PURIFICATION, CHARACTERIZATION AND MECHANISM OF ACTION OF THE PROTEIN ACTIVATOR OF CYCLIC AMP PHOSPHODIESTERASE OF BOVINE HEART

by

TIAN SENG TEO

A Thesis Submitted To The University of Manitoba In Partial Fulfillment For The Degree Of Doctor Of Philosophy

Department of Biochemistry

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ABSTRACT

A protein activator for cAMP phosphodiesterase has been purified from bovine heart by a simple procedure which involves ammonium sulfate fractionation, acid precipitation, heat treatment, DEAE-cellulose and Sephadex G-100 column chromatography. The purified protein activator appears to be homogenous by ultracentrifugal and disc gel electrophoretic criteria. The specific activity of the pure protein activator is approximately 3,200 fold that of the crude tissue extract. The protein activator appears to be a single polypeptide with a molecular weight of approximately 15,000 daltons. It exhibits a low isoelectric point of 4.0 since one third of its amino acid residues are acidic while only one tenth of its amino acid residues are basic. Cysteine and tryptophan residues are absent.

cAMP phosphodiesterase from the soluble fraction of bovine heart can be separated by DEAE-cellulose column chromatography into two isoenzymes: PDE-I and PDE-II. While PDE-I can be activated 6 to 10 fold by Ca^{2+} and the protein activator, PDE-II is insensitive to both agents. PDE-I has been purified 60 to 70 fold by a procedure involving ammonium sulfate fractionation and a unique affinity chromatographic technique which exploits the reversible binding of PDE-I to the protein activator on a column of DEAE-cellulose. The protein activator activates PDE-I by enhancing the

V_{max} and decreasing the K_m for cAMP. The extent of activation depends on the concentration of substrate. Maximum activation of cAMP hydrolysis is at 20 μM cAMP and is marked at other cAMP concentrations. On the other hand, only very slight activation of cGMP hydrolysis is observed at millimolar concentrations of cGMP but becomes very marked at micromolar concentrations of cGMP. PDE-I hydrolyzes cAMP faster than cGMP at substrate concentrations in excess of 40 μM but hydrolyzes cGMP faster than cAMP at substrate concentrations lower than 40 μM.

Kinetic studies indicate that (a) the activation of PDE-I by the protein activator is a slow process relative to catalysis and (b) PDE-I, the protein activator and PDE-I-protein activator complex exist in a state of equilibrium.

Numerous factors have been shown to affect the hydrolysis of cAMP by PDE-I in the presence of the protein activator. The effects of each of these factors on the $A_{50\%}$ were investigated. Of the factors studied, only the (a) substrate concentration and (b) Ca^{2+} concentration influence the affinity of the protein activator for PDE-I. The protein activator appears to exhibit greater affinity for PDE-I at micromolar than at millimolar concentrations of the substrate. This difference in affinity is much greater for the hydrolysis of cGMP than for cAMP. Activation of PDE-I by the protein activator is completely dependent on the presence of Ca^{2+}. Conversely, activation of PDE-I by Ca^{2+} is

completely dependent on the presence of the protein activator. The concentration of Ca^{2+} required to give half maximal activation [at a cAMP concentration of 1.2 mM] is 2.3 μM. An equilibrium binding study has shown that ^{45}Ca binds to the protein activator. A Scatchard plot exhibits two linear regions suggesting the presence of two sets of Ca^{2+} binding sites on the protein with different affinities: one high-affinity and two or three low-affinity sites per molecule of protein activator. The dissociation constants for Ca^{2+} bound at the high- and low-affinity sites are 3 and 12 μM, respectively.

Ca^{2+}, at very low concentrations, induces in the protein activator (a) an enhancement of tyrosyl fluorescence, (b) an increased resistance to heat inactivation, (c) an increased resistance to tryptic digestion and (d) perturbations in its UV absorption spectrum. These results suggest that the binding of Ca^{2+} by the protein activator induces a change in the conformation of the protein activator.

A model is proposed for the activation of PDE-I. The activation of PDE-I is depicted in this scheme as a stepwise process which is initiated by the binding of Ca^{2+} to the protein activator. Upon binding Ca^{2+}, the protein activator is converted from an inactive to an active conformation. The active protein activator then associates with the PDE-I enzyme to form a complex in which the protein activator and the enzyme exist as neighboring subunits in a protein molecule.

Through subunit interaction in this protein complex, the enzyme undergoes a conformational change with a concomitant increase in enzyme activity.

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LIST OF ABBREVIATIONS

$A_{50\%}$	concentration of protein activator required to give half maximal activation of PDE-I
5'AMP	adenosine 5'monophosphate
cAMP	adenosine 3':5' cyclic monophosphoric acid
cGMP	guanosine 3':5' cyclic monophosphoric acid
cIMP	inosine 3':5' cyclic monophosphoric acid
cUMP	uridine 3':5' cyclic monophosphoric acid
CRP	cAMP receptor protein
CTP	cytidine triphosphate
db-cAMP	dibutyryrl cAMP
db-cGMP	dibutyryrl cGMP
$D_{20,w}$	diffusion constant corrected to water at 20 ^{o}C
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol bis (β-aminoethyl ether) N, N'-tetraacetic acid
5'GMP	guanosine 5'-monophosphate
GTP	guanosine triphosphate
K_m	michaelis constant for substrate
Kg	kilogram
MW_g	molecular weight values determined by gel filtration

MW_{sd}	molecular weight values determined by sedimentation diffusion method
MW_{se}	molecular weight values determined by sedimentation equilibrium method
mg	milligram
mM	millimolar
M	molar
nm	nanometer
5' nucleotidase	5'ribonucleotide phosphohydrolase E. C. No. 3135
OD660	optical density measured at 660 nm
OD280	optical density measured at 280 nm
PDE	cyclic nucleotide phosphodiesterase
Pi	inorganic orthophosphate
pI	isoelectric point
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
$S_{20,w}$	sedimentation coefficient corrected to water at 20 ^{o}C
Tris	tris (hydroxymethyl) aminomethane
TCA	trichloroacetic acid
Troponin TN-C	subunit of troponin which confers calcium sensitivity on the interaction of actin and myosin

μM	micromolar
μL	microliter
V_{max}	maximum velocity
V_t	total volume of gel bed in gel filtration

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I. REVIEW OF LITERATURE

A. The Cyclic Nucleotide Systems

A. 1. The Cyclic AMP System

It is well established that cAMP mediates the actions of a variety of hormones (1). A list of such hormones includes epinephrine, glucagon, ACTH, TSH among others. It has been shown for these hormones that (a) the hormones raise the levels of cAMP in the target tissues, (b) the hormones activate adenyl cyclase in cell free preparations of the target tissues, (c) the rise in the intracellular levels of cAMP in the target tissues is very rapid and precedes the physiological responses and finally that (d) cAMP or one of its derivatives can mimic the hormones in evoking their physiological responses.

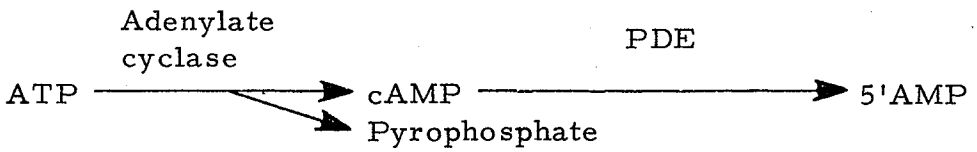
Sutherland (2, 3) proposed the concept of cAMP functioning as the "second messenger" to explain how the hormones listed above work. According to this hypothesis, the hormone circulating in the vascular system is the "first messenger." The hormone binds to specific receptors on the outer surface of the cells of the target tissues. Specificity for binding the hormones is built into these receptors. These hormone receptors are thought to be either in close association with or are subunits of the adenylate cyclases (4). The orientation is such that the hormone receptors are on the outer surface and the adenylate cyclases are on the inner surface of the plasma

membrane. Binding of the hormone to its receptors causes a change in the enzyme activity of the adjacent adenylate cyclase resulting in a change in the intracellular concentration of cAMP. Experiments using polypeptide hormones covalently bound to agarose beads have shown that these hormones can act without entering the cell.

cAMP is a stable compound. It is unaffected by exposure for short periods of time to high temperatures (up to 100 ^{o}C), extremes of acid or alkali. Space filling models show that cAMP can assume two conformations, either the anti or the syn conformations, with the ribose moiety either extended away or bent back underneath the purine ring. The conformation of cAMP, in which it is active, is not known.

cAMP is broken down to 5'AMP by cyclic nucleotide phosphodiesterases (PDE) within the cell. This is the only means known so far by which this important compound is destroyed <u>in vivo</u>. As is discussed below, there are multiple forms of these PDE enzymes.

The overall reaction catalysed by the system is:



In the steady state in most tissues, the cAMP concentration (10^{-6} M) is very low compared to the concentrations of its precursor, ATP (10^{-3} M), or its degradation product, 5'AMP (10^{-4} M).

A. 2. Adenylate Cyclase

Adenylate cyclase appears ubiquitous in nature. It is found in bacteria, higher plants and animals. Sutherland, Rall and Menom (5) found adenylate cyclase activity in every tissue of a dog examined except erythrocytes. The highest specific activity was in brain cortex. They examined numerous animal phyla and found adenylate cyclase activity in all of them. In all mammalian species, brain tissue has the highest adenylate cyclase activity. Adenylate cyclase is primarily associated with the plasma membrane. Adenylate cyclase has also been reported to be associated with other subcellular fractions including mitochondria [brain cortex (6), rabbit skeletal muscle (7)], endoplasmic reticulum [cardiac sarcoplasmic reticulum (8)], microsome (brain) (6) and pineal gland (9).

cAMP labelled with ^{32}P is formed from labelled ATP only if the radioactive label is in the alpha position. Greengard, Hayaishi and Colowick (10) reported that purified adenylate cyclase from Brevibacterium liquefaciens catalyzed the adenylation of pyrophosphate by cyclic AMP to yield ATP in an exergonic reaction. They concluded that the reaction catalyzed by adenylate cyclase was readily reversible. However, Cheung and Chiang (11) disagreed with this conclusion. They found that the conversion of ATP to cyclic AMP catalyzed by adenylate cyclase after prolonged incubation was essentially irreversible. Using isotopic techniques, Tao and Lipman (12) and Rosen and Rosen

(13) also failed to detect reversibility of the adenylate cyclase reaction. In vivo, the reaction probably proceeds strongly to the right. This is because the concentration of ATP is two orders of magnitude greater than cAMP, and the pyrophosphatase reaction is essentially irreversible.

The study of adenylate cyclase is complicated by the particulate nature of the enzyme. Solubilized preparations, while catalytically active, are generally unresponsive to hormones which suggest that the regulatory site is either part of the membrane or depends for its functioning on an association between enzyme and membrane. Levy and Klein (14) have noted that adenylate cyclase solubilized with lubrol was still catalytically active but lost its responsiveness to hormones. They observed that addition of phosphotidylserine to this preparation restored its ability to respond to glucagon.

A. 3. The Mechanism of Action of Cyclic AMP

cAMP mediates the action of a wide variety of hormones. The biological responses of these hormones range from the well characterized glycogenolysis (15) and glycogenesis (15) of liver and muscle and lipolysis in adipose tissue (15) to the myriads of as yet uncharacterized responses such as salivary secretion (16) and phosphaturia and calcium renal resorption (17). cAMP has been shown to be an allosteric effector of a small number of enzymes or biologically

active proteins, namely phosphofructokinase of muscle (18), protein kinase of a variety of tissues and the cAMP receptor protein (CRP) of Escherichia coli. In Escherichia coli, CRP activates the transcription of the genes for the enzymes such as β-galactosidase and Lac permease whose synthesis is repressed in the presence of glucose. It was shown by Pastan and his co-workers (19) that for the Lac operon, CRP must first bind cAMP before it can stimulate the synthesis of messenger RNA. The CRP-cAMP complex activates transcription presumably by binding to the promoter region of the Lac operon thereby enabling RNA polymerase to bind and initiate transcription.

The allosteric activation of protein kinase by cAMP is the first step leading to increased glycogenolysis in liver and muscle and the increased lipolysis in adipose tissue. In these tissues the protein kinase is primarily cAMP-dependent. In adipose tissue an elevation of cAMP results in increased protein kinase activity which in turn leads to a greater phosphorylation of lipase, activating it and turning on the degradation of triglycerides (20). In liver and muscle, the sequence of events leading finally to the enhanced glycogenolysis is more complicated. The process was elucidated by Krebs and his co-workers (1). Protein kinase phosphorylates phosphorylase kinase as well as glycogen synthetase, thereby activating the former and partially inactivating the latter. Phosphorylase kinase phosphorylates glycogen phosphorylase, activating it in the process. With an

increased glycogen phosphorylase activity on the one hand and a decreased glycogen synthetase activity on the other, the net result is an increased breakdown of glycogen.

Induction of enzyme synthesis by cAMP in mammalian systems has been demonstrated in a number of cases (21, 22, 23). cAMP-dependent protein kinase can phosphorylate histones (24) and ribosomal proteins (25, 26). It is postulated that in those tissues where cAMP induces enzyme synthesis, phosphorylation of histones and ribosomal proteins may somehow participate. There is, however, no direct evidence to support this hypothesis.

A. 4. The cGMP System

cGMP had been synthesized in laboratories many years before it was discovered in 1963 by Ashman and his co-workers to be a naturally occurring cyclic nucleotide (27). Information regarding the biological importance of cGMP has until recently remained limited compared to the vast body of knowledge concerning cAMP. cGMP has been detected in all phyla of the animal kingdom examined. Tissue cGMP levels range between 10^{-8} and 10^{-7} moles/Kg, and in a given tissue are usually 1/10 to 1/100 the level of cAMP (10^{-7} to 10^{-6} moles/Kg) (28). However, in some tissues, cGMP levels may be as high as 1/5 to 1/2 those of cAMP (28).

A. 5. Guanylate Cyclase

Guanylate cyclase catalyzes the generation of cGMP from GTP by a reaction that appears to be analogous to that catalyzed by adenylate cyclase (28). Guanylate cyclase has been detected in all mammalian tissues examined as well as in other animals and bacteria (29).

A number of properties of guanylate cyclase distinguish it from adenylate cyclase:

(a) Unlike mammalian adenylate cyclase which is considered to be totally particulate, the major portion of the guanylate cyclase activity after homogenization appears to reside in the soluble fraction (30). It is possible that guanylate cyclase activity in intact cells is particulate but becomes solubilized during homogenization.

(b) Whereas adenylate cyclase's cation requirement can be satisfied almost equally by either Mg^{2+} or Mn^{2+}, guanylate cyclase was shown to be ten times more active in the presence of Mn^{2+} than with the same concentration of Mg^{2+} (30).

(c) Hormones such as epinephrine, glucagon, ACTH and non-hormonal agents such as fluoride stimulate adenylate cyclase but do not stimulate guanylate cyclase in cell-free preparations of all mammalian tissue tested (28). The lack of an effect of these hormones in vitro is consistent with their ineffectiveness in stimulating tissue cGMP accumulation in vivo.

A. 6. cGMP as Second Messenger

A. 6. (a) Effects observed with exogenous cGMP on intact cells.

In order to define a biological role for cGMP, experiments were conducted whereby organs were perfused and tissue slices incubated with high concentrations of cGMP (0. 1 to 10 mM) or its dibutyryl derivatives. These concentrations represent as much as 10^6 times the concentrations known to occur in most tissues. In some tissues, these high concentrations of exogenous cGMP were found to mimic cAMP in its effects in promoting glucose output, glycogenolysis, phosphorylase activation, etc., but with much less potency (31, 32). cGMP is a poor substitute for cAMP in activating cAMP-dependent protein kinase. However, at very high concentrations, cGMP can nevertheless activate cAMP-dependent protein kinase. It is speculated that this activation may be sufficient to account for the cAMP-like effects of cGMP (28).

In other tissues, investigators found that cGMP or its dibutyryl derivatives could produce effects which were different from those of cAMP. For example, Puglisic et al. (33) reported that db-cAMP antagonized acetylcholine-induced contraction of isolated rat fundus strips whereas db-cGMP (8 mM) mimicked the effects of the cholinergic agent.

A. 6. (b) Effect of agents that promote cGMP accumulation in intact cells.

Hormones (such as epinephrine, glucagon, ACTH) which promote cAMP accumulation are ineffective in inducing the generation of cGMP in isolated cells (24, 35). On the other hand, perfusion of organs with low concentrations of acetylcholine or other cholinergic agents resulted in rapid (within seconds) accumulation of cGMP. George et al. (36) found that perfusion of isolated rat hearts with acetylcholine (0.37 μM) produced an elevation of myocardial cGMP (within 10 sec) coincident with the first sign of cholinergically induced effects in both cardiac function and elevation of myocardial cGMP. Other investigators have reported similar induction of cGMP accumulation by cholinergic agents in other biological systems; uterus (29), brain (37) and anterior pituitary (38).

A. 7. The Biological Role of cGMP

Until recently it was thought that regulation of cellular metabolism may be by bidirectional changes (increases or decreases) in the concentrations of intracellular cAMP alone. This "UNITARY" concept has been used to explain, for example, the regulation of cardiac contractility, the control of lipolysis, hepatic glycogen metabolism and cell proliferation. Although, in at least some of the examples cited, decreases in cellular cAMP can be demonstrated, the decreases in cAMP concentration have not actually been shown to precede the

physiological responses produced. In fact, evidence to the contrary have appeared. Agents promoting cAMP-opposing events can be shown in some cases to have no cAMP-lowering effect (39, 40).

This "UNITARY" concept of regulation is now superceded by the "DUALISTIC" concept of regulation whereby increases in cAMP concentrations induce cAMP-like responses and increases in cGMP concentrations induce cAMP-opposing events (28). Since it has been shown that low concentrations of cGMP do activate cAMP phosphodiesterases (41), a decrease in cAMP concentration could be viewed as an event secondary to the increase in cGMP concentrations. The "dualism" between cAMP and cGMP is speculated (28) to regulate biological systems modulated by opposing biological signals. These could be considered to be "bidirectionally" controlled systems. It is also possible that other biological systems exist that are controlled only "monodirectionally." In the adrenal cortex, steroidogenesis is stimulated by ACTH. No naturally occurring antagonist of this ACTH action is known to exist.

B. cAMP Phosphodiesterase

B. 1. General Distribution

cAMP phosphodiesterase is widely distributed. It has been detected in all mammalian tissues examined with the exception of avian erythrocytes (42). Among the lower forms of life, it has been

found in yeast (43), in bacteria (44), in slime mold (45) and in several marine organisms (46).

Surveys (42, 47) of different mammalian tissues show that brain has the highest cAMP phosphodiesterase activity. A lower specific activity was found in spleen, skeletal muscle and heart muscle while very low specific activity was found in intestinal mucosa and plasma.

B. 2. Subcellular Distribution

In general, cAMP phosphodiesterase is known to be distributed between soluble and particulate fractions in most sources. The proportion of the total enzyme activity in the soluble fraction varies depending on the source of the enzyme. Butcher and Sutherland (47) and Hrapchak (48) reported that 20-30% of total cAMP phosphodiesterase activity in beef heart is soluble. Goren (49) found that a greater proportion (60%) of the total beef heart enzyme activity is soluble. Nair (50) reported that prior freezing and thawing helps to increase solubilization of the dog heart enzyme. In tissues of rat the proportion of the total enzyme activity in the soluble fraction varies from 90% in liver to 36% in brain cortex and 10% in adipose tissue (51).

De Robertis et al. (51) noted that the majority of the enzyme in the particulate fraction of rat brain was found in the mitochondrial and microsomal fractions. Hypoosmotic shock treatment of the mitochondrial fraction led to the release of 50% of its particulate activity.

Cheung (52) reported that 0.4% Triton X-100 could expose all the latent activity of the microsomal enzyme of rat brain but only part of it became truly soluble.

Butcher and Sutherland (47) observed that the particulate enzyme of beef heart was similar to the soluble enzyme with respect to pH optimum, methyl xanthine inhibition, Mg^{2+} dependence, imidazole activation, thermal stability and stability to freezing and thawing.

B.3. pH Optimum

The pH optimum for cAMP phosphodiesterase from most tissues ranges from pH 7.5 to 8.5. Butcher and Sutherland (47) reported that the beef heart enzyme has a pH optimum of 8 in 0.08 M Tris buffer. In a buffer of 0.04 M Tris and 0.04 M Imidazole, the pH optimum was shifted to 7.5. Goren and Rosen (53) found that in 0.1 M Tris buffer, the beef heart enzyme hydrolyzed cAMP and cGMP with pH optimum of 8.0 and 8.5, respectively. The pH optimum for the hydrolysis of cAMP by the rat brain enzyme is 8 and that by the dog heart enzyme is between 8.5 and 9.0.

B.4. Temperature Optimum

The temperature optima of the beef heart enzyme for the hydrolysis of cAMP and cGMP are 35 ^{o}C and 42 ^{o}C, respectively (53). cGMP (53) protects the enzyme from inactivation to a greater extent than cAMP at temperatures above 35 ^{o}C. The energy of activation for

the hydrolysis of cAMP by rat brain phosphodiesterase (52) is 7.5 K cal/mole between 25 °C and 30 °C and 3.5 K cal/mole between 38 °C and 45 °C. The energy of activation for the hydrolysis of cAMP by dog heart phosphodiesterase (50) is 19 K cal/mole.

B.5. Stability

The relatively impure enzyme from bovine brain or heart is very stable (49, 52, 54). It can be stored at 4 °C for up to 4 weeks with very little loss in enzyme activity. At -20 °C, it can be kept for over 6 months with almost no loss in enzyme activity. The enzyme is not stable above 45 °C (53). The beef heart enzyme loses 50% of its enzyme activity when heated at 45 °C for 5 minutes and loses 100% of its activity when heated at 55 °C for 5 minutes.

Although the beef heart enzyme is very stable in the impure stage, it is labile when highly purified (48, 55). Conventional procedures for concentration (such as ultrafiltration) and freezing and thawing rapidly inactivate the highly purified enzyme. Hrapchak (48) recommended that the enzyme be best stored as a suspension in 60% saturated solution of ammonium sulfate in the presence of Mg^{2+} and dithiothreitol or 2-mercaptoethanol. Kakiuchi and Yamazaki (56) reported that bovine serum albumin protected the rat brain enzyme from inactivation at 30°. Goren (49), however, found that bovine serum albumin did not affect the thermal stability of the beef heart enzyme.

B. 6. Requirement for Metal Ions

cAMP phosphodiesterase requires Mg^{2+} for activity (54). Maximum activation is achieved by Mg^{2+} concentrations of around 1 to 3 mM, while concentrations of Mg^{2+} above 10 mM inhibit the enzyme activity (53, 57). The rat brain enzyme (52) exhibits an apparent K_m for Mg^{2+} of 1.3×10^{-5} M. The human blood lymphocyte enzyme (58) has an apparent K_m for Mg^{2+} of the order of 10^{-4} M. The bovine thyroid enzyme (59) exhibits two K_m for Mg^{2+}; the reciprocal plot exhibits a biphasic curve.

Co^{2+} and Mn^{2+} can replace Mg^{2+}. Goren and Rosen (53) reported that Mn^{2+} and CO^{2+} stimulated the beef heart enzyme to a lesser extent than Mg^{2+} and inhibited the enzyme activity to a greater extent at high concentrations. They also reported that 1 mM concentrations of Zn^{2+}, Ca^{2+}, Fe^{2+} and Cu^{2+} inhibited the enzyme activity in the absence of Mg^{2+}. The activity of the beef heart enzyme in the presence of 1 mM Mg^{2+} was reported to be inhibited by the addition of 1 mM Zn^{2+}, Fe^{2+} or Cu^{2+} and unaffected by the addition of 1 mM Ca^{2+}, Co^{2+} or Ni^{2+}. 0.5 mM EDTA and 0.5 mM EGTA inhibited the beef heart enzyme 100% and 60%, respectively. These inhibitions could be overcome by addition of excess Mg^{2+}.

In agreement with many other research workers, Kakiuchi and co-workers (60) found that for the rat brain enzyme, Ca^{2+} alone did not replace Mg^{2+}. However, they were the first to report that of

the two forms of cAMP phosphodiesterase in rat brain, the major form required Mg^{2+} and low concentrations (10^{-6} to 10^{-4} M) of Ca^{2+}, while the minor form of the enzyme had a requirement only for Mg^{2+} (56, 57, 60, 61, 62).

B.7. Substrate Specificity

Phosphodiesterases in general exhibit a preference for cyclic nucleotides with a purine rather than a pyrimidine base (54). The two naturally occurring cyclic nucleotides, cAMP and cGMP, are the best substrates for the enzymes. In general, the enzymes have greater affinity for cGMP than cAMP. However, different molecular forms of the enzyme exhibit different preference with respect to the two cyclic nucleotides. This is discussed in greater detail in the section "Multimolecular Forms" (page 17). Hardman and Sutherland (63) found a phosphodiesterase in the particulate fraction of dog heart that hydrolyzed cUMP faster than cAMP.

B.8. Kinetic Parameters

Table 1 compares the K_m values of cyclic nucleotide phosphodiesterases from different sources. The K_m values for cAMP vary between 10^{-6} to 10^{-4} M. Most of the work reported in Table 1 had been carried out before the discovery of the protein activator, and the activating effect of Ca^{2+}, and consequently the concentrations of both activators were not standardized to enable investigators to obtain meaningful comparisons of the kinetic parameters.

Table 1

K_m VALUES OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASES FROM DIFFERENT SOURCES

	K_m (cAMP) Low Affinity (μM)	K_m (cAMP) High Affinity (μM)	K_m (cGMP) (μM)	
Bovine Heart	60 - 100	-	-	Butcher & Sutherland (47)
Bovine Heart	36 , 69	-	-	Hrapchak & Rasmussen (55)
Bovine Heart	250 , 290	-	50	Goren & Rosen (53)
Dog Heart	490	-	-	Nair (50)
Rat Heart	87	4	22	Thompson & Appleman (68)
Guinea Pig Heart	130 - 210	-	-	Poch (127)
Rat Skeletal Muscle	20	5	4	Huang & Kemp (64)
Rat Uterus Myometrium	60	2	-	Kroeger, Teo, Ho & Wang (128)
Rat Brain	104	2	13	Thompson & Appleman (68)
Bovine Brain	140	-	-	Cheung (71)
Trout Brain	90	-	-	Yamamoto & Massey (129)
Rat Liver	92	-	7	Thompson & Appleman (68)
Rat Fat Pad	38	3	38	Thompson & Appleman (68)
Human Adipose Tissue	400	0.4	-	Solomon (130)
Rat Adrenal	100	-	-	Klotz et. al. (131)
Rat Thyroid	30 - 50	-	-	Bastomsky et al, (132)
Guinea Pig Islet	30	3	-	Sams & Montague (133)
Rat Thymic Lymphocytes	8	0.13	-	Frank & Macmanus (73)
Frog Erythrocytes	300	-	-	Rosen (66)

Phosphodiesterase preparations from beef heart, rat brain, rat and human lymphocytes, and bovine thyroid exhibit multiple K_m values for cAMP. In general, a single low K_m for cGMP is obtained for each of the tissues investigated, being of the order of 10^{-5} M or lower.

Huang and Kemp (64) reported that rabbit skeletal muscle cAMP phosphodiesterase exhibited a lower K_m when the pH was lowered. This was confirmed by Goren and Rosen (53).

B. 9. Multimolecular Forms

Monn and Christenson (65) developed a method of staining cAMP phosphodiesterase activity on starch gels. They found that the cAMP phosphodiesterases from tissue homogenates of rat and rabbit existed as isoenzymes when analyzed by starch gel electrophoresis. Although 7 isoenzymes were detected, no tissue showed more than 4 isoenzymes. Most tissues including heart had two isoenzymes. Spleen, lung and stomach had two main isoenzymes and one minor isoenzyme each; only brain had 4 isoenzymes: two main ones and two minor ones.

Many groups of researchers have separated cAMP phosphodiesterase into its respective isoenzymes and partially characterized these isoenzymes. Rosen (66) with frog erythrocytes, Kakiuchi and co-workers (57, 61) with rat brain and Appleman and co-workers (67, 68, 69) with rat tissues utilized gel filtration to separate the different

isoenzymes. DEAE-cellulose chromatography was used to fractionate the isoenzymes from beef heart [Hrapchak (55)] and rat liver [Appleman (41)]. The only group of investigators to purify the enzyme to apparent homogeneity is Goren and Rosen (53) who used preparative disc gel electrophoresis as the final step in the purification of the beef heart enzyme. The results obtained by each group of researchers is described in detail below.

B. 9. (a) Rosen.

Rosen (66) reported that two isoenzymes of cAMP phosphodiesterase could be separated from the soluble fraction (35, 000 x g supernatant) of frog erythrocytes by gel filtration on a column of Sephadex G-200. The Sephadex G-200 gel filtration profile revealed one major low molecular weight isoenzyme (MW of 100, 000) and one minor high molecular weight isoenzyme (MW of 20, 000). Both forms were eluted in their original volumes of elution when refiltered on Sephadex G-200 indicating that the two isoenzymes did not interconvert. No attempts were made to investigate the relationship between the two isoenzymes. The low molecular weight isoenzyme was purified 1000-fold. This highly purified enzyme preferred cAMP as substrate but exhibited considerable activity with respect to cGMP and cIMP when assayed with millimolar concentrations of substrate.

B. 9. (b) Appleman and co-workers.

Thompson and Appleman (67) reported the separation by gel filtration on a column of agarose A-5M of the cAMP phosphodiesterase from a sonicated 20,000 x g supernatant fraction of rat brain cortex into two main activity peaks: a high molecular weight species (MW of 400,000), a low molecular weight species (MW of 200,000) as well as a minor exclusion peak. The high molecular weight enzyme preferred cGMP as substrate (K_m for cAMP = 100 μM, K_m for cGMP = 13 μM). The low molecular weight enzyme preferred cAMP as substrate (K_m for cAMP = 5 μM, K_m for cGMP = 10.5 μM). Furthermore, while the high molecular weight enzyme obeyed Michaelis Menten Kinetics, the lower molecular weight enzyme displayed negative co-operativity. The exclusion activity peak had substrate specificity similar to that exhibited by the low molecular weight enzyme and the authors postulated that the exclusion peak enzyme may be derived by the association of the low molecular weight form wih particulate matter.

An analysis of extracts from other rat tissues indicated that they, too, had similar isoenzyme patterns. Heart, skeletal muscle, adipose tissue and kidney possessed multiple isoenzymes of cAMP phosphodiesterase (68). The exception was rat liver which exhibited a single activity peak (MW 400,000) on gel filtration on agarose A-1.5 M. It hydrolyzed cGMP and cAMP at approximately the same rate. This single activity peak was resolved into three different activity peaks on

chromatography on DEAE-cellulose (41). The first and second enzyme peak came mainly from the soluble fraction of the rat liver. The third enzyme peak was derived mainly from sonicated 100,000 x g particles of rat liver. Enzymes from all three activity peaks possessed the same molecular weight of 400,000. They exhibited different substrate specificities. The enzyme from the first activity peak preferred cGMP as the substrate; the enzyme from the third activity peak preferred cAMP as the substrate, while the enzyme from the second activity peak hydrolyzed cAMP and cGMP at approximately the same rate. At pH 7.4 cGMP markedly stimulated (by 100-800%) the hydrolysis of cAMP by the enzyme of the second activity peak. cGMP inhibited the hydrolysis of cAMP by enzymes of the other two activity peaks.

B.9. (c) Kakiuchi and co-workers

Many investigators (53, 54) have established that Ca^{2+} by itself does not stimulate cAMP phosphodiesterase. That is, Ca^{2+} cannot replace Mg^{2+}. Kakiuchi and co-workers (60) found that cAMP phosphodiesterase in the crude extract of rat brain could be partially inhibited by low concentrations of EGTA (10^{-4} M) in the presence of excess Mg^{2+} (3 mM). Gel filtration on Sepharose 6B of the 100,000 x g supernatant of rat brain revealed two isoenzymes: a high molecular weight enzyme requiring Mg^{2+} but independent of Ca^{2+}, and a lower molecular weight enzyme which required both Mg^{2+} and Ca^{2+} for

enzymic activity (57, 61). The two isoenzymes were different in many ways:

(a) The Ca^{2+}-dependent isoenzyme was less stable than the Ca^{2+}-independent isoenzyme (57, 61).

(b) A protein activator from rat brain activated the Ca^{2+}-dependent enzyme in the presence of low concentrations of Ca^{2+} but had no effect on the Ca^{2+}-independent enzyme (57, 61).

(c) The Ca^{2+}-independent enzyme hydrolyzed cUMP to a negligible extent but hydrolyzed cAMP and cGMP to approximately the same extent. The Ca^{2+}-dependent enzyme hydrolyzed cUMP at approximately the same rate as it hydrolyzed cGMP, but was twice as active with cAMP (57, 61).

Using Ca^{2+}/EGTA buffer to determine the concentration of free Ca^{2+}, Kakiuchi and Yamazaki (56, 57) were able to show that only very low concentrations of Ca^{2+} (10^{-6} to 10^{-5} M Ca^{2+}) were required to activate the Ca^{2+}-dependent cAMP phosphodiesterase from rat brain in the presence of excess Mg^{2+} (3 mM). When no protein activator was added, activation began at 1.25×10^{-5} M Ca^{2+}. Addition of excess protein activator decreased the threshold of activation to 1.25×10^{-6} M Ca^{2+}. In the absence of added protein activator, excess Ca^{2+} activated the enzyme substantially. Addition

of excess protein activator only increased this Ca^{2+} activation (in the presence of excess Ca^{2+}) slightly. Thus, although Kakiuchi and co-workers could demonstrate that activation by the protein activator was completely dependent on Ca^{2+}, they could only demonstrate a partial dependence on the protein activator for the activation of the enzyme by Ca^{2+}.

B. 9. (d) Hrapchak and Rasmussen

Hrapchak and Rasmussen (55) purified beef heart cAMP phosphodiesterase to high degrees of purity. Their purification procedure involved ammonium sulfate fractionation, protamine precipitation, gel filtration on Sephadex G-200 and DEAE-cellulose column chromatography. They found that DEAE-cellulose column chromatography could separate the enzyme into two active isoenzymes. The first activity peak (MW = 121,500), the major form, was purified 180-fold; overall yield was 3%. Their final products were not homogenous. They claimed that the total enzyme activity could be accounted for at all stages of purification and there was no indication that a cofactor, protein or otherwise, was removed during the purification. The two isoenzymes exhibited similar K_m for the hydrolysis of cAMP; the values for cAMP of the lower molecular weight form and of the higher molecular weight form were 36 and 69 μM, respectively.

B.9. (e) Goren and Rosen

Goren and Rosen (53) developed a new procedure for staining cAMP phosphodiesterase activity on disc gels. Using this method, they demonstrated that the crude homogenate of beef heart possessed only a single enzyme activity band (PDE-II) of disc gel electrophoresis. Exposure to high ionic strength during ammonium sulfate fractionation, chromatography on DEAE-cellulose, or aging at 4 ^{o}C for 1 to 4 weeks, resulted in the appearance of another enzyme activity band (PDE-1) on disc gel electrophoresis.

Goren and Rosen (53) purified isoenzyme PDE-II and PDE-I to apparent homogeneity. The purification procedure used by them was a modification of the procedure developed by Butcher and Sutherland (47) with the addition of gel filtration on Sephadex G-200 and preparative disc gel electrophoresis. PDE-I and PDE-II were found to have molecular weight values of 175,000 and 125,000, respectively. SDS disc gel electrophoresis of purified PDE-II revealed two main bands indicating two subunits of 48,000 and 38,000. A number of other minor bands appeared on the SDS gel electrophoresis pattern (49) and the author described them as impurities. On the basis of these findings, Goren and Rosen suggested that PDE-I was made up of 3 subunits of molecular weight of 38,000 and one subunit of molecular weight of 48,000, and that PDE-II was made up of two subunits of molecular weight of 38,000 and one subunit of molecular weight of 48,000.

Both crude and partially purified beef heart cAMP PDE possessed two K_m for the hydrolysis of cAMP, but homogenous PDE-I and PDE-II each exhibited single K_m values for cAMP. Only one K_m for cGMP (4×10^{-5} M) was displayed by both isoenzymes at all stages of purification. The ratio, $\frac{\text{Rate of cAMP hydrolysis}}{\text{Rate of cGMP hydrolysis}}$, remained consistently at 1.3 up to the hydroxyapatite chromatography stage of purification (49). Further purification by gel filtration on Sephadex G-200 and preparative disc gel electrophoresis decreased the ratio to 0.5. Homogenous PDE-I and PDE-II possessed ratios of 0.48 and 0.53, respectively.

The protein activator reduced the K_m for cAMP from 520 to 220 μM. The protein activator had no effect on the K_m for cGMP although it increased the V_{max} for cGMP hydrolysis.

B.10. Inhibitors

A variety of compounds inhibit the activity of cAMP phosphodiesterase (54, 69). Butcher and Sutherland (47) demonstrated the inhibition of beef heart cAMP phosphodiesterase by the methyl xanthines. Cyclic nucleotides that are good substrates are also good competitive inhibitors (49). A number of pharmacologically active agents are known to be effective inhibitors of cAMP phosphodiesterase. Phenothiazine, promethazine, proamide and tolbutamide have been shown to inhibit the enzyme (54, 69). One of the more potent inhibitors

reported is the vasodilator, papaverine.

cAMP phosphodiesterase is inhibited by mercurial agents such as p-hydroxymercuribenzoate (49, 70). The inhibition is reversible by 2-mercaptoethanol suggesting that sulfhydryl groups on the enzyme are necessary for activity.

B. 11. Activators

B. 11. (a) Protein activator

Cheung (71), and Kakiuchi, Yamazaki and Nakajima (62) independently discovered the existence of a heat stable protein activator of cAMP phosphodiesterase.

Cheung (71) noticed that purification of cAMP phosphodiesterase from bovine brain resulted in partial loss of enzyme activity due to the dissociation and separation of a heat stable non-dialyzable activator from the enzyme. The crude homogenate contained excess non-dialyzable activator. Ammonium sulfate fractionation resulted in partial separation of the non-dialyzable activator from the enzyme; the supernatant being richer in the activator and the pellet being richer in the enzyme. Excellent separation was achieved by chromatography on DEAE-cellulose owing to the large differences in their isoelectric points; the isoelectric points of the non-dialyzable activator and PDE enzyme were 4. 0 and 5. 7, respectively.

Most of the work to date on the characterization on the non-dialyzable activator may be credited to Cheung. The properties of

this non-dialyzable activator may be itemized as follows:

(a) It is protein in nature since it was found to be insensitive to digestion by DNAse, RNAse and lipase but was rapidly destroyed by proteolytic enzymes (71).

(b) Cheung (71) determined the molecular weight of the protein activator to be approximately 40,000 by gel filtration on a calibrated column of Sephadex G-100. Kakiuchi, Yamazaki and Nakajima (62) obtained a figure of about 60,000 for the molecular weight of the protein activator from rat brain using Sepharose 6B gel filtration. This figure is probably inaccurate since the protein activator was eluted close to the void volume.

(c) The protein activator possesses remarkable heat stability. Goren (49) reported that a preparation of this protein activator from beef heart lost only 33% of its activity upon heating at 90 ^{o}C for 5 minutes. Cheung (71) observed that the bovine brain protein activator was stable in 8 M urea, and to heating at 100 ^{o}C at pH 1.7 for short periods of time. However, boiling the protein activator at pH 12.3 rapidly obliterated all activity.

(d) Cheung (71) reported that the protein activator decreased the K_m by 3-fold and increased the V_{max} by the same

extent when cAMP was used as the substrate.

Proteolytic digestion of the cAMP phosphodiesterase could also activate the enzyme, reducing the K_m and increasing the V_{max} to approximately the same extent as that achieved by the protein activator. Activation by the protein activator and by proteolytic digestion were found to be non-additive and to proceed by different mechanisms; the activation being stoichiometric for the former and catalytic for the latter. The protein activator exhibited no proteolytic activity when assayed with three substrates: p-toluene sulphenyl-L-arginine methylester, N-benzoyl-L-tyrosine ethyl ester and casein.

(e) Activation of cAMP phosphodiesterase by the protein activator is specific (71). Proteins with approximately the same molecular weight and isoelectric point do not activate the enzyme.

(f) The protein activator was found in all mammalian tissues investigated by Cheung (54). A protein activator from one tissue cross activated effectively a partially purified enzyme from another tissue indicating a lack of tissue specificity.

(g) A heated partially purified preparation of the bovine brain cAMP phosphodiesterase (71) could still activate the enzyme activity of an unheated preparation of the same enzyme. There was thus residual protein activator in the partially purified enzyme preparation. The difficulty in removing this residual protein activator activity was viewed by Cheung (71) as evidence of very strong affinity of the protein activator for the enzyme. Goren and Rosen (53) confirmed the findings of Cheung. Beef heart cAMP phosphodiesterase (PDE-II) apparently homogenous on analytical disc gel electrophoresis contained residual protein activator activity.

(h) Cheung (71) determined that the protein activator did not bind cAMP.

(i) Distribution of the protein activator activity in different areas of the bovine brain follows the distribution of the cAMP phosphodiesterase (54).

B.11. (b) Activation by Ammonium Ions

According to Nair (50), ammonium salts in the concentration range of 10^{-2} to 10^{-1} M produced a maximum of 1.5-fold activation of crude and partially purified cAMP phosphodiesterase from dog heart in the presence of optimal levels of Mg^{2+}.

B.11. (c) Activation by Imidazole

Butcher and Sutherland found that 40 mM imidazole in 0.04 M Tris buffer pH 7.5 activated purified beef heart cAMP phosphodiesterase by 75% compared to enzyme activity in 0.08 M Tris buffer pH 7.5. This concentration of imidazole also shifted the pH optimum from 8.0 to 7.5.

B.11. (d) Activation by Reducing Agents

Goren (49) reported that dithiothreitol activated by 50% to 60% partially purified beef heart cAMP phosphodiesterase. Maximum activation was at the concentration of 5 mM dithiothreitol. Kinetic analysis revealed that dithiothreitol decreased the K_m for cAMP from 500 μM to 230 μM but did not affect the V_{max} for the hydrolysis of cAMP. Under the same conditions, 5 mM 2-mercaptoethanol activated the enzyme activity by 42%.

B.11. (e) Activation by 5'GMP

Goren and Rosen (72) reported that 5'GMP but not 5'AMP or 5'IMP, activated fresh beef heart cAMP phosphodiesterase to a maximum of 75%. 5'GMP increased the V_{max} but had no effect on the K_m for the hydrolysis of cAMP. 300 μM 5'GMP was required for half maximal activation.

The ability to be activated by 5'GMP was found to be very labile. It was lost when the enzyme was stored above -10 ^{o}C or when

exposed to pH greater than 7.5. It was not possible to retain this property beyond the DEAE-cellulose chromatography stage during the purification of the enzyme. For relatively crude enzyme, this property could be maintained for up to 2 months when the enzyme was stored at -150 ^{o}C in liquid nitrogen.

B.11. (f) Activation by cGMP

Frank and MacManus (73) found that a particulate fraction (20,000 x g pellet) of rat thymus lymphocytes possessed a cAMP phosphodiesterase with K_m values of 0.13 and 8 μM for the hydrolysis of cAMP. cGMP inhibited the enzyme when the substrate concentration was relatively low (1 μM cAMP). However, cGMP at a concentration range between 10^{-7} and 10^{-5} M activated the hydrolysis of cAMP at higher substrate concentrations (10 μM cAMP). cGMP concentrations higher than 10^{-5} M inhibited the enzyme activity.

Beavo, Hardman and Sutherland (74) investigated the effects of cGMP on the hydrolysis of cAMP by both soluble and particulate fractions of cAMP phosphodiesterase from a number of rat tissues. They found that cGMP within the concentration range of 0.08 to 20 μM activated the rat liver enzyme for the hydrolysis of cAMP at substrate concentrations between 0.5 to 40 μM. cIMP exhibited lesser activation while cGMP concentrations in excess of 20 μM inhibited the enzyme activity. Activation by cGMP was shown only by particulate fractions

of rat heart and kidney. For rat liver, brain and thymus, both particulate and soluble fractions of the enzyme could be activated by cGMP. cAMP did not activate the hydrolysis of cGMP by phosphodiesterase from rat tissues. cAMP at concentrations above 2 μM inhibited the hydrolysis of cGMP. Russell, Terasaki and Appleman (41) recently confirmed the findings of Beavo and co-workers.

B. 11. (g) Activation by Naturally Occurring Lipids

Bublitz (75) reported that several naturally occurring lipids activated the hydrolysis of cAMP by soluble rat brain cAMP phosphodiesterase. N-propanol, guanidine-HCl and a variety of synthetic detergents did not exhibit similar activation.

B. 11. (h) Activation by Insulin

Senft and his co-workers (76) reported that insulin at a level of 2×10^{-5} M activated by 2-fold the activity of beef heart cAMP phosphodiesterase *in vitro*. This concentration of insulin was much higher than the physiological level of insulin. However, Menahan, Hepp and Wieland (77), among others, found that insulin had no effect on cAMP phosphodiesterase from the soluble fraction (100,000 x g supernatant) of rat liver. In rat liver, 80 to 90% of the total enzyme activity was in the soluble fraction. Similar results were obtained for the enzyme from rat cells. Many other researchers working with cAMP phosphodiesterase from the soluble fractions of other tissues

have confirmed the results of Menahan and co-workers. Of significance is the recent report by House, Porlis and Weidemann (78) that isolated plasma membranes contained cAMP phosphodiesterase that was directly stimulated by physiological concentrations of insulin. It is quite possible that cAMP phosphodiesterase preparations of Senft and co-workers (76) contained similar membrane-bound phosphodiesterase activity which was activated by insulin.

C. The Relationship Between Ca^{2+} and cAMP in Muscle

It is well established that Ca^{2+} is the coupling agent between excitation and contraction in muscle. This requires that the concentration of Ca^{2+} in muscle cells be regulated very precisely.

The concentration of calcium of a typical mammalian cell is in the range of 1 to 8 mM if all the calcium is soluble and evenly distributed throughout the cell water (79). The concentration of free Ca^{2+} in the cytosol is not known. An educated guess is that the concentration of free Ca^{2+} in the cytosol is probably between 10^{-5} and 10^{-8} M (79). In muscle cells, the bulk of the cell's calcium is compartmentized in mitochondria, microsomes and sarcoplasmic reticulum (80). The extracellular fluid has a Ca^{2+} concentration of approximately 1 mM. Thus, there exists a gradient of calcium concentration across the plasma membrane.

In order to maintain this distribution of calcium within the cell, there exists an externally directed Ca^{2+} pump in the plasma membrane

and an internally directed Ca^{2+} pump in the mitochondria, microsomes and sarcoplasmic reticulum (80, 81). The maximal rate of Ca^{2+} flux across the mitochondrial membrane is 100 to 1000 times more rapid than that across the plasma membrane (82). Consequently, transient changes in the rate of Ca^{2+} entry across the plasma membrane will produce very little change in Ca^{2+} concentration in the cytosol of cardiac muscle cells. However, only a very small change in Ca^{2+} concentration is required to cause muscle contraction since it responds as a function of the fourth power of the Ca^{2+} concentration (83).

When epinephrine acts on the heart, it elicits (a) an increase in the heart rate, (b) an increase in the force of contraction, and (c) an increase in glycogenolysis. Although the mechanism underlying the first change is not well established, much is known about that of the other two changes.

Epinephrine activates heart adenylate cyclase resulting in an increase in cellular cAMP (1). This signal is amplified via a cascade of enzymes resulting finally in the activation of phosphorylase. However, in the absence of an adequate concentration of Ca^{2+}, the conversion of phosphorylase b to phosphorylase a does not take place because phosphorylase b kinase requires Ca^{2+} (10^{-7} M) for activity (84).

Epinephrine also stimulates an influx of Ca^{2+} into the heart. It is not known whether the Ca^{2+} influx is a result of a direct effect of epinephrine on membrane Ca^{2+} transport or is mediated by the effect of cAMP on one or more cellular membranes. There is evidence to support both concepts (83, 85).

The concept of the mediation by cAMP in the epinephrine-induced Ca^{2+} influx into the heart involves the inhibition by cAMP of the externally-directed Ca^{2+} pump of the plasma membrane and the internally-directed Ca^{2+} pump of the mitochondria and a stimulation by cAMP of Ca^{2+} uptake by the microsomes and the sarcoplasmic reticulum. The net result would be (a) a shift of Ca^{2+} to the sarcoplasmic reticulum and (b) a means of increasing the rate of relaxation (83).

In addition, there is a coupling between the surface membrane depolarization and the release of Ca^{2+} from the sarcoplasmic reticulum (86). When surface depolarization takes place, a small amount of Ca^{2+} (trigger Ca^{2+}) enters the cytosol from the extracellular fluid and/or is released from the surface membranes of the cell. This "trigger Ca^{2+}" serves to initiate the release of Ca^{2+} from the sarcoplasmic reticulum (83, 86). The subsequent increase in cytosolic calcium excites the cardiac muscle to contract as well as providing sufficient Ca^{2+} to activate phosphorylase kinase. Thus, cardiac glycogenolysis and cardiac contraction occur in step.

In skeletal muscle, epinephrine increases skeletal muscle glycogenolysis without causing contraction. In contrast to the situation in the heart, there are at least two independent means of stimulating glycogenolysis, one of which involves cAMP while the other does not. Electrical stimulation of skeletal muscle results in very rapid (within seconds) increases in phosphorylase a activity (87). This is mediated by Ca^{2+} and does not involve cAMP. In skeletal muscle (in contrast to heart) both the non-activated and the activated forms of phosphorylase b kinase are stimulated by increases in Ca^{2+} concentration. The only difference from the situation in the heart is that the K_m for Ca^{2+} is approximately 10^{-6} M for the non-activated form of the enzyme and is 10^{-7} M for the activated form (88). When skeletal muscle is electrically stimulated, the release of Ca^{2+} is sufficient to stimulate the non-activated form of the enzyme (88).

While cAMP regulates the metabolism of Ca^{2+} in the cell, Ca^{2+} in turn regulates the concentration of cAMP too. It is well known that Ca^{2+} inhibits adenylate cyclase. Marcus and Aurbach (89) and Robison et al. (90) have shown that adenylate cyclase preparations (disrupted membranes) were inhibited by Ca^{2+} and were activated by EGTA.

Rat brain cAMP phosphodiesterase is regulated by physiological concentrations of Ca^{2+}. 10^{-6} to 10^{-5} M concentrations of Ca^{2+} activates the enzyme (57). The protein activator enhances the Ca^{2+}

activation and enables the enzyme to be activated at a lower concentration of Ca^{2+}.

Thus, there is evidence that both the rate of production and the rate of degradation of cAMP are influenced by Ca^{2+}.

D. Regulatory Proteins

Regulatory proteins [such as the protein activator for cAMP phosphodiesterase] are made by the cell, not for any catalytic activity of their own, but solely for the control of the catalytic activity of certain enzymes. These regulatory proteins reversibly modify quantitatively or even qualitatively the activity of a variety of enzymes. They do so either as chemical signals in themselves (protein inhibitors and activators) or as mediators of other chemical signals (regulatory subunits).

An example of a regulatory protein of the second type is the regulatory subunit of aspartyl transcarbamylase. Aspartyl transcarbamylase can be separated by treatment with mercurials into catalytic subunits (MW = 48,000) which are insensitive to CTP, and regulatory subunits (MW = 28,000) which are devoid of catalytic activity but act as the mediator between the allosteric effector (CTP) and the catalytic subunits (91).

An example of regulatory proteins which are in themselves chemical signals is the protein inhibitor of cAMP-dependent protein kinase. Walsh et al. (92) partially purified and characterized the

protein inhibitor from rabbit skeletal muscle. It is a highly acidic protein, molecular weight of approximately 26,000 as determined by gel filtration, and is remarkably stable to heating at 96 °C and to precipitation with 5% trichloroacetic acid. The protein inhibitor does not bind cAMP.

α-Lactalbumin is interesting in that it causes a qualitative change in the catalytic activity of mammary gland galactosyl transferase (93). α-Lactalbumin changes the substrate specificity of the enzyme from N-acetyl-D-glucosamine to glucose, thereby converting the enzyme from an N-acetyllactosamine synthetase which catalyzes the reaction:

UDP-galactose + N-acetyl-glucosamine ⟶ UDP + N-acetyllactosamine

to a lactose synthetase which catalyzes the reaction:

UDP-galactose + glucose ⟶ UDP + lactose .

Thus, the dramatic appearance of large quantities of α-lactalbumin at parturition, diverts the biochemical apparatus from one that synthesizes glycoproteins to one that synthesizes lactose. Kinetic analysis of the lactose synthetase system by Morrison and Ebner (94) revealed that α-lactalbumin is a special type of modifier which combines with the enzyme only after the binding of the carbohydrate reactant by the enzyme and is released from the lactose synthetase - α-lactalbumin

protein complex upon the discharge of the product. Thus, α-lactalbumin acts in a manner analogous to a reactant (although the α-lactalbumin by itself does not undergo a chemical change) and not as a subunit of the enzyme.

Many research workers (95, 96) have successfully fractionated the troponin from skeletal muscle into three active components. Troponin TN-I, with a molecular weight of 24,000, inhibits actomyosin ATPase, both in the presence and absence of Ca^{2+}. Troponin TN-T, with a molecular weight of 37,000, interacts with tropomyosin. Troponin TN-C, with a molecular weight of 17,000, confers Ca^{2+} sensitivity to the tropomyosin system. Troponin TN-C possesses high Ca^{2+} binding affinity and exhibits a low isoelectric point. The dissociation constant for the Ca^{2+} bound at the high affinity site is consistent with the low Ca^{2+} concentrations required for activation of myofibrils, natural actomyosin and single muscle fibers. Reconstitution of troponin activity requires the simultaneous presence of all three components.

II. OBJECTIVE

The aims of this thesis were:

1. To purify the protein activator of cyclic AMP phosphodiesterase from bovine heart to homogeneity and to characterize it with reference to its physical structure.

2. To elucidate the mechanism by which the protein activator activates the cyclic AMP phosphodiesterase enzyme. This involves studying the factors that influence the interaction of the enzyme and the protein activator.

The thesis is divided into two parts. The first part deals mainly with the purification and characterization of the protein activator. The second part of the thesis investigates the mechanism of activation of the enzyme by the protein activator.

III. MATERIALS

Acrylamide	Eastman Organic
Adenosine 3':5' monophosphate	Sigma
(^{3}H) Adenosine 3':5' monophosphate (5 to 15 c/mMole)	Schwarz-Mann
1-amino-2-napthol-4-sulfonic acid (A. C. S. grade)	Fisher
Ammonium sulfate (A. C. S. grade)	Fisher
Ammonium molybdate (A. C. S. grade)	Fisher
Aminophylline (theophylline)	Sigma
Barium chloride (A. C. S. grade)	Fisher
Beef pancreas chymotrypsinogen (6 x crystallized)	Mann
Calcium chloride (A. C. S. grade)	Fisher
(^{45}Ca) Calcium chloride (0.037 mC/μg calcium)	Amersham Searle
Chelex-100 (minus 400 mesh)	BioRad
Cobalt Chloride (A. C. S. grade)	Baker
Cupric Chloride (A. C. S. grade)	Fisher
DEAE-cellulose (medium capacity)	BioRad
DEAE-cellulose (DE-52, micro-granular)	Whatman
2, 5 diphenyloxazole (scintanalyzed grade)	Fisher
1, 4 bis (2-(5-phenyloxazolyl) benzene (scintanalyzed grade)	Fisher
Fresh beef hearts	Burns Food Ltd. of Winnipeg

Ferrous sulfate (A. C. S. grade)	Baker
Guanosine 3':5' monophosphate	Sigma
(^{3}H) Guanosine 3':5' monophosphate	Schwartz-Mann
Imidazole	Sigma
Isoproterenol	Gift of Dr. Depak Bose
Lactate dehydrogenase (2 x crystallized) Type X	Sigma
Lysolecithin	Nutritional Research
Magnesium acetate (Analar grade)	British Drug House
Manganese sulfate (Analar grade)	British Drug House
Myoglobin (2 x crystallized from sperm whale)	Mann
Napthalene (scintanalyzed)	Fisher
Nickelous sulfate (A. C. S. grade)	Baker
5' nucleotidase (partially purified from venom of Crotaleus adamenteus) 27.6 units/mg protein	Sigma
Ovalbumin (2 x crystallized)	Mann
Periodic acid	Matheson Coleman and Bell
Ribonuclease A (3 x crystallized)	Sigma
Riboflavin	Fisher
Sephadex G-100, G-75, G-50 and G-25	Pharmacia
Sodium potassium tartrate (A. C. S. grade)	Fisher
Sodium sulfite (A. C. S. grade)	Fisher
Sodium bisulfite (A. C. S. grade)	Fisher

Sodium chloride (A. C. S. grade)	Fisher
Sodium dodecyl sulfate (SDS)	Schwarz Mann
Sodium hydroxide pellets (A. C. S. grade)	Fisher
Strontium chloride (A. C. S. grade)	Fisher
Troponin C. from rabbit muscle	Gift of Dr. Jim Stull
Ultrafiltration membranes UM 2	Amicon
Zinc chloride (analar grade)	British Drug House

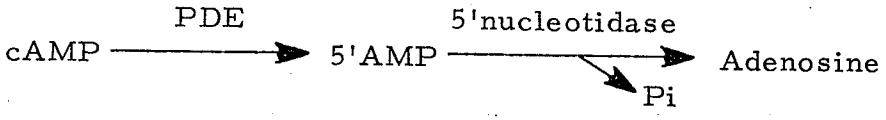
IV. METHODS

A. Assay of Cyclic Nucleotide Phosphodiesterase Activity

Cyclic nucleotide phosphodiesterase activity was routinely assayed by the method of Butcher and Sutherland (47) with slight modifications. This method monitors the inorganic orthophosphate released by the combined or sequential action of cyclic nucleotide phosphodiesterase (PDE) and 5'nucleotidase. This method was chosen for routine assays owing to its simplicity and convenience. It is, however, limited by the low sensitivity of its assay for inorganic phosphate; it requires substrate concentrations greater than 0.5 mM. For lower substrate concentrations, the radioassay method of Brooker et al. (97), as modified by Thompson and Appleman (67), was used.

A. 1. Phosphate Method of Butcher and Sutherland

The reaction involved in this method may be diagrammatically represented as follows:



The product of the cAMP phosphodiesterase reaction, 5'AMP, is converted to adenosine and inorganic orthophosphate (Pi) by 5'nucleotidase. In the original method described by Butcher and Sutherland (47), the venom of Crotalus atrox was used. In our method, partially purified

5'nucleotidase (purchased from Sigma) prepared from the venom of Crotalus adamanteus was used. The partially purified 5'nucleotidase was free of PDE activators which were reported to be present in crude venom (98). Incubation of PDE with the purified 5'nucleotidase did not result in a change in the catalytic activity of the PDE. This 5'nucleotidase preparation had a wide pH range (pH 6 - pH 9) of activity (99) and requires divalent cation (Mg^{2+}) for activity. Table 2 shows that this 5'nucleotidase was unaffected by the conditions or effectors that influenced cAMP phosphodiesterase activity.

The 5'nucleotidase reaction was carried out either concurrently with the cAMP phosphodiesterase reaction (a one-step assay) or after termination of the cAMP phosphodiesterase reaction by boiling for 2 minutes (a two-step assay). Both the one-step and the two-step assays gave similar results.

A. 1. (a) One-step phosphate method

In a routine assay, the reaction mixture, in a volume of 0. 8 ml contained, in addition to cAMP phosphodiesterase and the protein activator, 0. 16 μmoles of $CaCl_2$, 36 μmoles of Tris, 36 μmoles of imidazole, 2. 7 μmoles of magnesium acetate and 0. 2 unit of 5'nucleotidase, all at pH 7. 5. This mixture was incubated at 30 °C for 5 minutes, and then 0. 1 ml of 10. 8 mM cAMP was added to initiate the reaction. The reaction mixture was further incubated at 30 °C for

Table 2

ENZYME ACTIVITY OF 5' NUCLEOTIDASE UNDER DIFFERENT CONDITIONS

Addition	A_{660}
None (Control)	0.66
40 mM Imidazole	0.69
0.5 mM $CaCl_2$	0.69
0.1 mM EGTA	0.69
50 mM Mg^{2+}	0.69
0.4 M NaCl	0.67

The 5'nucleotidase enzyme activity was determined by measuring the inorganic phosphate released from 5'AMP. Experimental conditions and procedures were similar to that for the assay of cAMP PDE enzyme activity with the difference that 5'AMP was used as substrate instead of cAMP. The reaction mixtures contained 40 mM Tris-HCl buffer pH 7.5, 3 mM Mg^{2+}, 0.3 units of 5'nucleotidase and 0.35 mM 5'AMP.

30 minutes and then 0.1 ml of 55% trichloroacetic acid was added to stop the reaction. Following centrifugation at 8,000 x g for one minute, 0.5 ml of the supernatant was transferred to a 4-ml test tube containing 0.5 ml of 0.55% ammonium molybdate in 1.1 N H_2SO_4. A blue color was obtained by adding 0.05 ml of an aqueous solution containing 0.12% sodium bisulfite, 0.01% sodium sulfite and 0.0025% 1-amino-2-napthol-4-sulfonic acid. After it was allowed to stand at room temperature for 7 minutes, the optical density was read at 660 nm. The concentration of the cAMP phosphodiesterase used in the assay was so chosen that the OD660 readings did not exceed 0.9. This ensured that not more than 30% of the initial substrate was used up during the reaction.

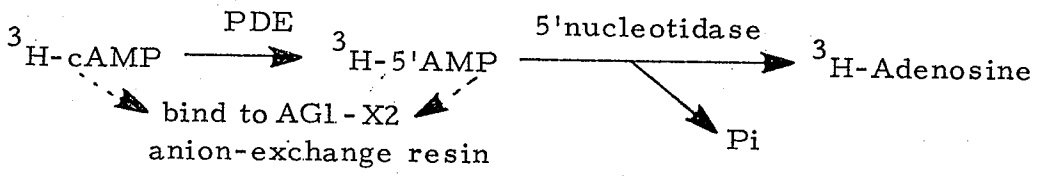
A. 1. (b) Two-step phosphate method

In certain situations, it was advantageous to use the two-step method rather than the one-step method. The procedure for the cAMP phosphodiesterase reaction of the two-step method was identical to the one-step method with the difference that 5'nucleotidase was omitted in the 1st stage reaction. After incubation at 30 °C for 30 minutes, the reaction mixture was heated at 95 °C for 2 minutes to stop the phosphodiesterase reaction. After the reaction mixture was cooled to 30 °C, 0.3 unit of 5'nucleotidase was added and the reaction mixture was further incubated at 30 °C for another 20 minutes. The 5'nucleotidase

reaction was stopped by the addition of 0.1 ml of 55% trichloroacetic acid and the inorganic phosphate released was assayed as described for the one-step assay method.

A.2. Radioassay of Phosphodiesterase Activity

The radioassay procedure may be diagrammatically represented as follows:



Tritium labelled cAMP or cGMP was used as substrate. The steps involved were essentially similar to the phosphate assay with the difference that the enzyme activity was monitored by the production of tritium-labelled adenosine rather than by the release of inorganic phosphate. A two-step incubation was carried out as for the two-step phosphate assay except that the 5'nucleotidase reaction was terminated by heating at 95 ^{o}C for two minutes, instead of by addition of trichloroacetic acid. 1 ml of a slurry of washed BioRad anion-exchange resin AG1-X2 (one part AG1-X2 to one part water) was added and after centrifugation, 0.5 ml of the supernatant was added to 10 ml of scintillation mixture in a 20 ml vial and counted in duplicate in a Beckman LS-250 liquid scintillation spectrometer. Blank reactions were run concurrently with the test reactions. These blank reactions were

identical to the test reactions with the exception that the blank reaction mixtures were heated at 95 ^{o}C for 3 minutes prior to the addition of the substrate. Total radioactivity (of ^{3}H-cAMP or ^{3}H-cGMP) was determined by measuring the ^{3}H-adenosine or ^{3}H-guanosine obtained by prolonged exhaustive incubation of the tritium-labelled substrate with large excess of PDE. The scintillation mixture was composed of 125 g naphthalene, 7.5 g of 2,5 diphenyloxazole and 0.375 g of 1,4 bis 2-(5-phenyloxazolyl)-benzene dissolved in one liter of dioxane.

One unit of cAMP phosphodiesterase enzyme activity is defined as the amount of enzyme which, when maximally activated by the protein activator and Ca^{2+}, hydrolyzes 1 μmole of cAMP per minute at 30 ^{o}C at a substrate concentration of 1.2 mM under standard conditions.

B. Assay of the Activity of Protein Activator of cAMP PDE

PDE-I preparations, largely free of protein activator by chromatography on DEAE-cellulose, always possessed low but significant PDE enzyme activity. When this activator-deficient PDE-I was titrated with protein activator, the activation curve (Fig. 1) showed a hyperbolic curve indicating that the enzyme saturation by the protein activator was approached asymptotically rather than abruptly.

One unit of protein activator activity is defined as the amount of protein activator which gives half maximal stimulation of 0.012

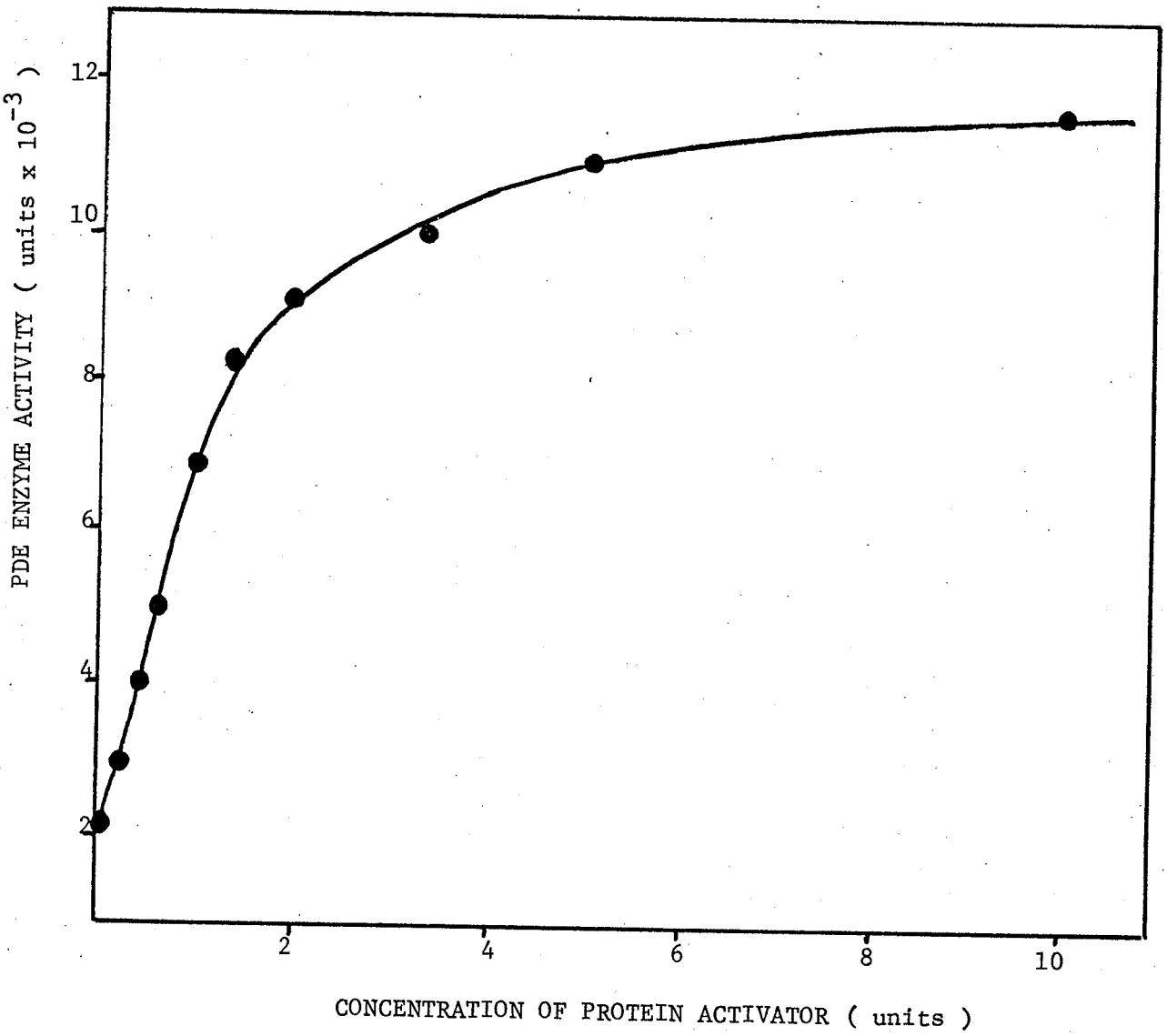


Fig.1 PDE-I ENZYME ACTIVITY AS A FUNCTION OF PROTEIN ACTIVATOR CONCENTRATION. PDE enzyme activity was measured by the two - stage phosphate method. The reaction mixtures contained 0.012 unit of PDE-I, 40 mM Tris-HCl, 40 mM imidazole pH 7.5, 3 mM Mg^{2+}, 0.1 mM Ca^{2+} and varying concentrations of protein activator. The PDE-I enzyme used had been rendered free of protein activator by prior chromatography on DEAE-cellulose. This curve is also referred to as the "PDE-I activity - protein activator titration curve".

unit of cAMP phosphodiesterase (PDE-I) in the presence of excess Ca^{2+} (0.1 mM) under standard conditions. Fig. 2 shows that the same concentration of protein activator gave half maximal activation of a wide range of PDE-I enzyme concentrations.

Protein activator activity was determined by plotting the enzyme activity of a fixed concentration of protein-activator-deficient PDE-I (0.012 units/ml) as a function of the volume of protein activator sample under standard conditions. For any given sample of protein activator, an activation curve similar to that in Fig. 1 could be obtained. From a knowledge of the volume of the protein activator sample required to give half maximal activation, the total activity of the protein activator sample can be calculated. Within limits, this method of assaying protein activator activity is independent of the enzyme activity used (see Fig. 2). Our experience showed that this method of assaying protein activator activity was reproducible. It was also very sensitive being able to detect protein activator activity down to about 5 ng/ml of protein activator.

When a high degree of accuracy was not required, the protein activator activity was assayed by measuring the extent of stimulation of a standard amount of PDE-I under standard conditions and then compared to a standard curve.

C. Protein Assay

Protein concentration was determined by the method of Lowry

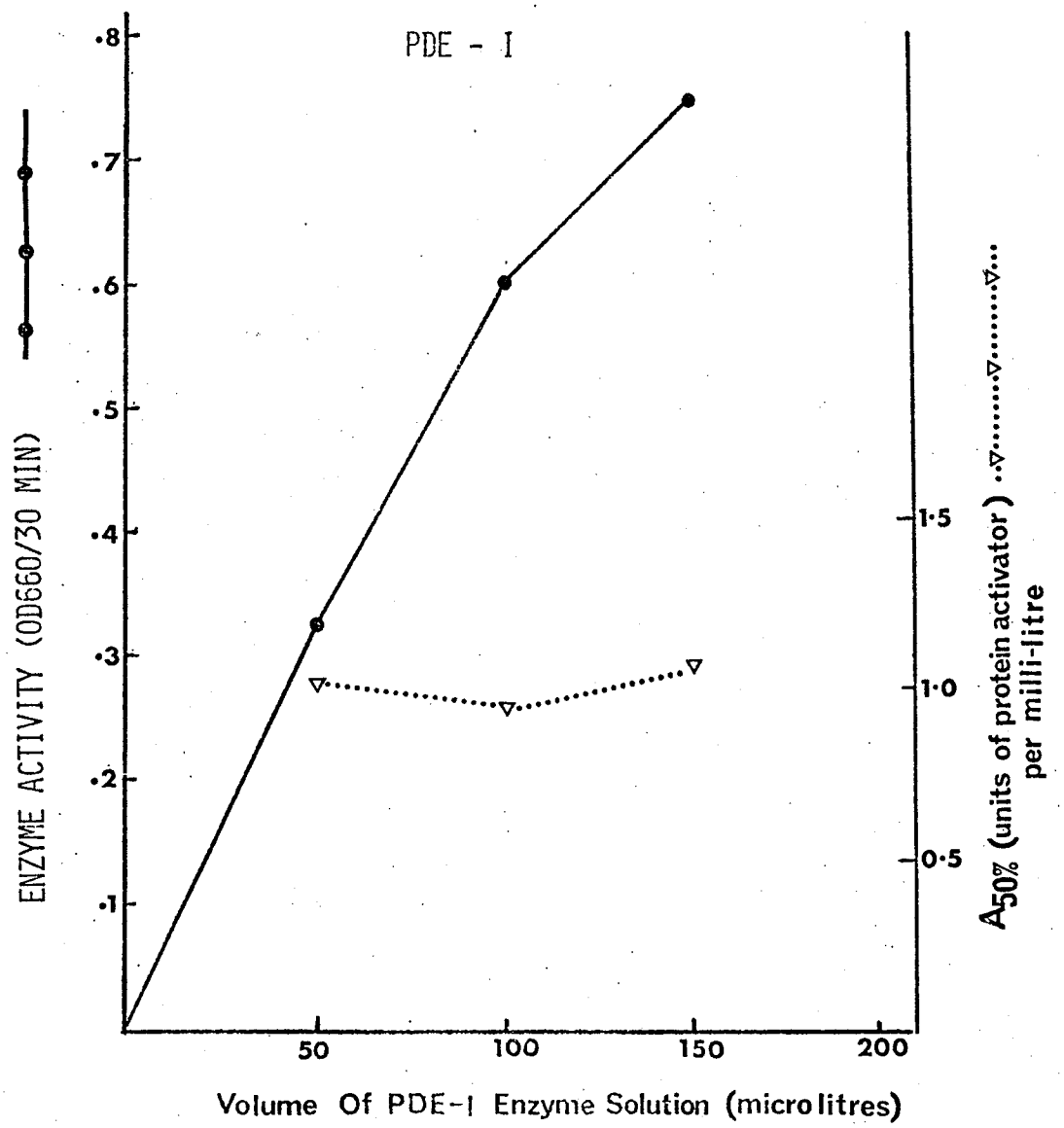


Fig.2 PDE-I ENZYME ACTIVITY AND $A_{50\%}$ PLOTTED AS A FUNCTION OF PDE-I ENZYME CONCENTRATION - PDE enzyme activity was measured by the one - stage phosphate method. The PDE-I enzyme used had been rendered free of protein activator by prior chromatography on DEAE-cellulose. The enzyme reactions were carried out with 40 mM Tris-HCl, 40 mM imidazole pH 7.5, excess protein activator (20 units), 3 mM Mg^{2+}, 0.1 mM Ca^{2+} and 1.2 mM cAMP. The values of $A_{50\%}$ were each obtained from seperate PDE-I activity - protein activator titration curves.

et al. (100) for most cases except for eluant from chromatographic columns when the optical density at 280 nm was utilized.

D. Gel Electrophoresis

D. 1. Disc Gel Electrophoresis at pH 8. 9

The method of Davis (101) was used. The concentration of acrylamide of the separating gel was 15% with acrylamide/bis ratio of 38 for most diagnostic runs. The concentration of acrylamide of the spacer gel was 1. 25% with acrylamide/bis ratio of 4.

D. 2. Disc Gel Electrophoresis at pH 7. 0.

The method of Williams and Reisfeld (102) was used. Concentration of acrylamide used was 15% with an acrylamide/bis ratio of 38.

D. 3. Staining for Protein on Disc Gel

The proteins in the gels were stained for 1/2 hour in amido black in 7% acetic acid. The gels were destained by successive washings with 7% acetic acid.

D. 4. Determination of Subunit Weight

The method of Weber and Osborn (103) was used for SDS gel electrophoresis. For the determination of subunit weight, 10% acrylamide gels were used with acrylamide/bis ratio of 38. 10 μg protein in 30% sucrose solutions containing bromophenol blue, 2-mercaptoethanol, SDS and buffer were layered on the gels. After electrophoresis

the gels were stained for one hour in a solution of 50% methanol containing 2.5% comassie blue and 9.2% acetic acid. The gels were destained by successive washings with 7% acetic acid containing 5% methanol.

D.5. Periodic Acid-Schiff Staining Method for Carbohydrate

Disc gels were stained for carbohydrates by the method of Kapitany and Zebrowski (104).

E. Removal of Ca^{2+} from Reagents

Chelex-100, a resin specific for chelating divalent cations, was used for removing Ca^{2+} from all stock solutions of reagents. 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. Double distilled water, Tris-HCl (0.3 M), and imidazole (0.3 M) were separately treated for the removal of Ca^{2+} by passage through Chelex-100 columns (20 x 3 cm). cAMP solutions (10.8 mM) were passed through a Chelex-100 column (6 x 1.5 cm) to remove Ca^{2+}. Plastic columns and connections were used in the column chromatography. The purified reagents were always stored in plastic containers and all reactions were carried out in plastic vessels. A Perkin-Elmer atomic absorption spectrophotometer model 303 was used to monitor the concentration of calcium in these stock reagents. The limit of detection of calcium by this instrument is 4

ppm. After Chelex-100 treatment, the calcium content of stock reagents was found to be below this limit of detection.

Calcium was removed from the protein activator and cAMP phosphodiesterase (PDE-I) samples by treatment with 0.5 mM EGTA for 20 minutes at 4 ^{o}C; these materials were then desalted by gel filtration through Sephadex G-25 columns (30 x 1.5 cm). Chelex-100 treated water and buffer were used at all steps. Atomic absorption spectrophotometry showed that the Ca^{2+} concentrations in both the enzyme and the protein activator samples were less than 4 ppm.

Magnesium acetate and cobalt chloride stock solutions used were essentially free of calcium contamination. Strontium chloride was contaminated with Ca^{2+} to the extent of 0.002%. Since the highest concentration of Sr^{2+} used was 0.22 mM, the resultant contribution of Ca^{2+} was less than 10^{-8} M.

F. Determination of Ca^{2+} Binding by Purified Protein Activator

The gel filtration method of Hummel and Dreyer (105), as modified by Fairclough and Fruton (106), was used to determine the binding of Ca^{2+} by the purified protein activator of cAMP phosphodiesterase. A column (60 x 0.9 cm) of Sephadex G-25 was equilibrated at 24 ^{o}C with buffer containing 25 mM Tris-HCl, 25 mM imidazole, and 3 mM magnesium acetate with a known concentration of Ca^{2+} plus trace amounts of ^{45}Ca. The column used was a plastic Pharmacia K9/60 column. Chelex-100 treated reagents were used throughout.

Desalted protein activator, 86.4 μg in 0.4 ml, was used for each calcium binding determination. The gel filtration was carried out at 24 °C at a flow rate of 3 ml per hour and 0.6 ml fractions were collected. Aliquots (80 μL) of each fraction were analyzed for radioactivity in duplicate in a Beckman LS-250 liquid scintillation spectrometer. The scintillation mixture used was composed of 125 g naphthalene, 7.5 g of 2,5-diphenyloxazole, and 0.375 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene dissolved in one liter of dioxane.

G. Determination of Molecular Weight by Gel Filtration

A column (1.5 x 90 cm) of Sephadex G-75, equilibrated with 0.02 M Tris-HCl buffer, pH 7.5, containing 1 mM imidazole, 1 mM Mg^{2+}, and 0.1 M KCl, was used to determine the molecular weight of the protein activator. 1 ml fractions were collected at the rate of 5 ml/hr. Dextran blue was used to mark the void volume and adenosine was used to mark the V_t. Molecular weight markers used were sperm whale myoglobin, ovalbumin, beef pancreas chymotrypsinogen and ribonuclease A.

H. Ultracentrifugation

A Beckman model E analytical ultracentrifuge was used. The molecular weight of the protein activator was determined by the conventional sedimentation equilibrium method as well as by the sedimentation diffusion method. In the first method, 1.2 mg/ml and 2.0 mg/ml

of purified protein activator were centrifuged at 22,000 rpm at 20 °C until equilibrium was reached. In the second method, purified protein activator samples of different concentrations were centrifuged at 60,000 rpm at 20 °C. In the determination of diffusion constant, the synthetic boundary cell was used, and the centrifugation was carried out at the low rotor speed of 6000 rpm at 20 °C. The diffusion constant was determined from the spreading of the boundary as a function of time. Both diffusion and sedimentation constants were corrected to the standard conditions, i.e. 20 °C in water. The density of the buffer used in the centrifugation studies was determined by the use of a pycnometer. The viscosity of the buffer was determined using an Oswald viscometer (Cannon Fenske, series 50).

I. Amino Acid Composition

A Beckman amino acid analyzer model 120 C was used for amino acid analysis. Lyophilized purified protein activator (800 μg) was hydrolyzed with 6N HCl at 110 °C for 24, 48, and 72 hours.

J. Isoelectric Focusing

The isoelectric point of the protein activator was determined by isoelectric focusing in a sucrose gradient in an LKB isoelectric focusing column (model 8101) with capacity of 110 ml. Sucrose density was from 0% to 40%. The pH of the ampholyte was 10 (top of column) to 3 (bottom of column). 2 mg of approximately 60% pure protein

activator was applied to the column and isoelectric focusing was carried on for 3 days at 4 °C. 1.5 ml fractions were collected using a fraction collector.

K. Concentration of Protein Activator Solutions

Concentration of protein activator solutions was carried out by ultrafiltration at 50 psi using Amicon ultrafiltration membrane UM-2.

L. UV Absorption Spectra of Protein Activator

Spectra of protein activator purified to homogeneity were recorded by means of a Cary spectrophotometer model 15.

M. Fluorescence Studies

A Aminco-Bowman spectrophotofluorometer model 4-8202 was used for fluorescence studies. The buffer used was 5 mM Tris-HCl pH 7.5 containing 0.1 M NaCl.

N. Handling of Beef Hearts for Subsequent Isolation of PDE or Protein Activator

As soon as the beef hearts were obtained from the local slaughterhouse, they were kept at 4 °C. The beef hearts were then cut into two-inch cubes and stored frozen at -20 °C before use.

V. RESULTS

A. Existence of Two Forms of Beef Heart cAMP Phosphodiesterase

Kakiuchi and Yamazaki (56) have found that low concentrations of EGTA inhibit rat brain cAMP phosphodiesterase. Our data (Fig. 3) show that this chelating agent, in the presence of excess Mg^{2+} (3 mM), inactivated cAMP phosphodiesterase of a crude extract of bovine heart to a maximum of 67% inhibition. This result suggests that a Ca^{2+}-dependent cAMP phosphodiesterase also exists in bovine heart. The finding that there was considerable cAMP phosphodiesterase activity in the presence of excess EGTA suggests that a Ca^{2+}-independent enzyme was also present. In the absence of EGTA, however, 30 μM Ca^{2+} activated the enzyme by less than 10% (Fig. 3). Presumably this is because a sufficient amount of endogenous Ca^{2+} was present in the assay mixture. These two forms of cAMP phosphodiesterase can be separated by chromatography on a column of DEAE-cellulose. A method has been developed by Dr. Honor Ho (107) in our laboratory for the partial purification of the two forms of beef heart cAMP phosphodiesterase.

B. Partial Purification of Beef Heart cAMP Phosphodiesterase

cAMP phosphodiesterase from beef heart was purified by a procedure adapted from the method of Butcher and Sutherland (47) and developed by Dr. Honor Ho (107) in our laboratory. It involved

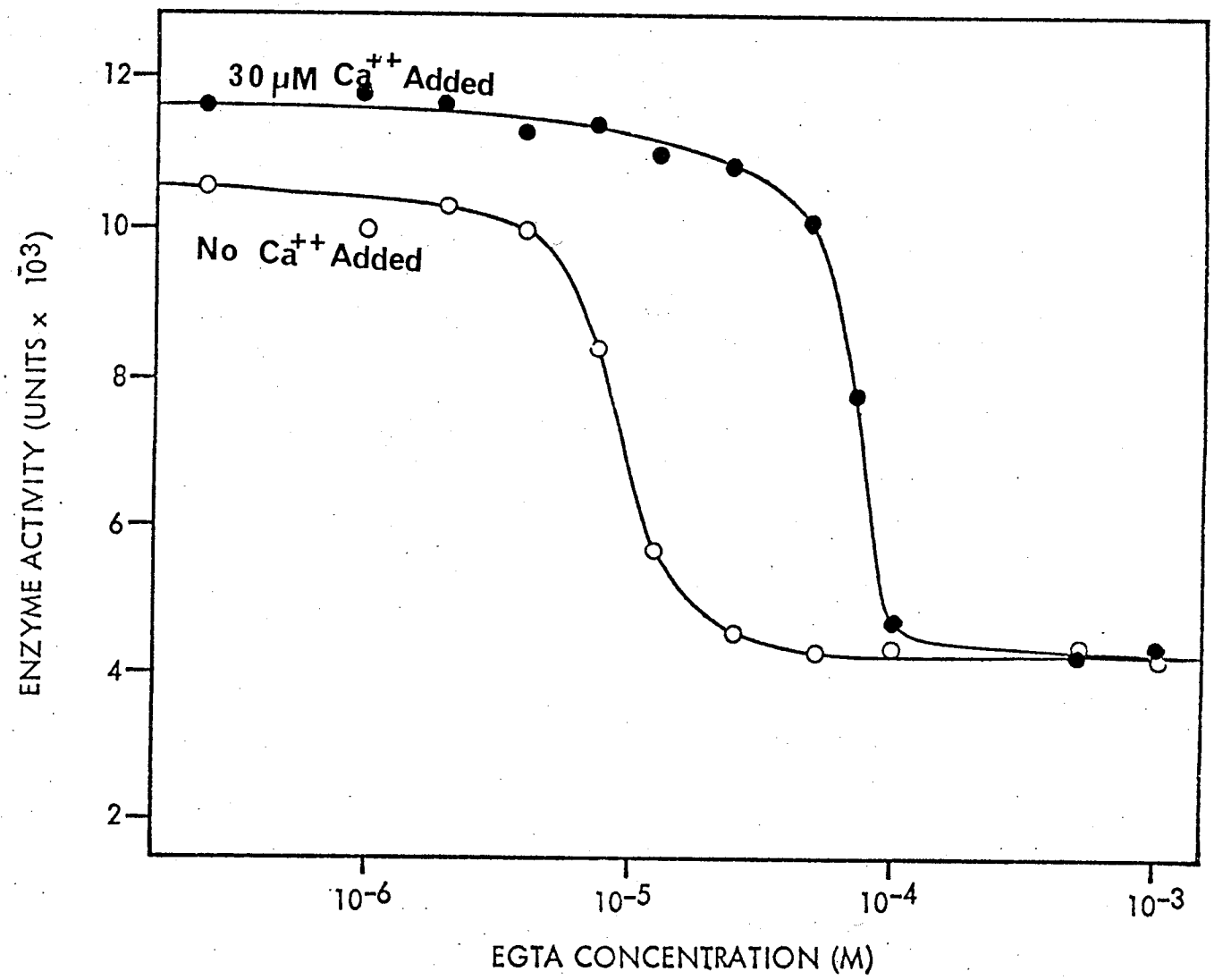


Fig.3 INHIBITION BY EGTA OF CAMP PHOSPHODIESTERASE IN CRUDE EXTRACT OF BEEF HEART

Dialyzed 10,000 x g supernatant of the homogenate of beef heart was used for the experiment. PDE enzyme activity was measured by the one - stage method. All reaction mixtures contained excess protein activator

(a) homogenization with Tris-HCl buffer, (b) ammonium sulfate precipitation, (c) centrifugation at 100,000 x g and (d) DEAE-cellulose column chromatography (see Fig. 4).

B.1. Homogenization with Tris-HCl Buffer

Frozen beef hearts (1 kilogram) were thawed, put through a meat mincer and homogenized in a Waring blender for 10 seconds at low speed with 2 1/2 volumes of 0.1 M Tris-HCl buffer containing 2 mM EDTA. The homogenate was centrifuged at 3000 x g at 4 ^{o}C for 15 minutes. The pellet was discarded.

B.2. Ammonium Sulfate Precipitation

The 3000 x g supernatant was titrated with 5 N NaOH to pH 8.8. Powdered ammonium sulfate was added to bring the 3000 x g supernatant rapidly to 50% saturation with respect to ammonium sulfate at 4 ^{o}C. Centrifugation at 4 ^{o}C was carried out immediately (within thirty minutes of addition of ammonium sulfate) at 10,000 x g for 20 minutes. The pellet (here referred to as pH 8.8 pellet) contained almost all the cAMP phosphodiesterase enzyme activity while the supernatant (here referred to as the pH 8.8 supernatant) contained the majority (60% to 80%) of the protein activator activity.

The pH 8.8 supernatant was further processed separately (discussed in Section C "Purification of the Protein Activator") by procedures designed for the isolation of the protein activator . The pH 8.8 pellet was used as a source of cAMP phosphodiesterase enzyme.

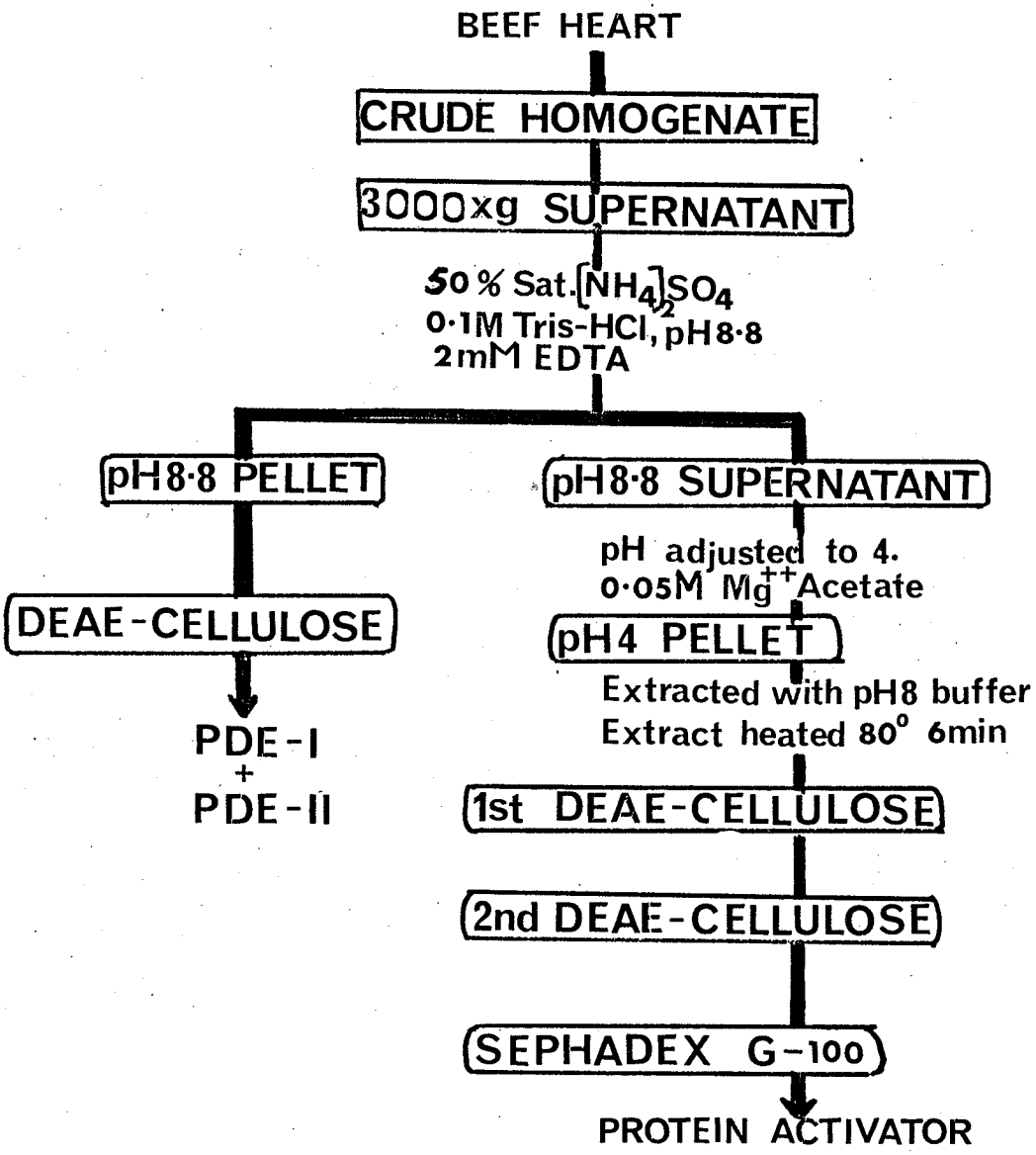


Fig.4 PURIFICATION SCHEME

The separation of the phosphodiesterase enzyme from its protein activator was found to be dependent on three factors:

(1) First of all, the time lapsed between addition of ammonium sulfate and centrifugation was a critical factor in determining the efficacy of the enzyme/activator separation. Immediate centrifugation after addition of ammonium sulfate resulted in high yields (60% to 80%) of the protein activator in the pH 8.8 supernatant. The longer the delay in centrifugation after ammonium sulfate addition, the lower was the recovery of protein activator activity in the pH 8.8 supernatant. The recovery of the enzyme activity in the pH 8.8 pellet was independent of time.

(2) Secondly, the more alkaline the pH (up to pH 9.0), at which the ammonium sulfate precipitation was carried out, the greater was the recovery of protein activator activity in the pH 8.8 supernatant.

(3) Thirdly, the inclusion of 2 mM EDTA increased the recovery of protein activator activity in the pH 8.8 supernatant.

B. 3. High Speed Centrifugation

The pH 8.8 pellet was dissolved in a minimal volume of Tris-HCl buffer pH 7.5 and was dialyzed overnight against 0.02 M Tris-HCl buffer pH 7.5 containing 0.05 M NaCl, 10 mM 2-mercaptoethanol, 20 μM $CaCl_2$ and 1 mM magnesium acetate. It was then centrifuged at 100,000 x g at 4 ^{o}C for one hour. The pellet was discarded.

This high-speed centrifugation step was necessary because of the presence of a considerable amount of particles in the pH 8.8 pellet which could not be removed by low-speed centrifugation. This particulate fraction was found to cause complications in the subsequent DEAE-cellulose chromatography steps.

B.4. DEAE-Cellulose Column Chromatography

The cAMP phosphodiesterase in the 100,000 x g supernatant was further purified by a unique type of affinity chromatography on columns of DEAE-cellulose. Conventional affinity chromatography utilizes a ligand (such as substrate, inhibitor, or activator) covalently bound to an inert support (such as agarose) to bind the desired protein. In our version of affinity chromatography we used protein activator* (which copurified with the cAMP phosphodiesterase) reversibly bound to DEAE-cellulose to bind cAMP phosphodiesterase. The protein activator (pI = 4.0), having a much lower isoelectric point than cAMP phosphodiesterase enzyme (pI 5.7), was bound more tightly to the DEAE-cellulose column than the enzyme.

B.4. (a) First DEAE-cellulose column chromatography of PDE

The dialyzed 100,000 x g supernatant was applied to a 4 x 40 cm DEAE-cellulose column. Elution was by a linear gradient from

*The pH 8.8 pellet, used as a source of PDE enzyme, usually contained sufficient protein activator for affinity chromatography.

0.05 M NaCl to 0.4 M NaCl in 0.02 M Tris-HCl buffer pH 7.5 containing 20 μM Ca^{2+}, 1 mM Mg^{2+} and 10 mM 2-mercaptoethanol. Inclusion of 20 μM Ca^{2+} in the elution buffer ensured maximal binding of the cAMP phosphodiesterase to the protein activator. Fig. 5 shows that the cAMP phosphodiesterase activity peaks were retarded relative to the main protein peak. Two cAMP phosphodiesterase activity peaks, PDE-I and PDE-II, were obtained. PDE-I and PDE-II were eluted at the ionic strength of 0.22 and 0.27, respectively. PDE-I was inhibited 85% by low concentrations of EGTA, while PDE-II was inhibited less than 10% by EGTA. PDE-I was purified 15-fold by this step alone over the homogenate (see Table 3). For most purposes, this purity was sufficient. For studies on the interaction between PDE-I and the protein activator and for measurements of kinetic parameters, PDE-I was further purified by rechromatography on a second column of DEAE-cellulose.

B.4. (b) Second DEAE-cellulose column chromatography of PDE-I

PDE-I was pooled, dialyzed and applied to a 2.5 x 30 cm DEAE-cellulose column which was equilibrated with 0.02 M Tris-HCl pH 7.5 buffer containing 10 mM 2-mercaptoethanol, 0.1 mM EGTA and 1 mM magnesium acetate. Elution was by the same buffer with a linear gradient from 0.05 M NaCl to 0.4 M NaCl. Only one cAMP phosphodiesterase activity peak was eluted (at ionic strength of 0.15). It was

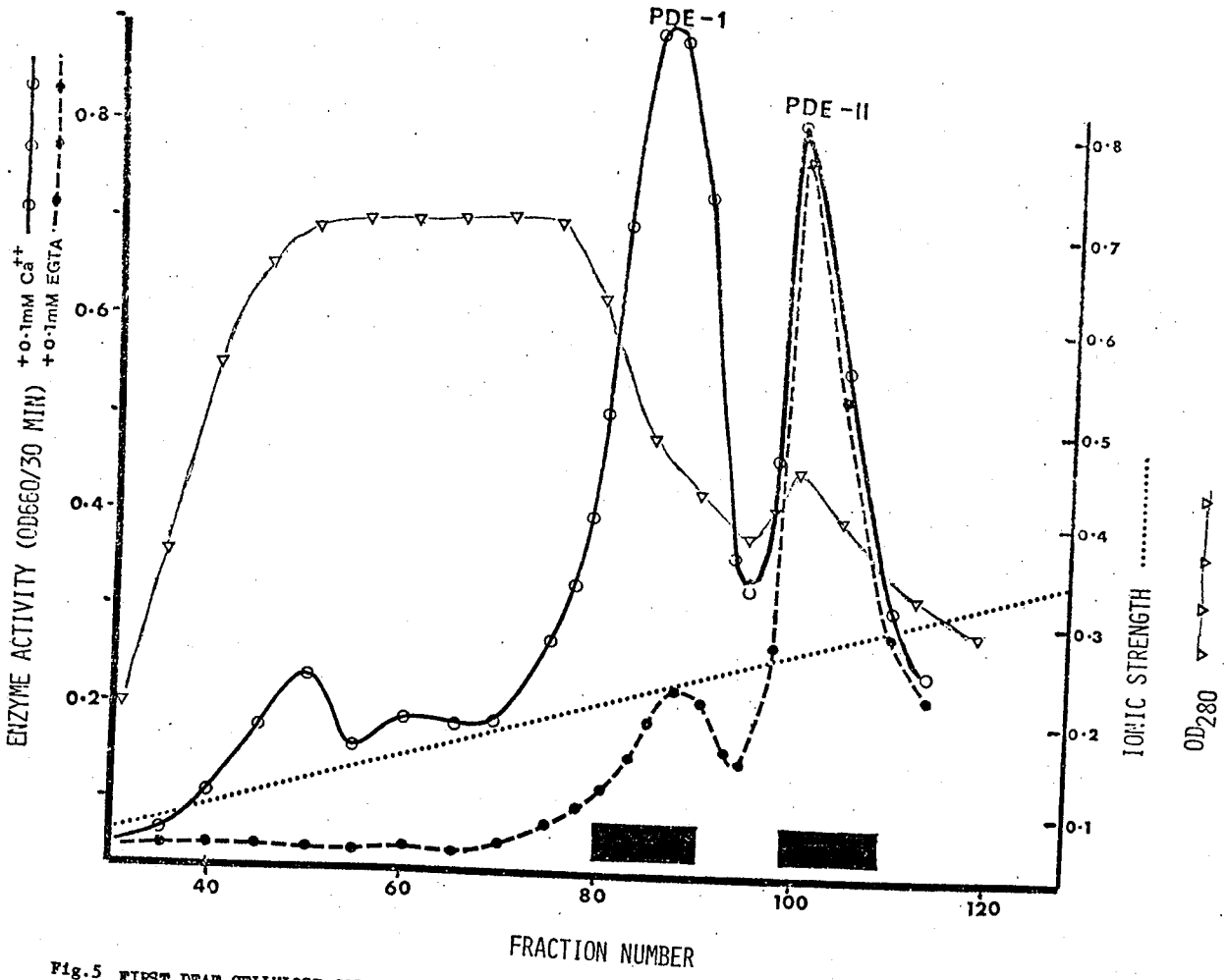


Fig.5 FIRST DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF PDE-I AND PDE-II. - A dialyzed solution of the pH 8.8 pellet was applied to a 40 x 4 cm DEAE-cellulose column and was eluted by a linear gradient from 0.05M to 0.4M NaCl in 20 mM Tris-HCl buffer pH 7.5 containing 10 mM 2-mercaptoethanol, 1 mM Mg^{2+} and 20 uM Ca^{2+}. Enzyme assays were carried out with excess protein activator and either with 0.1 mM Ca^{2+} (-⊖—⊖—⊖-) or with 0.1 mM EGTA (-●---●---●-). The fractions pooled for further purification are indicated by solid bars under the activity peaks. The data shown in this figure were from an experiment carried out in collaboration with Dr. Honor Ho.

eluted ahead of the main protein peak as is shown in Fig. 6. cAMP phosphodiesterase activity was inhibited 90% by 0.1 mM EGTA showing that Ca^{2+} was required for enzyme activity. The cGMP phosphodiesterase activity profile coincided with the cAMP phosphodiesterase activity profile. cGMP phosphodiesterase activity was only slightly inhibited by 0.1 mM EGTA. Hydrolysis of both cyclic nucleotides was measured at a concentration of 1.2 mM. The specific activity of PDE-I's cAMP phosphodiesterase activity was 1.7 units/mg protein. This represents a 65-fold purification over that of the homogenate (see Table 3).

In the second DEAE-cellulose column chromatography, inclusion of 0.1 mM EGTA in the elution buffer, resulted in very low concentrations of free Ca^{2+}. This weakened the interaction between the protein activator and cAMP phosphodiesterase enzyme and thereby allowed the cAMP phosphodiesterase enzyme to be eluted ahead of the main protein peak as is shown in Fig. 6. Fig. 6 also shows that PDE-I, on rechromatography on DEAE-cellulose, did not convert to PDE-II to any significant extent. PDE-II, on rechromatography on DEAE-cellulose, also did not convert significantly to PDE-I as is shown in Fig. 7.

PDE-I enzyme preparations obtained by DEAE-cellulose chromatography always possessed low but significant enzyme activity assayed in the absence of added protein activator or in the presence

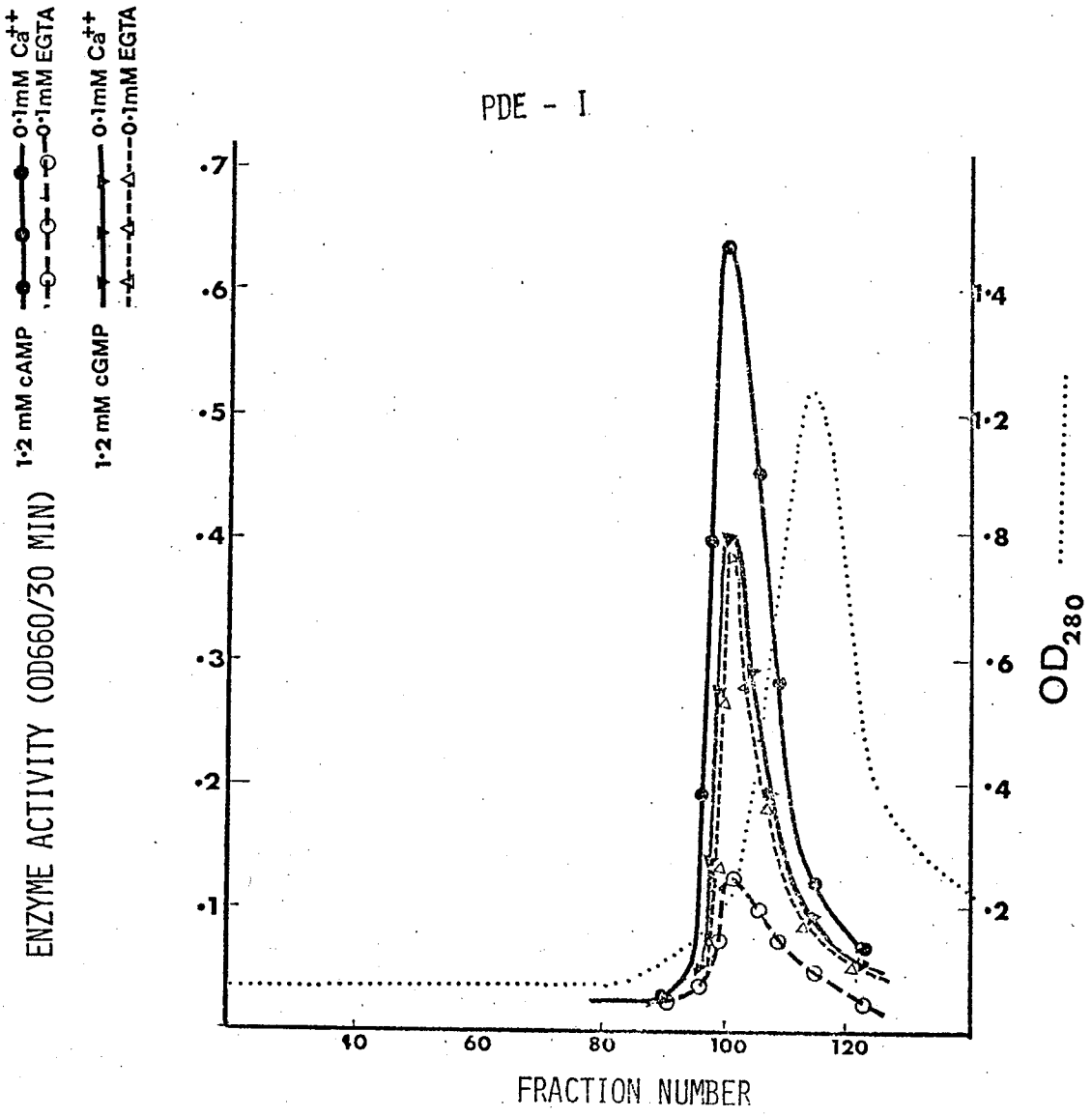


Fig.6 2nd DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF PDE-I. - Fractions 80 to 90 from the 1st DEAE-cellulose chromatography (see fig.3) were pooled, dialyzed and applied to a 2.5 x 30 cm DEAE-cellulose column (DE-52). Elution procedures and buffer used were identical to those of 1st DEAE-cellulose chromatography except that 0.1 mM EGTA was included in the elution buffer. PDE activity was assayed using cAMP and cGMP as substrate with excess protein activator added in the assay mixtures in all cases. The data shown in this figure were from an experiment carried out in collaboration with Dr. Honor Ho.

Table 3

PURIFICATION OF PDE-I FROM BEEF HEART

	PROTEIN		ENZYME ACTIVITY		SPECIFIC ACTIVITY	FOLD PURIFICATION
	(mg)	(% RECOVERY)	(Units)	(% RECOVERY)	(unit/mg)	
HOMOGENATE (100,000 x g supernatant)	17,300	100%	449	100%	0.026	-
$(NH_4)_2SO_4$ Pellet	7,873	45%	340	76%	0.043	1.6
1st DEAE-CELLULOSE	219	1.3%	93	20%	0.422	15.5
2nd DEAE CELLULOSE	11.6	0.07%	19.7	4.3%	1.70	65.5

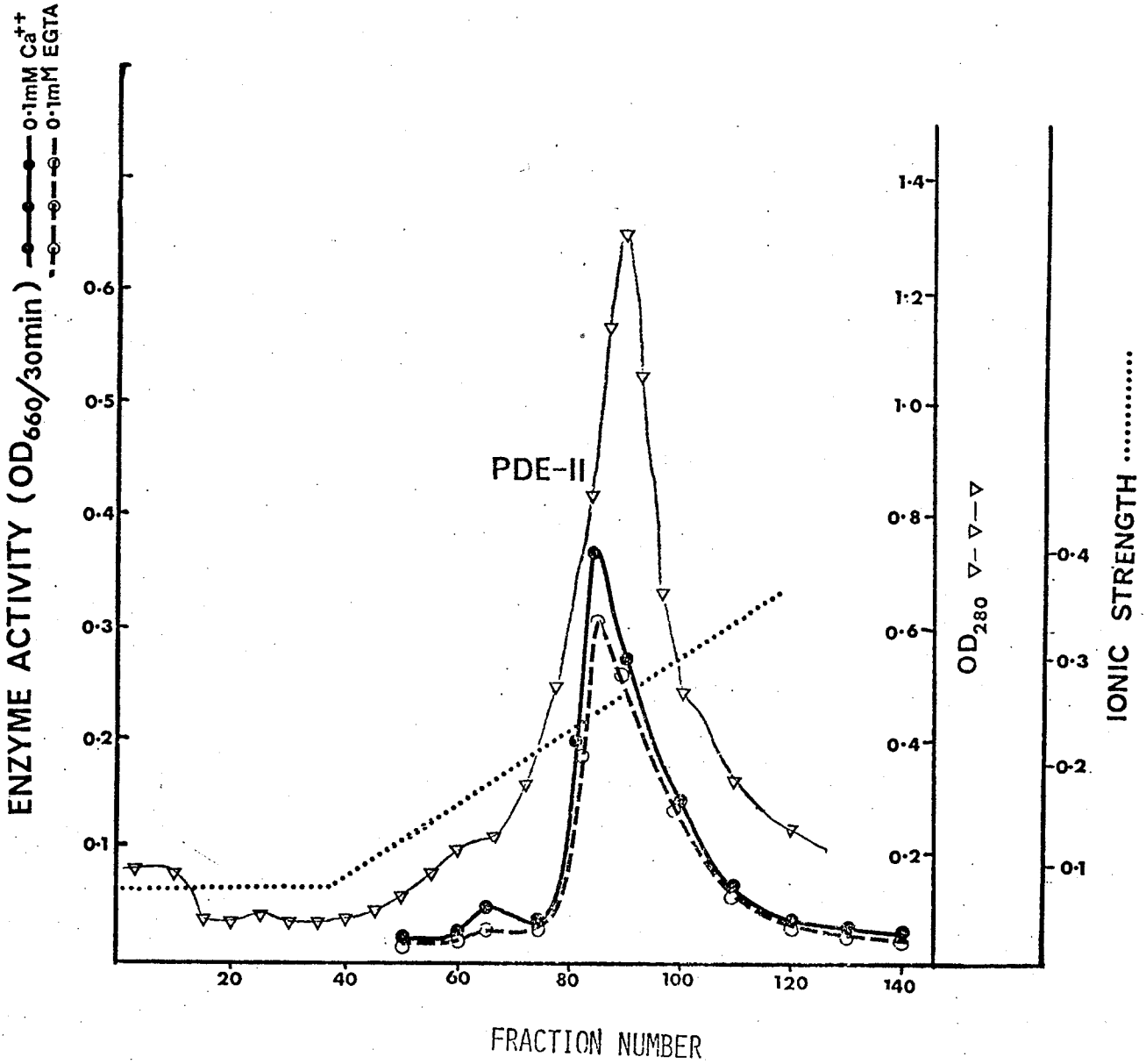


Fig.7 RECHROMATOGRAPHY OF PDE-II ON DEAE CELLULOSE. - Fractions 99 to 109 from the 1st DEAE-cellulose chromatography (see fig.3) were pooled, dialyzed and applied to a 2.5 x 30 cm DEAE-cellulose (DE-52) column and the chromatography was carried out as for fig.4. PDE enzyme activity assays were carried out using 1.2 mM cAMP as substrate with excess protein activator and with either 0.1 mM Ca^{2+} or 0.1 mM EGTA in the assay mixtures. The data shown in this figure were from an experiment carried out in collaboration with Dr. Honor Ho.

of EGTA. Fig. 37 shows that this low basal enzymic activity was not inhibited by EGTA in the presence of excess Mg^{2+} and was therefore independent of Ca^{2+}. These PDE-I preparations possessed negligible concentrations of protein activator. The concentration of protein activator in PDE-I preparations heated at 95 ^{o}C for 2 minutes (to inactivate the PDE activity) has been found to activate unheated PDE-I preparations by less than 3%. Hence the "basal PDE activity" of PDE-I preparations was not due to activation of the PDE-I enzyme by residual protein activator.

C. Purification of the Protein Activator of Beef Heart cAMP Phosphodiesterase

The procedure (see Fig. 4) for the purification of the protein activator of beef heart cAMP phosphodiesterase involves homogenization, ammonium sulfate fractionation, acid precipitation, heat treatment, ion-exchange chromatography on DEAE-cellulose columns, and gel filtration on Sephadex G-100 columns.

C.1. Homogenization

The procedure was identical to that described for the purification of cAMP phosphodiesterase enzyme.

C.2. Ammonium Sulfate Fractionation

The procedure was identical to that described for the purification of cAMP phosphodiesterase enzyme. The pH 8.8 supernatant

was used as a source of protein activator.

C. 3. Isoelectric Precipitation

More powdered ammonium sulfate was added to the pH 8.8 supernatant so as to bring it to 60% saturation with respect to ammonium sulfate. 1 M magnesium acetate solution was added to give it a final concentration of 0.05 M. The pH of the solution was adjusted to 4.0 by adding 10 N HCl. The solution was then allowed to stand at 4 ^{o}C overnight. There was formation of copious amounts of dark brown precipitate rich in protein activator. Precipitation of protein activator was essentially quantitative. This suspension was centrifuged at 10,000 x g at 4 ^{o}C for 15 minutes. A minimal volume (about 200 ml) of 0.04 M Tris-HCl buffer pH 8.0 was added to the precipitate and the pH of the slurry was adjusted to 8.0 by the addition of 2N NaOH. The mixture was then stirred vigorously for 10 minutes at room temperature.

C. 4. Heat Treatment

The mixture was heated at 80 ^{o}C for six minutes. Large amounts of inactive protein, precipitated out of solution by this heat treatment, was removed by centrifugation at 8000 x g at 4 ^{o}C for 10 minutes. This heat treatment resulted in the loss of less than 5% of the protein activator activity. The solution was dialyzed overnight at 4 ^{o}C against 10 volumes of 0.02 M imidazole pH 6.5 buffer containing 0.2 M NaCl and 1 mM magnesium acetate.

C. 5. DEAE-Cellulose Column Chromatography

The dialyzed heat-treated extract of the pH 4. 0 pellet was applied to a 4 x 40 cm DEAE-cellulose column which had been equilibrated with 0. 02 M imidazole pH 6. 5 buffer containing 0. 2 M NaCl and 1 mM magnesium acetate. The column was washed with the same buffer until no more protein was eluted. The protein activator was then eluted by the same buffer with a linear gradient from 0. 2 M NaCl to 0. 6 M NaCl. Fig. 8 shows the results of a typical DEAE-cellulose column chromatography. Approximately 80% of the total protein was washed out from the column as a huge inactive protein peak by the buffer containing 0. 2 M NaCl. The protein activator was eluted as a sharp peak at an ionic strength of 0. 36. The trailing edge of the activator peak overlapped with the leading edge of a protein peak which contained a group of largely acidic proteins. The leading and trailing edges of the protein activator peak were sacrificed and the fractions representing the principal portion of the activity peak were pooled and dialyzed at 4 ^{o}C for 12 hours against 5 volumes of 0. 02 M imidazole pH 6. 5 buffer containing 0. 2 M NaCl and 1 mM magnesium acetate. This DEAE-cellulose step alone resulted in a large increase in specific activity amounting to 26-fold purification (see Table 4).

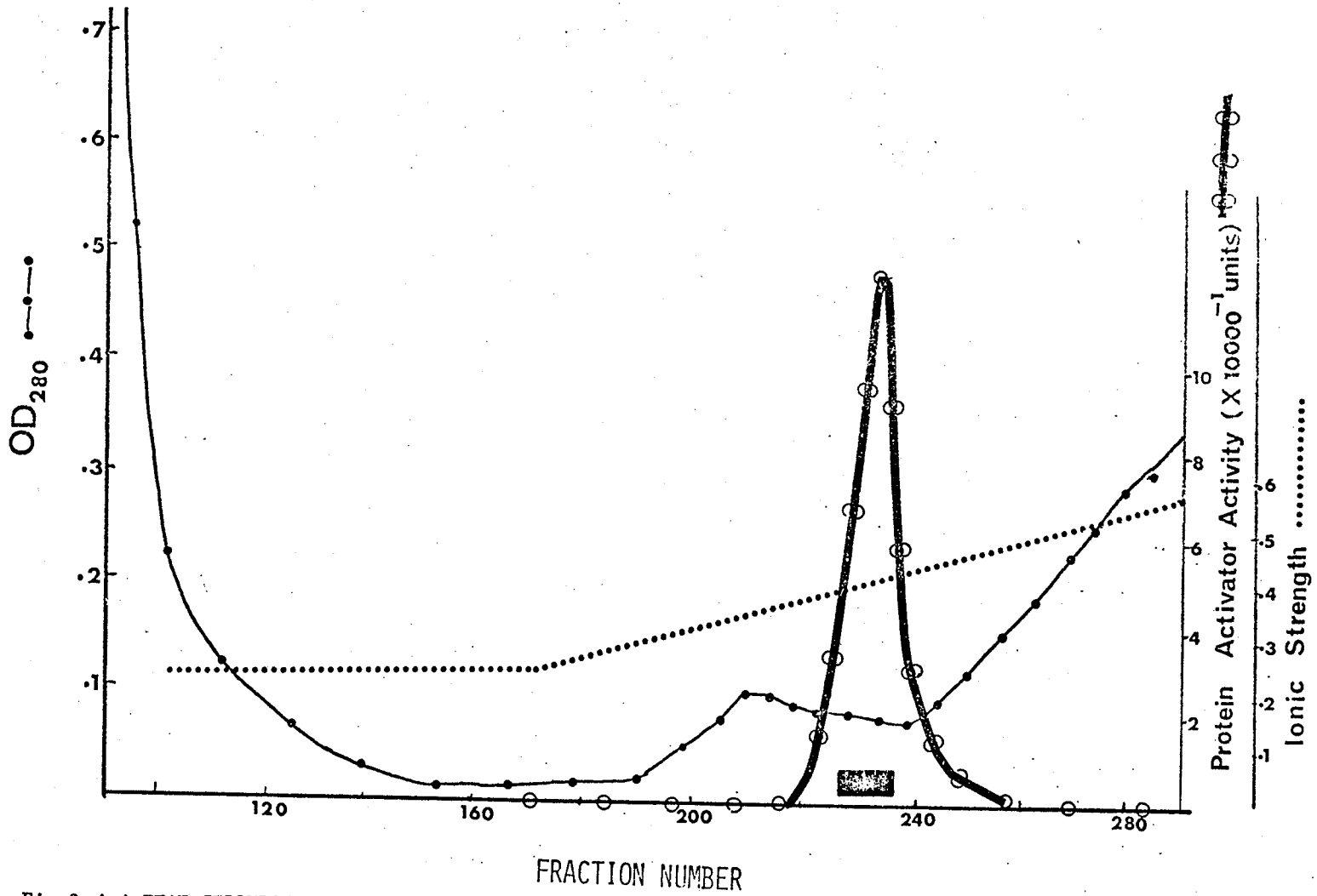


Fig.8 1st DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF PROTEIN ACTIVATOR. - The dialyzed heat-treated pH 4 pellet form 1 Kg of beef heart was used as a source of protein activator. A 4 x 36 cm column of DEAE-cellulose equilibrated with 20 mM imidazole buffer pH 6.5 containing 1 mM Mg^{2+} was used. The protein activator was eluted by the same buffer with a linear gradient from 0.2 to 0.6 M NaCl. The solid bar under the activity peak indicates the fractions pooled for further purification.

Table 4

PURIFICATION OF PROTEIN ACTIVATOR

	PROTEIN (mg)	(% Recovery)	ENZYME ACTIVITY (Units)	(% Recovery)	SPECIFIC ACTIVITY (Unit/mg)	FOLD PURIFICATION
HOMOGENATE (100,000 x g supernatant)	39,300	100%	1,354,000	100%	34	-
pH 4 PELLET (After Heat Treatment)	1,257	3.2%	1,056,100	78%	840	24
1st DEAE CELLULOSE	31.9	0.081%	686,480	51%	21,500	632
2nd DEAE CELLULOSE	7.4	0.019%	466,800	34%	63,040	1,854
SEPHADEX G-100	2.4	0.006%	259,200	19.1%	108,000	3,176

C. 6. Rechromatography on DEAE-Cellulose Column

The dialyzed protein activator from the first DEAE-cellulose column was applied to a smaller DEAE-cellulose column (2.5 x 30 cm) which had been equilibrated with 0.02 M imidazole pH 6.5 buffer containing 0.2 M NaCl and 1 mM magnesium acetate. Whereas the first ion-exchange column was packed with fibrous DEAE-cellulose of medium capacity (BioRad Cellex D), the second column was packed with microgranular DEAE-cellulose of higher capacity (Whatman DE52). The microgranular DEAE-cellulose gave better resolution though at a lower flow rate. The result of a typical rechromatography is shown in Fig. 9. The increase in specific activity was not as dramatic as that gained by the first DEAE-cellulose chromatography. However, this rechromatography was vitally necessary because of the abnormal behavior of the protein activator on gel filtration. On gel filtration, it was found that, although it has a molecular weight of 15,000 as determined by the sedimentation equilibrium method (see Table 6, page 107), it migrated as though it was of much higher molecular weight of 27,000 (see Table 6, page 107). Thus, if it were contaminated with proteins of molecular weights of 27,000, these contaminating proteins must be removed by ion-exchange chromatography first (assuming that they possessed charge characteristics different from that of the protein activator) since they cannot be eliminated by the subsequent gel filtration. At this stage, the protein

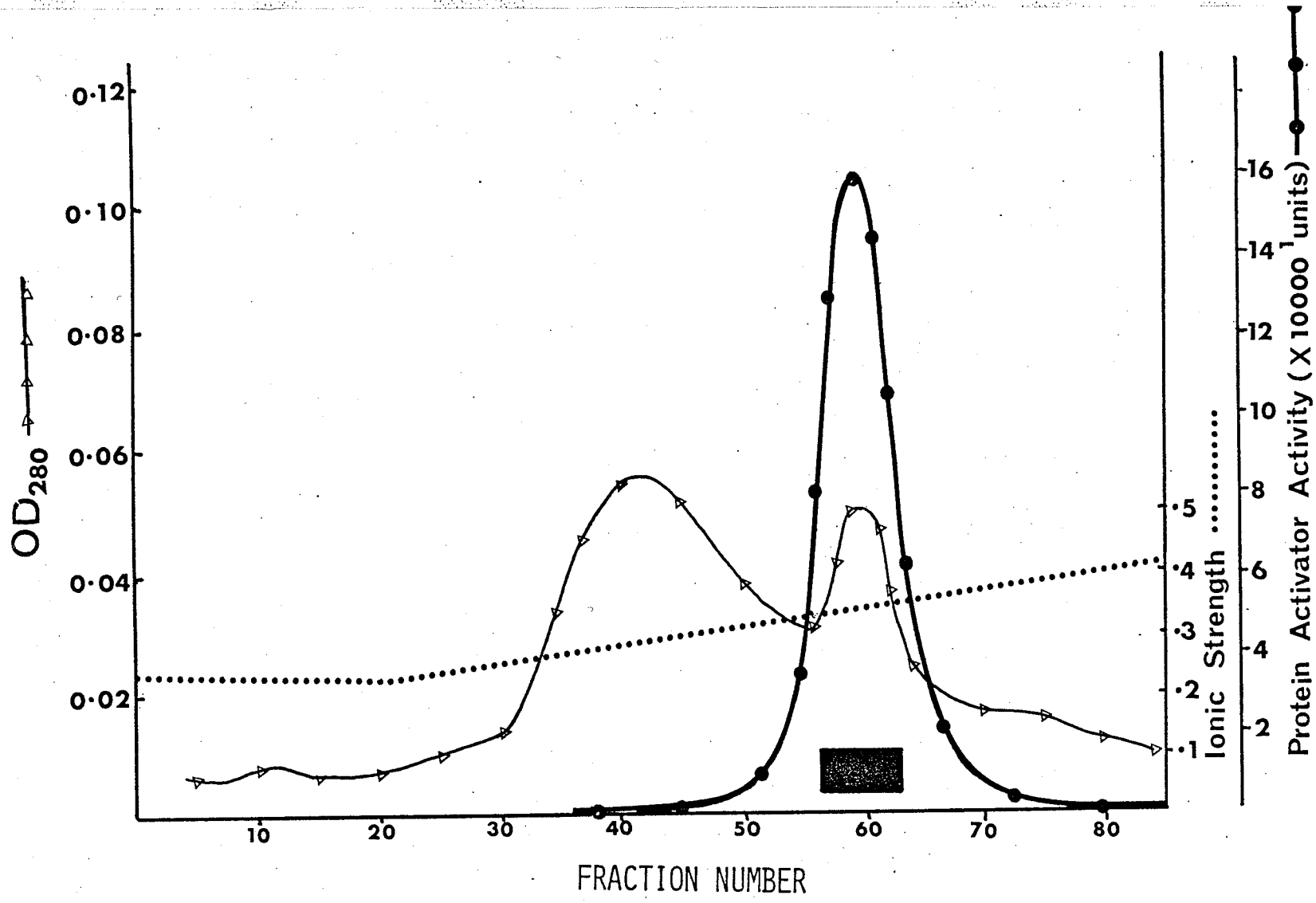


Fig.9 **2nd DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF PROTEIN ACTIVATOR.** - Fractions 226 to 236 of 1st DEAE-cellulose chromatography (see fig.6) of the protein activator were pooled, dialyzed and applied to a 2.5 x 30 cm column of DEAE-cellulose (DE-52). Buffer used and elution procedure were identical to those of fig.6. The solid bar under the activity peak indicates the fractions pooled for further purification.

activator preparation eluted from the second DEAE-cellulose column was at least 20% pure. For some preparations, their specific activity indicated that their purity was at least 60%.

C.7. Gel Filtration on Sephadex G-100

The fractions making up the main part of the activity peak were pooled and concentrated by ultrafiltration through a UM2 membrane to approximately 2 ml. This was applied to a 2.5 x 80 cm Sephadex G-100 column. The buffer used in developing the gel filtration was 5 mM Tris-HCl, pH 7.5 buffer containing 0.1 M NaCl and 0.02% sodium azide, the last constituent being included as a preservative. 1.2 ml fractions were collected at a flow rate of 7 ml per hour at 4 ^{o}C.

Fig. 10 shows the result of a typical gel filtration. A relatively large high molecular weight protein peak (which contained no activator activity) was eluted in the void volume and this was very well separated from a small protein peak which coincided with the activity peak. The specific activity was found to be constant throughout the activity peak. The fractions making up the main part of the activity peak were pooled and concentrated to approximately 1 ml. Disc gel electrophoresis at pH 8.9 on 15% acrylamide gels indicated that it was about 99% pure. The specific activity was 108,000 units/mg protein. The yield was 259,000 units of protein activator with an overall yield of 19%. The amount of protein obtained from 1 kilogram

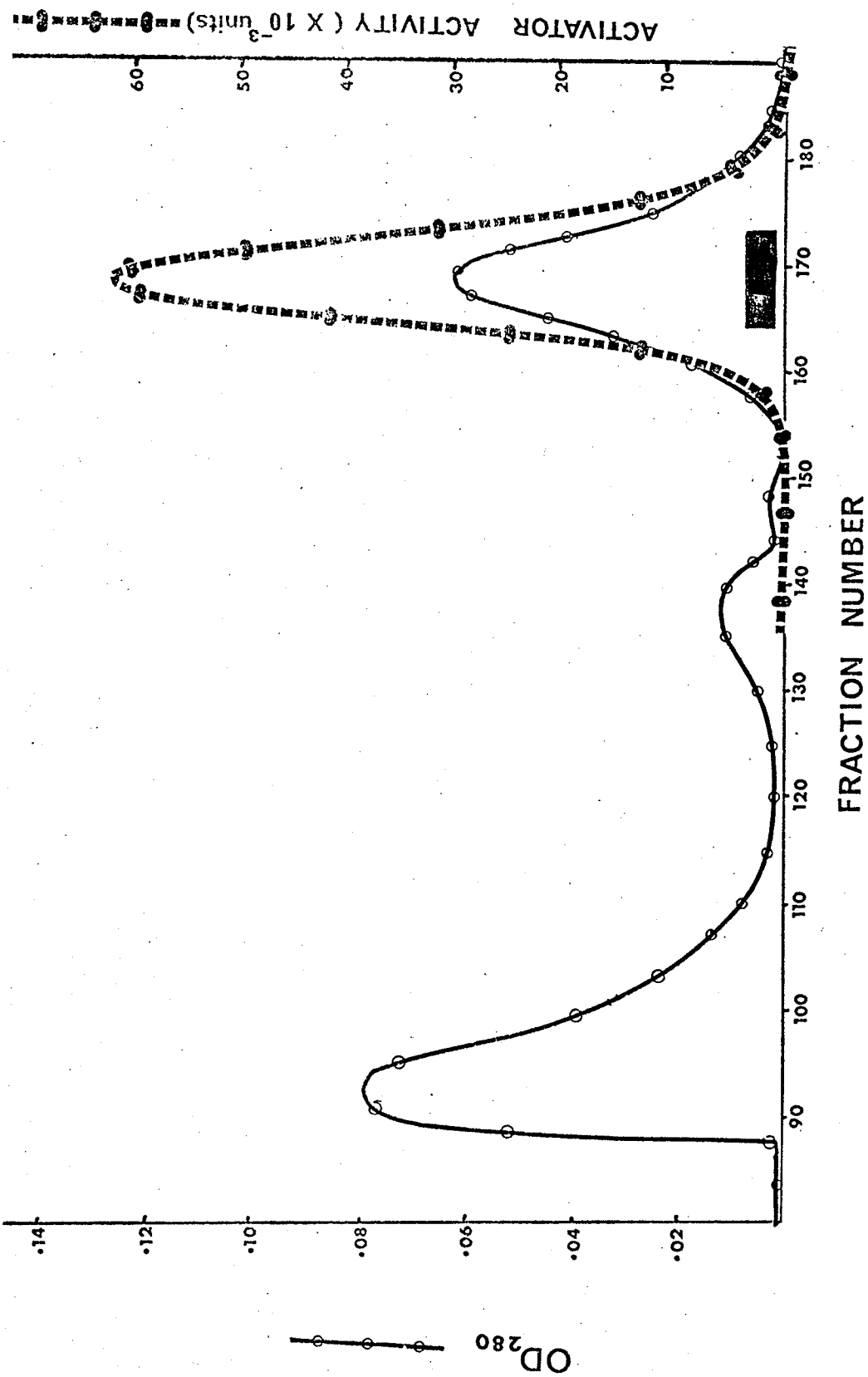


Fig.10 GEL FILTRATION OF PROTEIN ACTIVATOR ON SEPHADEX G-100 - Fractions 156 to 163 of 2nd DEAE-cellulose chromatography of the protein activator (see fig.7) were pooled, concentrated to 2 ml and then applied to 2.5 x 80 cm column of Sephadex G-100. Buffer used was 5 mM Tris-HCl pH 7.5 containing 0.1M NaCl and 0.02% azide. 2 ml fractions were collected at the rate of 10 ml/hr at 4°C.

of beef heart was 2.4 mg. In actual practice, protein activator purified to the stage of 1st DEAE-cellulose chromatography from two different batches was combined for further purification. This saved time and labor. On one occasion, 6 milligrams of protein activator eluted from the Sephadex G-100 column was obtained in an apparently homogenous state. Except for this preparation, all other protein activator samples obtained after the Sephadex G-100 stage had small amounts of impurity. The amount of impurity varied between 1% to 5% of the total protein as judged by the intensity of the amido black stain.

Attempts were made to remove the impurity by electrophoresis using a Canalco preparative disc gel instrument model PD-1. The protein activator samples so obtained were found to contain small amounts of the impurity. This method of purification was abandoned after two attempts.

Table 4 shows that the yield of protein activator was 15% to 20%.

D. Purity of Protein Activator

The protein activator preparations obtained by the methods discussed in the preceding pages were found to be homogenous in analytical disc gel electrophoresis and on SDS gel electrophoresis. Figs. 11 and 12 show that single bands were observed on analytical

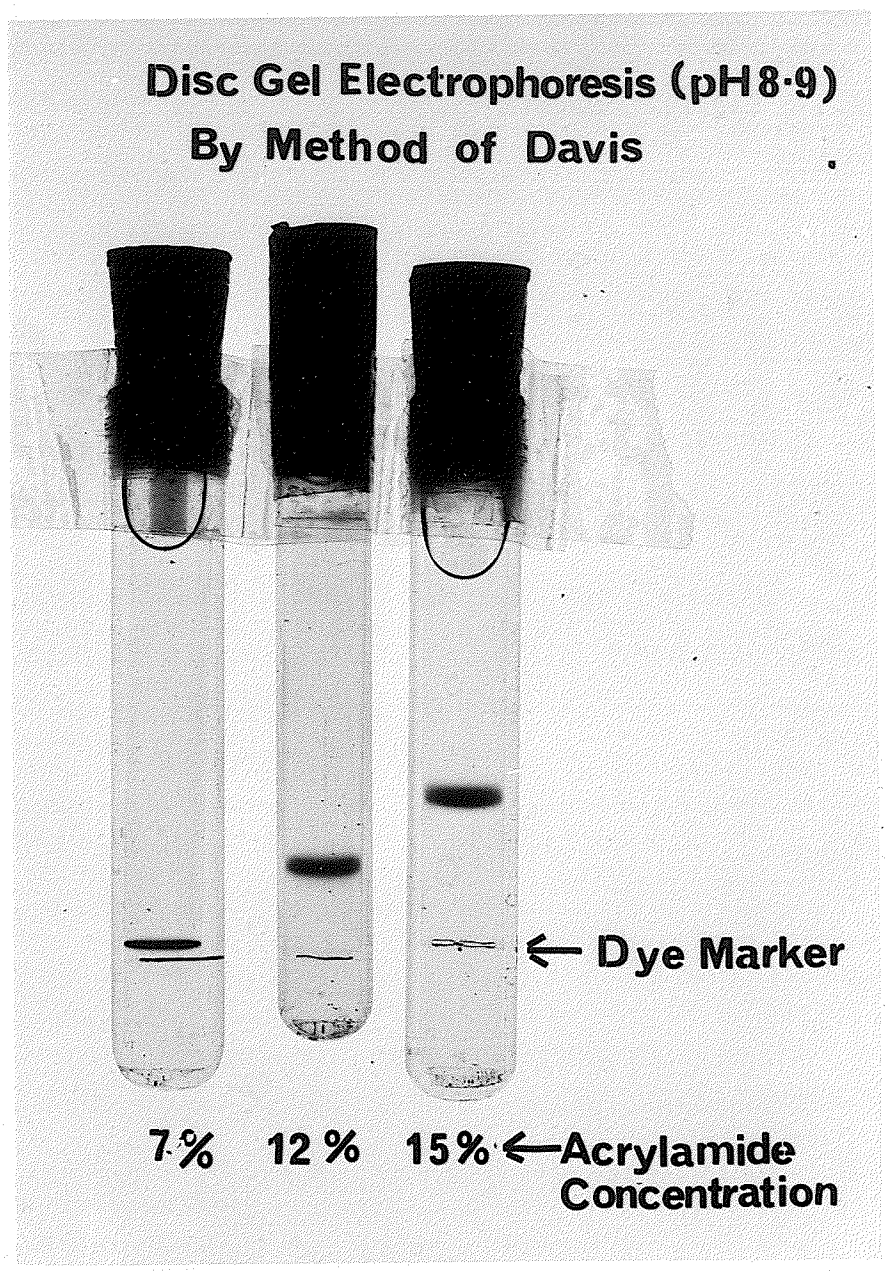


Fig.11 DISC GEL ELECTROPHORESIS AT pH 8.9 OF PROTEIN ACTIVATOR BY THE METHOD OF DAVIS (101) - Each gel was loaded with 15 to 20 µg of protein activator which had been purified to the stage of Sephadex G-100. After electrophoresis, the gels were stained with amido black

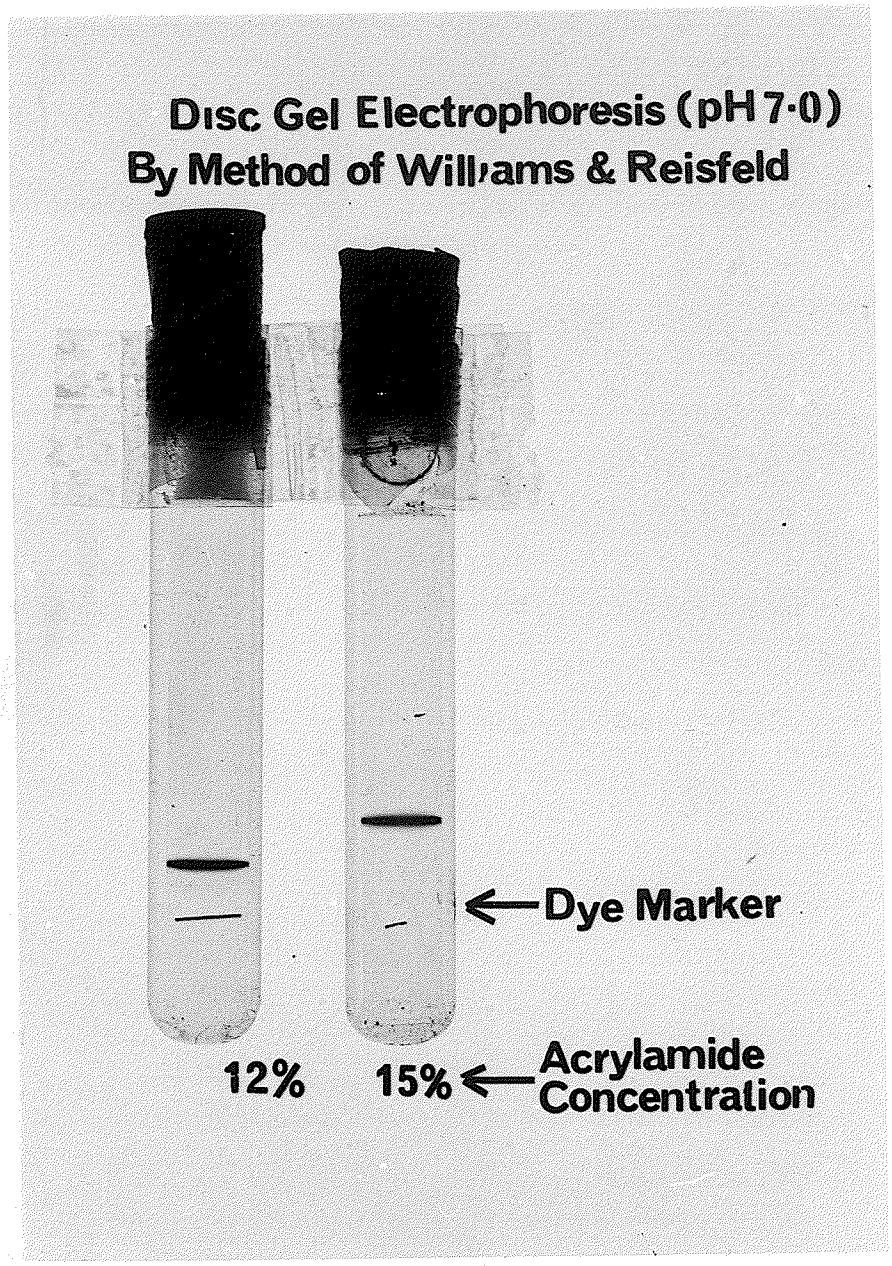


Fig.12 DISC GEL ELECTROPHORESIS OF PROTEIN ACTIVATOR AT pH 7.0 BY THE METHOD OF WILLIAMS & REISFELD (102) - Each gel was loaded with 15 µg of protein activator which had been purified to the stage of Sephadex G-100. After electrophoresis, the gels were stained with amido black.

disc gels of three different acrylamide concentrations (7%, 12% and 15%) at two different pH values (7 and 8.9). These gels were stained with amido black. A parallel gel was cut into 1 mm slices and the protein activator activity of these slices were measured. Fig. 13 shows that the activator activity profile coincided with the amido-black stained band. Fig. 14 shows also that single bands were obtained on SDS disc gels of two different acrylamide concentrations (12% and 15%). The SDS gels were stained with coomassie blue.

Purified protein activator preparations found to be homogenous on disc gels sedimented as single symmetrical peaks on ultracentrifugation at 60,000 rpm when visualized by Schlieren optics. A typical sedimentation pattern is shown in Fig. 15. These sedimentation patterns indicate that the protein activator preparations examined were free of contaminations by impurities which have molecular weights distinctly different from that of the protein activator. The method employing Schlieren optics is limited in its ability to reveal impurities which are similar in molecular weights to the protein under study.

Fig. 16 is a graphical representation of data from conventional equilibrium sedimentation determinations at a protein activator concentration of 2 mg/ml. The plot of log c versus r^2 is a straight line indicating that the protein activator sample analyzed was homogenous. (For a definition of c and r, see Appendix A).

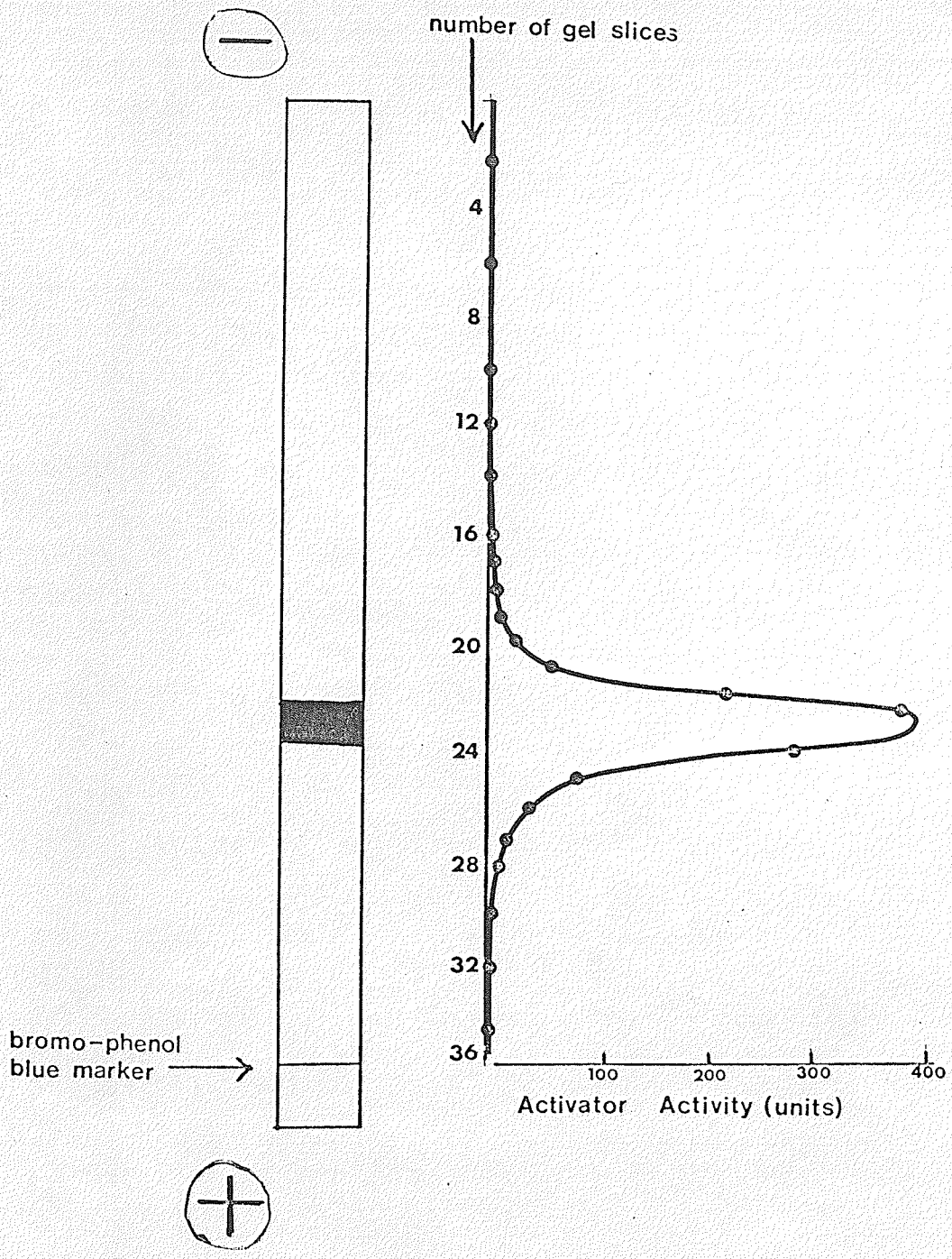


Fig.13 LOCALIZATION OF PROTEIN ACTIVATOR ON DISC GEL - 25 µg of protein activator, purified to the stage of Sephadex G-100, was subjected to electrophoresis by the method of Davis (101) on 15% acrylamide disc gel at pH 8.9. After electrophoresis, the disc gel was cut by the use of a razor blade into 1.5 mm thick slices. Each slice was immersed in 0.5 ml of 20 mM Tris-HCl buffer pH 7.5 containing 1 mM Mg^{2+}. Samples were taken for assay of protein activator activity.

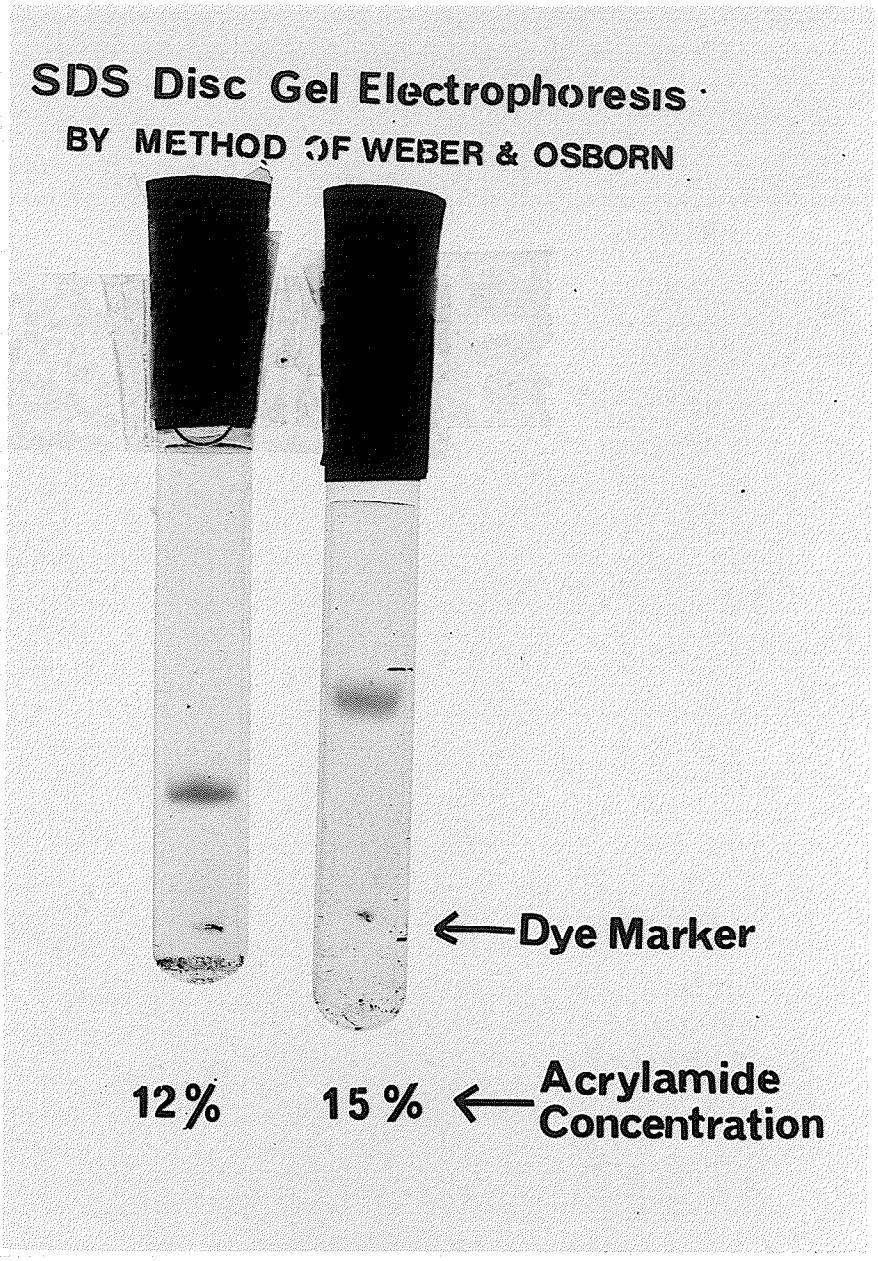


Fig.14 SDS GEL ELECTROPHORESIS OF PROTEIN ACTIVATOR BY THE METHOD OF WEBER & OSBORN (103) - Each gel was loaded with 20 µg of protein activator which had been purified to the stage of Sephadex G-100. After electrophoresis, the gels were stained with comassie blue.

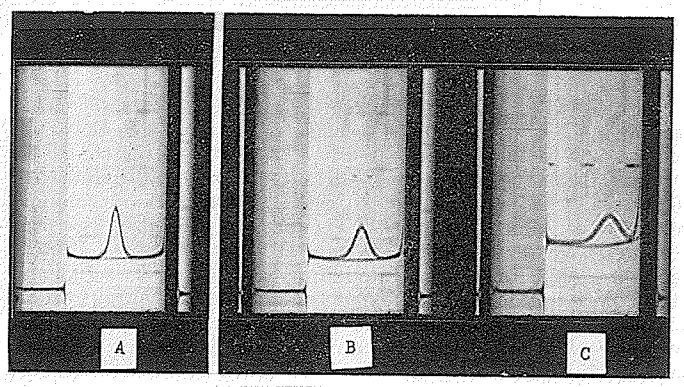


Fig.15 SEDIMENTATION PATTERNS OF PROTEIN ACTIVATOR - The protein activator used for this study had been purified to the stage of Sephadex G-100. The purified protein activator (2.2 mg/ml) in 20 mM Tris-HCl buffer pH 7.5 containing 1 mM Mg^{2+}, 1 mM imidazole and 0.1 M KCl, was centrifuged at 20°C in a synthetic boundary cell. Photographs shown were taken at 15 (A), 30 (B) and 60 (C) minutes after attainment of 60,000 rpm. The sedimentation is from left to right.

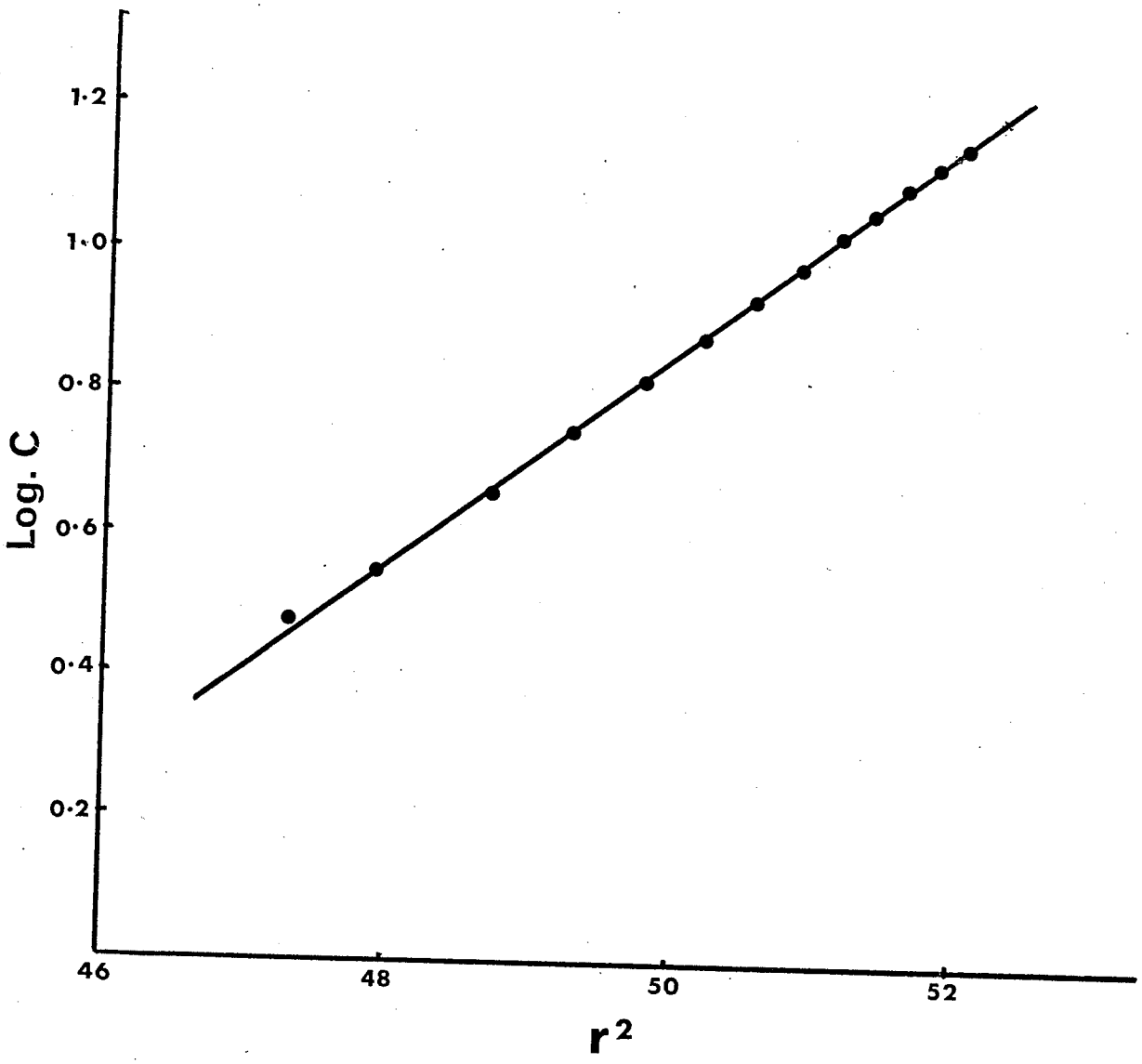


Fig.16 CONVENTIONAL SEDIMENTATION EQUILIBRIUM DETERMINATION OF THE MOLECULAR WEIGHT OF THE PURIFIED PROTEIN ACTIVATOR USING DATA OBTAINED BY INTERFERENCE OPTICS - Protein activator (2 mg/ml), which had been purified to disc gel electrophoretic homogeneity, was used for this study. The buffer used was 10 mM Tris-HCl pH 7.5 containing 0.1 M NaCl. " c " is the concentration of the protein activator in fringes at various radial distances " r " in cm. For the equation pertaining to this method see Appendix A.

Protein activator samples found to be homogenous by the above mentioned criteria had specific activity of 108,000 units per milligram of protein. The concentration of protein was determined by the method of Lowry et al. (100).

E. Structure of Protein Activator

E. 1. Molecular Weight

The approximate molecular weight of the protein activator was estimated by gel filtration on a calibrated column of Sephadex G-75. The protein activator sample used was partially purified with a specific activity of 750 units/mg. Fig. 17 shows that the protein activator behaved on Sephadex G-75 as though it had a molecular weight of 27,000.

Ultracentrifugation studies using the Beckman analytical ultracentrifuge model E were carried out using protein activator preparations which were shown to be homogenous by analytical disc gel electrophoresis. Fig. 18 shows that the sedimentation coefficient was dependent on concentration of the protein activator only to a slight extent. When extrapolated to zero protein concentration, the sedimentation coefficient (S_{20}, w) had a value of 2 Svedberg units.

The Beckman analytical ultracentrifuge Model E was used to determine the diffusion constant at three different protein activator concentrations. Diffusion constant (D_{20}, w) values of 8.73×10^{-7},

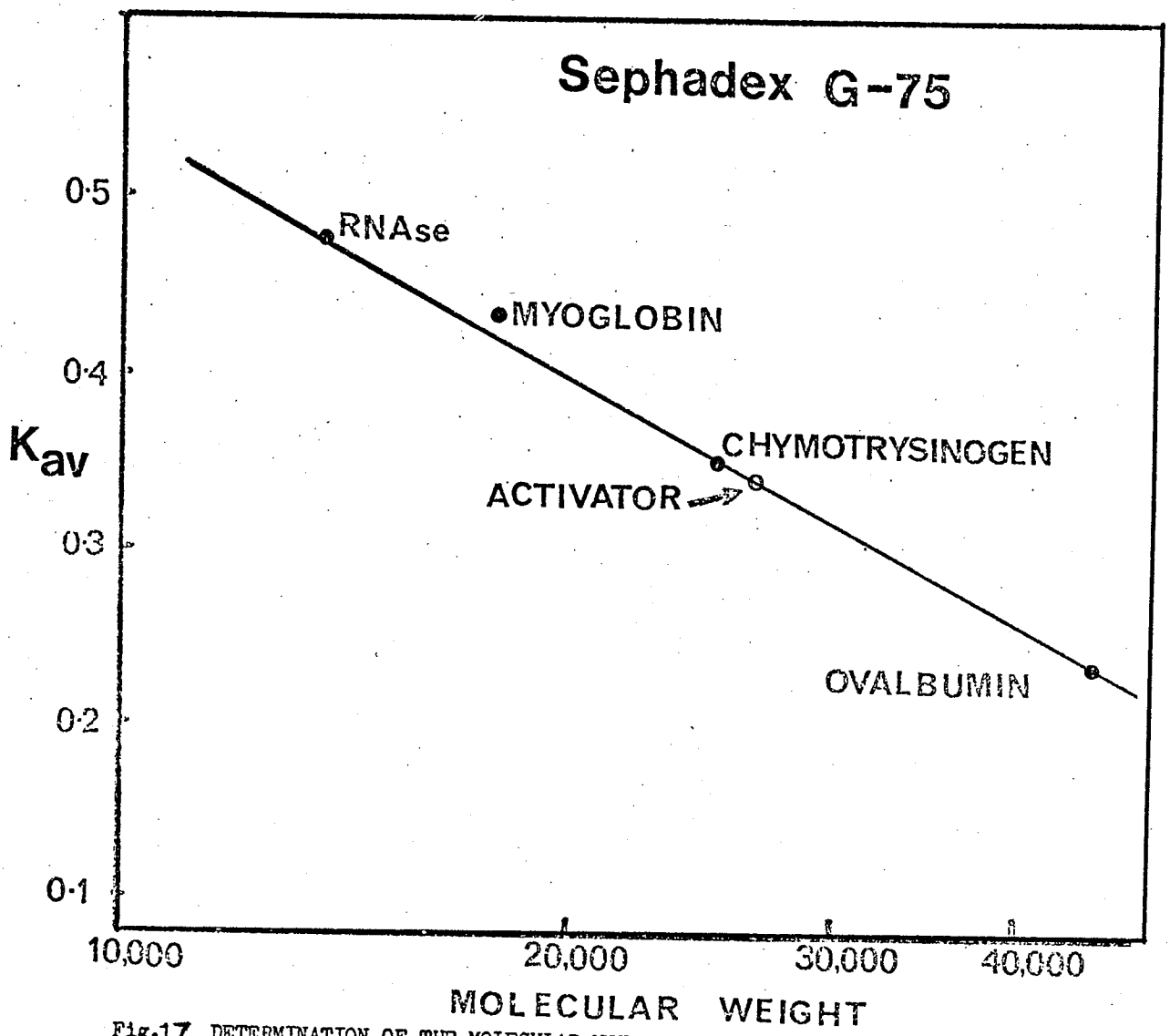


Fig.17 DETERMINATION OF THE MOLECULAR WEIGHT OF THE PROTEIN ACTIVATOR BY GEL FILTRATION ON A CALIBRATED COLUMN OF SEPHADEX G-75 - Protein activator used for this study had been purified to the stage of 1st DEAE-cellulose chromatography.

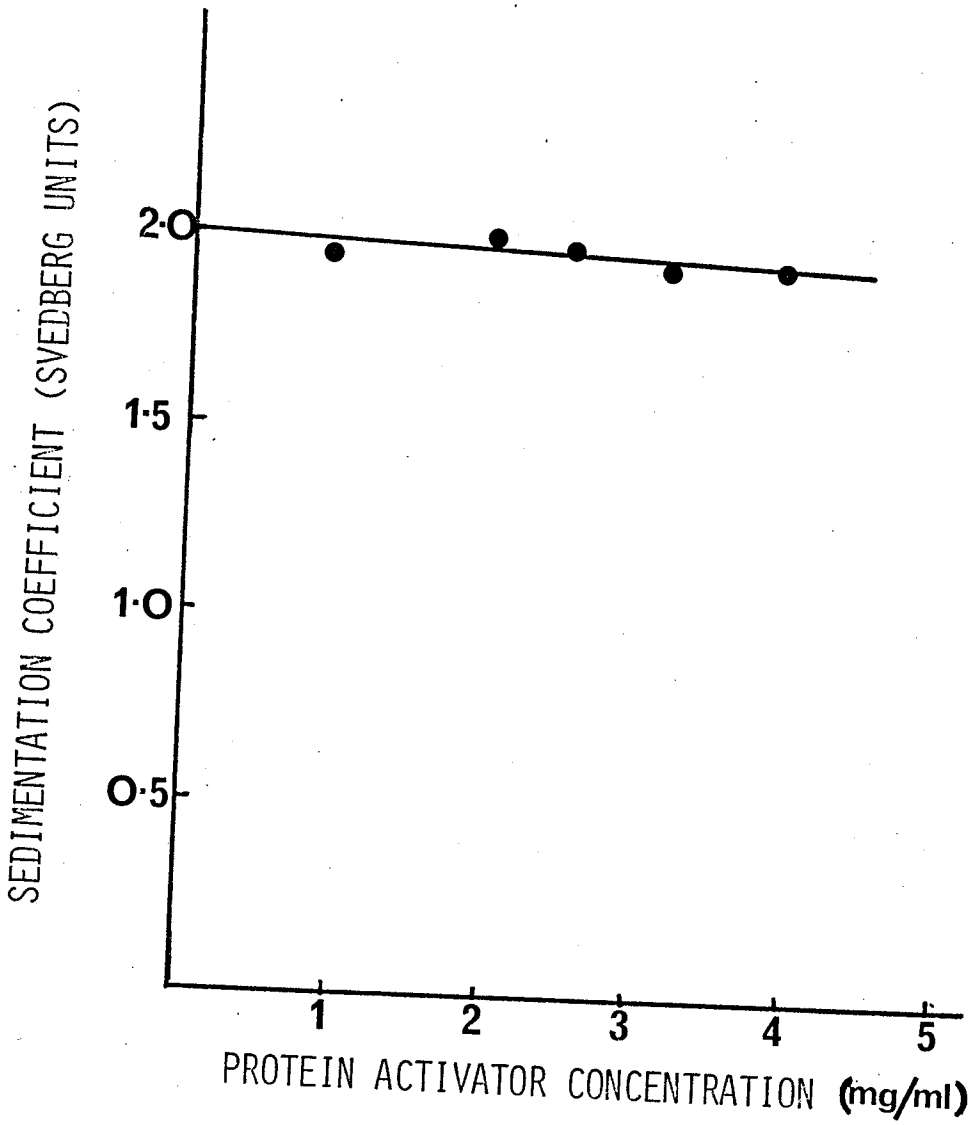


Fig. 18 CONCENTRATION DEPENDENCE OF THE SEDIMENTATION COEFFICIENT ($S_{20,w}$) OF THE PROTEIN ACTIVATOR - The protein activator used had been purified to disc gel electrophoretic homogeneity. The buffer used was 10 mM Tris-HCl pH 7.5 containing 0.1M NaCl. Other details are described in "Methods".

9.39×10^{-7} and 9.03×10^{-7} cm^2 per second were obtained at protein activator concentrations of 2.3, 3.45 and 4.6 mg/ml. Since the $D_{20,w}$ values were largely independent of protein activator concentration, a simple average (9.0×10^{-7} cm^2 per second) of the three experimentally determined values was taken. The partial specific volume ($\bar{v}$) of the protein activator was calculated from the amino acid composition (108). The molecular weight calculated from $D_{20,w}$, $S_{20,w}$ and ($\bar{v}$) was found to be 19,000.

The molecular weight of the protein activator was also determined by the method of conventional equilibrium sedimentation. The molecular weight, determined by this method, was found to be 14,800 and 15,200 determined on protein activator samples with concentrations of 1.2 mg/ml and 2 mg/ml. The average value of the molecular weight by this method was 15,000.

The subunit weight of the protein activator was determined by the method of Weber and Osborn (103). Fig. 19 shows that the subunit weight was numerically similar to the molecular weight as determined by ultracentrifugation (18,000 to 19,000).

E.2. Isoelectric Point

Partially purified protein activator with a specific activity of 60,000 units/mg protein was subjected to isoelectric focusing in a sucrose density gradient at 4 ^{o}C using an LKB isoelectric focusing

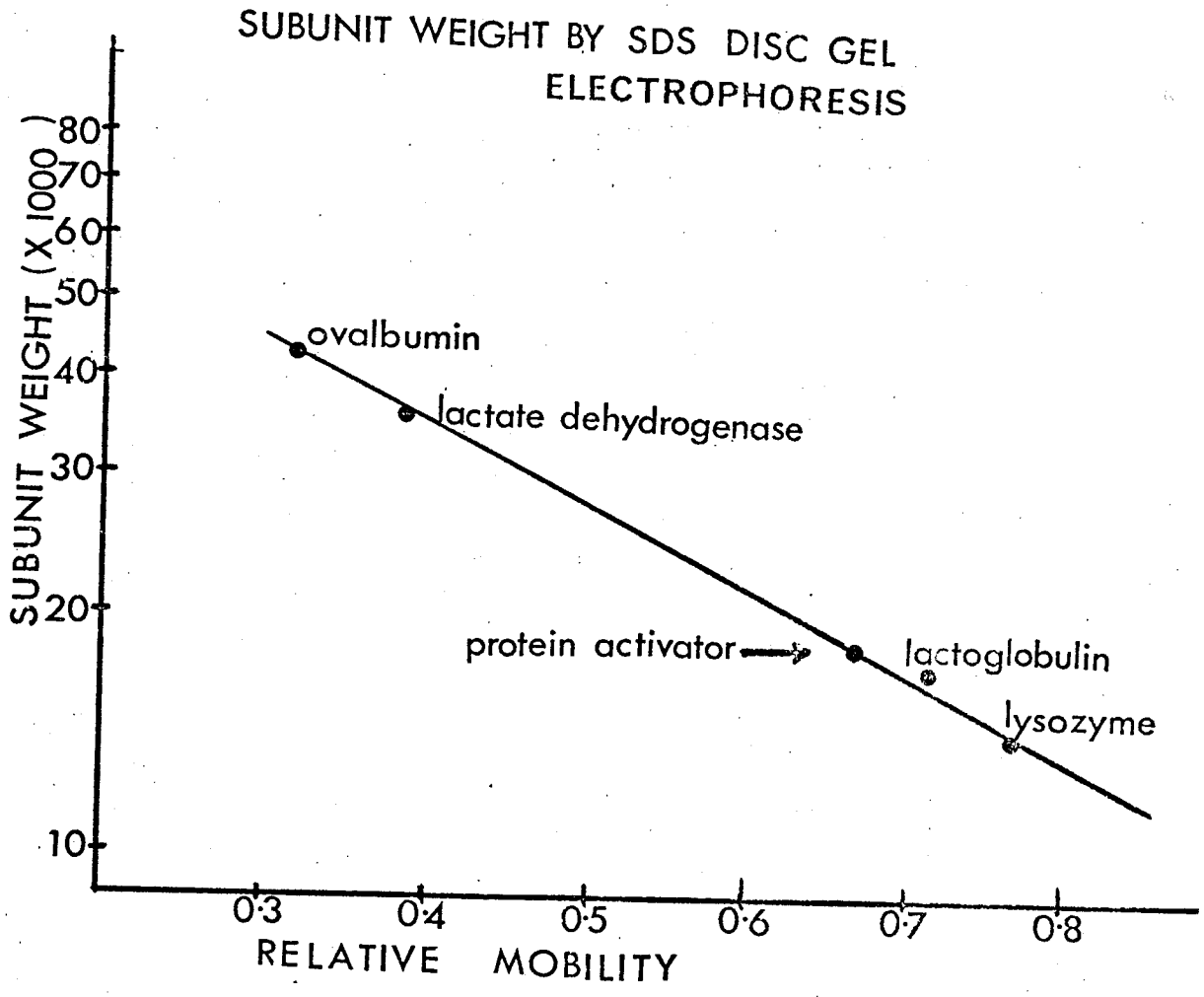


Fig.19 DETERMINATION OF THE SUBUNIT WEIGHT OF THE PROTEIN ACTIVATOR BY THE SDS GEL ELECTROPHORESIS METHOD OF WEBER &OSBORN (94) - Protein activator which had been purified to disc gel electrophoretic homogeneity was used for this study. SDS gel electrophoresis was carried out on 10% acrylamide SDS gel with acrylamide/bis ratio of 37. Other details are described in "Methods".

instrument model 8101. Fig. 20 shows that the protein activator possessed an isoelectric point of 4.0.

E.3. Periodic-Acid Schiff Stain

Disc gel electrophoresis of protein activator on 15% acrylamide gels at pH 8.9 was carried out according to the method of Davis (101). The gels were stained for carbohydrate by the method of Kapitany and Zebrowski (104). The protein activator showed no positive staining by this method, even when the gels were overloaded. Parallel gels run with ovalbumin showed positive bands stained by this method. According to Kapitany and Zebrowski, the method was sensitive to glycoprotein with carbohydrate content as low as 2 to 3 ng of bound carbohydrate.

E.4. UV Absorption Spectra

The UV absorption spectrum of the protein activator purified to disc gel electrophoretic homogeneity is shown in Fig. 21. The protein concentration as determined by the Lowry method was 3.47 mg/ml and the pH was 7.5. At this pH, the UV absorption spectrum shows a broad absorption band between 250 and 290 nm with peaks at 255, 258, 267, 271 and 278 nm. The absorbance at 260 nm, compared to that at 280 nm, is rather high, giving a A260/A280 ratio of 0.87. Other well characterized Ca^{2+}-binding proteins have been reported to exhibit similar UV absorption spectra. Porcine intestinal Ca^{2+}-binding

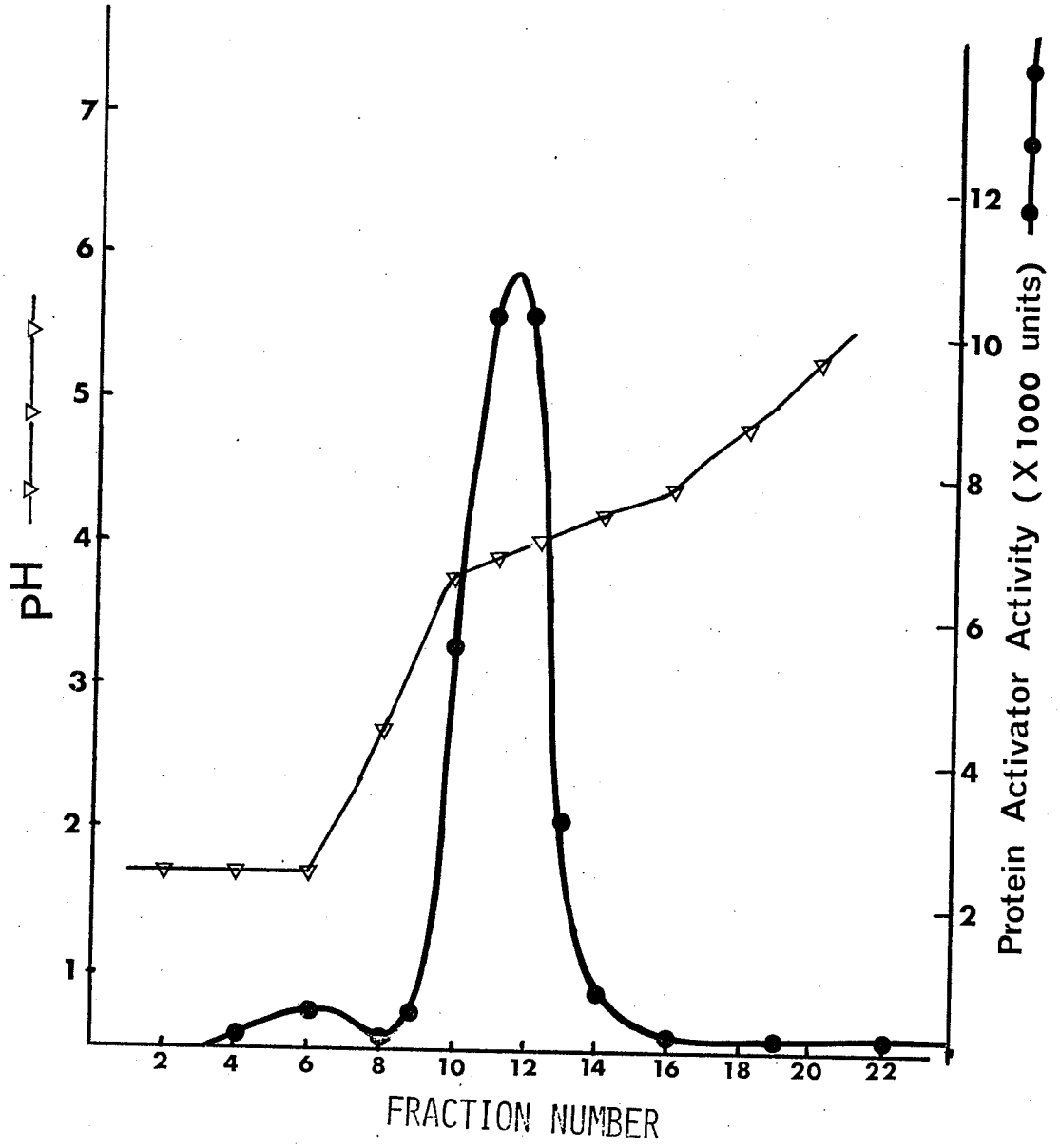


Fig.20 DETERMINATION OF THE ISOELECTRIC POINT OF THE PROTEIN ACTIVATOR BY ISOELECTRIC FOCUSING ON A SUCROSE GRADIENT. Details are described in "Methods"

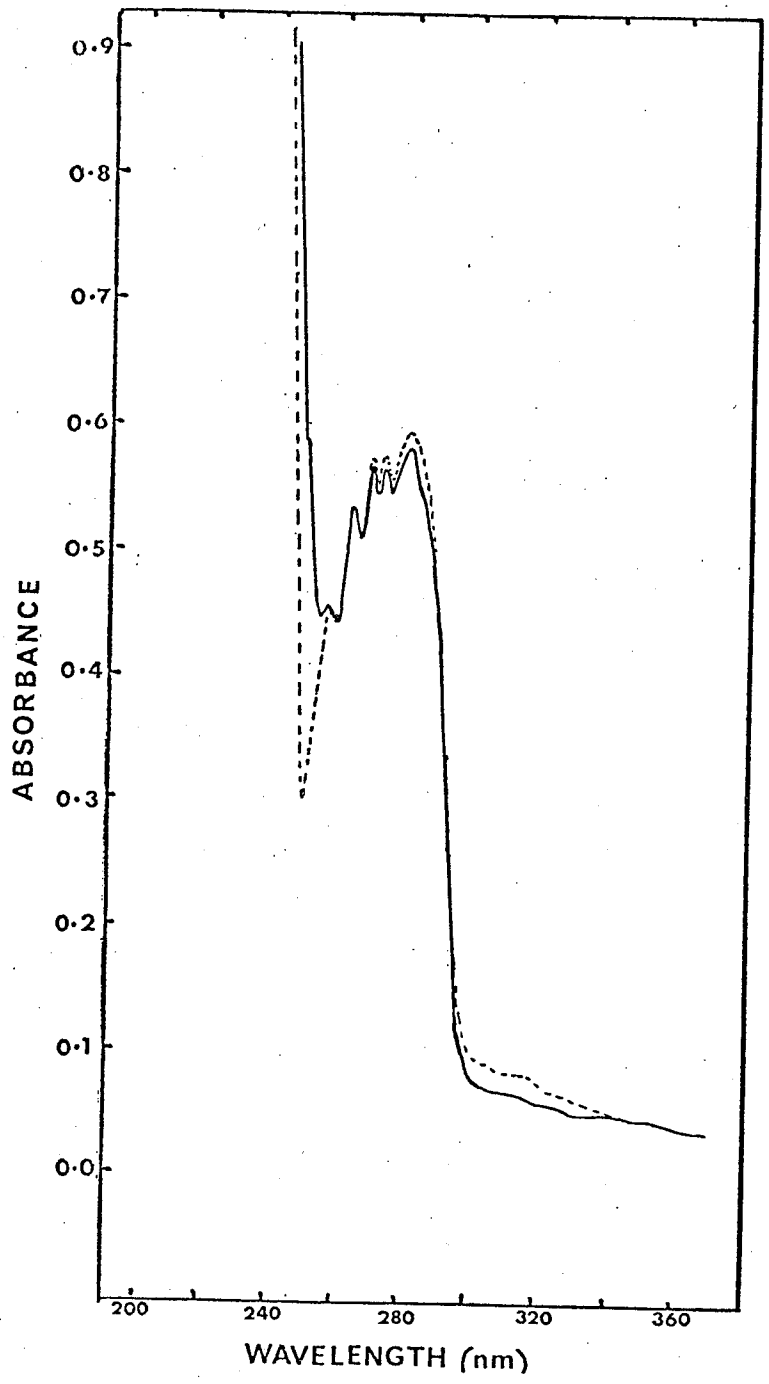


Fig.21 UV ABSORPTION SPECTRA OF PROTEIN ACTIVATOR - Pure protein activator (3.46 mg/ml) was used. The buffer used was 5 mM Tris-HCl pH 7.5. The solid line is the absorption spectrum in buffer containing 0.1 mM Ca^{2+}. The dashed line is the absorption spectrum of the same protein activator solution containing 0.2 mM EGTA and 0.1 mM Ca^{2+}.

protein (109) and the rabbit skeletal muscle troponin TN-C (110, 111) both possess similar UV absorption spectra with A260/A280 ratio of 1.0.

Ca^{2+} seems to have an effect on the UV absorption spectra of the protein activator. Fig. 21 shows the UV absorption spectra at pH 7.5 in the presence of 0.15 mM Ca^{2+} and 0.15 mM EGTA. Ca^{2+} decreased absorption between 270 and 280 nm (to the extent of 2% at 280 nm) and between 295 and 340 nm (to the extent of 26% at 340 nm). The same concentration of Ca^{2+} was observed to increase absorption between 243 and 257 nm with a maximum increase at 248 nm of 30%. Since only one spectral measurement was made, it is not possible to say if the perturbations in UV absorption spectra were reproducible.

Dorrington et al. (109) reported that low concentrations of Ca^{2+} induced similar changes in the UV absorption spectrum of the porcine intestinal calcium-binding protein. The perturbations in the UV absorption spectrum of the protein activator are of a magnitude similar to that of the porcine calcium binding protein (109). In the latter case, the effect of Ca^{2+} was attributed to conformational change arising from binding of Ca^{2+}.

Fig. 22 shows the UV absorption spectrum of the protein activator in 0.1 M NaOH at a protein concentration of 1 mg/ml. In the presence of 0.1 M NaOH the functional groups on the constituent amino acids of the protein activator were fully ionized giving rise to two

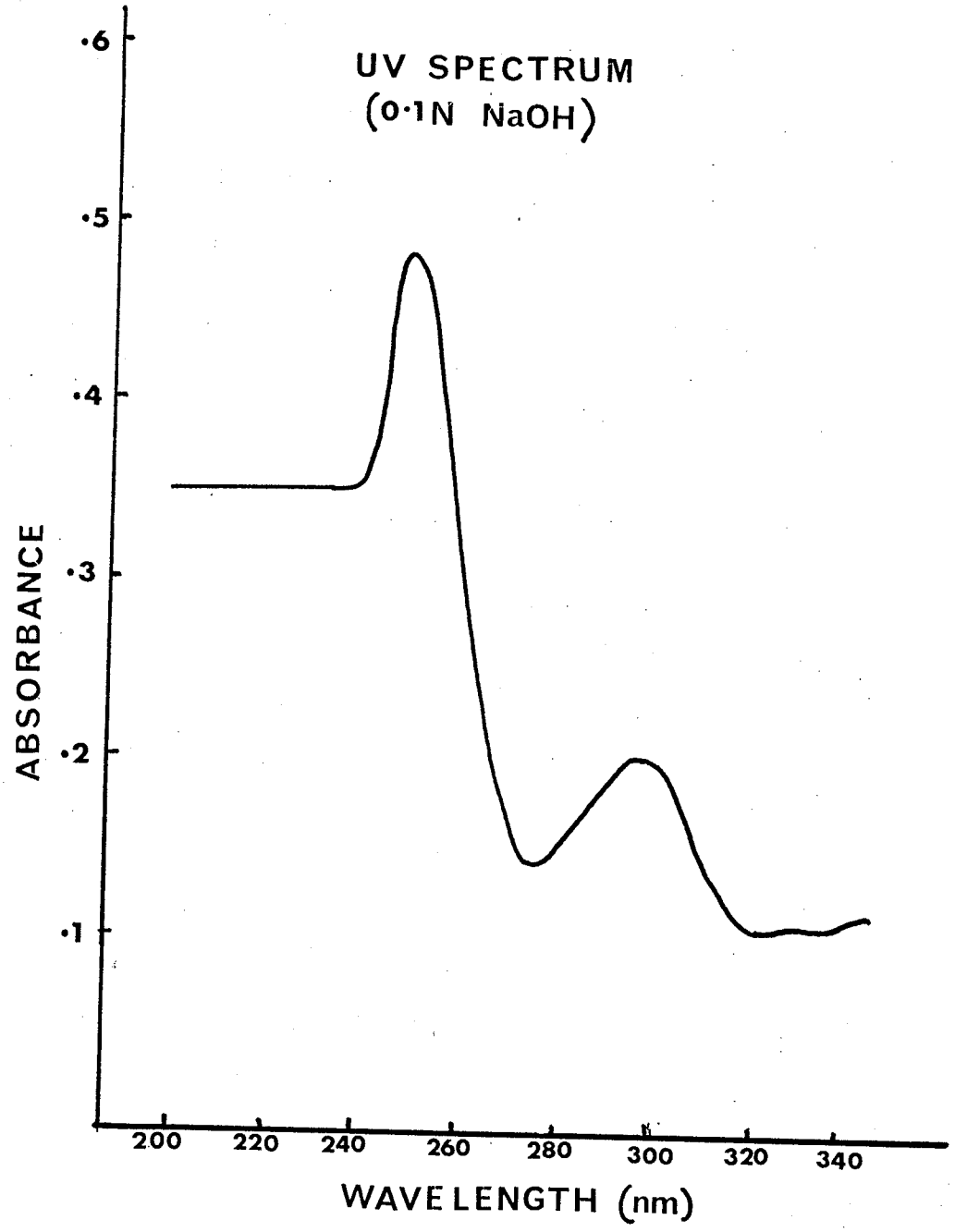


Fig.22 UV ABSORPTION SPECTRUM OF THE PURIFIED PROTEIN ACTIVATOR IN 0.1M NaOH. Protein activator (1mg/ml) which had been purified to disc gel electrophoretic homogeneity was used for this study. Other details are described in "Methods".

absorption peaks; one due to phenylalanine at 249 nm and a smaller peak to tyrosine at 297 nm. The greater size of the 249 nm peak indicates that phenylalanine is the predominant aromatic amino acid.

E. 5. Fluorescence Spectra

Amino acid analysis (Table 5) revealed that the protein activator molecule possessed 7 phenylalanine and 2 tyrosine residues. Typtophan residues were absent.

Excitation of the tyrosine residues of pure protein activator (buffered at pH 7. 1) at 278 nm resulted in a major fluorescence emission peak at 325 nm and a minor emission peak at 635 nm (Fig. 23). Excitation at 255 and 267 nm also resulted in identical emission spectra although at lower quantum yields. This indicates that photon energy absorbed by phenylalanine residues was transferred to the tyrosine residues before it was emitted as fluorescence energy.

The fluorescence of the protein activator at pH 7. 1 was found to be affected by low concentrations of Ca^{2+}. Fig. 23 shows the fluorescence emission spectrum of the protein activator in 5 mM Tris-HCl pH 7. 5 buffer containing 0. 1 mM EGTA. Addition of 0. 2 mM Ca^{2+} increased the emission intensity at 325 nm by 46%. Addition of excess EGTA (0. 8 nM) reversed the effect of Ca^{2+}. Fig. 24 shows that very low concentrations of Ca^{2+} were required to give maximum enhancement of fluorescence. 0. 1 mM EGTA decreased the emission

Table 5

AMINO ACID COMPOSITION OF PROTEIN ACTIVATOR [a] FROM BEEF HEART

Expressed as molar ratio with proline equal to 2

	24 Hours Hydrolysis	48 Hours Hydrolysis	72 Hours Hydrolysis	Corrected Molar Ratio [d]	Nearest Whole Number
Lysine	6.80	7.01	6.67	6.82	7
Histidine	0.98	1.11	0.86	1.17	1
Arginine	4.94	4.90	4.82	5.00	5
Aspartate	19.82	19.86	19.53	19.84	20
Threonine	8.61	7.66	6.75	9.57	10
Serine	2.41	1.82	1.38	2.97	3
Glutamate	23.77	23.73	23.11	23.75	24
Proline	1.89	1.75	1.64	2.00	2
Glycine	9.46	9.45	9.01	9.48	9 or 10
Alanine	9.31	9.34	9.04	9.34	9
Half-Cystine [b]	0.00	0.00	0.00	0.00	0
Valine	6.94	7.06	6.66	7.00	7
Methionine	7.24	6.91	7.08	7.30	7
Isoleucine	6.58	6.30	6.17	6.51	6 or 7
Leucine	7.75	7.70	7.56	7.84	8
Tyrosine	1.80	1.64	1.59	1.96	2
Phenylalanine	6.80	6.81	6.60	7.07	7
Tryptophan [c]	0.00	0.00	0.00	0.00	0

a Protein activator, which had been purified to disc gel electrophoretic homogeneity, was used for amino acid analysis

b Protein activator was reduced with dithiothreitol and alkylated with iodoacetate prior to acid hydrolysis.

c The tryptophan content of the protein activator was seperatedly determined by the spectrophotometric method of Goodwin & Morton (134) (and see fig.22) prior to acid hydrolysis.

d The figures for unstable amino acids were obtained by extrapolation to zero time of hydrolysis.

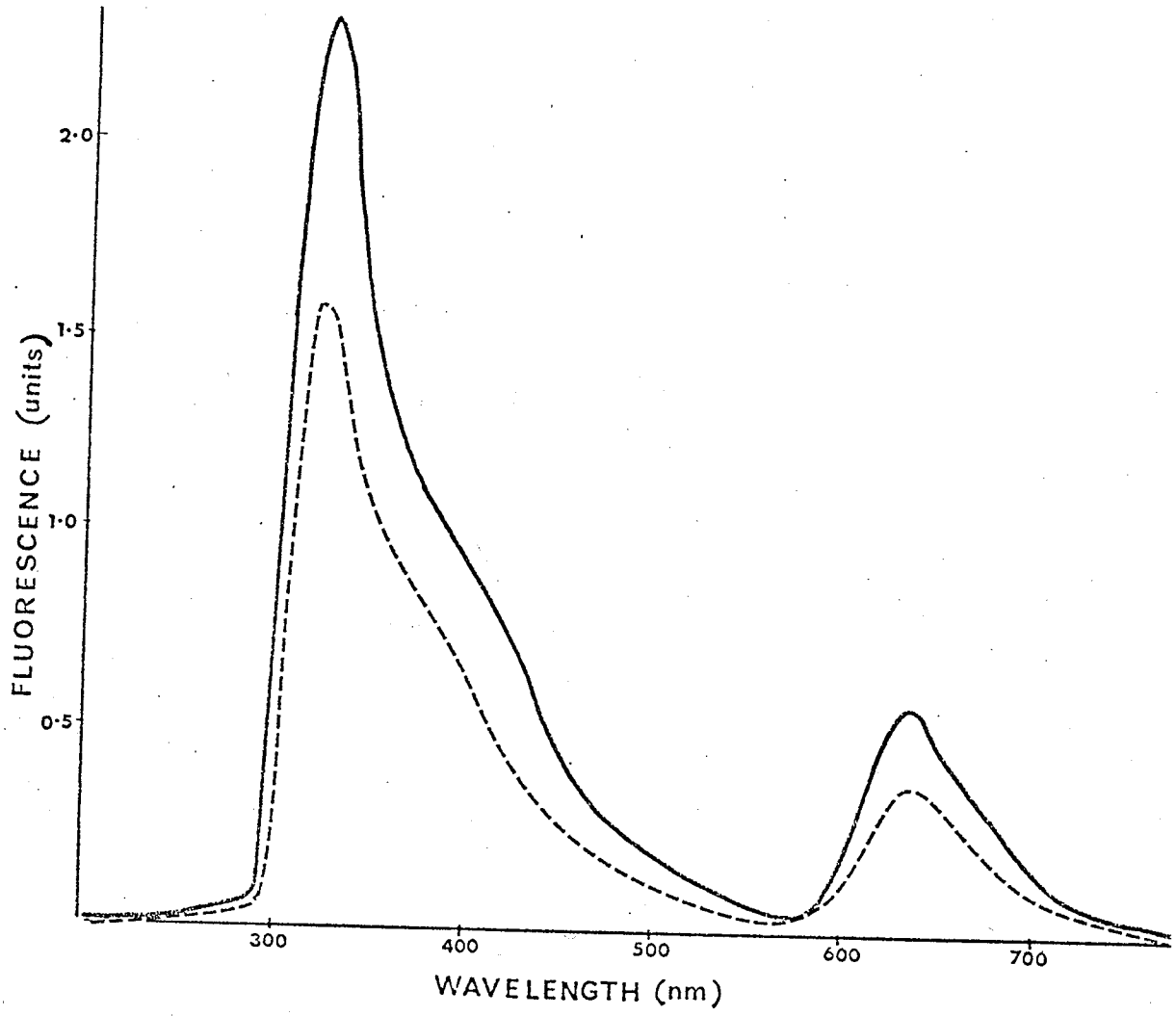


Fig.23 EMISSION FLUORESCENCE SPECTRA OF PURIFIED PROTEIN ACTIVATOR IN THE PRESENCE OF EITHER Ca^{2+} OR EGTA. - Protein activator (0.1 mg/ml), which had been purified to disc gel electrophoretic homogeneity, was used for this study. Excitation was at 278 nm. The solid line is the emission fluorescence spectrun in the presence of 20 μM $CaCl_2$. The dashed line is the emission fluorescence spectrum of the same solution of protein activator in the presence of 20 μM $CaCl_2$ plus 300 μM EGTA.

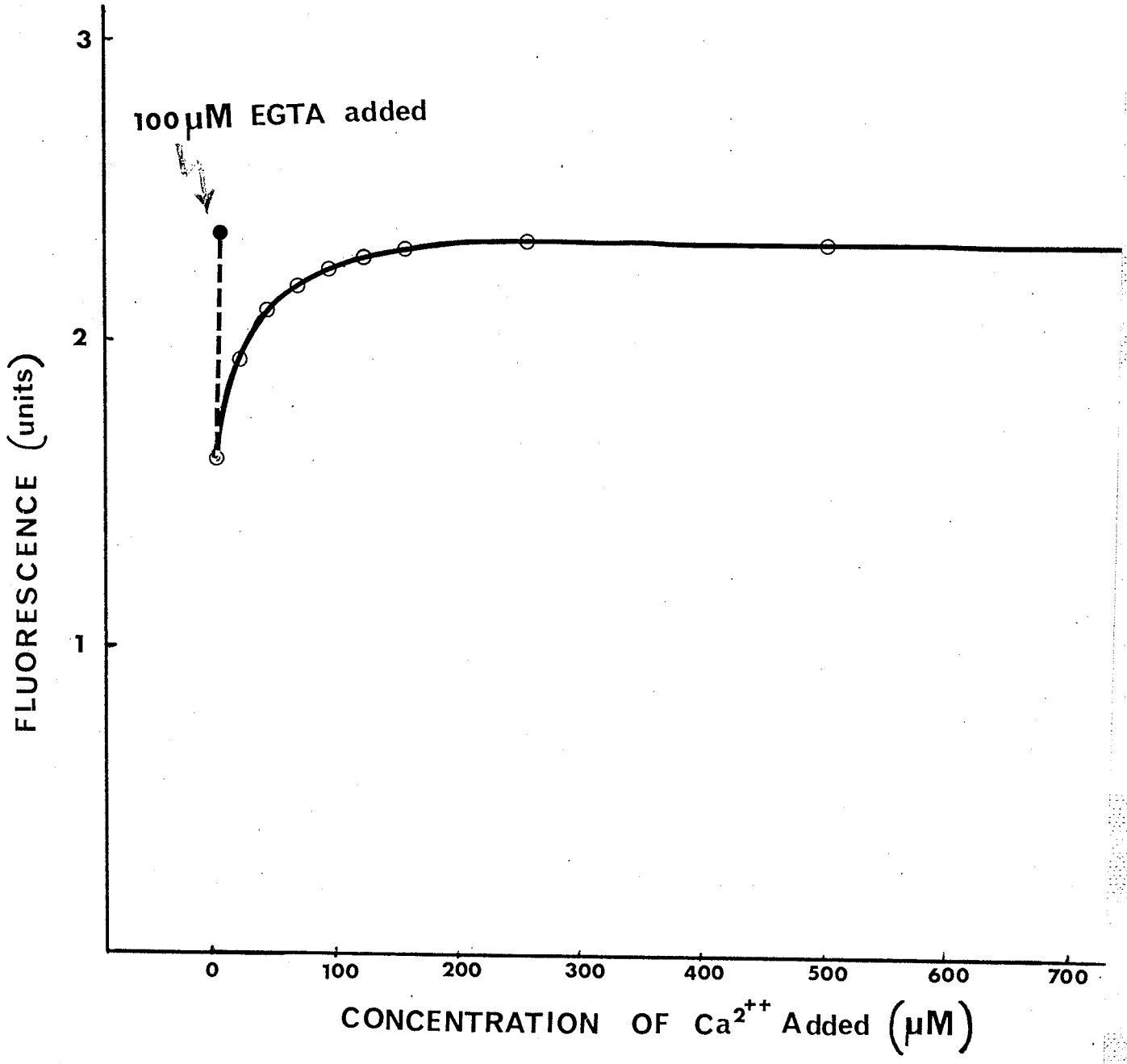


Fig.24 QUENCHING OF FLUORESCENCE OF PROTEIN ACTIVATOR BY THE ADDITION OF EGTA AND TITRATION OF THE FLUORESCENCE BY THE ADDITION OF Ca^{2+} - Protein activator (0.1 mg/ml), which had been purified to disc gel electrophoretic homogeneity, was used. The buffer used was 5 mM Tris-HCl pH 7.1. Excitation was at 278 nm and the emission fluorescence at 325 nm was recorded. As indicated in the figure, the emission fluorescence was recorded before and after addition of 100 uM EGTA. The fluorescence was then titrated with Ca^{2+}. The initial volume of the protein activator solution was 1.8 ml and each addition of $CaCl_2$ was 3 µL.

intensity at 325 nm by 31%. The fluorescence at 325 nm was titrated with Ca^{2+}. Half maximal and 90% enhancement of fluorescence intensity were attained by adding 24 and 126 μm Ca^{2+}, respectively. Mg^{2+} at the same concentration did not mimic the effects of Ca^{2+}.

Van Eerd and Kawasaki (111) have reported that a similar enhancement of the tyrosyl fluorescence of rabbit skeletal muscle troponin TN-C occurred upon binding Ca^{2+} at its high affinity Ca^{2+} binding site. Carboxylic groups in protein molecules have been demonstrated to quench the tyrosyl fluorescence if these carboxylic groups are bonded to or located near the tyrosyl side chain (112). If this quenching mechanism is operating in the case of troponin TN-C and the protein activator, the Ca^{2+} induced fluorescence enhancement could be explained by the removal of carboxylic groups from the vicinity of the tyrosyl side chains as a consequence of the postulated conformational change arising from binding Ca^{2+}.

E. 6. Heat Stability of the Protein Activator

The stability of the partially purified protein activator at different temperatures was investigated. The activity of the protein activator samples was measured after incubation in 10 mM Tris-HCl pH 7. 5 buffer containing 1 mM Mg^{2+} for various time periods at 0^{o}, 30^{o}, 55^{o}, 75^{o} and 95^{o}, and the results are shown in Figs. 25A, 25B and 25C. All three figures are different graphical representations of

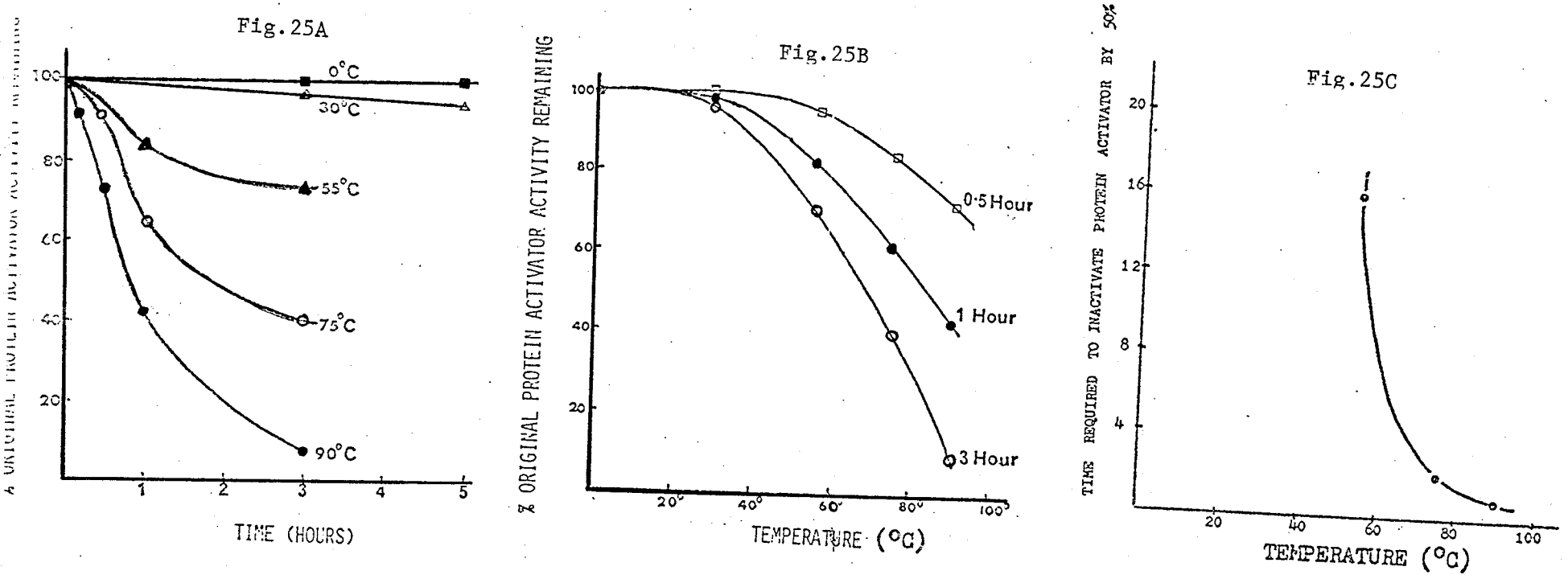


Fig.25 HEAT STABILITY OF PROTEIN ACTIVATOR - Protein activator purified to the stage of 1st DEAE-cellulose chromatography was used in this study. The protein activator samples, in 20 mM Tris-HCl pH 7.5 buffer containing 1 mM Mg^{2+}, were incubated at various temperatures for the indicated time intervals and their protein activator activity was then assayed by plotting PDE-I enzyme activity - protein activator titration curves for each individual sample. The same data was used for fig. 25A, 25B & 25C. Neither Ca^{2+} or EGTA was added to the incubation mixtures.

the same data. These results indicate that the protein activator is remarkably heat stable. Incubation at 95 °C for 10 minutes and 30 minutes caused a loss of only 9% and 28% of the original activity, respectively. Ca^{2+} was found to have a dramatic effect on the heat stability of the protein activator. When 0.1 mM Ca^{2+} was included in the buffer, the protein activator lost only 30% of its activity when heated at 95 °C for 30 minutes at pH 7.5. However, when 0.1 mM EGTA was included in the buffer, the protein activator lost 70% of its activity when heated in the same manner. Thus low concentrations of Ca^{2+} appear to protect the protein activator from heat inactivation.

The role of metal ions in stabilizing other proteins have been well established (113).

The experiments on heat stability were carried out in collaboration with Dr. Honor Ho.

E.7. Digestion of Protein Activator with Trypsin*

Fig. 26 shows that low concentrations of Ca^{2+} (10 μM) protected the protein activator from loss of activity arising from brief digestion by trypsin. Digestion of a sample of protein activator with trypsin for 20 minutes in the presence of 10 μM Ca^{2+} had very slight effect on the activity of the protein activator. However, digestion of a similar

*The experiments on trypsin digestion were carried out in collaboration with Dr. Honor Ho.

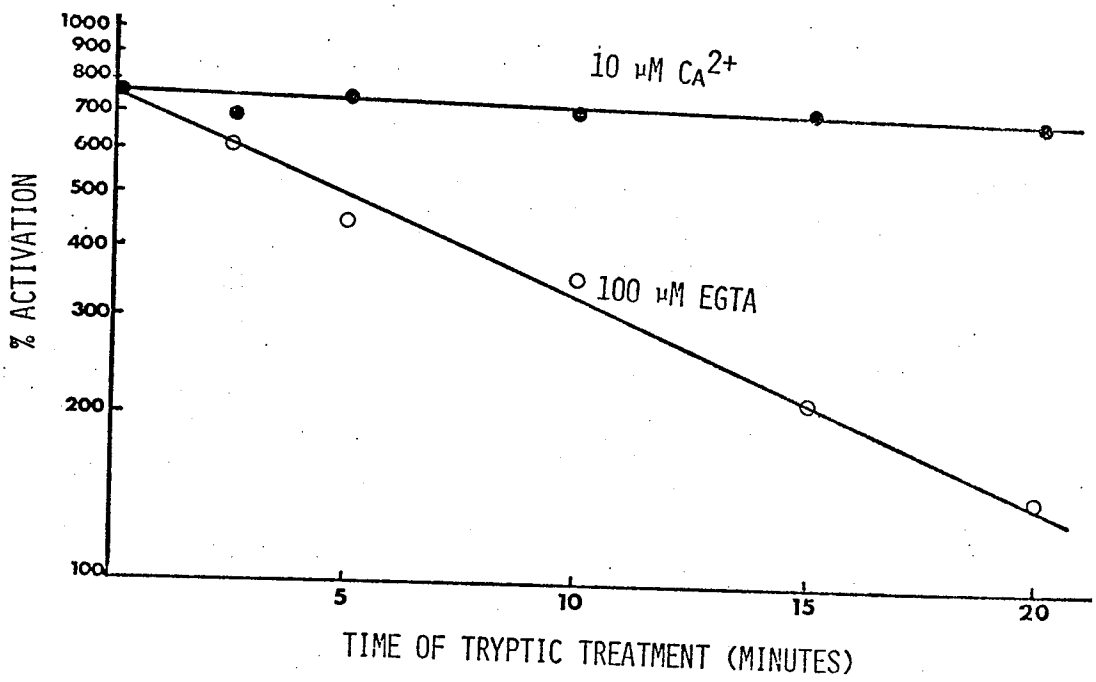


Fig.26 LOW CONCENTRATION OF Ca^{2+} PROTECTED PROTEIN ACTIVATOR FROM DIGESTION BY TRYPSIN - Protein activator (3 ug/ml), which had been purified to disc gel electrophoretic homogeneity, was incubated with 0.12 μg/ml trypsin in 20 mM Tris-HCl buffer pH 7.5 containing 1 mM Mg^{2+} and either 10 μM Ca^{2+} or 100 μM EGTA at 30°C for different time periods. The tryptic digestion was terminated by additions of ovamucoid as trypsin inhibitor. The protein activator samples were then assayed for its protein activator activity by measuring its activation of PDE-I enzyme under standard conditions. The data shown in this figure were from an experimant carried out in collaboration with Dr. Honor Ho.

sample of protein activator with trypsin in the same manner in the presence of 100 μM EGTA resulted in a much greater loss of protein activator activity. It must be pointed out that 10 μM Ca^{2+} has no significant effect on the activity of trypsin. The results of Fig. 26 can be explained by postulating that Ca^{2+}, on binding to the protein activator, induced it to assume a conformation such that less of its trypsin sensitive peptide bonds are exposed.

E. 8. Amino Acid Composition

Protein activator purified to homogeneity was reduced with dithiothreitol and alkylated with iodoacetate as described in "Methods." It was then hydrolyzed by 6N HCl at 110 °C for 24, 48 and 72 hours and the compositions of amino acids in the hydrolysates were determined by the use of a Beckman amino acid analyzer. The results are shown in Table 5 (see page 98).

Cysteine and carboxyl methyl cysteine were not detected in the acid hydrolysates.

The UV absorption spectrum of the protein activator in 0.1 N NaOH is shown in Fig. 22 (see page 96). Calculation of the content of tryptophan by the spectrophotometric method of Goodwin and Morton (114) using the data of Fig. 22 revealed that tryptophan is absent and that the absorption peak at 296 nm in Fig. 22 was due entirely to tyrosine. Table 5 shows that each molecule of protein activator possesses 7 phenylalanine and 2 tyrosine residues and this ratio of

the two aromatic amino acids is reflected in the UV absorption spectrum of Fig. 22.

A few other outstanding features of the amino acid composition of the protein activator are enumerated below.

1. Glutamic acid/glutamine and aspartic acid/asparagine residues together make up 1/3 of the total amino acid residues. The ratio of acidic to basic amino acid is 3.4. This high proportion of acidic amino acid residues account for the observed low isoelectric point (pI = 4) of the protein activator.

2. There are only two proline residues per molecule of protein activator.

Table 6 shows a list of the physical parameters of the protein activator.

F. Activating Properties of Protein Activator

F. 1. Hysteretic Activation of PDE-I

In a study of the interaction between cAMP phosphodiesterase (PDE-I) and its protein activator, it was observed that enzyme reactions often followed the course of non-linear lines. The characteristics of these progress curves were dependent on many factors. When an enzyme reaction (using PDE-I) was carried out in the presence of a saturating concentration of the protein activator, the rate of product formation was constant over 40 minutes (Fig. 27, curve A). At a

Table 6

PHYSICAL PARAMETERS OF PROTEIN ACTIVATOR FROM BEEF HEART

SEDIMENTATION COEFFICIENT ($S_{20,w}$)	2.0 S
DIFFUSION COEFFICIENT ($D_{20,w}$)	9.05×10^{-7} cm^2/sec
MOLECULAR WEIGHT (MW_g) by gel filtration	27,000
MOLECULAR WEIGHT (MW_{sd}) sedimentation/diffusion method calculated from $S_{20,w}$ & $D_{20,w}$	19,000
MOLECULAR WEIGHT (MW_{se}) sedimentation equilibrium method	15,000
SUBUNIT WEIGHT by SDS gel electrophoresis	18,000 to 19,000
FRICTIONAL RATIO ($f:f_o$) calculated from MW_{sd} & $D_{20,w}$	1.35
ISOELECTRIC POINT (pI)	4.0
PARTIAL SPECIFIC VOLUME ($\bar{\nu}$)	0.72
A_{280}/mg protein	0.169
A_{280}/A_{260}	0.78

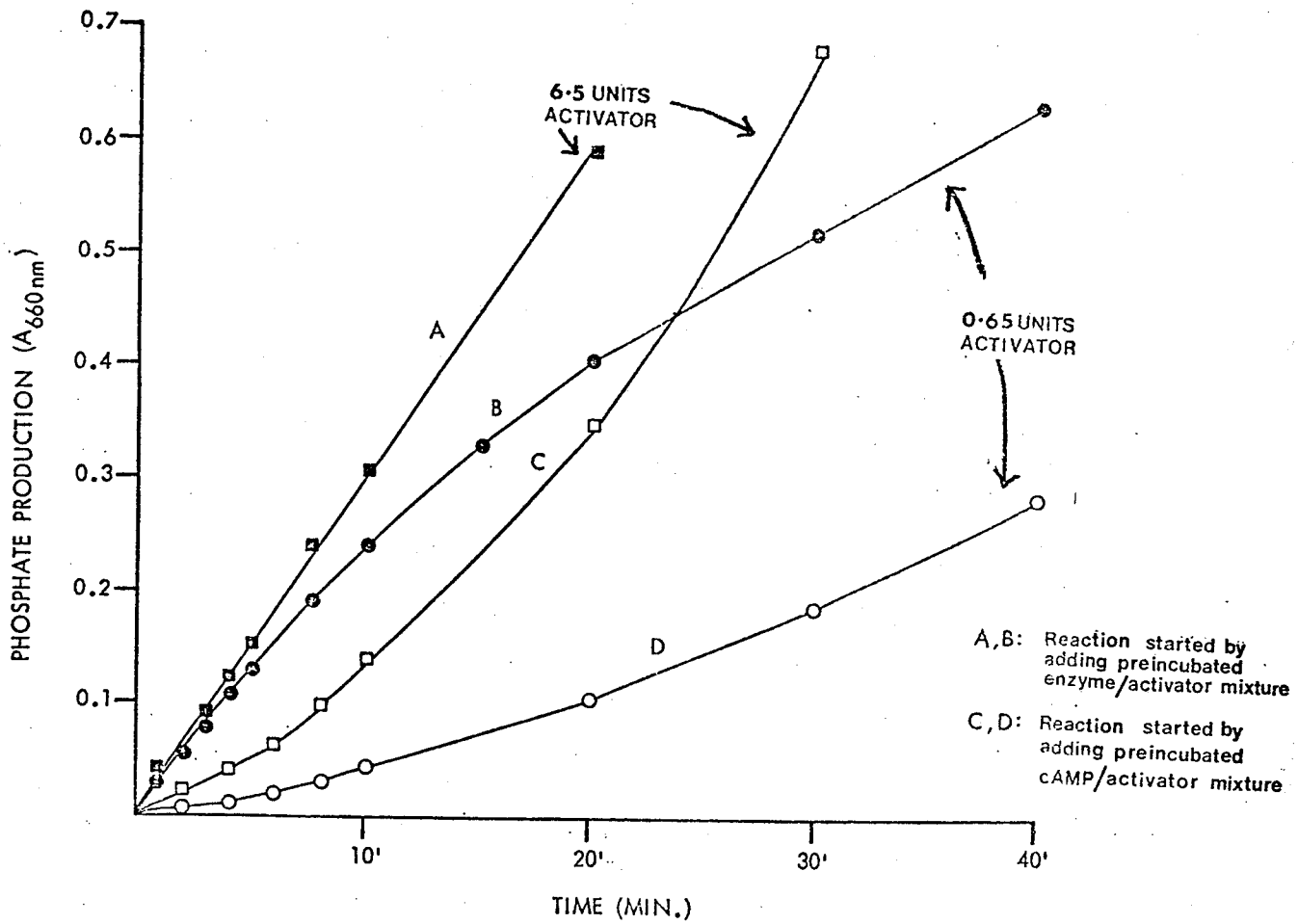


Fig.27 HYSTERETIC ACTIVATION OF PDE-I - Progress curves for cAMP phosphodiesterase reactions initiated by diluting mixtures of PDE-I and the protein activator by 9 fold into cAMP solutions (Curves A and B), or by adding mixtures of cAMP and the protein activator to PDE-I solutions (Curves C and D). After intiation of the reaction, aliquots were removed at various intervals and 0.45 ml of the aliquots were analyzed for phosphate content which was expressed as A660. Reaction mixtures contained 0.028 unit/ml of PDE-I and either 6.5 (Curves A and C) or 0.65 unit/ml of the protein activator (Curves B and D). Neither Ca^{2+} nor EGTA was added to the reaction mixtures.

suboptimal amount of the activator, the time course of the enzyme reaction showed a downward curvature (Fig. 27, curve B). In addition, the characteristics of the progress curves depended on the conditions of incubations of the reacting components prior to the enzyme catalysis. For reactions described by curves A and B in Fig. 27, the enzyme (PDE-I) and the protein activator had been mixed and incubated at relatively high concentrations prior to enzyme reactions. Enzyme reactions were initiated by direct dilution (9 fold) of the enzyme-activator mixture into solutions containing substrate. When the pre-incubation of the enzyme and the protein activator was omitted and the enzyme reaction was initiated by the addition of mixtures of the protein activator and cAMP to enzyme (PDE-I) solutions, the progress curves showed upward curvature irrespective of the protein activator concentrations (curves C and D of Fig. 27), thus suggesting a slow activation of the enzyme (PDE-I) by the protein activator. Cheung (71), however, showed that the activation of bovine brain cAMP phosphodiesterase by the protein activator was instantaneous. It should be noted that after 10 to 20 minutes of enzyme reaction, all the progress curves approach straight lines. The slopes of these linear regions depended only upon the concentrations of the activator. Thus, for Fig. 27 the slopes in the linear region of curves A and B are essentially the same as those for curves C and D, respectively.

The observed reaction curves in Fig. 27 may be readily understood by a simple scheme which assumes that; (a) the enzyme (PDE-I) which has low or no activity associates reversibly with the protein activator to form an active complex, and (b) the formation and dissociation of the protein complex are slow processes relative to the enzyme catalysis. According to this scheme and the law of mass action, dilution of a mixture of the enzyme and the protein activator will alter the equilibrium among the various protein species so as to result in a slow dissociation of the protein complex. This slow dissociation can account for the downward curvature observed in curve B of Fig. 27. When the concentration of the protein activator is sufficiently high so as to saturate the enzyme under the assay conditions, the dilution of the enzyme and protein activator mixture will not result in the dissociation of the complex and a linear progress curve will be expected (curve A). Similarly, the scheme also accounts for the upward curvature observed in curves C and D of Fig. 27. This upward curvature corresponds to the association of the enzyme and the protein activator. The linear region in all the curves may represent the rate of reactions catalyzed by the equilibrium concentrations of the active enzyme species.

Initiation of the cAMP phosphodiesterase reaction by the addition of cAMP to mixtures of the enzyme (PDE-I) and the protein activator was also found to give rise to curved progress curves. For

an experiment represented in Fig. 28, a solution of the enzyme (PDE-I) and the protein activator was diluted 9-fold into buffer and then incubated at 30 °C. At various intervals, aliquots (8.75 ml) of the diluted solution were withdrawn and mixed with 0.25 ml of 0.36 mM cAMP solution to initiate the enzyme reaction. Fig. 28 shows a family of progress curves (curves B, C, D and E) for samples preincubated for different time after the dilution. As the time of incubation increased, the progress curves changed gradually from downwardly curved to upwardly curved lines. Since the dilution of the enzyme-activator mixture may result in slow dissociation of the protein complex, samples incubated for different times prior to enzyme reaction are expected to exhibit different progress curves. The observation that progress curves for samples after 15 and 22 minutes of incubation are closely similar suggests that the equilibrium between the enzyme and the protein activator interactions can be attained after approximately 15 minutes. The pronouncedly upward curvature observed in the progress curves (curves D and E of Fig. 28) indicates that the enzyme may be slowly activated by cAMP. One possible mechanism of this activation is that the cyclic nucleotide enhanced the interaction between the enzyme (PDE-I) and the activator. This interpretation is supported by the observation that the activation was slow and most apparent when the diluted enzyme and protein activator had reacted to equilibrium (curves D and E of Fig. 28). Furthermore, when enzyme

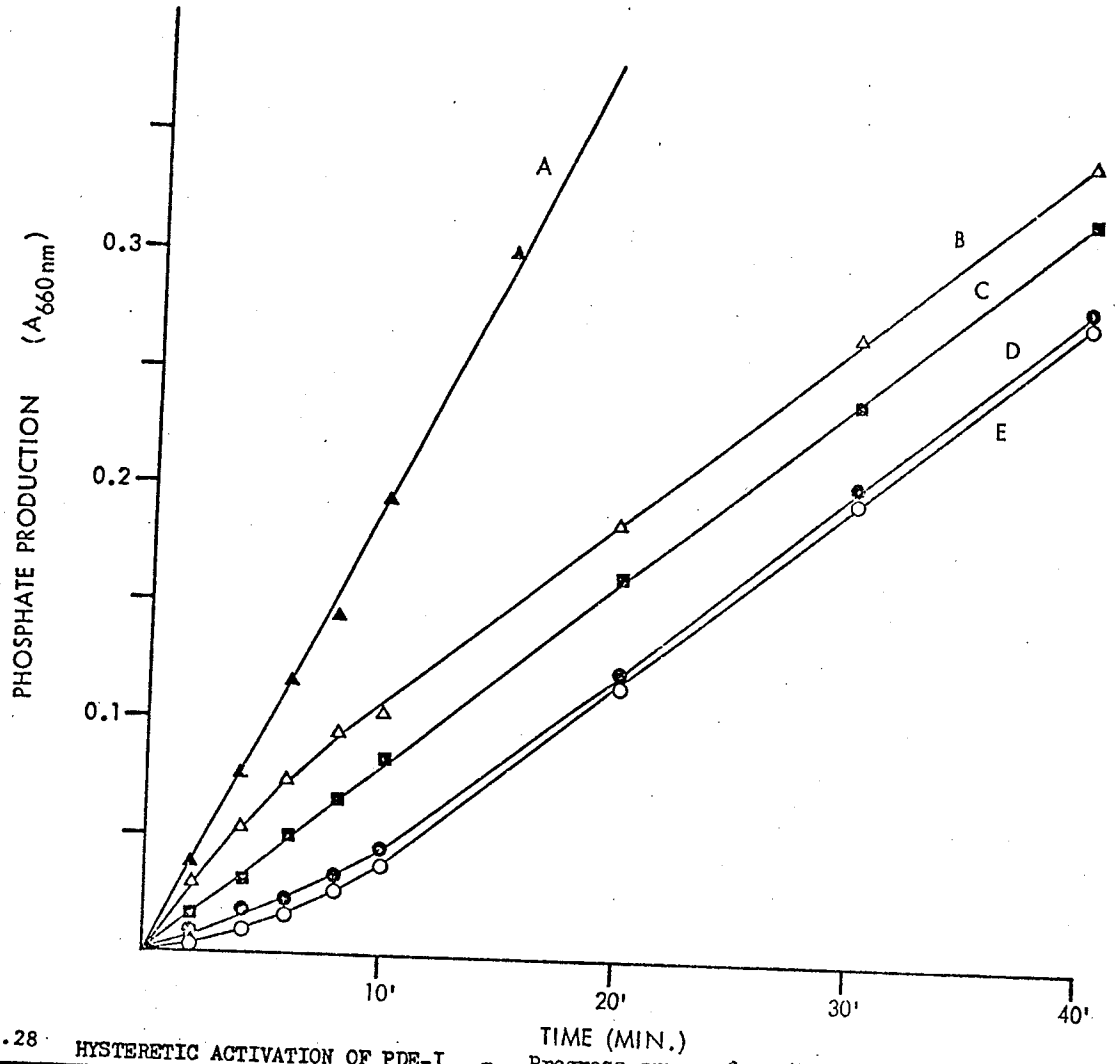


Fig.28 HYSTERETIC ACTIVATION OF PDE-I - Progress curves for cAMP phosphodiesterase reactions initiated by adding cAMP to the PDE-I and the protein activator mixtures. The PDE-I enzyme used had been rendered free of protein activator by prior DEAE-cellulose chromatography. A mixture of PDE-I (0.17 unit/ml) and the protein activator (7 unit/ml) was diluted 9 fold into a buffer solution at 30°C. Aliquots (8.75 ml) were removed after 1.5, 8, 15 and 22 minutes for Curves B,C,D and E, and mixed with 0.25 ml of cAMP solutions to initiate enzyme reactions. Experimental conditions for Curve A were the same as those for Curve E except that the protein activator concentration was 10 times more concentrated. Neither Ca^{2+} nor EGTA was added to the reaction mixtures.

reaction was carried out in the presence of a saturating amount of the protein activator, a linear progress curve was obtained (curve A of Fig. 28).

An alternative explanation for the results of Fig. 28 is that an "allosteric effector" of PDE-I (protein or otherwise) may be slowly dissociated from PDE-I during the 9-fold dilution. Addition of cAMP to start the PDE reaction could have induced a slow reassociation of this "allosteric effector" with PDE-1.

F. 2. Effects of the Protein Activator on the Kinetic Parameters of PDE-I

Other investigators (67, 68) have observed that the protein activator activates cAMP phosphodiesterase by increasing the V_{max} and by decreasing the K_m for cAMP. Fig. 29 shows that addition of small amounts (1. 7 units) of protein activator to PDE-I resulted in a more than 3-fold increase in the maximum velocity of the reaction. This low level of protein activator showed little effect on the K_m for cAMP, which remained at 1. 4 mM. When 3. 3 and 11 units of protein activator were added, the K_m values for cAMP decreased to 0. 67 and 0. 4 mM, respectively. However, no further increase in V_{max} was observed. Thus, although both V_{max} and K_m of PDE-I enzyme were influenced by the protein activator, the two effects were observed at different concentrations of protein activator.

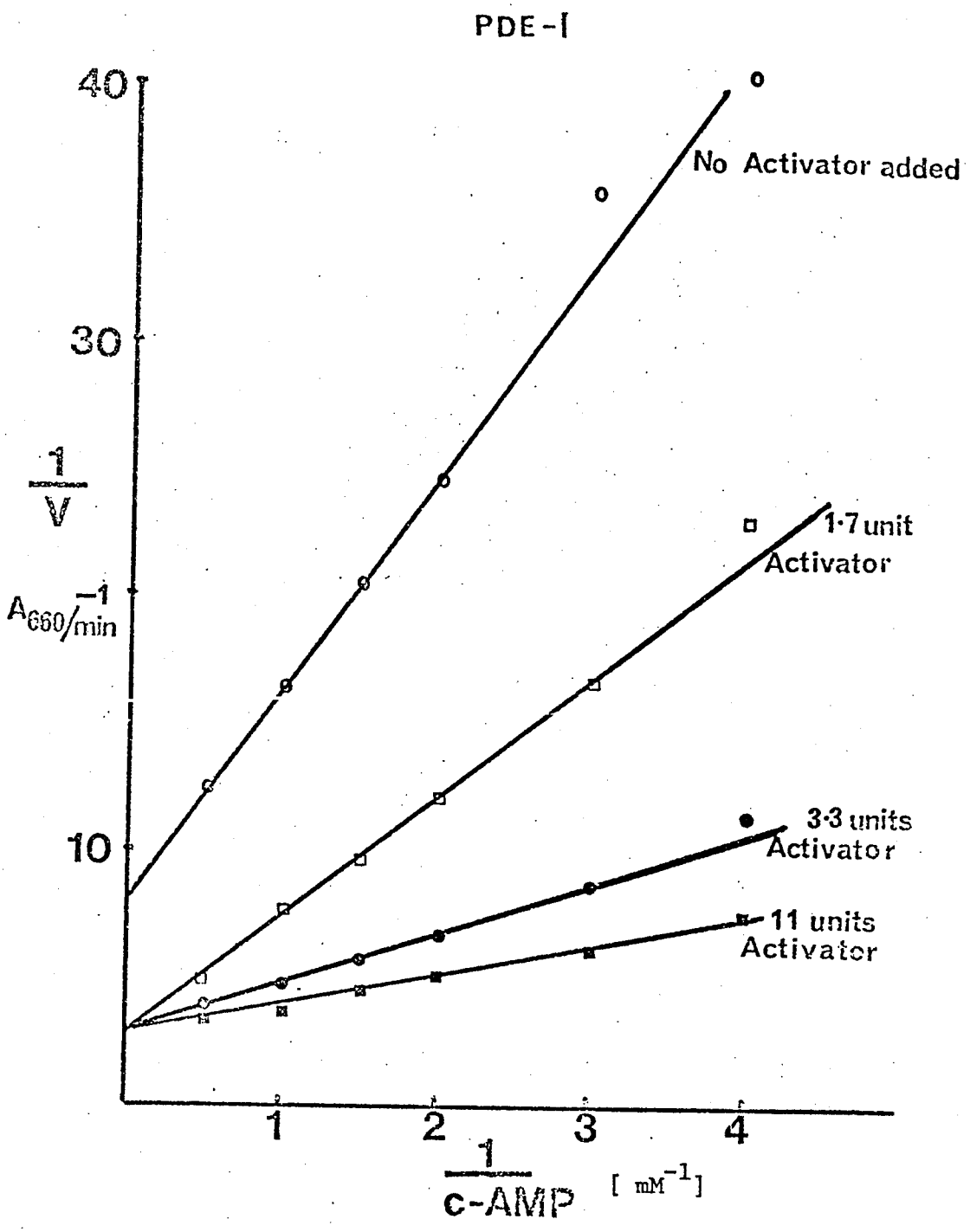


Fig.29 RECIPROCAL PLOT OF REACTION VELOCITY AND cAMP CONCENTRATION FOR PDE-I - The PDE-I enzyme used had been rendered free of protein activator by DEAE-cellulose chromatography. Each 0.9 ml reaction mixture contained 0.021 unit of this PDE-I enzyme, 40 mM imidazole and 20 mM Mg^{2+}. The reaction velocities are expressed as A_{660}/10 min. Neither Ca^{2+} nor EGTA was added to the reaction mixtures.

The extent of activation was found to be dependent on the concentration of the substrate. Fig. 30 shows that maximum activation (1400% increase) of the hydrolysis of cAMP by PDE-I was observed at 20 μm cAMP. At millimolar and micromolar concentrations of cAMP, the activation of cAMP hydrolysis were 500 - 600% and 800 - 900%, respectively.

The protein activator exhibited differential activation of hydrolysis of cAMP and cGMP. Fig. 30 shows that hydrolysis of cGMP by PDE-I was activated by only 30 - 40% by the protein activator at millimolar concentrations of cGMP. At lower concentrations of cGMP, the activation of cGMP hydrolysis was much greater; at micromolar concentrations of cGMP the activation was 700%.

Fig. 6 (see page 67) shows that at 1.2 mM substrate concentration, the "basal PDE activity"* of PDE-I hydrolyzed cGMP faster than cAMP. When the protein activator and Ca^{2+} were added to the assay mixture, PDE-I hydrolyzed cAMP faster than cGMP at 1.2 mM substrate concentration. Fig. 31 shows the relative rates of hydrolysis of cAMP and cGMP at different substrate concentrations. In the absence of protein activator, PDE-I hydrolyzed cGMP faster than cAMP at all substrate concentration investigated. However, when

*PDE-I activity in the absence of protein activator and in the presence of EGTA.

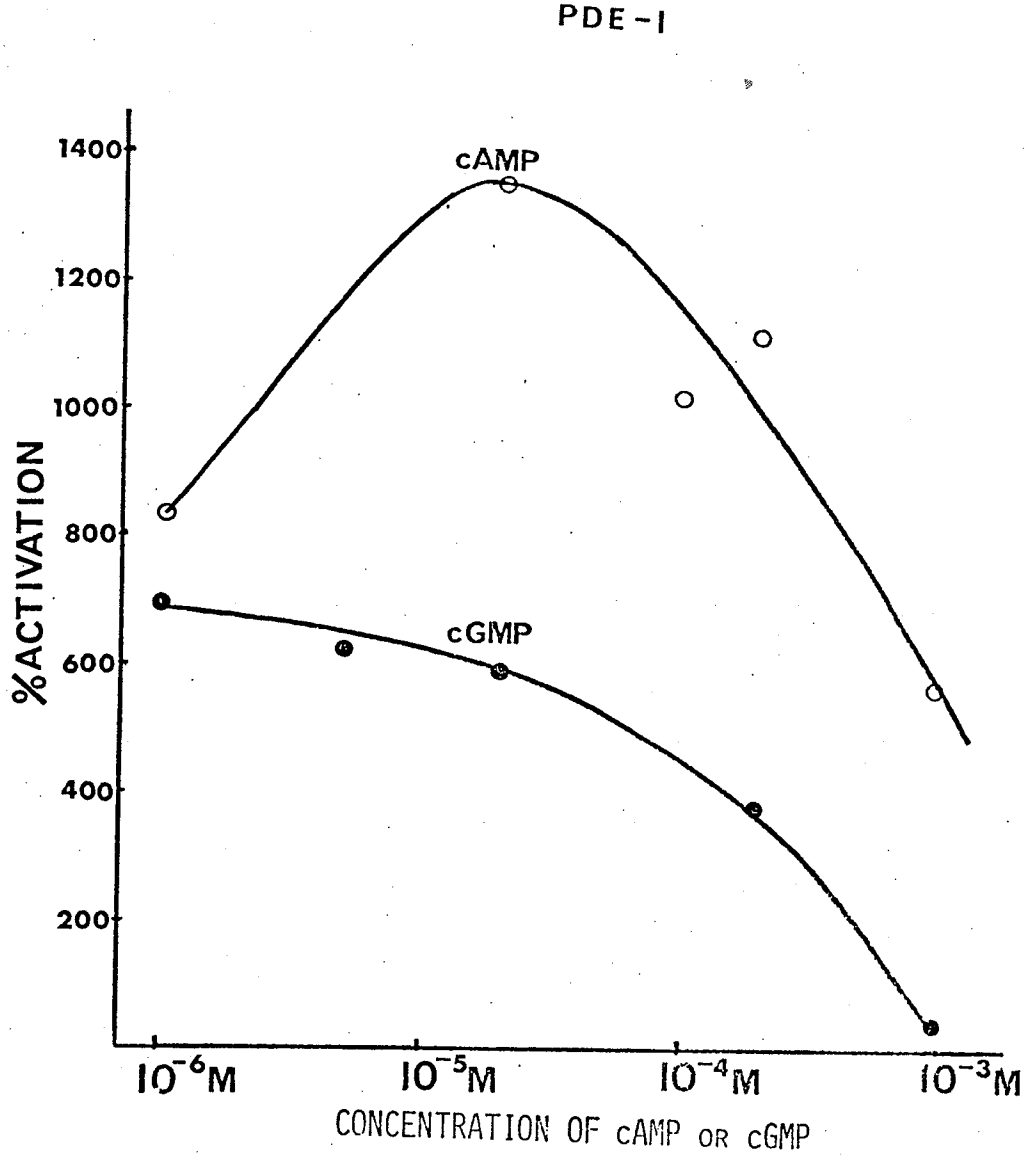


Fig.30 ACTIVATION OF PDE-I BY PROTEIN ACTIVATOR AS A FUNCTION OF SUBSTRATE CONCENTRATION - PDE enzyme activity was measured by the radioassay method. The PDE-I enzyme used was rendered free of protein activator by prior chromatography on DEAE-cellulose. The enzyme reactions were carried out with 40 mM Tris-HCl, 40 mM imidazole pH 7.5, 3 mM Mg^{2+}, 0.1 mM Ca^{2+} or 0.1 mM EGTA, excess protein activator and varying substrate concentration. The enzyme activity in the presence of 0.1 mM EGTA and in the absence of added protein activator was taken as the "basal PDE activity". Since the extent of activation varied so widely, the time of reaction was varied so that not more than 30% of the substrate was used up during the reaction.

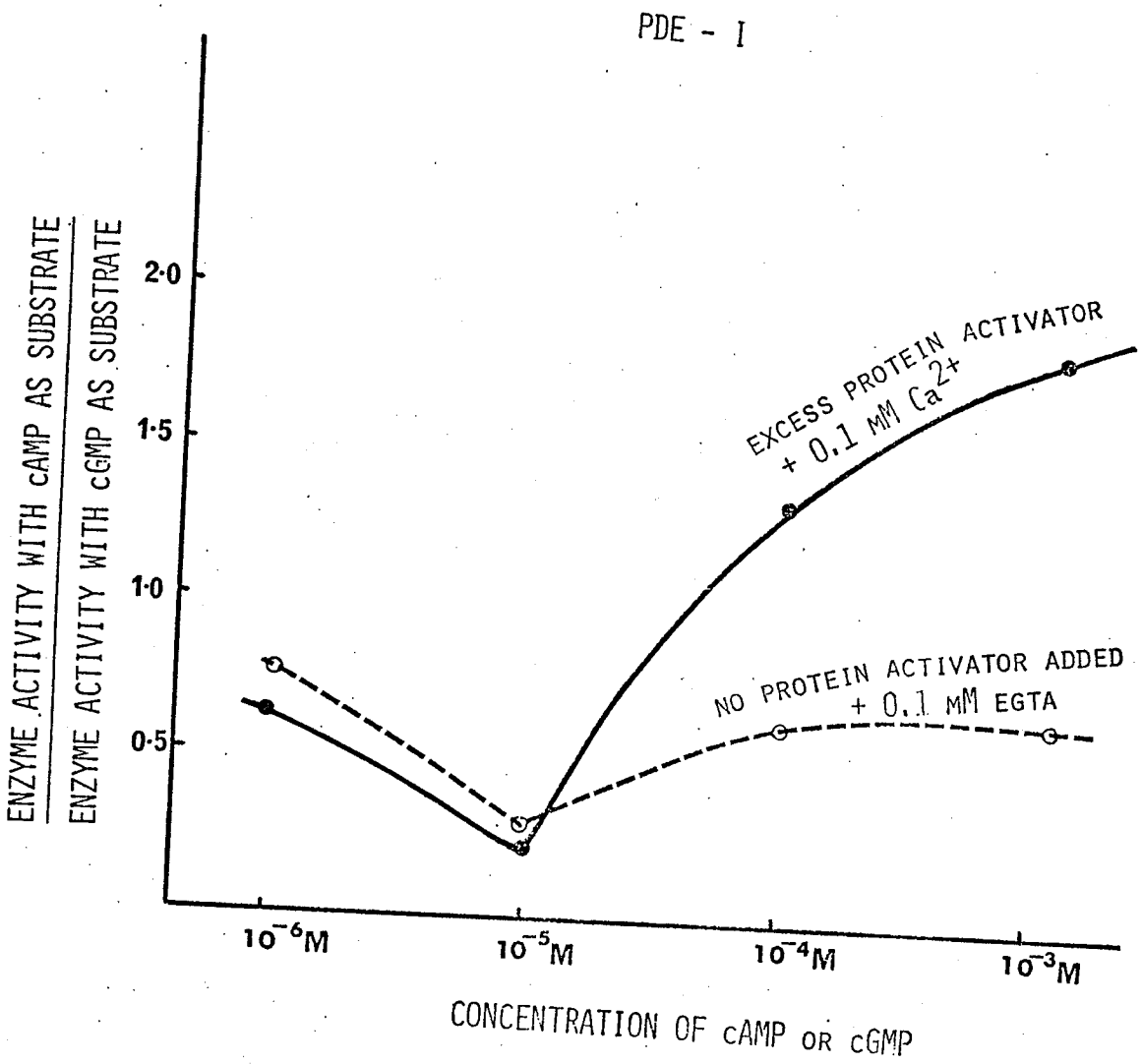


Fig.31 EFFECT OF PROTEIN ACTIVATOR ON THE SUBSTRATE SPECIFICITY OF PDE-I.

PDE enzyme activity was measured by the radioassay method. The PDE-I enzyme used was rendered free of protein activator by prior chromatography on DEAE-cellulose. The experimental conditions are described in "Methods"

excess protein activator and Ca^{2+} were added, PDE-I hydrolyzed cAMP faster than cGMP at a substrate concentration in excess of 40 μm but hydrolyzed cGMP faster than cAMP at substrate concentrations lower than 40 μm.

G. Factors that Affect the Interaction Between the Protein Activator and PDE-I

When a fixed amount of PDE-I (freed of protein activator by DEAE-cellulose chromatography) was reacted with increasing concentrations of protein activator, a PDE-I activity - protein activator titration curve such as shown in Fig. 1 (page 49) was obtained. The value of $A_{50\%}$ for a particular titration curve is the concentration of protein activator required to give half maximal activation of this fixed concentration of PDE-I under standard conditions. Under conditions when PDE-I exhibits greater affinity for the protein activator, a lower concentration of the protein activator is required to give half maximal activation of PDE-I and consequently a lower value of $A_{50\%}$ is recorded. Thus the $A_{50\%}$ can be considered to be a parameter analogous to the K_m for a substrate; the $A_{50\%}$ being an index of the affinity of PDE-I for the protein activator while the K_m can be an index of the affinity of an enzyme for its substrate. Many factors such as pH, ionic strength etc., are known to affect the activity of PDE-I. By empirically making a plot of $A_{50\%}$ as a function of these factors it is possible to determine if these factors exert their effect on the activity of PDE-I by influencing

the affinity of PDE-I for its protein activator.

G. 1. Concentration of PDE-I Enzyme

Fig. 2 (see page 51) shows that, as the concentration of PDE-I was increased in the presence of excess Ca^{2+} and the protein activator, the enzyme activity increased but not in a linear manner. The plot of enzyme activity curved downwards slightly at high concentrations of PDE-I. The reason for this is not clear. Preliminary experiments were unsuccessful in demonstrating the existence of an inhibitor in the PDE-I preparations. Fig. 2 also shows that the values of $A_{50\%}$ were not significantly affected by the concentration of PDE-I.

G. 2. pH

Fig. 32 shows that, under the conditions of the enzyme assay, PDE-I exhibited a pH optimum of 7.5 and that the pH did not affect the values of $A_{50\%}$.

G. 3. Temperature

PDE-I enzyme activity as measured by the one-step phosphate method was observed to increase linearly with respect to temperature between 20° and 36 °C (Fig. 33A). PDE-I activity-protein activator titration curves at 20°, 30° and 36 °C, shown in Fig. 33B indicate that the values of $A_{50\%}$ did not vary with temperature. However, since no controls were run on the effect of temperature on the 5'nucleotidase reaction, the results were not conclusive.

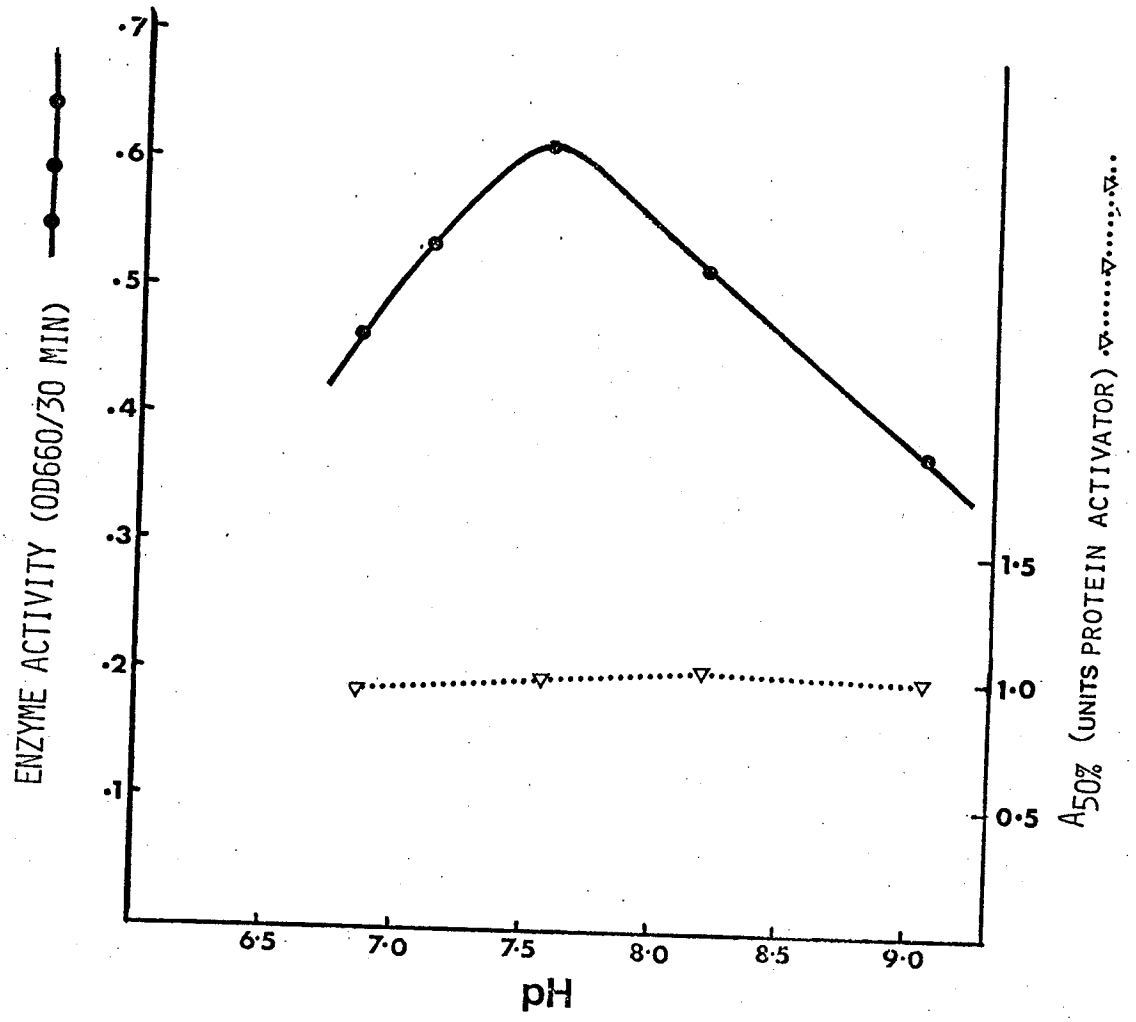


Fig.32 PDE-I ENZYME ACTIVITY AND $A_{50\%}$ PLOTTED AS A FUNCTION OF pH.

PDE enzyme activity was measured by the one-stage phosphate method. The PDE-I enzyme used had been rendered free of protein activator by prior chromatography on DEAE-cellulose. The enzyme reactions were carried out with 40 mM Tris-HCl, 40 mM imidazole, excess protein activator, 3 mM Mg^{2+}, 0.1 mM cAMP and 0.1 mM Ca^{2+}. The values of $A_{50\%}$ were each obtained from seperate PDE-I activity - protein activator titration curves.

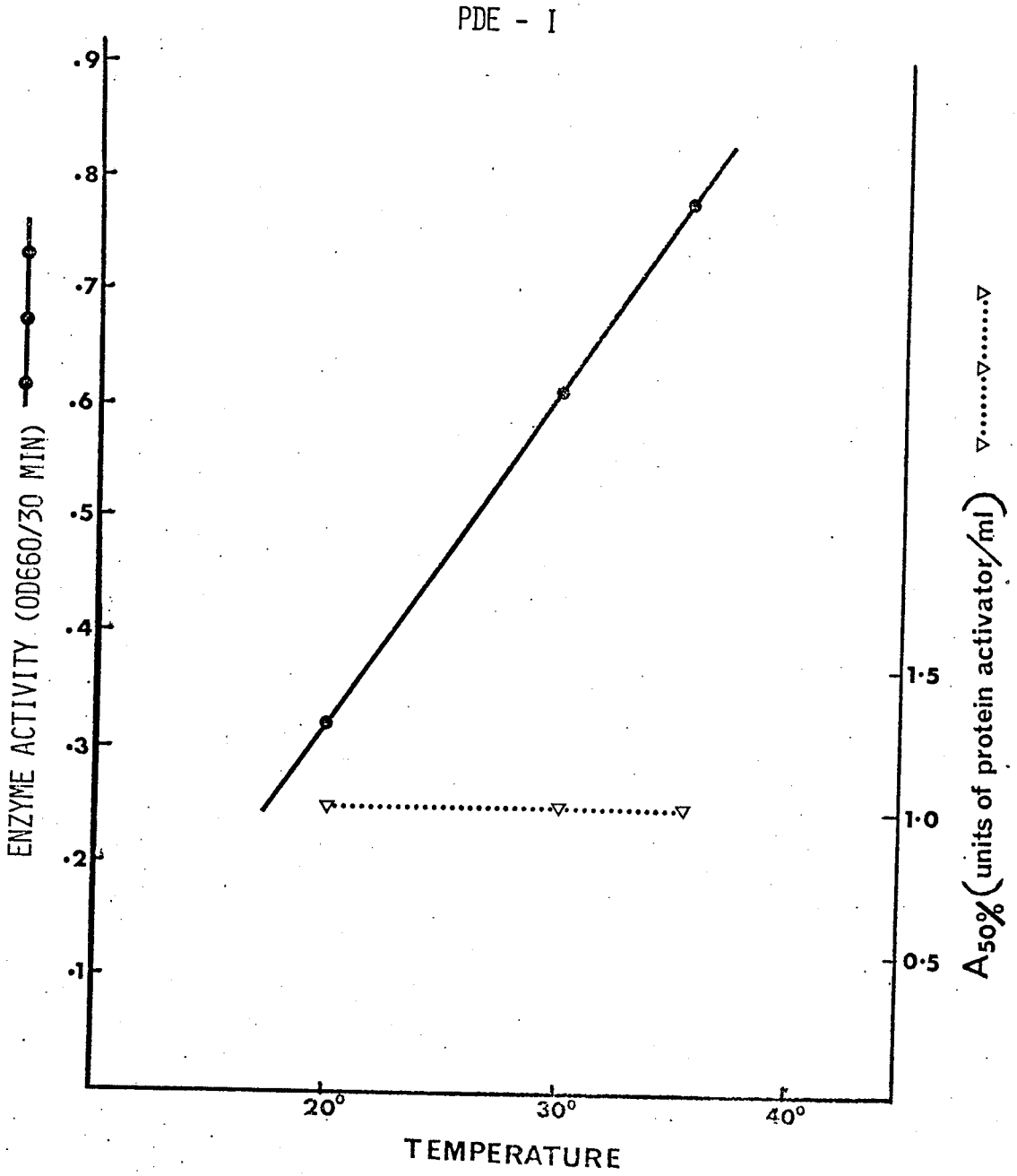


Fig.33A PDE-I ENZYME ACTIVITY AND $A_{50\%}$ AS A FUNCTION OF TEMPERATURE

PDE-I enzyme activity was measured by the one-stage method. The PDE-I enzyme used had been rendered free of protein activator by prior chromatography on DEAE-cellulose. The enzyme reactions were carried out with 40 mM Tris-HCl, 40 mM imidazole, pH 7.5, excess protein activator, 3 mM Mg^{2+}, 0.1 mM Ca^{2+} and 1.2 mM cAMP. The values of $A_{50\%}$ were each obtained from seperate PDE-I activity - protein activator titration curves. For limitations see page 119.

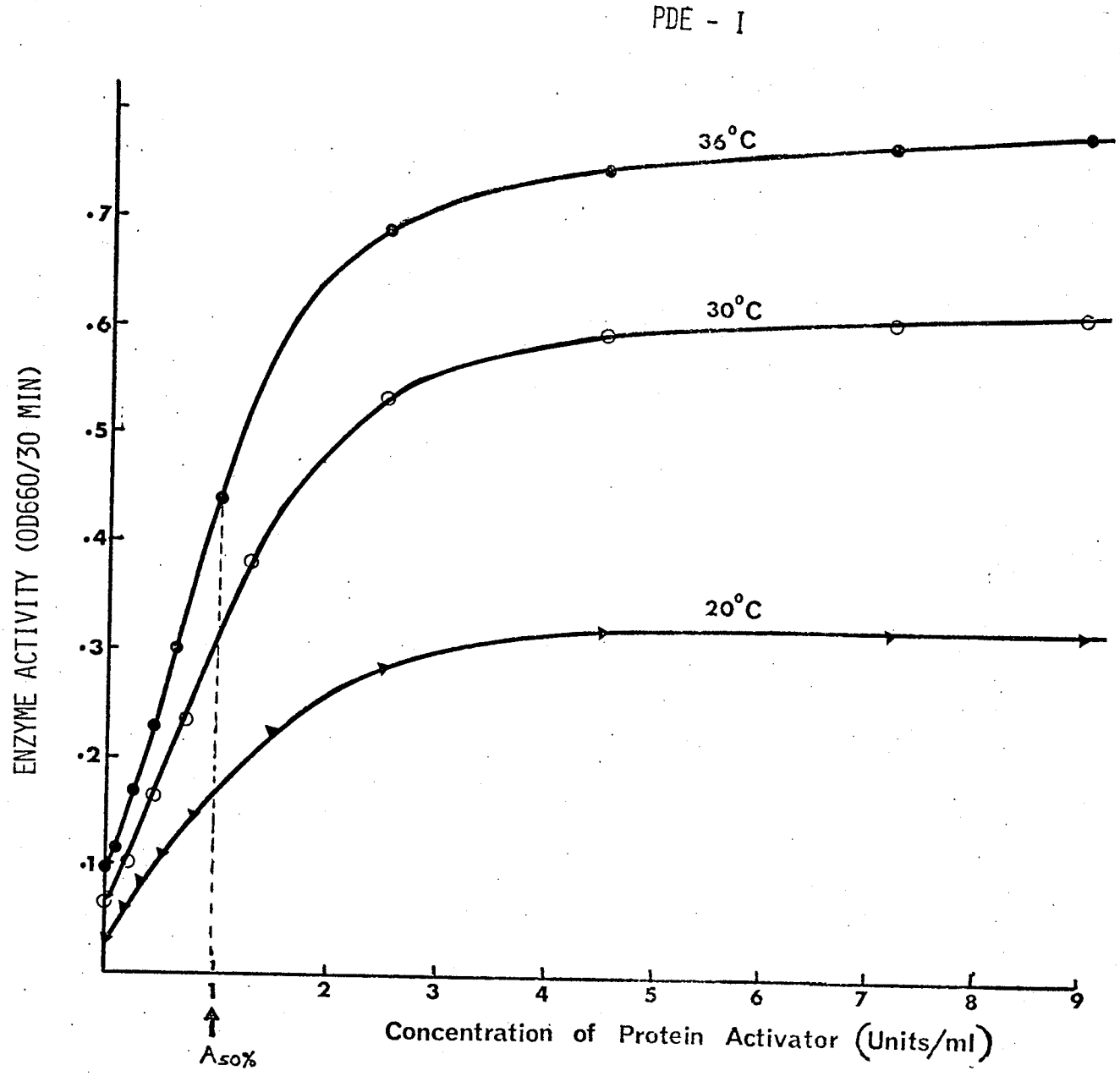


Fig.33B PDE-I ACTIVITY - PROTEIN ACTIVATOR TITRATION CURVES AT DIFFERENT TEMPERATURE . - Experimental conditions were identical to fig.33A

G.4. Ionic Strength

PDE-I enzyme activity was observed (Fig. 34A) to decrease in a non-linear manner with an increase in ionic strength. Fig. 34B shows that an increase of ionic strength from 0.09 to 0.28 M decreased the enzyme activity by 27% but had no effect on the value of $A_{50\%}$.

G.5. Imidazole Concentration

Fig. 35A shows that imidazole at relatively high concentrations enhanced the PDE-I enzyme activity assayed in the presence of excess Ca^{2+} and protein activator. Maximum activation was observed at imidazole concentrations in excess of 25 mM. Fig. 35B shows that, although 40 mM imidazole activated PDE-I enzyme activity by 95%, the value of $A_{50\%}$ was unaffected.

G.6. Substrate Concentration

As previously mentioned above (section on "Effects of Protein Activator on Kinetic Parameters of PDE-I" see page 113), the effects of the protein activator on PDE-I enzyme activity were found to be dependent on the concentration of the substrate as depicted in Fig. 30. Titration curves for the hydrolysis of cAMP and cGMP by PDE-I in the presence of excess Ca^{2+} were obtained at different concentrations of substrate. These titration curves were obtained in collaboration with Dr. Honor Ho. The plots of reciprocal of $A_{50\%}$ against the concentration of substrate is shown in Fig. 36. At millimolar concentrations of substrate, PDE-I exhibited greater affinity for the protein

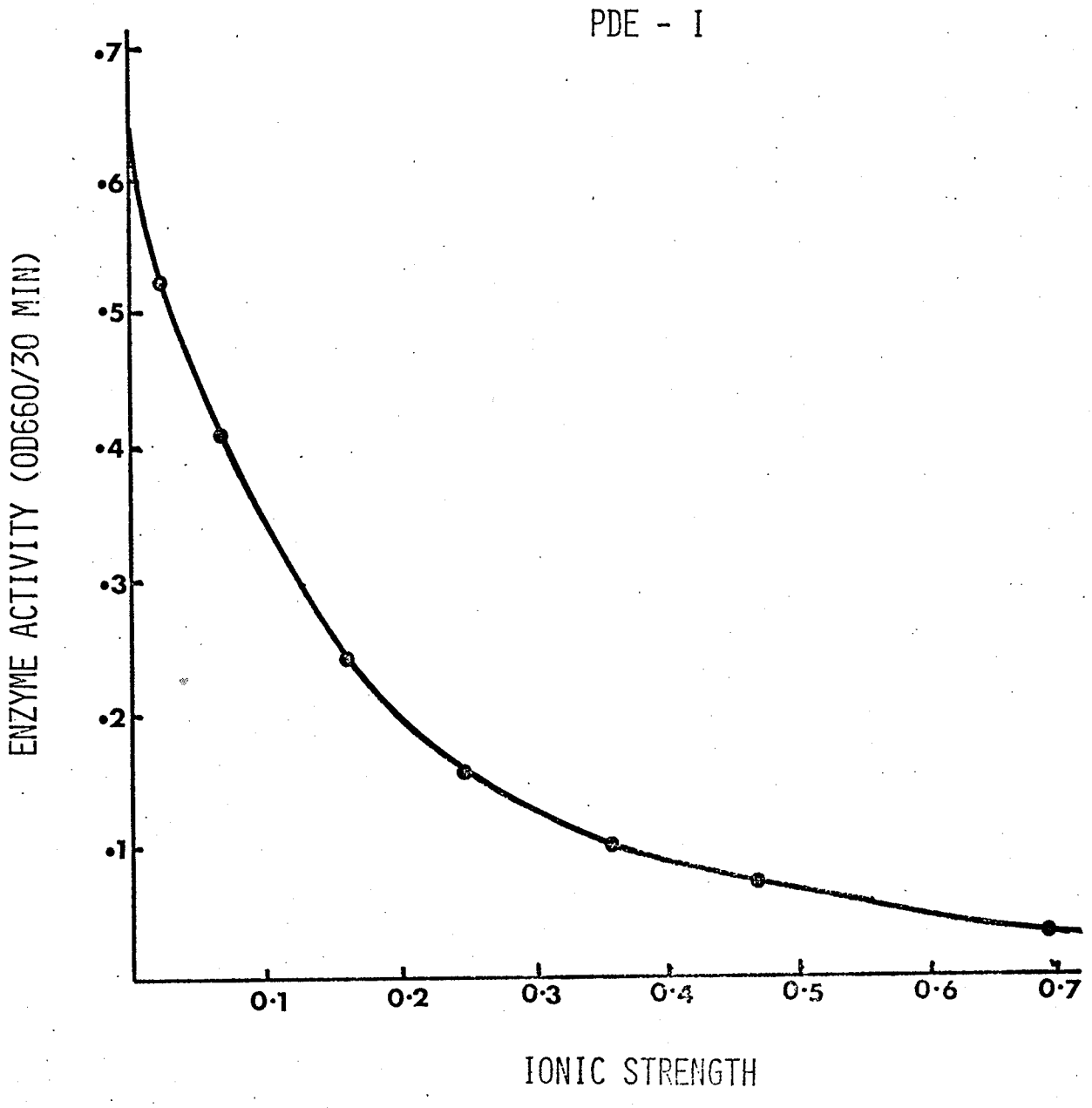


Fig.34A PDE-I ENZYME ACTIVITY AS A FUNCTION OF IONIC STRENGTH - experimental conditions were identical to those of fig.33A except that the ionic strength was varied by varying the NaCl concentration. For limitations see page 119.

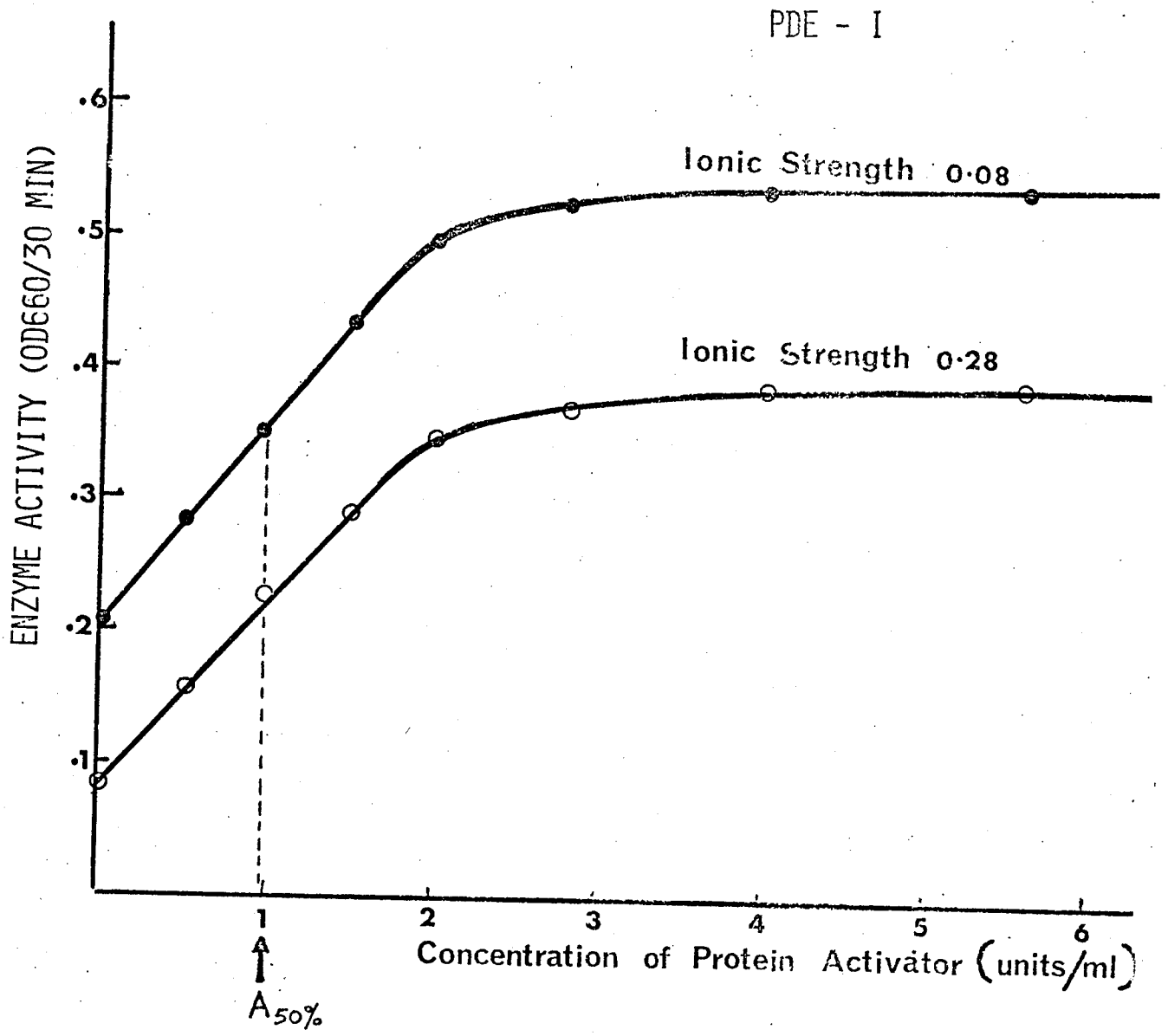


Fig.34B PDE-I ACTIVITY - PROTEIN ACTIVATOR TITRATION CURVES AT TWO IONIC STRENGTH - Experimental conditions were identical to those of fig.34A

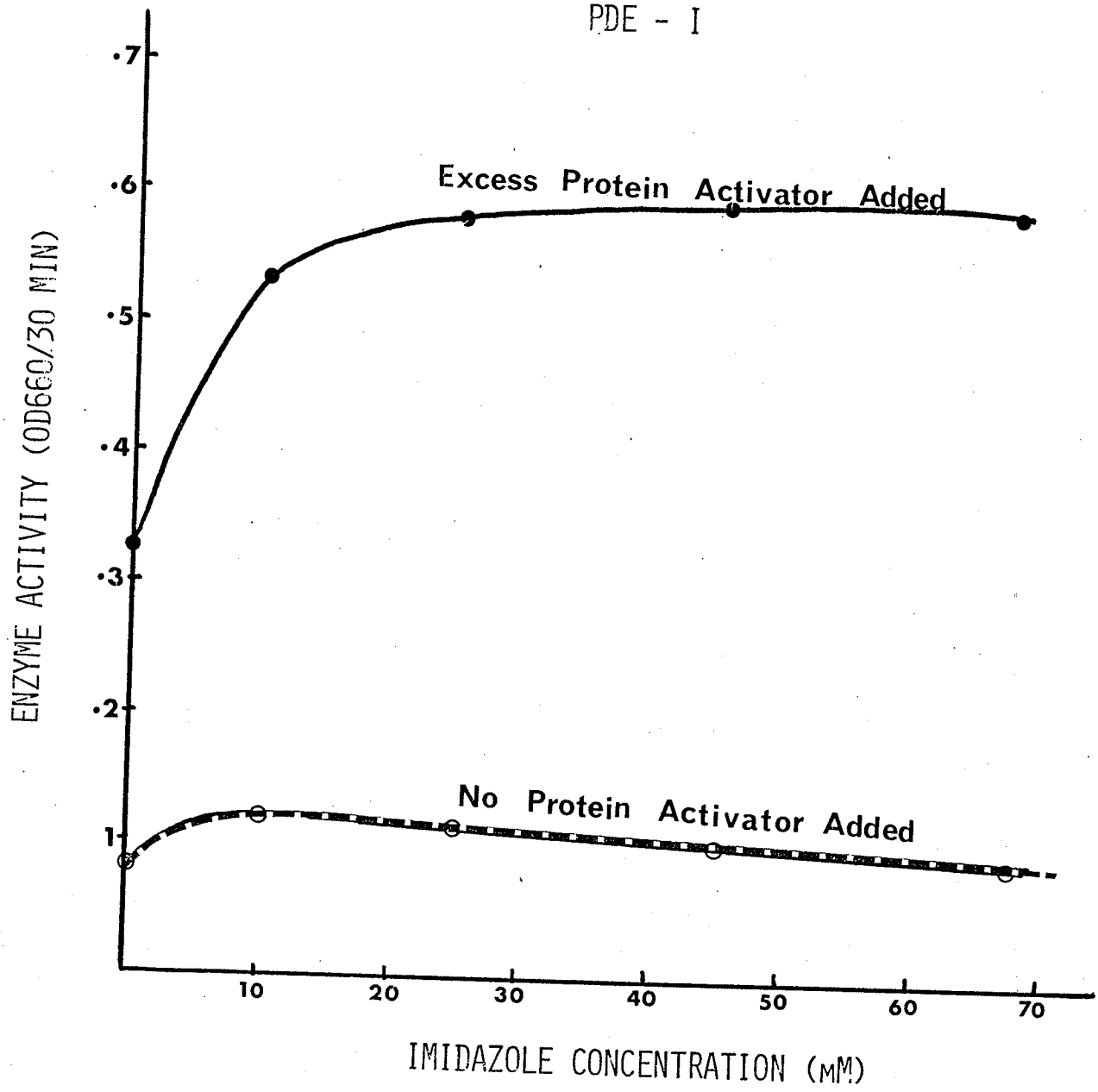


Fig.35A PDE-I ENZYME ACTIVITY AS A FUNCTION OF IMIDAZOLE CONCENTRATION

Experimental conditions were identical to that described for fig.33A except that reaction mixtures for determination of PDE basal activity (dashed line) contained no added protein activator. For limitations see page 119.

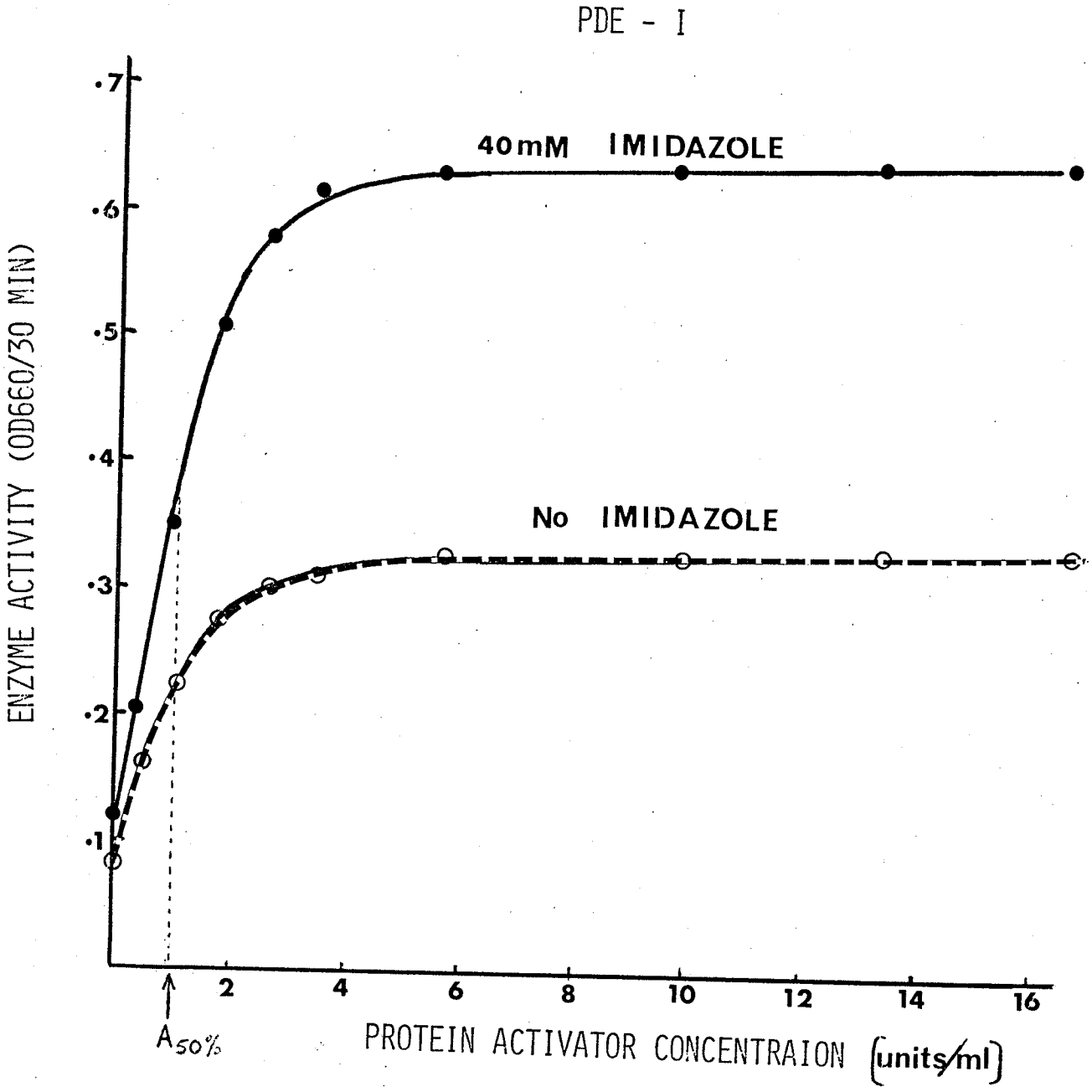


Fig.35B **PDE-I ACTIVITY - PROTEIN ACTIVATOR TITRATION CURVES IN THE PRESENCE AND ABSENCE OF IMIDAZOLE.** - Experimental conditions were identical to those for fig.35A

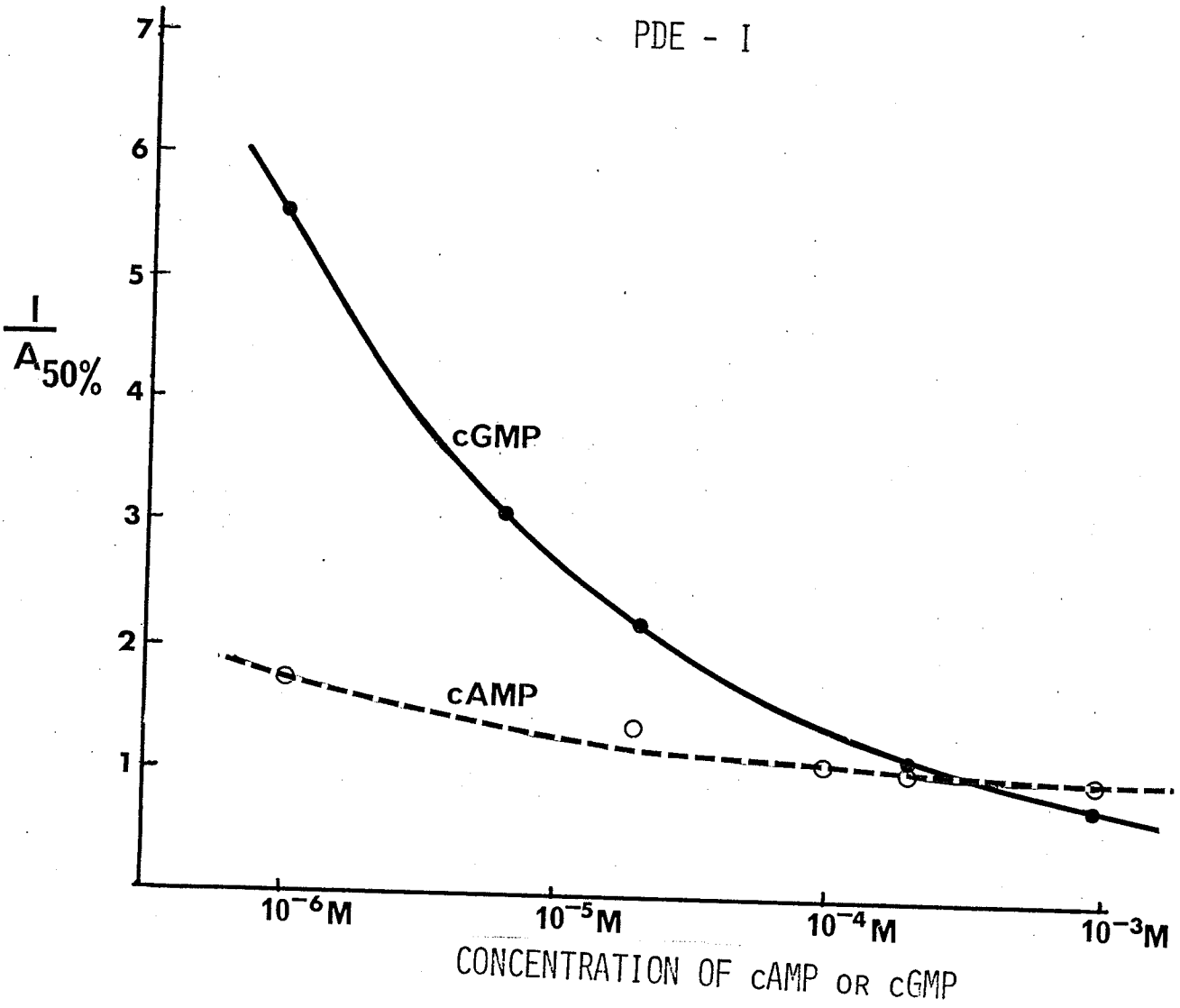


Fig.36 THE RECIPROCAL OF $A_{50\%}$ AS A FUNCTION OF THE SUBSTRATE CONCENTRATION

Each value of $A_{50\%}$ was determined seperately by plotting PDE-I activity - protein activator titration curves. PDE enzyme activity was measured by the radioassay method. The experimental conditions were identical to those described for fig.2. All reactions were carried out in the presence of excess protein activator and 0.1 mM Ca^{2+}.

activator in the presence of cAMP than in the presence of cGMP. For the hydrolysis of cAMP, decreasing the cAMP concentrations from millimolar to micromolar levels increased the values of $1/A_{50\%}$ by a mere 70%. For the hydrolysis of cGMP, decreasing the cGMP concentrations from millimolar to micromolar levels increased the values of $1/A_{50\%}$ by approximately 600%. Thus, the affinity of PDE-I for the protein activator varied slightly only with variation in cAMP concentrations but varied markedly with changes in cGMP concentrations. From the data of Fig. 30 and 36, it can be seen that the extent of activation of cGMP hydrolysis is parallel to the affinity of the protein activator for PDE-I. However, the extent of activation of cAMP hydrolysis does not parallel the affinity of the protein activator for PDE-I. The reason for this is not clear.

G.7. Ca^{2+} Concentrations

Fig. 37 shows that the activity of PDE-I in the absence of added protein activator ("basal PDE activity") was not inhibited by EGTA in the presence of excess Mg^{2+} and was therefore independent of Ca^{2+}. When excess protein activator was added, the enzyme activity was activated ten fold. This activation by the protein activator was completely abolished by low concentrations of EGTA in the presence of excess Mg^{2+}. This indicates that the activation of PDE-I required the simultaneous presence of protein activator and low concentrations of Ca^{2+}.

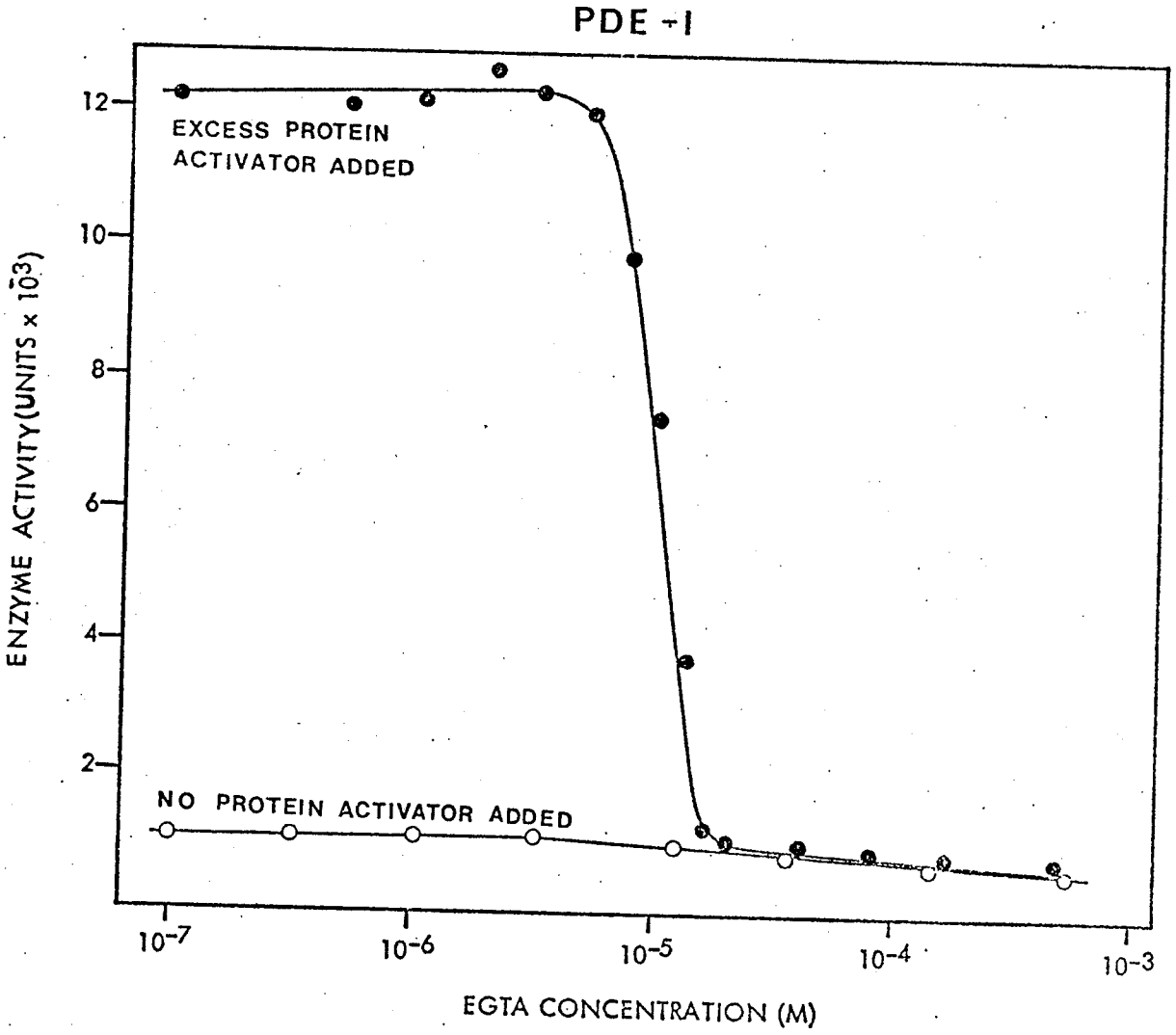


Fig.37 INHIBITION OF PDE-I BY EGTA - PDE enzyme activity was measured by the one-stage phosphate method. The PDE-I enzyme used was rendered free of protein activator by prior chromatography on DEAE-cellulose. The experimental conditions are described in "Methods". The concentration of cAMP was 1.2 mM.

Although the preceding results suggest that PDE-I is a Ca^{2+}-dependent enzyme, the possibility that EGTA had a direct effect on PDE-I or that a metal ion other than Ca^{2+} was responsible for the enzyme activation cannot be excluded. To establish unequivocally that Ca^{2+} activates the enzyme, removal of contaminating Ca^{2+} in the reaction mixture and direct demonstration of the Ca^{2+} activation are essential. Fig. 38 shows that when reagents and protein samples relatively free of the contaminating Ca^{2+} were used, activation of PDE-I by Ca^{2+} can be shown. In the absence of the protein activator, increasing the Ca^{2+} concentration to 0.2 mM resulted in little activation. At a saturating level of the protein activator (13 units/ml), Ca^{2+} could bring about a 10-fold increase in the enzyme activity. At a lower level of the protein activator, the maximal Ca^{2+} activation of PDE-I was also lowered. Furthermore, addition of protein activator shifted the activation curve towards lower concentrations of Ca^{2+}. In Fig. 38 in the presence of 1.4 and 13 units of protein activator, the concentrations of Ca^{2+} required to achieve half maximal activation were 3.6 and 2.3 μM, respectively.

The results presented above demonstrate that activation of PDE-I by Ca^{2+} was dependent upon the presence of the protein activator and the data of Fig. 39 show that the activation of PDE-I by the protein activator was dependent upon Ca^{2+}. In the absence of Ca^{2+}, PDE-I activity was not stimulated by the protein activator. At 100 μM Ca^{2+},

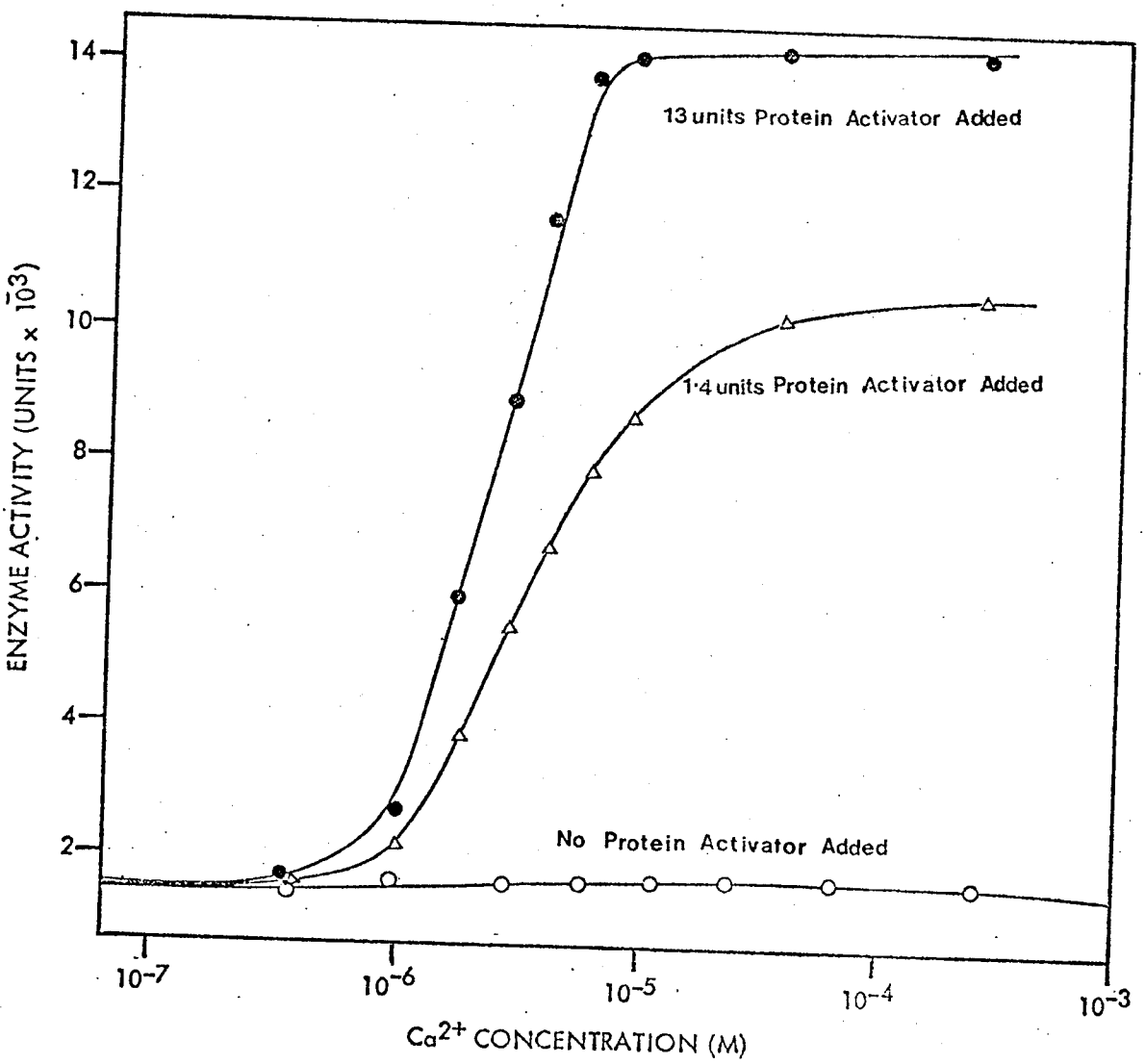


Fig.38 ACTIVATION OF PDE-I BY Ca^{2+} - PDE enzyme activity was measured by the two - stage phosphate method. The PDE-I enzyme used in this experiment had been rendered free of protein activator by prior chromatography on DEAE-cellulose. The PDE-I, protein activator and all reagents used were freed of calcium by procedures described in 'Methods'.

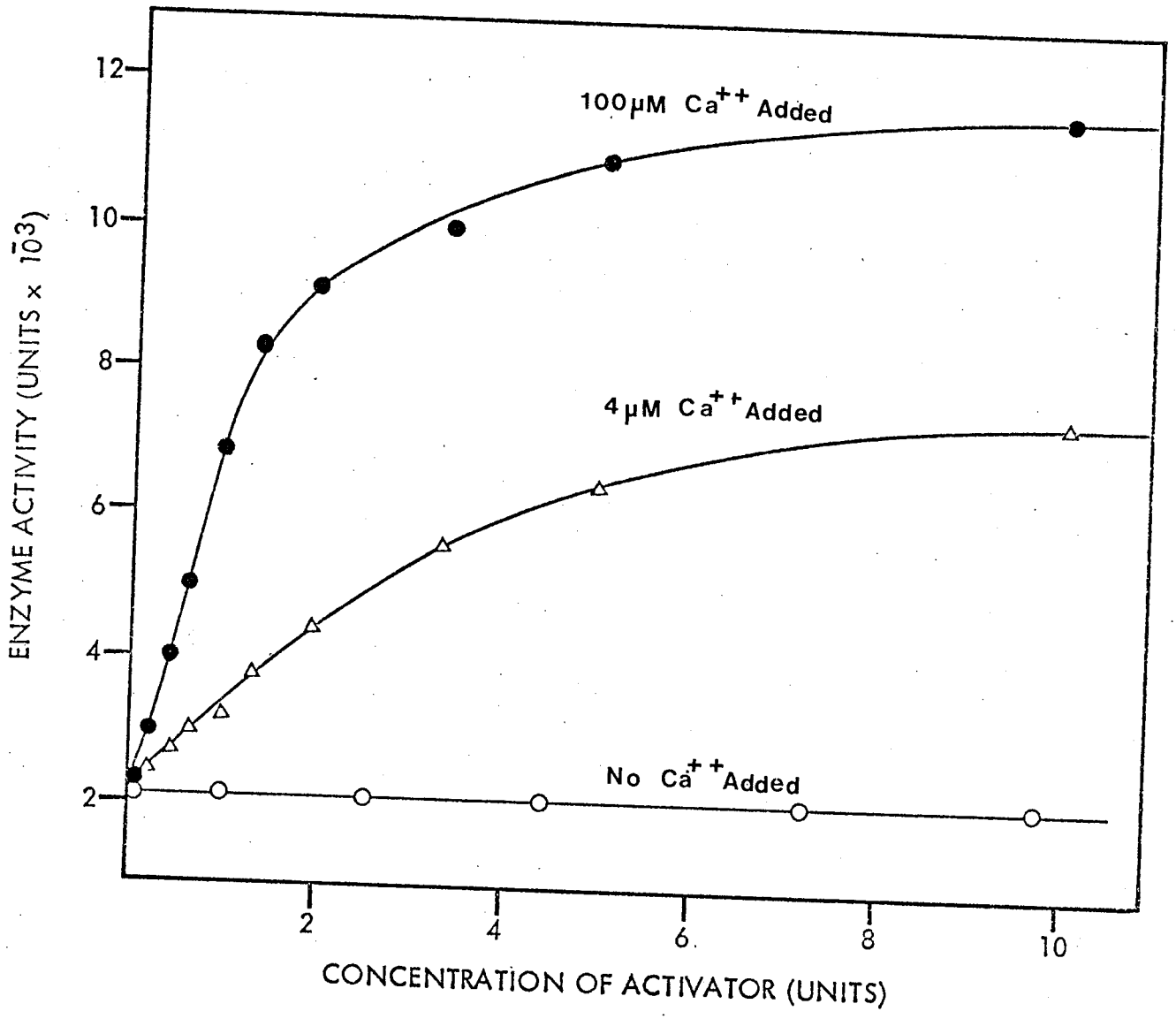


Fig.39 PDE-I ACTIVITY - PROTEIN ACTIVATOR TITRATION CURVES AT DIFFERENT CONCENTRATIONS OF CALCIUM - The experimental conditions were identical to those described for fig.38.

however, PDE-I was activated by increasing concentrations of the protein activator to a maximum of 600%. Both the extent of the enzyme activation and the concentration of the protein activator required for half maximal activation ($A_{50\%}$) were functions of Ca^{2+}. At a lower concentration of Ca^{2+}, PDE-I was activated by the protein activator to a smaller extent and more protein activator was needed to achieve 50% maximal activation of the enzyme. Thus the results of Fig. 38 and 39 indicate that activation of PDE-I was achieved only when both Ca^{2+} and the protein activator were present.

Since all enzyme assays in the present study were carried out in the presence of 3 mM Mg^{2+}, the demonstration of Ca^{2+} activation indicates that Mg^{2+} did not substitute for Ca^{2+} in the activation of PDE-I. However, Mg^{2+} was essential for the catalytic activity of PDE-I since the enzyme was inactive in the presence of Ca^{2+} alone. Thus, the enzyme is dependent on both Mg^{2+} and Ca^{2+} for its full activity. To further study the specificity of Ca^{2+} activation of PDE-I, the enzyme activity in the presence of 3 mM Mg^{2+} and one of several divalent cations has been examined. Table 7 shows that Sr^{2+}, Co^{2+} and Mn^{2+} were the only divalent cations which exhibited appreciable enzyme activation at a concentration of 27 μM. The magnitude of enzyme activation by these cations, were, however, much less than that by Ca^{2+}.

Table 7

ACTIVATION OF BEEF HEART cAMP PDE BY DIVALENT CATION[a]

Cation Added (27 μM)	PDE Activity (Units)	Stimulation (%)
None	0.00205	-
Ca^{2+}	0.0147	617
Sr^{2+}	0.00575	180
Mn^{2+}	0.00383	87
Co^{2+}	0.00360	76
Zn^{2+}	0.00287	40
Ni^{2+}	0.00243	18
Cu^{2+}	0.00242	18
Ba^{2+}	0.00227	11
Fe^{2+}	0.00217	5

a

The reaction mixtures contained 0.015 unit of PDE-I, 10 units of protein activator, 40 mM Tris-HCl, 40 mM imidazole pH 7.5 and 27 uM of a divalent cation in addition to 3 mM Mg^{2+}. PDE-I, the protein activator and all reagents used were freed of Ca^{2+} by the procedures described in 'Methods'. cAMP PDE enzyme activity was measured by the two-stage method.

In order to determine whether the low level of activation achieved by Sr^{2+} and Co^{2+} were due to low affinities of these cations for the binding sites or to low maximal enzyme activations, PDE-I activity in the presence of excess protein activator had been examined as a function of the cation concentration. Fig. 40 shows that PDE-I was maximally activated by Sr^{2+} and Co^{2+} to 900% and 300%, respectively. Under the same conditions, maximal Ca^{2+} activation of the enzyme was 1000%. Concentrations of Sr^{2+} and Co^{2+} required to provide half maximal activation were 36.3 and 19.2 μM, respectively; about 10 to 20 times higher than that for Ca^{2+}. Thus Ca^{2+} appeared to be the most effective divalent cation activator for PDE-I.

One possible explanation for the mutual dependence of Ca^{2+} and the protein activator in the activation of PDE-I is that the two activators have to combine to form a metal-protein complex in order to activate the enzyme. The possible formation of the Ca^{2+}-protein activator complex has been investigated by the equilibrium binding technique on a Sephadex G-25 gel filtration column (105, 106). Fig. 41 shows the elution profile for a typical binding experiment. The appearance of ^{45}Ca peak and troughs in the profile is indicative of the binding of Ca^{2+} to the protein activator. The radioactivity peak coincided exactly with the activity peak of the protein activator. In most experiments, double troughs have been observed in the elution profile, but the origin and significance of the double troughs are not clear. For the calculation of

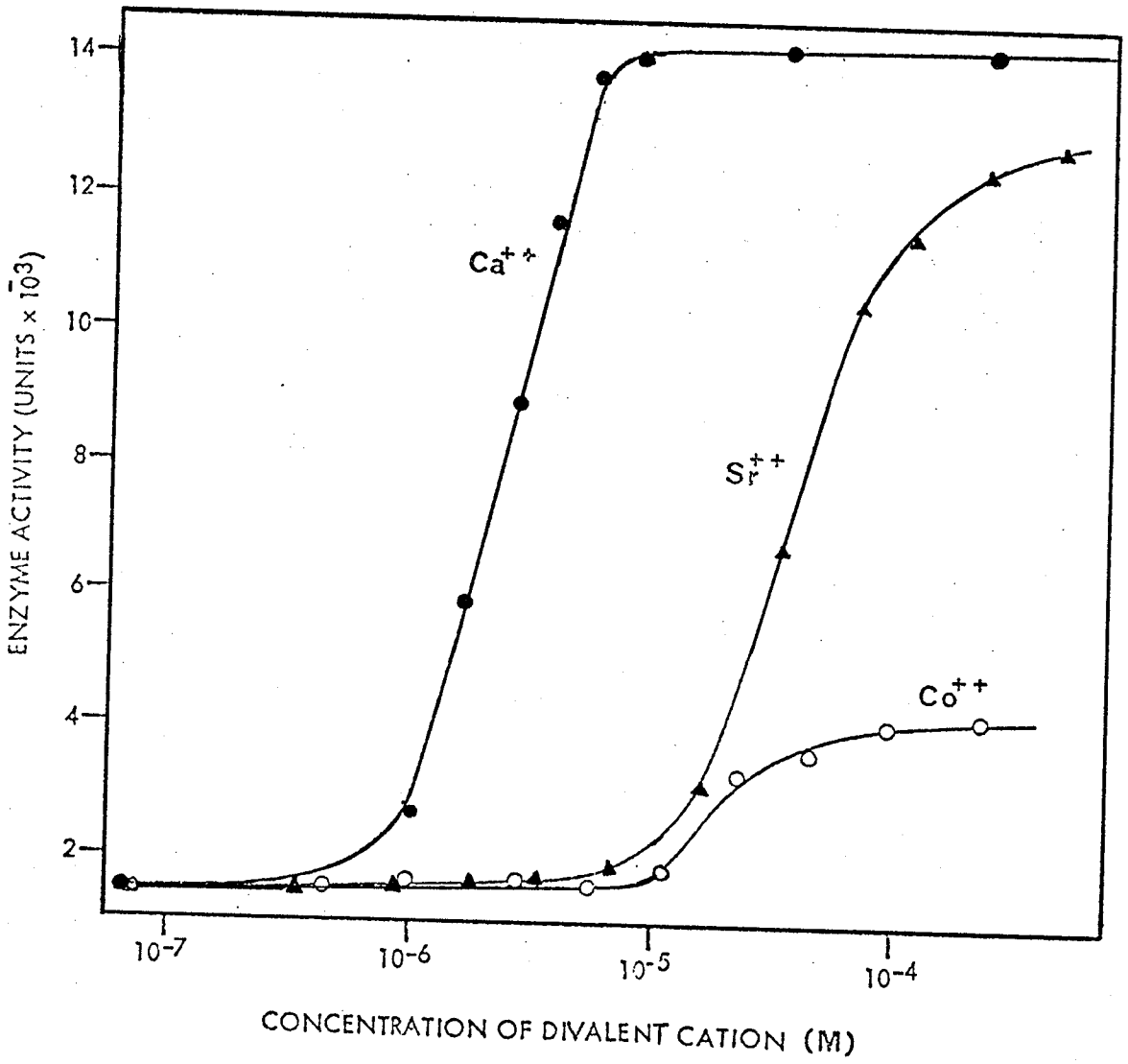


Fig.40 ACTIVATION OF PDE-I ENZYME ACTIVITY BY DIVALENT CATION IN THE PRESENCE OF EXCESS PROTEIN ACTIVATOR AND Mg^{2+} (3 mM). Experimental conditions were identical to those for fig.38.

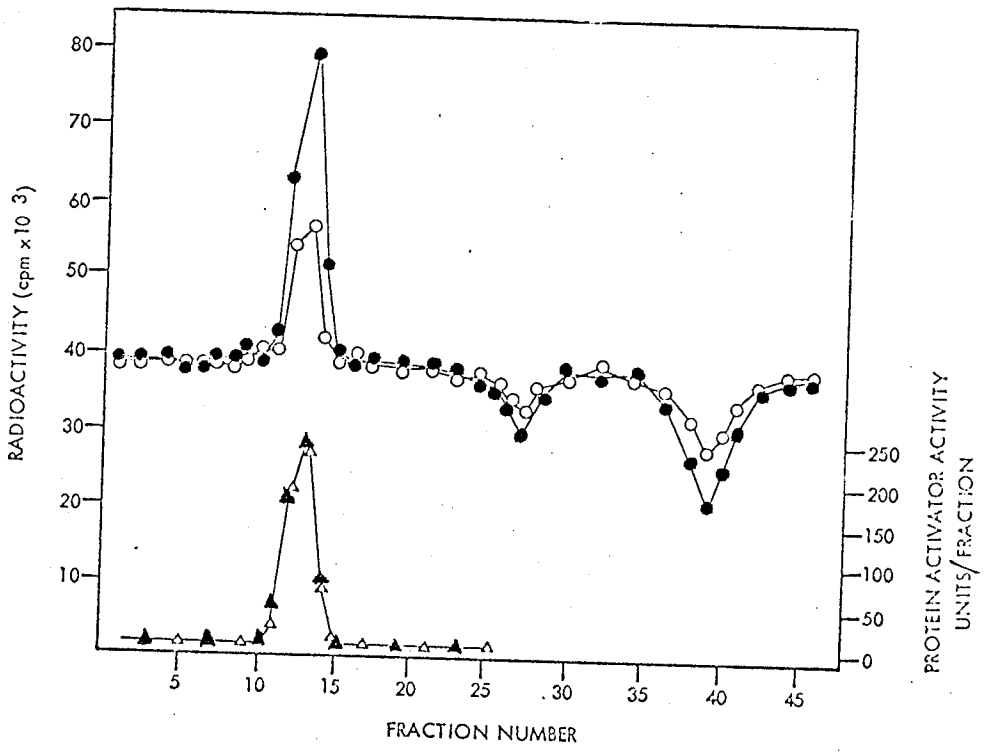


Fig.41 SEPHADEX G-25 ELUTION PROFILE FOR THE MEASUREMENT OF CALCIUM BINDING BY THE PROTEIN ACTIVATOR - The procedure used is described in "Methods". The radioactivity of ^{45}Ca in the fractions eluted with buffer containing 0.71 uM Ca^{2+}, ●—●; and that eluted with buffer containing 0.71 uM Ca^{2+} plus 500 uM Sr^{2+}, ⊖—⊖;. Protein activator activity in fractions eluted with buffer containing 0.71 uM Ca^{2+}, ▲—▲ ; and that in fractions eluted with buffer containing 0.71 uM Ca^{2+} plus 500 uM Sr^{2+}, △—△. The same amount of protein activator (86.4 ug) was used in both sets of experiments.

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

In a preceding section, it has been shown that Sr^{2+} can replace Ca^{2+} (see Table 7 and Fig. 40) in the activation of PDE-I. This metal ion, therefore, is expected to compete for Ca^{2+} binding sites on the protein activator if Ca^{2+} binding was indeed involved in the enzyme activation. As is shown in Fig. 41, binding of Ca^{2+} to the protein activator may be significantly reduced in the presence of 500 μM Sr^{2+}. At a Ca^{2+} concentration of 0.7 μM, the amount of Ca^{2+} bound per mole of protein was reduced from 0.205 in the absence of Sr^{2+} to 0.092 in the presence of Sr^{2+}. Although the result does not show that the two cations compete for the same binding site, it does agree with such an interpretation.

The stoichiometry of the interaction between Ca^{2+} and the protein activator and the dissociation constant for the complex have been determined from a Scatchard plot (Fig. 42). The Scatchard plot consists of two linear regions having two different slopes. The result suggests that there are two types of Ca^{2+} binding sites on the protein activator having different affinities. From the slopes, the dissociation constants of Ca^{2+} in the high- and low-affinity sites are calculated to be 2.9 and 11.9 μM, respectively. Since kinetic studies indicate that the Ca^{2+} concentration required for half maximal enzyme activation at a saturating concentration of the protein activator was 2.3 μM, it may

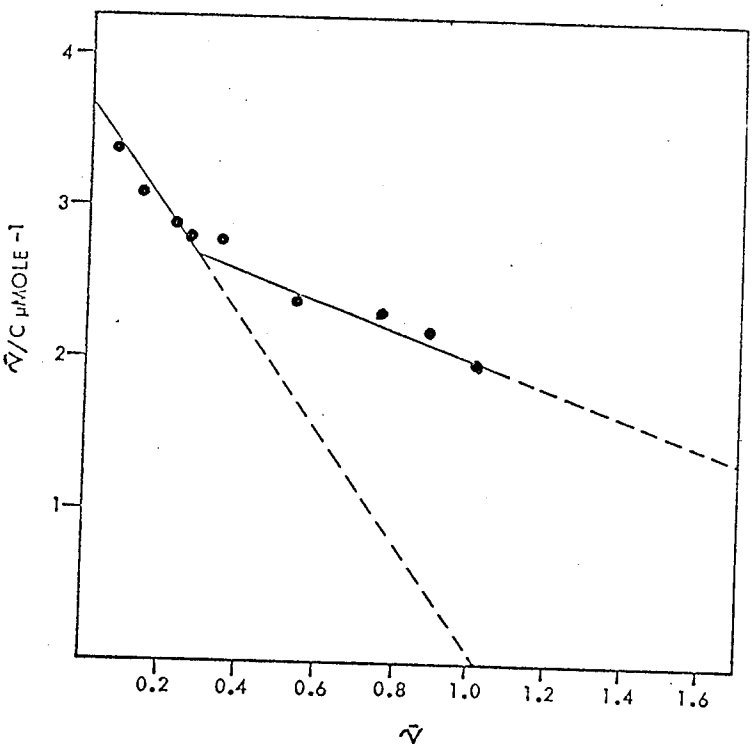


Fig.42 SCATCHARD PLOT FOR BINDING OF CALCIUM BY PURIFIED PROTEIN ACTIVATOR - $\bar{\nu}$, the mole of Ca^{2+} bound per mole of purified protein activator was calculated from the area under the peak region of the radioactivity profile (see fig.41). c is the concentration of Ca^{2+} in the buffer with which the protein activator was in equilibrium during the calcium binding experiments.

be suggested that only the high-affinity Ca^{2+} binding site is involved in the enzyme activation. The stoichiometry of the interaction between Ca^{2+} and the protein activator may be calculated from the intercepts on the horizontal axis of the Scatchard plot. Extrapolated lines for the high- and low-affinity sites intercepted at 1.04 and 3.25 moles per mole of the protein activator, respectively. This indicates that there is one high-affinity Ca^{2+} binding site and 2 to 3 low-affinity Ca^{2+} binding sites per molecule of the protein activator.

It has been observed by many investigators (53, 57) that high concentrations of Mg^{2+} (above 20 mM) inhibit PDE enzyme activity. High concentrations of Mg^{2+} were observed to inhibit PDE-I (Fig. 43A) but to have no effect on PDE-II (Fig. 43B). Fig. 43A also shows that additions of adequate amounts of Ca^{2+} can completely reverse this inhibition by high Mg^{2+} concentrations.

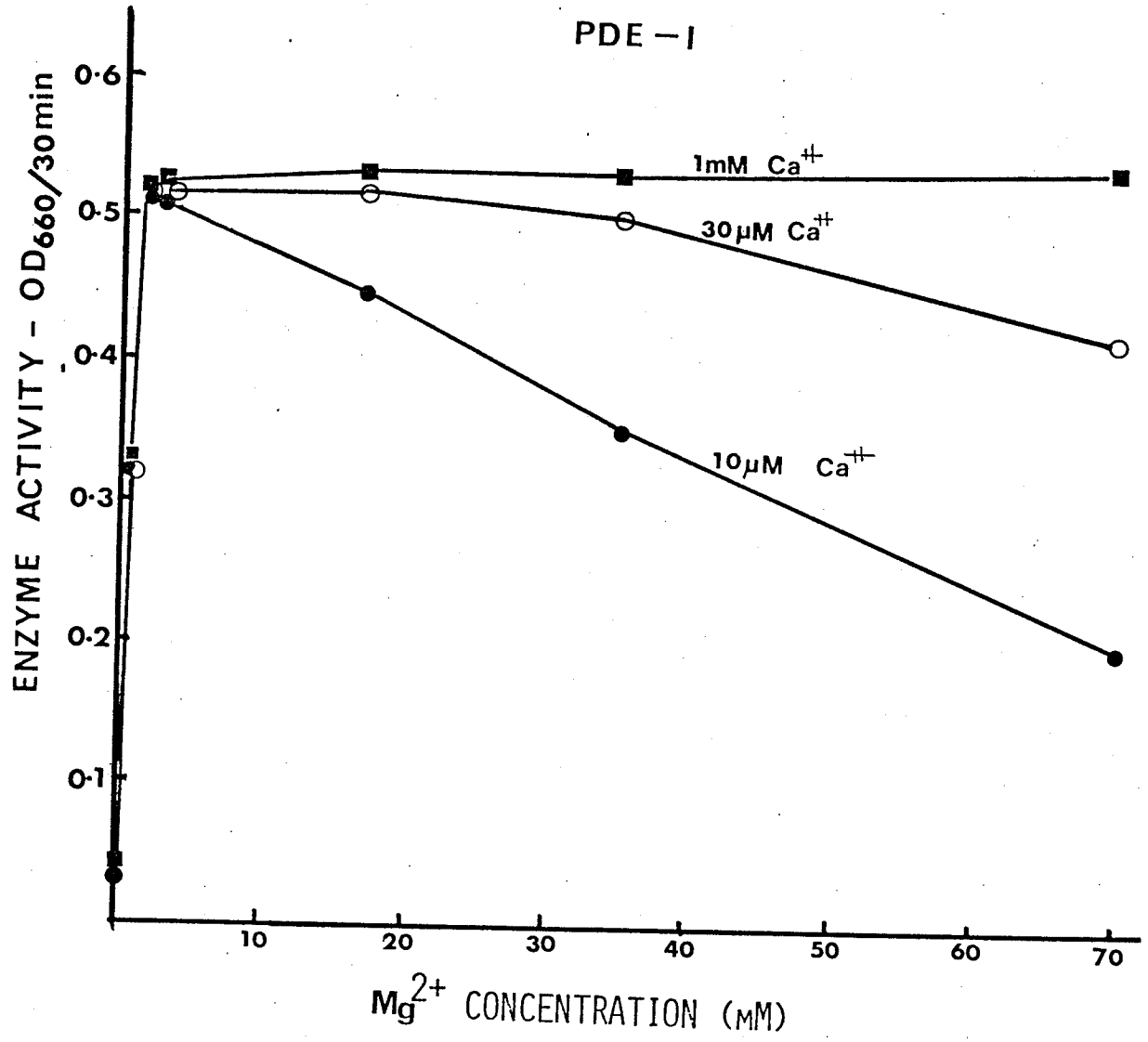


Fig.43A PDE-I ENZYME ACTIVITY IN THE PRESENCE OF DIFFERENT CONCENTRATION OF Ca^{2+} AND Mg^{2+} - PDE enzyme activity was measured by the one - stage phosphate method. The reaction mixtures contained 40 mM Tris-HCl, 40 mM imidazole pH 7.5, excess protein activator, 1.2 mM cAMP and the indicated concentrations of Ca^{2+} and Mg^{2+}. For limitations see page 119.

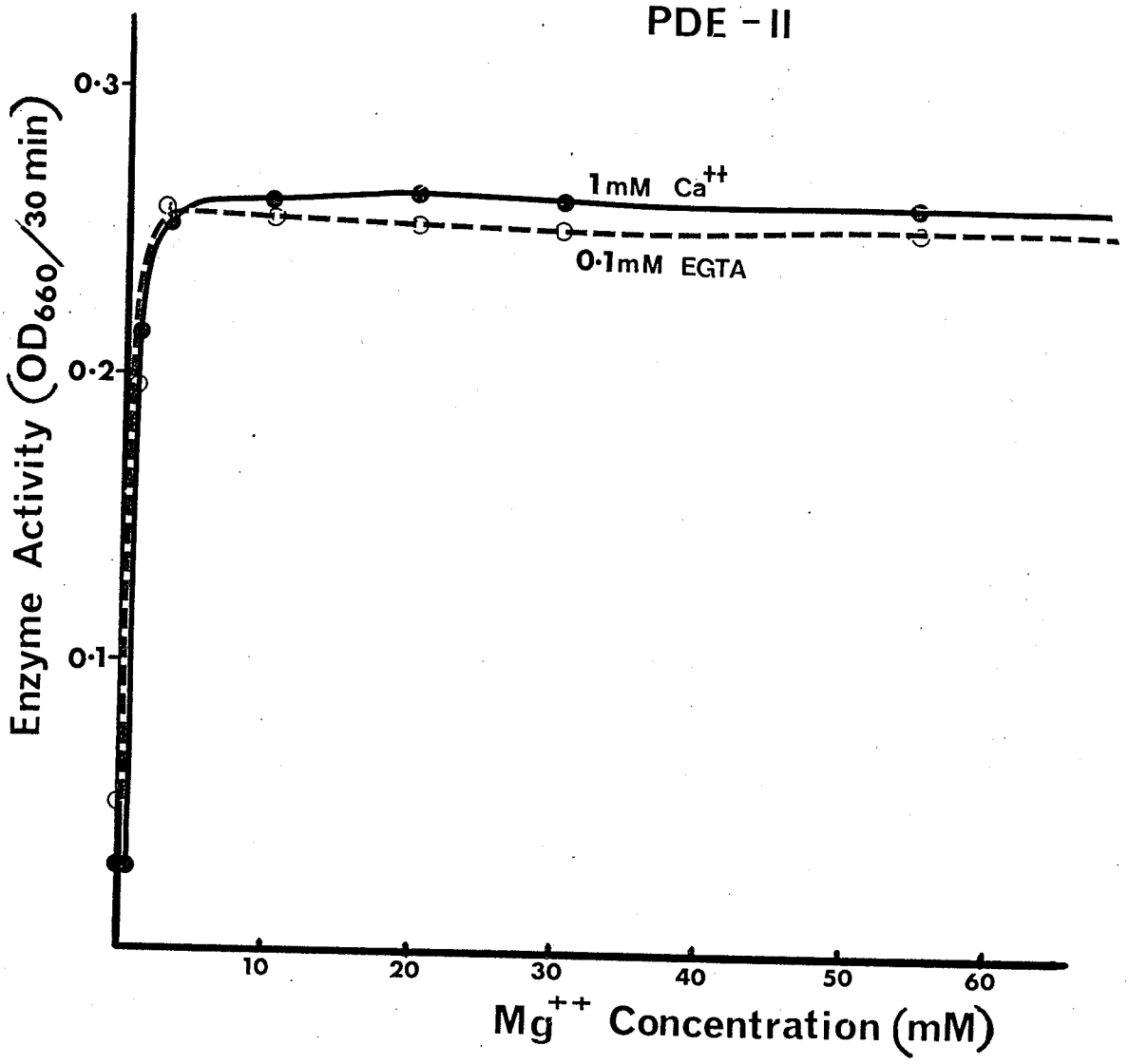


Fig.43B PDE-II ENZYME ACTIVITY IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF Ca^{2+} AND Mg^{2+} – The experimental conditions are identical to thsoe described for fig.43A

VI. DISCUSSION

The discussion is divided into six sections:

A. Multiple forms of PDE in beef heart.

B. Purification, structure and physical properties of the protein activator.

C. Interaction between PDE-I and the protein activator.

D. Molecular model of the activation of PDE-I.

E. Physiological implications.

A. Multiple Forms of PDE in Beef Heart

Hrapchak and Rasmussen (55) observed that the PDE activity in the soluble fraction of beef heart could be separated into two forms, PDE-I and PDE-II, when it was chromatographed on a column of DEAE-cellulose. We, too, found that the PDE activity in the soluble fraction of beef heart could be separated into two similar enzyme forms, PDE-I and PDE-II, by DEAE-cellulose column chromatography. While PDE-I could be activated 4 to 10 fold by Ca^{2+} and the protein activator, PDE-II was relatively insensitive to either Ca^{2+} or the protein activator. Hrapchak and Rasmussen (55) did not find any indication of a protein activator in beef heart mainly because very little effort was expended by them in looking for it. Kakiuchi and his co-workers (57) reported that the soluble PDE of rat brain could be separated by gel filtration into a lower molecule weight form of PDE (fraction II) which could be activated by Ca^{2+} and the protein activator and a higher molecular weight form of PDE (fraction I) which was insensitive to either Ca^{2+} or the protein activator. It appears that our PDE-I is similar to Hrapchak's PDE-I and to Kakiuchi's fraction II.

PDE-I, partially purified to the 1st or 2nd DEAE-cellulose stage, always possessed low but significant PDE enzymic activity in the absence of added protein activator or in the presence of excess EGTA. This "basal PDE activity" was not due to activation of the

PDE-I enzyme by residual protein activator as it was found that the concentration of protein activator in these PDE-I preparations were too low to account for it. Since the "basal PDE activity" of PDE-I preparations and the enzymic activity of PDE-II were both insensitive to Ca^{2+} or the protein activator, it would seem possible that contamination of PDE-I preparations by small amounts of PDE-II may explain the "basal PDE activity." However, PDE-II and the "basal PDE activity" of PDE-I preparations exhibited very different K_m for cAMP. Their K_m for cAMP are 54 and 1200 μM for PDE-II and for the "basal PDE activity" of PDE-I preparations, respectively (data of Dr. H. Ho, Table 8). Furthermore, our data indicate that beef heart PDE-I and PDE-II did not interconvert; they were eluted in their original volumes with no conversion into the other form when rechromatographed on DEAE-cellulose. Thus, it appears that PDE-I possessed intrinsic "basal PDE activity" which was independent of Ca^{2+} or the protein activator.

Our results show that the protein activator activated PDE-I by decreasing the K_m and increasing the V_{max} for cAMP. Dr. Honor Ho (107) in our laboratory has carried out a more comprehensive study of the kinetic parameters of PDE-I and PDE-II. Her kinetic data are shown, with her permission, in Table 8. They show that the two isoenzymes exhibited distinctly different kinetic constants. Both forms appear to have lower K_m values for cGMP than for cAMP. Furthermore,

Table 8

KINETIC CONSTANTS OF BEEF HEART PDE

(Data of Dr. Honor Ho (107))[a]

	K_m (μM)		V_{max} (relative units)[b]	
	cAMP	cGMP	cAMP	cGMP
Non-Activated PDE-I	1200	210	1	0.42
Activated PDE-I[c]	160	11	2.5	0.40
PDE-II	54	30	1	0.74

a The data shown in this table has been included in this thesis for the purpose of discussion with kind permission of Dr. Honor Ho.

b The PDE enzyme preparations used for these kinetic measurements were not pure and therefore the V_{max} values are given in arbitrary unit ; V_{max}'s of the non-activated PDE-I and PDE-II are assigned the arbitrary values of 1. Comparison of V_{max} values is valid only among the same enzyme form.

c Reactions were carried out at saturating concentrations of Ca^{2+} (0.1 mM) and the protein activator (30 units).

the protein activator decreased the K_m for cGMP by 20-fold while it decreased the K_m for cAMP by only 7-fold. These results are in agreement with the findings of Kakiuchi and his co-workers (115) who found that the protein activator from rat brain had a much greater effect on the hydrolysis of cGMP than it did on the hydrolysis of cAMP. Thus, it appears that PDE-I is more of a cGMP enzyme than a cAMP enzyme. Our results (Fig. 31, page 116) support this contention. The "basal PDE activity" of PDE-I preparations hydrolyzed cGMP faster than cAMP at all concentrations of substrates investigated. The "activated PDE activity" of PDE-I preparations hydrolyzed cGMP faster than cAMP at substrate concentrations below 40 μM and hydrolyzed cAMP faster than cGMP at concentrations above 40 μM. However, the concentrations of cAMP in the cell seldom exceed 10^{-5} M and the concentrations of cGMP are always one to two orders of magnitude below that of cAMP. Thus, the observed relative rates of hydrolysis of the two substrates observed *in vitro* may not reflect their relative hydrolysis rates *in vivo*.

B. Purification, Structure and Physical Properties of the Protein Activator

The protein activator exists in very low concentrations in beef heart; there being only 12 to 18 milligrams of protein activator present in each kilogram of beef heart. In spite of its very low specific activity in the crude extract, the protein activator could be purified

to a state of near homogeniety, owing partly to its remarkable heat stability.

The beef heart protein activator appears to be a single polypeptide made up of 127 to 129 amino acids and having a molecular weight of approximately 15,000. Cysteine residues are absent and consequently there are no disulfide linkages. There are only two proline residues per molecule of protein activator. It is well known that proline residues impede the formation of α-helix in polypeptides. It would be of interest in this context to determine the α-helix content of the protein activator by studying its circular dichroic spectrum. Cheung and his co-workers (116) have very recently reported the purification to homogeniety of the bovine brain protein activator. The bovine brain protein activator appears to possess an amino acid composition, molecular weight and isoelectric point very similar to that of the protein activator from bovine heart. Thus, the protein activator from the two tissues of the same animal appears to have the same or similar structure. It is known that the protein activator and PDE from different tissues of the same animal and even from different species can cross-react (53).

Wang et al. (107) have made a comparison of the physical and chemical properties of the beef heart protein activator and the rabbit skeletal muscle troponin TN-C (110, 111, 117). Both proteins have similar molecular weights and UV spectra, bind Ca^{2+} at high affinities

and possess remarkably similar amino acid compositions. Wang et al. (107) have determined that troponin TN-C and the protein activator are, however, not the same protein since (a) a sample of pure TN-C was found to be inactive towards the activation of beef heart PDE-I and (b) the two proteins did not have identical tryptic peptide maps. Although the tryptic peptide maps were not identical, they had similar patterns and 3 out of a total of sixteen tryptic peptides of TN-C were found to have identical amino acid composition to corresponding tryptic peptides of the protein activator. Thus, there is a good possibility that homology exists between the two Ca^{2+}-binding proteins. Recently, troponin TN-C, parvalbumins and alkali-light chain of myosin have been shown to be homologous (117, 118). Thus, these proteins, along with the protein activator, form a family of homologous proteins of diverse function.

C. Interaction Between the Protein Activator and PDE-I

The extent of activation of PDE-I and the affinities of the protein activator for PDE-I were found to be dependent on the concentration of the substrate. The mechanism by which the concentration of substrate influences the interaction of the protein activator and PDE-I is still a subject of speculation. Cheung (71) had determined that the bovine heart protein activator did not bind cAMP. Thus, the effect of substrate concentration on the interaction is not mediated via the protein activator. The possibility exists that cAMP or cGMP

binds to a regulatory site on the PDE-I enzyme, and this results in a conformational change in the enzyme molecule leading to a change in its affinity for the protein activator.

Based on experiments carried out in EGTA - Ca^{2+} buffer, Kakiuchi and his co-workers (60) have concluded that a Ca^{2+}-dependent cAMP phosphodiesterase is present in rat brain. In the present study, a similar enzyme, PDE-I, is also found in beef heart. Brostrom et al. (88), in their study of Ca^{2+} activation of phosphorylase kinase, have suggested the use of Ca^{2+}-free reagents rather than EGTA - Ca^{2+} buffer to control the concentrations of free Ca^{2+} in the study of Ca^{2+} effects on the enzymes. This is because EGTA also chelates other metal ions, and the possibility of a direct effect of EGTA on the enzyme activity is difficult to rule out completely. In addition, Ca^{2+} concentrations in enzyme assays can be more accurately determined if Ca^{2+}-free reagents are used. For these reasons, we have used a Ca^{2+}-free system in our study on the interaction of PDE-I and the protein activator.

Kakiuchi and his co-workers (56) observed that activation of rat brain PDE (fraction II) was absolutely dependent on low concentrations of Ca^{2+} but that the activation by Ca^{2+} was only partially dependent on the protein activator since activation of the enzyme by Ca^{2+} was shown to be quite appreciable in the absence of added protein activator. Furthermore, they demonstrated that, in the presence of

protein activator, a significantly lower concentration of Ca^{2+} was needed to activate the enzyme (56). These observations are confirmed and extended in the present study with the bovine heart PDE-I enzyme. Our data show that the activation of beef heart PDE-I required the simultaneous presence of both Ca^{2+} and the protein activator. These findings may be explained by postulating that PDE-I is activated by the complex of the protein activator and Ca^{2+}. Such a postulate is supported by the observation that the purified protein activator binds Ca^{2+}. It is significant that the observed dissociation constant for the Ca^{2+} bound at the high affinity site of the protein activator is very similar to the concentration of Ca^{2+} required to give half maximal activation of PDE-I in the presence of excess protein activator. Furthermore, Sr^{2+}, which can substitute for Ca^{2+} in the enzyme activation, was shown to reduce the Ca^{2+} binding to the protein activator. Binding of Ca^{2+} at the high-affinity site appears to induce a conformational change in the protein activator. The conformational change results in changes in its physical properties viz : UV spectra, fluorescence spectra, heat stability and resistance to tryptic digestion. Of the nine divalent cations investigated, Ca^{2+} was most effective in the activation of PDE-I, being able to activate the enzyme to the greatest extent at the lowest concentration.

Many investigators (53, 57) in the past have noticed that Mg^{2+}, at concentrations in excess of 20 mM, partially inhibited the activity

of cAMP phosphodiesterase. Beef heart PDE-I was inhibited to a considerable extent (60% inhibition at 70 mM Mg^{2+}) by high concentrations of Mg^{2+}. This inhibition could be completely reversed by the addition of Ca^{2+}. This suggests that Mg^{2+}, at very high concentrations, inhibits PDE-I activity by competing with Ca^{2+} for the calcium binding site on the protein activator. Whereas the binding of calcium to the protein activator results in the activation of the protein activator, the binding of magnesium does not result in a corresponding activation. This hypothesis is supported by the observation that high concentrations of Mg^{2+} had no effect on the activity of PDE-II (Fig. 43B) or on the "basal PDE activity" of PDE-I. (Both enzyme activities were independent of either Ca^{2+} or the protein activator.) Kakiuchi and his co-workers (57) have demonstrated that, when the concentration of Mg^{2+} was lowered from 10 mM to 0.3 mM, the concentration of Ca^{2+} required to give half maximal activation of rat brain PDE was lowered from about 9 μM to 1 μM.

From an analysis of the progress curves (Fig. 27 and 28) of the enzyme reaction of PDE-I under different conditions, it can be concluded that (a) PDE-I, the protein activator and the PDE-I - protein activator complex exist in equilibrium with each other and (b) the formation and dissociation of the PDE-I - protein activator complex are slow processes relative to catalysis. The experiments depicted in Figs. 27 and 28 were carried out at an early stage of our work when

the importance of Ca^{2+} was not yet realized. When these experiments were repeated with excess Ca^{2+} in the reaction mixture, lag phases in the progress curves were still observed although the lag times were shortened to approximately one to two minutes.

The reversible formation and dissociation of the PDE - protein activator complex have been demonstrated by direct means in a recent report by Kakiuchi and his co-workers (115). They showed that, when the soluble fraction of rat brain was filtered on a column of Sepharose-6B with EGTA in the eluting buffer, a PDE enzyme activity peak (molecular weight about 150,000) exhibiting only basal enzyme activity and a protein activator activity peak (molecular weight about 28,000) were obtained. When the gel filtration was repeated with Ca^{2+} in the buffer, the chromatographic profile was dramatically changed. The position of the protein activator activity peak was shifted to coincide with the PDE enzyme activity peak which, in turn, was shifted, eluting slightly earlier indicating an increase in molecular weight to approximately 200,000 suggesting that (a) PDE - protein activator complex formed in the presence of calcium and dissociated in the presence of EGTA and that (b) two molecules of protein activator binds to each molecule of PDE in the presence of Ca^{2+}. Cheung and his co-workers (116) recently reported that similar results were obtained when they repeated the experiments of Kakiuchi using PDE and protein activator from bovine brain.

D. Model of the Activation of PDE-I

Fig. 44 represents a model of the mechanism of activation of PDE-I by the protein activator. In this model, the protein activator molecule exists in two conformational states, PA (active) and PA (inactive) which are in equilibrium. Binding of one Ca^{2+} at its high-affinity binding site induces PA (inactive) to change its conformation to that of PA (active). This means that at Ca^{2+} concentrations below 1 μM, the protein activator exists mainly in the PA (inactive) form, while at Ca^{2+} concentrations above 5 μM, the protein activator exists mainly in the PA (active) form. Whereas PA (inactive) has very low affinity for PDE-I, PA (active) has high affinity for PDE-I.

Like the protein activator, PDE-I also exists in two conformational states which are in equilibrium. In the absence of the protein activator or at Ca^{2+} concentrations below 1 μM, it exists mainly in the PDE-I (basal) form. In this form it binds Mg^{2+} but not Ca^{2+} and exhibits low catalytic enzymic activity which is the "basal PDE activity." It has high affinity for the PA (active) form and very low affinity for the PA (inactive) form of the protein activator. Binding of the protein activator induces a conformational change in the enzyme giving rise to the activated form of the enzyme, PDE-I (activated), which has enhanced catalytic activity. The two forms of the enzyme are in equilibrium with each other. One molecule of enzyme binds two molecules of the activated form of the protein activator. When the

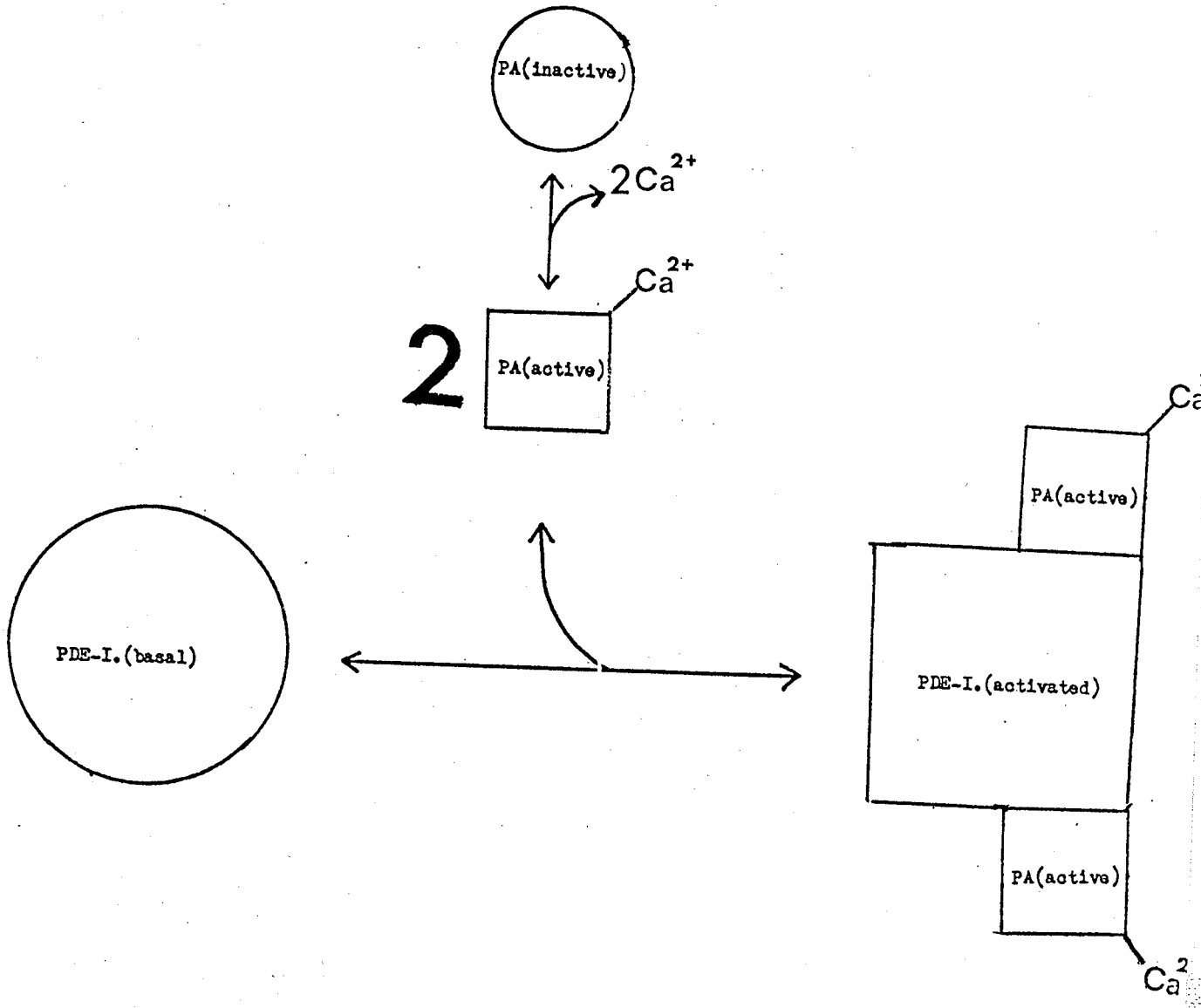


Fig.44 MODEL OF THE MECHANISM OF ACTIVATION OF PDE-I

PA(inactive) = inactive form of protein activator

PA(active) = active form of protein activator

PDE-I(basal) = Non-activated form of PDE-I enzyme which by itself exhibit slight PDE activity ; "basal PDE activity"

PDE-I(activated) = activated form of PDE-I (activated by protein activator and Ca^{2+})

Ca^{2+} of the protein activator, in the complex of the protein activator and the enzyme, is removed by chelation with EGTA, the complex breaks down into its component parts.

This model does not exclude the possibility that Ca^{2+} can activate PDE-I directly in the presence of the protein activator. Dr. H. Ho in our laboratory has determined that highly purified preparations of PDE-I did not bind Ca^{2+} (107). It therefore appears that the activation of PDE-I by Ca^{2+} is mediated in an obligatory manner by the protein activator.

Dr. Honor Ho has indirect evidence (107) to show that PDE-I undergoes a change in conformation upon binding of the protein activator. She found that PDE-I was relatively stable at 55 ^{o}C and pH 7.5 either in its free state or when one of the activators was present. In the presence of both Ca^{2+} and the protein activator, the enzyme rapidly lost its activity. The rate of thermal inactivation for the complexed enzyme was about 7 times higher than that for the free enzyme. Thus, indirect evidence is available to show that the protein activator upon binding Ca^{2+}, and PDE-I upon binding the protein activator, undergo conformational change.

E. Physiological Implications

The fact that the functions and the metabolism of cAMP and Ca^{2+} are closely related has been pointed out in a review by Rasmussen et al. (83). In the heart, both Ca^{2+} and cAMP have been implicated in

the hormone-regulated myocardial contraction. Several groups of investigators (119, 120, 121) have suggested that cAMP may control the free Ca^{2+} level in cardiac muscle by facilitating the inflow of Ca^{2+} into the cell. Kirchberger et al. (122) have shown that the uptake of Ca^{2+} may be stimulated by cAMP in the presence of cAMP-dependent protein kinase. This observation may suggest that the inotropic effect of epinephrine is mediated by an effect of cAMP on Ca^{2+} metabolism. The present observation that cAMP phosphodiesterase from bovine heart is activated by Ca^{2+} suggests that the concentrations of cAMP could in turn be regulated by Ca^{2+} in cardiac muscle. That this Ca^{2+} activation of the enzyme is operative in intact hearts is supported by the observations of Namm et al. (123) that cAMP concentrations in rat hearts can be increased or decreased upon perfusion of the hearts with a Ca^{2+}-free or a Ca^{2+}-rich medium, respectively. It should be pointed out that Ca^{2+} has only a slight effect (generally inhibition) on adenylate cyclase (124). The range of Ca^{2+} concentration effective in the activation of cAMP phosphodiesterase, 1 to 10 μM, also suggests that this Ca^{2+} activation may have an important regulatory role in the cardiac contraction. It has been suggested (125) that the Ca^{2+} level during the myocardial contraction cycle fluctuates in the range of 0.1 to 10 μm. Although it is not clear as to how Ca^{2+} activation of cAMP phosphodiesterase contributes to the regulation of myocardial contraction, it could be an important mechanism for the removal of the

cAMP used for the excitation of the muscle. In addition, the Ca^{2+} activation of cAMP phosphodiesterase could even be involved in the control of myocardial contraction in the absence of the hormonal stimulation. Brooker (126) has recently demonstrated the fluctuations of cAMP concentration during the contraction cycle of electrically stimulated frog ventrical strips.

Of the two forms of PDE in the soluble fraction of beef heart, activated PDE-I exhibited 2 to 3 times higher K_m for cAMP than PDE-II (Table 8). Furthermore, the in vivo concentrations of cAMP seldom rises above 10^{-5} M. The question therefore arises as to whether PDE-I and the protein activator play a significant role, if any, in controlling the in vivo levels of cAMP. To resolve this question, one must consider not just the relative K_m for cAMP but also the relative V_{max} for the hydrolysis of cAMP by the two isoenzymes. It is not possible, at this juncture, to make a meaningful comparison of the V_{max} of PDE-I and PDE-II from the data of Table 8 since the preparations of both PDE-I and PDE-II were not pure. Thus, a definitive answer to the above question awaits the purification of both isoenzymes to homogeniety.

On the other hand, there is no doubt that PDE-I and the protein activator play an important role in the in vivo metabolism of cGMP. First of all, the K_m for cGMP of PDE-I (11 μM) is three times lower than the corresponding K_m for cGMP of PDE-II (Table 8) and secondly,

the protein activator has been shown to have a greater effect on the hydrolysis by PDE-I of cGMP than cAMP. However, the biological role of cGMP in cardiac muscle is as yet not clear and consequently the role of PDE-I and the protein activator in this respect remains to be resolved.

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Appendix A

Sedimentation Equilibrium Method For The Determination Of Molecular Weight

The sedimentation equilibrium method studies the concentration distribution of molecules at equilibrium positions. At equilibrium, the total potential of any component in the centrifuged solution is constant. The total potential can be defined as the sum of the solution's chemical and centrifugal potentials, its chemical potential being a function of concentration and pressure and its centrifugal potential being a function of the centrifugal field and thus of the position in the cell.

If appropriate expressions are supplied for the effect of concentration (RT/c), pressure (Mv) and distance ($-Mw^2r$) and if the derivative of the total potential is set to zero, the following equation can be written :

$$M = \frac{2RT}{(1 - \bar{v}\rho)w^2} \frac{d\ln c}{d(r^2)}$$

where M = molecular weight

R = gas constant (8.313×10^7 ergs/degree mole)

T = absolute temperature

$\bar{v}$ = partial specific volume

ρ = density of solution

w = angular velocity

c = concentration

r = distance from axis of rotation (radial distance)

In fig.16, since the temperature was maintained at a constant level,

all the terms in the above equation were constants except for "c" and "r". Hence a plot of ln c against r^2 is a straight line if the protein activator sample was homogenous. The concentration of the protein activator in the centrifugal cell was expressed in terms of fringes.