 2.4 μM for the rat testes protein modulator. Wolff et al, (1977) has reported the existence of two classes of Ca^{2+} binding sites in the bovine brain modulator, one class bound three mol with K_d 0.2 μM and the other class bound one mol with K_d 1.0 μM. Binding studies were performed using equilibrium dialysis. Klee et al, (1977) observed two classes of Ca^{2+} binding sites, two high affinity sites (K_d 4 μM) and two low affinity sites (K_d 12 μM) using equilibrium dialysis of porcine brain protein modulator.

As pointed out by Wolff et al (1977) experimental determination of bound Ca^{2+} can be greatly affected by

trace contamination of buffers by Ca^{2+}. Variation in the concentration of Mg^{2+} in buffers from study to study could also produce confusing results. Dedman et al (1977b) have pointed out that ionic strength is an important factor when interpreting the effects of metal binding to protein modulator. It therefore stands to reason that many of the discrepancies reported in the literature could be composed of many factors including study to study variation in the concentration of Mg^{2+} and ionic strength of buffers, as well as trace contamination of Ca^{2+} in buffers, different methods of Ca^{2+} and protein concentration determination and denaturation of the protein modulator during the study.

A consensus of the reported literature suggests that the protein modulator binds a maximum of four mol Ca^{2+}/mol protein with μM dissociation constants when assayed at high ionic strength (> 40 mM). Concentrations of Mg^{2+} as high as 3 mM have no effect on this Ca^{2+} binding (in brain tissue free Mg^{2+} is believed to be about 1 mM, Veloso et al, 1973). While different classes of binding sites may be present there is no cooperativity in the binding of Ca^{2+} to protein modulator (as witnessed from the linearity of Scatchard plots). At low ionic strength cation binding properties become more complicated and competitive between Ca^{2+} and Mg^{2+} for the binding sites

may occur (Wolff et al, 1977).

3. Ca^{2+} Induced Conformational Changes

Considerable evidence has accumulated to suggest that the protein modulator undergoes changes in conformation as a consequence of Ca^{2+} binding. Wang et al, (1975) reported that upon binding Ca^{2+} the modulator undergoes changes in several of its physical properties; the ultraviolet absorption spectrum shows a small decrease in absorption and the tyrosine fluorescence emission at 315 nm exhibits a 30% enhancement in intensity. Ca^{2+} has also been shown to stabilize the protein modulator against tryptic or chymotryptic inactivation (Ho et al, 1975; Liu and Cheung, 1976) and against thermal inactivation (Wang et al, 1975).

Several investigators have examined the Ca^{2+} induced conformational changes in α helical content of the modulator. Optical rotary dispersion measurements made by Liu and Cheung (1976) have suggested that the helical content of the bovine brain modulator increases from 39% (Ca^{2+}-free media) to 57% in the presence of saturating Ca^{2+}. Based on ultraviolet circular dichroism measurements, Kuo and Coffee (1976) have suggested that the bovine adrenal medulla protein modulator undergoes a change in helical content from 20% to 40% in the presence of

saturating Ca^{2+}. Klee (1977) has estimated with far ultraviolet circular dichroism that the porcine brain modulator is composed of approximately 30 - 35% α helix, 50% random coil, and 15 - 20% pleated sheet. In the presence of Ca^{2+} a 5 - 8% increase in helical content and corresponding decrease in random coil occurs. Dedman et al (1977b) have also examined the far ultraviolet spectra of the modulator from rat testes, and calculated that the α helical content at 10^{-8} M Ca^{2+} was 45%. At 10 μM Ca^{2+} the α helical content increased to 54%. Similar measurements have been made by Wolff et al, (1977) who has determined that the α helical content of the bovine brain protein modulator changes from 28% in Ca^{2+} free media to 42% in the presence of saturating Ca^{2+}.

Kuo and Coffee (1976b) have observed an increase in the sedimentation coefficient of the adrenal medulla protein modulator, from 1.50S in the absence of Ca^{2+}, to 1.89S in the presence of Ca^{2+}. The Stokes radius determined on Sephadex G-100 was found to decrease from 21.9 ^{O}A in the absence of Ca^{2+} to 19.8 A^{O} in the presence of Ca^{2+}. From these studies Kuo and Coffee (1976b) concluded that removal of Ca^{2+} from the protein modulator results in a transformation from a compact symmetrical structure to one that is less ordered and more asymmetrical.

Wolff et al (1977) have examined the difference circular dichroism spectra of the bovine brain modulator in the presence and absence of Ca^{2+}. They reported that the difference spectra displayed maxima at 280, 269, 262, and 258 nm revealing contributions from both tyrosine and phenylalanine, and suggesting that the binding of Ca^{2+} modifies the environment of these residues. Similar results were reported by Klee (1976). Spectrophotometric titrations of the two tyrosine residues of the porcine brain protein modulator revealed that one tyrosine residue (pk'12) was buried in the hydrophobic core of the modulator and is not significantly affected by the conformational changes accompanying Ca^{2+} binding. The second tyrosine residue (pk' 10.4) is partially exposed to the solvent in the absence of Ca^{2+} and upon Ca^{2+} binding the pk' of this residue shifts to pk' 10.1 indicating increased exposure of this residue to the solvent. Changes in the environment of this residue are suspected to have resulted in the increased tyrosine fluorescence reported by Wang et al, (1975). Walsh and Stevens (1977) have reported that in the presence of Ca^{2+} the microenvironment of histidine may change. This result was based on the reactivity of the histidine residue to carboxymethylation in the presence and absence of Ca^{2+}.

In conclusion, it appears that Ca^{2+} binding causes

profound changes in the conformation of the protein modulator; α helical content is increased, the stokes radius decreases and the sedimentation coefficient increases suggesting that the protein modulator becomes a more compact symmetrical molecule. The microenvironment of one tyrosine, histidine, and one or more phenylalanine residues is altered by Ca^{2+} binding.

4. Ca^{2+} Dependent Stoichiometric Interactions of Protein Modulator and Phosphodiesterase.

The demonstration of the Ca^{2+}-binding properties of protein modulator (Teo and Wang, 1973), that both Ca^{2+} and protein modulator are required for enzyme activation (Teo and Wang, 1973; Kakiuchi et al, 1973), that phosphodiesterase does not bind Ca^{2+} (Wang et al, 1975), and that Ca^{2+} is required for the association of modulator and phosphodiesterase (Teshima and Kakiuchi, 1974) along with the reports of conformational changes in protein modulator induced by Ca^{2+} (Wang et al, 1975) has led to the suggestion that after Ca^{2+} binding the protein modulator undergoes a conformational change such that association with phosphodiesterase can subsequently occur (Wang et al, 1975).

That the Ca^{2+} binding to the protein modulator is related to enzyme activation is supported by the similarity between values reported for the apparent K_d of Ca^{2+} binding

and K_a of enzyme activation; 3 to 18 μM as compared to 2 to 8 μM (Teo and Wang, 1973; Lin et al, 1974). Klee (1977) has presented evidence that the bulk of conformational changes in the porcine brain protein modulator occur upon binding of the first two mol of Ca^{2+} (Klee reported two sites of Ca^{2+} binding of high affinity, K_d 4 μM and low affinity K_d 12 μM). Klee has suggested that the correspondence between the affinity of Ca^{2+} for the high affinity sites and the concentration required for half-maximal stimulation of phosphodiesterase (2 - 5 μM) further reinforces the idea that the high affinity sites rather than the low affinity sites are required for the formation of the ternary complex (Ca^{2+}-PM-PDE).

Dedman et al, (1977b) have examined the dependency on Ca^{2+} concentration of Ca^{2+} binding, conformational change (monitored by circular dichroism and tyrosine fluorescence) and phosphodiesterase activation. They concluded that the conformational changes induced by Ca^{2+} are required before protein modulator is capable of phosphodiesterase activation. In particular, approximately 80% of the tyrosine fluorescence enhancement and 95% of the α helical change occurred before the threshold activation of phosphodiesterase was obtained. Interestingly maximum phosphodiesterase activation corresponded to about 50%

Ca^{2+} binding (four equivalent sites in rat testes protein modulator reported by Dedman et al, 1977b).

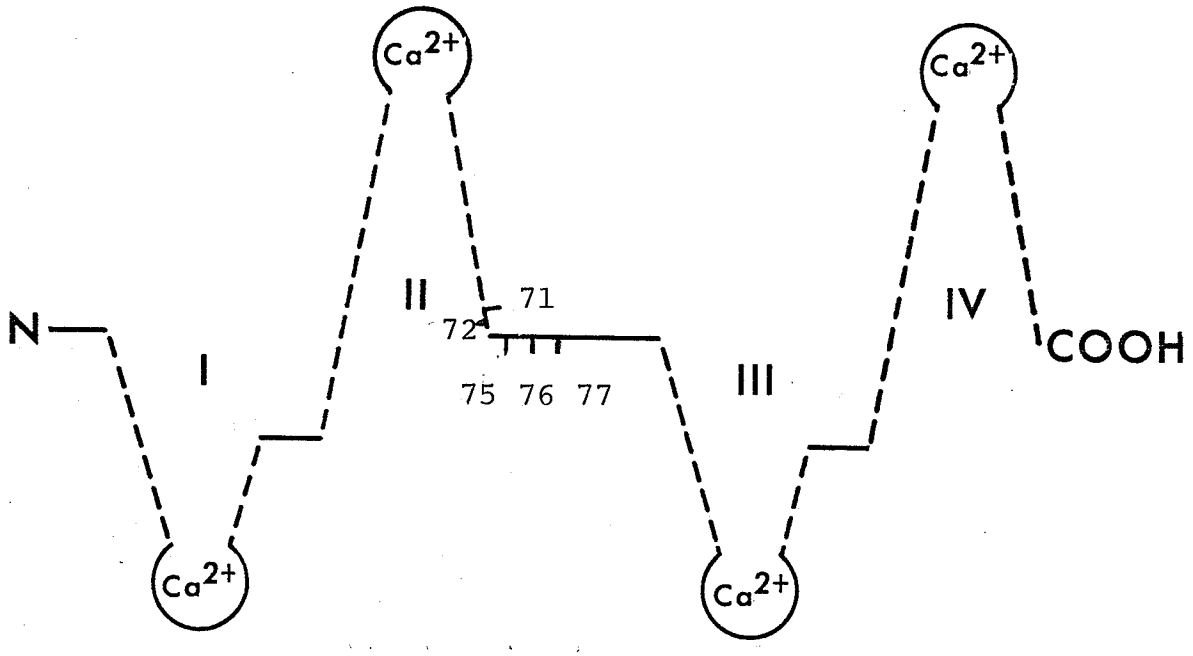
The stoichiometry of interaction between phosphodiesterase and protein modulator has been studied by Teshima and Kakiuchi (1974) and Lin et al, (1975) using gel filtration of partially purified phosphodiesterase. Values of 150,000 daltons for the free enzyme and 200,000 daltons for the enzyme modulator complex were obtained, however, the anomalous behavior of protein modulator during gel filtration has complicated interpretation of the results. Ho et al (1977) estimated a molecular weight (gel filtration) of 155,000 for the free enzyme and 230,000 for the modulator enzyme complex. The phosphodiesterase used in this study was 80% homogeneous. The results of the gel filtration experiments suggest that it is possible that more than one mol of modulator/mol enzyme may interact.

Dedman et al, (1977b) have determined the Hill coefficient of phosphodiesterase activation by increasing amounts of protein modulator is 2.0. This implies the interaction of multiple Ca^{2+}-PM complexes with a molecule of phosphodiesterase, however, impure phosphodiesterase was also used in this study. Determination of the exact stoichiometry of interaction of phosphodiesterase and protein modulator will have to await purification of phosphodiesterase.

Using the technique of chemical modification, Walsh (1978) has indicated that the site for interaction with phosphodiesterase is located between the second and third Ca^{2+}-binding regions of the protein modulator and is on the surface of the molecule. Walsh (1978) has also pointed out the importance of the integrity of both lysine and methionine residues for the expression of phosphodiesterase stimulating activity. Implicated in the interaction with phosphodiesterase was lysine 75 and 77 and methionine 71, 72, and 76. The position of these residues is illustrated in scheme I.

5. Protein Modulator Induced Conformational Changes in Phosphodiesterase.

The association between phosphodiesterase and protein modulator has been suggested to be accompanied by conformational changes in the enzyme. Wang et al, (1975) have determined that partially pure bovine heart phosphodiesterase is rapidly inactivated at 55^{o} when both Ca^{2+} and protein modulator are present, but is relatively stable in its free state. Other investigators have found similar results (Kakiuchi et al, 1975; Wolff and Brostrom, 1976; Liu and Cheung, 1976). The results suggest that the enzyme undergoes a conformational change upon association with the Ca^{2+}-PM complex. The failure of Ca^{2+} or protein



SCHEME I

DIAGRAMATIC REPRESENTATION OF THE STRUCTURE OF THE PROTEIN MODULATOR.

Dashed lines represent regions of α helix. The location of lysine 75 and 77 and methionine 71, 72, and 76 is indicated to show the possible site of interaction with phosphodiesterase (after Drabakowski et al, 1977a).

modulator alone to influence stability is in agreement with the observation that the enzyme does not interact with these ligands separately (Wang et al, 1975). That certain other activators (e.g. phospholipid) of phosphodiesterase also decrease the thermal stability of the enzyme (Wolff and Brostrom, 1976), the extent of this destabilization being identical to that produced by Ca^{2+} and protein modulator supports the view that the conformational change in phosphodiesterase induced by the Ca^{2+}-PM complex, as manifested by the change in enzyme stability is associated with enzyme activation.

Ho et al, (1977) have reported that purified phosphodiesterase can be stabilized by Ca^{2+} and protein modulator. An 80% homogeneous preparation was used in this study. The result suggest that the enzyme stability may depend on many factors.

The metal binding properties of the Ca^{2+}-PM-PDE complex have not been studied so that possible conformational changes in the Ca^{2+}-PM complex after binding to phosphodiesterase cannot be evaluated at this time.

6. Mechanism of Activation

A model has been proposed to explain the activation of cyclic nucleotide phosphodiesterase (Teshima and Kakiuchi, 1974; Wolff and Brostrom, 1974; Wang et al, 1975) and

adenylate cyclase (Brostrom et al, 1975; Cheung et al, 1975). This model is presented as follows: -

$$Ca^{2+} + PM \rightleftharpoons Ca^{2+}\text{-}PM \rightleftharpoons Ca^{2+}\text{-}PM^{*}$$

$$Ca^{2+}\text{-}PM^{*} + E \rightleftharpoons Ca^{2+}\text{-}PM^{*}\text{-}E \rightleftharpoons Ca^{2+}\text{-}PM^{*}\text{-}E^{+}$$

where the symbols denote conformational change (*, +). The stoichiometry of interaction has not been established, the equation simply depicts the necessary interaction of the two proteins. A similar model has been proposed by Dedman et al, (1977b) to compensate for the possible involvement of more than one Ca^{2+}-PM complex.

Klee, (1978) and Wallace et al (1978) have purified phosphodiesterase and suggested that phosphodiesterase consists of two subunits and that the Ca^{2+}-PM complex activates the enzyme by interaction with a specific Ca^{2+}-PM binding subunit. Recently, a heat stable inhibitor protein has been discovered and purified by Sharma et al (1978a, b) shown to undergo Ca^{2+}-dependent association with the modulator protein. This protein, unlike phosphodiesterase is monomeric. Similarly, Waisman et al (1978) have reported a monomeric structure for myosin light chain kinase. It therefore seems more likely that proteins capable of specific interactions with protein modulator contain a common structural domain rather than a common subunit

which is specific for the protein modulator.

An alternative mechanism of enzyme activation by the protein modulator has been proposed for rabbit skeletal muscle phosphorylase kinase by Cohen et al, (1978). These investigators reported the presence of protein modulator in near stoichiometric amounts with the other three subunits of phosphorylase kinase. The protein modulator appeared to be tightly associated with phosphorylase kinase and could not be removed from the enzyme by gel filtration in the absence of Ca^{2+}. Sharma and Wirch (1979) have reported the presence of tightly bound protein modulator associated with bovine lung phosphodiesterase. This tightly bound protein modulator could not be dissociated from phosphodiesterase by DEAE-cellulose or gel filtration chromatography in the absence of Ca^{2+}. Therefore, a second mechanism for the protein modulator mediated Ca^{2+}-linked regulation of enzyme activity may exist for certain enzymes; the mechanisms is presented as follows:

$$Ca^{2+} + E\text{-}MP \rightleftharpoons E\text{-}MP\text{-}Ca^{2+} \rightleftharpoons E^{*}\text{-}{}^{*}_{MP}\text{-}Ca^{2+}$$

where E and PM stand for enzyme and the modulator protein and the asterisk indicated the activated state of the protein species (after Sharma and Wirch (1979).

III. THE FAMILY OF HOMOLOGOUS Ca^{2+}-BINDING PROTEINS

Comparisons of the amino acid sequences of pike parvalbumins (Frankenne et al, 1973), rabbit skeletal muscle troponin-C (Collins et al, 1973; 1977), bovine brain protein modulator (Vanaman et al, 1977), rabbit skeletal muscle DTNB light chain (Collins, 1976), rabbit skeletal muscle alkali light chain (Frank and Weeds, 1974) and the mammalian intestinal calcium binding protein (Hofmann et al, 1977) have suggested that these proteins are homologous and may have been derived from a common ancestral protein. Together the proteins comprise a family of homologous proteins, the structural and functional interrelationships of the members of this family are discussed.

1. Parvalbumins

Parvalbumins are small (12,000 daltons), highly antigenic, acidic, Ca^{2+} binding proteins found in skeletal muscle of vertebrates. The amino acid sequence of parvalbumins from pike (Frankenne et al, 1973), carp (Coffee and Bradshaw, 1973), frog (Capony et al, 1975), rabbit (Enfield et al, 1975; Capony et al, 1976) and hake (Capony et al, 1973) has been reported. The amino acid sequence and chemical data indicate that all known parvalbumins are isostructural with that of carp (Kretsinger, 1979).

Kretzinger and Nockolds, (1973) first reported that the internal sequence repeats of parvalbumin suggested the existence of three homologous regions, and therefore parvalbumin may have evolved by gene triplication of a primitive calcium binding unit termed the "EF hand". The crystal structure of the carp parvalbumin has been determined and refined at 1.9 ^{o}A resolution (Kretsinger et al, 1971; Moews and Kretsinger, 1975). Based on X-ray crystallography work, Moews and Kretsinger, (1975) demonstrated the existence of six α-helical regions, A through F. Of the non helical loops between α-helices A and B, C and D, and E and F only the loops between C and D, and E and F bind Ca^{2+}. The Ca^{2+} ions are ligated to oxygen atoms donated by six amino acids, four of which are acidic. Each Ca^{2+} binding site lies in a pocket between two helices, and the structure is stabilized by the packing of apolar residues, present within these helices into a hydrophobic core. Helix E, loop EF, and helix F represent the basic structural and homologous domain, called the EF region (hand). The AB and CD regions are homologous to the EF region, although Kretsinger (1978) has pointed out that the AB region is barely recognized as a homolog. Each EF region contains about 33 residues. The Ca^{2+}-binding loops between the regions of α-helix are in β-antiparallel

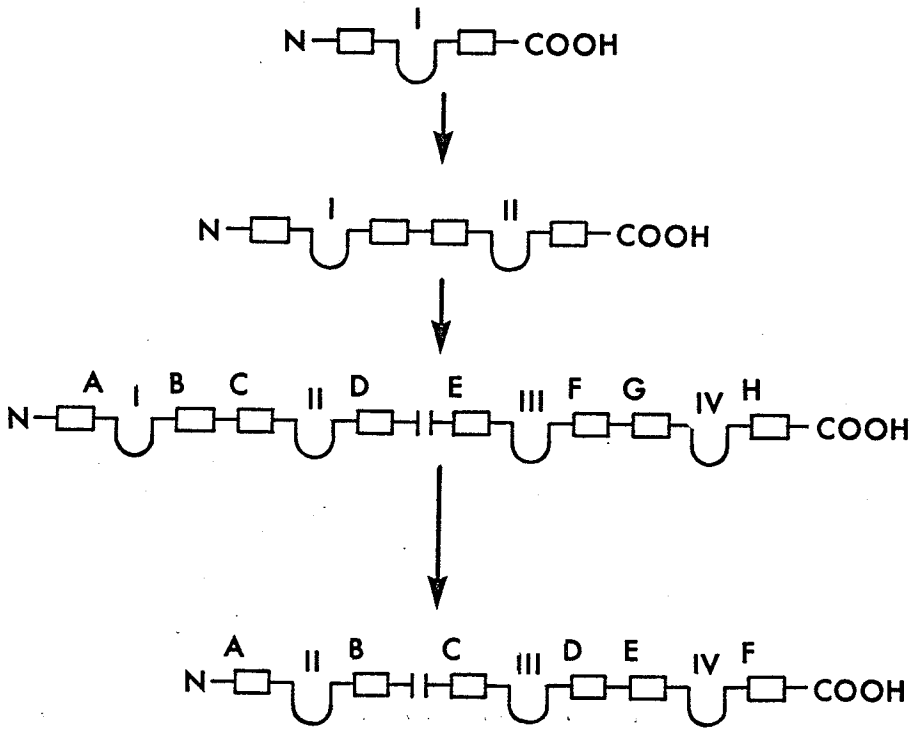
sheet conformation. The EF region is related to the CD region by an approximately two fold axis passing through the monomeric protein. The AB region lies over the EF and CD regions, does not bind Ca^{2+} but may function to cover and contribute to the hydrophobic core.

Reports in the literature of the existence of other proteins with homologous EF regions, such as troponin-C (Collins et al, 1973), alkali light chains of rabbit skeletal muscle (Weeds and McLachlan, 1974; Tufty and Kretsinger, 1975), DTNB light chains of rabbit skeletal muscle (Mutsuda et al, 1977) intestinal calcium binding protein (Hofman et al, 1977) and protein modulator (Vanaman et al, 1977) have suggested that the EF region represents the basic structural and homologous domain, repeated internally and shared by all members of the family of homologous proteins. Collins (1976a) has suggested that these homologous proteins were derived from an ancestral protein containing a single EF domain. This protein underwent two successive gene duplications and fusions to produce an ancestor containing four Ca^{2+}-binding sites. However, a comparison of the individual domains of the homologous proteins shows that regions two and four are more closely related to one another than they are to regions one and three. Vanaman et al, (1977)

and Kretsinger (1979) have suggested the existence of a primordial odd-even pair; the subsequent duplication would produce a pair of pairs, namely domains one and two and domains three and four. Gene fusion would complete the procedure. The model of the mechanism for the formation of the homologous proteins is presented in Scheme II (after Vanaman et al, (1977)).

In the case of parvalbumin it appears that domain one has been deleted after gene duplication, and domain two has lost the ability to bind Ca^{2+}. Parvalbumin contains two Ca^{2+}-binding sites with pK_d 6.5 (T = 25°C, pH 7.55, with 2 mM Mg^{2+}; Pechère, 1977) and in the absence of Mg^{2+} a pK_d of 8.4 was reported by Potter et al, (1977). A Hill coefficient of 1.05 suggests that at best only slight cooperativity in Ca^{2+}-binding may exist (Pechère, 1977),. Potter et al (1977)have reported that pK_d (Mg^{2+}) to be 4.0. Parvalbumin therefore contains two high affinity binding sites that bind Mg^{2+} competitively (Ca^{2+}-Mg^{2+} sites).

Skeletal muscle appears to be a rich source of parvalbumins. Amounts ranging from 11.5 g/Kg. (hake; Pechère et al, 1975) to 0.8 g/Kg (rabbit; Blum et al, 1977) have been reported. In addition to white skeletal muscle, values of less than 0.1 mg/Kg have been reported in carp brain (Gosselin-Rey et al, 1977), chicken red



SCHEME II

HYPOTHETICAL ANCESTRY AND STRUCTURAL FEATURES OF THE PROTEIN MODULATOR, TROPONIN-C AND PARVALBUMIN.

Kretsinger (1972) and Kretsinger and Nockolds (1973) first reported the internal sequence repeats of parvalbumin suggested gene triplication of a primitive Ca^{2+}-binding domain referred to as an "EF hand". Analysis of the sequence data of troponin-C, protein modulator ICBP, and myosin light chains have also demonstrated the existence of homologous internal repeat sequences of the EF region. Several investigators (Collins, 1976a; Vanaman et al, 1977; Kretsinger, 1979) have suggested that these homologous proteins are related to a common ancestral protein which arose from successive gene duplications of a 33 residue "EF" ancestor to form a protein of four "EF" regions. Analysis of the homology of the internal repeat sequences has suggested that the protein modulator is most closely related to the ancestral protein.

back muscle, breast, heart, stomach, brain, pancreas, small intestine and kidney but not spleen, liver, or blood (Heizmann et al, 1977). Baron et al, (1975) reported less than 0.2 mg/Kg of parvalbumin in rabbit uterus, bladder, small intestine, spleen, kidney, ovary, adipose tissue, lung, liver, and erythrocytes. Parvalbumin has also been found in the white leg muscle of the invertebrate Limulus (Anderson et al, 1978). Lowe et al, (1978) found by immunochemical techniques that in the mouse, parvalbumin is present only in white muscle. The results suggest that the majority of parvalbumin within an organism is located in the white muscle, but not restricted to this tissue.

The physiological function of parvalbumin is at present unknown. Pechère has suggested that parvalbumin may play a role in the relaxation cycle of white muscle as compensation for the kinetic insufficiencies of Ca^{2+} recaptured by the sarcoplasmic reticulum (Ebashi, 1976). Potter et al, (1977) suggested that parvalbumin may play a role in the activation of phosphodiesterase. The amount of parvalbumin necessary for half-maximal stimulation of phosphodiesterase (11 µg) was about 1400 x the amount of protein modulator required for similar activation. The fact that bovine serum albumin, also an acidic protein had no effect on the activity of phosphodiesterase at

concentrations up to 200 µg (per assay) suggested that the stimulatory nature of parvalbumin was not a non-specific ionic or acidic effect. The Ca^{2+} concentration required for half-maximal activation of phosphodiesterase by parvalbumin was determined to be 0.14 µM as compared to values of 1.2 µM for protein modulator activation, therefore suggesting that the activation of phosphodiesterase could not be due to a small contamination by protein modulator. Furthermore the Ca^{2+} concentration dependence of both Ca^{2+} binding and phosphodiesterase activation by parvalbumin were very similar, half-maximal stimulation of phosphodiesterase occurred with the binding of one mol Ca^{2+} (Potter et al, 1977).

2. Troponin-C

Comparative studies have shown that muscles in all animals are regulated by changes in Ca^{2+} concentration. Two distinctly different regulatory systems occur in different animals, one acting by means of the thin filaments (Ebashi, 1963) and the other by the thick filaments (Kendrick-Jones et al, 1970).

The term "thin filament control" refers to the fact that additional proteins required for the control mechanism -tropomyosin and troponin- are located on the thin filaments. Potter and Gergely (1974) have proposed a

mechanism for thin filament regulation of muscle contraction. In the presence of low levels of cytosolic Ca^{2+} ($10^{-7}M$) the troponin-tropomyosin complex blocks the interaction of actin and myosin. After stimulation the Ca^{2+} concentration increases ($10^{-5}M$), and the Ca^{2+}-binding subunit of troponin, troponin-C, binds Ca^{2+}, undergoes a conformational change ultimately resulting in the movement of tropomyosin into the long-pitch groove of actin, and allowing interaction of actin and myosin, thus, resulting in muscle contraction. A study of the properties of troponin-C is therefore fundamental in the understanding of the molecular mechanisms of the regulation of muscle contraction by Ca^{2+}.

The complete amino acid sequence of TN-C from rabbit (Collins et al, 1977) and chicken (Wilkinson, 1976) skeletal muscle and from cardiac muscle (van Eerd and Takahashi, 1976) has been determined. Several investigators (Collins et al, 1973; Weeds and MacLachlan, 1974; Tufty and Kretsinger, 1975) have noted the homology of TN-C to parvalbumin. Collins et al, (1974) first reported that the amino acid sequence of muscle TN-C could be divided into four homologous domains each of which contained a potential Ca^{2+}-binding site. Kretsinger and Barry (1975) have predicted a three dimensional model for TN-C, consisting of

two pairs of EF hands, each pair as found in parvalbumin. Since sequence analysis has suggested that parvalbumin evolved from a TN-C like ancestor with the C-terminal portion conserved, it is assumed that the two Ca^{2+} sites (CD and EF, scheme II) of parvalbumin correspond to sites III and IV of TN-C. Since the two parvalbumin sites are (Ca^{2+}-Mg^{2+}) sites this would mean that sites III and IV in skeletal muscle TN-C would also be (Ca^{2+}-Mg^{2+}) sites. This has been confirmed by Potter et al, (1976) and Leavis et al, (1977), using proteolytic fragmentation studies.

That skeletal muscle TN-C has two high affinity sites $pK_d(Ca^{2+})$ 7.3 which also bind Mg^{2+} pK_d 3.7 (Ca^{2+}-Mg^{2+} sites) and two low affinity sites $pK_d(Ca^{2+})$ 5.3, which do not bind Mg^{2+} (Ca^{2+} sites) was reported by Potter and Gergely (1975). In the presence of 2 mM Mg^{2+} the affinity of the (Ca^{2+}-Mg^{2+}) sites are lowered to $pK_d(Ca^{2+})$ of 6.3. Cardiac TN-C has two high affinity (Ca^{2+}-Mg^{2+}) sites (scheme II: III & IV) and one lower affinity site pK_d 4.3 in loop II (Potter et al, 1977). Loop I does not bind Ca^{2+}. The scatchard plot of the binding of Ca^{2+} by cardiac TN-C demonstrates positive cooperativity (Potter et al, 1977).

Studies of the myofibrillar ATPase activity as a function of Ca^{2+} concentration, at several Mg^{2+} concentrations have been examined by Potter and Gergely (1975)

who have concluded that since there is no difference in the Ca^{2+} dependence of myofibrillar ATPase at several Mg^{2+} concentrations, only the Ca^{2+} specific sites are involved in regulation of the myofibrillar ATPase. Using dansylaziridine (DANZ) labelled skeletal muscle TN-C as a probe of conformational changes due to Ca^{2+} binding by the Ca^{2+} specific sites, Potter et al, (1977) showed that the fluorescence of $TN\text{-}C_{DANZ}$ was enhanced approximately two fold when Ca^{2+} bound to the Ca^{2+}-specific sites, and this was accompanied by a 10 nm blue shift. The Ca^{2+} concentration required for half-maximal fluorescence change was 40 μM and was unaffected by the presence or absence of Mg^{2+}. Circular dichroism data have demonstrated that the Ca^{2+} dependent changes in ellipticity of skeletal muscle TN-C are biphasic with $K_{Ca}2+$ of 0.27 μM and 33 μM, representing binding to the (Ca^{2+}-Mg^{2+}) and Ca^{2+}-specific sites, respectively. The total change in α helix is from 34% to 50% (Kawasaki and van Eerd, 1972; Potter et al, 1977) and 35% of this change in ellipticity occurs with the binding of Ca^{2+} to the Ca^{2+} specific sites.

Troponin is a complex of three proteins (Greaser and Gergely, 1973). The components of the troponin complex are: troponin I (21,000 daltons) which is believed to inhibit the actin-myosin interaction, troponin C (18,000

daltons)which binds Ca^{2+} and relieves the inhibition caused by TN-I, and troponin T (30,500 daltons) which attaches the troponin complex to tropomyosin. Proteolytic studies (Leavis et al, 1977; Weeks and Perry, 1978; Drabikowski et al, 1977b) have indicated that the binding site of TN-C with TN-I is located between residues 83 - 104, and the binding sites for TN-T is located between residues 120 - 159. In terms of scheme I these binding sites are located in the III - IV domain.

TN-C has also been implicated in the Ca^{2+} dependent activation of phosphodiesterase (Dedman et al, 1977a) although 600 fold more TN-C (5 µg) is required to produce similar activation by protein modulator. The Ca^{2+} concentration required for half-maximal activation of phosphodiesterase by TN-C was 1.9 µM compared to 1.2 µM for the rat testes protein modulator (Dedman et al, 1977b). Since native muscle troponin at 100 µg concentration had no effect on phosphodiesterase activation, the activation of phosphodiesterase by TN-C was not a result of a 1/600 contamination by protein modulator.

The similarity in structure between TN-C and protein modulator has generated confusion in the literature, and made attempts to distinguish between these two proteins very difficult. As a result of this confusion reports have appeared in the literature concerning the isolation of soluble TN-C like proteins isolated from the extracts of

many tissues including bovine adrenal medulla (Kuo and Coffee, 1976), chick embryo brain (Fine et al, 1975), smooth muscle (Head et al, 1977), platelets (McGowan et al, 1976) and several vertebrate brains (Vanaman et al, 1975). Drabikowski et al, (1977b) has analyzed the 100,000 x g supernatant and pellet of the following tissues for the presence of TN-C, skeletal muscle (rabbit), cardiac muscle (bovine), smooth muscle (rabbit uterus and chicken gizzard), adrenal medulla (bovine), brain (bovine) and platelets (bovine). The partially purified cytosol or particulate fraction was subjected to preparative urea gel electrophoresis and the band revealing a common property of both TN-C and protein modulator namely the change in relative mobility in alkaline urea gel depending on the presence or absence of Ca^{2+} was isolated. TN-C and protein modulator were distinguished by three criteria, the difference in mobility on 15% SDS polyacrylamide gels, the difference in mobility of the complex with TN-I in urea gel in the presence of Ca^{2+}, and the ability of low concentration of protein modulator to stimulate Ca^{2+}-activatable phosphodiesterase. In all tissues examined TN-C was found only in the 100,000 x g pellet of skeletal and cardiac muscle, therefore indicating that the TN-C like proteins of smooth muscle (Head et al, 1977), brain (Vanaman et al, 1975), adrenal medulla (Kuo and Coffee, 1976) and platelet

(McGowan et al, 1976) are in fact the protein modulator. This suggestion has been confirmed; the TN-C like proteins isolated from bovine brain have been demonstrated to be the protein modulator (Stevens et al, 1976; Watterson et al, 1976) and the TN-C like protein isolated from soluble extracts of adrenal medulla has also been identified as the protein modulator (Crimaldi et al, 1978). Troponin however, may not be limited in distribution to vertebrate tissues; Goldberg and Lehman (1978) have reported the presence of troponin like proteins in the muscles of the scallop.

3. Protein Modulator

The amino acid sequence of the protein modulator from bovine brain has been determined by Vanaman et al (1977). The sequence of the protein modulator can be divided into four homologous domains each of which contains a potential Ca^{2+} binding site. The level of internal homology is greatest between domain one and domain three and between domain two and domain four. This level of internal homology appears greater than that observed within TN-C or parvalbumin (Collins, 1976a, b), and suggests that the protein modulator is more closely related to the ancestral protein (scheme II) than TN - C (Vanaman et al, 1977; Barker et al, 1977).

Alignment of the amino acid sequences of bovine

cardiac and rabbit skeletal muscle TN-C with that of bovine brain protein modulator has demonstrated that the modulator is closely related in structure to the muscle TN-C. Using those residues defined by Dayhoff (1976) as functionally conservative, Vanaman (1977) has determined that the total number of identical plus functionally conservative residues shared by bovine brain modulator and either TN-C, is 114 out of 148 positions compared (77% homologous), as compared with 82% homology between skeletal and cardiac TN-C. Walsh et al (1977) and Drabikowski et al (1977a) have demonstrated that tryptic cleavages in the protein modulator both in the presence and absence of Ca^{2+} occur in positions homologous to tryptic cleavage positions in TN-C. Collectively, these results confirm the original suggestion of Wang et al (1975) and Stevens et al (1976) that the protein modulator and TN-C are homologous proteins.

The Ca^{2+} binding properties of protein modulator from different tissues have been studied by many laboratories and there is no clear agreement about the affinity of the Ca^{2+} binding sites. However the literature supports the original discovery of Teo and Wang (1973) that the protein modulator binds four Ca^{2+} in the presence of 3 mM Mg^{2+}. This would suggest that unlike TN-C the protein modulator has four Ca^{2+} specific sites. Dedman et al (1977a) have

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demonstrated that the binding of Ca^{2+} to the Ca^{2+} specific sites can produce the conformational changes (change in α helix from 45% to 54%) before activation of phosphodiesterase. Klee (1977) has suggested that the bulk of the conformational changes in the porcine brain protein modulator occurs upon the binding of the first 2 mol of Ca^{2+}. Dedman et al (1977b) have suggested that the binding of only one mol of Ca^{2+} is sufficient to result in PDE activation. Since the literature has not resolved the question of the existence of one or two classes of Ca^{2+} specific sites, the possible role of the four Ca^{2+} binding domains in the Ca^{2+} induced conformational changes of protein modulator cannot be evaluated. Dedman et al (1977a) have demonstrated that the protein modulator was capable of substitution for TN-C in the troponin complex, and regulation of actin-activated myosin ATPase.

Taken collectively the results suggest that the structural and functional properties of TN-C and protein modulator have been highly conserved; according to sequence determinations (Vanaman et al, 1977) TN-C and protein modulator are 77% homologous and each can substitute for each other in their respective systems.

4. Intestinal Calcium Binding Protein

Two classes of vitamin D-dependent calcium binding

proteins have been demonstrated, a 9,7000 molecular weight class found in mammalian intestine, bovine kidney, and guinea pig kidney, and a 28,000 molecular weight class found in avian intestine kidney, shell gland, brain, as well as bovine brain, rat kidney and human kidney. Within the two classes the proteins are immunologically cross reactive, however there is no cross reaction between the two groups. The chick gut and smaller bovine gut protein have been shown to be acidic proteins, pI 4.2 and 4.7 respectively (Wasserman et al, 1977). Collectively these proteins have been referred to as intestinal calcium binding proteins (ICBP).

While the exact cellular location of ICBP has not been resolved, a partly particulate (5%) and partly soluble (95%) subcellular distribution has been reported for the chick gut protein (Feher and Wasserman, 1976). The detergent dependent release of the particulate ICBP has been demonstrated, and since sonication and hypoosmotic treatment of washed cellular debris released only a small portion of the bound protein it is likely that the particulate ICBP is not due to contamination by the soluble form.

The amino acid sequence of porcine ICBP (Hofmann et al, 1977) has established the existence of two EF regions. The evolution of the ICBP is unclear. Kretsinger (1979) has pointed out a slight and possibly not significant closer

relationship of the two domains of ICBP to the domain I and II of the four domain proteins, protein modulator and TN-c, than to the domains III and IV of these proteins. This suggests that ICBP may have evolved by a deletion of a pair of EF domains. Alternatively, ICBP may have diverged prior to the basic pair duplication event and evolved directly from the original odd-even pair. The 28,000 dalton class of ICBP may represent a four domain protein, however, sequence studies will be necessary to evaluate these ICBP.

Evidence for the involvement of ICBP in Ca^{2+} transport has come from correlative data in which the rate of absorption of Ca^{2+} was shown to be directly related to the concentration of ICBP under a wide variety of conditions. Wasserman (1977) has demonstrated a temporal relationship between ICBP synthesis and Ca^{2+} transport after Vitamin D (1,25 dihydroxycholecalciferol) is given to rachitic chicks. The appearance of ICBP, initially preceeded the enhancement of Ca^{2+} absorption, then a parallel increase in both parameters occurred. Embryonic chick intestine culture studies have demonstrated that inhibitors of protein synthesis (actinomycin, α-amanitin) inhibit both the vitamin D stimulated synthesis of ICBP and the vitamin D dependent Ca^{2+} uptake (Corradino, 1973). Corradino et al (1976)

demonstrated that accompanying the vitamin D induced appearance of ICBP in chick intestine cells is an increase in Ca^{2+} uptake. Alternatively Krawit and Stubbert (1972) have demonstrated hydrocortisone inhibited vitamin D mediated absorption of Ca^{2+} in the rat, without a corresponding effect on the concentration of ICBP.

The results suggest that ICBP may be responsible for the vitamin D induced increase in intestinal Ca^{2+} transport, but the concentration of the ICBP in intestinal tissue may not account for the total intestinal response to vitamin D.

5. Myosin Light Chains

In muscle cells there are two well-described mechanisms by which Ca^{2+} regulates actin-myosin interaction. First, in skeletal muscle and also cardiac muscle, Ca^{2+} initiates the contractile event by binding to the regulatory protein, TN-C (Greaser and Gergely, 1973) which results in removal of inhibition of the actin-myosin interaction imposed by the complex troponin-tropomyosin, in the absence of Ca^{2+} (thin filament regulation). A second type of regulatory mechanism, originally elucidated for the scallop myosin, has been described by Szent-Gyorgi et al (1973). In this system Ca^{2+} regulation of actin-myosin interaction is mediated by a particular light chain

of myosin (thick filament regulation). Both types of regulation have been demonstrated in certain worms and insects (Lehman and Szent-Gyorgi, 1975).

Myosin is composed of two heavy chains (200,000 daltons) and four light chains (15,000 - 25,000 daltons). The presence of two distinct classes of light chains was first demonstrated in vertebrate skeletal muscle (Weeds, 1969; Weeds and Lowey, 1971) by chemical methods. One class of light chain (2 mol, molecular weight 19,000 daltons) could be dissociated from myosin by reaction with 5,5' dithiobis (2-nitrobenzoic acid) (DTNB). The dissociation of light chains from myosin does not result in the loss in myosin ATPase activity. (Weeds and Lowey, 1971). This light chain was termed the DTNB light chain and was not required for ATPase activity. The second class of light chain required alkaline pH for dissociation from myosin which resulted in a loss of enzyme activity (Weeds and Lowey, 1971). This light chain was referred to as the alkali light chain. Rabbit fast-twitch muscle myosin contains two alkali light chains of 21,000 and 17,000 molecular weight. They are identical in amino acid sequence over their C-terminal 141 residues but differ significantly in their N-terminal residues (Frank and Wedds, 1974). Furthermore, the alkali light chains occur in unequal and non integral

ratios (Lowey and Risby, 1971) and the relative amounts change during development (Pinnoni-Muller et al, 1976) therefore suggesting the presence of myosin isozymes within a single homogeneous muscle type. All other muscles examined do not have the two forms of this type of light chain, but contain two mol of identical light chain.

The scallop myosin ATPase system has allowed classification of the myosin light chains into two functional categories. In the presence of Ca^{2+} the scallop myosin ATPase activity can only be activated by actin when a full complement of light chains is present. Removal of one mol of a possible two mol of the EDTA light chain (so called because this light chain is removed by EDTA) results in a loss of Ca^{2+} sensitivity and one Ca^{2+} binding site. The removal of this light chain is called desensitization. Ca^{2+} sensitivity can be restored by readdition of the scallop EDTA light chain or by substitution of the 20,000 dalton light chain of smooth muscle, the 19,000 dalton light chain of vertebrate slow-twitch muscle, and the 19,000 dalton (DTNB) light chain of vertebrate fast-twitch muscle in place of the scallop (EDTA) light chain. In contrast the remaining class of light chain (e.g. the alkali light chains of vertebrate fast-twitch muscle) were ineffective in the restoration of Ca^{2+} sensitivity to scallop myosin (Kendrick-Jones et al, 1976).

The scallop myosin ATPase system has allowed a classification of all the myosin light chains according to function, into two classes, the regulatory light chains (RLC) and the essential light chains (ELC).

The regulatory light chains of all myosins restore Ca^{2+} sensitivity to the desensitized scallop actomyosin ATPase. They have been identified in all myosins. Comparisons of the amino acid compositions of these RLC (Kendrick-Jones et al, 1976) has suggested that these proteins are homologous. Furthermore, with the exception of the EDTA light chain, all RLC can be phosphorylated (Frearson and Perry, 1975; Perrie et al, 1973; Adelstein et al, 1976).

The essential light chains of myosin do not restore Ca^{2+} sensitivity to desensitized scallop actomyosin ATPase. Their name is derived from the fact that frequently the removal of these light chains results in loss of enzyme activity. Grouped in this category are the alkali light chains of fast-twitch myosin, the guanidine-HCl light chains of scallop myosin, the 17,000 dalton light chain of smooth muscle, and the 21,000 dalton light chains of cardiac and slow-twitch muscle. Recent work by Wagner and Weeds (1977) and Winstanley et al, (1977) has suggested that the ELC's do not have a significant effect on myosin ATPase activity in the absence of actin but may effect the actin activated myosin

ATPase activity and also may be involved in actin binding.

The amino acid sequence of the RLC of cardiac myosin (Léger and Elsinga, 1977), rabbit fast-twitch muscle, (Collins, 1976a), and scallop muscle (Kendrick-Jones and Jakes, 1977) as well as the ELC of fast-twitch muscle (Frank and Weeds, 1974) has been determined and compared with the sequences of parvalbumin, ICBP, TN-C, and protein modulator (Kretsinger, 1979). The results demonstrate that the myosin light chains are homologous both to each other and to the other members of the family of homologous proteins. The RLC and ELC both contain four EF domains. The pattern of internal sequence repeats is very weak, and the pattern of hydrophobic residues predicted to form the integral core has been conserved.

Of all the myosin light chains only the DTNB light chain can bind Ca^{2+}. This binding is weaker (K_d 10 μM) when Mg^{2+} is present (Werber et al, 1972). The Ca^{2+} binding site of the DTNB light chain therefore appears to be a (Ca^{2+}-Mg^{2+}) site. The desensitized scallop myosin preparation has been used to provide evidence that Ca^{2+} binding by the RLC may only occur when the light chain associates with myosin. Upon desensitization of scallop myosin both Ca^{2+} sensitivity and one Ca^{2+} binding site is lost. However when this preparation is resensitized with the

scallop EDTA light chain or by the RLC of vertebrate smooth muscle both Ca^{2+} sensitivity and Ca^{2+} binding are restored to the actin-activated myosin ATPase. This would suggest that the RLC of the scallop and smooth muscle demonstrate Ca^{2+} binding only when associated with myosin. Interestingly the Ca^{2+} binding exhibited by scallop myosin (K_d 1μM) is unaffected by Mg^{2+}, therefore indicating the possible presence of two Ca^{2+} specific sites on this myosin.

Sequence analysis of the site of phosphorylation of the RLC has suggested a serine residue located on the N-terminal side of domain I (Jakes et al, 1976). The EDTA light chain also contains an equivalent serine residue, but is not phosphorylated. Kendrick-Jones and Jakes (1977) have proposed that the N-terminal region of the EDTA light chain necessary for recognition by the kinase, is missing in the EDTA light chain (EDTA MW, 17,000; other RLC MW 19,000 - 20,000). Proteolytic studies have supported this postulate (Jakes et al, 1976). The RLC appear to be the only members of the family of homologous proteins which can be phosphorylated.

The evolution of the myosin light chains away from a direct Ca^{2+} regulatory role is suggested from analysis of the essential light chains. They do not bind Ca^{2+} or restore Ca^{2+} sensitivity to desensitized scallop myosin, nor are

they phosphorylated. However, sequence analysis (Frank and Weeds, 1974) have shown that the essential features of the family of the homologous proteins are retained, they possess EF regions and the distribution of hydrophobic residues in the EF regions have been conserved.

It is therefore reasonable to conclude that the RLC and the ELC of myosin have evolved from a common ancestral protein, as have parvalbumin, TN-C, and protein modulator. Interestingly, analysis of the homology of the internal repeat sequences has suggested that the protein modulator is probably the most closely related member of this family of homologous proteins to the ancestral protein (Barker et al, 1977; Kretsinger et al, 1979).

IV. PHYSIOLOGICAL SIGNIFICANCE OF PROTEIN MODULATOR REGULATION

1. Cyclic Nucleotide Phosphodiesterase

Cyclic nucleotide phosphodiesterase was first identified by Butcher and Sutherland (1962) in bovine heart, and represents the only enzymatic mechanism for the hydrolysis of c-AMP and c-GMP. That rat cerebral cortex contained multiple forms of cyclic nucleotide phosphodiesterase was first reported by Thompson and Appleman (1971). Two or more forms of the enzyme have been found in every tissue examined. Methods that have been used to characterize these multiple

forms include gel filtration (Thompson and Appleman, 1971; Kakiuchi et al, 1971), anion exchange chromatography (Russel et al, 1973; Schubart et al, 1974; Wells et al, 1975) polyacrylamide gel electrophoresis (Goren et al, 1971; Uzunov and Weiss, 1972; Campbell and Oliver, 1972) starch gel electrophoresis (Monn and Christiansen, 1971), sucrose density gradient centrifugation (Thompson et al, 1973) and isoelectric focussing (Pledger et al, 1974).

Russel et al (1973) have demonstrated that DEAE-cellulose chromatography of liver extract prepared by homogenization, sonication, and centrifugation, exhibits three discrete active fractions of phosphodiesterase activity referred to as D-I, D-II, and D-III according to their elution from the column by a salt gradient. This chromatographic analysis has been applied to a number of mammalian tissues including heart, kidney, mammary gland, lung, and brain (Appleman and Terasaki, 1975) and while the relative amounts of the different forms of phosphodiesterase activities vary from tissue to tissue the basic pattern of three fractions of cyclic nucleotide phosphodiesterase activity is conserved. Other forms of phosphodiesterase activity in addition to the three described above may exist in mammalian tissues. For example, six fractions of phosphodiesterase activity have been isolated from sonicated cerebellar preparations by

polyacrylamide gel electrophoresis (Uzunov and Weiss, 1972) and by isoelectric focussing (Pledger et al, 1974). Hidaka et al, 1978 has reported the separation of five fractions of PDE activity by DEAE-cellulose chromatography. While these fractions could be separate enzymes it is also possible they could represent forms that arise from proteolysis or from aggregation of one or more of the original forms present.

Of the three forms of phosphodiesterase activity originally reported by Russel et al (1973), D-III appears to have a low Km (c-AMP), particulate enzyme. The activity of D-III is characterized by relative selectively toward c-AMP as substrate and in many studies by concave-downward Linweaver-Burk plots (Appleman et al, 1973). Furthermore, the activity of this enzyme may change in rat liver in response to insulin and glucagon (Loten et al, 1978). While the D-III enzyme of rat liver is particulate, forms D-I and D-II appear to be soluble. D-II has been identified as possessing approximately equal activity toward c-AMP and c-GMP. When c-GMP is used as substrate the Linewearer-Burk plot shows a pronounced upward curvature suggesting the presence of homotropic cooperativity in the enzyme with respect to this substrate, however no significant homotropic interactions were reported when c-AMP was the substrate. This enzyme

has been characterized in rat liver (Appleman and Terasaki, 1975) and bovine heart (Ho et al, 1976).

The separation of phosphodiesterase into a Ca^{2+} sensitive and Ca^{2+} insensitive form was initially reported by Kakiuchi et al (1971), in rat brain. Similar results have been obtained for bovine heart (Goren and Rosen, 1972; Hrapchak and Rasmussen, 1972; Wang et al, 1972) and rat liver and heart (Appleman and Terasaki, 1975). Using the nomenclature of Russel et al (1973), kinetic analysis has confirmed that the Ca^{2+} sensitive enzyme corresponds to D-I and the Ca^{2+} insensitive form to D-II. The activity of D-I is stimulated by the protein modulator and the activation is completely reversed by EGTA (Kakiuchi et al, 1973; Teo and Wang, 1973). All reports to date are consistant with the idea that a single protein modulator sensitive enzyme will catalyse the hydrolysis of both c-AMP and c-GMP. Reports that the hydrolysis of c-AMP is competitively inhibited by c-GMP and vice versa, with K_i values that are similar to the respective Km values strongly suggest that a single catalytic site is involved in the hydrolysis of both substrates (Wells et al, 1975b; Brostrom and Wolff, 1976; Ho et al, 1976). Ho et al (1977) have reported a specific activity of 120 μmol/min/mg for a partially pure (80%) phosphodiesterase preparation. Ca^{2+}-activable phosphodiesterase has been purified from

bovine brain by two groups of investigators (Wallace et al, 1978; Klee et al, 1978).

In all tissues examined thusfar, it has been shown that the Ca^{2+}-activated phosphodiesterase has a higher affinity for c-GMP than c-AMP. These tissues include rat brain (Kakiuchi et al, 1973), porcine brain (Brostrom and Wolff, 1976), bovine heart (Ho et al, 1975), rat heart (Appleman and Terasaki, 1975), porcine coronary arteries (Wells et al, 1975b), and human aorta (Hidaka et al, 1978). These observations have led to the suggestion that the Ca^{2+}-activated enzyme is primarily a c-GMP enzyme in vivo.

The kinetic mechanism of phosphodiesterase activation is at present unclear. Ho et al, (1976) have demonstrated that the activation of bovine heart phosphodiesterase by the protein modulator results in a five fold increase in the Vmax and a decrease of about 90% in the Km (from 1.5 mM to 0.2 mM) when c-AMP is used as substrate. Similar results have been reported by Klee et al, (1978). When c-GMP is used as substrate, a decrease in Km of about fifty fold (from 0.26 mM to 9 μM) and no change in Vmax (remains at about 30% of the rate of hydrolysis of c-AMP by the modulator stimulated enzyme) was reported by Ho et al, (1976). Brostrom and Wolff (1976) have reported that protein modulator activation of PDE results only in a decrease in Km when both substrates are used.

In contrast, Wickson et al (1975)have reported a change in Vmax without any change in Km when c-GMP is used as substrate. The reason for these conflicting observations is not at all apparent but may reflect tissue differences, different states of purity, or different assay conditions.

The Ca^{2+}-stimulated phosphodiesterase can also be activated by proteolysis (Cheung, 1971) and by certain lipids and phospholipids, particularly phosphatidyl inositol and lysolecithin (Wolff and Brostrom, 1976), but this stimulation is independent of Ca^{2+}. Hidaka et al, 1978 have reported stimulation of human aorta PDE by behenic acid (C_{22}). Imidazole and ammonium sulfate (Ho et al, 1976) have have been reported to specifically increase the maximal activation of phosphodiesterase by the protein modulator. Mg^{2+} has been demonstrated to inhibit PDE by competing with Ca^{2+} for the protein modulator (Ho et al, 1976). Levin and Weiss (1977) have demonstrated that the inhibition of PDE by the antipsychotic drug trifluoperazine results from direct binding of trifluoperazine to the protein modulator. The effects of other phosphodiesterase inhibitors e.g. theophylline, and papaverine have been suggested to act by competition with the substrate (Weiss, 1975), and to vary in effectiveness from tissue to tissue according to the pattern of phosphodiesterase activities found in these tissues.

2. Adenylate Cyclase

Adenylate cyclase derived from a variety of tissues has been shown to be strongly inhibited by low concentrations of Ca^{2+} (Birnbaumer, 1973). Brain tissue (Bradham et al, 1970) and one glioma cell line (Brostrom et al, 1976) however, possess adenylate cyclase activity which exhibits a biphasic response to Ca^{2+}; low Ca^{2+} concentrations activate and higher concentrations inhibit the activity. Using ECTEOLA-cellulose chromatography to partially purify detergent-dispersed extracts of brain tissue, several investigators have demonstrated the existence of PM activated adenylate cyclase in porcine cerebral cortex (Brostrom et al, 1975) and rat or bovine brain (Cheung et al, 1975b). The adenylate cyclase activity of a rat cerebral cortex preparation has been shown to be composed of two contributing components, only one of which requires the protein modulator for activity (Brostrom et al, 1977). The protein modulator component represented 80% of the basal activity of the cortex homogenate and comprised about half of the overall activity when each component was fully activated by NaF. Pretreatment of this preparation with Ca^{2+} and modulator for 1 hr at 37^{o} resulted in the selective inactivation of the protein modulator independent adenylate cyclase, and therefore allowed a study of the protein modulator dependent adenylate cyclase activity. The

PM-dependent enzyme was shown to be activated 10 to 30 fold by PM, to respond biphasically to free Ca^{2+} concentrations (in the presence of PM), to be stimulated by NaF only in the presence of Ca and PM, NaF stimulation being readily reversed by EGTA, to be inhibited by high ratios of Mg^{2+} to Ca^{2+} and to be inhibited by chloropromazine. In contrast, the PM-independent adenylate cyclase was inhibited with increasing free Ca^{2+} concentration, had elevated activity at high ratios of Mg^{2+} to Ca^{2+}, was not effected by chloropromazine, and was irreversibly activated by NaF.

The activation of adenylate cyclase by protein modulator is very similar to the activation of phosphodiesterase. The activation of adenylate cyclase by PM is Ca^{2+} dependent and reversible by EGTA (Brostrom et al, 1975). Activation depends on the simultaneous presence of Ca^{2+} and PM (Brostrom et al, 1975; Cheung et al, 1975), and the formation of the PM-adenylate cyclase complex is dependent on Ca^{2+} (Lynch et al, 1976). Lynch et al (1976b) have reported that the protein modulator increased the Vmax several fold but did not effect the Km for ATP. Stimulation required Ca^{2+}, with half-maximal effect at 15 μM. That the PM and Ca^{2+} conferred thermal stability to adenylate cyclase indicates that the protein modulator probably induced a conformational change in the enzyme, an increase in thermal stability of phosphodiesterase

preparations due to the protein modulator and Ca^{2+} has also been reported (Ho et al, 1976).

3. Cyclic Nucleotide Regulation

The fact that the protein modulator stimulates the activities of both adenylate cyclase (catalyzing the formation of c-AMP and cyclic nucleotide phosphodiesterase (catalyzing cyclic nucleotide degradation) appears paradoxical. A further complication is added by the observation of protein modulator sensitive and insensitive forms of phosphodiesterase and adenylate cyclase activity. While protein modulator stimulated phosphodiesterase has been reported in most mammalian tissues examined, protein modulator stimulated adenylate cyclase has only been reported in mammalian brain. For these reasons, the exact physiological mechanism of the regulation of cyclic nucleotide metabolism by the protein modulator remains speculative.

One of the earlier attempts to postulate a possible physiological function for protein modulator in the regulation of cyclic nucleotide metabolism was by Kakiuchi et al (1973). They proposed that based on kinetic evidence the modulator dependent phosphodiesterase is actually a c-GMP rather than a c-AMP hydrolyzing enzyme. Therefore the Ca^{2+} influx in response to a stimulus was suggested to result in the formation of an active Ca^{2+} protein modulator complex

resulting in the simultaneous stimulation of adenylate cyclase and phosphodiesterase, causing an increase in intracellular c-AMP and a decrease in c-GMP.

Another theory, proposed by Lynch et al (1976) suggests that the sequential activation of adenylate cyclase and phosphodiesterase causes a transient elevation of c-AMP in many tissues. Stimuli which result in an influx of Ca^{2+} through the plasma membrane, or release of membrane bound Ca^{2+} would activate the membrane bound adenylate cyclase resulting in an increase of intracellular c-AMP. The Ca^{2+} would then activate the soluble phosphodiesterase therefore decreasing the c-AMP to basal concentrations. Bartfai (1978) has proposed that protein modulator regulation of phosphodiesterase and adenylate cyclase may be of importance in the regulation of c-AMP and perhaps c-GMP in postsynaptic neurons. Uzunov et al (1975) have reported that stimulation of the nicotinic receptors of adrenal medulla by carbamylcholine resulted in elevation of the content of c-AMP and protein modulator, the increase in c-AMP preceeded the increase in protein modulator. Furthermore, when the increase of the protein modulator reached peak values, the c-AMP content of the medulla was declining. Subsequently Uzunov et al (1976) demonstrated that the protein modulator released from the cytosol interacted with the high K_m phosphodiesterase in the presence of Ca^{2+}, and lowered its K_m

thereby facilitating the hydrolysis of c-AMP. Gnegy et al (1976) demonstrated that protein modulator released from the membrane fraction of a brain or adrenal medulla homogenate could be stimulated by in vitro incubation with purified c-AMP dependent protein kinase. The c-AMP dependent protein kinase did not phosphorylate the protein modulator, therefore suggesting phosphorylation of the membrane preparation. Subsequently Gnegy et al (1977) reported that the release of protein modulator was mainly from membrane fractions enriched in synaptic membranes, and occurred due to the phosphorylation of membrane protein by the c-AMP dependent protein kinase. From this evidence Gnegy et al (1977) proposed that the protein modulator was directly involved in the transsynaptically elicited increase in c-AMP in neuronal tissue. It was postulated that when the concentration of c-AMP reaches a certain level it activates the c-AMP dependent protein kinase which phosphorylates a membrane protein that binds protein modulator, resulting in release of protein modulator, stimulation of phosphodiesterase, and a decrease to resting levels of c-AMP.

4. $(Ca^{2+}-Mg^{2+})$ ATPase

A soluble factor capable of the stimulation of the $(Ca^{2+}-Mg^{2+})$ ATPase of the human erythrocyte membrane was originally reported by Bond and Clough (1973). Luthra

et al (1976) partially purified this activator and reported that it was acidic, acid and heat stable, and had a molecular weight below 50,000. The interaction of the red blood cell activator with the membrane resulted in the stimulation of the (Ca^{2+}-Mg^{2+}) ATPase, and this interaction between the activator and the membrane was demonstrated to be dependent on the presence of Ca^{2+} (Farrance, 1976). Noting the similarity of the red blood cell activator with the Ca^{2+} binding proteins, parvalbumin troponin-C and protein modulator, Gopinath and Vincenzi (1977) tested these proteins as potential activators of the (Ca^{2+}-Mg^{2+}) ATPase. While parvalbumin and troponin-C produced a modest increase in activity at high concentrations, the biological activity of the red blood membrane activator and the bovine brain protein modulator (1.5 µg/ml) were indistinguishable, both proteins produced about a four fold activation, and increased the Vmax and apparent affinity of (Ca^{2+}-Mg^{2+}) ATPase for Ca^{2+}. Based on these observations Gopinath and Vincenzi suggested that the protein modulator and the red blood cell activator might be the same protein.

Independently, Janett and Penniston, (1977) also reported activation of the human erythrocyte (Ca^{2+}-Mg^{2+}) ATPase by a soluble endogenous activator. This activator was purified 475-fold by these investigators who reported

that the ATPase activator and the protein modulator were very similar, both stimulated (Ca^{2+}-Mg^{2+}) ATPase and phosphodiesterase and they co-electrophorised on SDS polyacrylamide gel.

Recently Jarrett and Penniston (1978) have reported purification of the human erythrocyte activator to homogeneity. A comparison of the protein modulator and the human erythrocyte activator was undertaken; the amino acid compositions of the proteins were nearly indistinguishable and both proteins contained one residue trimethyllysine. Furthermore, both proteins were indistinguishable as measured by their ability to activate the (Ca^{2+}-Mg^{2+}) ATPase (values for 50% of maximal activation of the (Ca^{2+}-Mg^{2+}) ATPase varied from 36 to 76 ng/ml, depending on the history of the ghosts).

The erythrocyte membrane (Ca^{2+}-Mg^{2+}) ATPase activity is thought to be an expression of the Ca^{2+} active transport system in whole cells (Schatzmann and Vincenzi, (1969) which functions to maintain low intracellular Ca^{2+} concentrations. Because of the Ca^{2+} dependent and reversible binding to the erythrocyte membrane, and specificity of the activation of the (Ca^{2+}-Mg^{2+}) ATPase it has been postulated that the protein modulator functions as a regulator of the plasma membrane Ca^{2+} pump (Farrance et al,

1977). MacIntyre and Green (1977) observed stimulation of Ca^{2+} transport of an inside-out vesicle preparation of human erythrocyte by lysed erythrocyte supernatant. Hinds et al (1978) have tested this hypothesis with inside-out membrane vesicles prepared from human red blood cells. The active transport of Ca^{2+} was increased by addition of the human erythrocyte activator or the bovine brain protein modulator. When Lanthanum, an inhibitor of active Ca^{2+} transport (Quist and Roufogalis, 1975) was added to the system in the presence of protein modulator, the uptake of Ca^{2+} was inhibited. Addition of the calcium ionophore A23187 caused a rapid effux of Ca^{2+} from the loaded vesicles. The results suggest that the activity of the human erythrocyte plasma membrane Ca^{2+} pump may be regulated by the protein modulator.

5. Modulator Binding Proteins

Recently two bovine brain proteins which exhibit specific inhibitory activity against the protein modulator activated cyclic nucleotide phosphodiesterase have been reported, one of the proteins is heat labile and is referred to as the modulator binding protein (MBP) (Wang and Desai, 1976; Klee and Krinks, 1978), the other protein is heat stable and referred to as the heat stable inhibitor protein (HSIP) (Sharma et al, 1978a).

A. Modulator Binding Protein

The MBP specifically counteracts the activation of phosphodiesterase by the protein modulator, and shows no inhibitory activity, against the basal activity of the Ca^{2+}-activatable enzyme, against the trypsin activated Ca^{2+}-activatable enzyme, or against the Ca^{2+}-insensitive enzyme (Wang and Desai, 1977). Two experimental approaches have suggested that the MBP interacts with the protein modulator in the presence of Ca^{2+} (Wang and Desai, 1977). First, kinetic studies of the mutual effects of protein modulator and the MBP in the phosphodiesterase reaction are characteristic of a competitive interaction; the inhibition of PDE by MBP, is reversed by the addition of excess PM.

Second, gel filtration analysis (Sephadex G-200) has revealed that in the presence of Ca^{2+} the protein modulator and MBP can associate to form a complex; no interaction between Ca^{2+}-activatable phosphodiesterase and the MBP in the presence of EGTA was observed. These results have suggested that the MBP is similar to other protein modulator regulated enzymes, such as Ca^{2+}-activatable phosphodiesterase and adenylate cyclase in forming a Ca^{2+} dependent complex with the protein modulator. While MBP might possess catalytic activity, at present no catalytic activity has been identified for this protein. Wang and Desai (1977)

have tested MBP for various enzyme activities, including ATPase, GTPase, 5'nucleotidase and adenylate cyclase. Results were negative. It therefore remains unsettled whether MBP represents a protein modulator regulated enzyme or protein with nonenzymatic activity such as specific binding or transporting activity. It also cannot be ruled out that MBP might represent *in vivo* regulation of PM.

The modulator binding protein of bovine brain has been purified, to near homogeneity by Sharma *et al* (1979b), by a procedure based on the Ca^{2+}-dependent association of this protein with the protein modulator. The MBP is shown to be a globular protein (stokes radius 40.5^{o}A) of molecular weight 85,000. It has been shown to contain two distinct subunits of molecular weight 60,000 (α) and 14,500 (β) daltons respectively. A mass ratio of subunits α/β of 2.3 has been interpreted to suggest the subunit structure of MBP is α/β. Sharma *et al* (1979b) have separated the subunits of MBP by gel filtration on Sephadex G-100 in the presence of 6 M urea. Characterization of the separated subunits has demonstrated that only the α subunit can interact with PM. Furthermore the interaction of a subunit of MBP and PM is unaffected by β subunit. The results suggest that subunit α of the MBP is responsible for association of the MBP and the protein modulator. The function of the β subunit is at present unresolved.

B. Heat Stable Inhibitor Protein

A heat stable inhibitory protein of Ca^{2+} activatable phosphodiesterase has been partially purified from bovine brain by Sharma et al, (1978a). The HSIP has a molecular weight of 70,000 daltons as determined by gel filtration on G-100 Sephadex, and therefore appears to be different from the heat stable phosphodiesterase inhibitor of bovine retina (Dumler and Etinof, 1976) (MW 40,000).

The mechanism of action of HSIP is similar to that of the MBP. Based on the competitive interaction between HSIP and modulator protein in the phosphodiesterase reaction, and the Ca^{2+} dependent association of HSIP and protein modulator on gel filtration columns (G-100 Sephadex), Sharma et al (1978b) has suggested that the HSIP inhibits phosphodiesterase by specifically interacting with the protein modulator.

The HSIP has been purified to electrophoretic homogeneity by Sharma et al (1978b). The HSIP had a molecular weight of 68,000 on SDS polyacrylamide gels therefore suggesting the HSIP is monomeric. The protein has no inhibitory activity toward the c-AMP dependent protein kinase or protein phosphatase. Furthermore, examination of HSIP for various enzyme activities includint ATPase, GTPase, c-AMP phosphodiesterase, c-GMP phosphodiesterase, 5'nucleotidase and protein kinase has proved to be negative. It

therefore appears that the physiological function of both MBP and HSIP is unknown. These proteins could represent *in vivo* regulators of protein modulator, protein modulator regulated enzymes of unknown catalytic function or protein modulator regulated proteins (non enzymatic activity).

6. Stimulus-Secretion Coupling

Considerable evidence suggests that Ca^{2+} plays a number of important roles in the functioning of the nervous system (Rubin, 1970; Baker, 1972). Electrical depolarization of nerve terminals and of other secreting structures has been shown to cause an influx of extracellular calcium through specific calcium channels (Baker *et al*, 1971) and this calcium influx appears to mediate the stimulus-coupled release of neurotransmitters and peptide hormones (Katz and Miledi, 1967; Douglas, 1968). There is also evidence that calcium may regulate the synthesis of catecholamines in presynaptic nerve terminals (Patrick and Barchas, 1974).

A possible mechanism for the Ca^{2+} regulated release of neurotransmitter from presynaptic nerve terminals has been suggested by several investigators. DeLorenzo (1976) has reported that in preparations of ruptured rat brain synaptosomes two proteins of molecular weight 60,000 and 50,000 daltons are phosphorylated by a mechanism which is stimulated by calcium and inhibited by diphenylhydantoin

(DPH). Krueger et al (1977) has similarly reported that agents known to increase Ca^{2+} transport across the plasma membranes of nerve terminals, stimulate the phosphorylation of two specific endogenous proteins in intact synaptosomes (80,000 and 86,000 daltons). Agents found to be stimulatory included veratridine, high K^{+} (60 mM), and the calcium ionophore A23187. The results were interpreted to suggest that conditions which cause an accumulation of Ca^{2+} by synaptosomes lead to a calcium-dependent increase in phosphorylation of specific endogenous proteins and that these phosphoproteins may be involved in the regulation of certain calcium-dependent nerve terminal functions such as neurotransmitter synthesis and release.

That the molecular mechanism mediating the effects of calcium on neurotransmitter release and synaptic vesicle function may be the action of calcium on the level of phosphorylation of specific synaptic vesicle-associated proteins was first suggested by DeLorenzo and Freedman (1977a, b). These investigators presented evidence to show that the Ca^{2+} specific phosphorylated proteins are present in the presynaptic nerve terminal. A direct correlation between Ca^{2+} specific synaptosomal neurotransmitter release and Ca^{2+} specific synaptosomal phosphorylation has been reported by DeLorenzo and Freedman (1978). Using highly purified synaptosomal preparation these investigators

have demonstrated that both Ca^{2+} and Mg^{2+} are necessary for synaptosomal phosphorylation, and norepinephrine release. Ca^{2+} or Mg^{2+} alone was ineffective. Ca^{2+} was also shown to cause the greatest increase in the levels of phosphorylation of synaptosomes that showed the greatest Ca^{2+} dependent norepinephrine release. DPH was demonstrated to inhibit both the Ca^{2+} dependent neurotransmitter release (Pincus and Lee, 1973) and Ca^{2+} dependent synaptosomal phosphorylation. Furthermore, it was shown that both the Ca^{2+}dependent phosphorylation and Ca^{2+} dependent neurotransmitter release were stimulated by a protein factor. The results are compatible with the hypothesis that the phosphorylation of specific synaptic vesicle proteins is the molecular mechanism mediating some of the effects of calcium on neurotransmitter release. De Lorenzo and Freedman (1978) have also reported similar results for the Ca^{2+} dependent release of dopamine, acetylcholine, and γ aminobutyric acid.

Schulman and Greengard (1978) have reported that the calcium-dependent phosphorylation of highly purified membrane fractions from rat cerebral cortex required an endogenous heat stable protein factor present in the synaptosomal cytoplasm. Calcium stimulated phosphorylation lost on purification of synaptic membranes could be effectively recovered by reconstitution with either the synaptosomal

cytoplasm or a purified preparation of protein modulator. The PM regulated kinase was localized in the synaptosomal membrane.

The results of De Lorenzo and Friedman (1978) and Schulman and Greengard (1978) have suggested that the Ca^{2+} dependent presynaptic release of neurotransmitter is dependent on the phosphorylation of specific presynaptic proteins by a particulate, protein modulator regulated protein kinase.

Ca^{2+} has also been shown to regulate the synthesis of catecholamines in presynaptic nerve terminals (Patrick and Barchas, 1974). Kuhn et al (1978) and Hamon et al (1978) have presented evidence that tryptophan hydroxylase in rat brainstem extracts is activated by a Ca^{2+} stimulated protein kinase. Phosphorylation increased the catalytic activity of the enzyme 2.5 - fold and decreased the apparent Km for cofactor. Morgenroth et al (1975) have observed a reversible four fold increase in tyrosine hydroxylase activity in rat brain extracts with an apparent pK_d (Ca^{2+}) of 5.9. However, only c-AMP dependent phosphorylation of tyrosine hydroxylase has been reported (Yamauchi and Fujisawa, 1978). The c-AMP dependent phosphorylation of tyrosine hydroxylase is compatible with the postulated stimulation of norepinephrine release by presynaptic β-adrenoreceptors (Pelayo et al, 1978).

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It is interesting to note that while the anticonvulsant drug diphenylhydantoin has been shown to inhibit Ca^{2+} dependent phosphorylation and Ca^{2+} dependent release of neurotransmitter from synaptosomes (De Lorenzo and Friedman, 1978) this drug has also been demonstrated to block several other Ca^{2+}-dependent release processes including the release of insulin from islet cells (Knopp et al, 1972), and of oxytocin (Mittler and Glick, 1972), and antidiuretic hormone release from the pituitary. It is within the realm of possibility that protein modulator regulated protein kinase phosphorylation represents a basic mechanism for the Ca^{2+} mediated process of stimulus-secretion coupling. It is however apparent that DPH appears to represent an agent capable of inhibition of many Ca^{2}— dependent secretory processes, at least one of which is regulated by protein modulator.

7. Stimulus-Contraction Coupling

Considerable evidence suggests that Ca^{2+} is the coupling agent between excitation and contraction in all forms of muscle (Bianchi, 1973). In different types of muscle or contractile systems the major source of calcium which initiates the contractile process differs. Recent developments in muscle biochemistry have suggested that the protein modulator may mediate the effects of Ca^{2+}, although

the role of the protein modulator appears to differ in different types of muscle.

A. Smooth Muscle

That myosin could be responsible for the regulation of actin-myosin interaction (thick filament regulation) by Ca^{2+} was first demonstrated in molluscam muscles (Kendrick-Jones et al, 1970) and from comparative studies (Lehman et al, 1972) was initially considered to be a property exhibited only by myosin from certain invertebrate species. More recently, studies of vertebrate smooth muscle have indicated that in this system, Ca^{2+} regulation is also a myosin-linked phenomena (Bremel, 1974). Sobieszek and Small (1976) have extended the observations of Bremel (1974). They have shown that smooth muscle myosin (chicken or turkey gizzard) binds Ca^{2+} and interacts with F-actin from either smooth or skeletal muscle to produce actomyosins that exhibit a Ca^{2+}-dependent actin-activated ATPase. Mixtures of smooth muscle thin filaments with skeletal muscle myosin were Ca^{2+} insensitive (ie. no thin filament regulation). Furthermore, the results of proteolytic digestion experiments suggested that the 20,000 dalton light chain (RLC) of the chicken or turkey gizzard myosin might be necessary for Ca^{2+} sensitivity. More direct evidence for the involvement of the RLC chain of smooth muscle in the Ca^{2+} regulatory process

has come from the demonstration (Sobieszek, 1977) that phosphorylation of this light chain is triggered by the same Ca^{2+} concentration required to activate the ATPase activity of actomyosin. The degree of phosphorylation of the myofibrils was proportional to their measured Ca^{2+} sensitivity. The phosphorylation process was very rapid and essentially completed before the rise in ATPase activity. While the enzyme responsible for the Ca^{2+} dependent phosphorylation was identified as a specific myosin light chain kinase, attempts at purification of the enzyme resulted in a loss of enzyme activity. Ikebe et al (1977) also reported Ca^{2+} dependent phosphorylation of the RLC of myosin, and in addition, dephosphorylation of myosin by a crude preparation of myosin light chain phosphatase.

The myosin light chain kinase has been purified by Dabrowska et al, (1977). They have reported that the kinase is composed of two proteins of molecular weights 105,000 and 17,000 daltons, none of which alone possessed any activity. Dabrowska et al (1978) have identified the 17,000 dalton component as the protein modulator. The results have therefore suggested that the protein modulator mediates the Ca^{2+} dependent phosphorylation of the RLC of smooth muscle. The results of Sobieszek (1977) and Dabrowska et al, (1978) suggest the mechanism of regulation of actin-myosin interaction in smooth muscle is based on the phosphorylation

and dephosphorylation of myosin. When a muscle cell is stimulated the Ca^{2+} concentration rises and forms an active Ca^{2+}-PM complex which serves to activate the myosin light chain kinase resulting in phosphorylation of the 20,000 dalton RLC of myosin, and activation of the actin-activated myosin ATPase. When the Ca^{2+} concentration is returned to resting levels, the myosin light chain kinase is inactive (the PM stimulation is removed) and the activity of an as yet uncharacterized phosphatase dephosphorylates myosin, resulting in loss of the actin activated myosin ATPase and muscle relaxation.

It is interesting to note that in scallop myosin, the first system to demonstrate myosin regulation of muscle contraction, phosphorylation of the RLC is not thought to occur. Instead it has been proposed that the binding of Ca^{2+} by the RLC of scallop myosin (in association with myosin) is the required event to allow actin and myosin interaction. The scallop myosin therefore represents a system in which Ca^{2+} exerts a regulatory role by direct interaction with the Ca^{2+} binding component of myosin, namely the regulatory light chain.

Adelstein et al,1977 have pointed out that Ca^{2+} dependent phosphorylation of the RLC of guinea pig vas deferens myosin results in increased actin-activated

myosin ATPase activity, while dephosphorylation decreases this activity. However, unlike vertebrate smooth muscle the actin-activated myosin ATPase activity of phosphorylated vas deferens myosin was inhibited by EGTA in the absence of kinase and phosphatase. The results suggest that the vas deferens myosin may be regulated by both Ca^{2+} dependent phosphorylation of the RLC (as is vertebrate smooth muscle) and direct interactions of Ca^{2+} with the RLC of myosin (as is molluscan myosin).

B. Skeletal Muscle

The original observation that myosin from rabbit skeletal muscle was a phosphoprotein (Perrie et al, 1972) sparked interest in the possible regulatory role of protein phosphorylation in myofibrils. Subsequently, Perrie et al (1973) identified that the RLC (the DTNB light chain) of myosin was phosphorylated at a single serine residue by the action of a kinase present in the sarcoplasm. That this 18,000 dalton RLC was phosphorylated by a specific Ca^{2+}-dependent light chain kinase was reported by Pires et al (1974). The myosin light chain kinase was purified to electrophoretic homogeneity by Pires and Perry (1977), who suggested that the enzyme was monomeric with a molecular weight of 77,000 daltons. In contrast, Yazawa and Yagi (1977) reported that rabbit skeletal muscle

myosin light chain kinase was composed of two protein components of molecular weight 100,000 and 20,000 daltons. The 20,000 dalton component was identified as a Ca^{2+}-binding protein. Independently Yagi et al (1978) and Waisman et al (1978) identified this Ca^{2+}-binding protein as the protein modulator. Therefore, in the presence of modulator protein, the activity of the myosin light chain kinase, and hence light chain phosphorylation is reversibly controlled by Ca^{2+}.

The phosphorylation of the RLC of rabbit skeletal muscle myosin in vivo has been investigated by Stull and High, (1977). They have reported that the light chains of myosin contained 0.50 mol phosphate per mol myosin in control muscles which increased to 0.90 with tetanic electrical stimulation. Intra-arterial injection of 1 n mol of isoproterenol increased cyclic AMP and phosphorylase a formation but had no effect on light chain phosphorylation. The results have indicated that phosphorylation of myosin can be stimulated in skeletal muscle in vivo in response to contractile activity. Similar results were reported in frog muscle by Barany and Barany (1977).

At present there is no direct evidence that the RLC of skeletal muscle are involved in Ca^{2+} regulation. In fact phosphorylation or removal of the RLC results in no change in the actin-activated myosin ATPase activity

(Weeds and Lowey, 1971). The only regulatory role of Ca^{2+} appears to be via its binding to troponin. Indirect evidence has been provided by Lehman (1978), who has demonstrated that skeletal muscle myosin can be activated by pure actin in a Ca^{2+}-dependent fashion when assayed at 120 mM NaCl. Considering the weak binding of Ca^{2+} by myosin as compared to troponin the possible significance of a potential myosin regulated system in skeletal muscle remains to be evaluated.

C. Non Muscle Myosin

The model system for investigation of non muscle myosin has been the human platelet. Human platelet myosin is similar to other non-muscle myosins, has a molecular weight of 460,000, and is composed of two heavy chains (200,000) and two different light chains (20,000 and 15,000) (Adelstein and Conti, 1972). The 20,000 dalton light chain can be phosphorylated and the phosphorylation results in increased actin-activated myosin ATPase, and an increase in the maximum isometric tension (Lebowitz and Cooke, 1978). Dephosphorylation decreases this activity (Adelstein et al, 1976). However the kinase catalyzing this phosphorylation is not Ca^{2+} sensitive (Daniel and Adelstein, 1976, Adelstein et al, 1977). It therefore appears that Ca^{2+} is not involved in the regulation of non muscle myosin.

8. Possible Involvement of Protein Modulator in Troponin

The regulation of skeletal muscle actin-activated myosin ATPase is thought to involve components tropomyosin and troponin. Troponin is composed of three subunits troponin-T, troponin-I, and troponin-C. The homology of the physical and chemical properties of troponin-C and protein modulator was initially reported by Wang et al (1975). Subsequently Dedman et al (1977a) have examined the possible functional homology of troponin-C and protein modulator, and have demonstrated that troponin-C can substitute for protein modulator in the activation of phosphodiesterase although at 600 fold the concentration of protein modulator Dedman et al (1977a) have also reported that protein modulator can form a complex with TN-I and TN-T (TN-T—TN-I—PM) and therefore substitute for TN-C. This hybrid complex was found to inhibit the actin-activated myosin ATPase activity of the reconstituted system 50% in the presence of Ca^{2+} and 20% in the absence of Ca^{2+}. In contrast the native troponin had little effect on the actin-activated myosin ATPase activity in the presence of Ca^{2+} but when Ca^{2+} was absent, the enzyme activity was decreased to 60%. These results suggested the existence of functional differences between native troponin and the hybrid complex.

Amphlett et al, (1976) have also reported that

protein modulator can substitute for TN-I and form a TN-T—TN-I—PM complex. Of interest was the observation that the protein modulator also possessed the property of interacting with troponin I to form a TN-I—PM complex which in the presence of tropomyosin could restore Ca^{2+}-sensitivity to the reconstituted actomyosin ATPase of skeletal muscle. The TN-C—TN-I complex was capable of restoration of only a small degree of Ca^{2+} sensitivity. It therefore appears that protein modulator can form functional complexes with TN-I or with TN-I and TN-T.

The substitution of TN-C by protein modulator appears to have doubtful *in vivo* significance, in muscle cells, It has been suggested however that protein modulator might have a role in the regulation of non-muscle contractile systems, similar to the role of troponin-C in muscle cells. As yet no direct evidence in favor of this suggestion has been reported.

9. Protein Modulator Regulated Glycogenolysis

Meyer *et al* (1964) first postulated that Ca^{2+} might couple muscle contraction and glycogenolysis. This theory was based on the observation that EGTA, a Ca^{2+} chelating agent was a potent inhibitor of phosphorylase kinase. Subsequently, the Ca^{2+} concentration required for

half-maximal activation of phosphorylase kinase was determined by Ozawa et al, (1967) to be about 10^{-7} M Ca^{2+}.

Equilibrium binding studies have established that phosphorylase kinase binds Ca^{2+} with high affinity. Brostrom et al, (1971) determined that at low ionic strength and in the absence of Mg^{2+} two classes of Ca^{2+} binding sites exist on phosphorylase kinase with K_d 0.3 X 10^{-7} M and 3 X 10^{-6} M. More recently Kilimann and Heilmeyer (1977) reported the existence of a single class of Ca^{2+} binding sites, K_d (Ca^{2+}) of 5.5 X 10^{-7} M capable of binding 8 mol Ca^{2+} per mol phosphorylase kinase at an ionic strength of 0.10 M NaCl. When 20 mM Mg^{2+} was included at constant ionic strength two classes of Ca^{2+} binding sites were reported, a high affinity class binding 8 mol Ca^{2+} with K_d (Ca^{2+}) of 3.0 X 10^{-6} M and a second lower affinity class binding 4 mol Ca^{2+} with K_d (Ca^{2+}) of 3.5 X 10^{-5} M. The K_d (Mg^{2+}) was determined to be 1.7 x 10^{-3} M. It therefore appears that in the presence of Mg^{2+} the phosphorylase kinase holoenzyme binds 12 mol of Ca^{2+}.

Several studies have suggested that Ca^{2+} stimulates phosphorylase kinase activity by affecting the interaction between phosphorylase kinase and its substrate phosphorylase. Heilmeyer et al, (1970) reported that the Km for phosphorylase b decreased approximately 25-fold when the concentration of Ca^{2+} was increased from about

10^{-8} to 10^{-5}M. In support of this work Gergely et al, (1975) found that the protein complex between phosphorylase and phosphorylase kinase was dissociated by the addition of EGTA.

Cohen (1973) and Hayakawa et al (1973) have established that phosphorylase kinase is composed of three types of subunits termed α, β and γ which have molecular weights of 145,000, 128,000 and 45,000 respectively. Since the native enzyme has a molecular weight of 1.28×10^{-6} daltons the subunit structure has been proposed to be $(\alpha\ \beta\ \gamma)_4$. Cohen et al, (1978) have recently reported the existence of a fourth subunit of phosphorylase kinase termed the δ subunit. This subunit was identified as the protein modulator. Unlike the Ca^{2+}-dependent interaction between protein modulator and phosphodiesterase, the protein modulator could not be dissociated from phosphorylase kinase by gel filtration in the absence of Ca^{2+}. This tightly bound protein modulator was reported in near stoichiometric amounts with the other three subunits of phosphorylase kinase (0.7 mol/mol subunit). This would suggest 3 mol of protein modulator per mol of phosphorylase kinase holoenzyme. Assuming 4 mol of Ca^{2+} bound per mol of protein modulator this would result in the binding of 12 mol of Ca^{2+} per mol of phosphorylase kinase; a value in agreement

with the Ca^{2+} binding studies of Kilimann and Heilmeyer (1977). The observation that exogenous protein modulator is capable of stimulation of phosphorylase kinase activity (Cohen et al, 1978) has been interpreted to suggest that protein modulator may also interact with phosphorylase kinase by a Ca^{2+}-dependent and reversible mechanism. The stoichiometry of interaction of this "loosely" bound protein modulator with phosphorylase kinase has not been determined. As will be presented in the Results section, the effect of modulator protein on phosphorylase kinase has also been observed in our laboratory.

10. Possible Protein Modulator Regulation of Mitosis

Using immunofluorescence technique Welsh et al, 1978 have shown that protein modulator decorates the stress fibers of non-muscle interphase cells. As the cells enter prophase, the distinct cytoplasmic localization of the protein modulator disappears commensurate with the dissolution of the cytoskeleton, and the protein modulator assumes a random distribution. At prometaphase it is localized in association with the half-spindles of the mitotic apparatus, and through metaphase and most of the anaphase the protein modulator remains localized between the chromosomes and the poles of the spindle. During the late anaphase this protein is found in the interzone region but rapidly condences into two small regions one on each side of the

midbody that separates the daughter cells. The protein modulator was not localized in the cleavage furrow during telophase. Based on these results Welsh et al, (1978) have suggested a possible role of protein modulator in the mediation of the Ca^{2+} effects on the mitotic apparatus and hence chromosomal movement.

Fujiwara and Pollard (1976) have used indirect immunofluorescence to demonstrate myosin-specific fluorescence in the cleavage furrow but not the spindle. These results and others have suggested the role of actomyosin during mitosis might be cytokinesis. The observation that protein modulator is not localized in the cleavage furrow would appear to preclude a role of protein modulator in association with actomyosin. Alternately, protein modulator could be involved with the Ca^{2+} dependent inhibition of microtubule assembly (the depolymerization of kineticope-associated microtubules is necessary for chromosomal movement to the poles). The distinctive localization of protein modulator in the mitotic half-spindle where microtubule depolymerization occurs during anaphase chromosome movement has further suggested an involvement of protein modulator in the control of the state of microtubule assembly during mitosis.

The Ca^{2+}-dependent regulation of microtubule

assembly-disassembly by the protein modulator has been studied *in vitro* by Marcum *et al*, (1978). It was determined that stoichiometric concentrations of protein modulator both inhibited and reversed microtubule assembly in a Ca^{2+}-dependent manner; in the absence of protein modulator Ca^{2+} (10 μM) caused only a slight reduction in polymerization whereas in the presence of both Ca^{2+} and the protein modulator inhibition of microtubule assembly was observed. Troponin-C was found to also inhibit and reverse microtubule assembly. The biochemical and immunofluorescent localization results therefore provide evidence for the Ca^{2+}-dependent regulation of microtubule assembly by protein modulator.

V. EXPERIMENTAL PROCEDURES

1. Purification Procedures

A. Preparation of Crude Animal Extracts - Animals were stored frozen at -20° before use. The frozen animals were chopped into small pieces and homogenized with a polytron for one minute in five volumes of 40 mM Tris/HCl, ph 7.5. After homogenization, samples were strained through cheesecloth, centrifuged at 10,000 x g for thirty minutes, and the supernatant dialyzed overnight against two changes of four liters of 40 mM Tris/HCl, ph 7.5. The extracts were then heated in a boiling water bath (95-100°C) for six minutes; precipitated protein was removed by centrifugation, and analysis performed on the supernatant. An aliquot of undialyzed homogenate supernatant was usually adjusted to 75% ammonium sulfate, centrifuged, and the precipitate dialyzed against 40 mM Tris/HCl. This dialyzed 75% ammonium sulfate precipitate was analyzed for phosphodiesterase activity.

B. Preparation of Crude Plant Extracts - Extracts of plant specimens were obtained by homogenization of chopped frozen plants for three minutes with a Waring blender, in three volumes of 100 mM Tris/HCl, 2 mM EDTA, ph 7.5 (the algae extract only was sonicated for one minute at 45% intensity). The slurry was centrifuged at 750 x g for

forty-five minutes and the supernatant strained through cheesecloth, adjusted to 75% ammonium sulfate, and allowed to stir slowly for thirty minutes, then centrifuged at 10,000 x g for thirty minutes. The precipitated protein was solubilized and dialyzed against 40 mM Tris/HCl, 1 mM Mg^{2+}, 50 μM Ca^{2+} overnight, then heated in a boiling water bath for five minutes: precipitated protein was removed by centrifugation and analysis performed on the supernatant. The tissues examined included root (beet, turnip, and onion), fruit (cucumber), whole plant except root (broccoli).

C. Protein Preparations - Modulator binding protein was prepared according to the procedure of Wang and Desai (1977). N-tosyl-phenylalanine, chloromethyl ketone-treated trypsin was obtained from Worthington Biochemicals. Snake venom 5'-nucleotidase was obtained from Sigma. Type II histone and protamine sulphate were obtained from Sigma Chemical Co. Casein was obtained from Nutritional Biochemicals Corp. Glycogen phosphorylase b, phosphorylase b kinase, mixed myosin light chains, glycogen synthase, and the inhibitor protein of c-AMP-dependent protein kinase were all prepared from rabbit skeletal muscle according to the procedure of Fisher and Krebs (1958), Hayakawa et al

(1973), Pires and Perry (1977), Soderling et al (1970), and Walsh et al (1971) respectively. Tubulin was prepared according to Weisenberg et al (1976). Acetyl CoA carboxylase (chicken liver) was a generous gift from Dr. K. Dakshinamurti, Department of Biochemistry, University of Manitoba. Troponin, troponin I, and troponin-C were a generous gift of Dr. Cyril Kay, Department of Biochemistry, University of Alberta. Bovine brain protein modulator was prepared according to Wang and Desai (1977).

2. PHYSICAL AND CHEMICAL METHODS

A. Reduction and Alkylation - Protein samples were dissolved to a concentration of 2 mg/ml in 0.2 M Tris/HCl, ph 8.5, containing 6 M guanidine HCl,EDTA (1 mg/ml), and 0.1 M dithiothreitol. After two hour incubation at room temperature an equal volume of 0.2 M Tris/HCl, pH 8.5, containing 0.05 M iodoacetic acid was added to the reaction mixture. Following two hour incubation at room temperature the sample was extensively dialyzed versus distilled water and freeze-dried.

B. Amino Acid Analysis - Samples (0.3 to 0.6 mg) of reduced and alkylated protein were hydrolyzed with 6 N HCl containing 2 µl of thioglycolic acid and 50 µl of 5% phenol at 110°, in sealed evacuated tubes for 21, 48, and 72 hours. Analysis was performed on a Spinco 120/139

amino acid analyser as outlined in the Spinco manual.

C. Digestion with Trypsin and Peptide Mapping - The protein sample (0.5 mg) was dissolved in 100 µl of 0.1 M ammonium bicarbonate, 0.1 M EGTA. After saturation with nitrogen and the addition of 5 µl of N-tosyl-L-phenylalanine chloromethyl ketone trypsin (16 mg/ml in 0.1 M ammonium bicarbonate) the tube was covered and incubated at 37^{o} for two hours. The reaction was stopped by the addition of 10 µl of 0.2 N HCl, applied to Whatman No. 3 MM paper and subjected to peptide mapping. High voltage electrophoresis was performed in a Savart electrophoresis tank at pH 4.7 according to Tan and Stevens (1971) using methyl green (1%) as marker. Descending chromatography was carried out in the other dimension using 1-butanol/pyridine/acetic acid/water (120:80:24:96), V/V)) as the solvent. After drying, peptide spots were detected with the ninhydrin-collidine reagent (Margoliash and Smith, 1962).

D. Trypsin Digestion of Crude Extracts - The effect of trypsin on crude extract protein modulator activity was performed at a trypsin concentration of 0.1 mg/ml. The crude extract was incubated with the trypsin for one hour at 30^{o}, pH 7.5 in the presence of 0.1 mM EGTA. The reaction

was stopped with the addition of lima bean trypsin inhibitor (10/1) and assayed according to Teo et al (1973).

E. Analytical Ultracentrifugation - Analytical ultracentrifugation was carried out with a Beckman-Spinco model E analytical ultracentrifugation. Sedimentation velocity experiments were run at 53,000 rpm and 19.8° using Schlieren optics. Sedimentation equilibrium runs were carried out at 19.8°, and at a rotor speed of 12,933 rpm. Both Rayleigh and Schlieren optics were used. The buffer density was measured with a pycnometer. The partial specific volume, V of the protein sample was calculated from the amino acid composition of the protein according to Cohn and Edsall, (1943).

F. Removal of Ca^{2+} from Reagents - Chelex 100, a resin specific for chelating divalent cations, was used for removing contaminating Ca^{2+} from the reagent. The resin was washed once with 1 N HCl and then with 1 N NaOH prior to the packing of the column. The packed columns were then washed with double-distilled water. Tris/HCl (0.5 M), imidazole (1.0 M) and double-distilled water were separately passed through Chelex 100 column (6 x 1.5 cm) to remove Ca^{2+}. Purified reagents were stored in plastic vessels, and plastic columns and connections

were used in the chromatography. Calcium concentration was monitored by a Perkin-Elmer atomic absorption spectrophotometer, model 303 and the calcium content of stock reagents, after Chelex 100 treatment was below the limit of detection (4 ppm). Calcium was removed from the protein modulator and phosphodiesterase by treatment with 1.0 mM EGTA for thirty minutes at 4^{o} followed by gel filtration on Sephadex G-25 (45 x 1.5 cm) to remove the chelating agent. Chelex-100 treated water and buffer was used in all steps.

G. Equilibrium Ca^{2+} Binding - The gel filtration method of Hummel and Dreyer (1962) as modified by Fairclough and Fruton (1966) was used. A column (45 x 0.9 cm) of Sephadex G-25 was equilibrated at 22^{o} with buffer containing 25 mM Tris/HCl, 25 mM imidazole, and 3 mM magnesium acetate with a known concentration of Ca^{2+}. A sample of desalted protein modulator (4.8 µg in 0.6 ml) was then applied to the column and the column was eluted with the equilibrating buffer. Gel filtration was carried out at 22^{o} at a flow rate of 5 ml/hr and 0.6 ml fractions were collected. Aliquots (100 µl) of each fraction were analyzed for radioactivity in a Beckman LS-R50 liquid scintillation spectrometer. The column used was a plastic Pharmacia K9 column Chelex 100 treated reagents were used throughout. Scintillator mixture was composed of 125 g

of naphtalene, 7.5 g of 2,5-diphenyloxazole and 0.375 g of 1,5-bis [2-(5-phenyloxazolyl)] benzene/liter of dioxane.

3. ASSAY PROCEDURES

A. Assay of Phosphodiesterase - The activity of cyclic nucleotide phosphodiesterase was measured according to Teo et al (1973). The procedure involved coupling of the phosphodiesterase reaction to a 5'nucleotidase reaction (c-AMP $\xrightarrow{PDE}$ 5'AMP $\longrightarrow$ adenosine + Pi) followed by analysis of the resulting phosphate. Phosphodiesterase was incubated with 40 mM Tris, 40 mM imidazole, 5 mM magnesium acetate 1.2 mM cyclic AMP, and 0.3 U 5'nucleotidase.

At low concentrations of cyclic nucleotide, the assay was performed according to Wickson et al (1975). Essentially, each reaction mixture (0.2 ml) contained 44 mM Tris/HCl, 3 mM magnesium acetate, and 1 mM or 10 µM cyclic nucleotide. The reaction was started by the addition of substrate. After incubation at 30° for 30 minutes, the reaction was terminated by addition of 0.01 ml of 55% trichloroacetic acid; an aliquot of 50 µl was spotted along with appropriate carriers (0.1 µ mol of cyclic nucleotide, nucleotide, and nucleoside on Whatman No. 3 MM paper and chromatographed (descending) for 19 hours

using 1 M ammonium acetate, 95% ethanol (3:7, V/V). The papers were air dried and the areas containing cyclic nucleotide and nucleoside (visualized by ultraviolet light) were cut out and the radioactivity was determined by scintillation spectrometry. Reaction velocity was determined from the percent conversion of cyclic nucleotide to nucleotide and nucleoside.

B. Assay of Protein Modulator - The activity of the modulator protein was measured on the basis of its activation of cyclic nucleotide phosphodiesterase under conditions where the amount of modulator protein was limiting (Teo et al, 1973). One unit was defined as the amount of modulator protein which gave rise to half-maximal activation of a standard amount of the enzyme.

C. Assay of Modulator Binding Proteins - Modulator binding proteins are assayed by their inhibitory activity against Ca^{2+}-activated phosphodiesterase. The extent of inhibition by the test sample of a standard phosphodiesterase reaction containing 4 U/ml of the modulator protein and 0.012 - 0.016 U/ml of the enzyme when fully activated. The amount of inhibitor giving rise to 50% inhibition was defined as 2 units. To measure modulator binding proteins in crude extracts a batchwise DE procedure for assaying modulator binding proteins in the presence of contaminating

protein modulator was used (Wang and Desai, 1977).

D. Protein Kinase Assay - The filter paper method of Reimann et al (1971) was used for the assay of protein kinase. Myosin light chain kinase was assayed in a reaction mixture containing 1.0 mg/ml of histone or light chain fraction, 20 mM Tris/HCl, 0.25 mM [γ^{-32}P]ATP of specific activity 20 to 100 cpm/pmol, 40 mM β-mercaptoethanol, either 0.8 mM EGTA or 0.4 mM $CaCl_2$ plus 20 µg/ml of purified protein modulator, and 10 mM magnesium acetate. Typically in time course experiments, 0.020 ml of a total volume of 0.2 ml was spotted while in other experiments 0.035 ml of a total volume of 0.050 ml was spotted and analyzed for protein bound phosphate. Phosphorylase kinase activity was determined by following ^{32}P incorporation into phosphorylase according to the method of Hayakawa et al (1973). One unit of phosphorylase kinase activity was defined as the amount of the enzyme catalyzing the incorporation of 1 µ mol of phosphate into phosphorylase b per minute at 30°.

4. OTHERS

A. Protein Concentration - Protein concentration was determined by the method of Lowry et al (1964) or Bradford (1976) using bovine serum albumin as standard. The concentration of pure phosphorylase and phosphorylase kinase was

determined spectrophotometrically at 280 nm using absorbance indices of 13.1 and 12.4 respectively for 1% solutions (Cohen, 1973).

B. Acrylamide Gel Electrophoresis - Discontinuous gel electrophoresis was carried out according to Davis (1964). Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborne (1969) or Hayakawa et al (1973). Sodium dodecyl sulfate-urea gels were carried out according to Swank and Munkres (1971). In all gel systems 0.05% Coomassie Blue in 25% isopropyl alcohol and 10% acetic acid was used as stain (Fairbanks et al, 1971). Gels were destained in 50% methanol, 7 1/2% acetic acid. The isoelectric point was measured by analytical isoelectric focussing in polyacrylamide gels according to Vesterberg (1971).

C. Electrophoretic Analysis of Protein Bound Phosphate - Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Weber and Osborne (1969). Samples of phosphorylated protein were boiled for three minutes in the presence of 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. For the determination of ^{32}P incorporation into protein a modification of the procedure of Basch (1968) was used. After

electrophoresis and staining with Coomassie Blue the gel was sliced manually and dried overnight at 50°. Each slice was then digested with 0.6 ml of NCS solubilizer in the presence of 0.1 ml of double-distilled water at 50° for 3 ½ hours. Radioactivity (^{32}P) was then determined in a Beckman LS250 scintillation spectrophotometer after the addition of 0.02 ml of glacial acetic acid followed by 12.5 ml of Omnifluor scintillation mixture.

D. Preparation of Protein Modulator Affinity Column - A sepharose 4B bound protein modulator conjugate was prepared by a slight modification of the method of Watterson and Vanaman (1976). Washed sepharose 4B was adjusted with distilled H_2O to produce a 1/1 (V/V) slurry. The slurry (80 mls) was allowed to slowly stir at 0° while two grams of cyanogen bromide crystals were added; the pH was maintained at pH 11.0 by addition of 10 M NaOH. After thirty minutes, the gel was washed with two liters of water followed by two liters of 0.5 M sodium bicarbonate pH 8.5 and 1 mM $CaCl_2$. To the slurry (1/1) was added 15 mg of protein modulator in bicarbonate-calcium buffer, followed by incubation at 4° with gentle agitation, overnight. After washing the slurry with one liter of bicarbonate-calcium buffer, the gel was dried and added to 50 ml of 1 M ethanolamine/HCl, pH 8.5. After two hours of slow stirring, the gel was throughly washed with distilled water and storage buffer

(40 mM Tris, 1 mM Mg, 50 μM Ca^{2+}, pH 7.5).

5. Materials - $^{45}CaCl_2$[^{3}H] c-AMP, [^{3}H] c-GMP, and γ^{32}P] ATP were purchased from New England Nuclear Corporation. DEAE-cellulose (DE52) was obtained from Whatman and Sephadex G-200, G-100, G-75 from Pharmacia. Live earthworms were obtained from Conroy Live Bait of Toronto, Ontario. Cyanogen bromide was obtained from Pierce Chemicals (Rockford, Ill). Iodoacetic acid, EGTA, Chelex 100 resin, PMSF, c-AMP and c-GMP were purchased from Sigma Chemical Co. All other chemicals were reagent grade.

EXPERIMENTAL RESULTS

VI. PHYLOGENETIC DISTRIBUTION OF PROTEIN MODULATOR

1. Animal Studies

In order to study the phylogenetic significance of protein modulator mediated Ca^{2+} regulation, the distribution of the protein modulator was investigated. Animals representative of the major phyla were chosen for the study. Crude extracts consisting of heat treated, dialyzed, homogenate supernatants were prepared and analyzed for their ability to activate protein modulator deficient mammalian cyclic nucleotide phosphodiesterase. Representative data are presented in figure 1. The protein modulator deficient bovine heart cyclic nucleotide phosphodiesterase was activated by the crude extracts to approximately the same maximal enzyme activity, as the activation of phosphodiesterase by the purified bovine heart protein modulator. Furthermore, activation appears to depend on the presence of free Ca^{2+}, since EGTA at 300 μM inhibits these activations.

In addition to the Ca^{2+} activation by the protein modulator, mammalian cyclic nucleotide phosphodiesterase may also be irreversibly activated by limited proteolysis (Cheung, 1967). To exclude the possibility that the activation of the bovine heart enzyme by animal extracts is due to proteolysis, the reversibility of enzyme

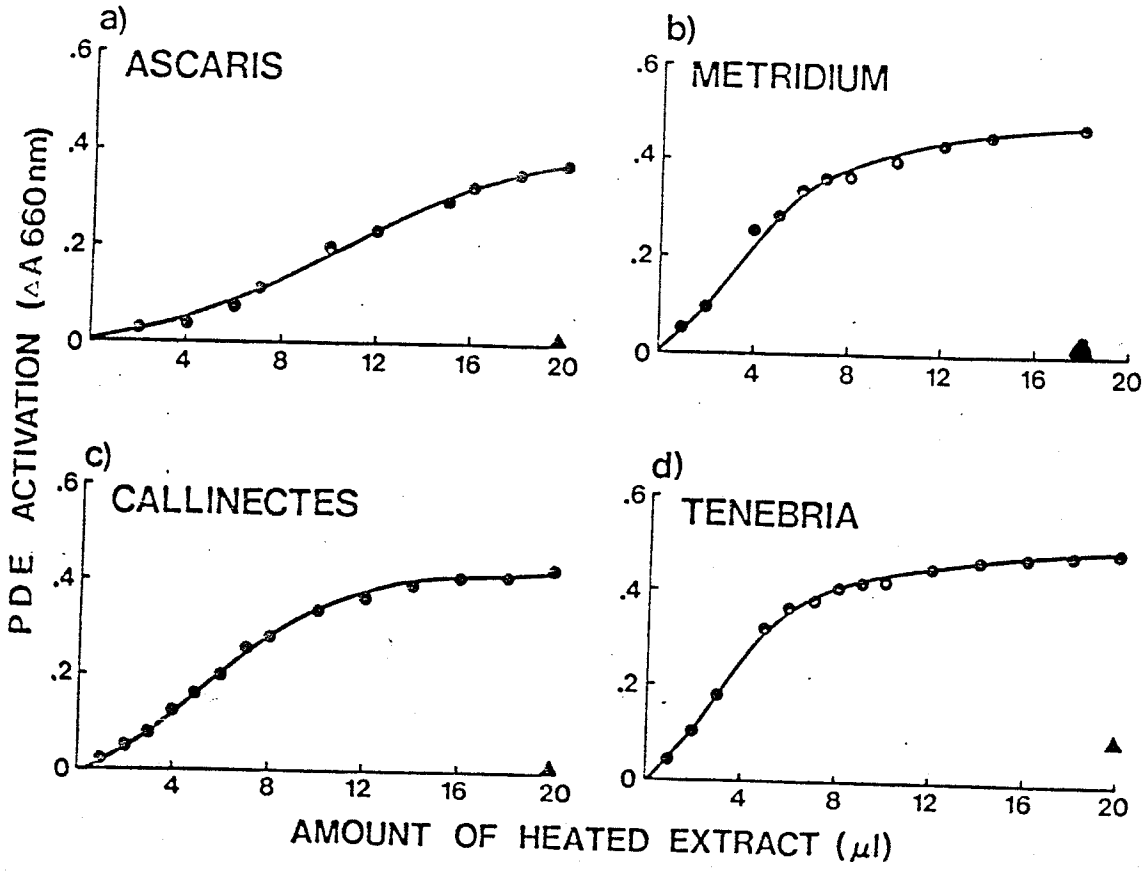


Figure 1. Activation of mammalian cyclic nucleotide phosphodiesterase by crude animal extracts. Phosphodiesterase was assayed in the presence (▲) or absence (●) of 300 μM EGTA with various amounts of the heat-treated and dialyzed homogenate supernatants of a) Ascaris, the round worm b) Metridium, the sea anemone; c) Callinectes, the blue crab and d) Tenebria, the meal worm. Enzyme activation is expressed as the difference in activity between the activated and control sample (no added homogenate).

activations was examined . Results are presented in figure 2. Since activation of the enzyme by the crude extracts could be inhibited by the addition of EGTA, and this inhibition could be reversed by addition of excess Ca^{2+}, proteolytic activation of mammalian cyclic nucleotide phosphodiesterase by the animal extracts could be ruled out.

In view of the fact that the mammalian protein modulator is rapidly inactivated by trypsin, especially in the presence of EGTA (Ho et al, 1975), the susceptibility of the crude extract protein modulator activity to proteolysis was examined. Results are presented in Table 1. Trypsin treatment resulted in total destruction of protein modulator activity in the extracts, suggesting that the activating factors are protein. No loss in modulator activity was detected in control samples where trypsin was not added or where the addition of trypsin inhibitor preceeded the addition of trypsin.

An estimate of the relative amounts of protein modulator in the crude animal extracts was obtained. Table II shows that all the lower animal crude extracts contain higher levels of protein modulator activity, than the bovine heart crude extract. To facilitate the documentation of protein modulator in crude animal extracts

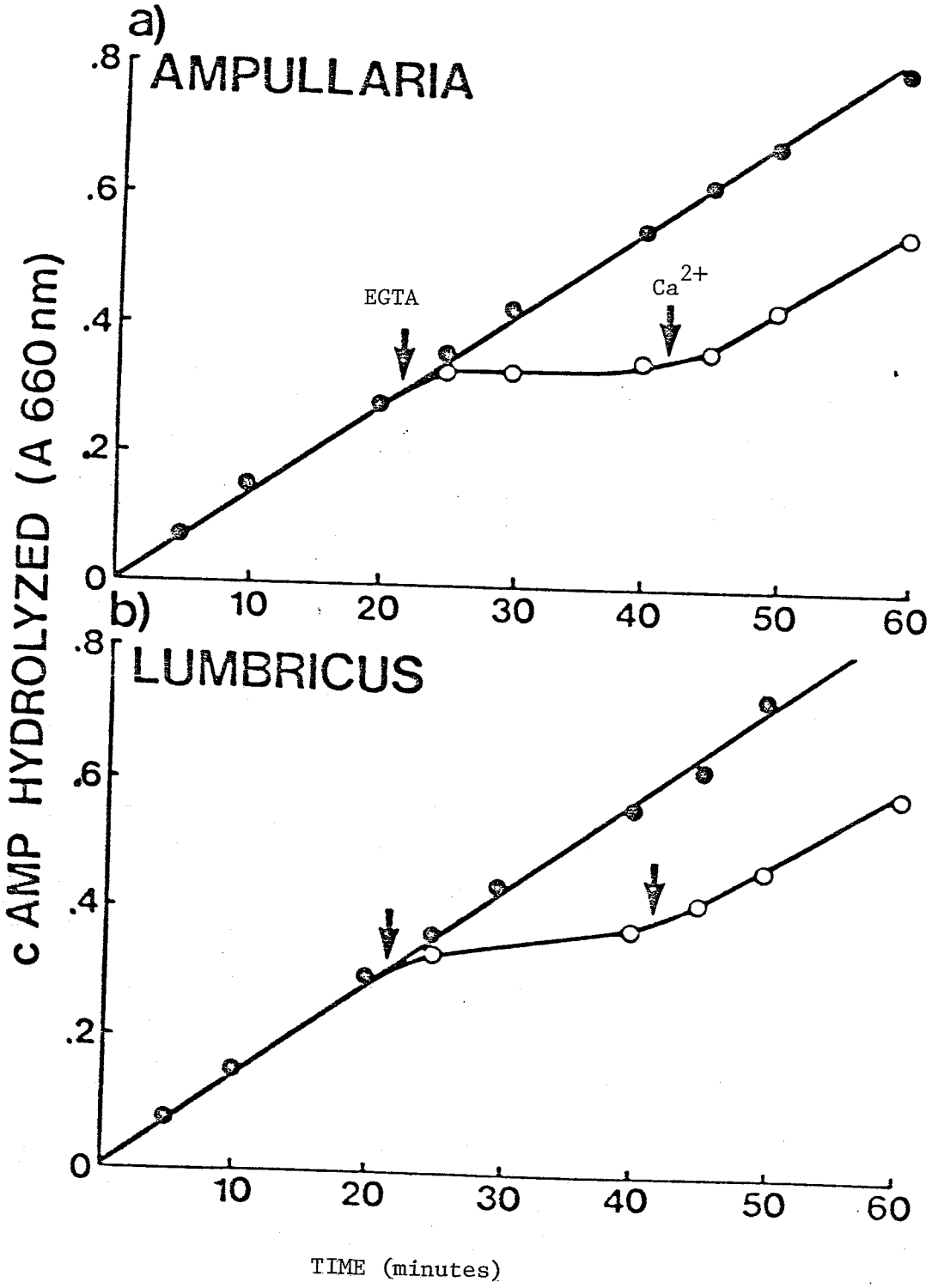


Figure 2. Reversibility of phosphodiesterase activation. Time course of bovine heart phosphodiesterase reactions in the presence of 100 μM Ca^{2+} and 50 μl of heat treated and dialyzed extracts of a) Ampullaria, the mystery snail and b) Lumbricus, the earthworm. For each experiment two identical incubation mixtures were prepared, one (o), was adjusted to 0.48 mM EGTA at twenty-two minutes and to 0.8 mM Ca^{2+} at forty-two minutes, the other (●) was maintained as a control. At various times after initiation of the reaction, aliquots were removed and analyzed for phosphate production.

TABLE 1

Effect of Trypsin on Crude Extract Protein Modulator

	PROTEIN MODULATOR ACTIVITY (A660nm)	
	BEFORE TRYPSIN	AFTER TRYPSIN
EARTHWORM	.534	0
BLUE CRAB	.545	0
STAR FISH	.510	0
SNAIL	.400	0
BOVINE HEART	.500	0

Heat treated and dialyzed extracts were incubated with 0.1 mg/ml trypsin for sixty minutes at 30^{o}, pH 7.5. The protein modulator activity is expressed as the difference in phosphodiesterase activity in the presence and absence of the animal extract. Controls (before trypsin) were incubated for sixty minutes at 30^{o}.

TABLE II

Estimation of the relative amounts of protein modulator in crude extracts.

ANIMAL			MODULATOR ACTIVITY (A660nm)	
COMMON NAME	GENUS	PHYLUM	TOTAL ACTIVITY unit/g tissue	SPECIFIC ACTIVITY units/mg protein
SEA ANEMONE	METRIDIUM	CNIDARIA	813	51
CLAM	ANODONTA	MOLLUSCA	500	27
SNAIL	AMPULLARIA	MOLLUSCA	1259	41
EARTH WORM	LUMBRICUS	ANNELIDIA	2376	110
ROUND WORM	ASCARIS	NEMATHELMINTHES	400	10
BLUE CRAB	CALLINECTES	ARTHROPODA	704	11
MEAL WORM	TENEBRIA	ARTHROPODA	960	20
STAR FISH	ASTERIAS	ECHINODERMA	2205	161
SPONGE	EUSPONGIA	PORIFERA	930	133
BEEF HEART	BOS	CHORDATA	45	2

a set of criteria for the demonstration of protein modulator in crude extracts was developed and is presented in Table III. If a crude extract satisfies all the criteria of Table III, then it is considered to possess protein modulator activity.

The results suggest that crude animal extracts contain heat stable, non dialysable protein factors capable of Ca^{2+} dependent and reversible activation of mammalian cyclic nucleotide phosphodiesterase. The activation of mammalian phosphodiesterase by crude extracts, or purified bovine heart protein modulator, to the same maximal enzyme activity suggests structural and functional homology between primitive and bovine heart protein modulator.

2. Plant Studies

Heat treated dialyzed, ammonium sulfate precipitated extracts of several higher plants and a single lower plant were examined for protein modulator activity. Figure 3 presents representative data of the activation of mammalian phosphodiesterase by crude plant extracts. In all cases the activations were found to be inhibited by EGTA, The activations were also susceptible to proteolysis (data not shown).

CRITERIA FOR THE PRESENCE OF PROTEIN MODULATOR ACTIVITY IN CRUDE EXTRACTS

1. THERE IS ACTIVATION OF MAMMALIAN CYCLIC NUCLEOTIDE PHOSPHODIESTERASE BY THE CRUDE EXTRACT.

2. THE PHOSPHODIESTERASE ACTIVATION IS BOTH Ca^{2+} DEPENDENT AND REVERSIBLE BY EGTA.

3. THE PROTEIN MODULATOR ACTIVITY IS SUSCEPTIBLE TO PROTEOLYSIS.

4. THE PROTEIN MODULATOR ACTIVITY IS RETAINED AFTER HEAT TREATMENT AND DIALYSIS.

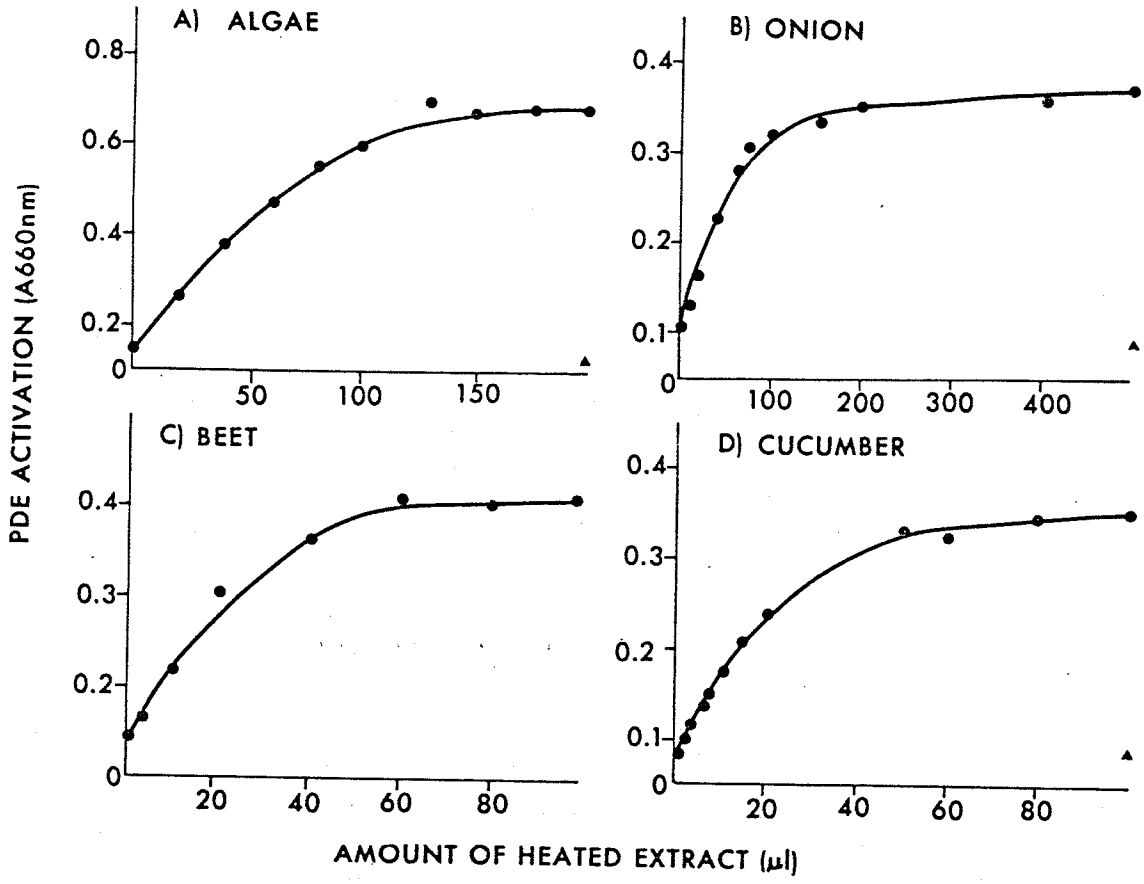


Figure 3. Activation of mammalian cyclic nucleotide phosphodiesterase by crude plant extracts. Phosphodiesterase was assayed in the presence (▲) or absence (●) of 400 µM EGTA with various amounts of the heat-treated and dialyzed plant extracts.

Furthermore, the activations were found to be Ca^{2+} dependent and reversible. Figure 4 presents typical data. An estimation of the relative amounts of protein modulator is summarized in Table IV. A comparison of the amount of protein modulator activity in plant extracts with a similarily prepared earthworm extract is provided. Of interest is the observation that protein modulator activity is present in the root, leaf, and fruit of the corn plant, and the tuber and sprout of the potato, therefore suggesting that in these plants, protein modulator is not localized in one specific region of the plant. Of possible correlation is the observation that in the mammal the protein modulator lacks tissue specificity (Smoake et al, 1974).

In conclusion, the plant specimens examined in this study satisfy the criteria for the demonstration of protein modulator in crude extracts, and therefore are suggested to possess protein modulator activity. The fact that plant extract protein modulator is capable of activation of mammalian phosphodiesterase to the same extent as the activation by purified bovine heart protein modulator further reinforces, and extends the suggestion that the structure and function of the protein modulator has been conserved during evolution.

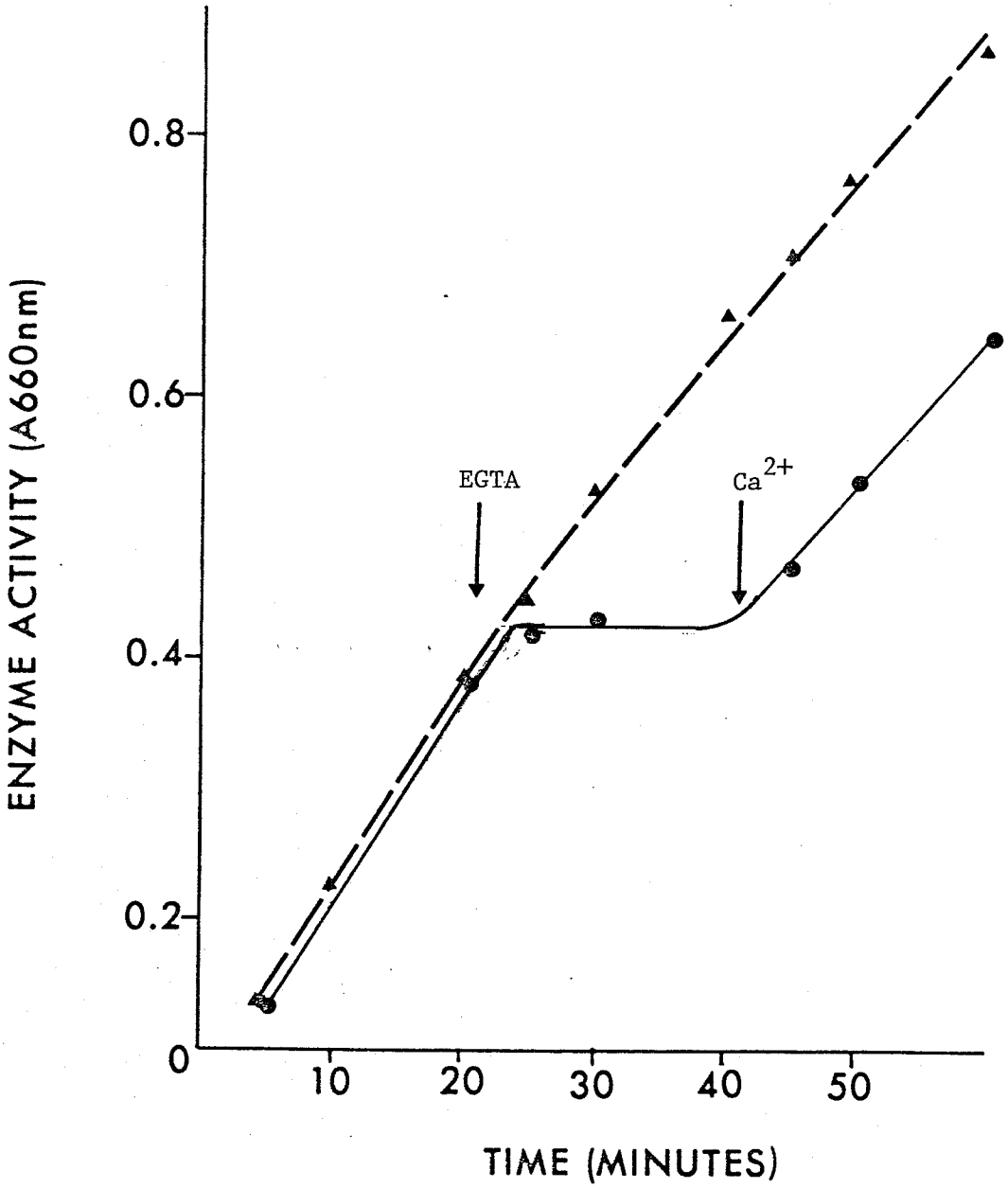


Figure 4. Reversibility of phosphodiesterase activation. Time course of bovine heart phosphodiesterase reaction in the presence of 100 µM Ca^{2+} and 200 µL of heat treated and dialyzed extract of Solanum, the potato. For each experiment two identical incubation mixtures were prepared, one (●) was adjusted to 1.0 mM EGTA at twenty-two minutes and to 2.0 mM Ca^{2+} at forty-two minutes, the other (▲) was maintained as a control. At various times after initiation of the reaction aliquots were removed and analyzed for phosphate production.

PROTEIN MODULATOR IN CRUDE PLANT EXTRACTS

COMMON NAME	PROTEIN MODULATOR ACTIVITY	
	TOTAL ACTIVITY (unit/g tissue)	SPECIFIC ACTIVITY (units/mg protein
BROCCOLI	380	494
TURNIP	140	1080
BEET	173	480
CABBAGE	180	1130
ONION	65	621
CUCUMBER	200	1250
CORN		
i) root	14	140
ii) leaf	34	195
iii) fruit	10	40
POTATOE		
i) tuber	21	23
ii) sprout	1	16
ALGAE	—	2000
EARTHWORM		2300

3. General Conclusions

The presence of protein modulator activity in crude plant and animal extracts suggests that the protein modulator is widely distributed in the animal kingdom and in higher plants. While the presence of protein modulator activity in the algae demonstrates that the protein modulator can exist in lower unicellular plants, further studies are necessary before the distribution of protein modulator in the lower plants can be evaluated.

The Ca^{2+} dependent and reversible activation of mammalian cyclic nucleotide phosphodiesterase by the crude extract protein modulator suggests that the ability of modulator to interact with Ca^{2+} and in particular, the role of the protein modulator in the mediation of the Ca^{2+}-linked regulation of enzyme activity has been conserved during evolution. However, the regulatory role in these lower lifeforms of ions other than Ca^{2+} cannot be ruled out.

It is important to point out the limitations of this study. The general conclusion that the structural and functional properties of the protein modulator are conserved is valid only as far as these properties relate to activation of mammalian phosphodiesterase. Conclusions regarding the structure of the protein modulator itself

can only be made, in so far as these structural elements are necessary for phosphodiesterase activation. The analysis of possible conservation of protein modulator structure, in general, is presented in section VII.

In conclusion, it appears that protein modulator mediated Ca^{2+} regulation may be of widespread physiological significance. Certainly the protein modulator is the most widely distributed member of the family of homologous Ca^{2+}-binding proteins.

VII. CONSERVATION OF THE STRUCTURAL AND FUNCTIONAL PROPERTIES OF THE PROTEIN MODULATOR DURING EVOLUTION

1. Analysis of the Physical-Chemical Properties of Crude Extract Protein Modulator.

The observation that crude extracts were capable of Ca^{2+} dependent and reversible activation of mammalian cyclic nucleotide phosphodiesterase has been interpreted to suggest that the structural and functional properties of the protein modulator, related to Ca^{2+} dependent activation of phosphodiesterase, have been conserved during evolution. To further test this hypothesis, a method for comparing the properties of crude extract protein modulator without purification has been utilized. Since the relative mobility of crude extract protein modulator activity

(measured as mammalian phosphodiesterase activation) on discontinuous polyacrylamide gel electrophoresis is dictated by both size and charge properties of the proteins, this method has the advantage of comparing the physical-chemical properties of the crude extract protein modulator, without purification.

To compare the physical-chemical properties of the crude extract protein modulator, crude extracts were subjected to discontinuous gel electrophoresis according to Davis (1964). The gel was sliced and the relative mobility of the protein modulator activity was determined, representative data are presented in figure 5. In addition to the data presented in figure 5, extracts of sponge, blue crab, mystery snail, and potato were analyzed on polyacrylamide gels and similar results were obtained. In all species examined the protein modulator activity could be located on the gel as a single activity band. Furthermore the relative mobilities of the crude extract protein modulator activity were all similar, with values ranging from 0.58 to 0.62. The relative mobility of the purified bovine heart activity band, or stained protein band was found to be 0.60.

A comparison of the physical-chemical properties between the crude extract protein modulator activity, and

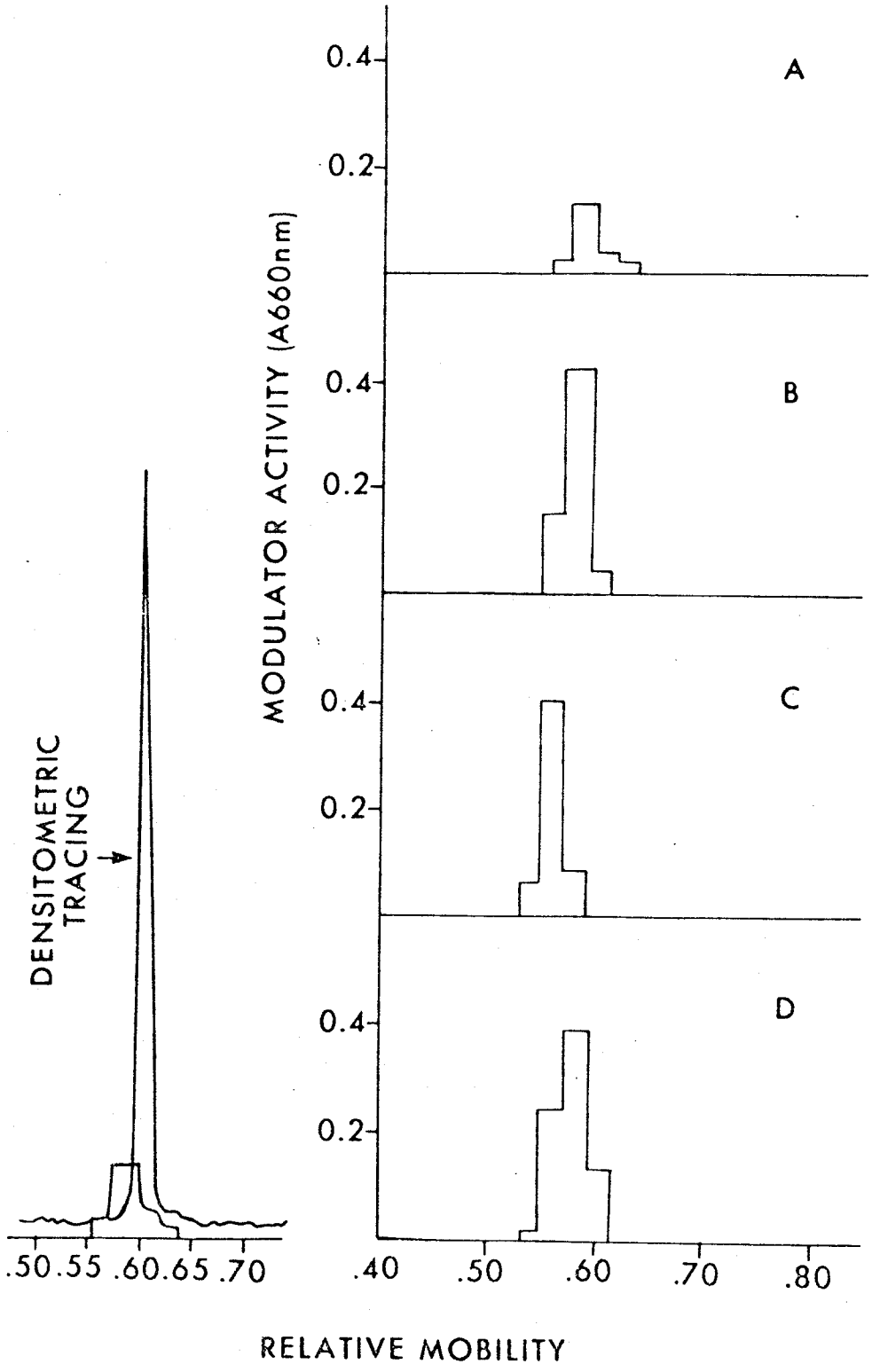


FIG. 3. Discontinuous gel electrophoresis of (*A*) crude earthworm (*B*) sea anemone, (*C*) lobster, and (*D*) starfish extracts according to Davis (26) for 15% polyacrylamide gels. Approximately 100 units of heat-treated dialyzed homogenate supernatants were applied to each gel; gels were sliced into 2-mm slices and each slice was extracted with 100 μl of pH 7.5, 20 mM Tris/HCl buffer, then assayed for Ca^{2+}-dependent bovine heart phosphodiesterase activation. This activity is plotted as $A_{660\,nm}$. *E*, densitometric trace of purified bovine heart modulator compared with crude earthworm modulator activator in relative mobility.

between the crude extract protein modulator and purified protein modulator from bovine heart has suggested that the physical-chemical properties of the protein modulator have been conserved during evolution, therefore confirming our initial hypothesis. The conservation of the physical-chemical properties of higher plant protein modulator is indicated since electrophoretic analysis of the potato yield typical results (Rm 0.61), however further studies are needed to test this possibility.

2. Purification and Characterization of the Protein Modulator of Lumbricus terrestris.

Studies of crude extracts have suggested that the physical-chemical properties of the protein modulator have been conserved during evolution. A more detailed analysis of conservation of protein modulator properties was undertaken. The protein modulator from a non vertebrate source (the earthworm) was purified and the physical, chemical and Ca^{2+} binding properties were examined. From a comparison of the properties of purified earthworm protein modulator and the purified modulator from bovine heart a more detailed analysis of conservation of protein modulator properties could be presented.

3. Purification of Earthworm Protein Modulator

Live earthworms were rinsed in distilled water to remove particulate materials, and then stored at -20^{o} until use. A typical preparation of earthworm modulator consumed one kg.

Extraction and Heat Treatment - Frozen earthworms were chopped into 2 to 4 cm. pieces, ground in a Hobart meat mincer and homogenized for twenty seconds with a Waring blender in three volumes of ice cold buffer containing 40 mM Tris/HCl, 1 mM magnesium acetate and 50 μM calcium chloride (buffer A) plus 5% phenylmethylsulfonylfloride solution (PMSF 6 mg/ml in 95% ethanol). The homogenate was centrifuged at 750 x g for forty-five minutes and the resulting supernatant was filtered through cheese cloth. The filtered extract was divided into 300 ml aliquots which were each placed into a 500 ml flask and then immersed in a boiling water bath for five minutes. After cooling in ice-water the heated extract was centrifuged at 10,000 x g for thirty minutes to remove denatured protein.

Ammonium Sulphate Fractionation - To the supernatant of the heat treated extract, solid ammonium sulfate was added in a stepwise manner with continual stirring to a final concentration of 60% saturation. The mixture was allowed

to stir slowly for about forty-five minutes and then centrifugated at 10,000 x g for thirty minutes. The supernatant was adjusted to 80% saturation of ammonium sulfate and then centrifuged at 10,000 x g for thirty minutes. The precipitate was suspended in buffer A, homogenized in a glass homogenizer and then slowly stirred for twenty minutes. The resulting solution was dialyzed overnight against buffer A.

DEAE-Cellulose Column Chromatography - To a DEAE-cellulose column (2.5 x 35 cm) pre-equilibrated with buffer A was applied the 80% ammonium sulfate precipitated fraction. After sample application the column was eluted with two bed volumes of buffer A then with 1 liter of salt gradient from 0.03 to 0.5 M NaCl in buffer A. Figure 6 shows that the modulator activity is separated from the main bulk of proteins, and peaks at 0.3 M NaCl. Fractions #120-160 were pooled and dialyzed overnight against buffer containing 40 mM Tris/HCl, 40 mM imidazole, 50 μM $CaCl_2$ pH 6.5 (buffer B). The dialyzed sample was then applied to a second DEAE-cellulose column (1.7 x 40 cm) pre-equilibrated with buffer buffer B. The column was first eluted with two bed volumes of buffer B, then with a linear salt gradient of 0.03 to 0.5 M NaCl in buffer B (500 mls). Results are presented in Figure 7. The

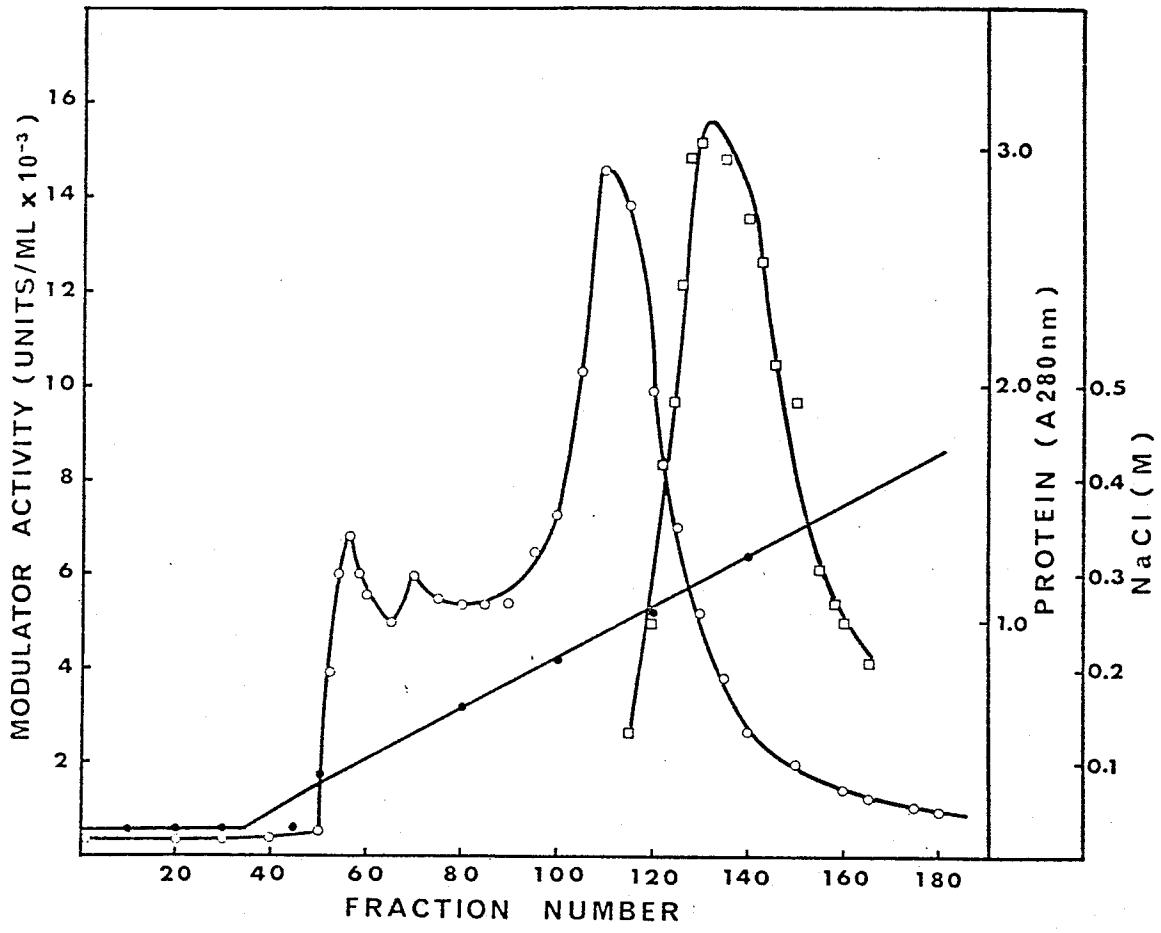


Figure 6. Chromatography of earthworm modulator on DEAE-cellulose. The dissolved $(NH_4)_2SO_4$ pellet obtained from one kilogram of frozen earthworms was chromatographed on the DEAE-cellulose column (2.5 x 35 cm) according to the text. The column fractions were analyzed for protein concentration (O), modulator activity (□), and ionic strength (●).

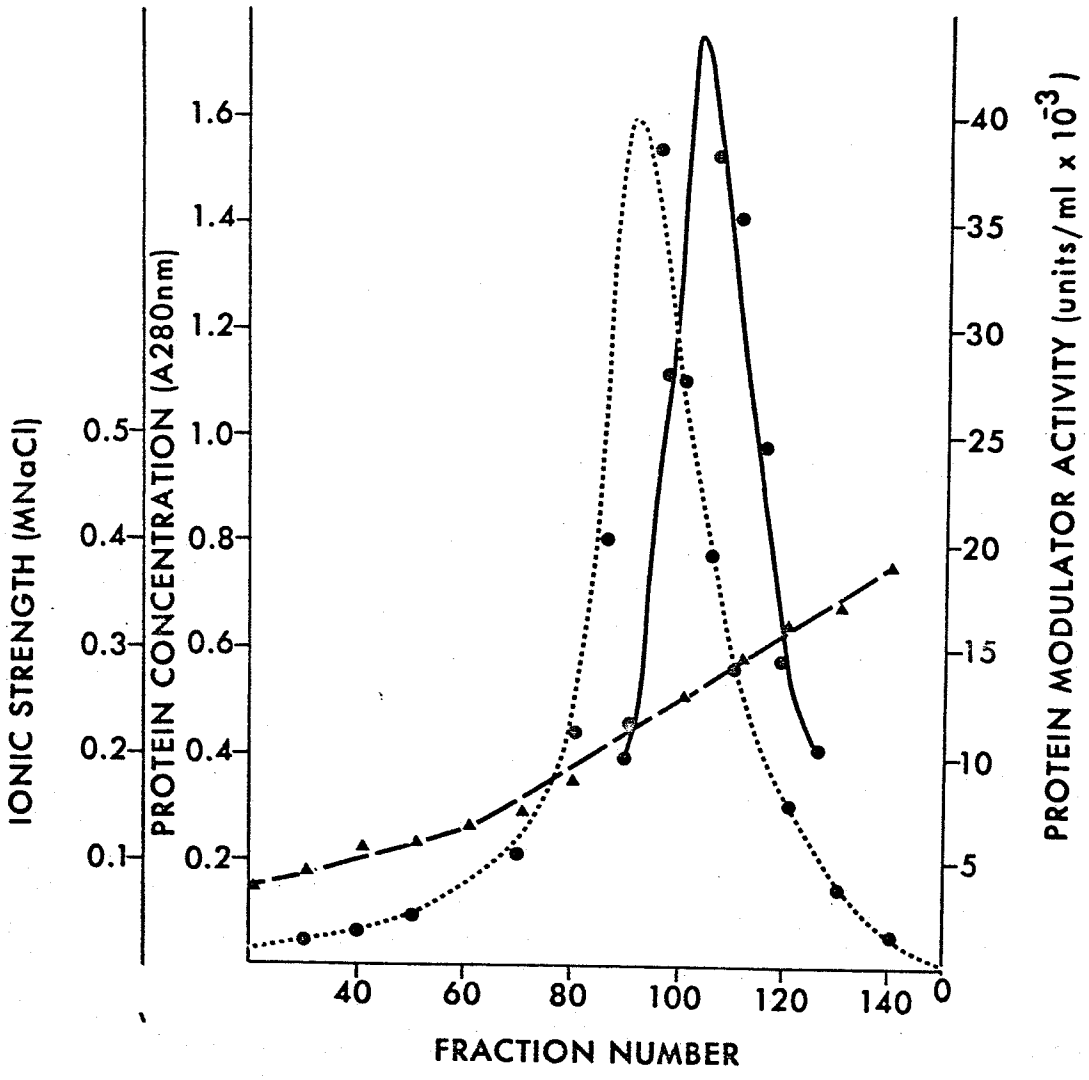


Figure 7. Second chromatography of earthworm modulator on DEAE-cellulose.
The pooled, dialyzed fractions of the first DEAE-cellulose column were reapplied to a second DEAE-cellulose column (1.7 x 40 cm) according to the text. The column fractions were analyzed for protein concentration (-●-) modulator activity (—●—), and ionic strength (▲).

main protein peak was eluted at 0.22 M NaCl and the protein modulator activity peak at 0.27 M NaCl. Fractions 92 to 122 were pooled and dialyzed overnight against double-distilled water and lyophilized.

Molecular Sieving Chromatography - The lyophilized modulator protein sample was dissolved in 2 ml of buffer A containing 0.1 M NaCl. The solution was applied to a Sephadex G-100 column (2.5 x 90 cm) which had been equilibrated with buffer A containing 0.1 M NaCl. Figure 8 shows the elution profile of the G-100 Sephadex column chromatography. While the G-100 Sephadex column was very effective in removal of most contaminating proteins, the elution profile suggests that all contamination has not been removed. The pooled protein modulator (fractions 94-106) from this column was dialyzed against water and lyophilized. The lyophilized sample was dissolved in buffer A containing 0.1 M NaCl and chromatographed on a G-75 Sephadex column. Figure 9 shows the elution profile of the G-75 Sephadex column chromatography. It can be seen that the protein modulator activity peak corresponds to the main protein peak. Furthermore, the profile suggests removal of small amounts of contaminant, even though a loss of specific activity occurred during the chromatography. The protein modulator activity

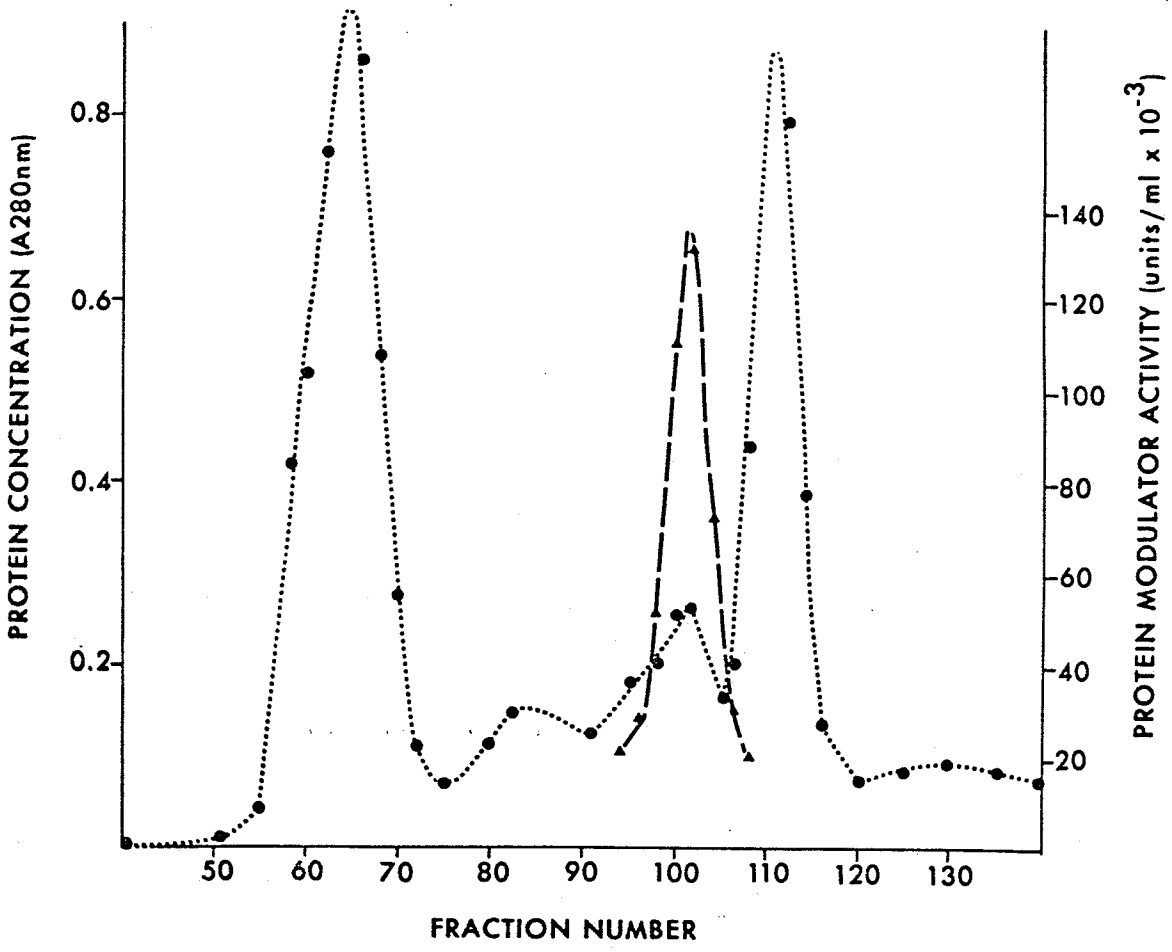


Figure 8. Chromatography of earthworm modulator on Sephadex G-100. The pooled, dialyzed, and lyophilized activity fractions from the second DEAE-cellulose chromatography were applied to a Sephadex G-100 column (2.5 x 90 cm). Fractions were assayed for protein concentration (●) and modulator activity (▲).

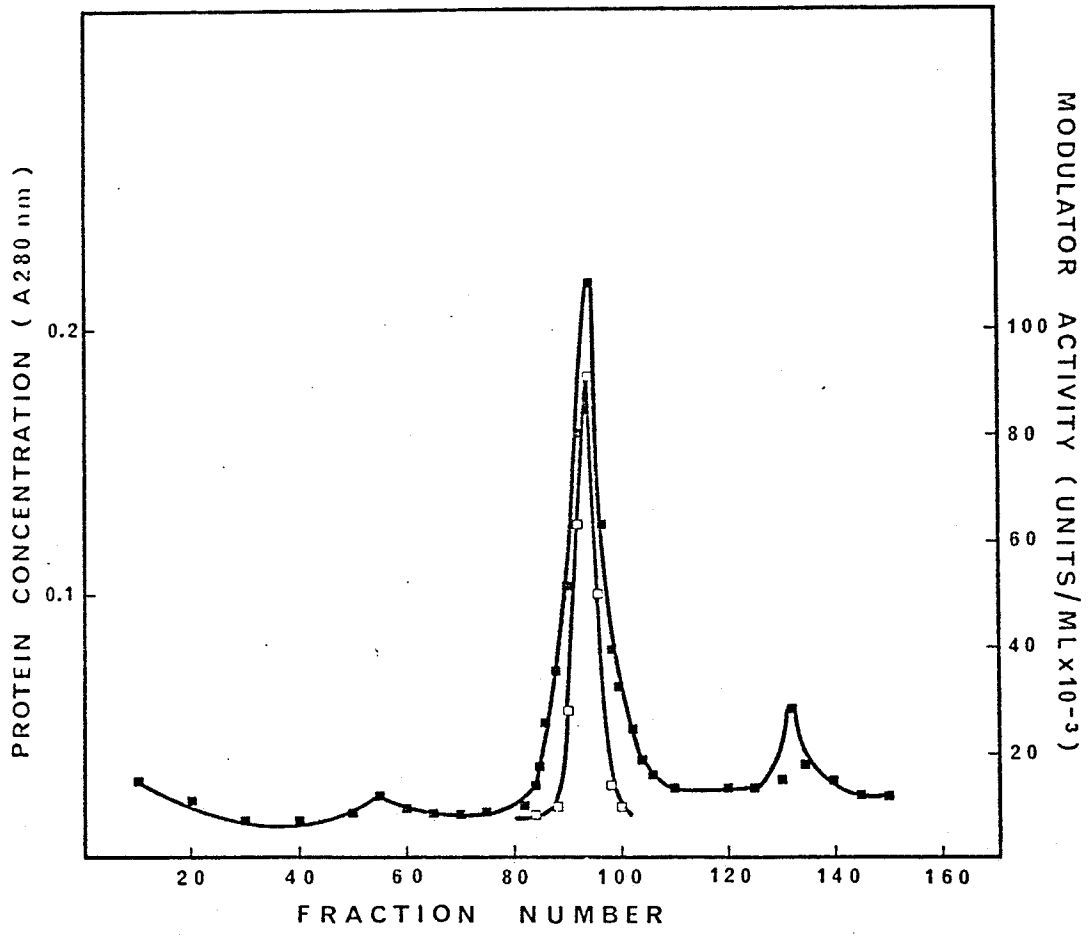


Figure 9. Chromatography of earthworm modulator on Sephadex G-75. The pooled, dialyzed and lyophilized fractions from the Sephadex G-100 column were applied (2 ml of 10 mg/ml) to a Sephadex G-75 column (2.5 x 90). Fractions were analyzed for protein concentration (■) and modulator activity (□).

(fractions 88-98) was pooled, concentrated, and stored frozen at -20^{o} until use.

Summary of Purification - Table V summarizes the data for a typical purification of the earthworm protein modulator. The purification resulted in a 650 fold increase in specific activity and a yield of 27%. The specific activity of the purified earthworm protein modulator was 76,000 units/mg which compares with a value of 80,000 - 100,000 units/mg reported for the bovine heart protein modulator (Teo et al, 1973).

CHARACTERIZATION OF PROTEIN MODULATOR FROM THE EARTHWORM

Purity of Earthworm Protein Modulator - The purified protein modulator appears essentially homogeneous on urea-sodium dodecyl sulphate-polyacrylamide disc gel electrophoresis (Figure 10, F. G), however faint bands of impurity were observed when 75 µg of sample was electrophorised in 15% polyacrylamide (Figure 10, C), these impurities could not be detected on 7.5 or 10% polyacrylamide gel (Figure 10, A,B). Densitometric tracing of this gel suggests the impurity to be less than 5%. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the purified earthworm sample (Fig. 10 D) according to Weber and Osborne (1969) revealed the presence of two

TABLE V

PURIFICATION OF EARTHWORM MODULATOR PROTEIN

FRACTION	TOTAL ACTIVITY UNITS×10^6	SPECIFIC ACTIVITY UNITS/MG	RECOVERY %	PURIFICATION -fold
Homogenate supernatant	4.9	117	100	1
Heat treated supernatant	4.4	621	90	5.3
0-60% $(NH_4)_2SO_4$ supernatant	4.2	929	86	7.9
60-80% $(NH_4)_2SO_4$ precipitate	3.25	2,300	66	19.7
1st DEAE cellulose	3.20	19,600	65	168
2nd DEAE cellulose	1.85	24,000	38	205
Sephadex G-100	1.6	80,000	33	684
Sephadex G-75	1.31	76,000	27	650

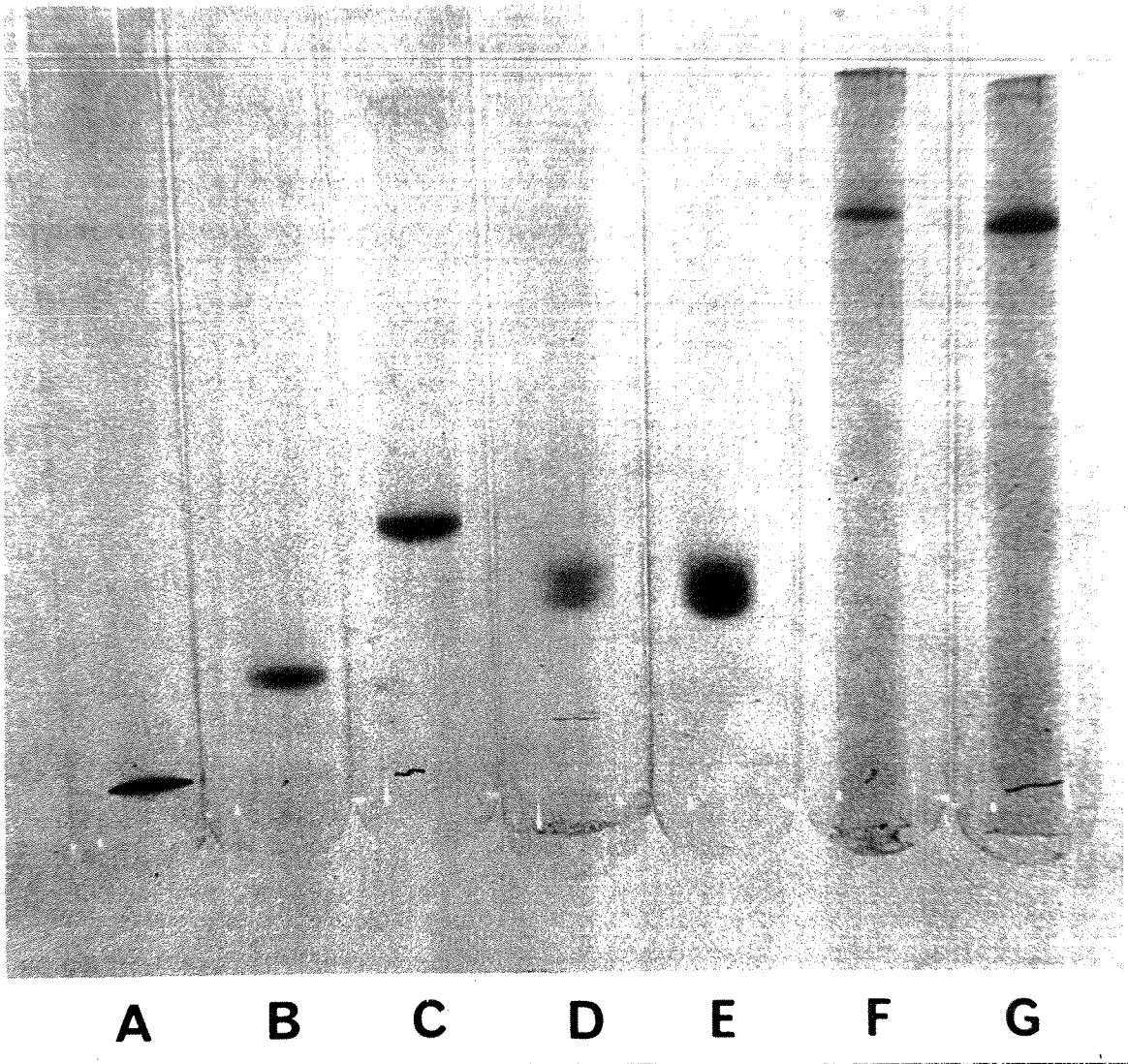


Figure 10. Acrylamide gel electrophoresis of purified earthworm protein modulator.
A, B, and C represent standard polyacrylamide disc gel electrophoreses in 7.5, 12, and 15% polyacrylamide for 75 µg of sample.D and E sodium dodecyl sulfate-gel electrophoresis in 7.5% polyacrylamide for 20- and 50-µg samples respectively. F and G, sodium dodecyl sulfate-gel electrophoresis in the presence of 8 M urea for 50- and 100-µg modulator samples respectively.

major bands of equal intensity. Since electrophoresis of earthworm modulator on sodium dodecyl sulfate according to Hayakawa et al (1973) or on sodium dodecyl sulfate gels in the presence of urea (Figure 10 F and G) showed only one band, anomalous behavior for the earthworm modulator on sodium dodecyl sulfate-polyacrylamide gels (Weber and Osborne, 1969) is suggested. Further evidence of homogeneity was suggested by the presence of a single band on polyacrylamide gel isoelectric focusing.

Physical Properties - Figure 5 compares the relative mobility of crude extract protein modulator activity with a densitometric tracing of the purified bovine heart modulator, and suggests similar relative mobility. To compare the relative mobility of the purified protein modulator from earthworm and bovine heart, electrophoresis of purified samples was performed and is presented in Figure 11. Since a single protein band results when earthworm and bovine heart protein modulators are co-electrophorised on a single polyacrylamide disc gel (Figure 11A), or on a single polyacrylamide gel in the presence of sodium dodecyl sulfate (Figure 11 B) it would appear that the purified protein modulator of earthworm and of bovine heart are electrophoretically identical, therefore confirming and extending the results of

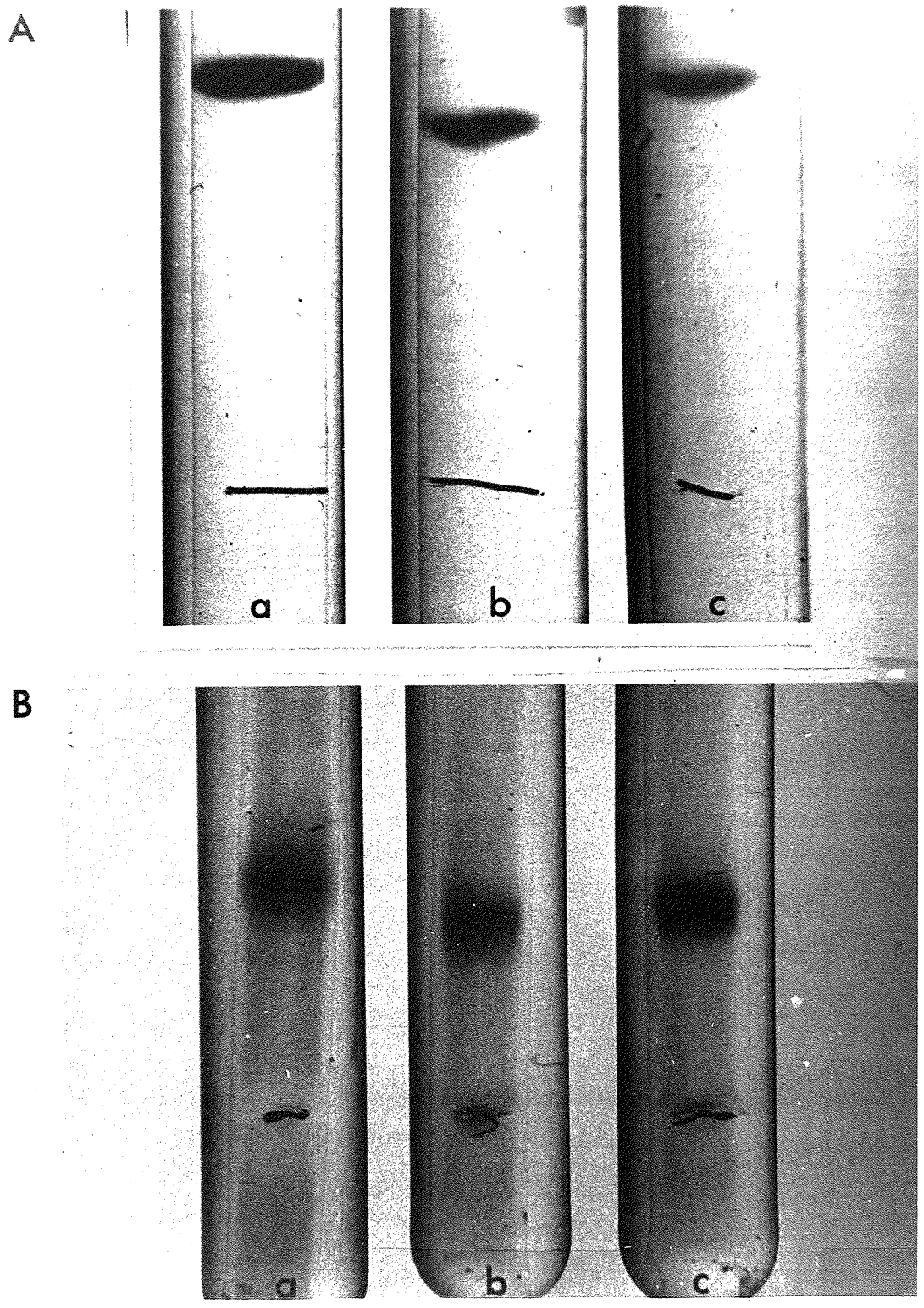


Fig. 11. Electrophoretic comparisons of purified earthworm and bovine heart protein modulator. A. 15% PAGE of a, 25 µg of earthworm modulator, B, 10 µg and 2 µg or C, 2 µg and 10 µg of earthworm and bovine heart, protein modulator. B. 10% PAGE SDS gel electrophoresis of a, 50 µg of earthworm modulator. B. 10 µg and 2 µg or C, 10 µg of earthworm and bovine heart protein modulator.

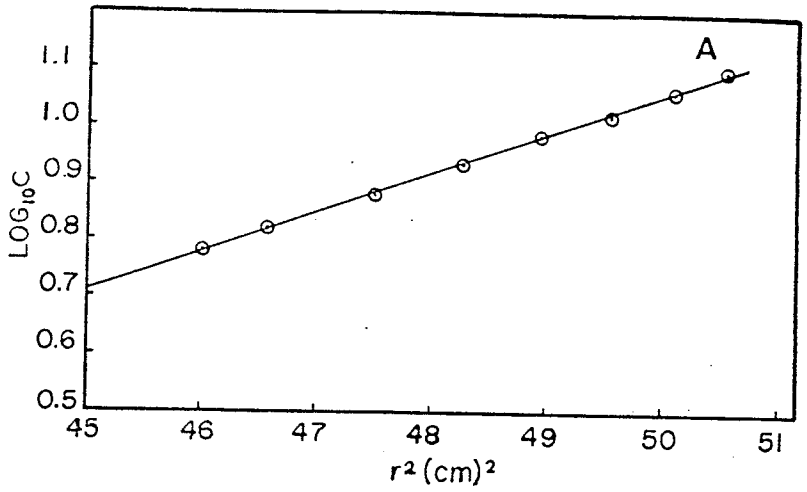
fig 11

electrophoresis of crude extracts (Figure 5).

Figure 12 A shows the result of a sedimentation equilibrium experiment with purified earthworm protein modulator. The plot of log protein concentration versus the square of the radial distance from the center of rotation gives a straight line. A molecular weight of 15,700 was calculated from this result. On sodium dodecyl sulfate-gel electrophoresis the earthworm modulator migrates with a mobility corresponding to a molecular weight of 18,000 (Figure 12 B). This value is similar to the molecular weight as determined by ultracentrifugation suggesting the protein modulator is a monomeric protein.

In sedimentation velocity experiments the purified protein modulator exhibited a single symmetrical peak in its schlieren pattern with an extrapolated sedimentation constant of 1.95 S. The diffusion constant determined during the ultracentrifugation had a value of 9.25×10^{-7} cm $^{2}/S$. Using these values and a partial specific volume of 0.72 ml/g as calculated from the amino acid composition (Table VII) the molecular weight is calculated to be 18,200.

The ultraviolet spectra of the purified earthworm modulator is presented in Figure 13. The spectrum is atypical of common globular proteins but similar to those



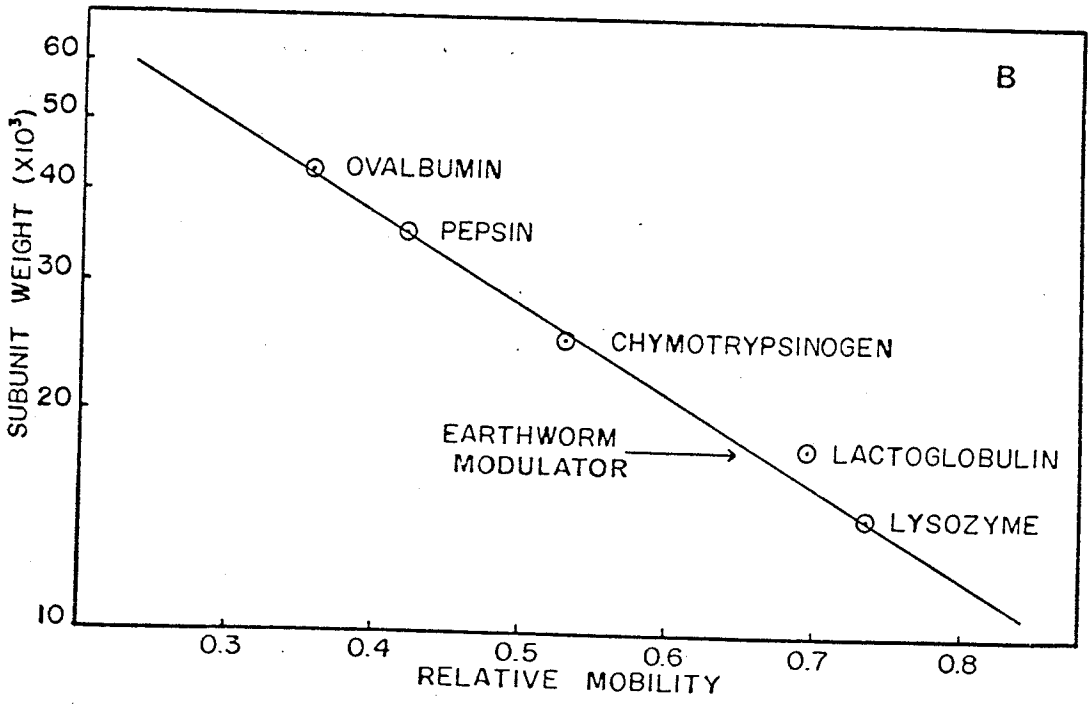


Figure 12 A. Sedimentation equilibrium of purified earthworm modulator. Centrifugation was performed at 12,933 rpm and 19.8°. Radial distance (r) in cm and fringe displacement (c) in microns.

B. Determination of apparent molecular weight of earthworm modulator by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Mobility relative to the dye front is plotted against known subunit molecular weight of each protein.

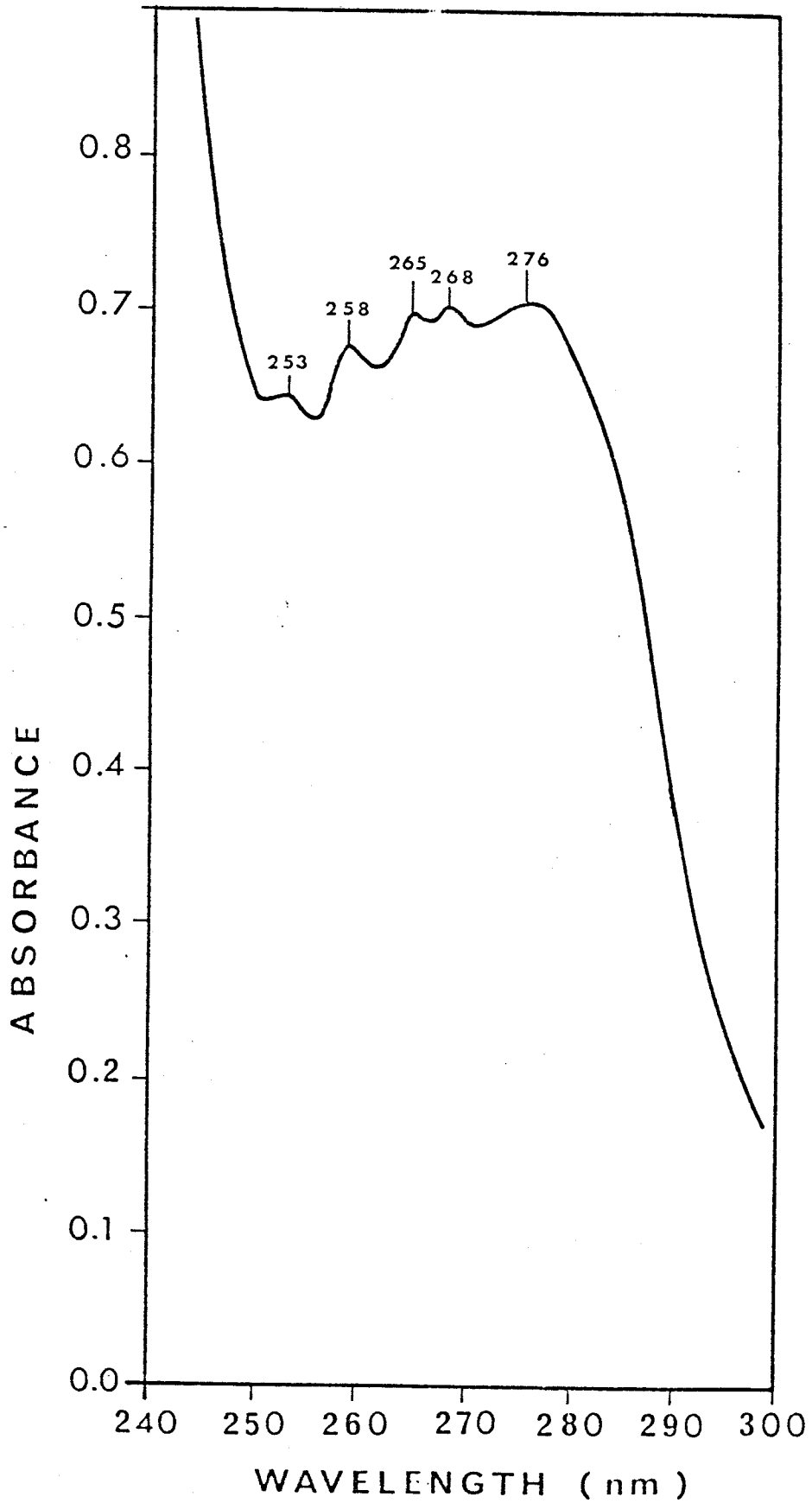


Figure 13. Absorption spectra of earthworm modulator. Earthworm modulator at a protein concentration of 1.89 mg/ml in a pH 7.5 buffer containing 40 mM Tris/HCl, 1 mM $MgAc_2$ and 50 μM$CaCl_2$ was used.

TABLE VI

PHYSICAL PARAMETERS OF EARTHWORM AND BOVINE HEART PROTEIN MODULATORS

Parameter	Earthworm Modulator	Bovine Heart* Modulator
Sedimentation constant $S^{\circ}_{20,W}$	1.95	2.0
Diffusion coefficient $D^{\circ}_{20,W}(\times 10^{7} cm^{2}/s)$	9.25	9.0
Molecular weight; analytical ultracentrifugation	15,700-19,500	16,800-19,000
Sodium dodecyl sulphate-gel electrophoresis	18,000	18,500
pI (pH)	4.0	4.1
$E_{275-278}$ (1% protein, 1 cm)	3.2	1.9
$\bar{v}$	0.72	0.72

* From Wang et al, (1975) and Stevens et al, (1976)

TABLE VII

AMINO ACID COMPOSITION OF EARTHWORM AND BOVINE HEART PROTEIN MODULATORS

Amino Acid	21 hr. hydrolysate	48 hr. hydrolysate	72 hr. hydrolysate	Average or extrapolated value	Nearest Integer Earthworm	Bovine heart
Lysine	7.12	7.03	7.28	7.14	7	9*
Histidine	1.48	1.41	1.48	1.46	1-2	1
Arginine	5.28	5.26	5.39	5.31	5	6
Aspartic acid	23.98	23.79	23.86	23.88	24	25
Threonine	11.54	10.84	10.75	11.7	12	12
Serine	6.00	5.71	5.24	6.3	6	3
Glutamic acid	29.06	28.92	28.86	28.95	29	30
Proline	5.39	6.01	6.18	5.86	6	2
Glycine	13.08	12.92	13.05	13.02	13	12
Alanine	10.78	10.93	10.91	10.87	11	12
Cysteine+	.73	.71	.65	.70	1	0
Valine	7.13	7.51	7.44	7.36	7	9
Methionine	8.63	9.21	8.96	8.93	9	9
Isoleucine	8.23	8.47	8.33	8.34	8	8
Leucine	9.70	9.97	9.84	9.84	10	10
Tyrosine	1.89	1.78	1.84	1.84	2	2
Phenylalanine	7.92	7.70	8.08	7.9	8	9
Tryptophan ▲	—	—	—	0		0

* this value for lysine includes 1 residue of 3 methyl-lysine which does not separate from lysine in the acid hydrolysate method. From Stevens et al. (1976).

\+ carboxymethylcysteine was detected in the hydrolysate of the reduced carboxymethylated protein.

▲ tryptophan was absent as judged by the spectrophotometric method of Goodwin and Morton (1946)

exhibited by the bovine heart modulator. Absorption peaks are seen at approximately 253, 258, 265, 268, and 276 nm. The maximum of the spectrum is in the region of 275 to 278 nm. The absorbance for the earthworm protein modulator in the region ($E_{275-278}$, for a 1% solution) has been calculated to be 3.2.

The isoelectric point was determined by polyacrylamide gel isoelectric focussing to be 4.0.

Table VI summarizes some of the physical parameters of the bovine heart and earthworm protein modulators. The two proteins are similar in almost all parameters compared. Only the absorbance ($E_{275-278}$) appears to be significantly different.

Amino Acid Composition - The amino acid composition of the earthworm modulator is presented in Table VII. The notable features of the amino acid composition include the low content of histidine and tyrosine, the high content of acidic residues, and the high phenylalanine to tyrosine ratio.

The comparison of amino acid composition of earthworm and bovine heart modulator indicate that the two proteins are remarkably similar. They have identical numbers of residues of threonine, methionine, isoleucine, leucine and tyrosin and both lack tryptophan. Furthermore, both proteins contain approximately 35% acidic

residues, consistent with the pI (pH) of 4.0. The major differences between the earthworm and bovine heart modulator appear to be the presence of 1 residue of cysteine and a significantly higher proline content in the earthworm modulator. The similar content of tyrosine, tryptophan, and phenylalanine (one residue less in the earthworm) shared by both protein modulators appears to be inconsistent with a $E_{275-278}$ value of 3.2 for the earthworm modulator, compared with the lower value of 1.9 for the bovine heart modulator. The possibility of a contaminant in the earthworm preparation would necessitate a $E_{275-278}$ value of 24 (1% solution) for the contaminant (assuming 5% contamination).

Activation of Mammalian Phosphodiesterase - The activation of phosphodiesterase by the purified earthworm protein modulator was characterized using modulator-deficient phosphodiesterase from bovine heart. The extent of phosphodiesterase activation by the earthworm modulator was identical to that achieved by the bovine modulator. Furthermore, the activation is dependent on Ca^{2+}, since the enzyme activation by the earthworm modulator was inhibited by EGTA (Figure 14 A). The results are consistent with the observations made with crude extract protein modulator

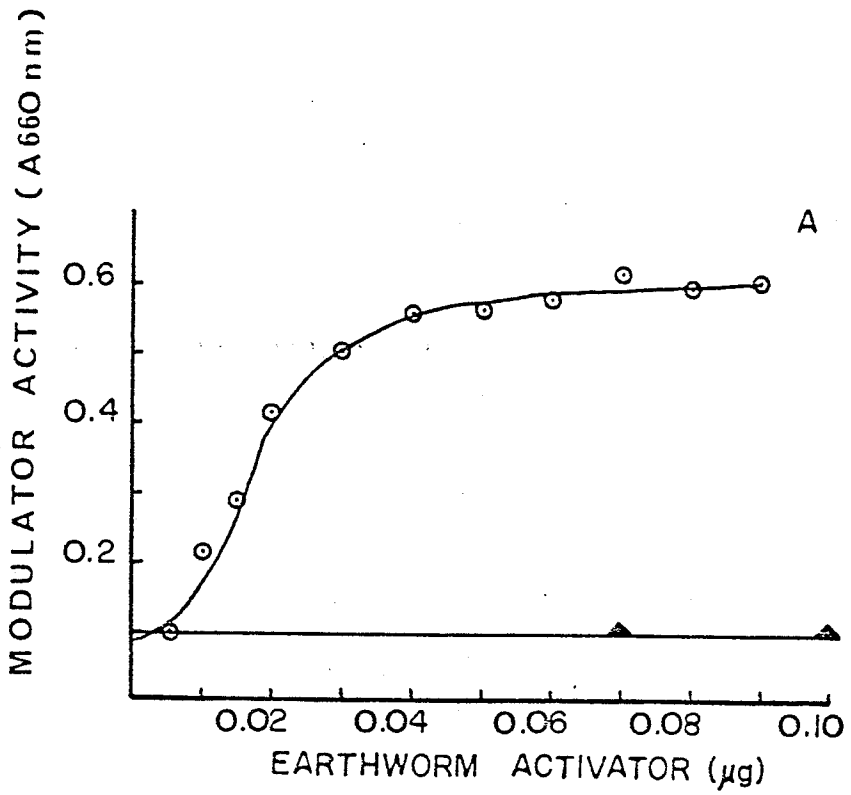


Figure 14 A. Dose-response curve for the activation of bovine heart phosphodiesterase by purified earthworm modulator in the presence of (▲) or absence of (⊙) 800 μM EGTA. EGTA was used to chelate the Ca^{2+} normally present in the enzyme assay (100 μM Ca^{2+}).

(Figure 1 and 3). The similarity in specific activity of the earthworm and bovine protein modulator suggests that the two proteins have similar affinity toward mammalian phosphodiesterase.

The activation of modulator-deficient bovine heart phosphodiesterase by Ca^{2+}, in the presence and absence of added earthworm protein modulator is presented in Figure 14 B. The enzyme activation is dependent on both the presence of protein modulator and calcium; in the absence of protein modulator Ca^{2+} has no stimulatory effect. The amount of Ca^{2+} required for 50% activation of mammalian phosphodiesterase (in the presence of 25 units of earthworm modulator) was determined to be 2.0 μM, this value may be compared with that obtained for the enzyme activation by bovine heart protein modulator under similar conditions, 2.3 μM (Teo and Wang, 1973).

Equilibrium Ca^{2+} Binding - The equilibrium interaction between the purified earthworm protein modulator and Ca^{2+} has been studied by the gel filtration method of Hummel and Dreyer (1962). Figure 15 shows the elution profile for a typical binding experiment. The appearance of ^{45}Ca peak and trough in the profile is indicative of the binding of Ca^{2+} to the protein. The radioactivity peak coincides exactly with the activity peak of the protein

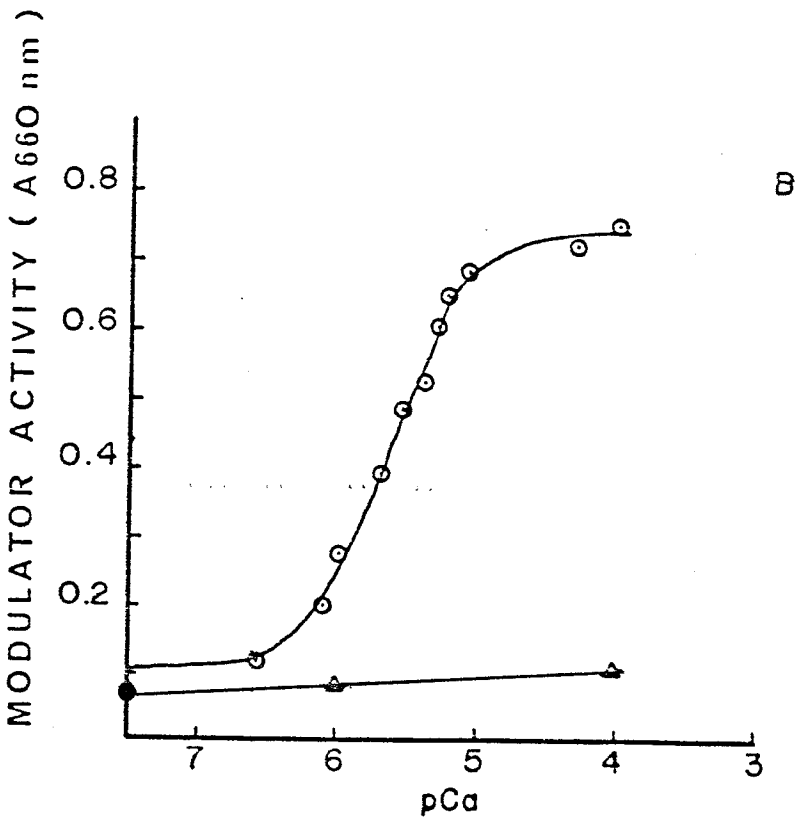


Figure 14 B. Activation of bovine heart phosphodiesterase by varying concentrations of Ca^{2+}. The activation was assayed in the presence of (O), or in the absence of 25 units of earthworm modulator (▲) or in the presence of modulator and 0.4 mM EGTA (●).

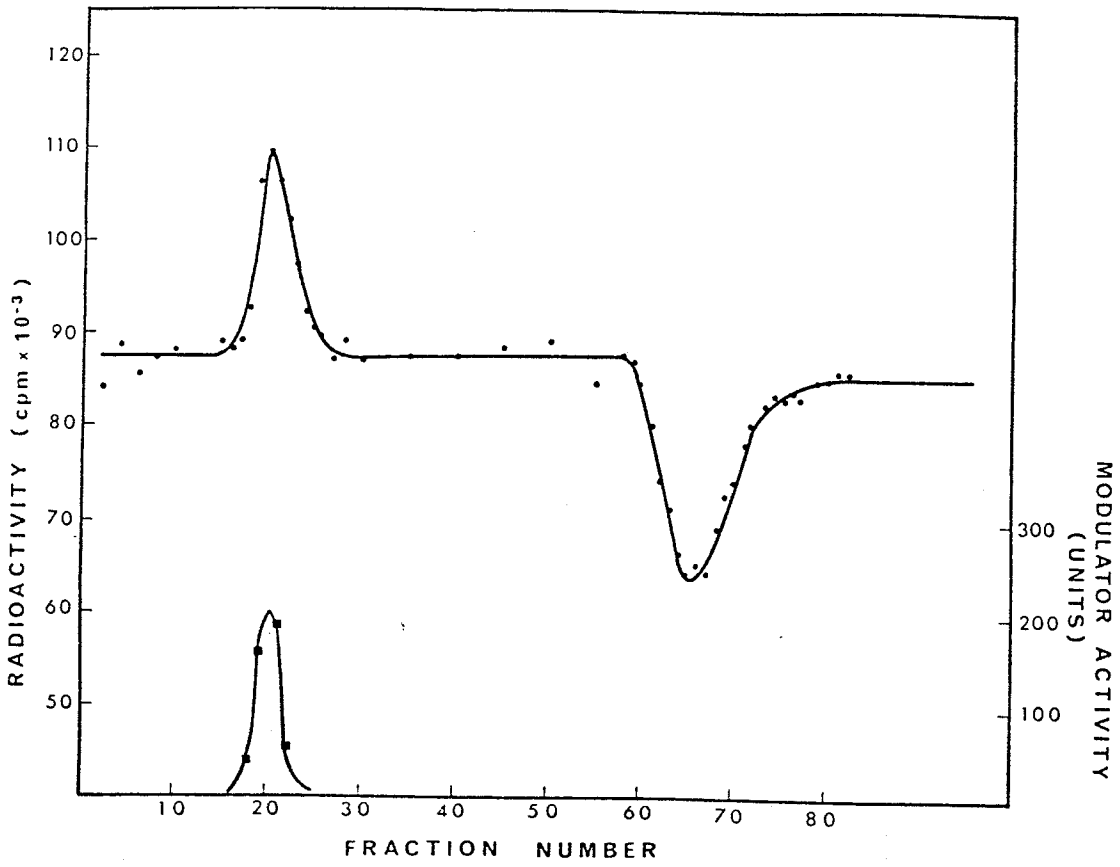


Figure 15. Elution profile for the measurement of Ca^{2+} binding by the earthworm modulator.
Radioactivity of ^{45}Ca in the fractions eluted with buffer containing 0.50 µM Ca^{2+} (●). Protein modulator (48 µg in 0.6 ml) was applied to the column (0.9 x 45 cm) and the modulator activity determined (■) in the column fractions.

modulator. For the calculation of the amount of bound Ca^{2+}, only the data at peak regions have been used.

Figure 16 presents the Scatchard plot of the stoichiometry of the Ca^{2+} binding and dissociation constant of the Ca^{2+}-protein complex. The plot consists of a straight line and from the slope of this line the dissociation constant was calculated to be 6 μM. The intersection of the line at 1.7 mol/mol of modulator indicates two Ca^{2+} binding sites per molecule of the modulator. Furthermore, the linearity of the line suggests that the binding sites behave as independent, non-cooperative sites. These results are contrasted with the bovine heart protein modulator which binds four mol of calcium with differing affinities (Teo and Wang, 1973).

Interaction with Modulator-Binding Protein - The bovine heart protein modulator has been shown to form a Ca^{2+}-dependent complex with a protein from bovine brain of unknown function, called the modulator binding protein (Wang and Desai, 1977; Klee and Krinks, 1978). The modulator protein is assayed on the basis of its ability to counteract the activation of phosphodiesterase by the protein modulator. Figure 17 shows that the protein modulator from earthworm is capable of Ca^{2+} dependent interaction with the modulator binding protein, in fact,

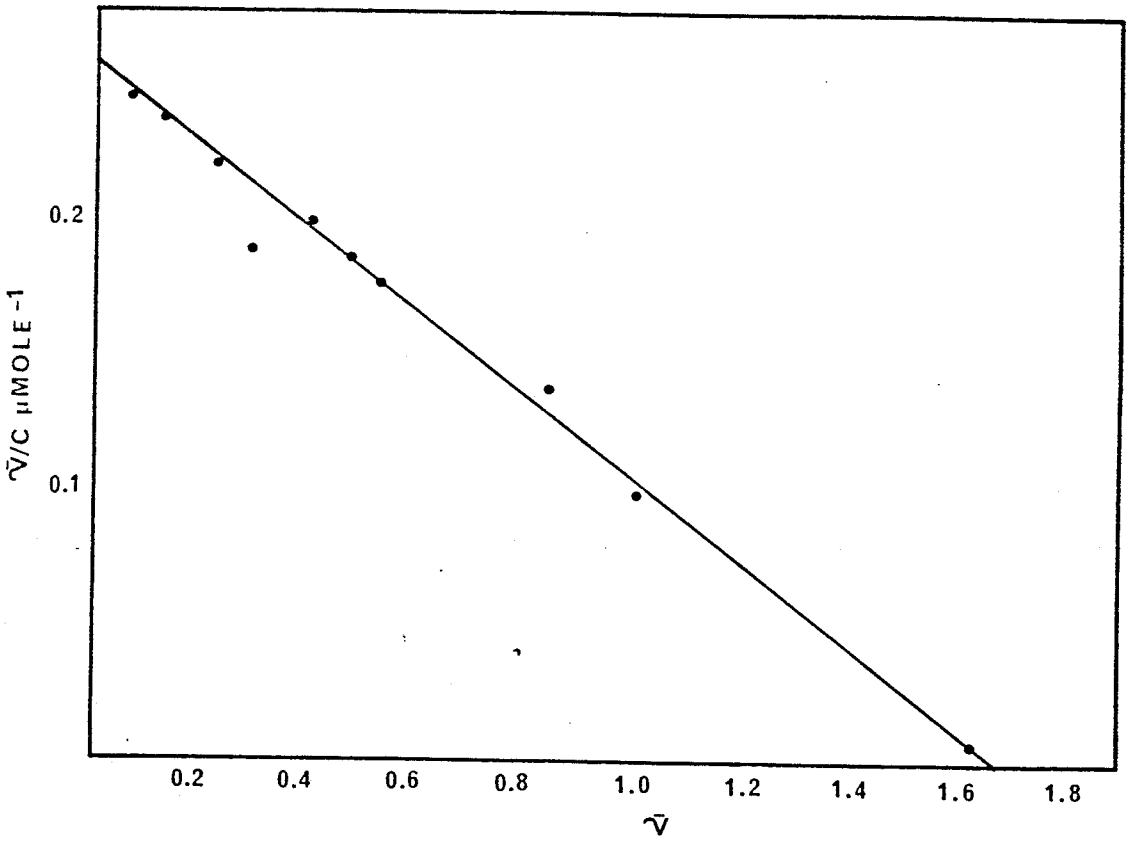


Figure 16. Scatchard plot for the binding of Ca^{2+} by purified earthworm modulator. $\bar{\nu}/C$, moles of Ca^{2+} bound per mol of purified earthworm modulator; C, concentration of Ca^{2+} in buffer with which the protein modulator is in equilibrium during the Ca^{2+} binding experiments.

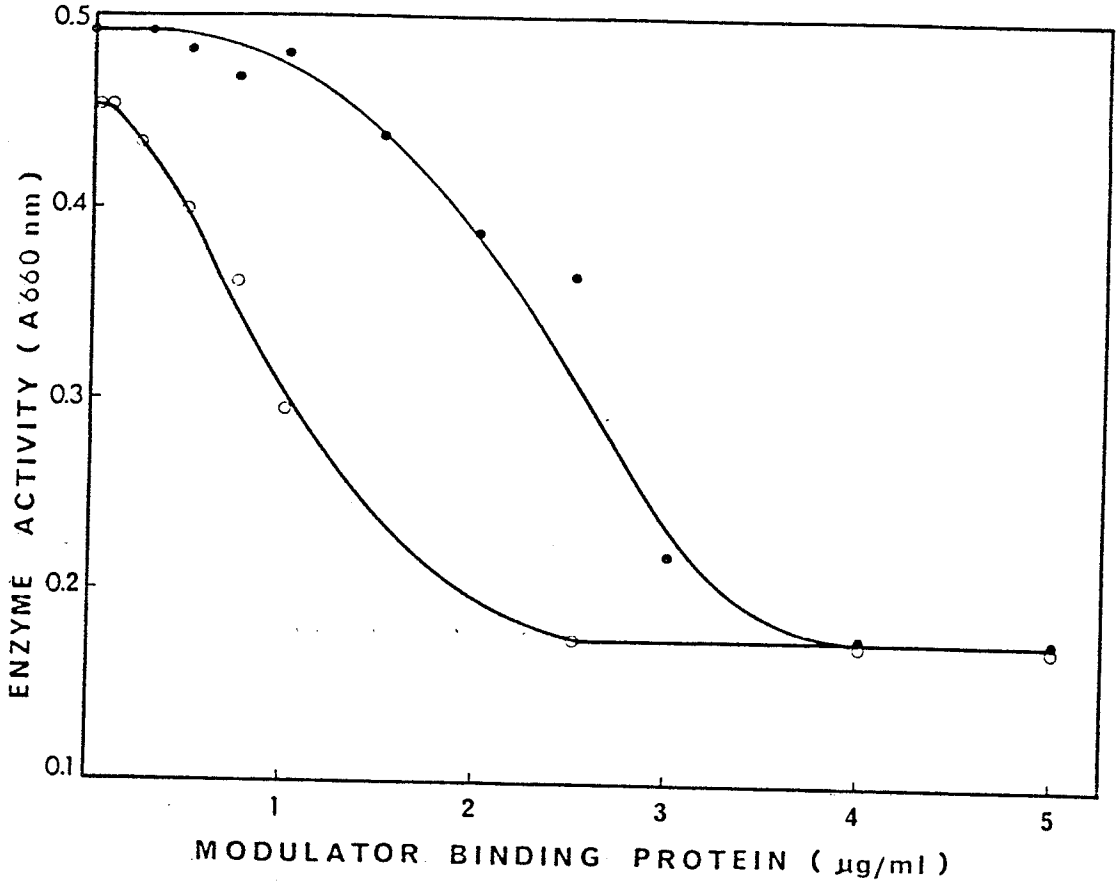


Figure 17. Effect of bovine brain modulator binding protein on the activation of phosphodiesterase by earthworm modulator.
Phosphodiesterase reaction contained either 4 (O) or 8 (●) units of earthworm modulator and was assayed in the presence of varying concentrations of bovine brain modulator binding protein as indicated.

the activation of mammalian phosphodiesterase by the earthworm protein modulator can be completely overcome by bovine brain modulator binding protein. The results suggest that the earthworm modulator, like the bovine modulator, is capable of specific interaction with the modulator binding protein.

Tryptic Peptide Mapping - As a further test of the similarities between the purified protein modulators from bovine heart and earthworm, tryptic peptide maps were prepared, and are presented in Figure 18. The peptide maps are similar in only six of twenty peptides. This apparent lack of similarity between the peptide maps of protein modulator from earthworm and bovine heart is in contrast to the striking similarities in amino acid composition and physical properties previously described. It should however be emphasized that closely related homologous proteins do not necessarily give very similar peptide maps. For example, sequence studies (van Eïd and Takahashi, 1975) have shown that there is 65% homology between rabbit skeletal and bovine heart troponin C; yet based on amino acid sequence comparison one would predict only three out of a possible eighteen tryptic peptides identical.

The number of clearly visible peptides on the earthworm peptide map was observed to be twenty, as compared

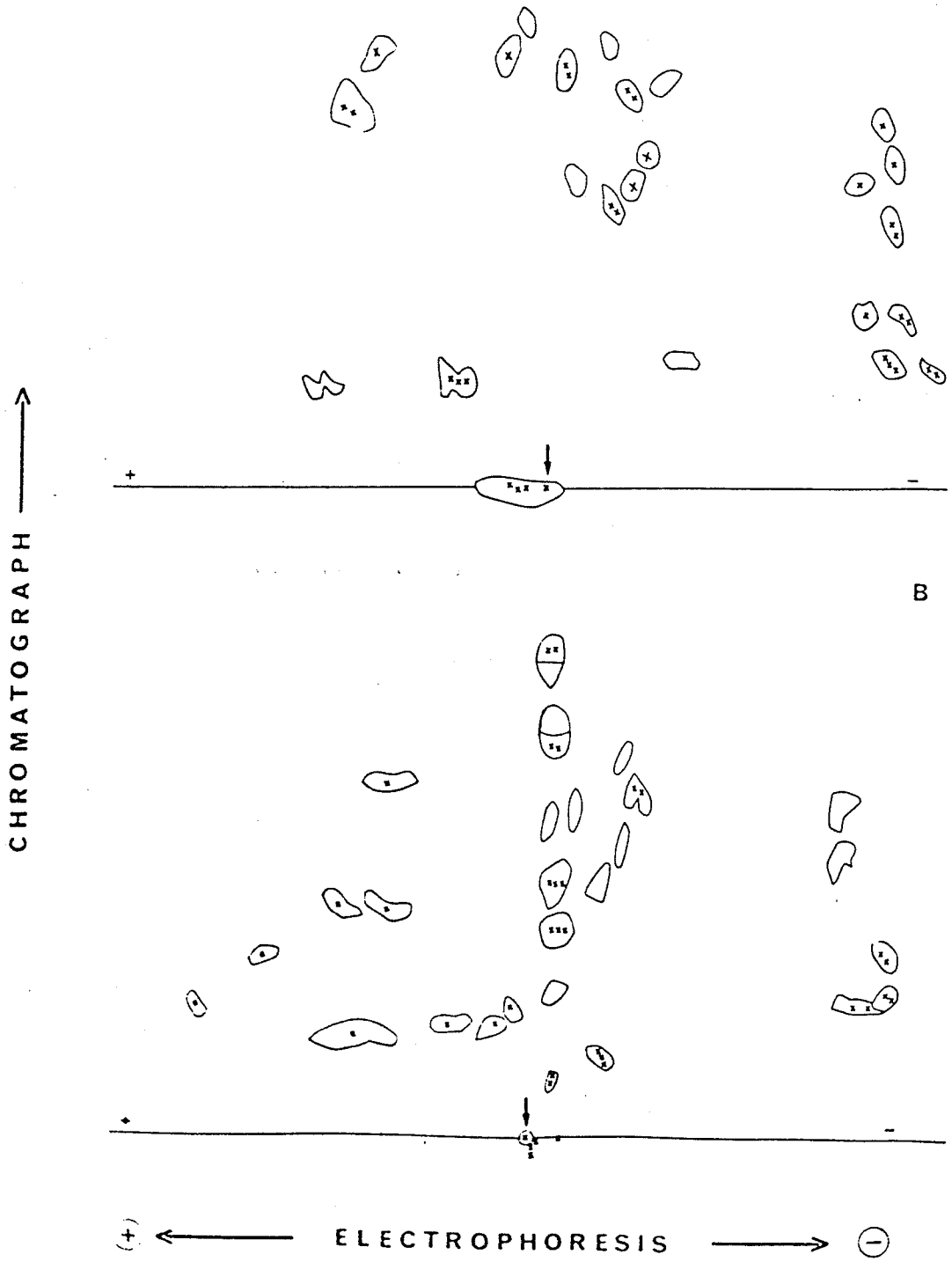


Figure 18. Tryptic peptide map of earthworm and bovine heart protein modulator.
XXX, strongly stained peptides; XX, faint but visible peptides; X very weakly stained spots. A) bovine heart protein modulator; B) earthworm protein modulator.

to a maximum of thirteen peptides predicted by the amino acid analysis (12 residues of lysine and arginine), therefore suggesting that some nonspecific peptide bond hydrolysis has taken place during digestion with trypsin. This has been shown to be the case for the bovine brain modulator (Stevens et al, 1976)

3. GENERAL CONCLUSIONS

The activation of mammalian phosphodiesterase by crude extract protein modulator (Figure 1 and 3) has suggested that the structural and functional properties of the protein modulator have been conserved during evolution, however, the inability to demonstrate Ca^{2+}-dependent phosphodiesterase (section VIII, 1) in lower forms of animals and in plants (Table VIII and IX), appears to indicate that the function of the protein modulator in lower lifeforms may be quite different than in mammalian systems. In a general context, the crude extract studies would suggest conservation of structural and functional properties as related to the role of the protein modulator in the mediation of Ca^{2+}-linked regulation of enzyme activity.

The comparisons of the relative mobility of crude extract protein modulator activity with purified modulator from bovine heart has also suggested conservation of the structural and functional properties of protein modulator. In addition, this study has demonstrated that the physical-chemical properties of the protein modulator itself, have been conserved during evolution. In order to test the suggestion of conservation of physical-chemical properties, the protein modulator from the earthworm has

been purified and characterized. A comparison of the physical parameters of bovine heart and earthworm protein modulators has revealed striking similarities (Table VI). Both proteins exhibit ultraviolet spectra atypical of common globular proteins, and have molecular weights of about 18,000 and isoelectric points of about 4.0. An examination of the amino acid compositions of the proteins (Table VII) has demonstrated a preponderence of acidic residues (35%). Furthermore, the earthworm and bovine heart protein modulators have identical numbers of leucine, isoleucine, methionine, threonine, tyrosine, and both lack tryptophan. The only major differences appear to be a residue of cysteine and a higher proline content in the earthworm modulator. The dissimilarity of tryptic peptide maps of earthworm and bovine heart protein modulator has been interpreted to suggest that the two proteins are homologous but not identical.

The conservation of the functional properties of the protein modulator has also been investigated. The purified earthworm protein modulator has essentially the same potency as bovine modulator in the activation of mammalian cyclic nucleotide phosphodiesterase. In addition, earthworm modulator appears capable of specific interaction with the bovine brain modulator binding protein

(Fig. 17). Another striking similarity between the bovine heart and earthworm protein modulators is that the concentration of Ca^{2+} required for 50% activation of mammalian phosphodiesterase in the presence of protein modulator was determined to be about 2.0 μM for both proteins (Figure 14 B). Although equilibrium Ca^{2+} binding studies have shown that the earthworm protein modulator binds two mol of calcium ions compared with four mol of calcium ions for the bovine heart modulator, the loss of two functional calcium binding sites, due to lability during desalting cannot be entirely ruled out. If purification of other primitive protein modulators substantiate the existence of only two Ca^{2+} binding sites, then it will be possible to speculate that four functional calcium binding sites may be a recent evolutionary development. If the similar protein potency, and similar calcium concentration requirement for 50% activation of phosphodiesterase in the presence of protein modulator are considered along with the observation of Klee (1977) that about 85% of the conformational change (monitored by UV-absorption) occurs when bovine modulator binds only two mol of Ca^{2+}, then it is possible to speculate that only two functional calcium binding sites might be necessary for phosphodiesterase activation by either

modulator. Following this speculation further, it follows that the addition of 2 functional calcium binding sites may have been the result of the development of a new function for protein modulator during evolution.

In conclusion, the results suggest that the protein modulator is a ubiquitiously distributed, highly conserved protein. The conservation of the functional properties of the protein modulator further suggest that the role of the protein modulator as a mediator of Ca^{2+}-linked regulation of enzyme activity is conserved during evolution.

VIII. ROLE OF PROTEIN MODULATOR REGULATION

1. The Role of the Protein Modulator in Non-Mammalian Systems

Previous studies (section I) have demonstrated that protein modulator of non mammalian organisms is capable of Ca^{2+} dependent and reversible mammalian phosphodiesterase activation, and based on these studies it has been suggested that the structural and functional properties of the protein modulator, related to phosphodiesterase activation have been conserved. It was at first considered that the explanation for the ability of non mammalian crude extract protein modulator to activate

mammalian phosphodiesterase might be that in non mammalian systems protein modulator also serves to regulate a similar phosphodiesterase. Therefore, it would be expected that the non mammalian crude extracts contain protein modulator regulated phosphodiesterase.

As a test of this suggestion non mammalian crude extracts were prepared and examined for the presence of Ca^{2+} activatable (protein modulator regulated) cyclic nucleotide phosphodiesterase. Table VIII shows that crude animal extracts contain cyclic nucleotide phosphodiesterase activity. A comparison of the phosphodiesterase activity of each specimen examined indicates that EGTA has little if any, inhibitory effect on enzyme activity, therefore suggesting that the crude animal extracts contain little if any, Ca^{2+}-activatable cyclic nucleotide phosphodiesterase. In some extracts both c-AMP and c-GMP were used as substrates and similar results were found. While the low to negligible level of EGTA inhibition may suggest that most if not all of the non mammalian phosphodiesterase is not regulated by protein modulator the possibility that protein modulator dependency of the enzyme becomes pronounced under different assay conditions cannot be excluded. To test this possibility a crude earthworm extract was prepared and assayed under several

TABLE VIII

Ca^{2+} REGULATED CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

IN CRUDE ANIMAL EXTRACTS

COMMON NAME		PHOSPHODIESTERASE ACTIVITY (A660nm) Ca^{2+}	EGTA
SNAIL		0.180	0.180
CLAM	(c-AMP)	0.390	0.330
	(c-GMP)	0.210	0.200
ROUND WORM		0.180	0.220
SEA ANEMONE		0.370	0.370
BLUE CRAB		0.360	0.340
MEAL WORM	(c-AMP)	0.030	0.030
	(c-GMP)	0.100	0.380

different pH and at several concentrations of substrate. Results are presented in Table IX. While both c-AMP and c-GMP phosphodiesterase activities can be demonstrated in the crude earthworm extract, the phosphodiesterase activity does not appear to be inhibited by EGTA. Furthermore EGTA appears to stimulate the enzyme at millimolar concentrations of c-AMP.

To further test the possibility of protein modulator regulated cyclic nucleotide phosphodiesterase in the earthworm, an earthworm extract was chromotographed on a DEAE-cellulose column (2.6 x 30 cm), pre-equilibrated with 40 mM Tris/HCl, 1 mM magnesium acetate, 0.50 mM calcium chloride and 10 mM β-mercaptoethanol. The column was eluted with 750 ml of a NaCl gradient of 0.1 to 0.8 M in the same buffer. Two peaks of phosphodiesterase activity were eluted at salt concentrations of 0.25 and 0.65 M. Analysis of both peaks with 10 μM c-AMP or 10 μM c-GMP at pH 8.5 and in the presence of added protein modulator was unable to detect protein modulator regulated phosphodiesterase. The results together with the findings of other workers who examined the phosphodiesterase activity of Neurospora crassa (Scott and Solomon, 1973), pea seedling (Lin and Varner, 1972), potato tuber (Ashton and Polya, 1975), baker's yeast (Fujimoto et al, 1974)

TABLE IX

Cyclic Nucleotide Phosphodiesterase of Lumbricus terrestris

pH	Substrate	Rate of Hydrolysis (pmoles/min/mg)*	
		$+Ca^{2+}$	+EGTA
5.5	10 μM cAMP	0.2	0.2
7.5	1 mM cAMP	120.0	160.0
	10 μM cAMP	1.8	1.9
8.5	1 mM cAMP	260.0	330.0
	10 μM cAMP	2.0	2.0
7.5	1 mM cGMP	10.0	10.0

*500 μM EGTA or 50 μM $CaCl_2$ was added.

and silkworm (Morishima, 1974) suggest that protein modulator regulated cyclic nucleotide phosphodiesterase has a limited phylogenetic distribution. The function of the protein modulator in non mammalian lifeforms remains unresolved.

B. The Function of the Protein Modulator in Rabbit Skeletal Muscle.

The physiological function of the protein modulator in rabbit skeletal muscle has been investigated. Rabbit skeletal muscle was chosen because of the well defined role of calcium in that tissue. In particular it has been suggested that calcium is responsible for the coordinated regulation of muscle contraction and glycogenolysis (Brostrom et al, 1971) and also the stimulation of phosphorylation of the 18,000 dalton myosin light chain (Pires et al, 1974; Stull and High, 1977). The regulation of glycogenolysis and myosin light chain phosphorylation is achieved by virtue of the fact that phosphorylase kinase and myosin light chain kinase, the enzymes responsible for regulation of glycogenolysis and for myosin light chain phosphorylation respectively, are dependent on the presence of calcium ions for activity. Since the role of the protein

modulator is the mediation of the Ca^{2+}-linked regulation of enzyme activity, it seems reasonable to suspect that the protein modulator might be involved in the regulation of one or both of these kinases. Alternatively, the existence of other protein modulator regulated kinases could not be ruled out. Rather than the separate purification, and characterization of potential modulator regulation of myosin light chain kinase and phosphorylase kinase, a more general approach was chosen, involving purification of all protein modulator stimulated protein kinase activity. Therefore the rationale of the search for the function of protein modulator in rabbit skeletal muscle was to purify kinase activity which could be stimulated by the protein modulator. After purification of the protein modulator regulated protein kinase(s) characterization of this kinase(s) could be undertaken.

Isolation of the Modulator Dependent Protein Kinases

Typically two rabbits were used for each preparation. The following procedure was performed with 1.5 kg of rabbit muscle. All buffers were adjusted to pH 7.5.

Extraction - New Zealand White rabbits were killed by a blow on the neck, exsanguinated and rapidly skinned. The back and hind-leg muscles were quickly dissected out and

placed on ice. The muscle was ground in a Hobart meat mincer and homogenized for one minute at low speed with a Waring blender in three volumes of ice cold pH 7.5 buffer containing 20 mM Tris/HCl, 4 mM EDTA, 5% phenylmethylsulfonylfluoride (PMSF, 6 mg/ml in 95% ethanol), and 40 mM β-mercaptoethanol. The homogenate was centrifuged at 6,000 x g for thirty minutes and the resulting supernatant was filtered through glass wool.

Ammonium Sulfate Fractionation - To the filtered supernatant solid ammonium sulfate was added in a stepwise manner, with continual stirring to a final concentration of 60% saturation. The mixture was allowed to stir slowly for about thirty minutes and then centrifuged at 6,000 x g for thirty minutes. The precipitate was solubilized and dialyzed overnight against two changes of 20 mM Tris/HCl, 0.1 mM EGTA, and 40 mM β-mercaptoethanol.

DEAE-Sephacel Column Chromatography - The dialyzed 60% ammonium sulfate precipitate was adjusted to 20 mM benzamidine and 1 mM magnesium acetate before application to a 500 ml DEAE-Sephacel column pre-equilibrated with buffer of the same. After sample application the column was eluted with two bed volumes of buffer and then with a four liter linear salt gradient from 0.02 to 0.3 M NaCl. Figure 19 presents the elution profile of DEAE-Sephacel chromatograph. The presence of proteins capable of association

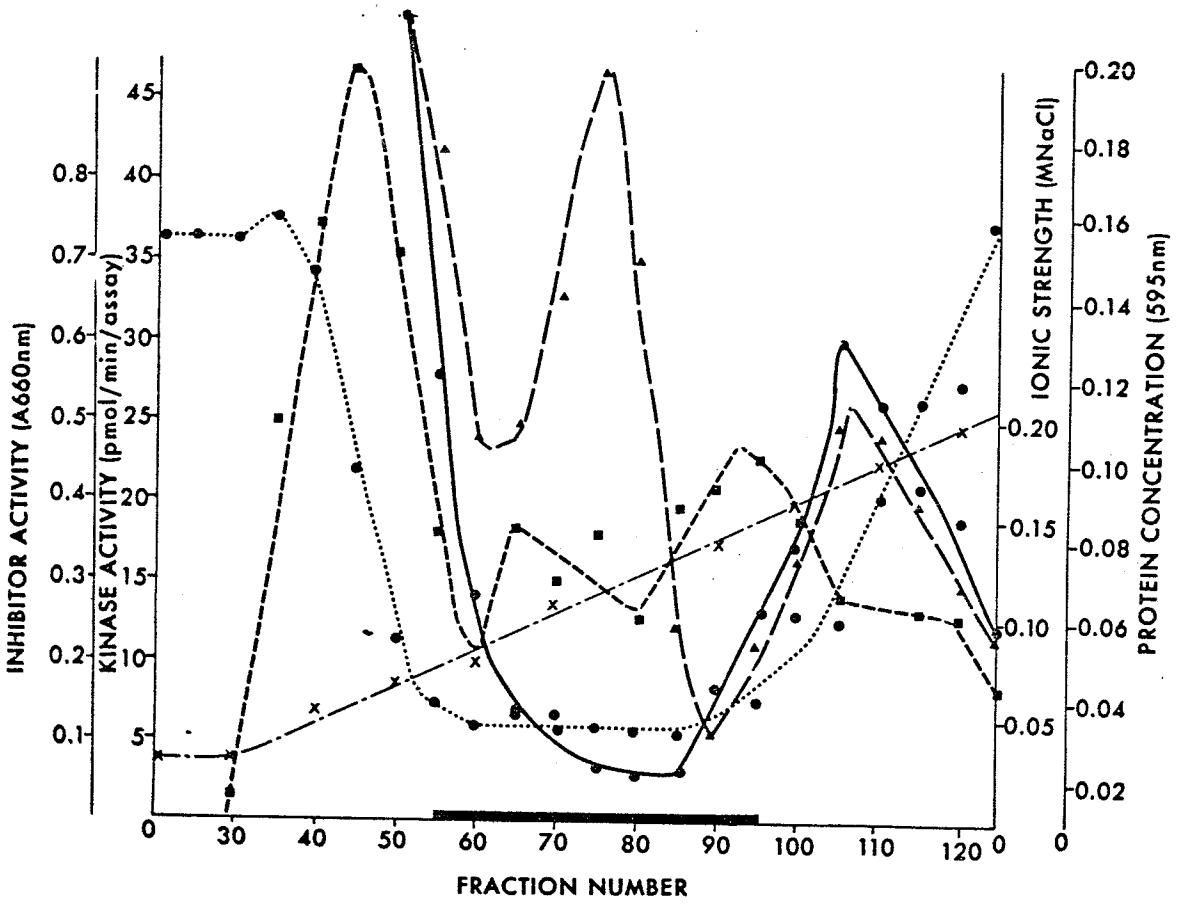


Figure 19. Chromatography of rabbit skeletal muscle extract on DEAE-Sephacel.
The dissolved $(NH_4)_2SO_4$ precipitate was applied to a 500 ml DEAE-Sephacel column. The column fractions were assayed for protein concentration (----■----), ionic strength (——X•——), inhibitor activity (....●....) and histone kinase activity in the presence (—▲—) or absence of protein modulator (—●—).

with the protein modulator is suggested by the presence of a broad trough of inhibitor activity (i.e. proteins capable of binding protein modulator and therefore inhibiting the activation of phosphodiesterase by modulator). A comparison of protein kinase activity assayed in the presence or absence of protein modulator suggests the presence of modulator dependent protein kinase activity, eluting in about the centre of the inhibitor activity trough. To avoid omission of MDPK activity, responsive to substrates other than histone, the entire inhibitor activity through representing fractions 55 and 95 were pooled and dialyzed overnight against 20 mM Tris/HCl, 1 mM magnesium acetate and 20 mM β-mercaptoethanol.

Affinity Column Chromatography - The dialyzed fractions were adjusted to 0.2 mM calcium chloride and 20 mM benzamidine and applied to a 40 ml protein modulator affinity column pre-equilibrated against 20 mM Tris/HCl, 1 mM magnesium acetate, 20 mM β-mercaptoethanol, 0.2 mM calcium chloride (Ca^{2+} buffer) plus 20 mM benzamidine (protease inhibitor). After the sample application the affinity column was washed with ten bed volumes of Ca^{2+} buffer, followed by washing with Ca^{2+} buffer plus 0.1 mM NaCl. Washing continued until the concentration of protein eluted from the column, as monitored by A 280 nm was negligible. The the elution

buffer was changed to buffer of 20 mM Tris/HCl, 1 mM magnesium acetate, 20 mM β-mercaptoethanol, 0.1 M NaCl and 0.4 mM EGTA (affinity column profile not shown). This fraction containing proteins capable of Ca^{2+}-dependent association with the protein modulator was found to be enriched in histone kinase activity. Since modulator stimulated histone kinase activity also was measured in the Ca^{2+} wash, the affinity column procedure was repeated with the Ca^{2+} and Ca^{2+} plus salt fractions. It was found that the second affinity column chromatography was necessary to recover the MDPK in these fractions. The EGTA fractions were pooled together, adjusted to 10% sucrose and concentrated to 6.0 mls by diaflo concentrations using a PM-10 membrane. Figure 20 presents the electrophoretic analysis of the EGTA fraction on 5% polyacrylamide gels in the presence of sodium dodecyl sulfate. The subunit molecular weights have been determined and from these determinations it was concluded that the main protein bands (figure 20) have a molecular weight of 128,000, 90,000, and 50,000. Minor bands are observed with molecular weights of 190,000, 80,000 and 32,000.

G-200 Sephadex Column Chromatography - The diaflo concentrated affinity column purified fraction (EGTA fraction) was applied to a 2.5 x 90 cm G-200 Sephadex column equilibrated with 20 mM Tris/HCl, 1 mM magnesium acetate,

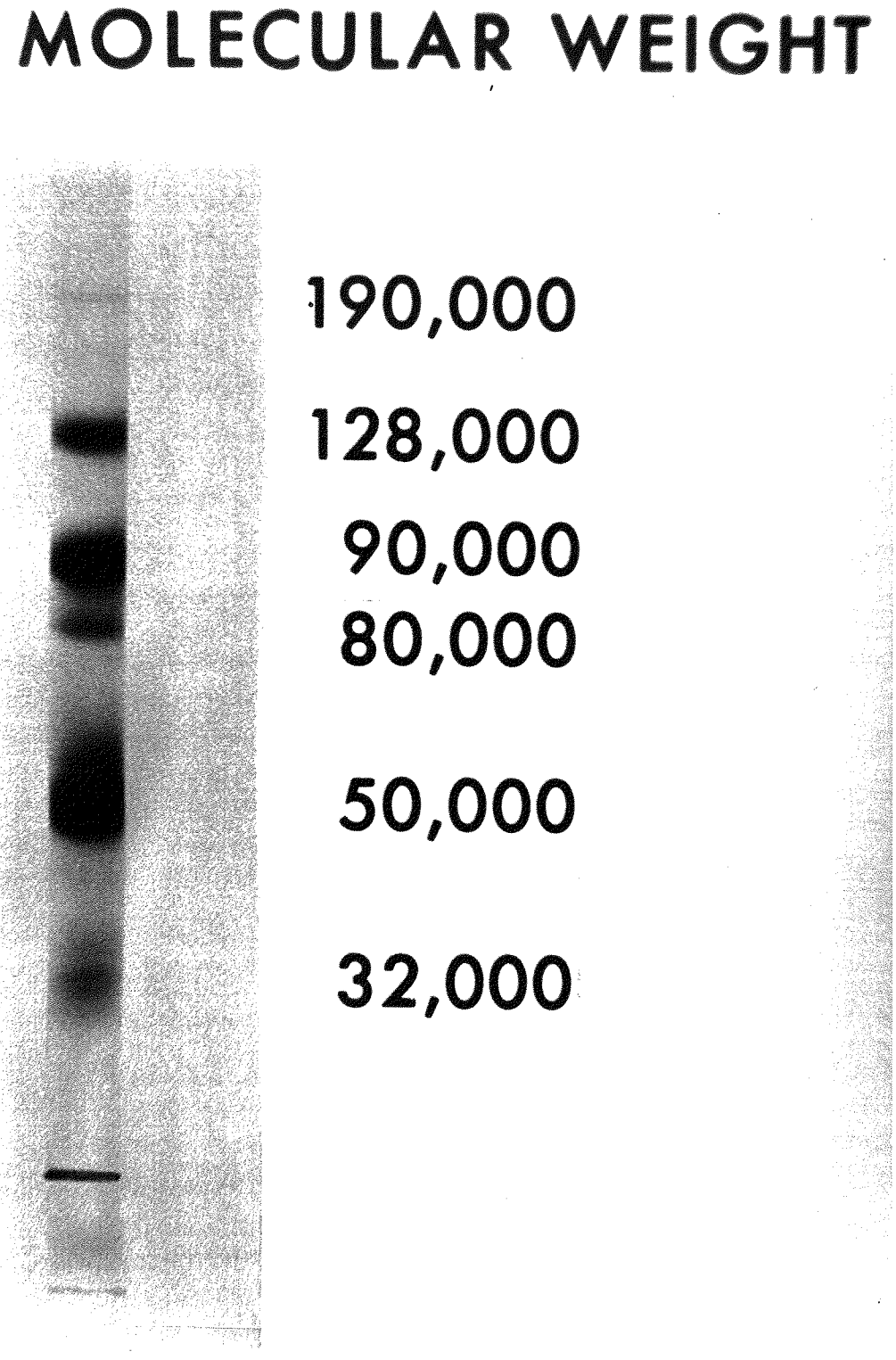


Fig. 20. Electrophoretic analysis of protein modulator affinity column purified fraction.
The concentrated, pooled, EGTA eluted fractions from the protein modulator affinity column were subjected to 5% PAGE in the presence of sodium dodecyl sulfate, according to Weber and Osborne (1969). About 100 µg of sample was applied. Molecular weights of protein bands are provided.

fig 20

20 mM β-mercaptoethanol, 0.1 M NaCl, 0.1 mM EGTA, and 10% sucrose. Figure 21 presents the elution profile of the Sephadex G-200 chromatograph. Three peaks of histone kinase activity were resolved. Peak I corresponded to the void volume of the column. Peak II and Peak III kinase activity corresponds to the two peaks of protein (A_{595}nM) which were resolved by the chromatography. Since all three peaks of histone kinase activity correspond with the trough of inhibitor activity it is suggested that modulator dependent protein kinase activity represents a major component of the proteins of rabbit skeletal muscle capable of interaction with the protein modulator.

The three peaks of kinase activity were pooled and analyzed for homogeneity on 5% polyacrylamide gels in the presence of sodium dodecyl sulfate (Weber and Osborne, 1969). Results are presented in the insert of figure 21. The presence of one major protein band and three minor bands in peak I is noted. Peak II and Peak III appear essentially homogeneous.

The subunit molecular weight of the peak I, II and III was determined with sodium dodecyl sulfate gel electrophoresis. Results are presented in figure 22. The molecular weight of the major band of Peak I was determined to be about 128,000 daltons. The molecular

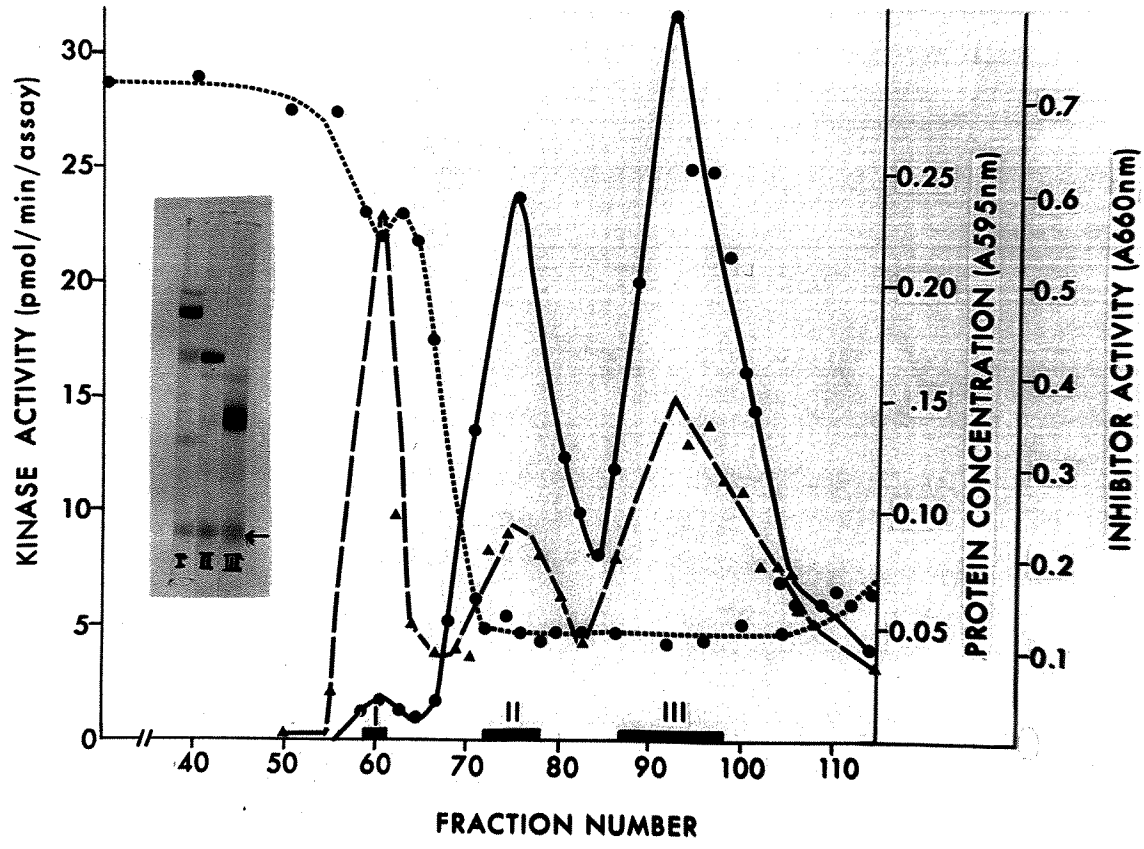


Figure 21. Chromatography of affinity column purified proteins on Sephadex G-200.
The EGTA eluted fractions from the protein modulator affinity column were concentrated and applied to a Sephadex G-200 column (2.5 x 90 cm). The column fractions were assayed for protein concentration (▲), inhibitor activity (---●---), and histone kinase activity (←—●—→). The histone kinase activity was assayed as outlined in "experimental procedure" with the addition of protein modulator (20 µg/ml) and Ca^{2+} (0.4 mM).

Insert. Electrophoretic analysis of pooled Sephadex G-200 fractions.
Peaks I, II, and III were pooled and subjected to 5% PAGE in the presence of sodium dodecyl sulfate. Approximately 20 µg of the samples was applied to the gels. The arrow indicates the dye front (cytochrome-C).

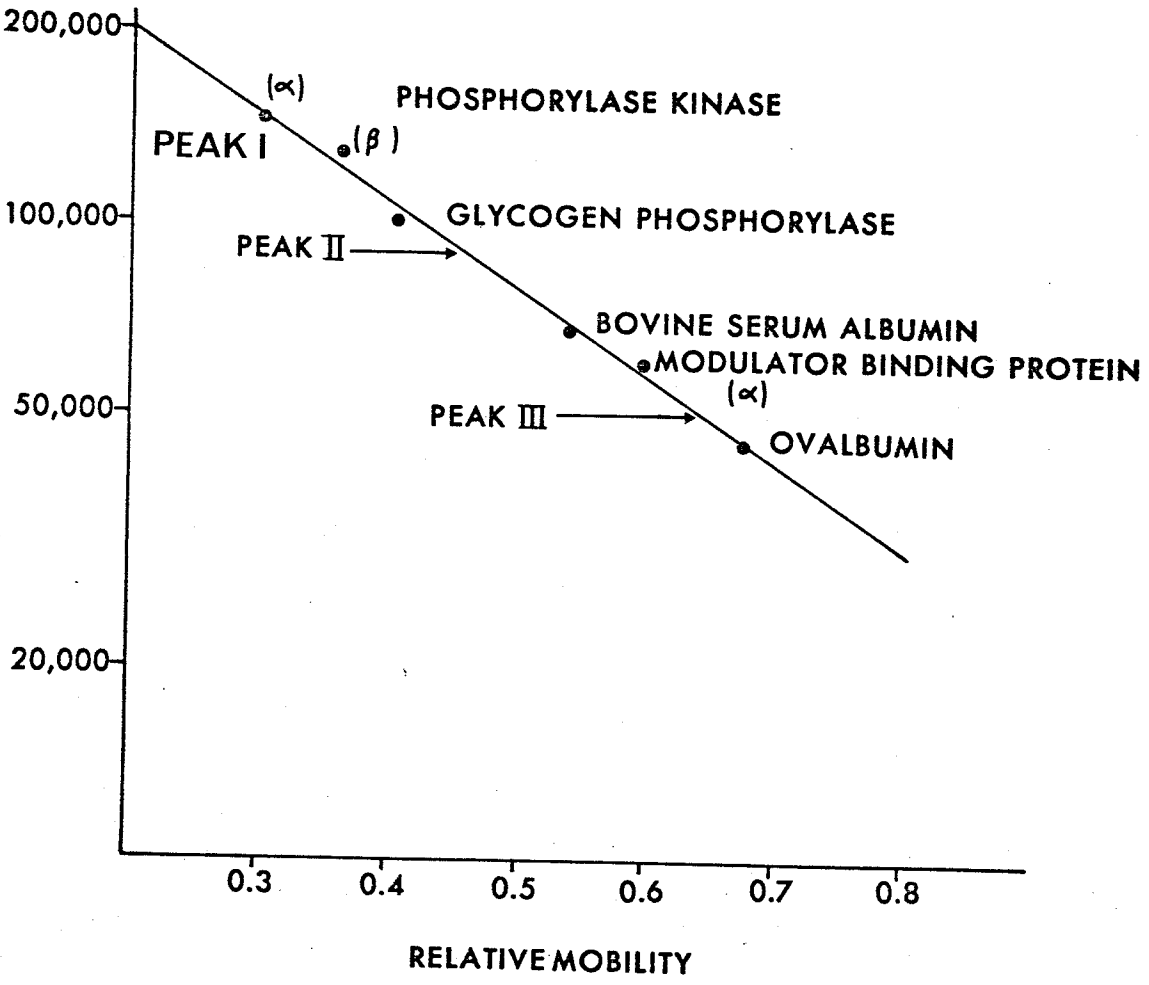


Figure 22. Determination of the apparent molecular weight of the Sephadex G-200 kinase activity peaks. Mobility relative to the dye front is plotted against known subunit molecular weight of each protein. The two protein bands of peak I were found to coincide to molecular weights 145,000 and 128,000 daltons.

weights of the minor bands of peak I were also determined (not shown), and of particular interest was the presence of a minor band at 145,000 daltons. The molecular weight of this minor band and the major band correspond to the α and β subunits of phosphorylase kinase. The molecular weights of peak II and peak III were determined to be 90,000 and 59,000 daltons. From the position of peak II and peak III on the G-200 elution profile it is suggested that they are monomeric.

CHARACTERIZATION OF THE MODULATOR DEPENDENT PROTEIN KINASES

Analysis of Sephadex G-200 Peak I

Substrate Specificity - Because of the similarity of the molecular weight of the two bands of peak I with the α and β subunits of phosphorylase kinase, peak I was analyzed for phosphorylase kinase activity. Results are presented in figure 23. Since peak I catalyses the phosphorylation of phosphorylase b, the identity of peak I as phosphorylase kinase is confirmed. Calculation of the phosphorylase kinase activity ratio (pH 6.8/pH 8.2) was determined to be 0.23. The appearance of a faint α and broad β band on SDS gel electrophoresis (Figure 21) suggests that peak I consists of proteolytically activated phosphorylase kinase (Cohen, 1973). However as will be shown in the following

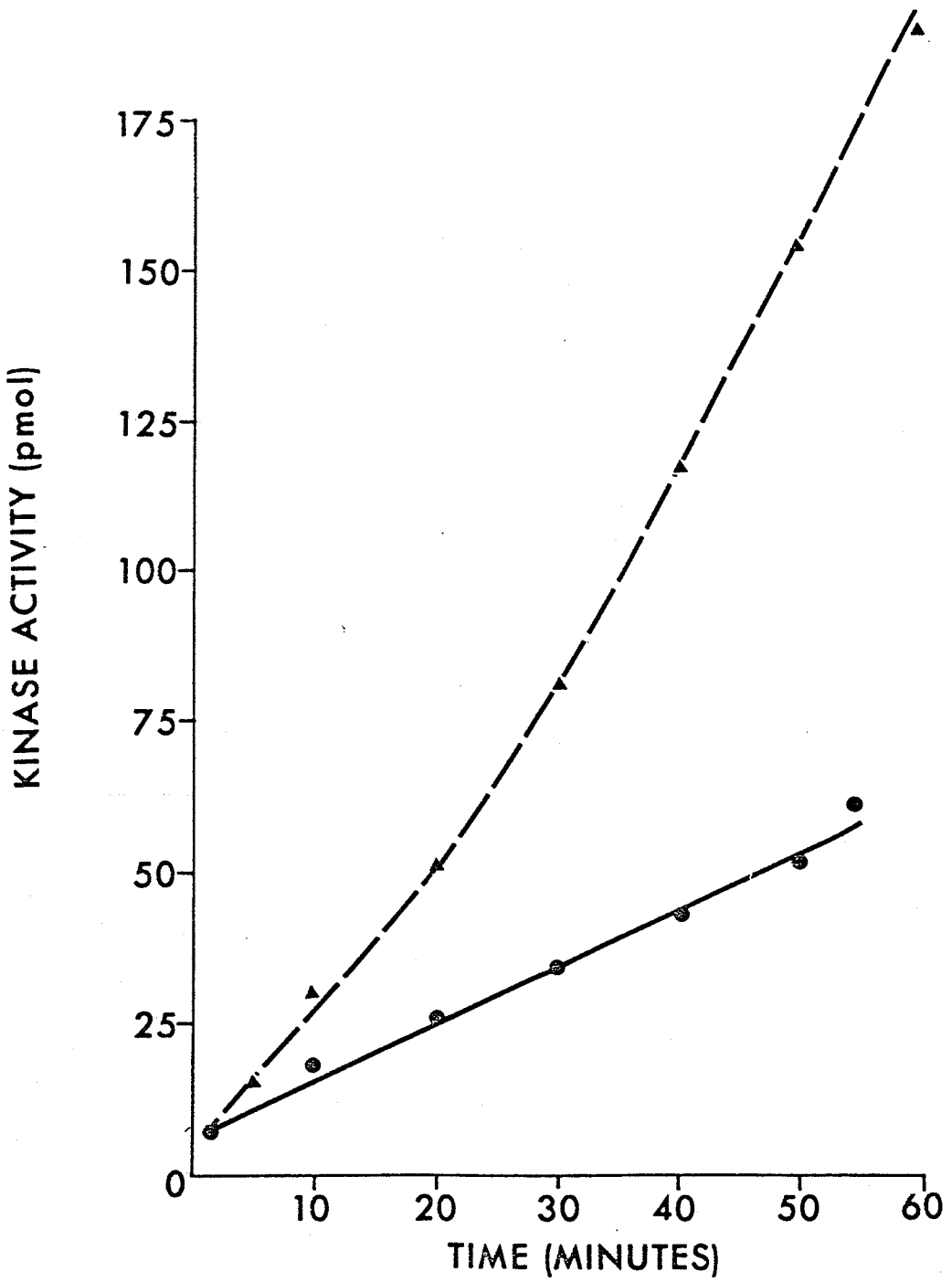


Figure 23. Phosphorylase kinase activity assay of Sephadex G-200, peak I.
Peak I (0.22 µg) was incubated in 12.5 mM Tris/HCl, 12.5 mM β-glycerol phosphate (pH 6.8), 10 mM Mg^{2+}, 20 mM β-mercaptoethanol, 3.8 mg/ml phosphorylase, and 1 mM ATP, in the presence of either 0.1 mM Ca^{2+} (▲) or 0.4 mM EGTA (●). Aliquots (20 µL) were removed at intervals and analyzed for protein bound phosphate as in "experimental procedures".

section, the purified non activated phosphorylase kinase also shows Ca^{2+}-dependent interaction with the modulator affinity column. Phosphorylase kinase (non activated) purified to electrophoretic homogeneity according to Hayakawa et al, (1973) was used in all subsequent studies.

Physical Interaction with Protein Modulator - A protein modulator affinity column was used to study the possible interaction between protein modulator and phosphorylase kinase. Results are presented in table X. In the presence of Ca^{2+}, phosphorylase kinase binds to the protein modulator and is retained by the affinity column. When EGTA is used to elute the column, phosphorylase kinase is released into the eluant indicating that EGTA disrupts the protein modulator-phosphorylase kinase interaction. The results suggest a Ca^{2+}-dependent association between protein modulator and phosphorylase kinase.

The interaction between phosphorylase kinase and protein modulator was also studied using the modulator binding protein assay. This assay is actually a competition assay, proteins are assayed according to their ability to compete with Ca^{2+}-activated cyclic nucleotide phosphodiesterase for the protein modulator present in the assay (enough protein modulator is present to maximally activate the phosphodiesterase). If a protein is capable

CHROMATOGRAPHY OF PHOSPHORYLASE KINASE ON PROTEIN MODULATOR AFFINITY COLUMN

ELUANT COLLECTED	VOLUME OF ELUANT	PHOSPHORYLASE KINASE CONCENTRATION (mg/ml)	RECOVERY (%)
SAMPLE BREAKTHRU	7.1	0.07	18
Ca^{2+} WASH	17.0	0	0
EGTA WASH	5.2	0.28	53

Phosphorylase kinase (2.75 mg) was adjusted to 3 mM Mg^{2+}, 0.1 M NaCl, 0.5 mM Ca^{2+}, and 50 mM β-glycerol phosphate, and applied to a one ml protein modulator affinity column, equilibrated with Ca^{2+} buffer, 50 mM β-glycerol phosphate, 3 mM Mg^{2+}, 0.5 mM Ca^{2+}, 0.1 M NaCl, and 20 mM β-mercaptoethanol. Fractions were collected batchwise. After sample application the column was washed with a small volume of Ca^{2+} buffer. This fraction, containing the sample breakthrough and some Ca^{2+} wash is referred to as the sample breakthrough. The column was then thoroughly washed with Ca^{2+} buffer (fraction called the Ca^{2+}WASH) and finally washed with EGTA buffer (EGTA WASH fraction) containing 50 mM β-glycerol phosphate, 3 mM Mg^{2+}, 0.5 mM EGTA, 0.1 M NaCl, and 20 mM β-mercaptoethanol. Phosphorylase kinase concentration was determined by assuming a molar extinction coefficient of 12.4 (Cohen, 1973).

of competing with phosphodiesterase for protein modulator then the resulting decrease in the amount of protein modulator available to phosphodiesterase will result in a decrease in phosphodiesterase activity. Figure 24 shows the inhibition of Ca^{2+}-activated phosphodiesterase by phosphorylase kinase. Experimental conditions were identical to that of the modulator binding protein assay. The amount of phosphorylase kinase necessary for 50% inhibition of the phosphodiesterase was determined to be 4.0 µg/ml.

Activation of Phosphorylase Kinase - Since Ca^{2+}-dependent interaction between phosphorylase kinase and protein modulator was indicated, the possibility that protein modulator might regulate phosphorylase kinase activity has been investigated. Results are presented in figure 25. The presence of protein modulator in the assay medium results in a significant increase in the rate of phosphorylation of phosphorylase kinase. This stimulatory effect depends on the presence of both Ca^{2+} and the protein modulator. Furthermore the increased rate of phosphorylation results in an increased rate of activation of the enzyme (data not shown). The activation of phosphorylase kinase was not due to partial proteolysis since enzyme activation

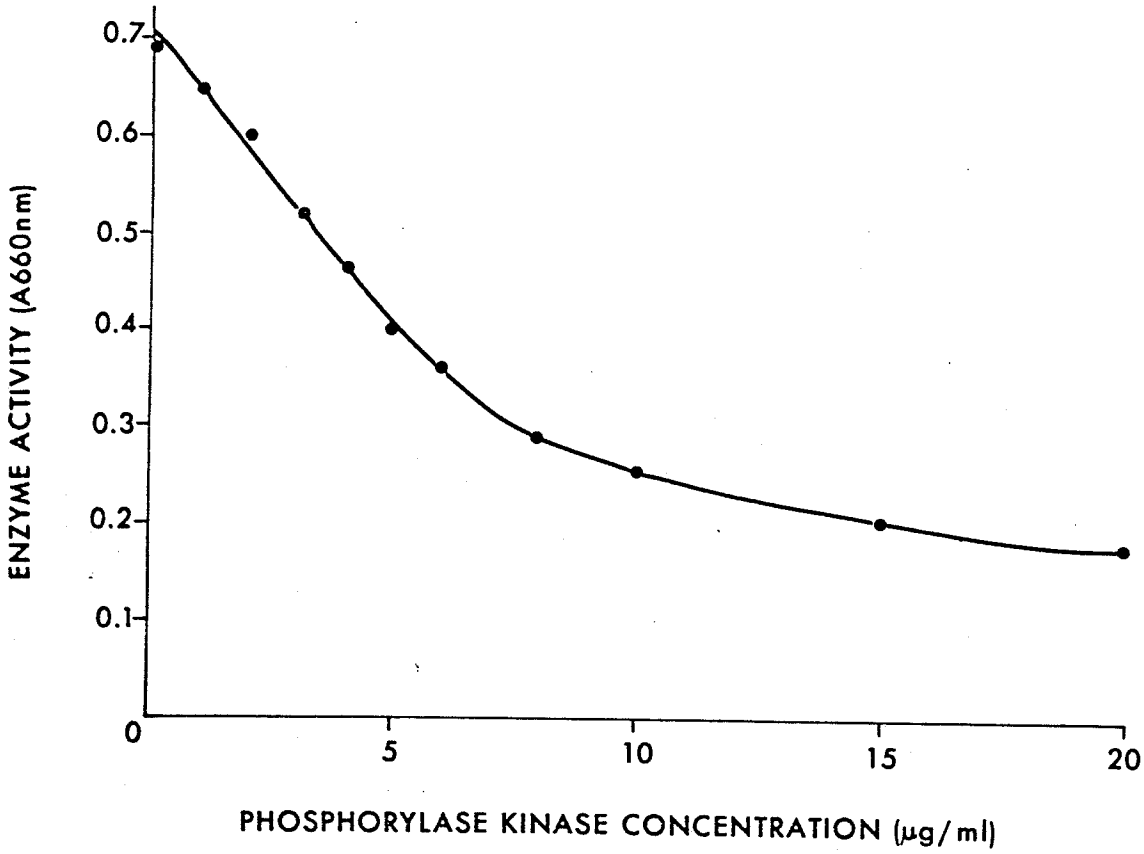


Figure 24. Effect of phosphorylase kinase concentration on phosphodiesterase activity.
Phosphodiesterase activity (0.012 units) was assayed in the presence of 4 units (3.5 µg/ml) of protein modulator and various concentrations of phosphorylase kinase.

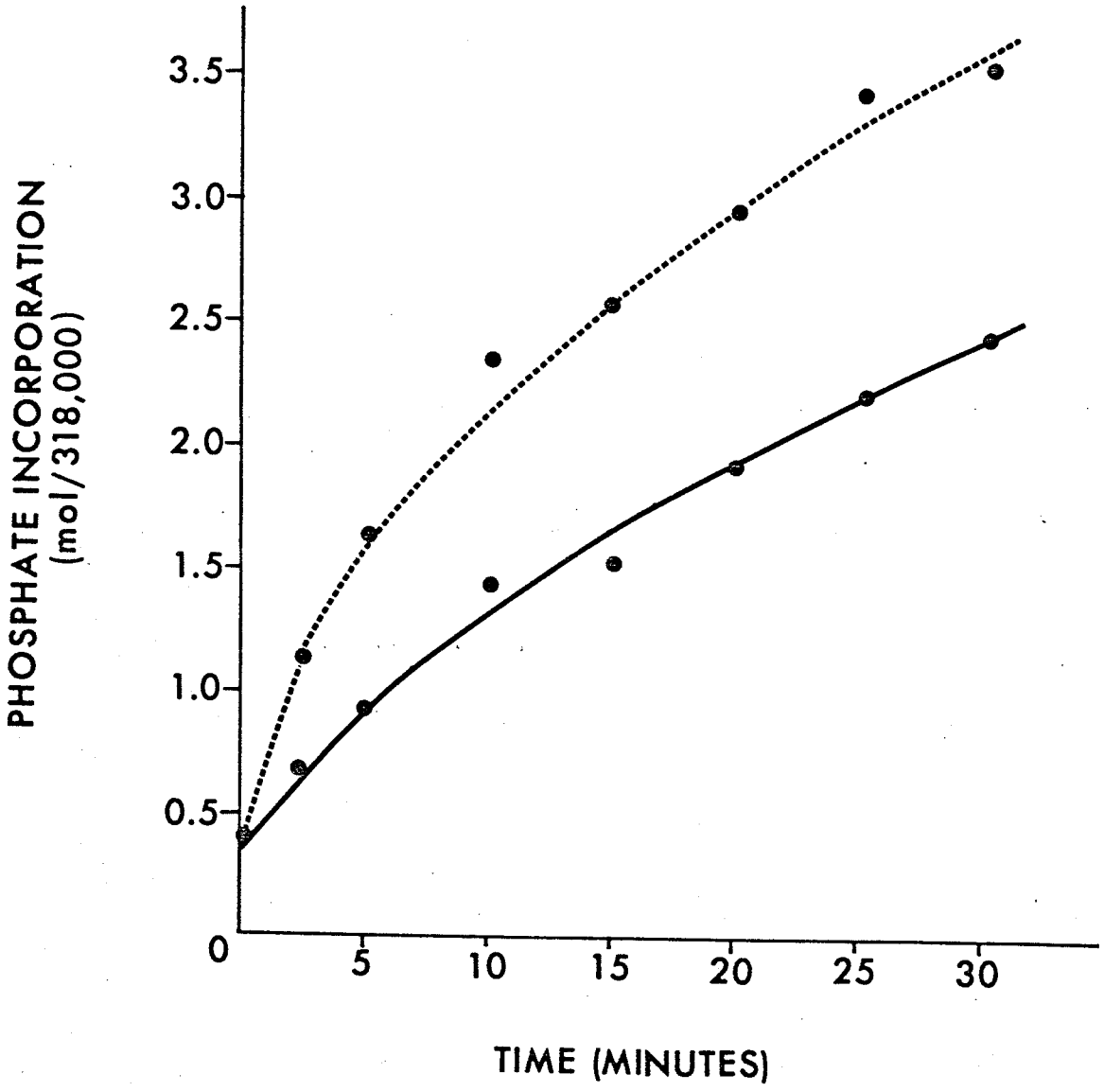


Figure 25. Effect of protein modulator on autocalytic reaction. Phosphorylase kinase (0.2 mg/ml) was incubated in 50 mM β-glycerol phosphate (pH 6.8), 10 mM Mg^{2+} 0.25 mM ATP, 40 mM β-mercaptoethanol and 0.4 mM Ca^{2+}, in the presence (---●---) or absence (——●——) of 20 µg/ml of protein modulator. At intervals, aliquots were removed and assayed for protein-bound phosphate.

was not observed in the absence of ATP. The phosphorylation and activation of phosphorylase kinase by the protein modulator could be due to direct stimulation of the autocatalytic reaction by protein modulator, or could be explained by the presence of a contaminating protein modulator stimulated protein kinase. Alternatively the protein modulator could induce a conformational change in phosphorylase kinase resulting in inhanced susceptibility to a contaminating protein kinase.

To clarify the possible mechanism of phosphorylation and activation of phosphorylase kinase by protein modulator, the effect of protein modulator on enzyme activity was investigated. Results are presented in figure 26. The stimulation of phosphorylase kinase activity by both protein modulator and Ca^{2+} alone appears to be better than two fold. This could be attributed to direct stimulation of the autocatalytic rate of phosphorylase kinase or by the presence of large amounts of a contaminating protein modulator regulated protein kinase, since catalytic amounts of a contaminant would be diluted out by the assay procedure. The data showing direct interaction between protein modulator and phosphorylase kinase, and the homogeneity of the phosphorylase kinase preparation on SDS gel electrophoresis is interpreted as favoring the former

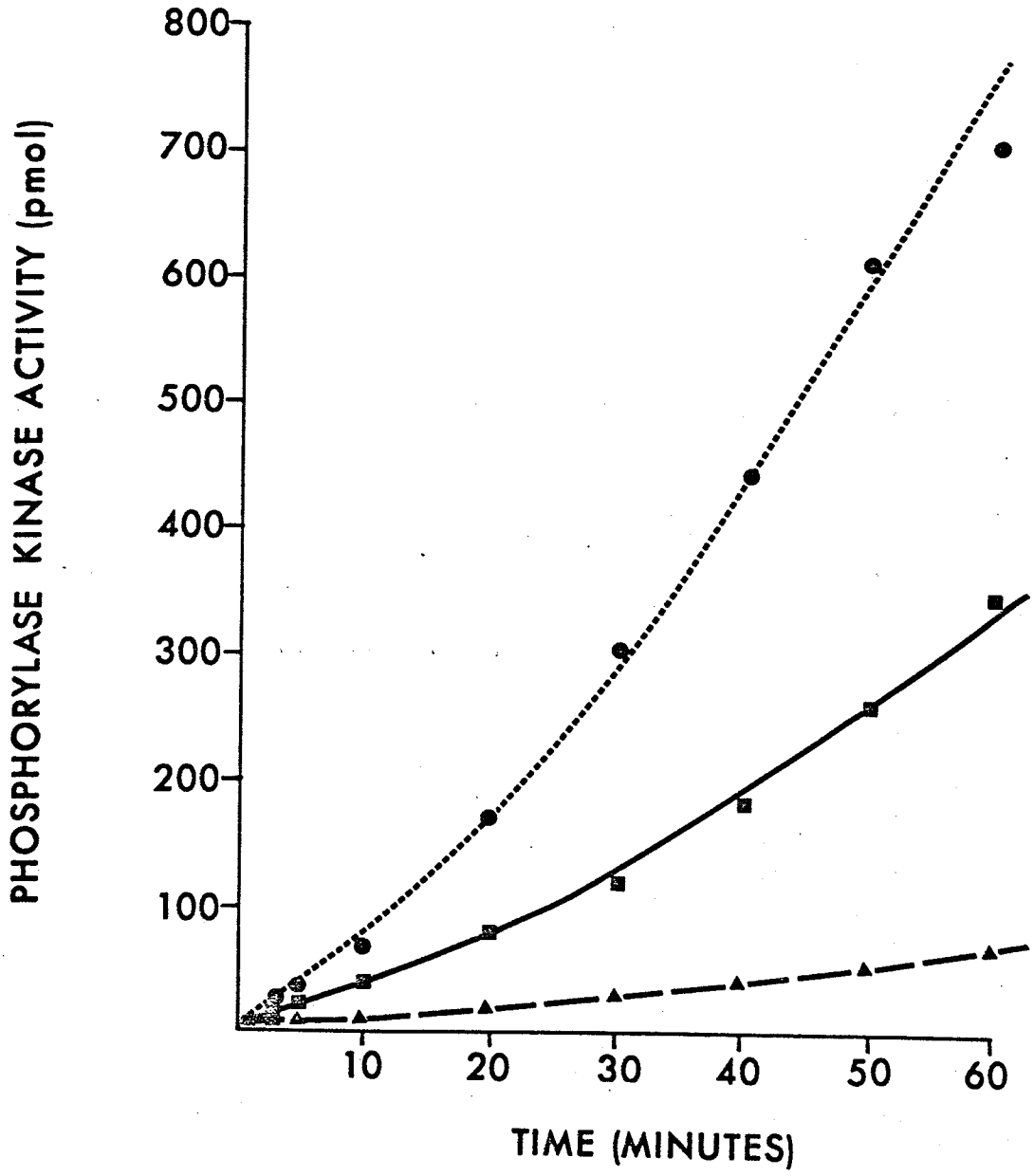


Figure 26. Effect of protein modulator on phosphorylase kinase activity.
Phosphorylase kinase (0.92 µg/ml) was incubated in 50 mM β-glycerol phosphate (pH 6.8), 10 mM Mg^{2+}, 1.9 mM ATP, 40 mM β-mercaptoethanol, 4.5 mg/ml of phosphorylase in the presence of 0.8 mM EGTA, and 20 µg/ml protein modulator (▲), 0.4 mM Ca^{2+} (■), or 0.4 mM Ca^{2+} and 20 µg/ml protein modulator (●). The addition of 7 µg/ml of bovine brain modulator binding protein to the phosphorylase kinase reaction(assayed in the presence of Ca^{2+}), had negligible effects on the phosphorylase kinase activity.

suggestion.

Contamination of Phosphorylase Kinase by Protein Modulator - Figure 26 shows that the activity of phosphorylase kinase in the presence of Ca^{2+} alone is very high. This could be attributed to the presence of protein modulator as a contaminant of the phosphorylase kinase preparation or to an intrinsic property of phosphorylase kinase. This question has been partly resolved by Cohen et al, (1978) who have demonstrated that purified preparations of phosphorylase kinase contain protein modulator as a tightly bound subunit. The observation of Cohen et al, (1978) is confirmed by the result of figure 27 which demonstrates the existence of protein modulator in the phosphorylase kinase preparation. From figure 27 it was calculated that the amount of protein modulator was about 1.1 mol per holoenzyme. The calculation was based on the assumption that one unit of the modulator protein in the phosphorylase kinase preparation corresponds to 9 ng/ml of the modulator and that the heat treatment of the original sample did not cause a significant loss in the modulator activity. Thus the value reported should be considered a tentative value. Cohen et al, (1978) have reported that their samples of purified phosphorylase kinase contained near stoichiometric concentration of protein

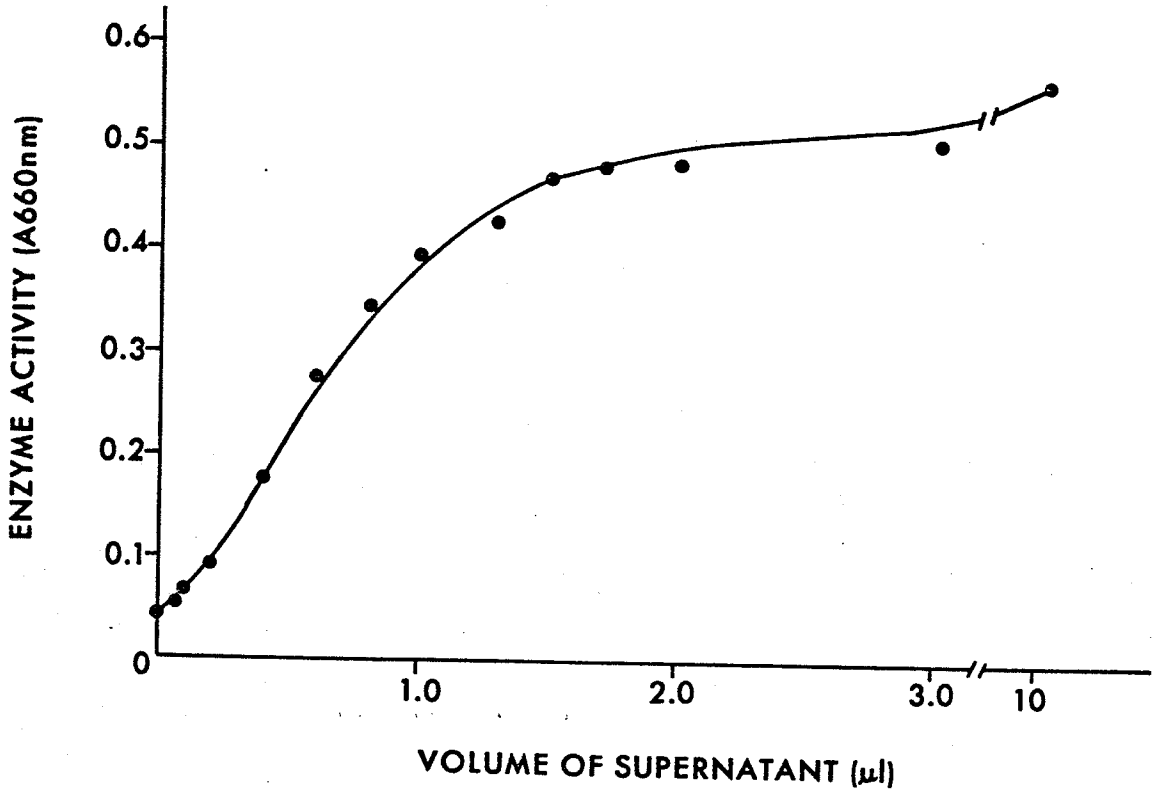


Figure 27. Analysis of phosphorylase kinase preparations for protein modulator activity.
Phosphorylase kinase (0.92 mg/ml) was adjusted to 0.4 mM Ca^{2+} then boiled for three minutes, centrifuged at 6,000 x g for thirty minutes, and the supernatant was analyzed for the presence of protein modulator.

modulator with the other three subunits of phosphorylase kinase. Based on the value of 0.7 mol of protein modulator per mol of subunit Cohen et al, (1978) have proposed that the phosphorylase kinase contains 3 mol of protein modulator per phosphorylase kinase holoenzyme. On the other hand data presented by Cohen et al, (1978) and in this thesis suggests that phosphorylase kinase is capable of Ca^{2+}-dependent interaction with protein modulator (figure 24, table X) and the Ca^{2+}-dependent and reversible interaction of protein modulator with these sites on phosphorylase kinase results in stimulation of enzyme activity (figure 25, 16).

The simplest explaination at present is that in vivo, phosphorylase kinase may contain tightly bound protein modulator and during purification of this enzyme some of the modulator is removed. The sites which have been stripped of protein modulator appear to retain the ability to interact in a Ca^{2+}-dependent and reversible manner with exogenous protein modulator but cannot bind the modulator as tightly as before removal of modulator from the modulator binding site. Alternatively it is possible that the protein modulator is capable of interaction with phosphorylase kinase according to two mechanisms, one involving a Ca^{2+}-dependent and reversible interaction and the other mechanism involving tightly bound modulator (section II, 6).

Whether or not the tightly bound protein is responsible for the high levels of activity when assayed with Ca^{2+} alone (figure 26) cannot at present be resolved. Since incubation of phosphorylase kinase with the modulator binding protein of bovine brain (figure 26) had no effect on the Ca^{2+}-stimulated phosphorylase kinase activity it is suggested that minor contamination of the enzyme with protein modulator which might interact with the reversible sites, is not responsible for this activation. Furthermore the ineffectiveness of the modulator binding protein suggests that if the tightly bound protein modulator mediates the Ca^{2+}-stimulated activity it is bound in such a manner by the phosphorylase kinase to render it incapable of interaction with the bovine brain modulator binding protein. If the stimulation of the activity of phosphorylase kinase by Ca^{2+} alone is shown to be mediated by the tightly bound protein modulator then the total stimulation of phosphorylase kinase activity by protein modulator (endogenous and exogenous) is suggested to be at least ten fold (figure 26).

At present two mechanisms exist for the activation of rabbit skeletal muscle phosphorylase kinase. The first mechanism involves the phosphorylation of phosphorylase

kinase by protein kinases. Four different protein kinases have been found to catalyse the phosphorylation of phosphorylase kinase; they are the c-AMP-dependent protein kinase (Walsh et al, 1971), c-GMP-dependent protein kinase (Khoo et al, 1977), Ca^{2+} protease activated protein kinase (Kishimoto et al, 1977) and phosphorylase kinase itself (Walsh et al, 1971). The phosphorylations result in stimulation of phosphorylase kinase activity. The second mechanism of activation of phosphorylase kinase involves the direct stimulation of phosphorylase kinase activity by the protein modulator. It appears that the direct stimulation of phosphorylase kinase activity (figure 26) by the protein modulator can, at present only by attributed to the Ca^{2+}-dependent and reversible binding of protein modulator by phosphorylase kinase. The possible involvement of the tightly bound protein modulator in the regulation of phosphorylase kinase activity remains to be elucidated.

ANALYSIS OF SEPHADEX G-200 PEAK II AND PEAK III. The purification of Peak II and Peak III kinase by the protein modulator affinity column suggests a Ca^{2+}-dependent interaction between the kinases and protein modulator. The effect of this interaction on kinase activity was investigated.

Effectors of Kinase Activity - Table XI shows that the purified kinase depends on the simultaneous presence of Ca^{2+} and the protein modulator for its histone phosphorylation activity. In the presence of EGTA the enzyme exhibited essentially no activity irrespective of the modulator protein addition. The addition of Ca^{2+} alone gave rise to small but significant histone phosphorylation activity. Analysis of kinase preparations has failed to reveal the presence of contaminating protein modulator activity, therefore suggesting that the kinase has basal activity in the presence of Ca^{2+}. In contrast to Ca^{2+} and the modulator protein, c-AMP and c-GMP did not activate the enzyme. Furthermore the enzyme was not significantly affected by the heat-stable inhibitor of c-AMP dependent protein kinase. Similar results have been found for peak II or peak III kinase activity. The data suggest that peak II and peak III do not contain the cyclic nucleotide-dependent protein kinases or the catalytic unit of c-AMP dependent protein kinase.

Figure 28 presents the rates of histone phosphorylation by peak II and peak III. The rate of the enzyme reaction is maximal when Ca^{2+} and protein modulator are present in the incubation medium. The rate of histone

TABLE XI

EFFECTORS OF KINASE ACTIVITY

Addition	Enzyme Activity pmol ^{32}P Incorporated/Assay
Protein Modulator	1.0
Ca^{2+}	10.0
Protein Modulator + Ca^{2+}	73.0
cAMP	1.0
cGMP	1.0
Protein MODULATOR + Ca^{2+} + Protein Kinase Inhibitor	67.0

The standard assay reaction (0.050 ml) contained 20 mM Tris/HCl (pH 7.5), 10 mM Mg^{2+}, 40 mM β-mercaptoethanol, 0.25 mM ATP (93 cpm/pmol), 1 mg/ml histone, 0.1 mM EGTA and 2.6 µg/ml of a mixed preparation containing peak II and peak III kinase (approximately equal molar ratio). The concentration of additions is c-AMP, 10 µM; c-GMP, 10 µM; protein modulator, 20 µg/ml; Ca^{2+}, 0.4 mM; and protein kinase inhibitor, 0.87 mg/ml. Reactions were carried out at 30° for ten minutes.

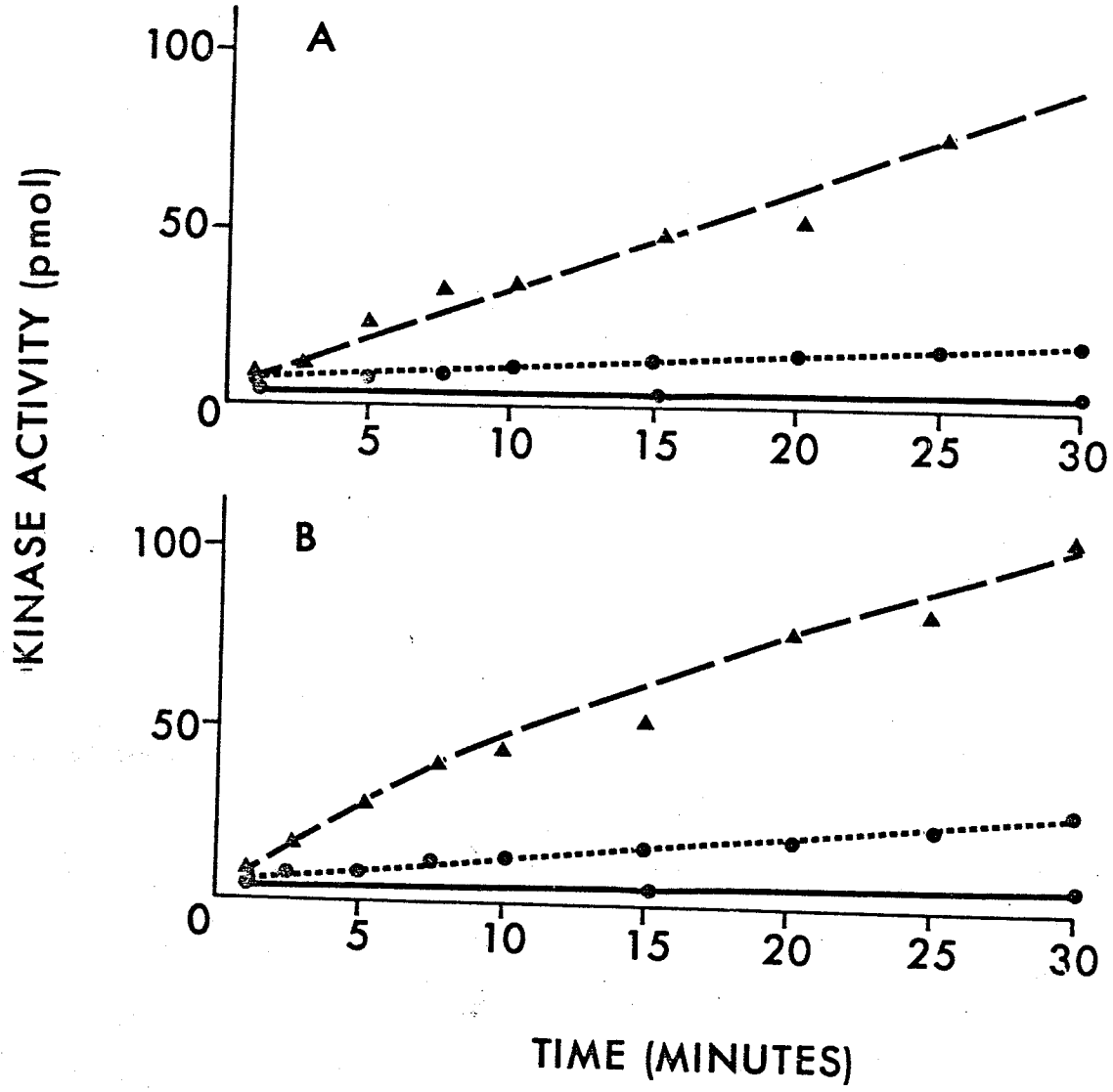


Figure 28. Time course of the rate of histone phosphorylation. A) Peak II kinase (2.55 µg/ml) or B) peak III kinase (2.7 µg/ml) was incubated with 20 mM Tris/HCl (pH 7.5), 10 mM Mg^{2+}, 0.8 mg/ml histone, 40 mM β-mercaptoethanol, 0.25 mM ATP, in the presence of 0.8 mM EGTA (—●—), 0.4 mM Ca^{2+} (---●---), or 20 µg/ml of protein modulator and 0.4 mM Ca^{2+} (▲). Aliquots (20 µl) were removed at intervals and analyzed for protein bound phosphate.

phosphorylation in the presence of Ca^{2+} is low but significant. There is essentially no phosphorylation in the presence of EGTA. The linearity of the reaction rate allowed calculation of the specific activities; peak II was determined to be 0.064 μmol/min/mg and peak III, 0.075 μmol/min/mg.

Substrate Specificity - The rates of phosphorylation of a number of possible substrates, by the histone kinases is presented in table XII. The substrate specificities of these kinases has been determined to be identical, both kinases catalyse the phosphorylation of histone and myosin light chain fraction. Furthermore the rate of phosphorylation of histone is only about 5% of the rate of phosphorylation of the myosin light chains. The addition of the protein modulator and Ca^{2+} in the absence and in the presence of peak II or peak III kinase produced no activation of chicken adipose tissue hormone-sensitive triglyceride lipase (pH 5.2 precipitate fraction; D.L. Severson, University of Calgary, personal communication). An earlier report (Waisman et al, 1978a) suggested that partially purified myosin light chain kinase phosphorylated and activated phosphorylase kinase. Attempts to reproduce this observation have been unsuccessful.

TABLE XII

SUBSTRATE SPECIFICITY

SUBSTRATE	RELATIVE ACTIVITY[+]
MYOSIN LIGHT CHAIN	100
HISTONE	5
GLYCOGEN PHOSPHORYLASE	< 0.1
GLYCOGEN SYNTHASE	< 0.1
PHOSPHORYLASE KINASE	< 0.1
TROPONIN	< 0.1
TROPONIN-I	< 0.1
PROTEIN MODULATOR	< 0.1
TUBULIN	< 0.1
CASEIN	< 0.1
PROTAMINE	< 0.1
ACETYL CoA CARBOXYLATE	< 0.1

INITIAL RATE OF MYOSIN LIGHT CHAIN PHOSPHORYLATION WAS TAKEN AS 100%.

Substrate specificity of kinase.
The standard assay reaction (0.050 ml) contained 20 mM Tris/HCl (pH 7.5, 10 mM Mg^{2+}, 40 mM β-mercaptoethanol, 0.25 mM ATP, 20 µg/ml protein modulator, 0.4 mM Ca^{2+} using the following amounts of substrate, myosin light chain, 1.2 mg/ml; histone, 1/0 mg/ml; glycogen phosphorylase, 1 mg/ml; glycogen synthase, 0.2 mg/ml; phosphorylase kinase, 0.18 mg/ml; troponin, 1 mg/ml; troponin-I, 1 mg/ml; protein modulator, 0.2 mg/ml; tubulin, 0.17 mg/ml; casein, 1 mg/ml; protamine, 1 mg/ml; acetyl CoA carboxylase, 0.5 mg/ml. Similar results were found for peak II (6.9 µg/ml) or peak III (7.3 µg/ml) kinase.

Pattern of Light Chain Phosphorylation - The phosphorylation of myosin light chains has been reported by Perry et al, (1974) to be catalysed by a Ca^{2+} activated myosin light chain kinase. The phosphorylation was found to be specific for the 18,000 dalton component of myosin, usually called the DTNB-light chain. The pattern of myosin light chain phosphorylation catalysed by peak II and peak III kinase has been determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Results are presented in figure 29. The peak of radioactivity corresponded to a relative mobility of 0.88. The relative mobility of the standard (15,000 daltons) was 0.90. Within the accuracy of this technique it is reasonable to suggest this represents phosphorylation of the DTNB light chain. The results suggest that peak II and peak III kinases are myosin light chain kinases. The rate of phosphorylation of myosin light chain by the myosin light chain kinase has been demonstrated to be stimulated by protein modulator (Yagi et al, 1978).

The report by Pires and Perry (1977) of the existence of enzymically active subfragments of myosin light chain kinase of molecular weights 50,000 and 30,000 daltons suggests that the peak III kinase is a subfragment of the 90,000 dalton peak II kinase. These investigators reported the

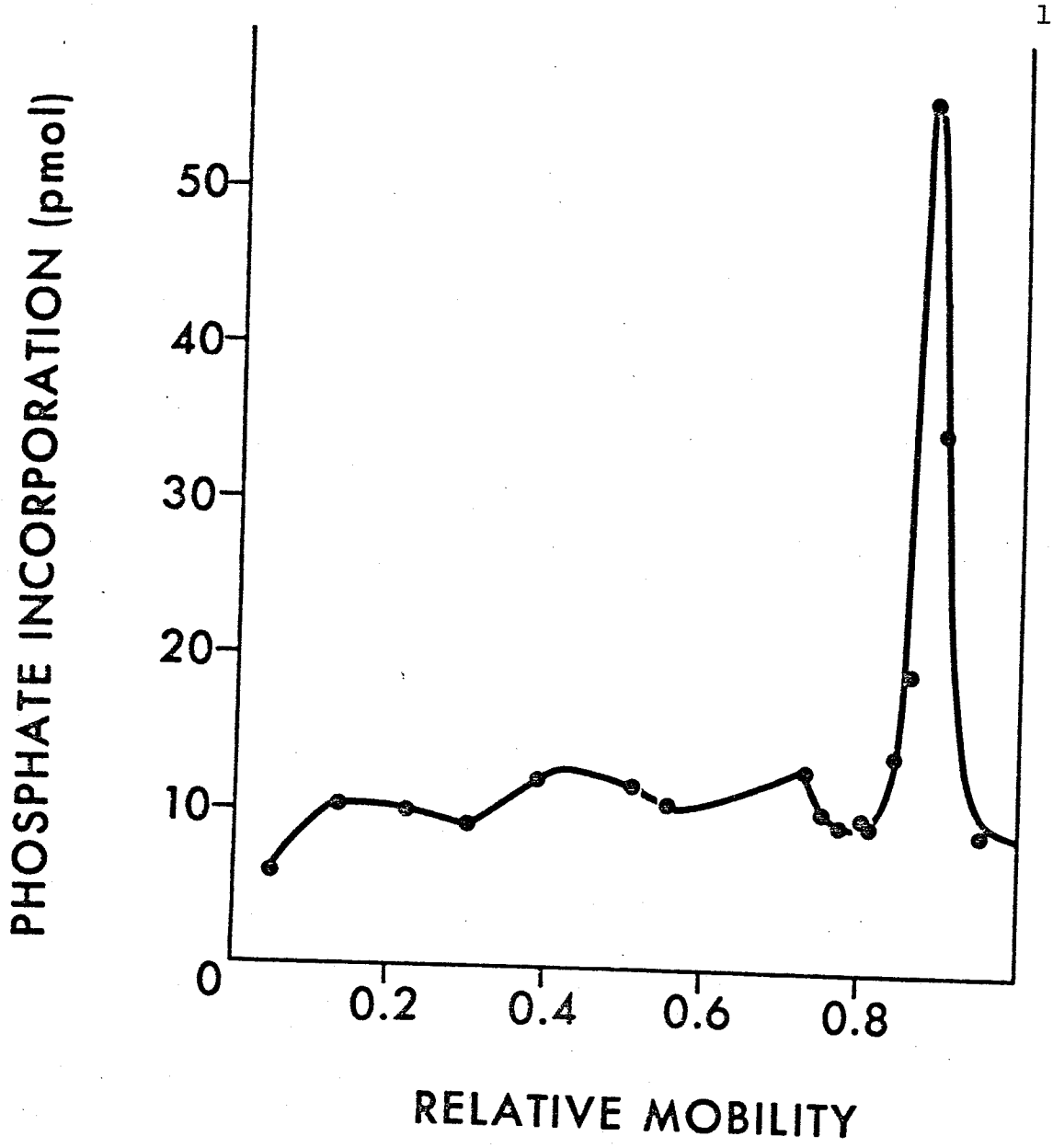


Figure 29. Electrophoretic analysis of myosin light chain phosphorylation.
Peak II kinase was assayed in 20 mM Tris/HCl (pH 7.5), 10 mM Mg^{2+}, 20 µg/ml protein modulator, 0.25 mM ATP (25 cpm/pmol), 0.4 mM Ca^{2+}, and 1.5 mg/ml myosin light chain. After twenty minutes the reaction was terminated by addition of an equal volume of 2% SDS, 40 mM EDTA. The mixture (54 µg) was then electrophoresed on 7 1/2% polyacrylamide gels in the presence of sodium dodecyl sulfate. The gel was sliced and assayed for radioactive bound phosphate. The peak of radioactivity corresponded to a Rm of 0.88. The molecular weight standard (15,000 daltons) had a Rm of 0.90.

molecular weight of the holoenzyme to be 77,000 daltons.

Interaction with Protein Modulator - The effect of protein modulator concentration on the two forms of myosin light chain kinase is presented in figure 30. The results suggest that both kinases have similar affinity for the protein modulator, the value for 50% activation of the kinase reaction was determined to be 0.60 µg/ml. This suggests that the protein modulator has a lower affinity toward the myosin light chain kinase than toward the Ca^{2+}-activatable cyclic nucleotide phosphodiesterase. Under standard assay conditions, 9 ng/ml of modulator protein can provide 50% activation of phosphodiesterase (Teo et al, 1973).

The interaction between protein modulator and myosin light chain kinase has been examined by the modulator binding protein assay. The concentration of kinase required for 50% inhibition of the modulator binding protein assay was determined to be 0.28 µg/ml for peak II and 0.24 µg/ml for peak III. It is interesting to note that while peak III kinase may be a subfragment of peak II kinase, it has retained its catalytic properties as well as its ability to interact with protein modulator. Quantitatively, these properties appear to have been unaffected.

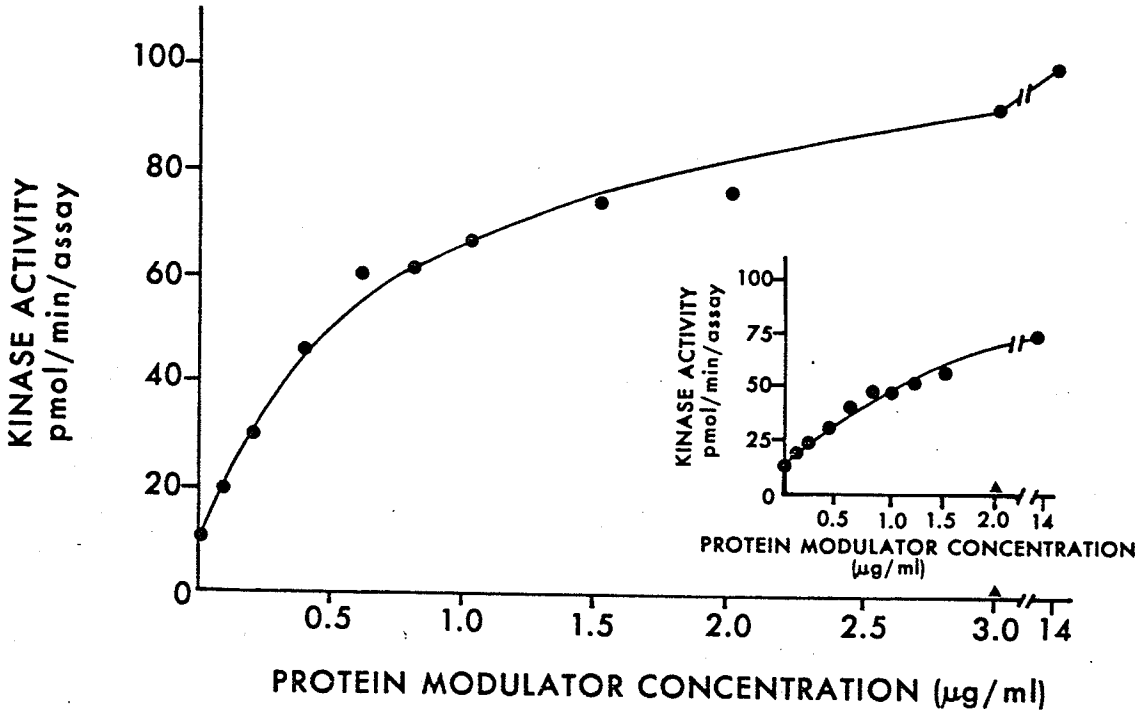


Figure 30. Activation of the modulator-deficient myosin light chain kinase by protein modulator. The assay contained 20 mM Tris/HCl (pH 7.5), 10 mM $MgAc_2$, 0.4 mM $CaCl_2$, 1 mg/ml histone, 0.25 mM ATP, and 5.98 µg/ml of peak II (insert), or 6.33 µg/ml peak III. (▲) represents the addition of 0.8 mM EGTA.

Effect of pH - Figure 31 presents the pH activity profile of peak II and peak III kinase. Both kinases have narrow pH curves and activity appears to decrease more rapidly at acidic pH than at alkaline pH. Differing pH optima are observed for the two kinases. A peak of activity is observed at pH 7.0 for peak II kinase and at pH 7.5 for peak III kinase.

The results of this study suggest that peak II and peak III kinase is actually the myosin light chain kinase, originally discovered by Pires et al, (1974). Although characterized as a Ca^{2+}-dependent enzyme (Pires and Perry, 1977), it is suggested that the myosin light chain kinase is actually a protein modulator regulated enzyme. This suggestion is supported by the observation that myosin light chain kinase is capable of Ca^{2+}-dependent association with the protein modulator, the result of this association is a seven to ten fold increase in enzyme activity. Yagi et al, (1978) have also reported stimulation of myosin light chain kinase by protein modulator.

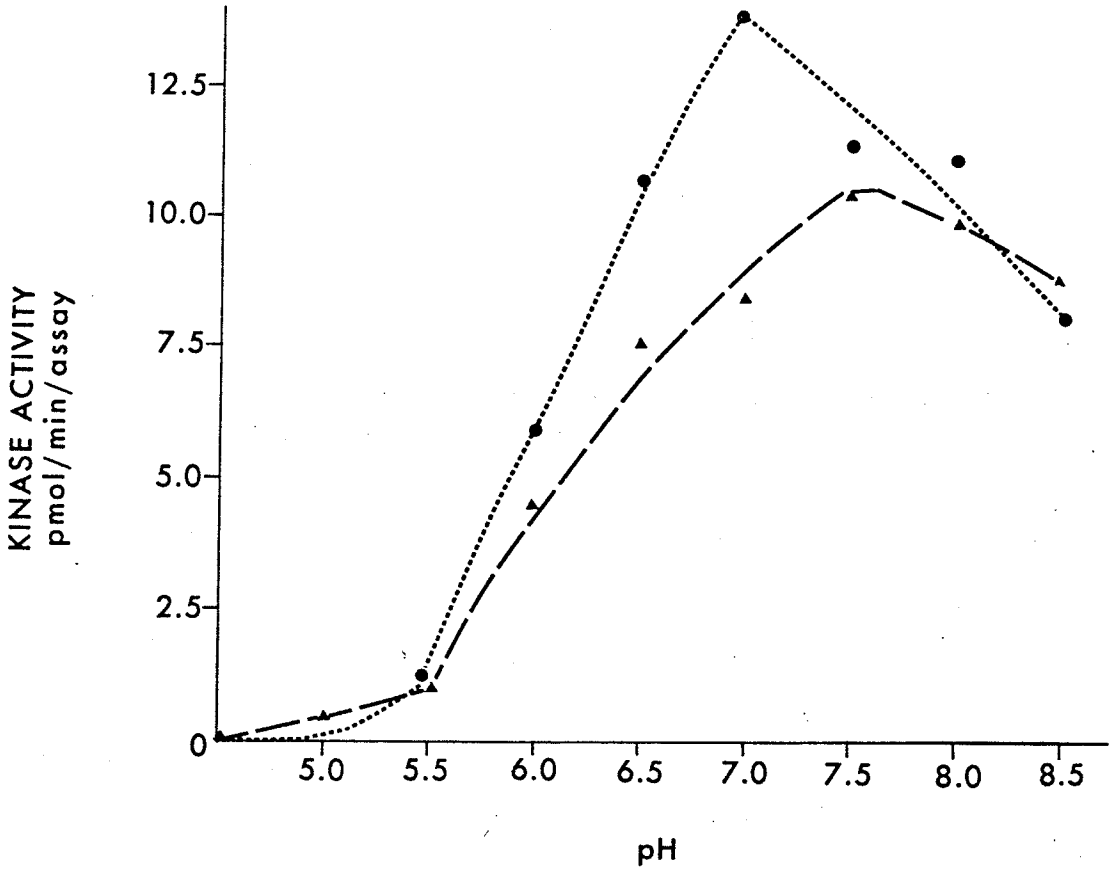


Figure 31. pH profile of myosin light chain kinase activity. 3.22 µg/ml of peak II (-●-) or 3.48 µg/ml of peak III (▲) was incubated with 40 mM β-glycerol phosphate, 20 mM Tris/HCl, 20 mM $NaAc_2$, 10 mM $MgAc_2$, 0.4 mM $CaCl_2$, 1.0 mg/ml histone, 40 mM β-mercaptoethanol, 20 µg/ml of protein modulator and 0.25 mM ATP.

PROTEIN MODULATOR REGULATION OF GLYCOGEN SYNTHASE

Protein Modulator Stimulation of Glycogen Synthase Phosphorylation - During the examination of the substrate specificity of peak II and peak III kinase it was observed that the protein modulator stimulated endogenous phosphorylation of the glycogen synthase preparation. This phenonema has received detailed examination, results are presented in figure 32. The addition of protein modulator results in a three fold increase in the rate of endogenous phosphorylation compared with the rate in the presence of Ca^{2+} alone. The significant rate of phosphorylation in the presence of Ca^{2+} alone could be explained by protein modulator contamination of the glycogen synthase preparation, or by the presence of a contaminating glycogen synthase kinase. The protein modulator stimulated endogenous phosphorylation of the glycogen synthase preparation has been investigated on sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the results are presented in the insert of figure 32. The result confirms the phosphorylation of glycogen synthase by a protein modulator stimulated glycogen synthase kinase. The protein modulator stimulated endogenous phosphorylation of glycogen synthase has been shown to correspond to a decrease in synthase activity (Srivastava et al, 1979).

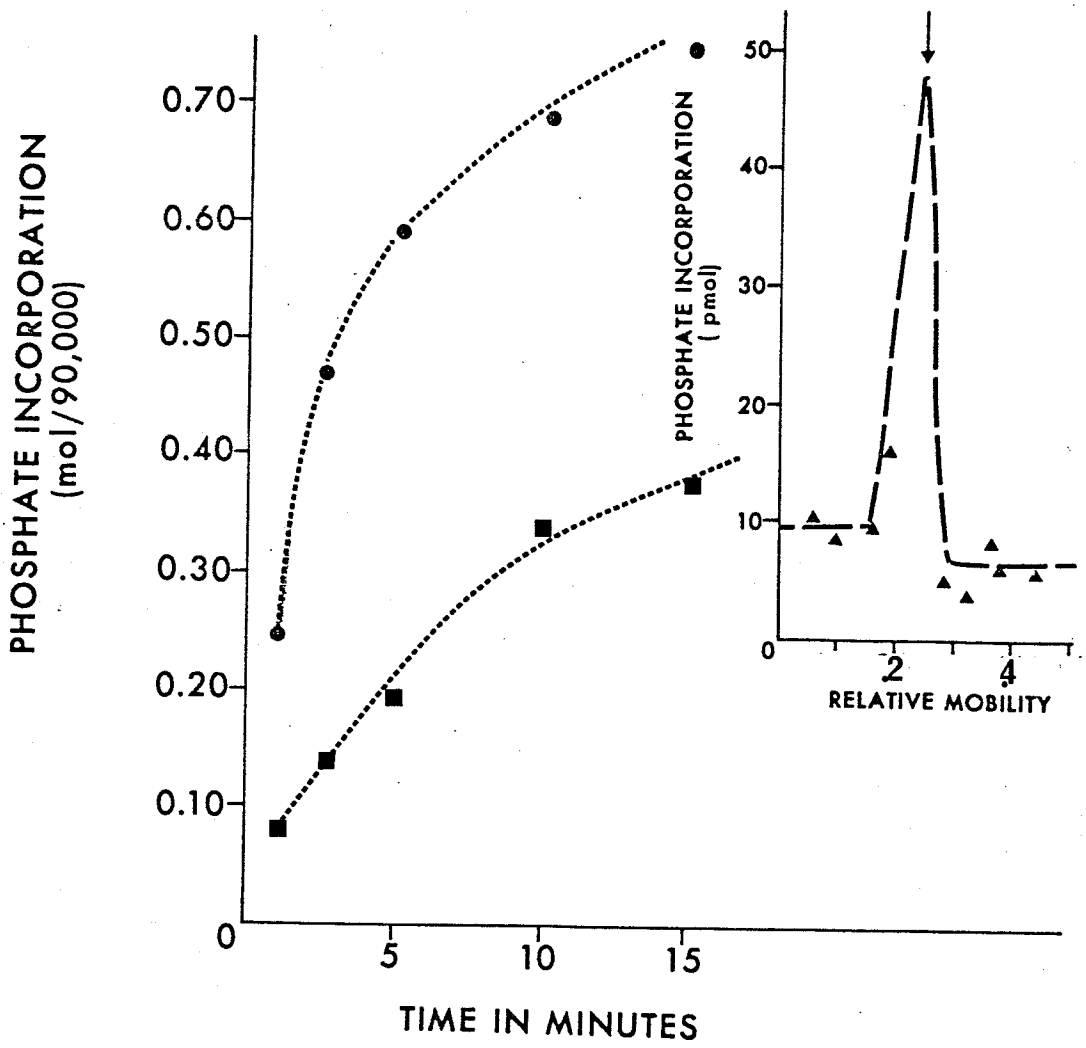


Figure 32. Effect of protein modulator on endogeneous glycogen synthase kinase activity.
Glycogen synthase (0.62 mg/ml) was incubated with 20 mM Tris/HCl (pH 7.5), 10 mM $MgAc_2$, 0.25 mM ATP, 40 mM β-mercaptoethanol, in the presence of 0.4 mM Ca^{2+} (■) or 0.4 mM Ca^{2+} and 20 µg/ml protein modulator (●).

Insert. Electrophoretic analysis of endogeneous glycogen synthase phosphorylation.
Glycogen synthase was incubated as indicated, in the presence of Ca^{2+} and protein modulator. After twenty minutes the reaction was terminated by the addition of an equal volume of 2% SDS, 40 mM EDTA and the sample was electrophoresed on 7.5 % polyacrylamide gels in the presence of sodium dodecyl sulfate. The gels were sliced and protein bound phosphate determined.

Glycogen synthase has been shown to be phosphorylated and converted from the a form to the b form of the enzyme by the c-AMP-dependent protein kinase (Soderling et al, 1970; Schlender et al, 1969), c-GMP-dependent protein kinase (Lincoln and Corbin, 1977), and a cyclic nucleotide independent protein kinase (Schlender and Reimann, 1975). The results of figure 32 suggest the existence of an as yet uncharacterized glycogen synthase kinase as a contaminant of the glycogen synthase preparation. This protein modulator regulated glycogen synthase kinase incorporates about 0.8 mol phosphate per monomer of glycogen synthase. Phosphorylation of glycogen synthase by this mechanism results in a conversion of the enzyme from the a to the b form (Srivastava et al, 1979).

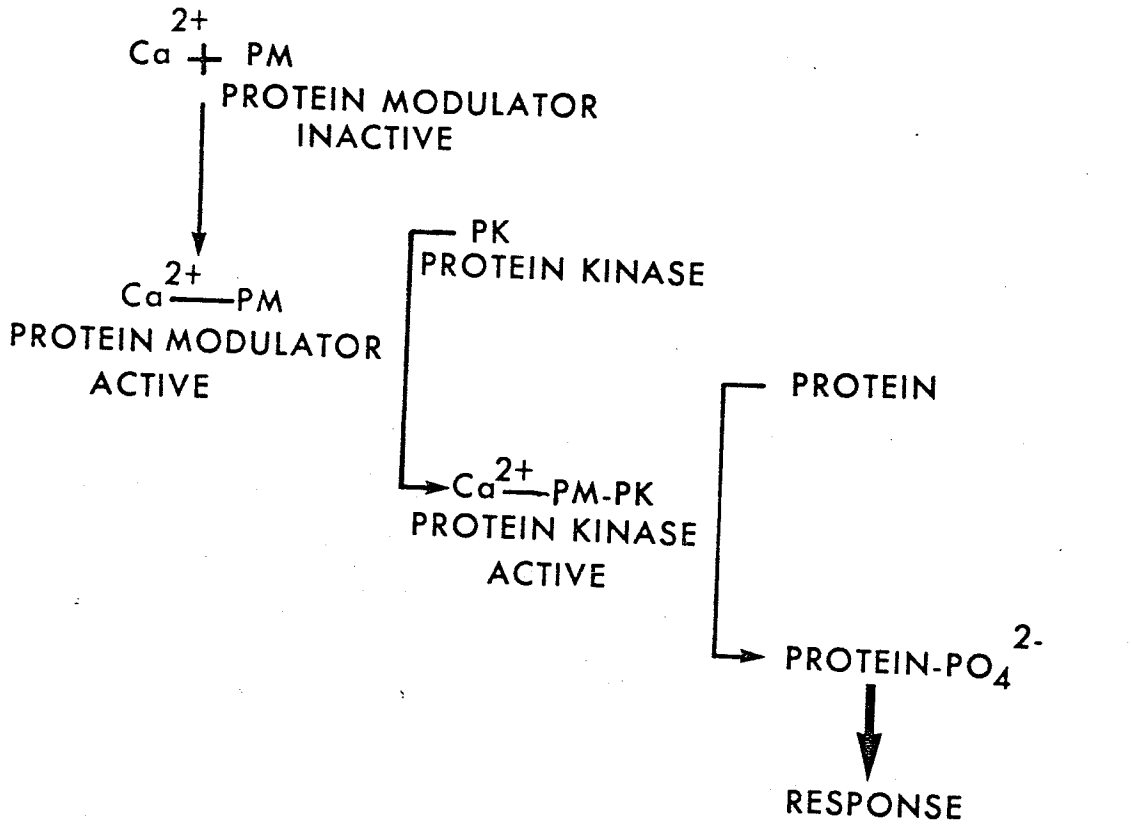
C) GENERAL CONCLUSIONS

The sodium dodecyl sulfate electrophoretic analysis of the affinity column purified fraction (figure 20) suggests the presence of major protein bands with molecular weights of 128,000, 90,000, and 50,000. Since electrophoretic analysis of the three peaks of protein kinase activity eluted from the Sephadex G-200 column demonstrate similar molecular weight components it is suggested that these modulator dependent protein kinases represent the major modulator regulated proteins in rabbit skeletal muscle. The presence of three minor protein bands in figure 20 could suggest the existance of other modulator regulated proteins or alternatively these protein bands could be artifactual, perhaps due to proteolysis or the presence of contaminating protein. It is important to note that the pooling of the DEAE-cellulose purified fraction according to the inhibitor activity as opposed to only kinase activity should result in partial purification of all the soluble proteins capable of interaction with the protein modulator. The further purification of the DEAE-cellulose fraction by protein modulator affinity chromatography should result in selective purification of only the modulator

regulated proteins. Figure 20 therefore represents the subunit molecular weight analysis of the proteins of rabbit skeletal muscle capable of Ca^{2+}-dependent interaction with the protein modulator.

The results of this study have suggested the existence in rabbit skeletal muscle of three protein modulator regulated kinases, namely, phosphorylase kinase, myosin light chain kinase, and glycogen synthase kinase. It is suggested that these kinases be referred to collectively as modulator dependent protein kinases. The mechanism of activation of the modulator dependent protein kinases by the protein modulator is presented in schemeIII.

The role of Ca^{2+} as a coupling agent between neuronal stimulation and muscle contraction has been well established (Ebashi et al, 1969). Studies of the dependence of phosphorylase kinase activity on Ca^{2+}, in vitro (Ozawa et al, 1967; Brostrom et al, 1971), and in glycogen granules (Meyer et al, 1970; Heilmeyer et al, 1970)have suggested that phosphorylase kinase activity is absolutely dependent on Ca^{2+}. Furthermore the observation by Danforth et al (1962) that isometric stimulation of frog sartorius muscle results in 50% conversion of phosphorylase b to phosphorylase a (in about one second) has reinforced the suggestion that activation of glycogenolysis during neuronal stimulation of muscle contraction may result from a Ca^{2+}-dependent stimulation of phosphorylase kinase activity. The mechanism of this stimulation is still unclear. Phosphorylase kinase may be phos-



SCHEME III

MECHANISM OF ACTIVATION OF MODULATOR DEPENDENT PROTEIN KINASES BY PROTEIN MODULATOR.

phorylated and activated by the Ca^{2+}-dependent autocatalytic reaction but the rate of autoactivation is slow and inhibited by millimolar concentration of phosphate (Wang et al, 1976). The work of Stull and Mayer (1971) and Drummond et al (1969) has demonstrated that tetanic electrical stimulation of rabbit graciles muscle results in the formation of phosphorylase a without activation of phosphorylase kinase. However the allosteric stimulation of non-activated phosphorylase kinase has been shown to be very low (Brostrom et al, 1971) and calculations of the time necessary for 50% conversion of phosphorylase b, by non-activated Ca^{2+}-stimulated phosphorylase kinase (Cohen, 1974) have been in disagreement with the in vivo data (Danforth et al, 1962) by more than an order of magnitude.

It is apparent from the discussion that the mechanism of activation of phosphorylase kinase by Ca^{2+} during neuronal stimulation of skeletal muscle is unclear, however, the future formation of a model to explain this mechanism will undoubtedly have to take into consideration the role of protein modulator in the Ca^{2+}-linked regulation of phosphorylase kinase.

The demonstration of protein modulator stimulated glycogen synthase phosphorylation presents a new dimension to the regulation of glycogen metabolism by Ca^{2+}. Since the protein modulator stimulation of glycogen synthase

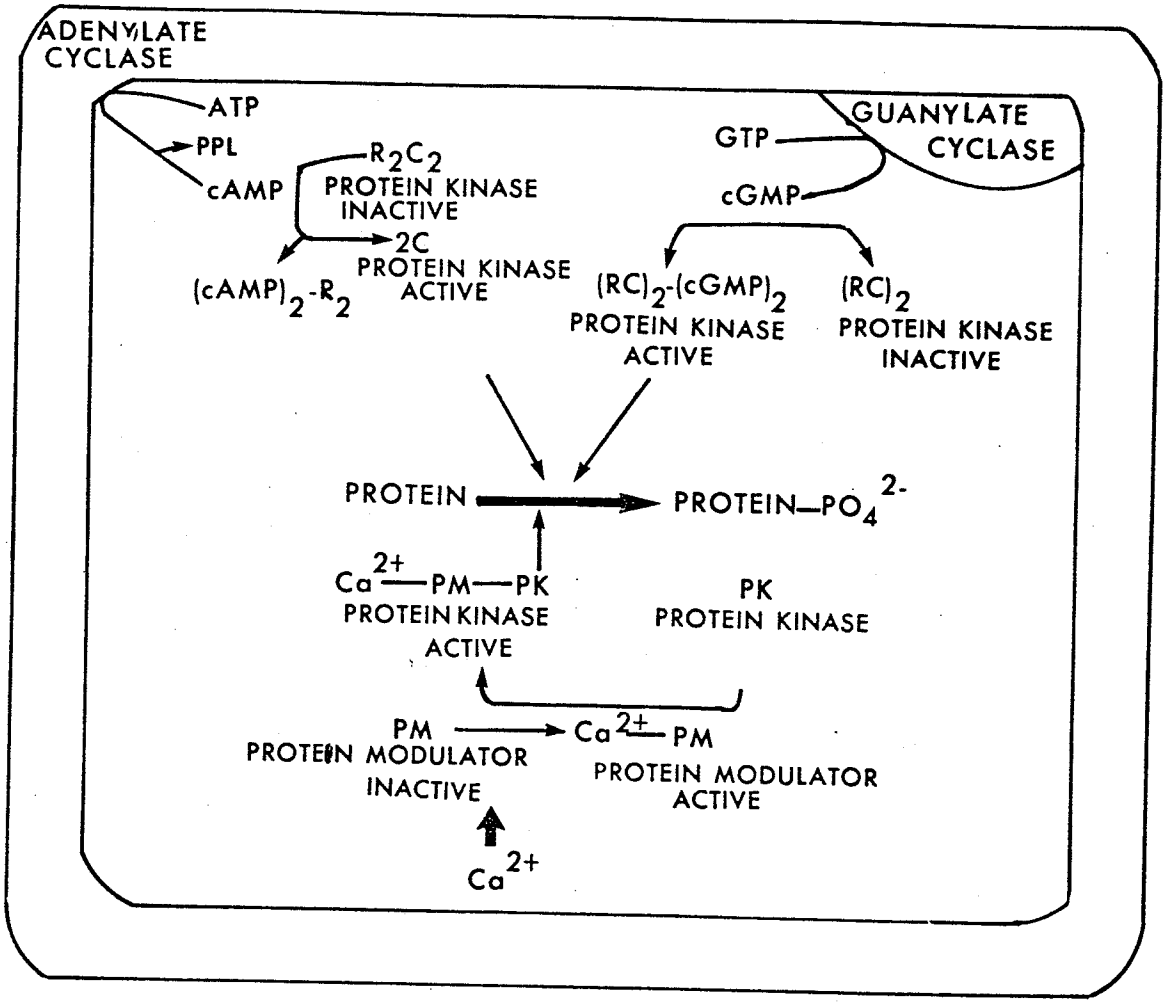
phosphorylation can be shown to result in a loss of synthase activity it is possible to demonstrate the regulation of glycogen metabolism by Ca^{2+} at the level of synthesis (glycogen synthase) and degredation (phosphorylase kinase). The identity of the protein modulator stimulated glycogen synthase kinase awaits further characterization.

That tetanic electrical stimulation of rabbit skeletal muscle resulted in phosphorylation of the 18,000 dalton light chain of myosin was demonstrated by Stull and High (1977), and appears to be consistant with the observation that the enzyme catalysing this phosphorylation, myosin light chain kinase, is a Ca^{2+}-dependent enzyme (Pires et al, 1974). Further characterization of this enzyme by Yagi et al (1978) and Waisman et al (1978a) have shown that myosin light chain kinase is a protein modulator regulated enzyme. The physiological significance of myosin light chain phosphorylation is at present unclear, although a regulatory funcion my exist (Lehman, 1978).

It is of interest to note that muscle glycogen metabolism appears to be regulated at the level of both synthesis and degradation by two extracellular mechanisms; neuronal and hormonal. Hormonal stimulation of muscle results in elevated intracellular c-AMP and subsequent activation of the c-AMP-dependent protein kinase. The c-AMP-

dependent protein kinase regulates glycogen metabolism by phosphorylation and activation of phosphorylase kinase and phosphorylation and inactivation of glycogen synthase. The neuronal stimulation of muscle results in elevated intracellular Ca^{2+} and activation of the modulator dependent protein kinases , resulting in phosphorylation and activation of phosphorylase kinase, phosphorylation and inactivation of glycogen synthase, and the phosphorylation of myosin light chains. It therefore appears that phosphorylase kinase and glycogen synthase provide a common focal point for the regulation of glycogen metabolism by Ca^{2+} and c-AMP. On the other hand myosin light chain phosphorylation appears to be solely regulated by Ca^{2+}. A generalized model is presented in scheme IV.

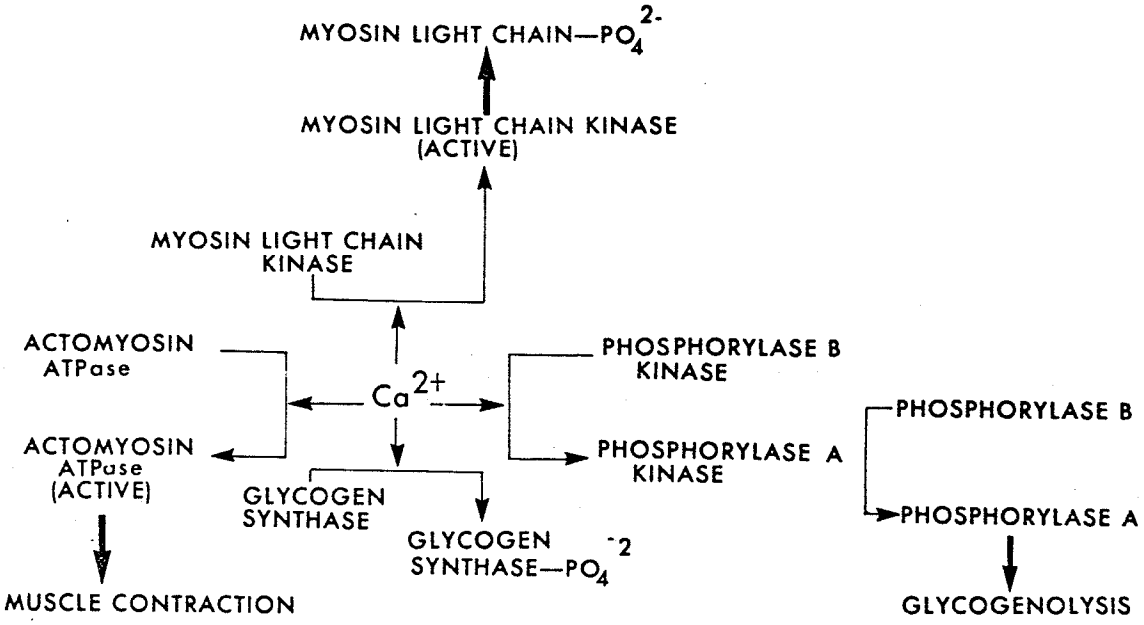
The results of this study suggest that the role of protein modulator in rabbit skeletal muscle is to mediate the Ca^{2+}-linked regulation of phosphorylase kinase (at least in part), myosin light chain kinase, and an as yet uncharacterized glycogen synthase kinase. The total dependence of kinase activity on Ca^{2+} is suggested to couple the activation of phosphorylase kinase, and phosphorylation of glycogen synthase and myosin light chains with the onset of muscle contraction (scheme V).



SCHEME IV

INTERRELATIONSHIPS OF HORMONAL AND NEURONAL REGULATION.

Hormonal stimulation of muscle tissue results in activation of adenylate cyclase, increase in intracellular c-AMP and activation of the c-AMP dependent protein kinase. Neuronal stimulation of muscle tissue results in elevated intracellular Ca^{2+}, which is suggested to activate the modulator dependent protein kinases. Phosphorylase kinase and glycogen synthase appear to represent common substrates for these kinases, therefore glycogen metabolism in muscle tissue is under dual regulation by c-AMP (hormonal regulation) and Ca^{2+} (neuronal stimulation). The protein modulator appears to be instrumental in the mediation of the regulatory effect of Ca^{2+} on the modulator dependent protein kinases as is the regulatory subunit of the c-AMP dependent protein kinase with c-AMP. The c-GMP dependent kinase may present a third mechanism of regulation however the physiological significance of c-GMP in muscle is at present unclear.



CENTRAL ROLE OF Ca^{2+} IN RABBIT SKELETAL MUSCLE

SCHEME V

CENTRAL ROLE OF Ca^{2+} IN RABBIT SKELETAL MUSCLE.

Of the four enzymes in this tissue regulated by Ca^{2+}, a role for protein modulator has been suggested for three of these enzymes, namely phosphorylase kinase, myosin light chain, kinase, and an unidentified glycogen synthase kinase. These modulator dependent protein kinases are coordinated with muscle contraction such that when muscle is stimulated to contract, glycogen degradation is stimulated, glycogen synthesis is inhibited and myosin light chain phosphorylation is stimulated.

IX. OVERALL CONCLUSIONS

Just as the regulatory subunit of c-AMP dependent protein kinase represents the biological receptor for c-AMP it appears that the protein modulator represents a biological receptor for Ca^{2+}. Although protein modulator, parvalbumin, troponin-C, intestinal calcium binding protein, and the DTNB myosin light chain represent the known Ca^{2+} receptors, based on the impressive number of biochemical processes that are protein modulator regulated it is likely that the protein modulator represents an important Ca^{2+} receptor of physiological significance.

The phylogenetic significance of the protein modulator has been investigated. Crude extracts were prepared of animals representative of the major phyla and these extracts were tested for protein modulator activity which involved measurements of the activation of mammalian phosphodiesterase by the extracts. This experimental approach has been successful in demonstrating the existence of protein modulator activity in all animals examined. Similar results have been documentated for several higher plants. The results of the phylogenetic survey have suggested that the protein modulator represents an universal Ca^{2+} receptor and therefore may be of importance in the mediation of the regulatory function of Ca^{2+} in the non-vertebrates.

The activation of mammalian phosphodiesterase by non-vertebrate crude extract protein modulator suggests conservation of those structural and functional properties of the protein modulator which are directly involved in the Ca^{2+}-dependent regulation of phosphodiesterase. The simplest explanation for the conservation of the structural and functional properties of the protein modulator is that the basic site of interaction between protein modulator and phosphodiesterase is phylogenetically conserved. The failure to demonstrate protein modulator regulated phosphodiesterase in non-vertebrates has suggested the possibility that the basic site of interaction between protein modulator and the protein modulator regulated enzymes may generally be conserved. This suggestion is consistent with the presence in non-vertebrates which are devoid of protein modulator regulated phosphodiesterase, protein modulator capable of the activation of mammalian phosphodiesterase. The Ca^{2+} dependent interaction of the earthworm protein modulator with the bovine brain modulator binding protein also provides further evidence for this suggestion. Studies of the site of interaction between protein modulator and the many protein modulator regulated enzymes will be necessary to further evaluate the possibility that this site of interaction may be conserved.

A comparison of the electrophoretic mobility of non-vertebrate crude extract protein modulator activity with the purified bovine heart protein modulator activity has suggested similar physical-chemical properties. A comparison of the properties of the purified protein modulator from the earthworm and bovine heart has also suggested that these proteins are similar. Both proteins have molecular weights of approximately 18,000 daltons, isoelectric points of about pH 4, similar and characteristic ultraviolet spectra, and similar amino acid compositions. Both proteins bind Ca^{2+} ions with high affinity. However, the protein modulator from the earthworm binds 2 mol of calcium ions with equal affinity, K_{diss} of 6 μM whereas the modulator from bovine heart binds 4 mol of calcium ions with differing affinities. The protein modulator therefore represents a ubiquitous, highly conserved protein, which probably serves to mediate the regulatory role of Ca^{2+} in many organisms.

In skeletal muscle the rise in intracellular Ca^{2+} concentration is suspected to coordinate muscle contraction with glycogenolysis and myosin light chain phosphorylation. Considering the role of protein modulator in the mediation of the regulatory influence of Ca^{2+} it is not unreasonable to suspect the involvement of

protein modulator in these Ca^{2+} regulated processes. As a result of the investigation of the physiological function of protein modulator in rabbit skeletal muscle the involvement of protein modulator in the regulation of phosphorylase kinase, myosin light chain kinase and glycogen synthase kinase has been documented.

The dependence of phosphorylase kinase activity on Ca^{2+} concentration was originally postulated to be the event coupling muscle contraction with glycogenolysis. The results of studies of the interaction of phosphorylase kinase with the protein modulator affinity column and of the competition between phosphodiesterase and phosphorylase kinase for protein modulator have suggested that phosphorylase kinase, and protein modulator are capable of Ca^{2+}-dependent and reversible interaction. The result of this interaction is a two fold stimulation of phosphorylase kinase activity. In contrast to the Ca^{2+}-dependent and reversibly interacting protein modulator, it appears that protein modulator may also be tightly bound by phosphorylase kinase and this interaction is unaffected by the presence or absence of Ca^{2+}. The role of this tightly bound protein modulator in the regulation of phosphorylase kinase activity is at present unclear.

The involvement of the protein modulator in the

the regulation of glycogen synthase has also been suggested. Protein modulator was found to stimulate the phosphorylation of glycogen synthase, presumably by the activation of an as yet uncharacterized endogenous glycogen synthase kinase. The phosphorylation was found to result in a decrease in glycogen synthase activity. The protein modulator stimulated glycogen synthase kinase is presently being characterized.

The increase in Ca^{2+} concentration responsible for the initiation of muscle contraction is also believed to be responsible for the stimulation of the phosphorylation of the DTNB myosin light chain. While phosphorylation of the myosin light chain has been demonstrated to occur in vivo during muscle contraction, the physiological significance of light chain phosphorylation is at present unclear. The enzyme responsible for the phosphorylation of the DTNB myosin light chain, myosin light chain kinase, has been shown to undergo Ca^{2+} dependent and reversible interaction with the protein modulator. As a result of this interaction, myosin light chain kinase activity is stimulated seven to ten fold. Unfortunately the physiological significance of protein modulator regulated myosin light chain kinase cannot be evaluated until the role of DTNB light chain phosphorylation is better understood.

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