ROLE OF PHOSPHODIESTERASE AS IT RELATES TO FLUID SECRETION IN *AMBLYOMMA AMERICANUM* (L.) SALIVARY GLANDS

By

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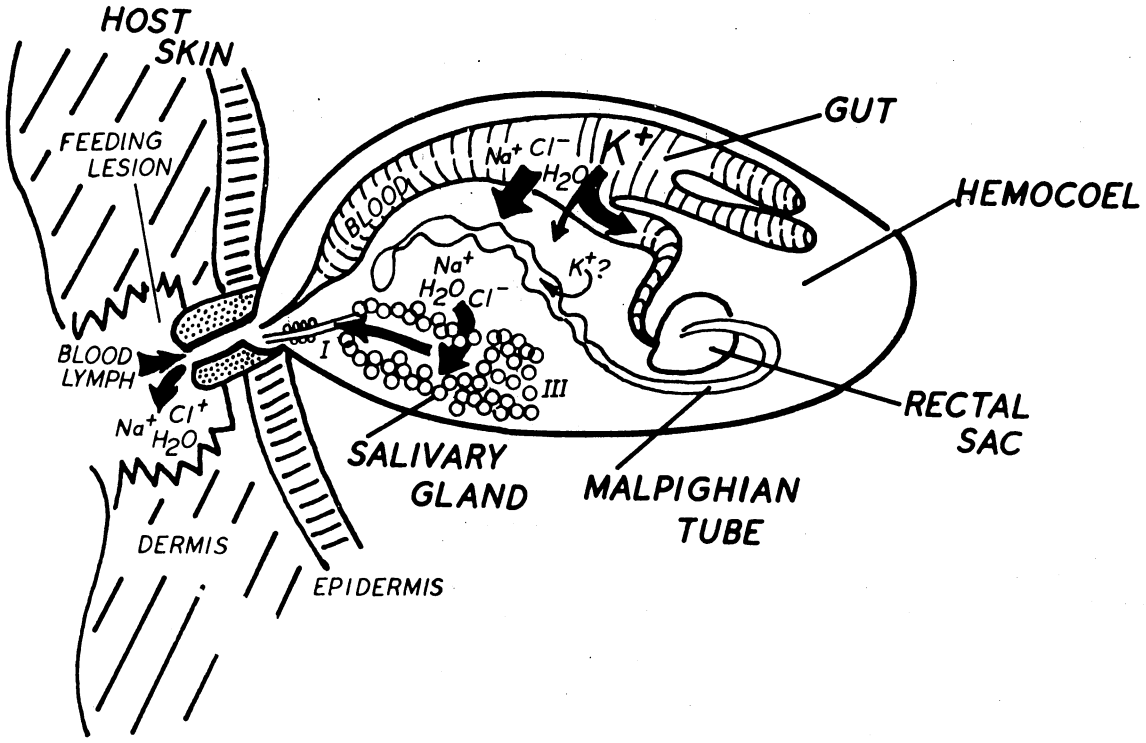
CHAPTER I

INTRODUCTION

The ixodid tick Amblyomma americanum (L.) is an animal of great economic importance. Losses to livestock as a result of lone star tick infestation total millions of dollars each year. If the biology and physiology of this animal were better understood, one might expect to devise better ways to control tick populations. Investigations into the various organ systems of ticks may reveal certain sites which are susceptible to disruption and provide a means of controlling ticks. The multifunctional nature of the salivary glands might well be such a site. Various functions of the salivary glands include its role as the primary organ of osmoregulation in feeding by ixodid ticks. Another role is the secretion of cement material, cytolytic products, anticoagulants, enzymes, pharmacological agents, toxic substances. They are also thought to aid in the process whereby nonfeeding ticks absorb water from unsaturated air (Sauer 1977).

We have found that as the female tick feeds on the host animal, (Fig. 1) the rates of fluid secretion increase greatly thus enabling the tick to concentrate its bloodmeal by returning excess water and ions to the host via the salivary glands. It seems likely that fluid secretion is controlled by nerves with a catecholamine being the secretagogue (Sauer 1977). There is increasing evidence that cyclic AMP (cAMP) and Ca^{2+} play important intracellular roles in mediating the catecholamine

Figure 1. Diagram showing a longitutional section of a feeding ixodid tick and detailing the move ment of fluid and ions; the bloodmeal is first concentrated in the gut by removal of much of the fluid and ionic content. Excess fluid and ions are then absorbed by the salivary glands and expelled back into the host. The salivary glands of hard ticks serve as their major osmoregulatory organs. (Diagram) courtesy Glen R. Needham, Dept. of Ent., Ohio State University.


HOST SKIN
FEEDING LESION
BLOOD LYMPH
Na+ Cl+ H2O
DERMIS
EPIDERMIS
SALIVARY GLAND
I
III
Na+ H2O Cl-
BLOOD
Na+ Cl- H2O
K+
K+?
GUT
HEMOCOEL
RECTAL SAC
MALPIGHIAN TUBE

regulation of fluid secretion (Needham and Sauer 1979). When the glands are stimulated by dopamine, cAMP levels increase (Sauer et al. 1979). In addition, adenylate cyclase activity increases when glands are stimulated by dopamine (Schmidt et al. unpublished). Fluid secretion is inhibited when Ca^{2+} is deleted or the Ca^{2+} antagonist verapamil is added to the bathing medium (Needham and Sauer 1979) suggesting a role for Ca^{2+} in stimulus-secretion coupling. If glands are stimulated with exogenous cAMP, fluid secretion is much greater if the glands are bathed in a medium with millimolar amount of free Ca^{2+}, as compared to bathing glands with micromolar levels of free Ca^{2+}. This difference is not observed in glands obtained from ticks in the slow phase of feeding (Sauer et al. 1979).

It is likely that cAMP plays an important role in regulation of salivary gluid secretion; therefore, knowledge of how cAMP levels are regulated is important in understanding the overall mechanism of fluid secretion. Cyclic AMP is hydrolized to 5'AMP by phosphodiesterase with a resulting termination of its effects. Cyclic nucletoide phosphodiesterase has been characterized in many different tissues but modulation of its activity during tissue or organ development is not well understood. Because ixodid salivary glands are known to undergo dramatic anatomical (Binnington 1978) and physiological changes while the tick is feeding on the hosts the glands provide a unique system in which to observe such changes in molecular events related to changes in a physiological process (Megaw and Beadle 1979). The present study on tick salivary glands was designed to characterize phosphodiesterase and determine its regulation and role in fluid secretion.

Cyclic Nucleotide Phosphodiesterase (PDE)

Regulation

The regulation of the PDE system is complex and may involve a number of different control mechanisms including hormones: endogenous protein activators; certain ions; genetic controls; cyclic nucleotides; enzyme interconversions (Pledger et al. 1976); inhibition by nucleotide triphosphates and pyrophosphate; protein-lipid interactions; and by other proteins such as trypsin (Albin et al. 1975).

Distribution

PDE activity is found in virtually all living cells, its relative activity varying greatly among different tissues (Weiss and Costa 1968). Although PDE is largely a soluble enzyme, some investigators have suggested that the particulate form may be of greater physiological importance because this fraction usually contains a high affinity (low Km) form of the enzyme (Amer and Krieghbum 1975). However, the soluble fraction may also contain a high affinity form of PDE (Weiss and Strada 1972). Additionally, there may be intracellular compartmentalization of cAMP and its hydrolyzing enzyme (Pan et al. 1974).

Substrate Specificity

PDE's are not completely substrate specific because some purified enzymes hydrolize other cyclic nucleotides (i.e. cGMP, cCMP) but the different isozymes of PDE do have different relative affinities for the various cyclic nucleotides (Brostrom and Wolf 1976).

Kinetic Properties

PDE exhibits anomolous kinetic behavior, suggesting the existence in some animals of a single enzyme form with the property of negative and positive cooperativity. Another possibility is the existence of multiple molecular forms with different affinities for cAMP (Strada et al. 1974). The evidence suggests that both possibilities exist, because different molecular forms of PDE have been isolated from some tissues and while in other systems a highly purified enzyme exhibits both negative and positive cooperativity (Weiss and Hait 1977). The latter finding suggests that there are allosteric sites on the enzyme that influence the catalytic site and effects the Km. It has been hypothesized that the low Km forms of the enzyme in either case are responsible for maintaining basal levels of cAMP and that the high Km forms control the cAMP concentration after hormonal stimulation of adenylate cyclase (Weiss and Hait 1977).

Metal Requirements

Drummond and Perrott-Yees (1961) found that PDE required Mg^{2+} ions. Metal ions such as Mn^{2+} were found to substitute for Mg^{2+} but usually only Mg^{2+} is present in high enough concentration in the cell to be of physiological significance. Calcium along with Mg^{2+} is also usually necessary for complete activation of PDE. Ca^{2+} concentrations of 1×10^{-6} to 1×10^{-5} M are usually sufficient for maximal PDE activity while Mg^{2+} levels of 3×10^{-4} to 3×10^{-3} M are usually required for maximal activity (Kakiuchi et al. 1972).

Multiple Molecular Forms

The existence of multiple molecular forms of PDE has been demonstrated in many tissues by numerous investigators using a variety of techniques (Wells et al. 1975). The pattern and ratio of the PDE isozymes vary with specific tissues and cell type and molecular weights range from 60,000 to 400,000 (Weiss and Hait 1977). In general, most investigators have found three types of phosphodiesterase in the tissue being studied (usually vertebrate).

The following discussion on molecular forms is general in nature and drawn from numerous sources. Some of the more recent reviews on phosphodiesterase are by Thompson and Strada (1978), Weiss and Hait (1977) and Wells and Hardman (1977).

Endogenous Activator

While purifying PDE from heart, Cheung (1970) noticed that PDE activity decreased as the enzyme became more pure, suggesting that a factor that activates PDE was being removed during the purification process. This activator (calmodulin) has now been isolated and has been the subject of numerous investigations (Cheung et al. 1975). The activator is heat stable and is a calcium dependent protein which has a molecular weight between 11,000 and 40,000.

Cyclic GMP Phosphodiesterase

This form has a lower Km for cGMP than cAMP and a larger Vmax for cAMP than cGMP. Cyclic GMP PDE is activated by calmodulin and Ca^{2+} and is considered the high Km form of the enzyme. The activation of PDE by calmodulin is poorly understood and may affect the Vmax, Km or both. It

is thought that two molecules of calmodulin are bound to each molecule of PDE during cell activation. This form of PDE has a molecular weight of around 400,000 and often appears to be the most abundant cellular PDE.

Because of the Km and Vmax of this enzyme and the known concentration of cAMP in cells (10^{-6}M), the PDE is not saturated with substrate and hydrolysis of cAMP occurs by a first order process. Cyclic AMP levels are commonly 10 to 100 times higher than levels of cGMP. This suggests that even though the enzyme has a lower Km for cGMP the higher concentration of cAMP in the cell make it important for hydrolysis of cAMP as well. One result is an ability to help maintain a ratio of cGMP to cAMP which may be more important than absolute concentrations of cyclic nucleotides in regulating cell activity. The high Km form has been considered by some to be the most important physiologically in maintaining cAMP levels following cell stimulation and lower Km forms of the enzyme are important in controlling basal or resting levels of cAMP. Further regulation of the Ca^{2+} dependent form may result from influxes of Ca^{2+} which activate calmodulin and calmodulin-dependent enzyme. Calmodulin does not bind to PDE unless Ca^{2+} is present.

The calmodulin dependent PDE may also be activated by certain phospholipids and fatty acids, or proteolysis independent of Ca^{2+}. It is thought that calmodulin binds to a regulator subunit on phosphodiesterase but protease and lipid activated forms cannot be further ativated by calmodulin (Wang and Waisman 1980).

Two other activators (Troponin C and Parvalbumin) of PDE have been reported but are required at centrations two to three orders of magnitude higher than calmodulin. Some antipsychotic drugs inhibit PDE and their mode of action is thought to be due to binding to calmoduin.

Potencies of inhibitor activity of drugs correlate roughly to clinical antipsychotic activity and the binding is Ca^{2+} dependent.

Cyclic AMP Phosphodiesterase

This form has a molecular weight ca 200,000 and is called the low Km form because it has a high affinity for cAMP. The enzyme exhibits biphasic (non-linear) kinetics. This PDE is membrane bound and can be released by sonication of tissue preparations enabling one to isolate and purify the membrane bound forms if desired. The biphasic kinetics may arise from:

1. One enzyme with multiple sites exhibiting negative cooperativity when occupied by cAMP. In the negative cooperitivy model, PDE has two sites for binding cAMP. After the cAMP becomes bound to the first site, a conformational change in the second site makes it more difficult to bind a second molecule of cAMP. For example, in unstimulated cells cAMP is hydrolyzed by PDE using only its high affinity site, mantaining a steady state level of cAMP. Upon stimulation by hormone there is an increase in cAMP. Since cAMP must exist for a finite period of time the concentration of cAMP should not return to basal levels rapidly. This biphase kinetics of the low Km enzyme at high cAMP would allow cAMP to remain at high enough levels to cause events in the cell related to its increase concentration. It is very characteristic of low Km forms to respond to hormones.
2. One enzyme with multiple sites.

3. One enzyme changes conformation thereby changing affinity - one site.

Other Characteristics of the low Km enzyme form are:

1. cAMP and cGMP noncompetitively inhibit hydrolysis of other cyclic nucleotide.
2. The ability to hydrolyze cGMP is lost when the enzyme is solubilized from the membrane.
3. Hormones affect Vmax of the enzyme.
4. Low Km cAMP PDE's have lower molecular weights than high Km forms of PDE.
5. An "aging" or increase in activity effect is seen that is similar to that seen when the soluble high Km PDE is stored in cold ("aging") resulting in the activation of PDE and is possibly caused by proteases. Trypsin also activated the Ca^{2+} dependent form of PDE. After trypsin activation, PDE was no longer susceptible to activation by the protein activator (calmodulin). They concluded that the high Km form of PDE was hydrolyzed by trypsin causing formation of a lower molecular form with a lower Km. Furthermore, these investigators speculated that multiple forms of PDE usually seen in other tissues may exist originally as a single molecular weight form but either upon tissue treatment or physiologically significant cellular events appear as multiple forms of the enzyme.

Cyclic GMP Dependent cAMP Phosphodiesterase

This enzyme form is characterized by cGMP acting as an allosteric activator of the enzyme.

It is not clear if all of the types of PDE's that are seen in tissues are present in all cells or if they are artificatual due to salt concentrations or proteolysis or effects caused by preparatory procedures. Many examples of this enzyme changing in response to hormones have been recorded but it is difficult to relate these to physiological processes.

Summary

The major properties of cyclic nucleotide phosphodiesterase include the following: (a) PDE may exist in several different forms (isozymes) of differing moclecular weights, (b) the isozymes may be unequally distributed among various tissues, (c) different isozymes have different kinetic properties and substrate affinities, (d) at least one form of the enzyme is selectively activated by an endogenous protein (calmodulin), (e) all forms of the enzyme can be selectively inhibited by drugs such as methyl-xanthine (i.e. theophylline, caffeine), (f) various forms possess allosteric sites that can influence their activity and (g) Mg^{2+} and in some cases Ca^{2+} are necessary for full activation.

Chapters III and IV of this thesis have already been published in Experientia 34, 1030-1031; McMullen & Sauer (1978) and Biochemical & Biophysical Research Communications 95, 1555-1562; McMullen et al. 1980). These prepublished papers are thus written in a deviated format following the style of the journal in which they are published. Chapter V will eventually be submitted for publication and also follows a deviated format.

CHAPTER II

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN THE SALIVARY GLANDS OF AN IXODID TICK

INTRODUCTION

The salivary glands of the ixodid tick serve as its primary organ of osmoregulation (Kaufman 1976, Sauer 1977). The tick concentrates its bloodmeal by moving excess fluid and ions across its gut epithelium and expelling the fluid back into the host via the salivary glands. The feeding of the adult lone star, Amblyomma americanum (L.), female occurs in two stages lasting about two weeks. The tick first goes through a slow phase of feeding (weight increases from 4-300 mg) lasting until about the final 12-24 hours of attachment. During the final rapid stage of feeding, the tick increases in weight from about 300 mg to its final engorgement weight of about 800 mg.

Results of recent investigations demonstrate the cAMP has a major role in regulation of tick salivary fluid secretion while it is feeding on the host animal (Sauer et al. 1974, 1976; Needham and Sauer, 1975, 1979). Hsu and Sauer (1975) and Sauer et al. (1979) demonstrated that catecholamines stimulate in vivo fluid secretion and in vitro glands and cause an increase in intracellular concentrations of cAMP. Schmidt et al. (unpublished) found that catecholamines stimulate tick salivary gland adenylate cyclase at doses similar to those necessary to initiate fluid secretion by isolated glands. The purpose of the present

experiments was to characterize preparations obtained from A. americanum (L.) salivary glands. (The only enzyme cyclic nucleotide phosphodiesterase (PDE) known to hydrolize cAMP). Although a more involved investigation of cyclic AMP-dependent PDE and its Ca^{2+}-dependent modulators in supernatant fractions (100,000 xg) of tick salivary glands has already been reported (McMullen et al. 1980), the data presented herein describe basic characteristics of crude salivary gland PDE and provide a basis of comparison to similar enzymes in other tissues.

METHODS AND MATERIALS

Adult female lone star ticks Amblyomma americanum (L.) were reared on female sheep and used as source of salivary glands. Glands were dissected from ticks in 0.05 M Tris-HCl buffer (pH 8) containing 0.005 mM Mg^{2+} within one hour of removal from sheep. Glands were then either stored at $-60^{o}C$ or placed in small glass tissue homogenizers containing 250 µl of the same buffer and homogenized. Each gland contained between 50 and 125 µg of protein depending on stage of tick feeding (weight) from which the glands were removed.

Phosphodiesterase Assays

Fifty µl of the preparation to be assayed for phosphodiesterase was placed in a ½ dram glass vial containing appropriate amounts of substrate (cAMP) in 10 µl of 0.05 M Tris-HCl buffer and trace amounts of (^{-3}H) and then terminated by addition of 10 µl of 2 M HCl at the appropriate time. Products of the reaction were separated and measured using the paper chromatographic method of Bielinsky and Piechowska (1975). Reactions were then terminated by boiling in water bath (1 min) and

measured by the bath technique (Thompson et al. 1979).

In the experiments in which the effect of changing pH on PDE activity was investigated, various buffers were used. Citric acid NaHPO4 was used at pH below 7 while Tris-HCl was used at pH between 7 - 9 and Glycine NaOH was used at pH 9. Chelex-100 (Biorad®) colums (2 cm X 2 cm) were used to remove divalent cation buffers in experiments where an effect of Mg^{2+} or Ca^{2+} was tested. Calcium concentrations were buffered as described by McMullen et al. (1980).

RESULTS AND DISCUSSION

At concentrations of 0.1mM cAMP, PDE activity was linear with time and tissue protein concentration (Figs. 2 and 3). The temperature optimum for the crude enzyme was between 35° and 45°C (Fig. 4). The pH optimum for the enzyme was approximately 7.5 and at pH's < 6.5 or > 8 the activity was significantly reduced (Fig. 5). The data points on Figs. 2 - 5 represent the means of 4 separate experiments. Ticks in Figs. 3 - 5 weighed between .05g and .8 grams, in Fig. 7 and 8 ticks weights .3 g and .05 g.

Magnesium concentrations of 2.5 mM or larger were necessary for full activation of PDE (Fig. 6). This is similar to the Mg^{2+} found by Kakiuchi et al. (1972), for full activation of PDE. The author has previously found that Ca^{2+} is also necessary for complete activation of supernatant (100,000 xg) PDE but at much lower concentrations (10^{-7}M) (McMullen et al. 1980). Calcium and Mg^{2+} effects on crude PDE appear to occur at different sites since no interaction between effects of the two metals was obvious in Fig. 7. The data points of Fig. 6 and 7 are the means of 3 separate experiments.

Figure 2. Effect of temperature on Cyclic AMP dependent phosphodiesterase ((PDE) activity in crude homogen- ates of salivary glands obtained from female Amblyomma americanum (L.)

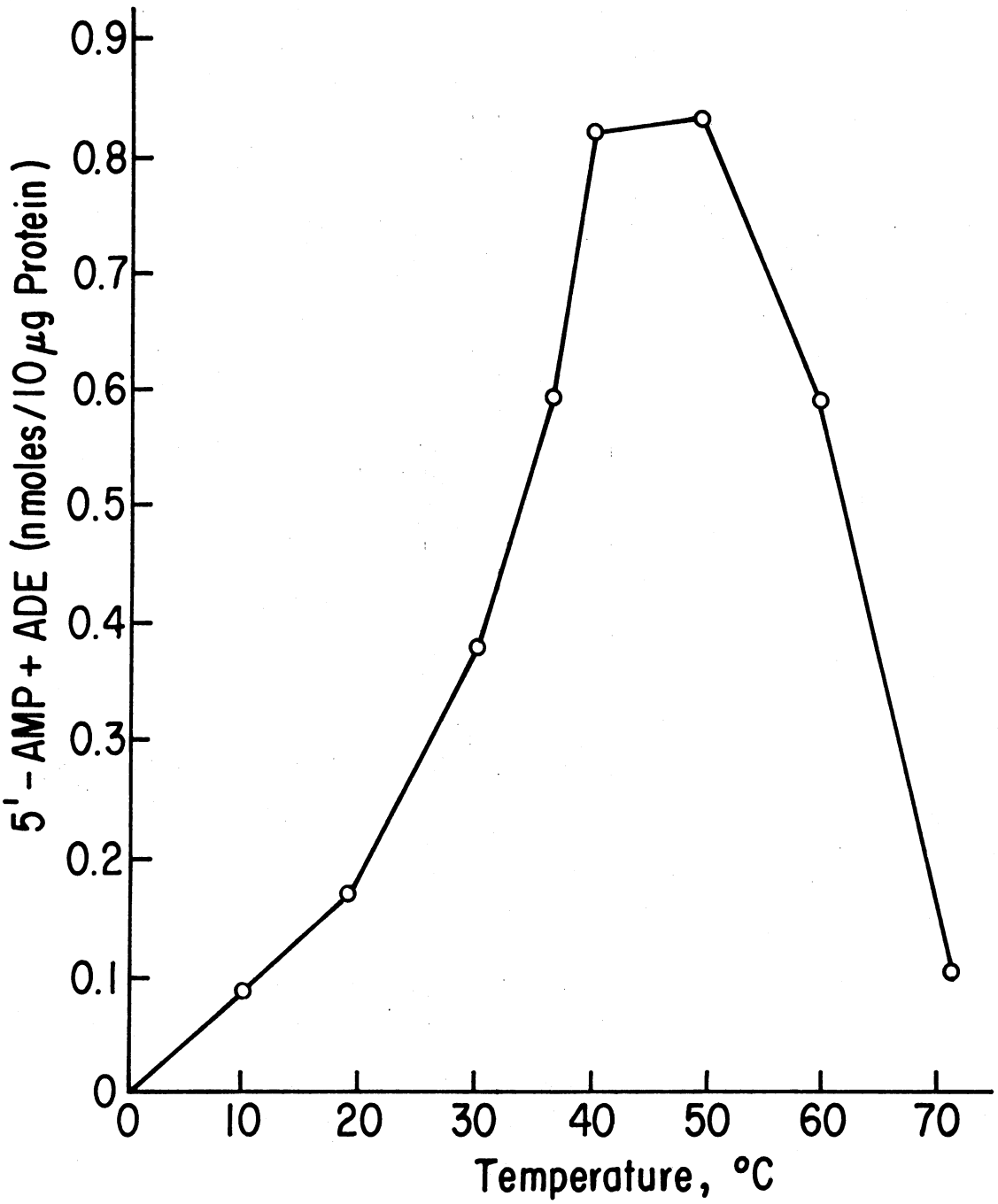

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5'-AMP + ADE (n moles/10 μg Protein)
0
10
20
30
40
50
60
70
Temperature, °C

Figure 3. Effect of Cyclic AMP dependent phosphodiesterase (PDE) pH on activity in crude hemogenate of salivary glands obtained from female Amblyomma americanum (L.)

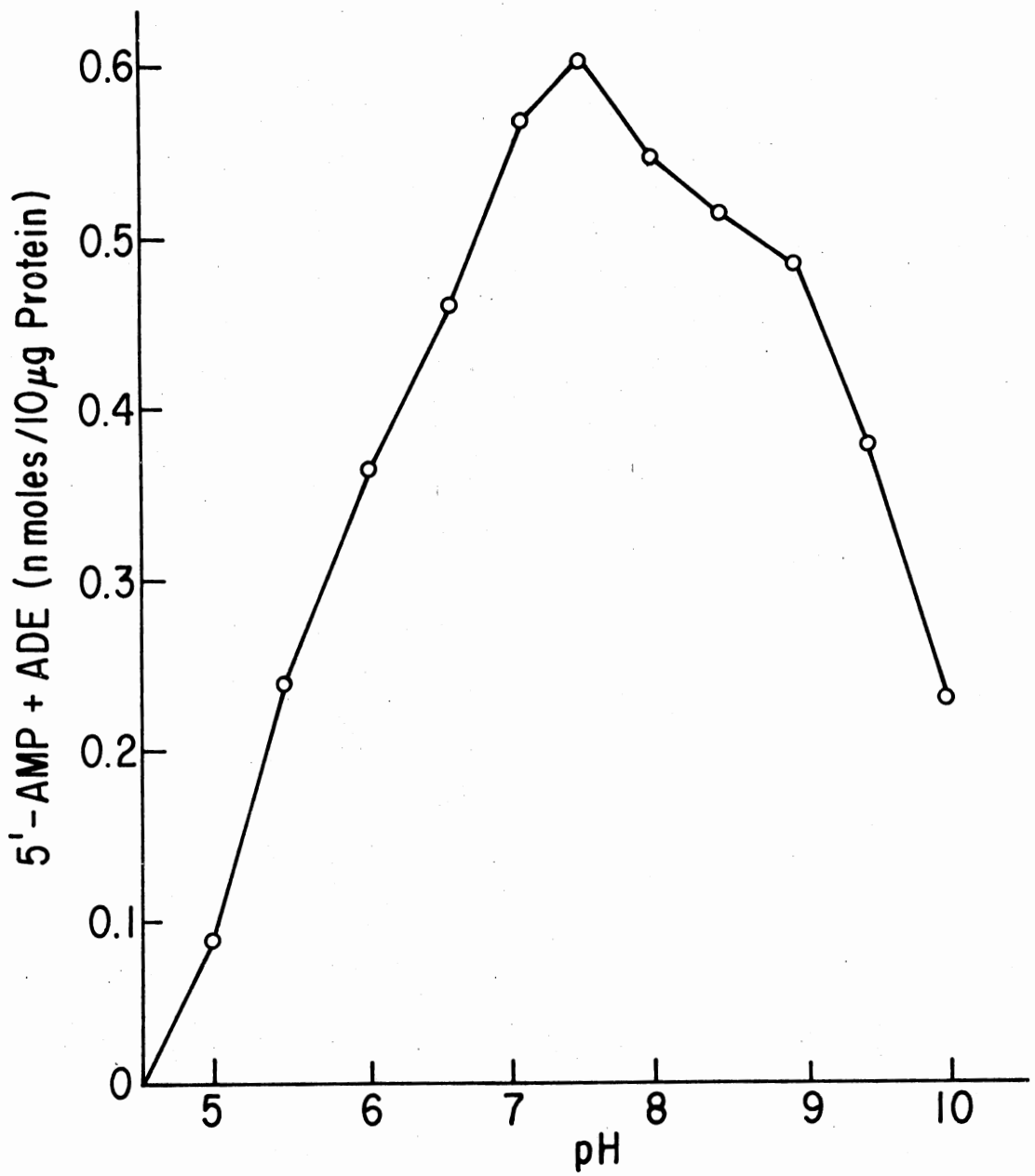

0.6
0.5
0.4
0.3
0.2
0.1
0
5'-AMP + ADE (n moles/10μg Protein)
5
6
7
8
9
10
pH

Figure 4. Time course of Cyclic AMP-dependent phosphodiesterase(PDE) on activity in crude homogenates of salivary glands obtained from female Amblyomma americanum (L.).

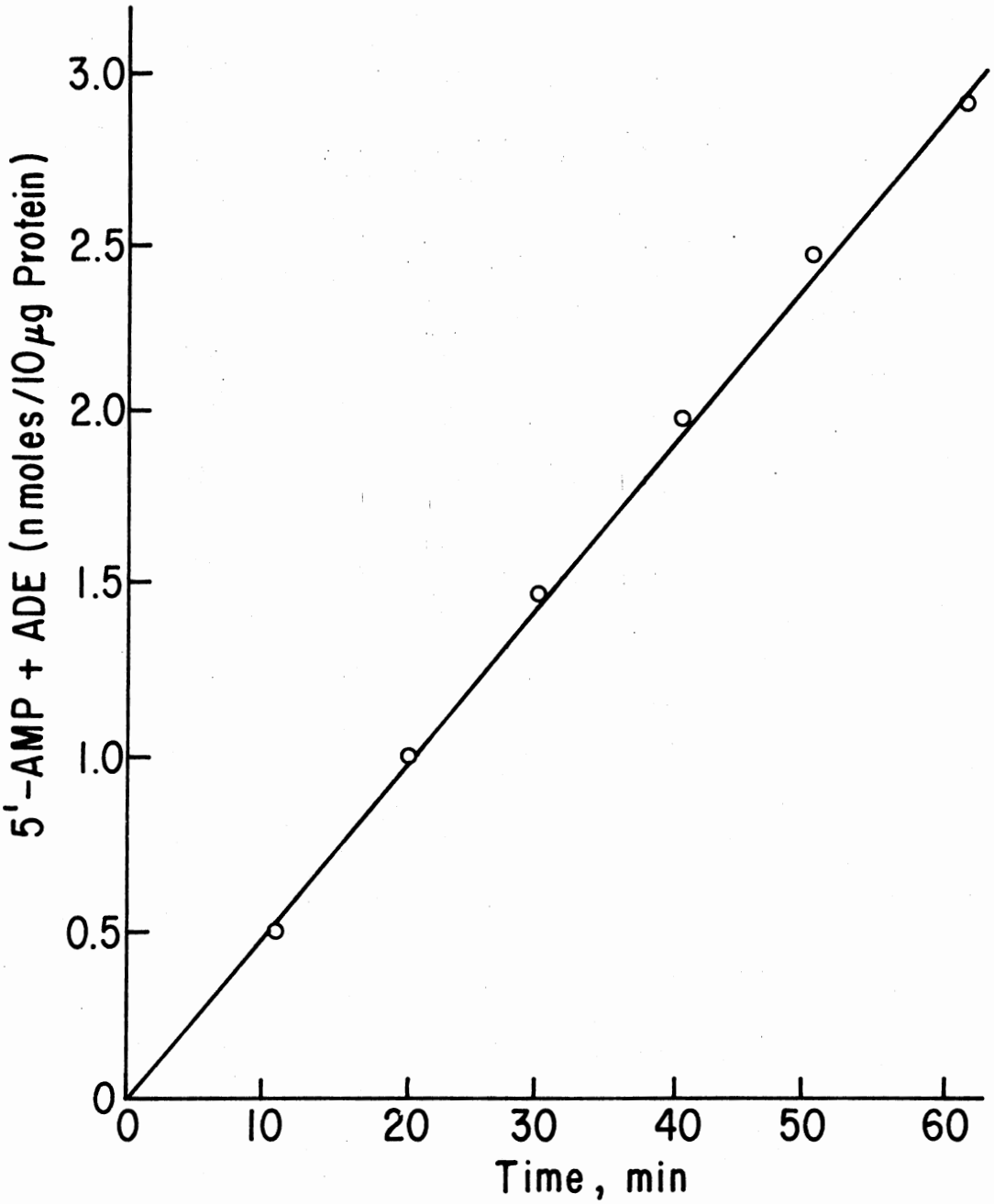

5'-AMP + ADE (n moles/10μg Protein)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
60
Time, min

Figure 5. Effect of protein concentration on Cyclic AMP dependent phosphodiesterase (PDE) activity of crude homogenates of salivary glands obtained from female Amblyomma americanum (L.).

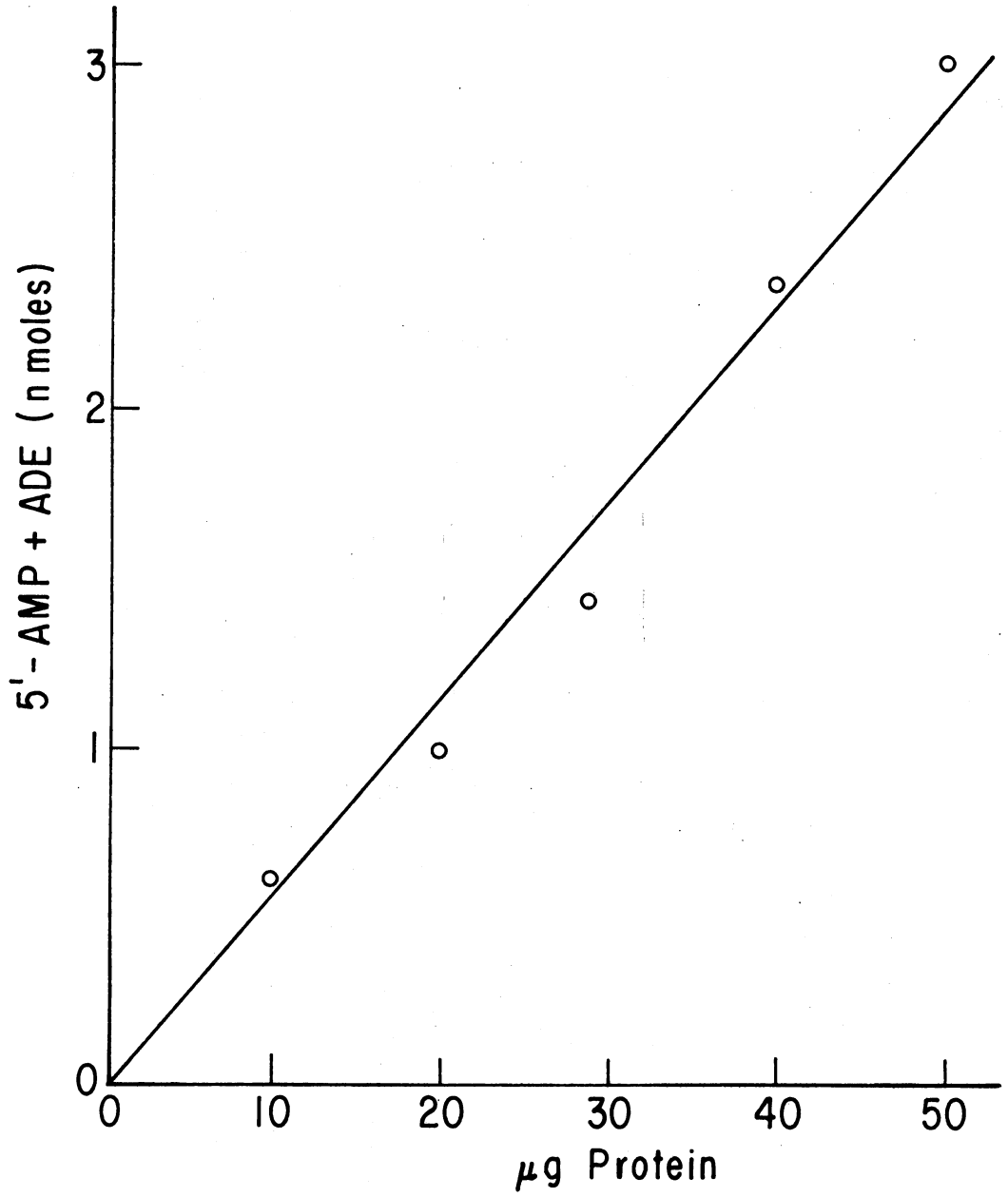

5'- AMP + ADE (n moles)
3
2
1
0
0
10
20
30
40
50
μg Protein

Figure 6. Effect of Mg^{2+} on Cyclic AMP dependent phosphodiesterase (PDE) on PDE activity in crude homogenates of salivary glands obtained from female Amblyomma americanum (L.).

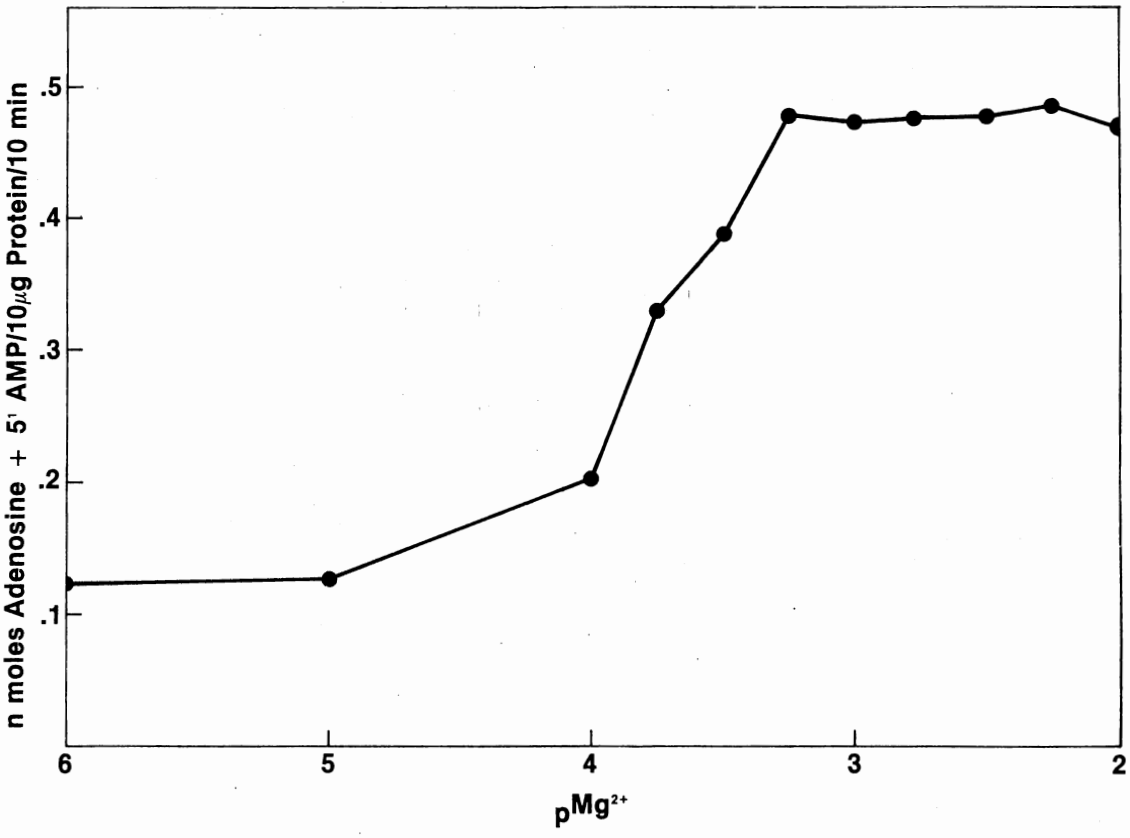
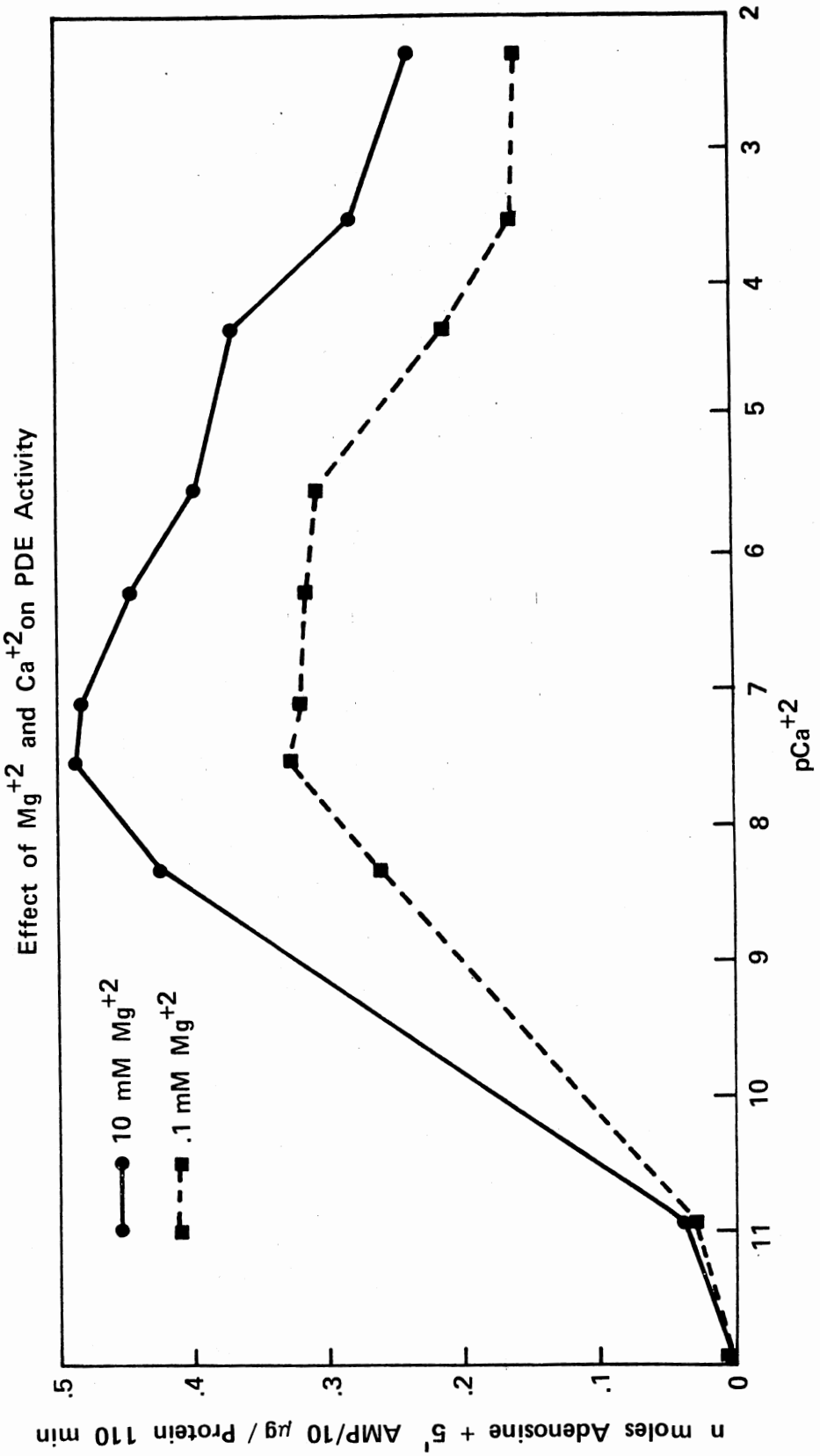

n moles Adenosine + 5' AMP/10μg Protein/10 min
.5
.4
.3
.2
.1
6
5
4
3
2
pMg2+

Figure 7. Interaction of Mg^{2+} and Ca^{2+} on Cyclic AMP dependent phosphodiesterase (PDE) activity in crude homogenates of salivary glands obtained from female Amblyomma americanum (L.).


Effect of Mg^{+2} and Ca^{+2} on PDE Activity
n moles Adenosine + 5' AMP/10 µg / Protein 110 min
pCa^{+2}
10 mM Mg^{+2}
.1 mM Mg^{+2}
0
.1
.2
.3
.4
.5
2
3
4
5
6
7
8
9
10
11

Kinetic analysis of PDE prepared from crude tissue homogenate (Table I) shows anomolous kinetics similar to that seen by others when working with crude preparations (Thompson and Strada 1979). The low Km value increases while the high Km value decreases as the tick goes from a slow to rapid phase of feeding. The same effect is seen when observing the Vmax values. These anomalous kinetics could indicate the possibility of one enzyme with multiple sites exhibiting negative cooperativity, or one enzyme with multiple substrate binding sites (Thompson and Strada 1979; Wells and Hardman 1977). It is known that cAMP is important in the regulation of fluid secretion of tick salivary glands (Sauer et al. 1974), (Sauer et al. 1975), (Sauer et al. 1979) and that the glands undergo very noticeable changes in anatomy when progressing from the slow to a rapid phase of feeding (Binnington 1978; Megaw and Beadle 1979); thus it seems possible that phosphodiesterase may play a critical role in the regulation of fluid secretion.

The changes observed between the PDE's taken from the tick salivary glands at various phases of feeding (Table I.) suggest that dramatic changes in PDE activity are occurring as the tick enters rapid engorgement; however, it remains to be seen if these changes may play a role in fluid regulation.

TABLE I. Km AND Vmax VALUES FOR PHOSPHODIESTERASE, TAKEN FROM CRUDE TISSUE HOMOGENATES.

		Km	Vmax
SLOW	1	55.6 ± 2.7	98.50*
	2	0.33 ± 0.7	3.90
RAPID	1	42.1 ± 0.36	10.33
	2	2.36 ± 0.71	7.80

*Vmax is expressed as p moles cAMP hydrolized per min. per mg protein. Due to anomolous kinetics, 2 Km and Vmax values are given for each stage of feeding.

CHAPTER III

THE RELATIONSHIP OF PHOSPHODIESTERASE AND CYCLIC AMP TO THE PROCESS OF FLUID SECRETION IN THE SALIVARY GLANDS OF THE IXODID TICK AMBLYOMMA AMERICANUM

INTRODUCTION

Phosphodiesterase (PDE) activity in the salivary glands of the female, Amblyomma americanum decreased as the tick progressed from a slow to a rapid phase of feeding, while the rate of fluid secretion increased when glands were stimulated with cyclic AMP and theophylline. Dopamine stimulated PDE activity and an 'inhibitory' factor was found in glands obtained from rapidly engorging ticks which decreased PDE activity. These findings are discussed as they relate to the process of fluid secretion by salivary glands of feeding ixodid ticks.

Female ixodid ticks must have effective osmoregulatory systems because of their ability to concentrate large blood meals, and are excellent animals in which to study the problem of ion and water balance. In the process of concentrating its bloodmeal, excess fluid moves across the gut epithelium and is expelled via the salivary glands back into the host (Tatchell, 1967; Kaufman and Phillips, 1973). The authors have recently obtained evidence that implicates both cyclic

AMP (cAMP) and Ca^{2+} in the process of salivary gland fluid secretion in females of the lone star tick Amblyomma americanum (Sauer et al. 1974; Needham and Sauer 1975; Sauer et al 1976; Sauer 1977; Needham and Sauer 1979; McMullen et al 1980) Kaufman (1976) and Sauer et al. (1979) have demonstrated that if salivary glands of female ixodid ticks are stimulated in vitro with catecholamines, the rate of fluid secretion is highest in glands obtained from rapidly engorging ticks. Catecholamines may stimulate fluid secretion by causing an increae in the steady state level of cAMP which acts as an intracellular second messenger mediating the signal of the primary transmitter (Sutherland et al. 1968). Recently, it was reported that cAMP mimics the stimulatory effect of catecholamines (Needham and Sauer 1975; Sauer et al. 1976). Furthermore, changes in levels or activity of cAMP, adenylyl cyclase and/or phosphodiesterase (PDE) may change as states of engorgement change. The author reports the relationship of phosphodiesterase and cAMP to the process of fluid secretion by salivary glands of the female lone star tick in this chapter.

METHODS AND MATERIALS

Salivary glands were dissected and homogenized as in chapter II. Some homogenates of salivary glands from the 2 feeding phases were pooled prior to assay. Additional tick salivary gland pairs were dissected in a Ringer's solution with MOPS buffer to retain maximal secretory ability (Needham and Sauer 1979). One gland was bathed in Ringer solution and pre-stimulated with 10^{-6}M dopamine for 5 min, rinsed with Tris buffer and homogenized as before. The other gland was incubated in the same solution but with dopamine and served as the control.

phosphodiesterase activity was measured as in Chapter II.

The technique of Needham and Sauer (1979) was used to measure the effect of 10^{-2}M cAMP and 10^{-2}M theophylline on *in vitro* salivary fluid secretion by glands obtained from ticks in various stages of engorgement using a support medium of modified TC-199 (Sauer et al. 1970; Kaufman 1976). Statistical analysis was performed using Student's t-test. All data are expressed $\pm$ SD.

RESULTS

Figure 8 shows the effect of tick weight upon salivary gland PDE activity. Activity drops sharply in glands obtained from ticks in slow phase of engorgement and reaches a stable level in glands obtained from ticks in the rapid phase of feeding. The figure also shows that cAMP and theophylline stimulated secretion more in rapidly feeding ticks (average maximum 240 nl/min) than in slow feeding ticks (average maximum 105 nl/min). These differences are significant at $p < 0.001$ level.

When salivary gland homogenates from ticks, either in a rapid or slow phase of feeding were pooled (Table II), the combined PDE activity was near that observed in glands from ticks in a rapid phase of feeding prestimulation of glands with 10^{-6}M dopamine (Table III) caused in all cases higher PDE activity.

DISCUSSION

These experiments establish that PDE activity drops sharply (70%) just prior to the tick entering rapid engorgement and thereafter, remains at low levels. These results correlate well with the observation that the secretory rates of salivary glands increase greatly

Figure 8. Effect of tick weight upon salivary gland PDE activity (0) and maximal secretion rate by in vitro salivary glands stimulated with cyclic AMP (10^{-2}M) and theophylline (10^{-2}M). Horizontal lines represent weight range of ticks from which glands were obtained (0.105-0.289 g in slowly feeding ticks and 0.364-0.825 g in rapidly feeding ticks). Vertical lines represent $\pm$ SD of the maximum rate of secretion. Numerals in parenthesis indicate numbers of experiments.

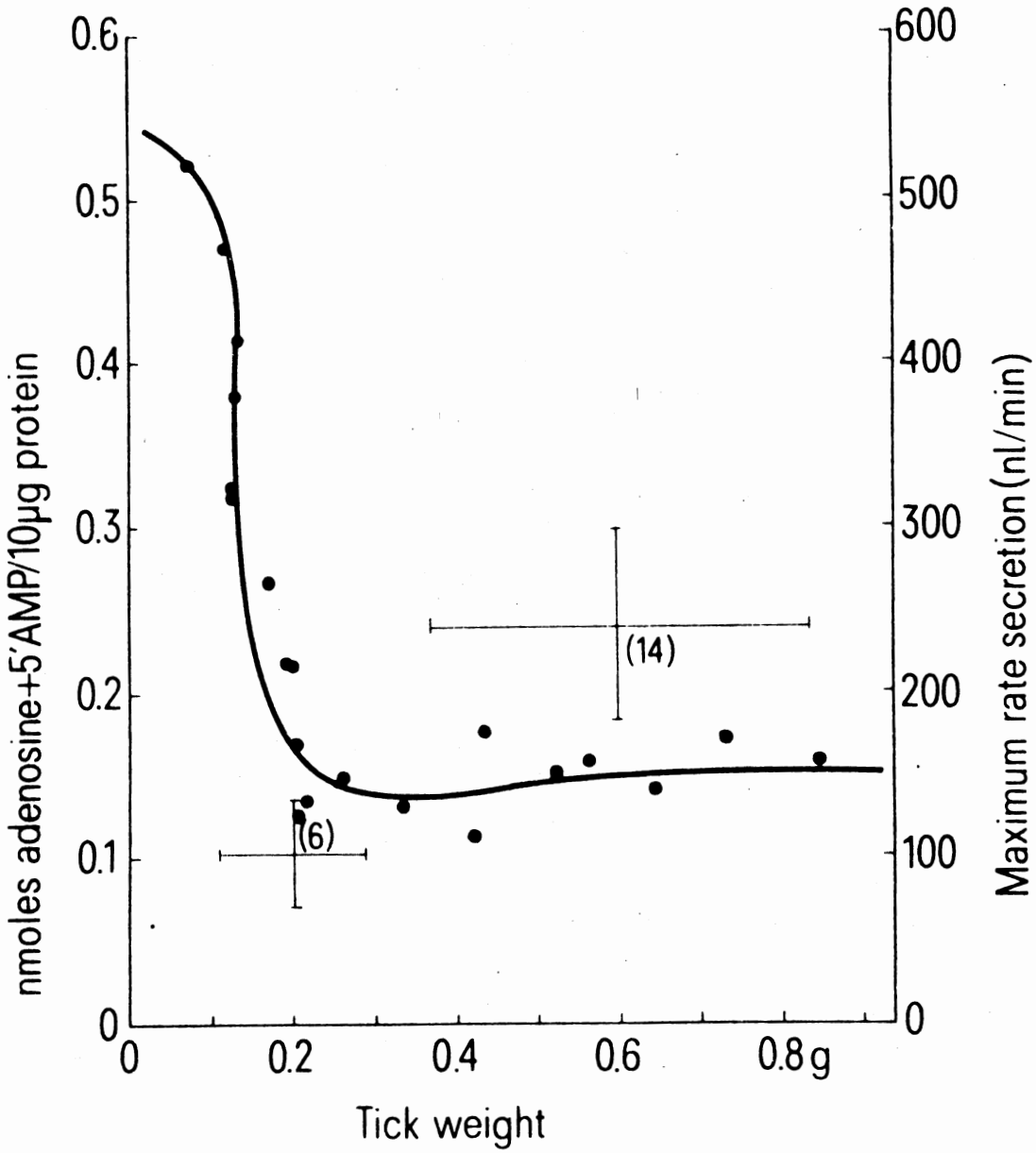

0.6
0.5
0.4
0.3
0.2
0.1
0
600
500
400
300
200
100
0
0
0.2
0.4
0.6
0.8 g
Tick weight
nmoles adenosine+5'AMP/10µg protein
Maximum rate secretion (nl/min)
(14)
(6)

TABLE II. PDE ACTIVITY IN POOLED HOMOGENATES OF SALIVARY GLANDS FROM TICKS IN 2 PHASES OF FEEDING.

TICK WEIGHT	5'-AMP+ADE (nmoles)/ 10 g protein	
> 0.3 g (rapid phase of feeding)	0.19 ± 0.07	(3)*
< 0.3 g (slow phase of feeding)	0.56 ± 0.13	(3)
Pooled homogenate	0.17 ± 0.09	(3)

PDE activity in glands from ticks < 0.3 g varied significantly from PDE activity in glands from ticks < 0.3 g and pooled homogenates ($p < 0.05$; t-test) *Numerals in parenthesis indicate numbers of experiments.

TABLE III. PDE ACTIVITY IN SALIVARY GLANDS PREINCUBATED IN A RINGER SOLUTION WITH OR WITHOUT 10^{-6} M DOPAMINE.

Tick Weight	Activity in gland pairs 5'-AMP + ADE (nmoles/μ0 g protein) Control	Prestimulated gland
0.14 g	0.41	0.62
0.20 g	0.44	0.67
0.21 g	0.35	0.60
0.23 g	0.16	0.23
0.26 g	0.28	0.29
0.32 g	0.28	0.40
0.52 g	0.16	0.36

Control and prestimulated gland PDE activity are significantly different ($p < 0.05$; t-test).

during the same period when stimulated by cAMP and theophylline. The same type of secretory response is noted when salivary glands are stimulated by catecholamines (Sauer et al 1976; Sauer 1977). These findings lend credence to the hypothesis that cAMP plays an important role during fluid secretion. The high PDE activity in glands obtained from the smaller ticks may be a reason that the secretory rate is correspondingly low. The PDE activity in smaller ticks may be at such high steady state levels that even if maximal stimulation of glands by catecholamines occured, the induced cAMP would be destoryed before it is able to substantially affect fluid secretion.

The deactivation of PDE which occurs in the salivary glands of rapidly engorging tick (Figure) and the results of the pooling experiments (Table I) suggest that an inhibitor of PDE may be present in glands from rapidly feeding ticks. It is worth noting that Wang and Desai (1977) have reported a protein in bovine brain that acts as a competitive inhibitor of the CA^{2+} activated activator of PDE.

The fact that both PDE activity and fluid secretion increase when tick salivary glands are stimulated by dopamine suggests an effective feedback mechanism for control of secretion. Recently Needham and Sauer (1979) suggested that cytoplasmic levels of Ca^{2+} may rise in salivary glands after stimulating glands of A. americanum with dopamine. An increased cytoplasmic level of Ca^{2+} may help account for the rise in PDE activity seen when glands are stimulated by dopamine by activating a Ca^{2+} dependent protein activator of PDE, a factor often associated with PDE (Wang 1977). Also Revuelta et al. (1976) have shown that when dopamine stimulates formation of cAMP the newly formed cAMP activates PDE by causing release of a membrane-bound protein activator of PDE in

rat brain tissue. The increased PDE then returns cAMP to basal levels.

Kaufman et al. (1976) have demonstrated that Na^+, K^+-ATP levels in Amblyomma habraeum salivary glands rise significantly during the rapid phase of feeding which correlates with increased secretory rates seen at the same time. Na^+, K^+-ATPase in many cases are phosphorylated before activation and phosphorylation of some component of a cell is the only known mechanism through which cAMP is known to regulate cellular activity (Schwartz et al. 1975). Thus it seems possible that fluid secretion, cAMP, and the Na^+, K^+-ATPase 'pump' are all closely related.

In conclusion, the results of the present experiments show an inverse relationship between tick salivary gland PDE activity and gland fluid secretory capability. Pooled enzyme experiments suggest the presence of an inhibitor of PDE in glands from rapidly engorging ticks, and results indicate that a primary effector of fluid secretion (dopamine) also affects PDE activity.

CHAPTER IV

REGULATION OF FLUID BY CALCIUM-DEPENDENT MODULATOR PROTEINS OF 3':5'-CYCLIC-AMP PHOSPHODIESTERASE

INTRODUCTION

Endogenous inhibitor proteins of 3':5'-cyclic-AMP phosphodiesterase (EC 3.1.4.17) (PDE) have been found in several tissues but little is known about their physiological role (Wang and Desai 1977; Liu and Wang 1979; Sharma et al. 1978). Sharma et al. (1978) have speculated that if these proteins are physiological inhibitors they must be compartmentalized or their concentration in the tissue must increase markedly under certain conditions. We report here changes in phosphodiesterase inhibitors which correspond to changes in rates of cyclic AMP mediated fluid secretion by tick salivary glands. The paired salivary glands of female ixodid ticks serve as their primary organs of osmoregulation. As the adult female feeds, the glands undergo rapid tissue differentiation in addition to important physiological and biochemical changes (Sauer 1977). After attachment to the host (sheep in these experiments), the female lone star tick Amblyomma americanum undergoes a slow phase of feeding lasting 8-14 days during which weight increases from 4-30mg followed by a rapid phase lasting 12-24 hours

during which the weight increases from 300 to 800mg. The salivary glands enable the tick to concentrate its bloodmeal and return excess water and ions to the host. There is increasing evidence that during feeding, salivary secretion is controlled by nerves and that the synapse at the glands is catecholaminergic (Sauer 1977). Cyclic AMP and Ca^{2+} have been shown to play key roles in regulating salivary fluid secretion (Sauer 1975; Sauer et al. 1979; Kaufman 1976). The levels of cAMP were found to increase when glands were stimulated with dopamine (Sauer et al. 1977). Kaufman (1976) and Sauer et al. (1979) demonstrated that when salivary glands of female ixodid ticks are stimulated in vitro with catecholamines or exogenous cAMP, fluid secretion is highest in glands from rapidly engorging ticks. Needham and Sauer (1979) have found that removing Ca^{2+} or adding the calcium antagonist verapamil to the bathing medium reduced catecholamine and cAMP/theophylline stimulated fluid secretion in glands from rapidly feeding ticks. Sauer et al. (1979) found that if glands from rapidly engorging ticks were stimulated with cAMP/theophylline, fluid secretion by glands was much greater than if glands were bathed in a medium with millimolar levels of free Ca^{2+}. This difference was not observed in glands taken from ticks in the slow phase of feeding. Recently McMullen and Sauer (1978) reported that activity of phosphodiesterase per unit protein drops sharply just prior to rapid engorgement and remains at low levels thereafter. In addition they found that homogenates of glands from ticks in the rapid phase of engorgement contained a factor that inhibited phosphodiesterase activity in homogenates of glands from ticks in the slow phase of feeding. These experiments indicated that inhibitors of the enzyme may play an important role in the regulation of fluid secretion

in the salivary glands of A. americanum.

METHODS AND MATERIALS

Isolation of phosphodiesterase, activator, inhibitors and effects of Ca^{2+} factors were isolated either from glands excised from 200 rapidly feeding lone star tick females Amblyomma americanum (>0.3g) or 200 slowly feeding females (<0.3g). Glands were homogenized in 0.05M Tris-HCl buffer (pH 8.0) and centrifuged at 100,000 xg for 1 hour. The supernatant was added to two gel filtration columns (Ultro-gel® AcA 34 and 54) in series. The columns were 1.5 X 85 and 95 cm, respectively. Columns were equilibrated and eluted at 22 ± 2°C with 0.05 M Tris-HCl (pH 8.0), 1 mM EGTA and 0.05 M NaCl, at a flow rate of 20 ml/hr. Three ml fractions from the column were collected and held at 3 ± 1°C. Fractions were analyzed for phosphodiesterase activity using the ATP, luciferin-luciferase firefly assay after converting 5' AMP to ATP with appropriate enzymes (Weiss et al. 1972) in a total reaction volume of 220 µl. All fractions eluted from the columns were analyzed for phosphodiesterase. Following isolation of phosphodiesterase, other fractions were assayed for ability to activate the enzyme. Activator and inhibitor activities were tested in these experiments in the presence of 10 M Ca^{2+} for maximal activation of 50 µl of phosphodiesterae + activator + inhibitor I or II. The factors were incubated for 10 min at 37° in 0.05 M Tris-HCl and 0.005 M $MgCl_2$ at pH 8.0 containing 1 mM cyclic AMP. Fifty 1 of peak fractions for phosphodiesterase from the column was added to each reaction vessel along with 20 µl of fractions containing activator where indicated. When inhibitor activity was tested, 20 µl of fractions with inhibitor was also added. The reactions

were stopped by placing vessels in a boiling water bath for 1 min. Enzyme activity was determined as described above. Free Ca^{2+} levels were set by using different concentrations of $CaCl_2$ in the presence of 0.5 mM EGTA and were calculated from the affinity constant for EGTA (Sillen and Martell, 1964).

Effect of Ca^{2+} on activity of phosphodiesterase in crude homogenates. Pairs of salivary glands were dissected from ticks in either slow or rapid phase of feeding and PDE activity was measured as in Chapter II. Calcium concentrations were adjusted as mentioned. The luciferin-luciferase technique were not applicable in these experiments because of high amounts of endogenous ATP.

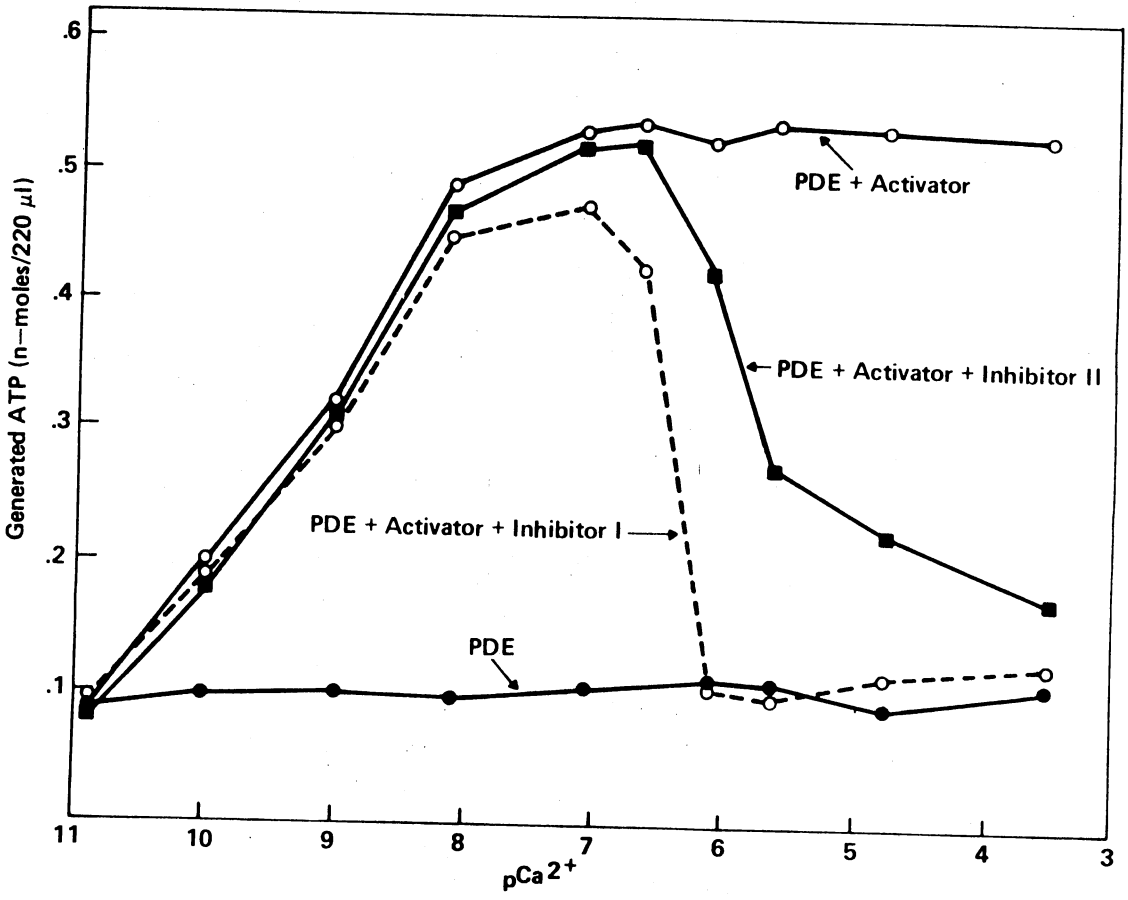
RESULTS AND DISCUSSIONS

To further determine the nature of 3':5'-cyclic-AMP phosphodiesterase inhibitor activity seen by McMullen and Sauer (1978), salivary gland supernatant (100,000 xg) was subjected to gel filtration. The eluate from the gel column showed separate peaks of a single PDE enzyme (136,400 MW), two heat-stable Ca^{2+}-dependent inhibitor proteins (inhibitor I, 43,500 MW and inhibitor II, 11,200 MW) and a Ca^{2+}-dependent activator protein (17,880 MW) (McMullen et al. 1980). Subsequent chromatography with diethylaminoethyl-sephacel and hydroxylapatite failed to resolve further forms of phosphodiesterase in the supernatant fractions. The activity of all four factors was destroyed when applied to a column containing proteinase-K coupled to CNBr-activated sepharose. The activator and inhibitor fractions remained active but phosphodiesterase activity was destroyed after placement for 5 min. in a

boiling water bath. These inhibitors, as in several other cases, (Wang and Desai 1977; Liu and Wang 1979; Sharma et al. 1978) were unable to inhibit phosphodiesterase unless the Ca^{2+}-dependent activator was present. Although not all criteria have as yet been met, it is highly possible that the activator is calmodulin (Cheung 1980). Inhibition was not reduced by increasing the level of activator protein or by adding a great excess of activator prior to addition of inhibitors at high levels of Ca^{2+} (5 mM). These results would seem to indicate that the inhibitors must bind with the activator's protein only after the activator has complexed with phosphodiesterase.

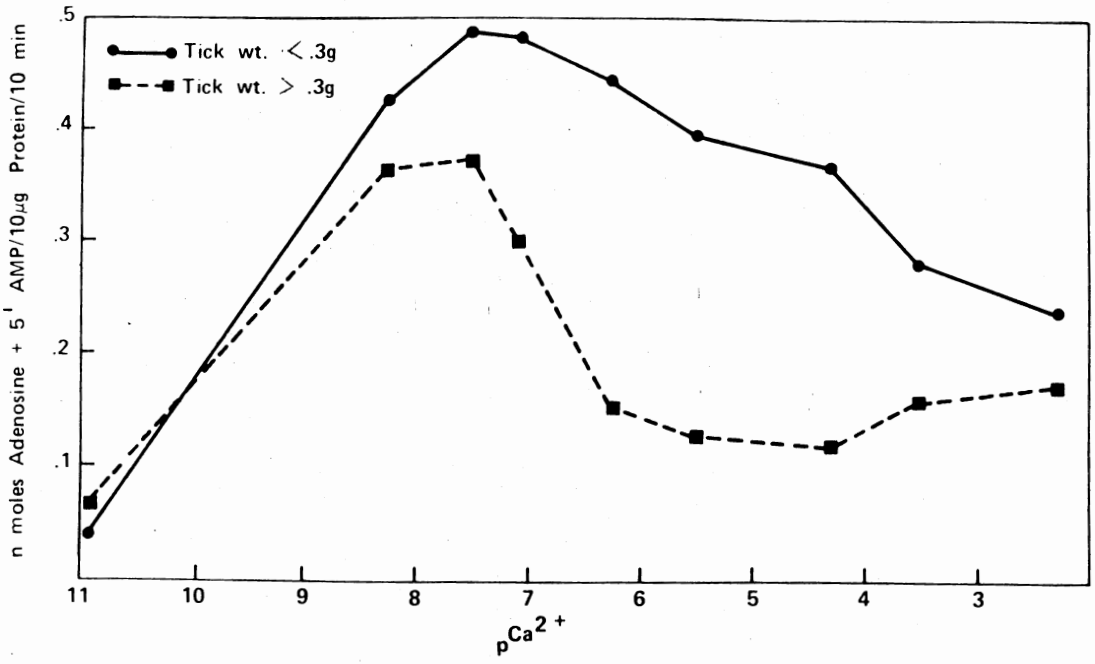
The specific activities of activator protein and phosphodiesterase (activity/µg protein), were found to be the same in glands obtained from ticks in either the slow or rapid phase of feeding. However, glands from ticks in the rapid phase of feeding contained approximately 2000 and 200 times more inhibitor I and II activity, respectively, as compared to that found in glands obtained from ticks in the slow phase of feeding. Fractions collected from salivary glands of ticks in the rapid phase of feeding contained approximately twice as much inhibitor I as inhibitor II activity. Thus, fractions collected from ticks in the slow phase of feeding expressed only about 0.2 as much inhibitor I as inhibitor II activity. Inhibitor activity in these experiments (amount of protein, µg, required to inhibit activated phosphodiesterase 50%) was assayed at high levels of Ca^{2+} (5 mM)for reasons that follow. The first involved level of calcium affected activator and inhibitor activities. A wide range of Ca^{2+} concentrations was tested with purified phosphodiesterase + activator either with or without inhibitor (Fig. 9). Calcium was found to have no effect upon

Figure 9. Effect of Ca^{2+} concentration on activity of a purfied 3':5'-cyclic-AMP phosphodiesterase from tick salivary glands in the presence and absence of its activator and inhibitors I and II. Each point represents the mean of three separate experiments.


.6
.5
.4
.3
.2
.1
Generated ATP (n—moles/220 μl)
PDE + Activator
PDE + Activator + Inhibitor II
PDE + Activator + Inhibitor I
PDE
11
10
9
8
7
6
5
4
3
pCa 2+

phosphodiesterase by itself but greatly enhanced the ability of the protein modulators to regulate phosphodiesterase activity. Activity of phosphodiesterase + activator reached a maximum at a pCa^{2+} of about 7. With increasing Ca^{2+}, activity in the presence of either inhibitor dropped sharply, but remained high if the inhibitors were absent. Inhibitor I was found to required lower Ca^{2+} levels than inhibitor II. These results demonstrate that a higher level of Ca^{2+} is necessary for inhibition of phosphodiesterase than for its activation, but the sensitivities of all 3 protein modulators are within ranges of Ca^{2+} believed to be present in cell cytoplasm (Rassmusen et al. 1975). Because inhibitor I is active at lower Ca^{2+} levels and its activity relative to inhibitor II increases more sharply when the tick enters rapid engorgement, it seems possible that inhibitor I is the more physiologically significant inhibitor. To assess more fully the physiological significance of the Ca^{2+} effects seen with purified protein, phosphodiesterase activity was similarly tested in crude homogenates of glands from slow and rapidly feeding ticks (Fig. 10). Enzyme activity increased with increasing Ca^{2+} to a pCa^{2+} of 7. Phosphodiesterase activity declined sharply in glands from rapidly feeding ticks. However, phosphodiesterase activity in crude homogenates of glands from slowly feeding ticks remained relatively constant and decreased only slightly at higher Ca^{2+} concentrations. Thus, the heat-stable protein inhibitors of phosphodiesterase seem to represent an important control mechanism in the regulation of salivary gland fluid secretion in A. americanum (L.). When the adult tick initiates slow feeding, inhibitors are at low levels, helping keep levels of phosphodiesterase high, cAMP low and rates of fluid secretion low. Towards the end of the slow phase of feeding, levels of the

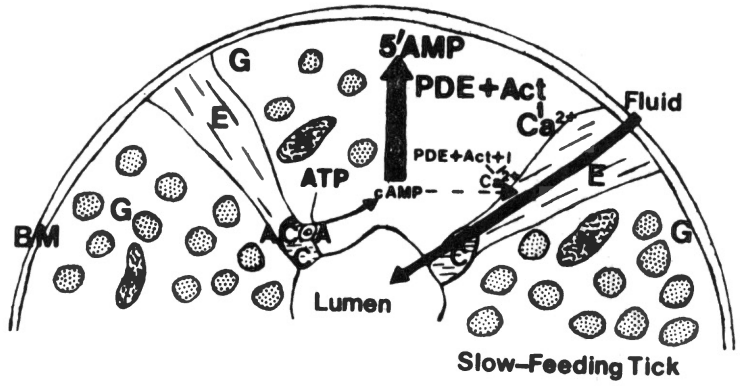
Figure 10. Effect of Ca^{2+} concentration on activity of 3':5'-cyclic-AMP phosphodiesterase in crude homogenates of glands from ticks in slow and rapid phase of tick feeding. Each point is the average of three experiments.

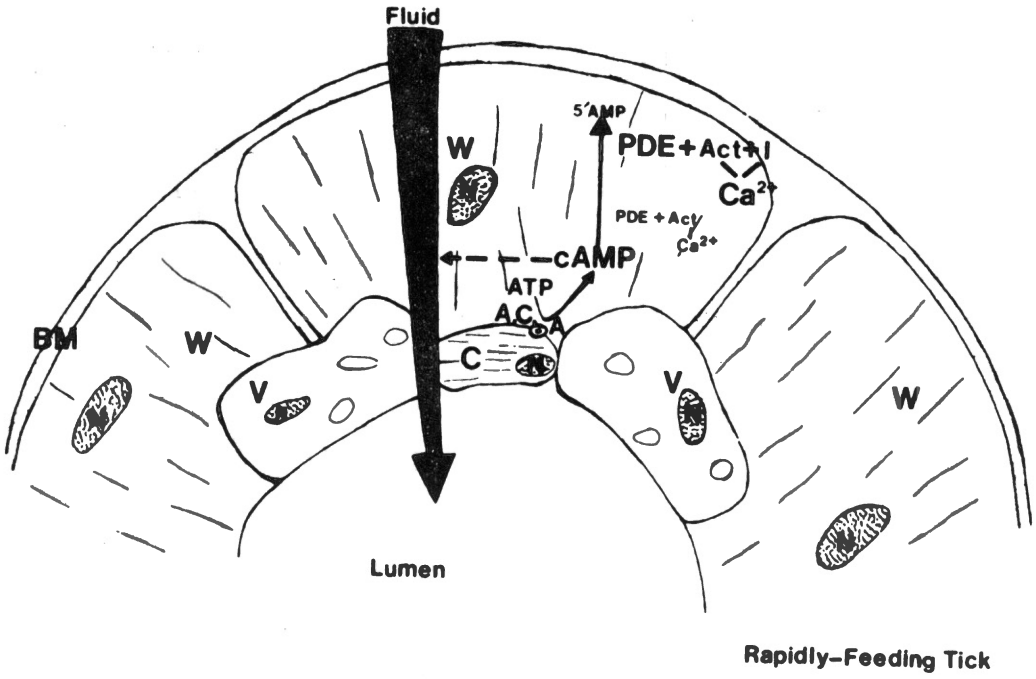

Tick wt. < .3g
Tick wt. > .3g
n moles Adenosine + 5' AMP/10μg Protein/10 min
.5
.4
.3
.2
.1
11
10
9
8
7
6
5
4
3
pCa2+

inhibitors increase, phosphodiesterase activity is depressed and rate of fluid secretion is increased (Fig. 11). Furthermore, since inhibitors are active only at relatively high Ca^{2+} and at levels which may be attained only during gland stimulated, a critical role of changes in cell Ca^{2+} seems likely.

Whether the inhibitors present the primary mechanism for modulating the action of normal secretagogues is not known. Studies in progress are aimed at obtaining information about levels and control of adenylate cyclase, levels of cAMP, cAMP dependent protein-kinases and protein phosphorylation in glands of slow and rapdily feeding ticks.

Figure 11. Schematic diagram showing proposed morphological and physiological changes in ixodid female salivary gland in Type II and III alveoli during feeding. The alveoli swell and increase in size during feeding. Alveoli are populated mostly by granular cells (G) in pre-fed and slowly feeding ticks but relatively inconspicuous epithlial cells (#) with features common to fluid transporting cells (i.e., numerous basal infoldings of the plasmamembrane, intercellular spaces and mitochondrial) are present. (Megaw, 1979). In rapidly feeding ticks very prominent cells, "water-cells", (W) particularly in Type III alveoli are evident; cells with granular material are much less common. The "Water-cells" possess the usual features seen in fluid transporting cells and they probably play a key role in fluid secretion. "Water-cells" are cut off from the alveolar lumen by vacuolar (V) and cap cells (C) (Megaw, 1979), and the route(s) of fluid transport are unknown. Details of how individual cells in alveoli change morphologically during progression of the tick from the slow to rapid stage during feeding are also mostly unknown. Secretion is likely controlled by nerves (A) which stimulate production of the "second messenger" cyclic AMP and ATP following activation of adenylate cyclase (A.C.). In slow-feeding ticks, 3':5'-cyclic AMP phosphodiesterase activity is high aided by the presence of activator (Act) and low levels of inhibitor proteins (I); factors are thus present for causing relatively low levels of cyclic AMP low rates of fluid secretion. When the tick enters rapid engorgement, protein activator is still present but is prevented from activating phosphodiesterase because of much higher levels of inhibitor proteins; factors are thus present with the potential for causing higher levels of inhibitor proteins; factors are thus present with the potential for causing higher levels of cyclic AMP and higher rates of fluid secretion. Both the activator and inhibitor proteins require Ca^{2+} for activity. The inhibitor protein has no effect upon basal phosphodiesterase activity and appears to bind with activator only after it has become bound to phosphodiesterase (see text).


5′AMP
G
PDE+Act
Fluid
E
Ca2+
PDE+Act+I
Ca2+
ATP
cAMP
E
G
BM
G
AC
A
C
Lumen
Slow–Feeding Tick


Fluid
5′AMP
W
PDE+Act+I
Ca2+
PDE + Act
Ca2+
cAMP
ATP
AC
A
BM
W
C
V
V
W
Lumen
Rapidly–Feeding Tick

CHAPTER V

CHARACTERIZATION AND PURIFICATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE AND ITS CALCIUM DEPENDENT MODULATORS IN THE SALIVARY GLANDS OF FEMALE *AMBLYOMMA AMERICANUM* (L.)

INTRODUCTION

Modulation of cyclic nucleotide activity during development has been noted in many different tissues but the mechanisms responsible for these changes in activity and physiological significance are not well understood (Thompson & Strada 1978). The salivary glands of the ixodid tick *Amblyomma americanum* (L.) provide a unique system in which to observe changes in enzyme activity and to relate these to changes in physiological events.

The paired salivary glands of the adult female ixodid tick *A. americanum* (L.) are its principal organs of osmoregulation. As the female feeds, the rate of salivary fluid secretion increases greatly and enables the tick to concentrate its bloodmeal by returning excess water and ions to host *via* the salivary ducts. It seems likely that fluid secretion is controlled by nerves (Megaw & Beadle 1979) with a catecholamine being the secretagogue (Megaw & Beadle 1979, Sauer 1977). There is increasing evidence that cAMP and Ca^{2+} play important roles in

the regulation of fluid secretion. When the glands are stimulated by dopamine, cyclic AMP levels increase (Sauer et al. 1979) and adenylate cyclase activity increases when glands are stimulated by dopamine Schmidt et al. (unpublished). Also, fluid secretion is highest when glands with low levels of phosphodiesterase are stimulated in vitro with catecholamines or exogenous cAMP.

Cyclic AMP induced secretion is inhibited when Ca^{2+} is deleted or the Ca^{2+} antagonist verapamil is added to bathing medium (Needham & Sauer 1979). When glands are stimulated with cAMP, fluid secretion is much greater when glands are bathed in a medium with millimolar levels of free Ca^{2+} as compared to glands bathed with micromolar amounts of free Ca^{2+}. This difference was not observed in glands taken from ticks in the slow phase of feeding.

Phosphodiesterase activity levels in salivary glands decrease greatly as the tick progresses from a slow to a rapid phase of feeding while the rate of salivary fluid secretion greatly. In addition, an inhibitor factor is found in glands obtained from rapidly engorging ticks which decreases phosphodiesterase activity (McMullen & Sauer, 1978). Further experiments have shown that this inhibition is due to an increase (>200 fold) during a 24 hour period of two inhibitor proteins antagonize calmodulin activation of phosphodiesterase (McMullen et al. 1980). Both calmodulin and the protein inhibitors are Ca^{2+} dependent but approximately 10 times more Ca^{2+} is necessary for activation of the inhibitors than for activation of calmodulin. These protein inhibitors are thought to serve as important intracellular regulators of salivary fluid secretion by their involvement in regulating cAMP levels in the gland. The present study was designed to further characterize salivary

gland supernatant phosphodiesterase, its activation by calmodulin and modulation of this activation by the inhibitory proteins. In addition, the properties of particulate phosphodiesterase and cyclic GMP phosphodiesterase were investigated.

METHODS AND MATERIALS

Adult female lone star ticks were reared on female sheep as the source of salivary gland tissue. The ticks were collected while in either the slow phase of feeding which lasts 6-14 days (weight increases from ca 4mg to ca 300mg) or in the rapid phase of feeding which lasts 12-24 hours (weight increases from ≃300mg to ≃800mg). Glands were excised from the tick in 0.05M Tris-HCl buffer containing 0.005M $MgCl_2$ adjusted to pH 8, within one hour after removing the tick from the sheep. After dissection, glands were either stored at -60°C or placed in small glass homogenizers containing 250µl of the same buffer and homogenized. Each gland contained between 50 and 125 µg of protein depending on the stage of tick feeding.

Phosphodiesterase Assays

Because enzyme assays were performed in crude and partially purified preparations it was necessary to use different techniques to assay cAMP and cGMP phosphodiesterase. Purified, soluble cAMP phosphodiesterase from which endogenous ATP was removed was assayed using the luciferin-luciferase firefly assay after converting 5' AMP to ATP with appropriate enzymes (Weiss et al. 1972).

Crude gland homogenate enzymes were assayed as described in Chapter II. Particulate (100,000 xg pellet) phosphodiesterase were

measured using the batch method of Thompson et al. (1979) and the paper chromotographic methods as described above.

Isolation of Supernatant Phosphodiesterase, Calmodulin and Inhibitors

Six separate groups of glands (≃100-250 gland pairs in each group) excised from rapidly or slowly feeding ticks were homogenized as before and centrifuged at 100,000 xg for 1 hour. The supernatant was added to two gel filtration columns, and chromatographed as described in Chapter IV. All fractions were assayed for cyclic AMP phosphodiesterase. Following isolation of phosphodiesterase, other fractions were assayed for ability to affect enzyme activity.

Phosphodiesterase, calmodulin and the inhibitors were further purified by applying 2 ml samples of fractions from the Ultrogel column to a DEAE-Sephacel® column, 1.5cm x 15 cm and eluted with 0.1mM Tris-HCl with a linear NaCl gradient (0-.0.15M) at a flow rate of 21 ml/hr for 10 hours.

Isolated active fractions from the DEAE-Sephacel column were further chromatographed on a 1.5cm x 51 cm column of spheroidal hydroxylapatite (Gallard-Schlesinger®) and eluted with a linear sodium phosphate buffer gradient (0.05-0.5M) at pH 6.8 with a flowrate of 10 ml/hr for 6 hr. Molecular weights of isolated fractions were determined by comparing elution profiles following gel filtration to those of pure proteins of known molecular weight, obtained from Pharmacia.

Inhibitor I Reduction with Dithiothreitol

One ml each of Inhibitor I and II were collected from eluate of

the hydroxylapatite column and treated with 1 ml 0.1 mM dithiothreitol flushed with nitrogen and maintained at 50°C for 4 hours. The solution was then cooled to room temperature and treated with 1 ml 0.2 mM iodoacetic acid (20 min. in the dark) and run over a 1.5 x 25 cm Ultrogel AcA 54 column as before.

Dopamine Stimulation

Some glands were stimulated by 10^{-6}M dopamine they were placed in Tris buffer pH 7.5 for 10 min., rinsed in buffer and homogenized as before.

Localization and Isolation of Particulate PDE

The pellet from the 100,000 xg centrifugation is referred to as the particulate fraction. Removal of phosphodiesterase from particulate membranes was accomplished by taking the 100,000 xg pellet of the salivary gland homogenate resuspended in 0.05M Tris-HCl (pH 8.0) and disruption Biosonic IV sonicator at a setting of 80W for 30S. The resulting suspension was centrifuged at 100,000 xg for 60 min. and the supernatant used as source of particulate phosphodiesterase.

Separation of Type I Alveoli from Types II & II

The salivary glands of ixodid females consist of three alveoli types (Krolak et al. 1981). Type I is easily distinguished from the others by its anterior location and direct attachment to the main salivary duct. Glands were removed and dissected further to give two fractions: Types II and III alveoli + ducts and Type I alveoli + duct based on these differences in location. The two groups of tissue were

then analyzed for phosphodiesterase activity as described.

RESULTS

Gel filtration of the supernatant (100,000 xg) (Fig. 12) yielded one peak of phosphodiesterase, one activator and 2 inhibitory fractions of phosphodiesterase. The results of a representative separation are shown in Fig. 12 and separately in Fig. 13-15. The inhibitors were found to inhibit only activator stimulated phosphodiesterase activty. The isolated enzyme was denatured if placed for two min. in a boiling water bath while the activator and inhibitory fractions were stable under the same high temperature conditions. Previous observations have shown that all four isolated fractions are proteins (McMullen et al. 1980). The inhibitor proteins were found in salivary gland homogenates from ticks in the rapid phase of feeding but not in gland homogenate from ticks in the slow phase of feeding if assayed as they were eluted from the column and not concentrated. Phosphodiesterase and its activator were found equally in glands regardless of stage of tick feeding.

The Ultrogel® column was calibrated with proteins of known molecular weight (Fig. 16) and the approximate molecular weight of phosphodiesterase and its modulating fractions was determined by the method of Andrews (1965). Phosphodiesterase was estimated to have a molecular weight of 136,400, and its activator had an apparent molecular weight of 17,000 and the two inhibitor proteins had molecular weights of 43,500 and 11,900.

Table IV shows the results of all purification steps upon phosphodiesterase and its modulators. The modulators isolated from either

Figure 12. Fractionation of supernatant cyclic nucleotide phosphodiesterase, its activator, and 2 inhibitor proteins on gel filtration columns of Ultrogel AcA 34 and 54 in a series of described under "Methods". Three ml fractions were collected and .05 ml of each fraction was assayed for cyclic nucleotide phosphodiesterase activity. Following isolation of phosphodiesterase, other fractions were assayed for their ability to modulate phosphodiestrase activity in the presence of 10 M Ca^{2+}. Localization of modulator activity was performed by assaying 50 l PDE fraction with 20 l of each other fraction. Inhibitor activity was located by adding 20 l of remaining fractions to phosphodiesterase plus activator mixture.

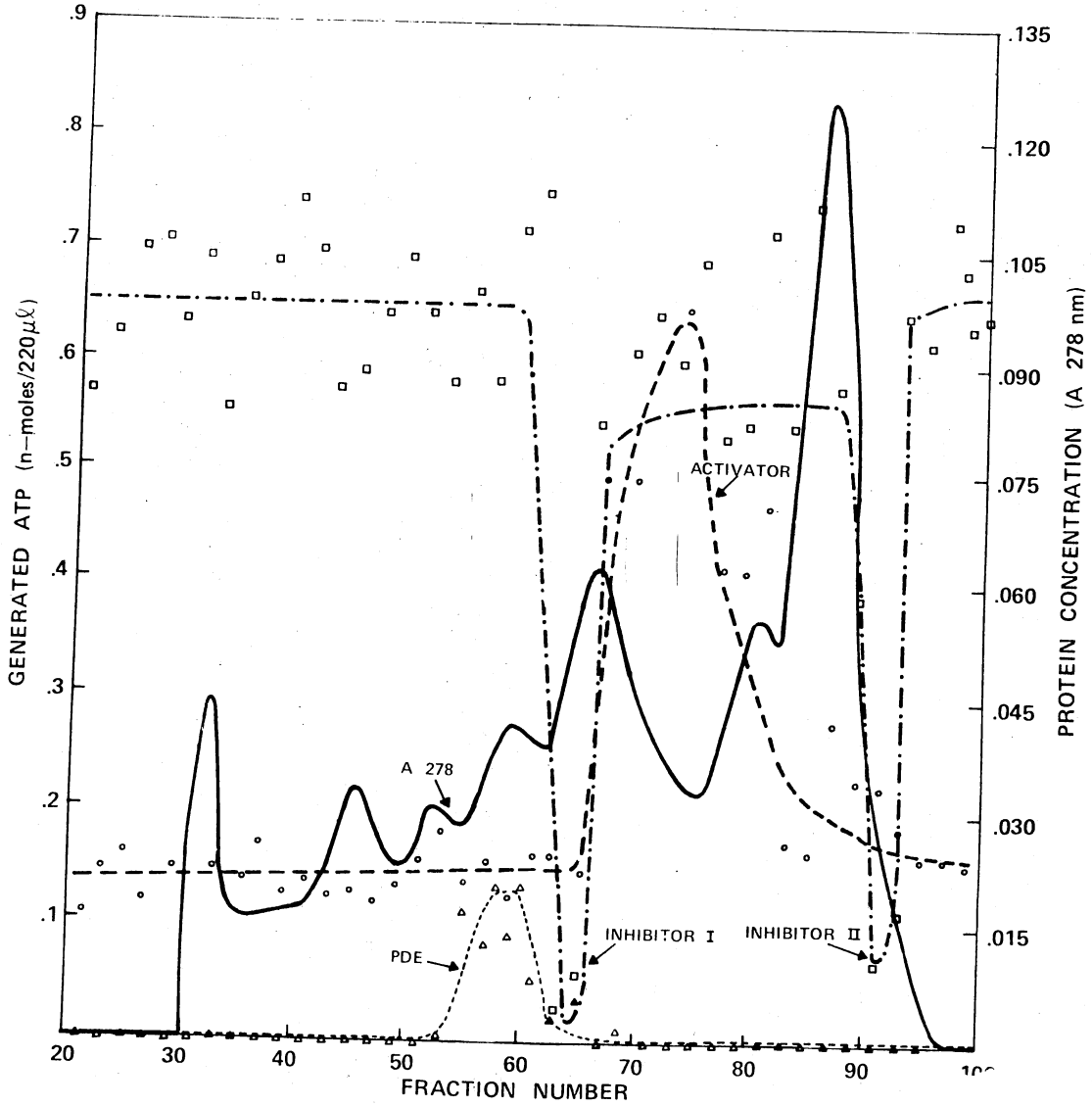

GENERATED ATP (n-moles/220μℓ)
PROTEIN CONCENTRATION (A 278 nm)
FRACTION NUMBER
A 278
ACTIVATOR
PDE
INHIBITOR I
INHIBITOR II
.9
.8
.7
.6
.5
.4
.3
.2
.1
.135
.120
.105
.090
.075
.060
.045
.030
.015
20
30
40
50
60
70
80
90

Figure 13. Fractionation in Figure 1 showing only PDE activity and absorbance of fractions at 278 mn.

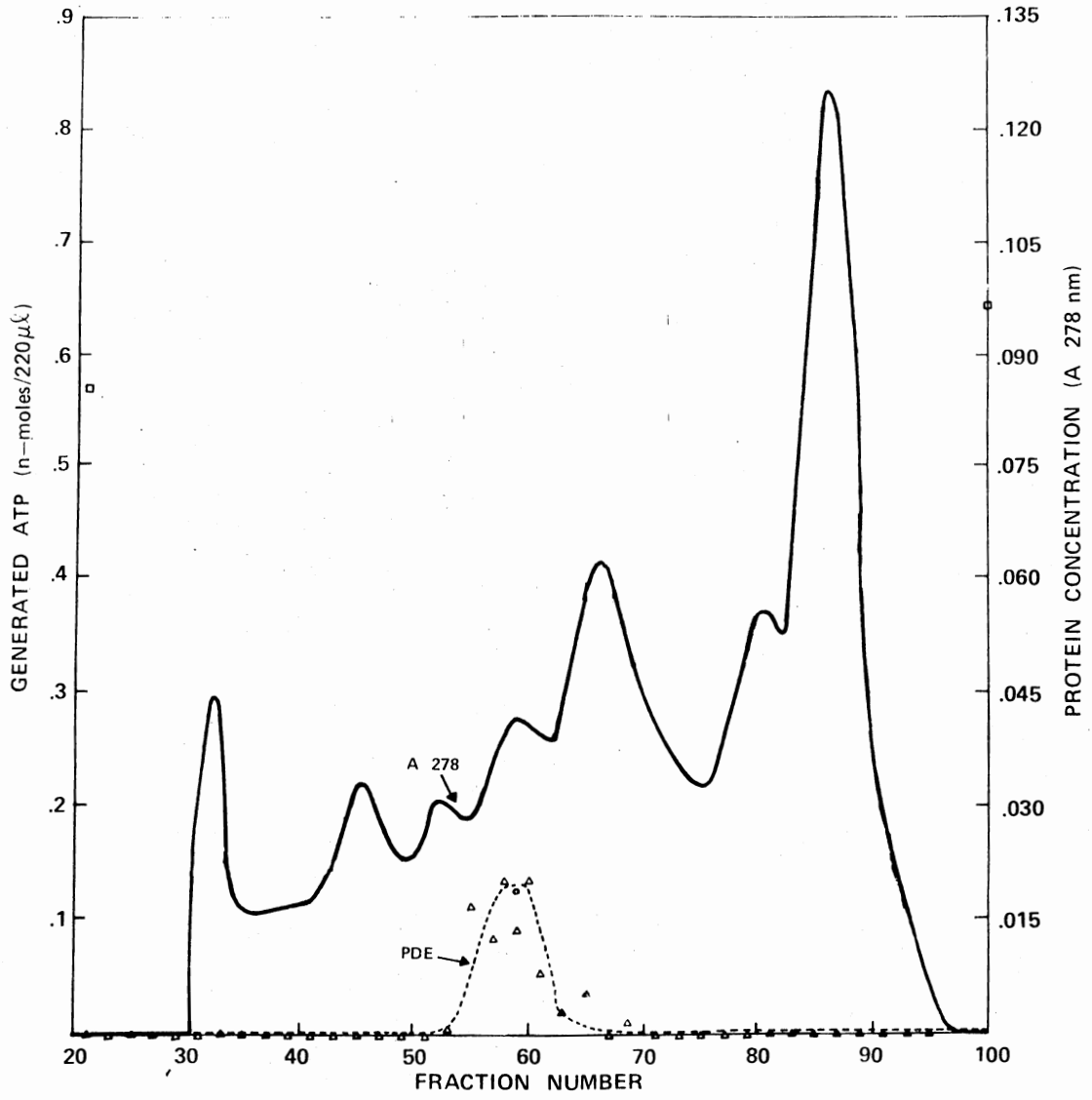

GENERATED ATP (n-moles/220μℓ)
PROTEIN CONCENTRATION (A 278 nm)
FRACTION NUMBER
A 278
PDE
.9
.8
.7
.6
.5
.4
.3
.2
.1
.135
.120
.105
.090
.075
.060
.045
.030
.015
20
30
40
50
60
70
80
90
100

Figure 14. Fractionation as in Figure 1 showing Ca^{2+}-dependent activator (calmodulin) peak.

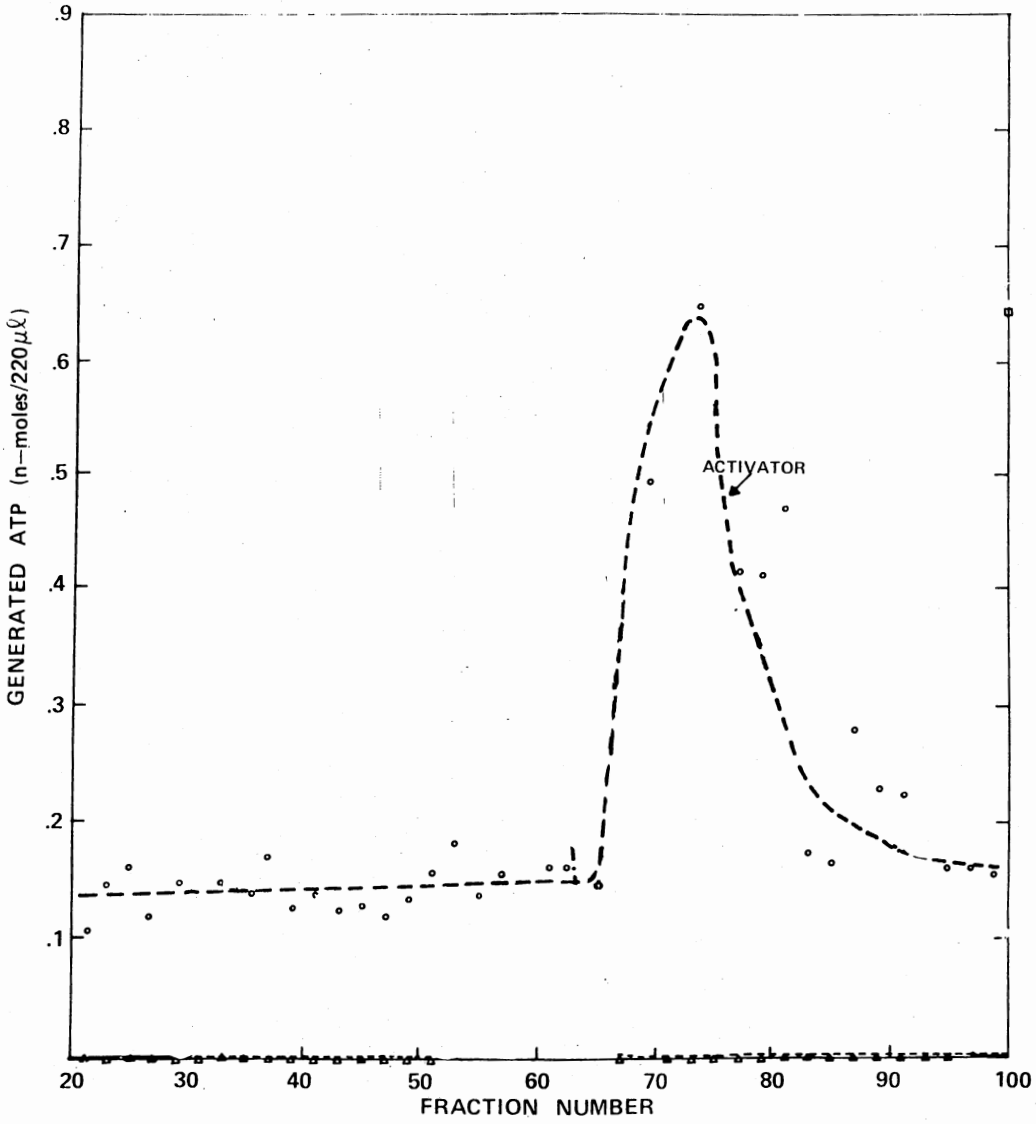

.9
.8
.7
.6
.5
.4
.3
.2
.1
GENERATED ATP (n-moles/220μℓ)
ACTIVATOR
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 15. Fractionation as in Figure 1 showing 2 Ca^{2+}-dependent inhibitor peaks.

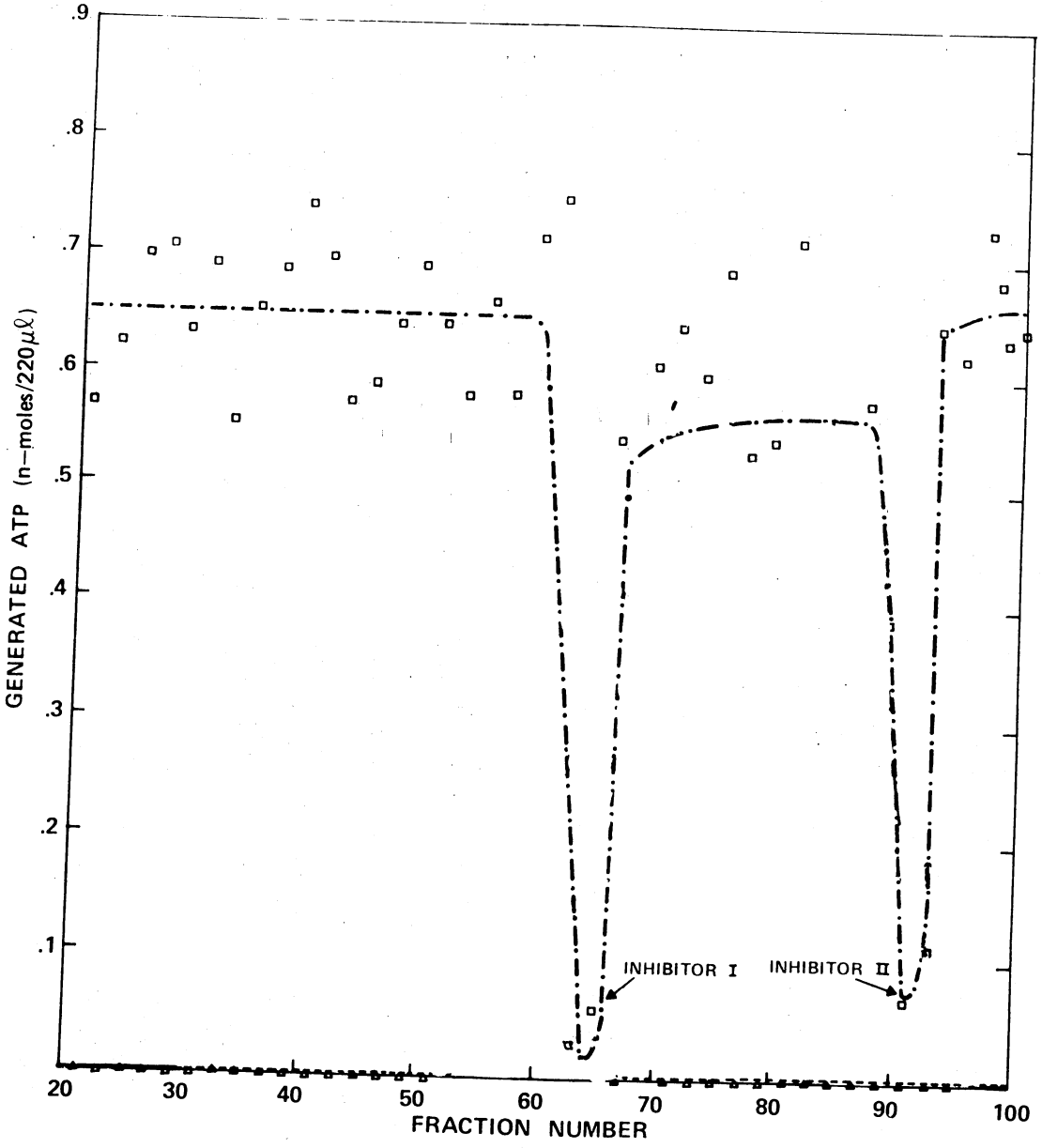

.9
.8
.7
.6
.5
.4
.3
.2
.1
GENERATED ATP (n-moles/220μℓ)
INHIBITOR I
INHIBITOR II
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 16. Molecular weight determination of tick salivary gland supernatant phosphodiesterase and its modulators by Ultrogel AcA 34 and 54 gel filtration with proteins of known molecular weight. Ca+= catalase; BSA = bovine serum albumin; oval = oval bumin; chy = chymotrypsin; Ril = ribonuclease; ve = elution column; Vv = Void volumn.

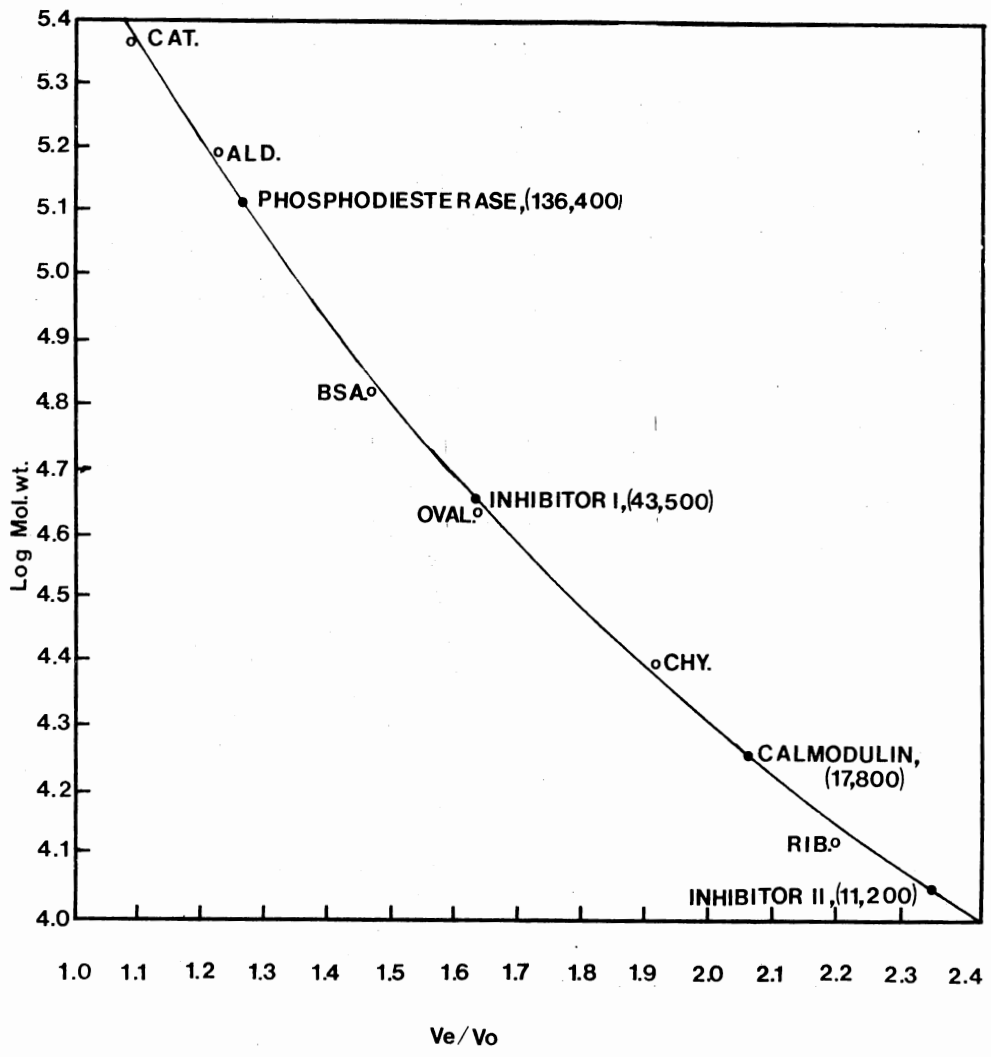

CAT.
ALD.
PHOSPHODIESTERASE,(136,400)
BSA.
OVAL.
INHIBITOR I,(43,500)
CHY.
CALMODULIN,
(17,800)
RIB.
INHIBITOR II,(11,200)
Log Mol.wt.
5.4
5.3
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4.0
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
2.4
Ve/Vo

TABLE IV. SUMMARY OF VARIOUS PROCEDURES USED TO PURIFY PDE AND ITS MODULATORS

Preparation*	Volume (ml)	Total Protein	Total Activity Units/ml	Specific Activity Units/mg Protein	%Yield (Overall)	Fold Purification
Phosphodiesterase crude extract	5	45	100	11.1	100	1
100,000 sg, supernatant	1	34.4	400	11.6	80	1.1
Ultrogel Chromotography	1	2.15	850	372.1	160	33.5
Dead-sephacel	1	0.230	450	1952.5	90	176
Hydroxyapatite	1	0.030	270	9000.0	54	810
Inhibitor I Ultrogel Chromotography	1	5	100	20.0	100	1
Deae-sephacel	1	0.310	72	232.3	72	11.6
Hydroxyapatite	1	0.015	28.8	1920.0	29	96
Inhibitor II Ultrogel Chromotography	1	1.0	100	100.0	100	1
Deae-sephacel	1	0.070	60	857.1	60	8.6
Hydroxyapatite	-	-	-	-	-	-
Activator (calmodulin) Ultrogel chromotography	1	7.5	100	13.3	100	1
Boil, Remove 30,000 XG ave. supernatant	1	2.0	95	47.5	95	3.6
Deae-sephacel	1	0.325	61	187.6	61	14.1
Hydroxyapatite	1	0.020	39	1950.0	39	146.6

*Prepared from ≃ 400 salivary glands of ticks in rapid phase of engorgement.

stage of feeding were active on PDE from the other stage of feeding. EGTA was found not to inhibit PDE activity but it did prevent its activation (Table V).

The activator protein is probably calmodulin because the molecular weight is very similar to that known for calmodulin, is calcium dependent and it activates phosphodiesterase in the same way that bovine brain phosphodiesterase is activated by calmodulin in bovine (Cheung 1980). The author will refer to this protein as calmodulin in the remainder of the paper.

Reduction of inhibitor I with dithiothreitol (Fig. 17) resulted in three separate peaks of inhibitors A, B, and C. The greatest amount of inhibitory activity was observed within fraction C. When inhibitor II was incubated with dithiothreitol no change in behavior was observed. Fig. 18 shows estimated molecular weights of various inhibitor fractions. The reduced inhibitor C fraction of inhibitor I has a molecular weight (11,900) which is very close to that of inhibitor II (12,100).

Kinetic analysis of purified phosphodiesterase and its modulator proteins (Fig. 19-20) shows that phosphodiesterase from either phase of feeding has approximately the same Km. If calmodulin is added, there is a reduction in the Km and an increase in Vmax is observed (Figs. 19 & 20), Table VI. When inhibitors are added to phosphodiesterase and calmodulin together no effect on Km is observed but the Vmax decreases considerably.

Kinetic analysis of particulate PDE shows that the Km of the enzyme is the same regardless of stage of feeding from which the glands were obtained but the Vmax increases both when the tick enters the rapid stage of feeding and when glands are pre-stimulated with dopamine

TABLE V. CROSS REACTIVITY OF PDE AND MODULATORS ISOLATED FROM TICKS IN DIFFERENT STAGES OF FEEDING.

	Generated ATP (n-moles/220 µl)*	
	PDE FROM	
	Slow-feeding ticks	Rapid-feeding ticks
PDE	0.09 ± 0.05	0.10 ± 0.06
PDE + Slow feeding Act.	0.70 ± 0.16	0.43 ± 0.14
PDE + Rapid feeding Act.	0.29 ± 0.12	0.60 ± 0.18
PDE + Slow feeding Act. + Inh. I	0.12 ± 0.01	0.08 ± 0.03
PDE + Slow feeding Act. + Inh. II	0.14 ± 0.10	0.07 ± 0.03
PDE + Rapid-feeding Act. + Inh. I	0.14 ± 0.06	0.07 ± 0.04
PDE + Rapid-feeding Act. + Inh. II	0.12 ± 0.06	0.08 ± 0.05
PDE + 1mM EGTA	0.11 ± 0.05	0.10 ± 0.06
PDE + Rapid feeding Act + 1mM EGTA	0.13 ± 0.08	0.13 ± 0.07

* Results are means ± S.D. of four experiments.

Figure 17. Dithiothreitol reduction of Inhibitors I and II. Treated fractions (as described under "Methods") were chromotographed on an Ultrogel® AcA 54 column. and show where untreated fractions of Inhibitor I and II were present in fractions coming off the column. Ve = elution volume; Vo = void volume of column.

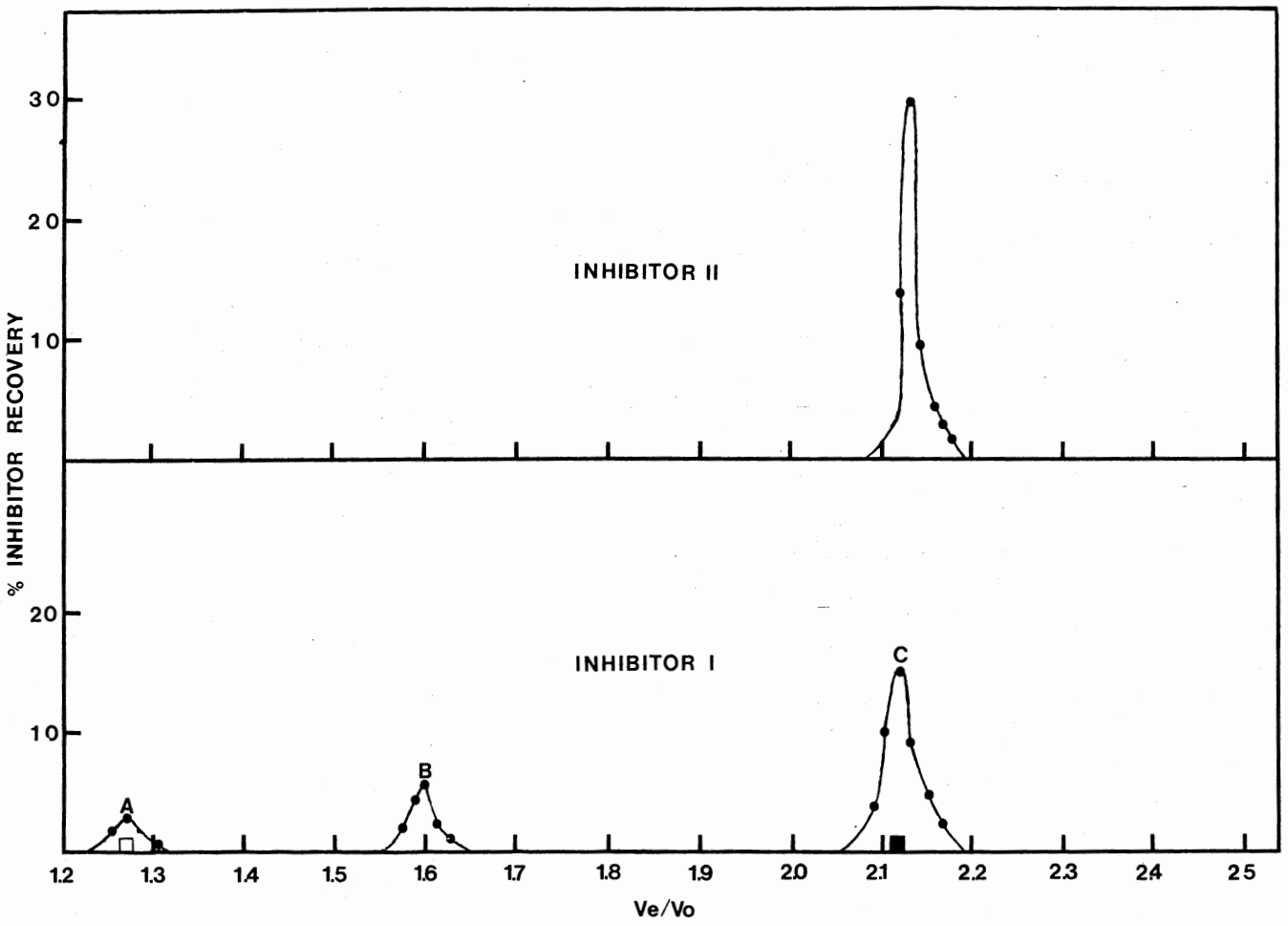

30
20
10
% INHIBITOR RECOVERY
INHIBITOR II
20
10
INHIBITOR I
A
B
C
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
2.4
2.5
Ve/Vo

Figure 18. Molecular weight determination of reduced fractions of inhibitors I and II by comparing to Ultrogel® AcA 54 gel filtration with proteins of known molecular weight. BLac = B lactose; Chy = chymotrypsin; Myog = globin, Rib = ribonuclease; Oval = oval bumin.

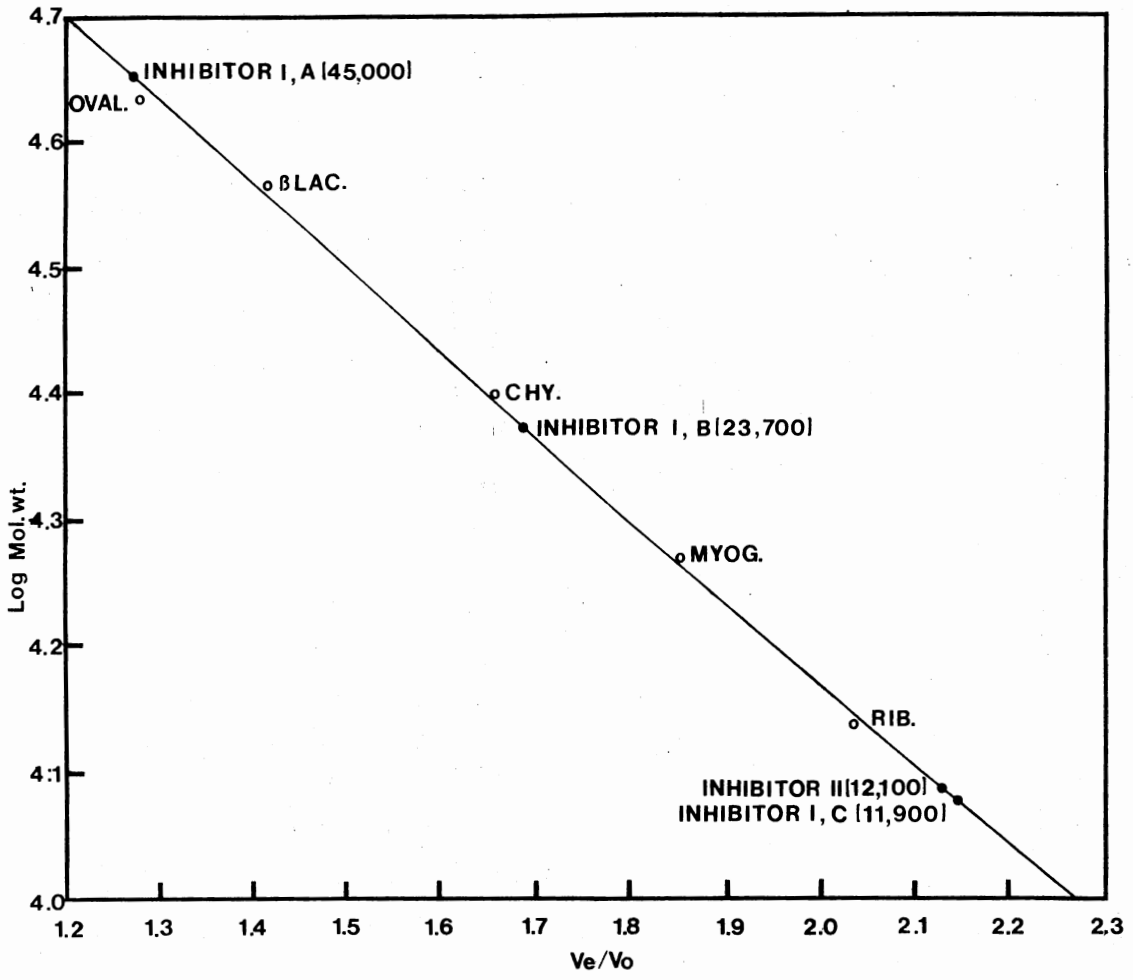

4.7
4.6
4.5
4.4
4.3
4.2
4:1
4.0
Log Mol.wt.
INHIBITOR I, A [45,000]
OVAL.
ßLAC.
CHY.
INHIBITOR I, B [23,700]
MYOG.
RIB.
INHIBITOR II [12,100]
INHIBITOR I, C [11,900]
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
Ve/Vo

Figure 19. Representative Lineweaver-Burk plot of 100,000 xg ave. supernatant cAMP-dependent phosphodiesterase (purified, see methods and materials) and the effect various protein modulators have on its activity.

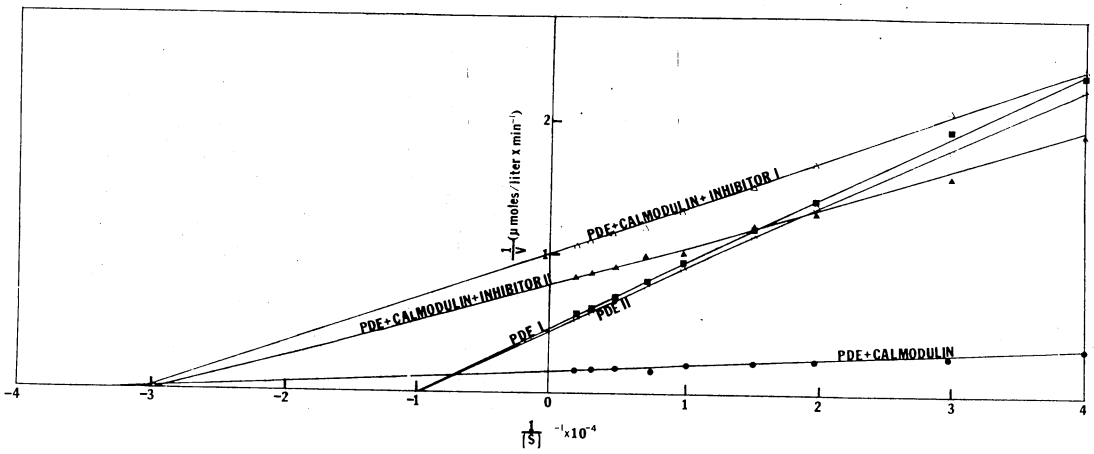

PDE+CALMODULIN+INHIBITOR I
PDE+CALMODULIN+INHIBITOR II
PDE I
PDE II
PDE+CALMODULIN
$\frac{1}{V}$ (μ moles/liter x min^{-1})
$\frac{1}{[S]}$ $^{-1}$x10^{-4}
-4
-3
-2
-1
0
1
2
3
4
1
2

Figure 20. Lineweaver-Burk plot of linetics of supernatant PDE from gland from slow and rapid feeding ticks.

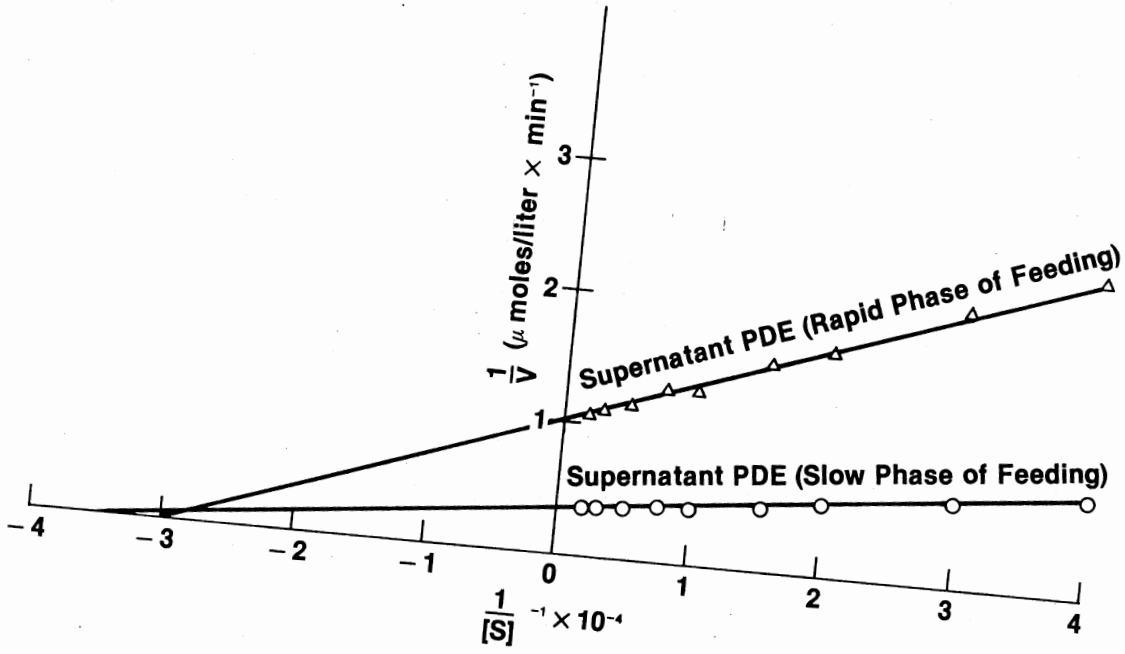

$\frac{1}{V}$ (μ moles/liter $\times$ min^{-1})
3
2
1
Supernatant PDE (Rapid Phase of Feeding)
Supernatant PDE (Slow Phase of Feeding)
−4
−3
−2
−1
0
1
2
3
4
$\frac{1}{[S]}^{-1} \times 10^{-4}$

TABLE VI. KINETIC COMPARISONS OF SUPERNATANT PDE AND ITS MODULATORS.

	PDE + Calmodulin	PDE + Calmodulin + Inh I	PDE + Inh II	Purified PDE, Rapid Phase	Purified PDE, Slow Phase	Crude PDE, Slow Phase	Crude PDE, Rapid Phase
Km X 10^{-6}M	30.9±0.4	33.1±0.3	34.1±0.2	99.6±0.9	100.6±0.7	29.4±0.2	33.7±0.3
Vmax n moles cAMP hydrolyzed per min per mg protein	12.50±.38	1.84±.02	2.42±.40	3.68±.37	3.77±.38	.107±.02	.036±.009*

*Magnitude of Vmax values in Crude preperation vary from others due to these fractions containing much higher quantity of total protein. Modulators were prepared as described in "Methods" except for crude which is supernatant from 100,000 xg centrifugation. Results are the means ± S.D. of four experiments.

Pre-stimulation of glands with dopamine has no effect on supernatant cAMP phosphodiesterase activity or on cGMP phosphodiesterase (Table VIII & IX) but causes an increase in particulate activity. The effect of dopamine on particulate activity is greater when glands are removed from ticks in the slow phase of feeding. Dopamine had no effect on particulate activity if glands were homogenized prior to the addition of dopamine. As can be seen in Table VIII, total gland phosphodiesterase activity drops considerably in the glands as the tick progressed from a slow to a rapid stage of feeding. This drop in total activity is due to a decrease in supernatant (PDE) activity (Table VIII). The particulate fraction, which contains 26% of total cell protein, contains only 7% of total cAMP phosphodiesterase activity in glands removed from ticks in the slow phase of feeding (Table X) but because of the decrease in supernatant phosphodiesterase activity, the particulate enzyme activity is 58.7% of the total gland protein in glands of rapidly feeding ticks even though particulate activity increased only slightly. Calmodulin had no effect on particulate activity.

These changes in phosphodiesterase activity with tick growth occured entirely in the Type II and III alveoli. Type I alveoli + duct showed no change in phosphodiesterase activity during feeding (Table XI). The Type I alveoli comprise 1% of total gland tissue and there was not enough tissue to easily separate into particulate and supernatant fractions.

Cyclic GMP phosphodiesterase was found entirely in the supernatant fraction. The measured Km of the cGMP phosphodiesterase is 7.3 $\pm$ 0.1 M and the Vmax 2.6 $\pm$ 0.1. These values did not change as the tick progressed from the slow to rapid phase of feeding nor did dopamine

TABLE VII. KINETIC ANALYSIS OF PARTICULATE PDE, WITH OR WITHOUT PRIOR STIMULATION OF GRANDS WITH DOPAMINE, 10^{-6} M.

	Control		Dopamine	
	Slow	Rapid	Slow	Rapid
Km X 10^{-6} M	2.72±.065	2.89±.071	3.03±.056	2.76±.036*
Vmax p moles cAMP hydrolized per min per mg protein)	21.1±1.6	43.5±.8	28.3±2.0	74.2±2.6

*PDE was prepared from ticks in either the slow or rapid phase of feeding. Results are the means ± S.D. of two experiments.

TABLE VIII. LOCALIZATION AND EFFECT OF 10^{-6}M DOPAMINE ON cAMP HYDROLYSIS.

	Slow		Rapid	
	Control	Dopamine	Control	Dopamine
Total	0.47±0.06	0.56±0.05	0.16±0.14	0.33±0.16*
Particulate	0.08±0.05	0.25±0.06	0.19±0.09	0.28±0.09
Supernatant	0.44±0.03	0.42±0.07	0.07±0.04	0.06±0.06

*PDE activity is expressed as n-moles nucleotide hydrolized/ 10 µg protein/10 min at 37°C and .1mM cAMP. Results are the means ± S.D. of three experiments.

TABLE IX. LOCALIZATION AND EFFCT OF PRIOR STIMULATION OF GLANDS WITH 10^{-6} M DOPAMINE ON cGMP HYDROLYSIS.

	Slow		Rapid	
	Control	Dopamine	Control	Dopamine
Total	0.03±0.02	0.04±0.03	0.02±0.03	0.03±0.01*
Particulate (100,000xg)	0.00±0.00	0.00±0.00	0.00±0.01	0.00±0.00
Supernatant (100,000xg)	0.03±0.04	0.02±0.02	0.03±0.03	0.04±0.02

*PDE activity is expressed as n-moles nucleotide hydrolized/10µg protein/10 min at 37°C and 0.1mM cGMP). Results are means ± S.D. of three experiments.

TABLE X. RELATIVE % OF TOTAL cAMP PDE ACTIVITY FOUND IN SUPERNATANT AND PARTICULATE FRACTIONS AT VARIOUS STAGES OF FEEDING.

	Percent	
	Slow	Rapid
Particulate (100,000xg)	7.7 ± 4.7	58.7 ± 11.1*
Supernatant (100,000xg)	92.3 ± 6.3	41.3 ± 8.3

*Results are means ± S.D. of three experiments.

TABLE XI. EFFECT OF STAGE OF FEEDING ON PDE ACTIVITY IN SALIVARY GLAND ALVEOLI TYPES AND COMPARTMENTS AND AGAINST cAMP AND cGMP.

	cAMP (n moles)		cGMP (n moles)	
Stage of Feeding	Slow	Rapid	Slow	Rapid
Type I alveoli + Duct	0.41±0.23	0.37±0.26	0.03±0.02	0.05±0.07*
Particulate Type II & III alveoli	0.09±0.04	0.21±0.06	0.00±0.00	0.00±0.00
Supernatant Type II & III alveoli	0.39±0.12	0.05±0.03	0.03±0.05	0.04±0.04
Total (Whole gland homogenates	0.48±0.16	0.14++0.05	0.04±0.05	0.03±0.04

*Activity is expressed on n moles nucleotide hydrolized/10 μg protein/ 10 min at 37°C and .1mM cAMP or cGMP. All experiments were repeated in triplicate.

have an effect on whole gland activity (Table VI & VIII). Cyclic GMP phosphodiesterase never exceeded more than 6% of total gland or cyclic nucleotide phosphodiesterase activity.

DISCUSSION

It is thought that cAMP plays a major role in the regulation of salivary fluid secretion by feeding processed by ixodid ticks (Sauer et al. 1974, 1976; Needham and Sauer 1975, 1979). The type II and III alveoli of the salivary glands are thought to secrete the bulk of the fluid (Binnington 1978; Megaw & Beadle 1979; Krolak et al. unpublished) and it has been demonstrated that when the tick enters the rapid stage of feeding the salivary glands secrete at maximum rates when stimulated with cAMP or dopamine (Sauer et al. 1979). Also, adenylate cyclase activity is highest in glands of rapidly feeding ticks (Schmidt et al. unpublished) and we have previously demonstrated that the only enzyme known to hydrolyze cAMP, phosphodiesterase, is lowest in glands of rapidly feeding ticks (McMullen & Sauer 1979).

Tick salivary glands were found to contain both particulate and supernatant forms of PDE. Similar distributions of PDE have been demonstrated by others in other tissues (Thompson & Strada 1979). The supernatant PDE (Figs. 12, 13) was found to be influenced by various endogenous protein modulators which may be physiologically significant. Calmodulin (Figs. 12, 14, 16) was isolated and found to be present in all salivary gland preparations. Calmodulin increased the supernatant PDE activity by increasing the Vmax and decreasing the Km (Fig. VIII, Table XI). Two protein inhibitors which inhibited the activating effect on the Vmax by calmodulin but had no effect on the Km (Fig. 19, Table VI) were isolated in glands from rapidly feeding ticks. Inhibitor I is

likely composed of 4 subunits of inhibitor II and inhibitor II is possibly due to inhibitor I breakdown during purification procedures which may be held together by disulfide bonds (Figs. 17, 18). Alternatively, it is possible that proteases of physiological significance are present with the ability to hyrolyze the tetramer to monomeric subunits.

A low Km form of PDE was found in the particulate fraction, (Table VII) and dopamine was found to increase its Vmax but not Km. These findings agree with the observation by others that the particulate or membrane-bound enzyme is affected by hormones and the kinetic affect is on the Vmax (Thompson & Strada 1979). These multiple forms of PDE are possibly the reason for the anomolous kinetics seen in kinetic plots of crude enzyme preparations (Chapter II).

When cAMP and cGMP PDE activity was compared during the various phases of feeding, several obvious changes were noted (Tables VIII, IX, XI). The total gland cAMP PDE activity decreases (Table VIII) as the tick progresses from the slow to rapid stage of engorgement. This same effect is seen in the supernatant PDE (Table VIII) and is confined to type II and III alveoli (Table XI). There is an increase in total gland particulate PDE activity and in particulate activity in type II and III alveoli as the tick enters rapid engorgement (Tables VIII and XI). No change in PDE activity was observed in type I alveoli during feeding. It is thought that type I alveoli are not involved in fluid secretion (Binnington 1978; Megaw & Beadle 1979). Changes in both particulate and supernatant cAMP-dependent PDE during feeding would seem to suggest that both are important in the regulation of salivary secretion in feeding ticks. There is little evidence for a role for cGMP phosphodiesterase

in salivary function. Cyclic GMP phosphodiesterase levels are low (never more than 6% of total PDE activity) and its activity does not change during the various phases of engorgement nor does pre-stimulation of glands with dopamine have any effect upon its activity. In previous experiments, Sauer et al. (1979) found exogenous cGMP able to stimulate fluid secretion, but the rate was only 7% that obtained when glands were stimulated with exogenous cAMP.

Total gland cAMP PDE is highest when the tick is in the slow phase of feeding. This may cause the relatively low levels of salivary fluid secretion seen in ticks in the slow phase of feeding by reducing basal levels of cAMP. However, as the tick enters rapid engorgement two changes in salivary gland PDE occur. The supernatant PDE activity drops sharply while the particulate enzyme increases slightly. Particulate activity in glands of slow feeding ticks totalled only 7% of all activity and 58% in glands from ticks in the rapid phase of feeding. Because of a much higher percentage of supernatant PDE in glands from ticks in the slow phase of feeding, the changes in percentage of distribution of PDE seen is due mostly to the drop in supernatant activity and not a rise in particulate PDE activity. This suggests that PDE in the cell may be under dual control. Particulate enzyme could be regulated by effectors of membrane receptors such as dopamine (Megaw & Robertson 1974). As the tick begins to feed more rapidly the gland may be increasingly stimulated by dopamine leading to a rise in particulate PDE activity. The drop in supernatant PDE activity is likely explained by the appearance of Ca^{2+} dependent inhibitors. Hormonal stimulation also causes activation of these proteins by changes in cytoplasmic levels of free Ca^{2+} (McMullen et al. 1980). In this way, the two PDE's

the particulate and the supernatant could interact through several different mechanisms to regulate cAMP levels and therefore fluid secretion. This agrument is further strengthened by the fact that all changes in gland PDE during tick feeding occurs in alveoli types II and III and not type I which are thought not to be involved in the fluid secretion process of feeding ixodid ticks.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Cyclic AMP (cAMP) phosphodiesterase (PDE) the only enzyme known to hydrolyze cell levels of cAMP, has been studied in many animals and there are few investigators who have found a correlation between biochemical regulation of the enzyme and physiological events in the cell. This researcher has studied the relationship of PDE activity to known biochemical, physiological, and morphological events in the salivary glands of an ixodid tick, Amblyomma americanum (L.) at the time of feeding.

The salivary glands of the tick serve as a primary organ of osmoregulation in the following manner: After concentrating the bloodmeal by moving excess fluid across its gut epithelium and the tick then expells the fluid back into the host via the salivary glands. We have recently obtained evidence that implicates both cAMP and Ca^{2+} in the process of fluid secretion. We have demonstrated that catecholamines stimulate fluid secretion and cause an increase in intracellular levels of cAMP. This probably relates to the fact that rates of fluid secretion increase greatly while ticks are feeding. Additional results of experiments from our lab suggest that cytoplasmic levels of Ca^{2+} may rise in salivary glands after stimulation with dopamine.

My experiments established that the tick salivary glands contained two different cyclic nucleotide dependent phosphodiesterases,

one high Km enzyme located in the cytosol and another low Km enzyme likely bound to membranes and located in the particulate fraction following centrifugation procedures.

These experiments established that total PDE activity drops sharply just prior to the ticks entering rapid engorgement and remains at low levels thereafter. These results correlate well with the observation that the secretory rates of salivary glands from rapidly feeding tick increase greatly when stimulated by cAMP and theophylline or catecholamines. This high PDE activity in glands obtained from lighter ticks may be one reason why the secretory rate is correspondingly low. This deactivation of PDE in the salivary glands of ticks in the rapid phase of engorgement and the results of the pooling experiments suggested that an inhibitor of PDE may be present in glands from rapidly feeding ticks. Further experiments in which pre-stimulation of glands with dopamine caused an activation of PDE suggested the presence of an activating factor of PDE. In later experiments I was able to isolate Ca^{2+}-dependent inhibitory and activating fractions from the supernatant fraction (100,000 xg) of gland homogenates.

After applying supernatant fractions of salivary gland homogenates to Ultrogel column chromatography, one PDE peak (136,400 M.W.), an activator (17,800 M.W.), and two inhibitors of PDE (inhibitors I and II 43,500 and less than 11,200 M.W.) were isolated. Inhibitor activity was found mostly in the salivary glandds from rapidly feeding ticks and was found to inhibit this form of PDE when added back to this fraction along with the activator.

Calcium ions were found to be necessary for the action of both the activator and inhibitory modulators of PDE, but the levels of Ca^{2+}

necessary for stimulation of the activator was found to be approximately 100 times less than that necessary for activation of the inhibitor. In addition Mg^{2+} ions were found necessary for activation of PDE.

The author has concluded that the activating protein is likely calmodulin and in later experiments determined that inhibitory protein I is composed of 4 subunits of inhibitor II. Calmodulin activated the high Km form of PDE by lowering the Km and raising the Vmax. The inhibitors prevented the complete effect of calmodulin on PDE from being seen by interferring with the Vmax. The inhibitors had no effect on PDE if calmodulin was absent and may serve as important intracellular regulators of salivary fluid secretion by their involvement in regulating cAMP levels in gland cells. In other tissues, endogenous inhibitor proteins of cyclic nucleotide PDE have been isolated but little is known about their physiological role. It has been suggested that if these proteins are physiological inhibitors they must be restricted, localized or their concentrations in the tissue must increase markedly under certain conditions. My experiments are the first that implicate a physiological role for the inhibitors of PDE.

In more recent experiments the author located a low Km PDE in the particulate fraction of tick salivary glands. The author also found that dopamine activated this PDE by raising the Vmax but had little effect on the Km. The activity of this enzyme was higher in glands obtained from rapidly feeding ticks. One possible explanation for this increase in particulate activity is that when glands are stimulated by nerves the increased transmitter release possibly causes an increase in PDE activity in glands of rapidly feeding ticks. It is interesting that one sees

an increase in particulate PDE both after in vitro stimulation of glands with dopamine and in glands of rapidly feeding ticks as compared to that seen in slowly feeding ticks. It is also interesting that the increase in both instances is caused by an increase in Vmax and not Km.

Other experiments suggest that cAMP dependent PDE may play an important role in regulation of fluid secretion. The author has found that the above changes in PDE activity, as the tick progresses from the slow to rapid phase of feeding, are observed only in type II and III alveoli and not in type I alveoli + duct. Only type II and III alveoli are thought to be involved in the process of fluid secretion by feeding ixodid ticks.

Cyclic GMP phosphodiesterase activity does not change as the tick progresses from the slow to rapid phase of feeding but is is Ca^{2+} dependent and activated by calmodulin. The results were inconclusive as to whether the protein inhibitors had any effect on cGMP phosphodiesterase. Cyclic GMP PDE levels were never greater than 6% of total salivary gland PDE. At this time it is difficult to speculate on the role, if any, of cGMP PDE in tick salivary glands.

In slowly feeding ticks, 93% of total gland PDE is located in the supernatant fraction (high Km PDE) but in the rapidly feeding tick only 50% is found in this fraction. This was not due to an increase in particulate activity (low Km PDE) but to a substantial decrease in supernatant PDE activity.

These facts suggest a possible mechanism for the control of fluid secretion in tick salivary glands. During early feeding, the inhibitors of soluble PDE are not active or not present and high basal PDE activity is seen keeping cAMP levels low thus helping to maintain low rates of

fluid secretion. As the tick enters the later stages of feeding, inhibitors of PDE appear to become active allowing cAMP levels to rise, causing high rates of fluid secretion. Additionally, it seems likely that an increase in cytoplasmic levels of Ca^{2+} occur during the period of rapid engorgement causing for activation of the inhibitors of PDE.

Gland PDE could also be under the control of the possible natural secretagogue dopamine. Dopamine is known to stimulate gland adenylate cyclase at doses similar to those which cause in vitro glands to secrete fluid. It may be that an additional role for dopamine is to the activation of a specific PDE receptor or possibly other receptors which change membrane-bound PDE activity especially in situations when gland stimulation is high (i.e. salivary glands of rapidly feeding ticks). There could then be interaction between the 2 PDE's (particulate and supernatant) in help regulating cAMP levels and therefor fluid secretion. Of the 2 forms of PDE, it would seem to be the more abundant supernatant PDE and as seen by its resulting drop in activity during rapid engorgement is the more important for helping regulate salivary fluid secretion during tick engorgement. The drop in total gland activity could lead to increased cAMP and the known increase in fluid secretion. If these conclusions are correct, this appearance of the Ca^{2+} dependent inhibitors of supernatant PDE in rapidly feeding ticks are very important for the physiological regulation of fluid secretion. Other investigators have shown that activation of PDE by calmodulin is not a very species specific reaction but the action of inhibitors is a very specific reaction. It would seem that if one could interfer with the appearance activation or activity of the inhibitors of PDE one might have a good target for possible inhibition in a tick control program.

SELECTED BIBLIOGRAPHY

Albin, E. E., S. J. Davison and R. W. Newburg. 1974. Properties of cyclic nucleotide phosphodiesterase in the central nervous system of Manduca sexta. Biochim. Biophys. Acta. 377: 364-380.

Amer, M. S., W. E. Kreighbau. 1975. Cyclic nucleotide phosphodiesterase: Properties, activators, inhibitors, structure-activity relationships, and possible role in drug development. J. Pharmacol. Sci. 64: 1-36.

Andrew, P. 1965. The gel-filtration behavior of proteins related to their molecular weights over a wide range. Biochem. J. 96: 595-606.

Bielinska, M. and M. J. Piechowska. 1975. Metabolism of exogenous cyclic adenosine 3'5'-monophosphate in tissues of adult locust, Schistocerca gregaria. Insect Biochem. 5: 47-51.

Binnington, K. C. 1978. Sequential changes in salivary gland structure during attachment and feeding of the cattle tick, Boophilus microplus. Int. J. Parasitology. 8: 97-115.

Brostrom, C. W., D. J. Wolff. 1976. Calcium-dependent cyclic nucleotide phosphodiesterase from brain: comparison of adenosine 3'5'-monophosphate and guanosine 3'5'-monophosphate as substrates. Arch. Biochem. Biophys. 172: 301-311.

Cheung, W. Y. 1970. Cyclic 3'5'-nycleotide phosphodiesterase. Demonstration of an activator. Biochem. Biophys. Res. Commun. 38: 533-38.

Cheung, W. Y., Y. M. Lin, Y. P. Liu and J. A. Smoake. 1975. Regulation of bovine brain phosphodiesterase in Cyclic 3'5'-Nucleotides in Disease (Edited by B. Weiss), pp 321-350. University Park Press, Baltimore.

Cheung, W. Y. 1980. Calmodulin plays a pivital role in cellular regulation. Science 207: 19-27.

Drummond, G. I. and Perrett-Yeess. 1961. Enzymatic hydrolysis of adenosine 3',5' phosphoric adid. J. Bio. Chem. 236: 1126-29.

Hsu, M. H. and J. R. Sauer. 1975. Ion and water balance in the feeding lone star tick. Comp. Biochem. Physiol. 52A: 269-76.

Kakiuchi S., Yamazaki, R., and Y. Teshima. 1972. In Advances in Cyclic Nucleotide Research. (Edited by P. Greengard et al.) 1: 455-78. Raven Press. New York.

Kaufman, W. R. and J. E. Phillips. 1973. Ion and Water balance in the ixodid tick Dermacentor andersoni. I. Routes of ion and water excretion. J. Exp. Biol. 58: 523-36.

Kaufman, W. 1976. The influence of various factors on fluid secretion by in vitro salivary glands of ixodid ticks. J. Exp. Biol. 64: 727-42.

Kaufman, W. R., P. A. Diehl and A. A. Aeschlimann. 1976. Na, K-ATPase in the salivary gland of the ixodid tick, Amblyomma hebraeum (Koch) and its relation to the process of fluid secretion. Experientia 32: 986-87.

Liu, Y. P. and V. G. Wang. 1979. A heat stable protein inhibitor of cyclic 3'5' nucleotide phosphodiesterase. Biochim. Biophys. Acta. 583: 273-78.

Megaw, M. J. W. and D. J. Beadle. 1979. Structure and function of the salivary glands of the tick Boophilus microplus Canestrini (Acarinia: Ixodidae). Int. J. Insect Morph. and Emboyol. 8: 67-86.

McMullen, H. L., J. R. Sauer and R. L. Burton. 1976. Possible role in uptake of water vapor by Ixodid tick salivary glands. J. Insect. Physiol. 22: 1281-85.

McMullen, H. L. and J. R. Sauer. 1978. The relationship of phosphodiesterase and cyclic AMP to the process of fluid secretion in the salivary glands of the ixodid tick Amblyomma americanum. Experientia. 34: 1030-31.

McMullen, H. L., J. R. Sauer, J. Bantle and R. C. Essenberg. 1980. Regulation of fluid secretion by calcium-dependent modulator protein of 3'5'-cyclic-AMP phosphodiesterase. Biochim. and Biophys. Res. Commun. 95: 1555-62.

Needham, G. R. and J. R. Sauer. 1975. Control of fluid secretion by isolated salivary glands of the lone star tick. J. Insect. Physiol. 21: 1893-98.

Needham, G. R. and J. R. Sauer. 1979. Involvement of calcium and cyclic AMP in controlling Ixodid tick salivary fluid secretion. J. Parasitology. 65: 531-42.

Pan, P., J. T. Bonner, H. J. Wedner and C. W. Parker. 1974. Immunofluorescence evidence for the distribution of cyclic AMP in cells and cell masses of the cellular slime molds. Proc. Natl. Acad. Sci. USA. 71: 1623-25.

Plegder, W. J., W. J. Thompson and S. J. Strada. 1976. Serum modification of cyclic nucleotide phosphodiesterase form independent of protein synthesis. Biochim. and Biophys. Res. Comm. 70: 58-65.

Rasmussen, H., P. Jenson, W. Lake, N. Friedman and D. B. P. Goodman 1975. Cyclic nucleotides and cellular calcium metabolism. In Advances in Cyclic Nucleotide Research (Edited by G. I. Drummond et al.), Vol. 5, pp. 375-94. Raven Press, New York.

Revuelta, A., P. Uzunov, and E. Costa. 1976. Release of PDE activator from the particulate fraction of cerebellum and Straitum by putative neurotransmitters. Neurochem. Res. 1: 217-27.

Sauer, J. R., J. H. Frick and J. Hair, 1974. Control of ^{36}Cl uptake by isolated salivary glands of the lone star tick. J. Insect Physiol. 20: 1771-78.

Sauer, J. R., P. Mincolla and G. R. Needham. 1976. Adrenaline and cyclic AMP stimulated uptake of chloride and fluid secretion by isolated salivary glands of lone star tick. Comp. Biochem. Physiol. 53: 63-5.

Sauer, J. R. 1977. Acrine Salivary glands physiological relationships. J. Med. Ent. 14: 1-9.

Sauer, J. R., G. R. Needham and H. L. McMullen. 1979. Cyclic Nucleotides, calcium and salivary fluid secretion in ixodid ticks. In Recent Advances in Acarology. (Edited by J. G. Rodriguez) Vol. I, pp. 365-73. Raven Press. New York.

Schwartz, A., G. E. Linden Mayer and J. C. Allen. 1975. The sodium-potassium adenosine triphosphate pharmacological, physiological and biochemical aspects. In Pharmacological Reviews (Edited by P. Greengard et al.), Vol. 1: pp 357-74. Raven Press, New York.

Sharma, R. K., R. Desai, R. T. Thompson and J. H. Wang. 1978. Purification of the heat-stable inhibitor protein of the Ca^{2+} - activated cyclic cyclic nucleotide phosphodiesterase by affinity chromatography; Amer. J. Biochem. 56: 598-604.

Sillen, L. G. and E. Martell. 1964. Stability constants of metal ions complexes. The Chemical Society of London. Special Publ. # 17.

Strada, S. J., P. Uznov and P. Weiss. 1974. Onogenetic development of a phosphodiesterase activator and the multiple forms of cyclic AMP phosphodiesterase of rat brain. J. Neurochem. 23: 1097-1103.

Sutherland, E. W., G. A. Robison and R. W. Butcher. 1968. Some aspects of the biological role of adenosine 3',5'-monophosphate (cyclic AMP). Circulation 37: 279-83.

Tatchell, R. J. 1967a. Salivary secretion in the cattle tick as a means of water elimination. Nature 213: 940-41.

Thompson, W. J. and S. J. Strada. 1978. Hormonal Regulation of cyclic nucleotide-phosphodiesterase. In Receptors and Hormone Action. (Edited by L. Birn baumer and B. W. O'Malley) Vol. 3: pp. 553-77. Academic Press, New York.

Thompson, J. P., W. L. Terasaki, P. M. Epstein and S. J. Strada. 1979. Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular form of the enzyme. In Advances in Cyclic Nucleotide Research (Edited by G. Brooker et al.), Vol. 10: pp. 69-92. Raven Press, New York.

Wang, J. H. 1977. Calcium-regulated protein modulators in Cyclic Nucleotide systems. In Cyclic 3'5'-Nucleotides: Mechanism of Action (Edited by H. Crammer and J. Schultz). pp. 37-56. John Wiley and Sons, New York.

Wang, J. H. and R. Desai. 1977. Modulator binding protein. Biol. Chem. 252: 4175-84.

Wang, J. H. and D. M. Waissman. 1979. Calmodulin and its role in the second messenger system. In Current Topics in Cellular Regulation (Edited by B. L. Horecker and E. R. Stradtman), 15: 47-107. Academic Press, New York.

Weiss, B. and E. Costa. 1968. Regional and subcellular distribution of adenyl cyclase and 3',5'-cyclic nucleotide phosphodiesterase in brain and pineal gland. Biochem. Pharmacol. 17: 2107-16.

Weiss, B. and S. J. Strada. 1972. Neuroendocrine control of the cyclic AMP sysem of brain and pineal gland. In Advances in Cyclic Nucleotide Research (Edited by P. Greengard et al.),1: pp. 357-74. Raven Press, New York.

Weiss, B., R. Lehne, and S. Strada. 1972. Rapid microassay of adenosine 3':5'-monophosphate phophodiesterase activity. Anal. Biochem. 45: 222-35.

Weiss, B. and N. H. Hait. 1977. Selective cyclic nucleotide phosphodiesterase inhibitors as potential therapeutic agents. Ann. Rev. Toxicol. 17: 441-77.

Wells, J. N., C. N. Baird, Y. J. Wu, and J. G. Hardman. 1975. Cyclic nucleotide phosphodiesterase activities of pig coronary arteries. Bio chim. Biophys. Acta. 384: 430-42.

Wells, J. N. and J. G. Hardman. 1977. Cyclic nucleotide phosphodiesterase. In Advances in Cyclic Nucleotide Research (Edited by P. Greengard and G. A. Robison), Vol. 8: pp. 119-44. Raven Press, New York.

VITA[2]

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